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A polydnavirus-encoded ANK protein has a negative impact on steroidogenesis and development

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## TITLE

# **A polydnavirus-encoded ANK protein has a negative impact on steroidogenesis and development**

Authors and Affiliations

**Marilena Ignesti <sup>a</sup>, Rosalba Ferrara <sup>b</sup>, Patrizia Romani <sup>a,c</sup>, Luca Valzania <sup>a,d</sup>,  
Giulia Serafini <sup>a</sup>, Francesco Pennacchio <sup>b,\*</sup>, Valeria Cavaliere <sup>a,\*\*</sup>, Giuseppe  
Gargiulo <sup>a,\*\*\*</sup>**

<sup>a</sup> Dipartimento di Farmacia e Biotecnologie, Università di Bologna, Via Selmi 3  
Bologna, Italy.

<sup>b</sup> Dipartimento di Agraria – Laboratorio di Entomologia “E. Tremblay”, Università di  
Napoli ‘Federico II’, Portici (NA), Italy.

<sup>c</sup> Present address: Dipartimento di Medicina Molecolare, Università di Padova,  
Padova, Italy.

<sup>d</sup> Present address: Department of Entomology, University of Georgia, Athens, GA  
30602, USA.

\* Corresponding author. Dipartimento di Agraria – Laboratorio di Entomologia “E.  
Tremblay”, Università di Napoli ‘Federico II’, Portici (NA), Italy.

\*\* Corresponding author. Dipartimento di Farmacia e Biotecnologie, Università di  
Bologna, Via Selmi 3 Bologna, Italy.

\*\*\* Corresponding author. Dipartimento di Farmacia e Biotecnologie, Università di  
Bologna, Via Selmi 3 Bologna, Italy.

## **Abstract**

Polydnaviruses (PDV) are viral symbionts associated with ichneumonid and braconid wasps parasitizing moth larvae, which are able to disrupt the host immune response and development, as well as a number of other physiological pathways. The immunosuppressive role of PDV has been more intensely investigated, while very little is known about the PDV-encoded factors disrupting host development. Here we address this research issue by further expanding the functional analysis of *ankyrin* genes encoded by the bracovirus associated with *Toxoneuron nigriceps* (Hymenoptera, Braconidae). In a previous study, using *Drosophila melanogaster* as experimental model system, we demonstrated the negative impact of *TnBVank1* impairing the ecdysone biosynthesis by altering endocytic traffic in prothoracic gland cells. With a similar approach here we demonstrate that another member of the viral *ank* gene family, *TnBVank3*, does also contribute to the disruption of ecdysone biosynthesis, but with a completely different mechanism. We show that its expression in *Drosophila* prothoracic gland (PG) blocks the larval-pupal transition by impairing the expression of steroidogenic genes. Furthermore, we found that *TnBVank3* affects the expression of genes involved in the insulin/TOR signaling and the constitutive activation of the insulin pathway in the PG rescues the pupariation impairment. Collectively, our data demonstrate that *TnBVANK3* acts as a virulence factor by exerting a synergistic and non-overlapping function with *TnBVANK1* to disrupt the ecdysone biosynthesis.

## **Keywords**

*Bracovirus; Drosophila; ANK proteins; Ecdysone biosynthesis; insulin/TOR signaling*

1 **1. Introduction**

2 Parasitic wasps develop on a wealth of insect species, on which they induce a number  
3 of physiological and developmental alterations, which are essential to create a suitable  
4 environment for the development of their progeny (Pennacchio and Strand, 2006).

5 These changes are currently denoted as host regulation, which is a complex process  
6 mediated by a network of molecular interactions, triggered and controlled by factors  
7 produced and released into the host by the ovipositing females (i.e. venom, microbial  
8 symbionts, ovarian secretions) and/or by the embryo (i.e. teratocytes, cells deriving  
9 from the dissociation of the embryonic membrane) or larvae (Pennacchio and Strand,  
10 2006). Among microbial symbionts, polydnaviruses (PDVs) are potent

11 immunosuppressive agents associated with ichneumonid and braconoid wasps  
12 parasitizing larval stages of moth larvae, and able to induce a number of pathological  
13 alterations in the host (Pennacchio and Strand, 2006; Strand and Burke, 2015). PDVs  
14 are integrated as proviruses in the wasp genome and replicate only in the epithelial  
15 cells of the ovarian calyx to produce free virions that are injected into the host at the  
16 oviposition. During this process they infect and express virulence factors in several  
17 host tissues, without undergoing replication (Herniou et al., 2013; Strand and Burke,  
18 2015). The segmented genome of PDVs consists of multiple circles of DNA of  
19 different size, characterized by large non-coding segments and by genes showing an  
20 eukaryotic structure, often organized in gene families (Herniou et al., 2013; Strand  
21 and Burke, 2015). One of the most widespread gene family encodes ankyrin motif  
22 proteins (ANK), which are virtually expressed in all host tissues and found associated  
23 with a number of different pathological symptoms, ranging from immune to  
24 developmental alterations (Falabella et al., 2007; Strand and Burke, 2013). The viral  
25 ANK proteins have sequence similarity with members of I $\kappa$ B protein family, which

26 control the NF- $\kappa$ B signaling in insects and vertebrate innate immunity (Silverman and  
27 Maniatis, 2001). Due to the lack of the regulatory sequences needed for their signal-  
28 induced and basal degradation, these ANK proteins appear to irreversibly bind to host  
29 NF- $\kappa$ B factors and block their transcriptional activity. Therefore, a function as  
30 suppressors of the host immune system has been proposed and demonstrated for some  
31 members of PDV *ank* genes (Thoetkiattikul et al., 2005; Falabella et al., 2007; Bitra et  
32 al., 2012). In contrast, we know comparatively much less on the role of *ank* genes,  
33 and more in general of PDV-encoded factors, in the induction of host developmental  
34 alterations.

35 The host-parasitoid association *Heliothis virescens-Toxoneuron nigriceps*  
36 (Lepidoptera, Noctuidae - Hymenoptera, Braconidae) provides a valuable  
37 experimental model system to study the molecular bases of developmental arrest of  
38 mature larvae, which is due to a combined action of PDV and teratocytes, disrupting  
39 the biosynthetic activity of prothoracic glands (Pennacchio et al., 1997, 1998) and the  
40 ecdysteroid metabolism (Pennacchio et al., 1994a) respectively. Since the ecdysone  
41 biosynthesis is well conserved in insects (Niwa and Niwa, 2014), to identify whether  
42 *TnBV* genes can disrupt this biosynthetic pathway, we took advantage of the  
43 *Drosophila melanogaster* model system that allow to design experiments that are not  
44 doable in *Heliothis*. The powerful molecular genetics techniques that can be applied  
45 in *Drosophila* (del Valle Rodriguez et al., 2011) allow the study of the effect that the  
46 expression of virulence genes has on specific tissues during development. Indeed, this  
47 model system has been even employed for studying human viral pathogens (Hughes  
48 et al., 2012).

49 Using this approach, in our previous work, we have gained insights on the role of a  
50 member of the viral *ank* gene family of *TnBV*, *TnBVank1* (Duchi et al., 2010;

51 Valzania et al., 2014). We found that it functions as a virulence gene disrupting  
52 ecdysteroidogenesis in prothoracic gland by interfering with the endocytic trafficking  
53 of steroidogenic cells (Valzania et al., 2014). *TnBV* genome carries two other  
54 members of the *ank* gene family (Falabella et al., 2007). In the present study we  
55 analyzed the effect of the expression of the *TnBVank3* in *Drosophila* steroidogenic  
56 cells. We found that also this gene contributes to the disruption of ecdysone  
57 biosynthesis by altering the expression of steroidogenic genes.

58

## 59 **2. Materials and methods**

60

### 61 *2.1. Fly strains*

62 Fly stocks were raised on standard cornmeal/yeast/agar medium at 18°C. *y w<sup>67c23</sup>* was  
63 used as the wild type stock in this study. We used the following Bloomington stocks:  
64 #5138 (*y<sup>1</sup> w<sup>\*</sup>*; *P[tubP-Gal4]LL7/TM3, Sb<sup>1</sup> Ser<sup>1</sup>*); #7019 (*w<sup>\*</sup>*; *P[w<sup>+mC</sup>=tubP-*  
65 *Gal80<sup>ts</sup>]*20; *TM2/TM6B, Tb<sup>1</sup>*); #8263 *y<sup>1</sup> w<sup>1118</sup>*; *P[UAS-InR.A1325D]*2. *phm-Gal4*  
66 (Ono et al., 2006) was a gift from C. Mirth (*phm-Gal4,UAS-mCD8::GFP/TM6B*).

67

### 68 *2.2. Crosses*

69 For the *tub-Gal80<sup>ts</sup>*; *phm-Gal4* experiments, *tub-Gal80<sup>ts</sup>*; *phm-Gal4/TM6B* females  
70 were crossed at 21°C to *UAS-TnBVank3* males, or to *y w<sup>67c23</sup>* males as control. Larvae  
71 were raised at 21°C and transferred at 29°C at specific time points after egg laying  
72 (AEL). For the *UAS-InR<sup>CA</sup>* expression, *tub-Gal80<sup>ts</sup>*; *phm-Gal4/TM6B* females were  
73 crossed at 21°C to *UAS-InR<sup>CA</sup>*; *UAS-TnBVank3* males, and to *UAS-InR<sup>CA</sup>* males as  
74 control. Larvae were raised at 21°C and transferred at 29°C after 3 days AEL.

75

76 2.3. *Generation of TnBVank3-HA-Myc transgenic line*

77 A construct containing the epitope tags hemagglutinin (*HA*) and *Myc* at the 3' end of  
78 of *TnBVank3* gene was produced (Biomatik) and cloned into the pUAST-attb vector  
79 (Bischof et al., 2007). The transgenic *Drosophila* line carrying the UAS-*TnBVank3*-  
80 *HA-Myc* chimeric gene was obtained by phiC31 integrase-mediated insertion into the  
81 attP2 landing-site locus on the third chromosome by BestGene Inc (USA).

82

83 2.4. *Immunofluorescence microscopy*

84 Immunostaining on ring glands was performed as described previously (Valzania et  
85 al., 2016). The *TnBVANK3-HA-Myc* protein was detected using a polyclonal rabbit  
86 anti-*HA* 1:50 (Santa Cruz Biotechnology, USA) and anti-rabbit Cy3-conjugated  
87 1:2000 (Invitrogen, USA). The glands were mounted in Fluoromount G (Electron  
88 Microscopy Sciences, USA) and analyzed with TCS SL Leica confocal system.  
89 Images were processed using Adobe Photoshop CS6.

90

91 2.5 *Protein Extracts and Western Blot Analysis*

92 *UAS-TnBVank3-HA-Myc* or control *y w<sup>67c23</sup>* males were crossed at 25 °C to *tub*-  
93 *Gal4/TM3*. Third instar larvae were collected and the total protein extraction and blot  
94 analysis were performed as already described (Romani et al., 2016). The *TnBVANK3*-  
95 *HA-Myc* protein was detected using a monoclonal mouse anti-*HA* 1:100 (Santa Cruz  
96 Biotechnology, USA) and ECL Plex anti-mouse Cy3 1:2500 (GE Healthcare, USA).

97

98 2.6. *20-E rescue experiments*

99 Two groups of ten *tub-Gal80<sup>ts</sup>; phm-Gal4/ UAS-TnBVank3* larvae, initially raised at  
100 21°C for 3 days AEL and then transferred at 29°C for other 3 days were collected and



101 placed in new tubes with yeast paste supplemented with 20-hydroxyecdysone (Sigma)  
102 1 mg/ml and kept at 29°C. As a control the same experiments were carried out on  
103 larvae of the same genotype fed with yeast paste containing an equal amount of  
104 ethanol.

105

#### 106 2.7. *Quantitative Real-Time PCR (qRT-PCR)*

107 For *E74A*, *E75A* and steroidogenic gene expression experiments, total RNA was  
108 isolated from 3 independent biological samples of 5 larvae or prepupae. Total RNA  
109 was isolated using TRIzol reagent (Thermo Scientific), and DNA was removed by  
110 RNase-Free DNase Set (Ambion). qRT-PCR was performed on an ABI PRISM 7900  
111 Real-Time PCR system (Applied Biosystems) by means of the Power SYBR-Green  
112 RNA-to-Ct-1-Step Kit (Applied Biosystems).

113 For the expression analysis of insulin and TOR pathway components, 15 brain-ring  
114 gland complexes (BRGCs) were dissected, in PBS buffer, from four independent  
115 biological samples. Total RNA was isolated using TRIzol reagent (Thermo Scientific),  
116 and contaminant DNA was removed by RNase-Free DNase Set (Ambion). cDNA  
117 synthesis was carried out with dT-primed M-MLV Reverse Transcriptase  
118 (LifeTechnologies). Quantitative PCR was carried out with FastStart SYBR Green  
119 Master Mix (Roche) on a QuantStudio 6 real-time thermal cycler.

120 The qRT-PCR primers used are listed in Table S1 in the supplementary material. For  
121 all of the genes examined, the reactions were conducted in technical triplicates. All  
122 transcript expression values were normalized to *Rpl23* gene.

123

#### 124 2.8. *Prothoracic gland size measurements*

125 For measurements of the PG area, confocal images of PGs taken at 40X magnification  
126 were quantified with Photoshop CS6.

127

## 128 2.9. Statistical analysis

129 GraphPad Prism software was used for statistical analysis. Statistical significance was  
130 determined on the basis of unpaired *t*-test performed on the means and *p* values were  
131 calculated (\*=*p*<0.05; \*\*=*p*<0.01 and \*\*\*=*p*<0.001). *p*<0.05 was considered  
132 statistically significant. All results are expressed as the mean ± standard deviation  
133 (SD).

134

## 135 3. Results and Discussion

### 136 3.1. Expression of *TnBVank3* in the prothoracic gland induces developmental arrest 137 at third instar

138 *TnBVANK3* is 168 aa long and contains 3 ankyrin repeats (Fig. 1A). To test the effect  
139 of the viral *TnBVANK3* protein on *Drosophila* development we used the GAL4/UAS  
140 binary expression system (Brand and Perrimon, 1993). We produced *Drosophila*  
141 transgenic lines carrying a UAS transgene encoding *TnBVANK3* protein tagged at the  
142 C terminus with the hemagglutinin (HA) and c-Myc epitopes. The expression of the  
143 *TnBVank3-HA-Myc* gene (hereafter abbreviated as *TnBVank3*) was assessed using the  
144 ubiquitous *tubulin-Gal4* driver (*tub-Gal4*, hereafter abbreviated as *tub*>). The  
145 *TnBVANK3* protein was detected in third instar *tub*>*TnBVank3* larvae by western  
146 blot on whole cell lysate, using anti-HA antibody. A band in the size range of 23 kDa  
147 was detected, as expected for the *TnBVANK3-HA-Myc* protein (Fig. 1B). No signal  
148 was observed in protein extracts from the control larvae *tub*>+. The ubiquitous  
149 expression of *TnBVank3* driven by the *tub*> driver did not affect larval development.

150 However, no *tub>TnBVank3* adult flies were obtained, since after pupariation the  
151 pupae degenerate (data not shown). This phenotype suggested that *TnBVank3*  
152 expression could affect metamorphosis, without any impact on larval molts, as  
153 observed in host larvae parasitized by *T. nigriceps* (Pennacchio et al., 1994b). Pulses  
154 of the hormone ecdysone (E) dictate the precise timing of the developmental  
155 transitions in *Drosophila*, such as larval molts, pupariation and metamorphosis  
156 (Warren et al., 2006). Ecdysone is synthesized in the steroidogenic cells of the  
157 prothoracic gland (PG) and secreted into the hemolymph, to reach peripheral tissues  
158 where it is converted to its active form, 20-hydroxyecdysone (20E). To test whether  
159 *TnBVANK3* affects *Drosophila* development impairing the prothoracic gland  
160 function, we specifically targeted the expression of *TnBVank3* in this gland using the  
161 *phantom-Gal4* driver (*phm-Gal4*, hereafter abbreviated as *phm>*), which allows high  
162 expression level of UAS transgene in the PG. We expressed *TnBVank3* in PG cells of  
163 larvae, at specific time points after egg laying (AEL) using a temperature sensitive  
164 form of the Gal4 repressor Gal80, Gal80<sup>ts</sup> (McGuire et al., 2003), which allows  
165 modulation of Gal4 activity. We used *tub-Gal80<sup>ts</sup>; phm-Gal4 (Gal80<sup>ts</sup>; phm>)* to  
166 control the timing of *TnBVank3* expression in the PG cells. *Gal80<sup>ts</sup>; phm>TnBVank3*  
167 and control *Gal80<sup>ts</sup>; phm>+* larvae were initially raised at 21°C, and at the early L2  
168 stage (3 days AEL) were shifted to the restrictive temperature (29°C) to promote Gal4  
169 activity. The temperature shift did not affect the normal development of control  
170 individuals. Conversely, at 29°C the larvae expressing *TnBVank3* exhibited a fully  
171 penetrant phenotype showing a block of larval-pupal transition, as observed in *H.*  
172 *virescens* larvae parasitized by *T. nigriceps* (Pennacchio et al., 1994b). After 2 days  
173 the *Gal80<sup>ts</sup>; phm>TnBVank3* larvae are similar in size to control larvae (Fig. 2A).  
174 However, at the third day, while control larvae pupate, *Gal80<sup>ts</sup>; phm>TnBVank3* do

175 not. During their prolonged L3 larval life that extended up to 3-4 weeks, the *Gal80<sup>ts</sup>*;  
176 *phm>TnBVank3* larvae continue to increase in size (Fig. 2A). When the *Gal80<sup>ts</sup>*;  
177 *phm>TnBVank3* larvae are shifted from 21°C to 29°C at 4 days AEL, some of them  
178 pupariated, while all the larvae shifted at 29°C, at 5 days AEL, regularly pupariated.  
179 Thus, when *TnBVank3* expression is triggered in the PG cells of L2 larvae it causes  
180 the block of pupariation.

181

### 182 3.2. *TnBVank3* affects ecdysone activity

183 At the end of larval development a high peak of ecdysone triggers pupariation. In our  
184 experimental conditions (larvae initially raised at 21°C for 3 days AEL and then  
185 shifted to 29°C) in wild type larvae pupariation occurs after 3 days at 29°C with the  
186 formation of white prepupae. To investigate whether the block of the transition to  
187 pupal stage caused by the expression of *TnBVank3* was due to a low level of 20E, we  
188 carried out ecdysone-feeding rescue experiments. Third instar *Gal80<sup>ts</sup>*;  
189 *phm>TnBVank3* larvae after 3 days at 29°C were transferred to new vials containing  
190 yeast paste supplemented with 20E dissolved in ethanol or just ethanol. After 24 h at  
191 29°C all the larvae fed with 20E had developed into pupae (100%, *n*=20) (Fig. 2B).  
192 Conversely, the *Gal80<sup>ts</sup>*; *phm>TnBVank3* larvae fed with yeast and ethanol, as a  
193 control, did not form any puparia and all of them persisted as third instar (*n*=20). This  
194 result indicates that the expression of *TnBVank3* in the PG impairs the biosynthesis of  
195 ecdysone. We therefore investigated the ecdysone activity by measuring the  
196 expression levels of two 20E-inducible transcription factors, *E74A* and *E75A*, which  
197 are required to undertake metamorphosis (Karim and Thummel, 1992) and can be  
198 used as readout for ecdysone levels. We induced *TnBVank3* expression in PG of  
199 larvae and, after 2 and 3 days at 29°C, we analyzed by qRT-PCR the expression levels

200 of *E74A* and *E75A* genes in *Gal80<sup>ts</sup>*; *phm>TnBVank3* larvae and in *Gal80<sup>ts</sup>*; *phm>+*  
201 control larvae/white prepupae of the same age (Fig. 2C). The expression of both  
202 *E74A* and *E75A* was significantly reduced in *TnBVank3* larvae (after 2 days at 29°C),  
203 as well as in larvae after 3 days at 29°C compared to the control white prepupae.  
204 Collectively these data indicate that in the *TnBVank3* larvae the ecdysone biosynthesis  
205 is impaired causing the block of larval development.

206

### 207 3.3. *TnBVANK3* affects the expression of steroidogenic genes

208 Ecdysone is synthesized from cholesterol in the steroidogenic cells of the PG (Fig.  
209 3A). Cholesterol, which cannot be synthesized by insects (Gilbert and Warren, 2005),  
210 enters the steroidogenic cells through a receptor-mediated low-density lipoprotein  
211 endocytic pathway (Rodenburg and Van der Horst, 2005), which delivers cholesterol  
212 to the endosomes. A number of ecdysone biosynthetic genes have been identified and  
213 characterized in *Drosophila* (Fig. 3A) (Gilbert and Warren, 2005; Niwa and Niwa,  
214 2014). The first enzymatic reaction of the pathway, the conversion of cholesterol to 7-  
215 dehydrocholesterol (7dC) is catalyzed by Neverland (Nvd) (Yoshiyama et al., 2006;  
216 Yoshiyama-Yanagawa et al., 2011). 7dC is then converted to 5β-ketodiol (KD)  
217 through the ‘Black Box’, a biosynthetic step not yet characterized, in which Shroud  
218 (Sro), Spook (Spo) and Spookier (Spok) are involved (Namiki et al., 2005; Ono et al.,  
219 2006, 2012; Niwa et al., 2010). Phantom (Phm) transforms KD in ketotriol (KT),  
220 Disembodied (Dib) converts KT in 2-deoxyecdysone (2dE) and Shadow (Sad)  
221 converts 2dE to ecdysone (E) (Chavez et al., 2000; Warren et al., 2002, 2004; Petryk  
222 et al., 2003; Niwa et al., 2004). After release from the PG into the hemolymph, E is  
223 converted in peripheral tissues to its active form 20-hydroxyecdysone (20E) by Shade  
224 (Shd) enzyme (Petryk et al., 2003).

225 We investigated whether expression of steroidogenic genes was affected by  
226 *TnBVank3* expression in the PG cells. We compared by qRT-PCR the expression  
227 levels of *nvd*, *spok*, *sro*, *phm*, *dib* and *sad* genes in *Gal80<sup>ts</sup>; phm>TnBVank3* larvae  
228 kept of 2 and 3 days at 29°C with that in *Gal80<sup>ts</sup>; phm>+* control larvae kept of 2  
229 days at 29°C and white prepupae of 3 days at 29°C (Fig. 3B). Expression of  
230 *TnBVank3* in the PG resulted in a down-regulation of steroidogenic genes, with a  
231 more pronounced effect on *nvd*, *spok* and *sro*, which catalyze early steps in the  
232 ecdysone biosynthetic pathway.

233 These data further support our finding that *TnBVANK3* impairs the ecdysone  
234 biosynthesis.

235

#### 236 3.4. *TnBVANK3* affects PG size

237 To investigate the *TnBVANK3* distribution in PG cells expressing the *TnBVank3*  
238 gene, we performed immunostaining experiments using an anti-HA antibody on  
239 *Gal80<sup>ts</sup>; phm>TnBVank3* PGs of third instar larvae (after 2 days at 29°C).

240 Interestingly, *TnBVANK3* was localized only in the nucleus of PG cells (Fig. 4A,B).  
241 Since, the *phm-Gal4* stock that we used carries the *UAS-mCD8::GFP* construct, the  
242 detection of the mCD8::GFP cell membrane marker allowed us to visualize the PGs  
243 (Fig. 4B). *TnBVank3* expression did not alter the gross morphology of the PG  
244 (compare Videos S1 and S2), although the PG size was smaller than in control PGs  
245 (-17.7% Fig. 4C), as observed also in parasitized tobacco budworm larvae  
246 (Pennacchio et al., 1997).

247

#### 248 3.5. *TnBVANK3* reduces the expression of the insulin/TOR signaling components

249 In *Drosophila*, as in the other holometabolous insects, metamorphosis can start after  
250 the larvae have reached the appropriate size, known as critical weight (CW). In  
251 *Drosophila* CW is attained in the early half of the L3 instar larvae. The achievement  
252 of CW is associated with the activation of steroidogenesis, which is controlled by a  
253 complex regulatory network of cross-modulating molecular events (Niwa and Niwa,  
254 2016).

255 The prothoracicotropic hormone (PTTH) produced by the brain stimulates the  
256 synthesis of ecdysone by targeting its receptor Torso in the PG cells, which influences  
257 both the activation of ecdysone biosynthesis and CW control (McBrayer et al., 2007;  
258 Rewitz et al., 2009). The insulin/TOR (target of rapamycin) signaling, which controls  
259 growth rate and body size, do also promote growth of PG cells and their biosynthetic  
260 activity (timing and amount) (Yamanaka et al., 2013), is part of the complex  
261 molecular network assessing the CW and controlling the downstream developmental  
262 events (Koyama et al., 2014). It has been shown that increasing insulin signaling in  
263 the PG causes an increase of gland size and ecdysone biosynthesis, which results into  
264 a precocious metamorphosis, leading to pupae and adults of reduced size (Caldwell et  
265 al., 2005; Colombani et al., 2005; Mirth et al., 2005). Conversely, a down-regulation  
266 of the insulin signaling has a negative impact on gland size and ecdysone  
267 biosynthesis, which determines a delayed pupariation, giving rise to larger pupae and  
268 adults. A more severe phenotype is produced in response to the knock down of TOR,  
269 controlling the progression of PG endocycle required for activation of ecdysone  
270 biosynthesis, which determines a reduction of PG size and a down-regulation of  
271 ecdysteroidogenic genes associated with a developmental arrest of third instar larvae  
272 (Ohhara et al., 2017)

273 The negative effect of *TnBVank3* on the ecdysone biosynthesis and the PG size,  
274 coupled with a severe developmental arrest phenotype, produced when this gene is  
275 expressed from the L2 stage, before the CW is reached, suggested that *TnBVANK3*  
276 impairs the insulin/TOR signaling. To test this hypothesis, we investigated the mRNA  
277 levels of the insulin/TOR signaling components in the BRGCs of *Gal80<sup>ts</sup>*;  
278 *phm>TnBVank3* and *Gal80<sup>ts</sup>* ; *phm>+* control larvae kept for 2 days at 29°C. For the  
279 insulin pathway we found a significant reduced expression of *InR*, *Pi3K* and *Akt* genes  
280 (Fig. 5A). The analysis of the TOR pathway also revealed a significant decrease of the  
281 mRNA levels of *Tor* and the key downstream effector *S6 kinase (S6k)* (Fig. 5A).  
282 We next investigated whether the activation of InR pathway could rescue the  
283 pupariation defect induced by *TnBVANK3*. We found that the expression in the PG  
284 cells of a constitutively active form of the insulin receptor (*InR<sup>CA</sup>*) was able to restore  
285 pupariation (Fig. 5B). Accordingly to the constitutive activation of insulin pathway,  
286 the control larva expressing only *InR<sup>CA</sup>* advanced the onset of metamorphosis giving  
287 rise to small pupae (Caldwell et al., 2005; Colombani et al., 2005). This phenotype  
288 was also produced in the *InR<sup>CA</sup>; TnBVank3* larvae.

289 Collectively, our data suggest that in the *TnBVank3* larvae the reduction of  
290 insulin/TOR signaling contributes to the negative effect on ecdysone biosynthesis.  
291 Although our findings indicate that a reduction of the expression of steroidogenic  
292 genes underlies the *TnBVANK3* developmental arrest phenotype, we cannot assert  
293 that this down-regulation is only due to the reduction of insulin/TOR signaling or  
294 whether *TnBVANK3* might also act directly on disrupting biosynthetic enzyme gene  
295 expression. However, if and how *TnBVANK3* may have an impact on other  
296 transduction pathways controlling steroidogenesis remains to be studied.

#### 297 **4. Conclusions**



298 Our study on the viral *ank* gene *TnBVank3* clearly points out its role in blocking  
299 ecdysone biosynthesis. A similar effect was produced by the expression of another  
300 member of the same gene family, *TnBVank1* (Valzania et al., 2014). Interestingly,  
301 these two genes target different parts of the ecdysone biosynthetic pathway, while  
302 *TnBVANK3* localizes into the nucleus and causes a reduced expression of  
303 steroidogenic genes, *TnBVANK1* acts in the cytoplasm, by blocking the cholesterol  
304 trafficking. The high similarity of natural host phenotypes induced by the PDV  
305 infection with those we produced in the *Drosophila* model system, by expressing  
306 specific PDV genes, paves the way for further experiments on the natural host, aiming  
307 to shed light if the complementary and synergistic effects of these two virulence  
308 factors are adopted by parasitic wasps to ensure a complete block of host ecdysone  
309 biosynthesis and its larval development.

310

### 311 **Figure captions**

312 **Fig. 1.** Inducible expression of *TnBVANK3*-HA-Myc chimeric protein.

313 (A) Scheme showing the amino acid sequence of *TnBVANK3* and the HA and Myc  
314 epitopes fused at its carboxy terminus. The underlined Ankyrin repeat domains were  
315 predicted by searching the sequences using the SMART database (Simple Modular  
316 Architecture Research Tool; <http://smart.embl-heidelberg.de/>), using the default  
317 parameters (Schultz et al., 1998). (B) Western blot of third instar larvae cell lysate  
318 using anti-HA antibody. Larvae expressing the *UAS-TnBVank3-HA-Myc* transgene by  
319 the ubiquitous driver *tub-Gal4* show a band in the size range of 23 kDa that  
320 corresponds to the predicted *TnBVANK3*-HA-Myc protein. This band is absent in  
321 control larvae *tub*>+ carrying only the Gal4 driver.

322

323 **Fig. 2.** *TnBVank3* expression in the PG cells affects the ecdysone biosynthesis  
324 causing the block of the transition from larval to pupal stage.  
325 (A) Light micrographs of *Gal80<sup>ts</sup>; phm>TnBVank3* and *Gal80<sup>ts</sup>; phm>+* larvae of  
326 different ages at 29°C. (B) Rescue experiments of *Gal80<sup>ts</sup>; phm>TnBVank3* with 20-  
327 hydroxyecdysone (20E). After 3 days at 29°C *Gal80<sup>ts</sup>; phm>TnBVank3* larvae fed  
328 with medium supplemented with 20E induces the pupariation (red), while larvae fed  
329 with medium containing ethanol (EtOH) do not pupate (green). (C) qRT-PCR  
330 analyses of the mRNA levels of the 20E-inducible transcriptional factors (*E74A*,  
331 *E75A*) of *Gal80<sup>ts</sup>; phm>+* and *Gal80<sup>ts</sup>; phm>TnBVank3* of individuals kept at 29°C  
332 for the indicated days. Graphs represent mean  $\pm$  SD;  $n=3$ ;  $**=p<0.01$ ;  $***=p<0.001$ .  
333

334 **Fig. 3.** *TnBVANK3* reduces the expression of genes of the ecdysone biosynthetic  
335 pathway. (A) Scheme showing the steps in the conversion of cholesterol to ecdysone  
336 (E). (B) qRT-PCR analysis of the transcript levels of the ecdysone biosynthetic  
337 enzymes of individuals of the reported genotypes kept at 29°C for the indicated days.  
338 Graphs represent mean  $\pm$  SD;  $n=3$ ;  $*=p<0.05$ ;  $**=p<0.01$ ;  $***=p<0.001$ .  
339

340 **Fig. 4.** *TnBVANK3* localization in the PG cells and its effects on PG size.  
341 (A,B) Immunolocalization of *TnBVANK3*-HA-Myc with anti-HA antibody (red) in  
342 PG cells of third instar *Gal80<sup>ts</sup>; phm>TnBVank3* larvae (marked with mCD8::GFP,  
343 green, B). *TnBVANK3* shows a nuclear localization. (C) The *Gal80<sup>ts</sup>;*  
344 *phm>TnBVank3* PGs are significantly smaller (-17.7%) than PGs from control  
345 *Gal80<sup>ts</sup>; phm>+* larvae. The graph represents the mean  $\pm$  SD; 16 *Gal80<sup>ts</sup>;*  
346 *phm>TnBVank3* PGs and 10 *Gal80<sup>ts</sup>; phm>+* PGs analyzed;  $**=p<0.01$ .

347 **Fig. 5.** *TnBVANK3* affects the expression of the insulin/TOR signaling components.  
348 (A) qRT-PCR analysis of the transcript levels of *InR*, *Pi3K*, *Akt*, *Tor* and *S6k* in the  
349 BRGC of the control and *TnBVank3* larvae that were raised at 21°C for 3 days AEL  
350 and then kept at 29°C for 2 days. Graphs represent mean  $\pm$  SD;  $n=4$ ;  $*=p<0.05$ ;  
351  $**=p<0.01$ ;  $***=p<0.001$ . (B) Coexpression in the PGs of *TnBVank3* and the  
352 constitutively active form of insulin receptor (*InR<sup>CA</sup>*). Activation of insulin signaling  
353 restores pupariation in *TnBVank3* larvae. The described results were obtained by  
354 analyzing larvae raised at 21°C for 3 days AEL and then were shifted to the 29°C  
355 restrictive temperature.

356

### 357 **Video captions**

358

#### 359 **Video S1**

360 Morphology of a PG gland expressing *TnBVank3* and the cell membrane marker

361 mCD8::GFP.

362 QuickTime movie of 50 confocal optical z stack sections each with a scan step size of

363 0.33  $\mu$ m through the entire PG. The detection of the mCD8::GFP protein allow to

364 visualize the PG morphology.

365

#### 366 **Video S2**

367 Morphology of a PG gland expressing the cell membrane marker mCD8::GFP.

368 QuickTime movie of 41 confocal optical z stack sections each with a scan step size of

369 0.33  $\mu$ m through the entire PG. The detection of the mCD8::GFP protein allow to

370 visualize the PG morphology.

371

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379

380 **Authors' contribution**

381 VC, GG and FP conceived the research. MI, PR, LV and GS performed *Drosophila*  
382 experimental work and morphological analyses. RF and PR performed qRT-PCR  
383 analyses. MI conceived and designed part of the experiments. VC, FP and GG wrote  
384 the manuscript.

385

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Table S1. The primers used for qRT-PCR experiments

Gene	Forward	Reverse
<i>nvd</i>	5'-ACCTCCCCCTTATCCAAATG-3'	5'-AGCAACGCTTCCACCAATAC-3'
<i>sro</i>	5'-ATGAGCGGCAGTCAACTTCT-3'	5'-CAGGAAATCACGGTCATGTG-3'
<i>spok</i>	5'-TATCTCTTGGGCACACTCGCTG-3'	5'-GCCGAGCTAAATTTCTCCGCTT-3'
<i>phm</i>	5'-TCGTCGTGGGCGATTATTTTA-3'	5'-AAGGCCACTGGGTCCATGT-3'
<i>dib</i>	5'-TGCCCTCAATCCCTATCTGGTC-3'	5'-ACAGGGTCTTCACACCCATCTC-3'
<i>sad</i>	5'-AAGGAGCGAGCTACCAATGA-3'	5'-GCTGCTCAAAGTGTGATGGA-3'
<i>E74A</i>	5'-GCCCTTTATCGACGATGCAC-3'	5'-GCTCCATTCAGTTCGTTGCC-3'
<i>E75A</i>	5'-ACGATATCAGCAGGCCAATC-3'	5'-GAATGCACGCCGTAATGGAAAC-3'
<i>Rpl23</i>	5'-GCTCAGGAAGAAGGTCATGC-3'	5'-GGCTATAGAGCTTGCATTGGA-3'
<i>Akt</i>	5'-GCCAGATCATGACCGTCGAT-3'	5'-GTCATAGCCACCTCACCCAC-3'
<i>InR</i>	5'-TTCTCTGGGAAATGGCCACC-3'	5'-TCGCCGAAGACCTATGATGC-3'
<i>Pi3K</i>	5'-GCCAGAACTGTCCTCCGAAA-3'	5'-CTTCGCTGAATTTGCTCGG-3'
<i>Tor</i>	5'-GCTATGACGAGGCGAATGGA-3'	5'-TCTTGGGGAACAGCGTCTTC-3'
<i>S6k</i>	5'-GCCAGGAGACCATACAGCTC-3'	5'-TGCCATAACCACCTTTGCCA-3'



Figure 1

**A**

MMASNYSLNDFFKMYSRYGHNYFHHVCCWGNYKLLCVARPFIDLSNSHLLLEDV  
DYQGMSCIHLAVICNPKNAREILQVLISWGVNINRQDEVTGESILHLAIKFHHLKL  
TKWIIKYSGINLNIRNYNDESPYELAYKLNYYVMWLLRKNGAICEYRLTSSEES  
SSED **HA** **Myc**

**B**

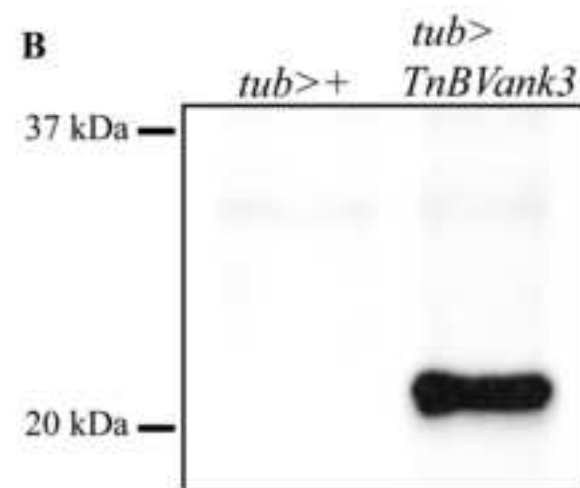


Figure 2

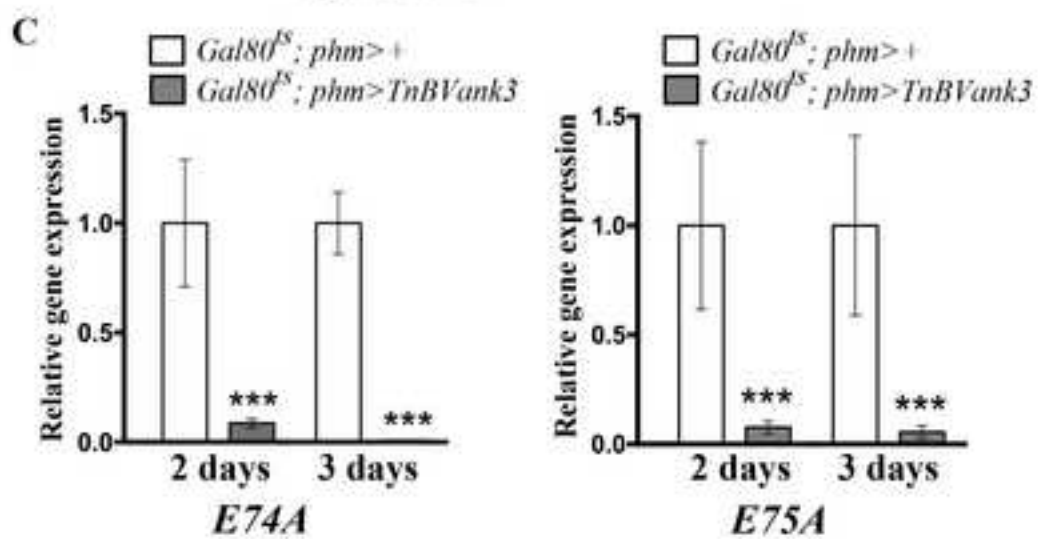
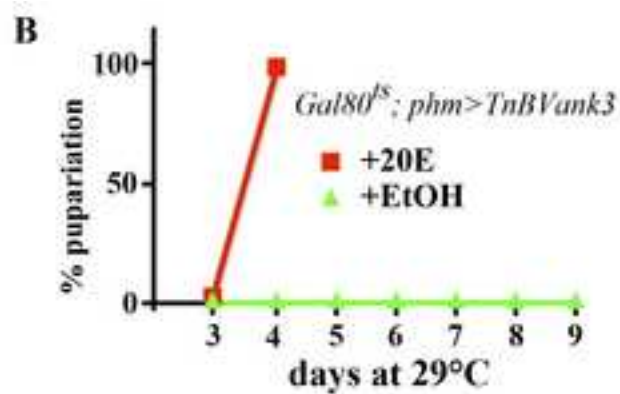


Figure 3

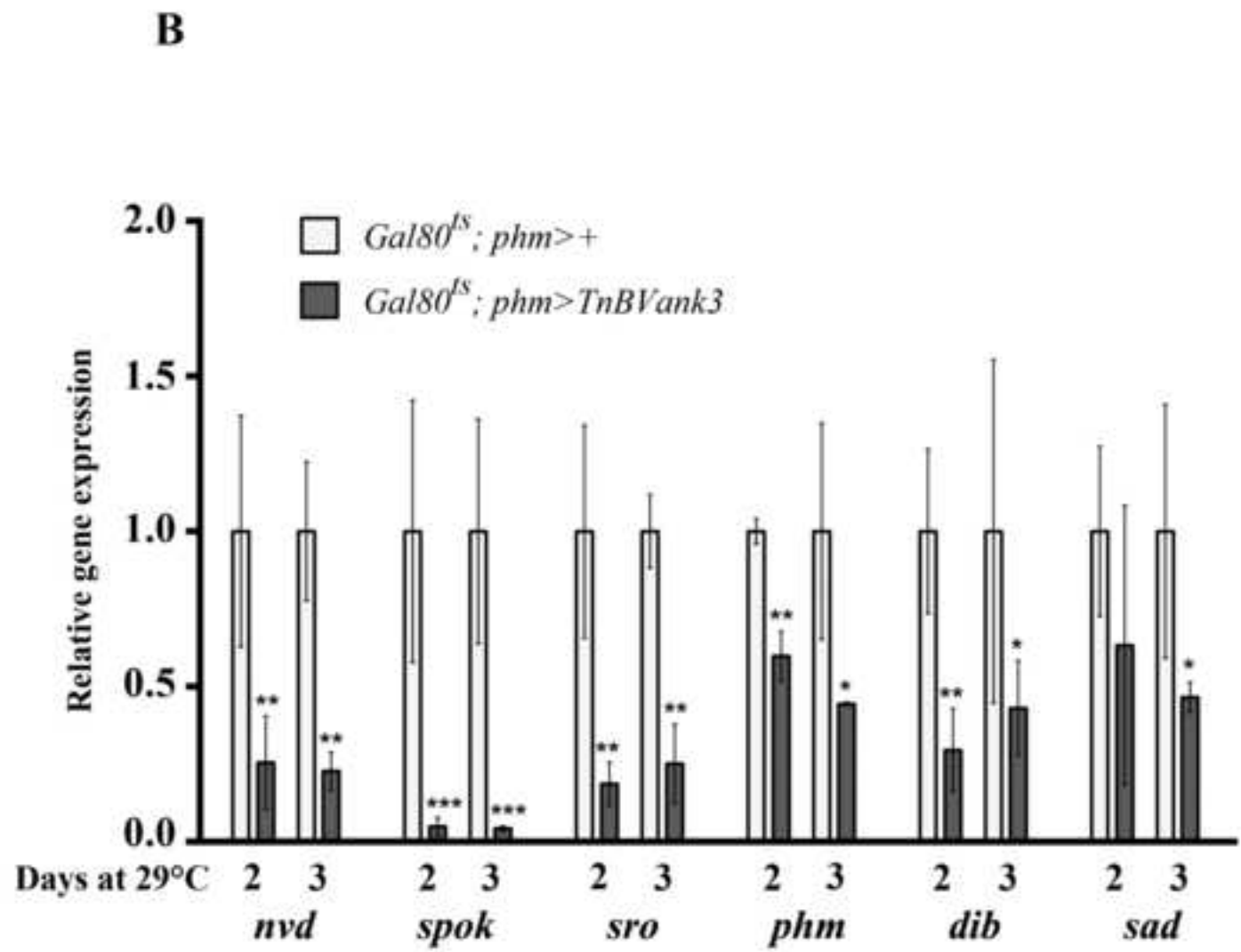
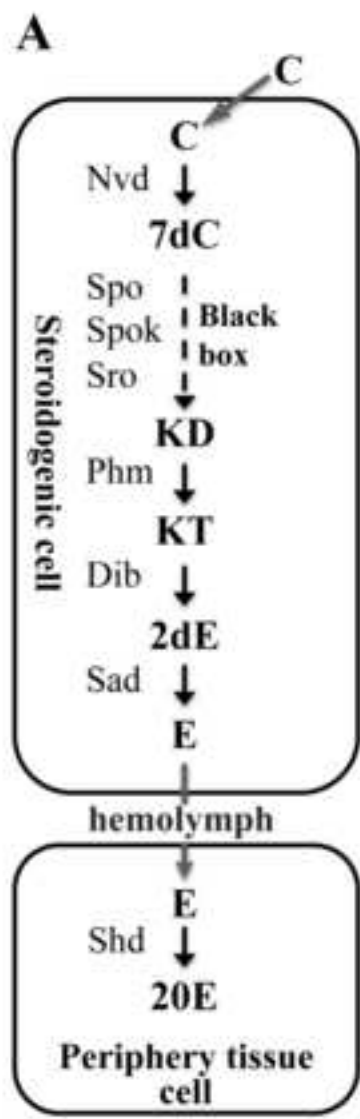


Figure 4

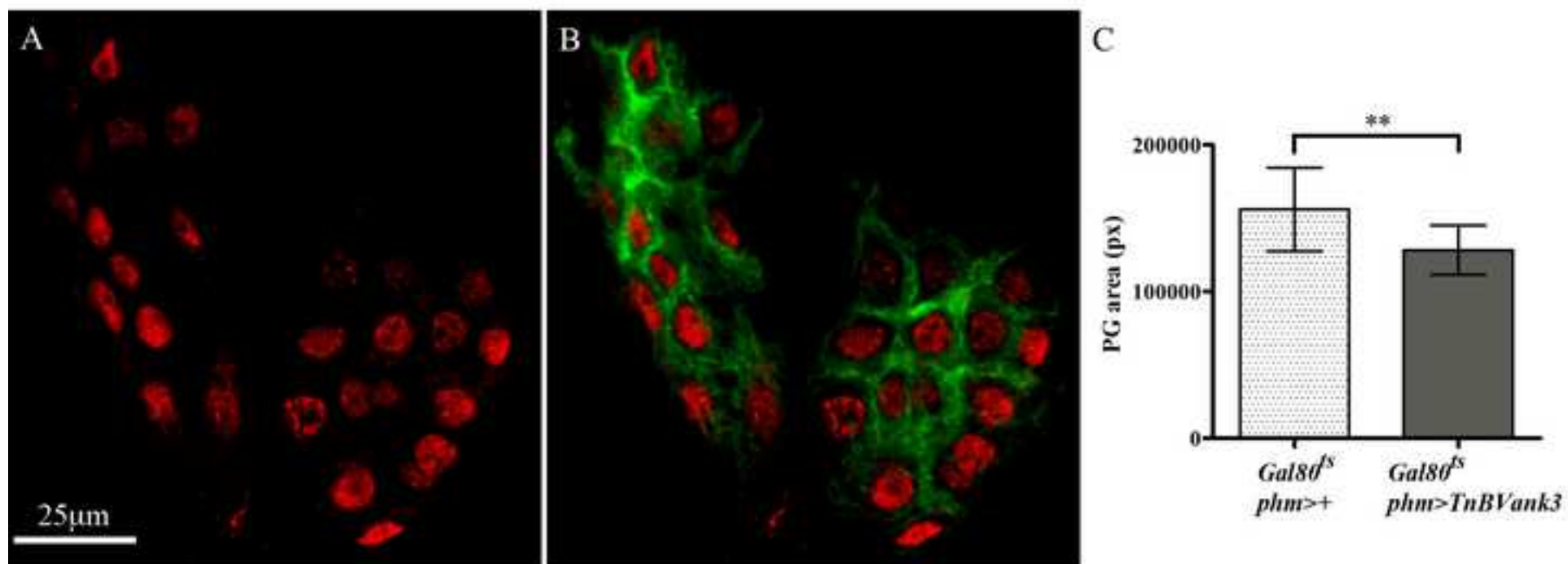


Figure 5

