A Polydnavirus ANK Protein Acts as Virulence Factor by Disrupting the Function of Prothoracic Gland Steroidogenic Cells

Luca Valzania1*, Patrizia Romani1*, Ling Tian2, Sheng Li2, Valeria Cavaliere1, Francesco Pennacchio3, Giuseppe Gargiulo1*

1 Dipartimento di Farmacia e Biotecnologie, Università di Bologna, Bologna, Italy, 2 Key Laboratory of Insect Developmental and Evolutionary Biology, Institute of Plant Physiology and Ecology, Shanghai, China, 3 Dipartimento di Agraria – Laboratorio di Entomologia “E. Tremblay”, Università di Napoli Federico II, Portici (NA), Italy

Abstract

Polydnaviruses are obligate symbionts integrated as proviruses in the genome of some ichneumonoid wasps that parasitize lepidopteran larvae. Polydnavirus free viral particles, which are injected into the host at oviposition, express virulence factors that impair immunity and development. To date, most studies have focused on the molecular mechanisms underpinning immunosuppression, whereas how viral genes disrupt the endocrine balance remains largely uninvestigated. Using Drosophila as a model system, the present report analyzes the function of a member of the ankyrin gene family of the bracovirus associated with Toxoneuron nigriceps, a larval parasitoid of the noctuid moth Heliothis virescens. We found that the TnBVank1 expression in the Prothorophila prothoracic gland blocks the larval-pupal molt. This phenotype can be rescued by feeding the larvae with 20-hydroxyecdysone. The localization of the TnBVANK1 is restricted to the cytoplasm where it interacts with Hrs and Alix marked endosomes. Collectively, our data demonstrate that the TnBVANK1 protein acts as a virulence factor that causes the disruption of ecdysone biosynthesis and developmental arrest by impairing the vesicular traffic of ecdysteroid precursors in the prothoracic gland steroidogenic cells.

Introduction

Parasitic wasps represent the largest group of parasitoid insects which attack and parasitize a number of insect species, exploiting different developmental stages [1]. These parasitic insects have a peculiar injection device, the ovipositor, which is used to deliver the egg along with host regulation factors that primarily disrupt the host immune reaction and endocrine balance to create a suitable environment for the development of their progeny [2,3]. These host regulation factors include viruses of the Polydnaviridae family, obligate symbionts of ichneumonid and braconid wasps suitable for the development of their progeny [2,3]. These host regulation factors include viruses of the Polydnaviridae family, obligate symbionts of ichneumonid and braconid wasps attacking larval stages of lepidopteran hosts, and respectively classified in the genera Ichneumovirus (IV) and Bracovirus (BV) [4].

Polydnaviruses (PDVs) [5,6] are integrated as proviruses in the genome of parasitoid wasps and their transmission to offspring is strictly vertical, through the germine. The genome encapsidated in the viral particles is made of multiple circular dsDNA segments, which have an aggregate size ranging between 190 and 600 kb. PDVs only replicate in the epithelial cells of the calyx, a specific region of the ovary, where they accumulate to a high density to be injected at oviposition along with the venom and the egg. Free PDV particles infect the host tissues without undergoing replication, and express virulence factors that alter host physiology in ways essential for offspring survival [5].

Evolutionary convergence of independent host-virus associations has favored the selection of gene families shared by both IV and BV [7–9]. For example, protein tyrosine phosphatases (PTP) and ankyrin motif proteins (ANK) are widely distributed in many PDVs and expressed to different degrees in virtually all host tissues analyzed so far, indicating that they play a key role in successful parasitism [10].

We know more about the functional bases underlying the immune disguise in parasitized hosts than we do about how the host developmental alteration is induced [3,11]. This is due to the complexity of the developmental mechanisms and to the concurrent action of various virulence factors, which often have redundant and overlapping effects on the regulating gene networks [2,3,12].

One of the best characterized developmental syndromes has been described in the host-parasitoid association Heliothis virescens-Toxoneuron nigriceps (Lepidoptera, Noctuidae - Hymenoptera, Braconidae) [13]. Briefly, in this experimental model the last instar larvae fail to pupate and show a higher nutritional suitability for parasitoid larvae. The developmental arrest is partly due to a
marked depression of ecdysone (E) biosynthesis by the prothoracic gland (PG), induced by the infection of the baculovirus associated with *T. niigispep* (TvBV). The inhibition of E biosynthesis is further reinforced by the conversion of the very low amounts of 20-hydroxyecdysone (20E) produced to inactive polar metabolites, a transformation mediated by teratocytes, special cells deriving from the parasitoid’s embryonic membrane.

The active transcription of *TvBV* genes in the PG of parasitized tobacco budworm larvae is required to disrupt their ecdysteroid biosynthesis, which remains very low and fails to increase in response to prothoracotropic hormone (PTTH) stimulation [13,14]. Unraveling the functional role of a specific virulence factor at molecular level is not easy when the natural host is used for these studies, due to the limited availability of genomic information and molecular tools. Therefore we used *Drosophila melanogaster* as an ideal experimental model to study *TvBV* genes. With this approach, we started the functional characterization of a member of the viral *ankyrin* (ank) gene family of *TvBV*, *TvBVank1* [15], showing that the expression of this gene in *Drosophila* germ cells alters the microtubule network function in the oocyte [16].

In the present study we analyze the effect of *TvBVank1* gene expression during *Drosophila* development. Interestingly, we found that *TvBVank1* expression in the PG cells blocks the transition from larval to pupal stage, mimicking the developmental arrest observed in *H. virescens* larvae parasitized by *T. niigispep*.

**Materials and Methods**

**Fly strains**

Stocks were raised on standard cornmeal/yeast/agar medium at 21°C and crosses were made at 25°C unless otherwise stated. *yw* was used as the wild-type stock in this study. The *UASp-TvBVank1* strain (genotype: *UASp-TvBVank1/UASp-TvBVank1; UASp-TvBVank1/UASp-TvBVank1; +/+ ) was generated in our laboratory [16].

The following stocks were obtained from the Bloomington Stock Center: hairy-Gal4 (#1734: w*, P[GawB]111), *UASp-z-tubulin-GFP* (#7374: v1 w*; P[Usp-GFP56S;z-Tub42B]14-6-II), UAS-p35 (#5073: w*; P[UAS-p35;H1B12]) and tub-Gal80b (#7019: w*; P[w1; tubP-GAL80b(20); TM2; TM6B, Tb]), phanto-Gal4 and P2906-Gal4 were a gift from C. Mirth (phantom-Gal4, UAS-mCD8::GFP/TM6B and P2906-Gal4, UAS-mCD8::GFP); august21-Gal4 (light green) was kindly provided by M. Jindra (august21-Gal4; CyO;phantom-Gal4/phantom-Gal4).

The stocks used for Gal4 driven expression of *UASp-TvBVank1* referred in Figure S2 and listed in Table S1 are from Bloomington Stock Center.

**Crosses**

Females *UASp-TvBVank1* were crossed to males of the different Gal4 lines. As control, females *yw* were crossed to males of the same Gal4 lines. For microtubules analysis, females *UASp-TvBVank1* were crossed to males *UASp-z-tubulin-GFP*; *phantom-Gal4*.

For Gal80b expression, females *UASp-TvBVank1* were crossed to males tub-Gal80b; *phantom-Gal4, UAS-mCD8::GFP/TM6B* and females *yw* were crossed to males tub-Gal80b; *phantom-Gal4, UAS-mCD8::GFP/TM6B* as control.

To coexpress p35 and *TvBVank1* in PG cells females *UASp-TvBVank1*; *UASp-p35* were crossed to males *phantom-Gal4, UAS-mCD8::GFP/TM6B*.

**Larval length measurements**

Five *UASp-TvBVank1/+; UASp-TvBVank1/+* larvae at different days after egg deposition (AED) and five control larvae were ice-anesthetized and photographed using a Nikon Eclipse 90i microscope. Images were taken at 4X magnification and the larval length was measured with NIS-Elements Advanced Research 3.10 software.

**20E titer**

Five larvae at different developmental stages were collected and washed with PBS and immediately frozen by liquid nitrogen. Samples were added 200 µl of methanol, homogenized and transferred into 1.5 ml plastic tubes. After 10 minutes centrifugation (12,000 rpm at 4°C) the supernatant was collected into a new tube, the precipitate was re-extracted with 200 µl of methanol and the supernatant was added to the previous one. After 30 minutes on ice, the samples were centrifuged following the same conditions. Samples were dried to remove methanol and then dissolved in the borate buffer. The standard curve was generated according to the standard process of the RIA protocol [17] and then the 20E titer in samples was calculated.

**Rescue experiment**

Five larvae at different developmental stages were collected and washed with PBS and immediately frozen by liquid nitrogen. Samples were added 200 µl of methanol, homogenized and transferred into 1.5 ml plastic tubes. After 10 minutes centrifugation (12,000 rpm at 4°C) the supernatant was collected into a new tube, the precipitate was re-extracted with 200 µl of methanol and the supernatant was added to the previous one. After 30 minutes on ice, the samples were centrifuged following the same conditions. Samples were dried to remove methanol and then dissolved in the borate buffer. The standard curve was generated according to the standard process of the RIA protocol [17] and then the 20E titer in samples was calculated.

**Immunofluorescence microscopy**

Larvae were dissected at room temperature in 1xPBS pH 7.5 (PBS) and fixed in 4% formaldehyde for 20 minutes at room temperature. After three washes in PBS, larvae were permeabilized in PB-T (PBS pH 7.5+0.3% Triton X-100) for 1 h, washed three times 5 minutes each in PB-T and 10 minutes in PB-T+2%BSA solution. After that, the larvae were incubated, overnight at 4°C, with primary antibodies diluted in PB-T+2%BSA. Larvae were washed three times 10 minutes each in PB-T, 10 minutes in PB-T+1%BSA solution and incubated 2 hours at room temperature on a rotating wheel with secondary antibodies diluted in PB-T+1%BSA. After several washes in PBS, the ring glands were dissected and mounted on microscopy slides in Fluoromount G (Electron Microscopy Sciences). Subsequently samples were analyzed by conventional epifluorescence with a Nikon Eclipse 90i microscope or with TC-11 SL Leica confocal system. Images were processed using Adobe Photoshop CS4 and Adobe Illustrator CS4.

TRITC-Phalloidin staining was carried out, after incubation with secondary antibodies, by washing larvae three times with PBS and then by incubating larvae for 20 minutes with TRITC-Phalloidin (40 µg/ml in PBS, Sigma).

For Propidium Iodide nuclear counterstaining, the larvae were treated with RNase A (400 µg/ml in PB-T, Sigma) overnight at 4°C. After three washes in PB-T, the larvae were labeled for 2
hours with Propidium Iodide (10 μg/ml in PBT, Molecular Probes).

The following primary antibodies were used: polyclonal rabbit anti-DiI 1:200 [18], anti-Cleaved Gaspase-3 1:25 (9661, Cell Signaling Technology), anti-Rab7 1:2000 [19] and anti-Rab11 1:5000 [19] were detected with DyLight 649-conjugated goat anti-rabbit 1:500 (Jackson). Polyclonal rabbit anti-TnBVANK1 1:200 [16] was detected using Cy3- (1:1000) and DyLight 649- (1:500) conjugated goat anti-rabbit (Jackson). Monoclonal mouse PI4 anti-Dynein heavy chain 1:200 [20], anti-Rab5 1:25 (610281, BD Biosciences) and anti-Alx 1:100 [21] were detected with Cy3-conjugated goat anti-mouse 1:1000 (Jackson). Polyclonal guinea pig anti-Hrs 1:1000 [22] was detected with DyLight 649-conjugated goat anti-guinea pig 1:500 (Jackson).

Terminal deoxynucleotidyl transferase-mediated dUTP Nick End Labeling (TUNEL)

Five days AED larvae were dissected at room temperature in PBS, fixed in 4% formaldehyde for 20 minutes according to a protocol previously described [23]. After TUNEL incubation, anti-Digoxigenin 1:100 (Roche) was detected with Cy3-conjugated goat anti-mouse 1:1000 (Jackson).

Filipin and Oil Red O staining

Ring glands were fixed in 4% formaldehyde for 20 minutes and washed three times in PBS for 5 minutes each. Samples were stained with 50 μg/ml of filipin (Sigma) for 1 h or incubated in an Oil Red O (Sigma) solution at 0.06% for 30 minutes. After incubation tissues were washed twice with PBS before mounting in Fluormount-G. Samples were analyzed by conventional epifluorescence with a Nikon Eclipse 90i microscope or with a Nikon Eclipse 90i confocal microscope. Images were processed using Adobe Photoshop CS4 and Adobe Illustrator CS4.

Colocalization analysis

Thresholds of confocal images were set in Adobe Photoshop CS4 to exclude background staining. 509 Hrs positive vesicles were analyzed per TnBVANK1 and Hrs staining, 443 TnBVANK1 positive vesicles were analyzed per TnBVANK1 and Alx staining, 118 Hrs positive vesicles were analyzed per Alx and Hrs staining.

Images were processed with the CDA plugin of ImageJ to obtain Pearson’s coefficient (from +1 = complete correlation, to −1 = anti-correlation with 0 = no correlation) [24].

Results

Expression of TnBVank1 in the prothoracic gland induces developmental arrest at third instar larvae

TnBVank1 gene expression during Drosophila development was targeted with the GAL4/UAS binary system [25]. We used a transgenic Drosophila stock carrying two copies of the TnBVank1 gene under the control of the UASp sequences [16]. Expression of this transgene was induced using different GAL4 drivers. Our first analysis expressed the TnBVank1 transgene during embryonic and larval development using the hasy-Gal4 driver (hasy-Gal4) [25]. TnBVank1 expression did not appear to affect embryonic and larval development but, interestingly, all larvae failed to pupate and died after an extended third instar larval life, which lasted up to three weeks (Figure 1A). By measuring larval size, we found that four days AED the larvae expressing TnBVank1 did not significantly differ from control yach-Gal4 (n = 5; t = 0.8557; NS) (Figure 1B). Moreover, they continued to feed and significantly increased in size during their prolonged larval life, reaching at eighteen days the maximal length (Figure 1AC, n = 5; t = 6.765; p < 0.0001), while control regularly pupated on day six AED (Figure 1A). Since hasy-Gal4 is expressed in various larval tissues, the observed developmental arrest suggested us that TnBVank1 expression could have reasonably affected the ring gland function, the major site of production and release of developmental hormones.

The Drosophila ring gland (Figure 2A) consists of the prothoracic gland (PG), which is composed of steroidogenic cells synthesizing the E, the corpora allata (CA) that produce the juvenile hormone, and the corpora cardiaca (CC), which play a key role in the regulation of metabolic homeostasis [26]. We assessed if the targeted expression of the TnBVank1 gene using different ring gland Gal4 drivers (Figure 2B) was able to reproduce the effect observed when the transgene was expressed using the hasy-Gal4. When the TnBVank1 gene was expressed in both CA and PG cells, using the P0206-Gal4 driver, all the larvae failed to pupate and showed the same phenotype obtained with hasy-Gal4. Conversely, when the augst21-Gal4 (augst21-Gal4) driver specifically targeted the expression of TnBVank1 in the CA, no effects on developmental timing were observed and regular progeny were obtained. Moreover, we specifically induced expression of the TnBVank1 gene in the PG using the phantom-Gal4 (phantom-Gal4) driver, which is strongly expressed in this gland. None of the larvae pupated and they had an extended larval life as shown using the P0206-Gal4 driver (Figure 2B). These data indicate TnBVANK1 impairs PG function causing the block of larval-pupal transition.

We specifically expressed TnBVank1 in several other tissues, using different Gal4 drivers, and monitored the timing of development and the adult phenotype, which were in all cases not affected (Table S1).

Collectively, our data suggest that the expression of TnBVANK1 has the potential to interfere with the steroid biosynthesis, as further indicated by the targeted expression of this viral ANK protein in PG, which is characterized by developmental arrest of mature larvae and the absence of a systemic injury response [27].

Ecdysteroid biosynthesis is impaired in TnBVank1 larvae

To assess whether the developmental arrest induced by TnBVANK1 was due to a reduced level of 20E, we measured the whole body 20E titer in larvae expressing TnBVank1 by the phantom-Gal4 driver and in control larvae (Figure 2C). At 110 h AED, wild type third instar larvae enter the wandering stage and, at 25°C, they become white pre-pupae at 120 h, after the surge of a 20E peak [17]. At 120 h AED and during their abnormal extended larval life, the 20E levels measured in phantom-Gal4>TnBVank1 larvae are extremely reduced and significantly lower than that measured both in UASp-TnBVank1 larvae (n = 5; t = 10.12; p < 0.0001) and in phantom-Gal4/TM6B larvae (n = 5; t = 8.196; p < 0.0001).

To further demonstrate that the block of the transition to pupal stage showed by the phantom-Gal4>TnBVank1 larvae (hereinafter TnBVank1 larvae) was actually due to a low level of 20E, we carried out a 20E-feeding rescue experiment. Third instar TnBVank1 larvae were fed with yeast paste containing 20E dissolved in ethanol at 106 h AED, just before the onset of the ecdysteroid peak occurring in the wild-type. As expected, at 120 h AED, 70% of control larvae started to pupate and within the following 20 h all of them reached the pupal stage (n = 30). Pupation of TnBVank1 larvae fed with 20E followed almost an identical pattern, with 100% pupation (n = 30) attained only 1 day later, but failed to progress to the pharate stage (Figure 2D). Instead, TnBVank1 larvae treated only with yeast and
ethanol persisted as third instar (n = 30). This result confirms that the developmental arrest of TnBVank1 larvae is due to a reduced level of 20E. However, the rescued pupae failed to develop into adult flies. This may be due to the fact that the large peak of 20E required to trigger metamorphosis is not generated by TnBVank1 pupae and cannot obviously be supplied with food at this developmental stage.

It has been reported that a positive feedback is required for the transcriptional up-regulation of enzymes acting at late steps in the ecdysone biosynthetic pathway [28]. Therefore we analyzed the expression of Disembodied (Dib), the downstream step enzyme C22 hydroxylase, which appeared strongly reduced in all TnBVank1 PGs analyzed (n = 60) compared to control (Figure 2E,F). This data is in agreement with low levels of 20E detected in TnBVank1 larvae.

**Figure 1. TnBVank1 expression causes the block of the transition from larval to pupal stage.** (A) Light micrographs of ywch-Gal4 larva and pupa (control) and h-Gal4>TnBVank1 larvae at different days AED. The scale bar is 500 μm. (B) Larval length of different genotypes, at 96 h AED. Five larvae of each genotype were analyzed and as control we measured larval length of yw and h-Gal4 and UASp-TnBVank1 stocks and yw:h-Gal4. Graph represents mean ± standard deviation (s.d.); there is no significant (NS) length difference between h-Gal4>TnBVank1 (2680 ± 83 μm) and yw-Gal4 larvae (2580 ± 82 μm). (C) Larval length of h-Gal4>TnBVank1 increases during the extended larval life. Five h-Gal4>TnBVank1 larvae were measured at different days AED; values are the mean ± 1.d. of three independent experiments. The mean values of h-Gal4>TnBVank1 larval length at four and eighteen days AED are shown above the bars.

doi:10.1371/journal.pone.0095104.g001

TnBVANK1 affects PG morphology

Using a polyclonal antibody raised against two synthetic peptides of TnBVANK1 [16], we detected the distribution of TnBVANK1 protein in TnBVank1 PGs of five days AED larvae. As shown in Figure 3A–C, the protein was strongly expressed and present only in the cytoplasm of PG cells, confined to stroke-shaped particles. We next analyzed the TnBVank1 PG gross morphology. To visualize the PG we used phm-Gal4,UAS-mCD8::GFP stock. PGs from control larvae (Figure 3D) were significantly larger (n = 50; t = 50.41; p < 0.0001) (Figure 3F) than TnBVank1 PGs (Figure 3E). In addition, the TnBVank1 PG cells showed a cytoplasmic rather than the expected membrane distribution of mCD8::GFP (Figure 3C, E) [29]. Measurements of the PG cell area did not show any reduction induced by TnBVank1 expression (n = 50; t = 1.262; NS) (Figure 3G). Therefore, the
observed size difference of PG can be attributed to a reduction of the cell number. We then assayed if apoptosis occurs, using Cleaved Caspase-3 antibody [30] and TUNEL labeling [31]. The Caspase-3 activity (Figure 3I,J; n = 60) and TUNEL positive staining (Figure 3L,M; n = 60) found in a few cells of TnBVank1 PGs suggested that the occurrence of cell death during development can partly account for this difference, which could be related to the developmental arrest induced by TnBVANK1. However, the possibility that this protein can also disrupt PG activity cannot be ruled out. Therefore, to assess the relative contribution of these two effects, not mutually exclusive, we expressed TnBVank1 in PG cells at different time points during larval life, using a temperature sensitive form of the Gal4 repressor Gal80, Gal80ts [32], that allows to regulate the phm-Gal4 activity. TnBVank1 and control larvae were initially raised at 21°C, and then shifted to the restrictive temperature (31°C) at specific time points (96 h, 72 h and 48 h AED) to promote Gal4 activity. The temperature shift did not affect the proper development of the control larvae, which pupate normally. Conversely, the larvae expressing TnBVank1 failed to pupate, increased their size and survived for an extended period.

For each time point we also analyzed the PG size at 120 h AED (Figure 3N). When the TnBVank1 expression was triggered at 96 h or 72 h, the PGs size was not significantly different from controls (respectively n = 10; t = 0.07636; NS and n = 10; t = 1.336; NS). While the earlier induction of the transgene expression, at 48 h AED, strongly affected the PG size, which appeared significantly reduced (n = 10; t = 11.68; p<0.0001). In addition, we examined whether by inhibiting apoptosis with ectopic expression of p35 [33] it would be possible to rescue the phenotype produced by the expression of TnBVank1 in the PG. Coexpression of UAS-p35 and UASp-TnBVank1 in the same PG cells with phm-Gal4 driver did not rescue the developmental arrest phenotype (n = 58). Collectively, these data indicate that the developmental arrest induced by TnBVank1 does not depend on the reduced PG size triggered by apoptosis, but on its capacity to disrupt PG functioning when expressed before the production of the 20E peak.

Figure 2. The expression of TnBVank1 in prothoracic gland affects the E biosynthesis. (A) Ring gland includes the prothoracic gland (PG; yellow), the corpora allata (CA; orange) and the corpora cardiaca (CC; red). (B) The expression of the TnBVank1 gene is driven in the different ring gland compartments, highlighted in green, by three Gal4 drivers. P0206-Gal4>TnBVank1, expressed in PG and CA, causes the developmental arrest at the last larval stage; aug21-Gal4>TnBVank1 (CA) does not induce any developmental defects; phm-Gal4>TnBVank1 (PG) blocks the transition from larval to pupal stage. (C) Total 20E titer in five larvae of UASp-TnBVank1 stock (white bars), phm-Gal4/TM6B (grey bars) and phm-Gal4>TnBVank1 (black bars), at different time (hours AED). In the control stocks UASp-TnBVank1 and phm-Gal4/TM6B, the 20E peak which induces the pupariation is present at 120 h AED. Instead, this peak is absent in phm-Gal4>TnBVank1 larvae at 120 h AED and during the extended larval life. Error bars represent s.d.; *** = p<0.0001 versus controls (UASp-TnBVank1 and phm-Gal4/TM6B). The mean values of total 20E at 120 h AED of different genotype larvae are shown above the bars. (D) Feeding TnBVank1 larvae with medium supplemented with 20E induces the pupariation (red), while TnBVank1 larvae fed with medium containing ethanol (EtOH) do not reach the pupal stage (green). Values are the mean ± s.d. of three independent experiments. The yw;phm-Gal4 larvae serve as background control (blue). Immunostaining with anti-Dib in yw;phm-Gal4 (E) and TnBVank1 (F) PG reveals that the expression of Dib is strongly reduced in all TnBVank1 PGs analyzed. Panels E,F are at the same magnification and the reference scale bar is 25 μm indicated in E.

doi:10.1371/journal.pone.0095104.g002
TnBVank1 affects the cytoskeletal network in the PG cells

The altered TnBVank1 PG cell morphology and the associated mislocalization of mCD8::GFP prompted us to analyze the cytoskeletal network in these cells.

We investigated F-actin and α-tubulin distribution in TnBVank1 PGs and we observed an altered cytoskeletal organization in all analyzed glands (n = 60). As shown by phalloidin staining (Figure 4A-D), cortical actin did not appear regularly distributed in TnBVank1 PG cells, in which thick masses of actin filaments were detected (Figure 4C,D). The microtubule network was investigated by analyzing the distribution of α-tubulin-GFP fusion protein, which was coexpressed with TnBVank1 in the PG. Compared to control, expressing only α-tubulin-GFP protein (Figure 4E,F), the cytoskeleton of the TnBVank1 PG cells appeared strongly affected, as shown by the formation of thick bundles of microtubules (Figure 4G,H). The dynamic function of the microtubule network was then analyzed in TnBVank1 PGs (n = 60) by assessing the distribution of the minus-end-directed microtubule motor dynein, using an anti-Dynein heavy chain antibody [20]. Compared to the control (Figure 4I,J), cells of TnBVank1 PG displayed a reduced cortical distribution of dynein, along with some large dynein dots (Figure 4K,L). These data indicate that the whole cytoskeletal network is markedly altered in the PG cells expressing TnBVank1. We also analyzed Gal80ts-TnBVank1 PG cells at different time points (96 h AED, 72 h AED and 48 h AED) and we observed that F-actin organization is strongly altered when larvae were shifted to restrictive temperature at 48 h AED compared to PG from control larvae (** = p < 0.0001). Graph represents mean ± s.d.; 10 PGs were analyzed for each experiment.

doi:10.1371/journal.pone.0095104.g003
TnBVANK1 interferes with proper microtubule and microtubule-motor protein functions [16], and does not affect the overall cytoskeletal structure.

TnBVANK1 expression alters the cholesterol trafficking endocytic pathway of PG cells

The observed negative impact of TnBVANK1 on the cytoskeleton of PG cells may reduce the level of ecdysteroid biosynthesis by disrupting the uptake, transport and trafficking of sterols, essential steps for ecdysteroid biosynthesis [34]. Cholesterol, which cannot be synthesized by insects [35], enters the steroidogenic cells through a receptor-mediated low-density lipoprotein (LDL) endocytic pathway [36], which targets cholesterol to the endosomes. It is then transformed into 7-dehydrocholesterol in endoplasmic reticulum and transported to other subcellular compartments through further metabolic steps of the ecdysteroidogenic pathway [35]. We analyzed lipid vesicular internalization and trafficking in the TnBVank1 PG cells with a staining procedure using Oil Red O. Conversely to control (Figure 5A), in all TnBVank1 PGs analyzed (n = 60), we observed a varying level of evident increased accumulation of lipid droplets (Figure 5B,C). Then, using filipin, which specifically stains non-esterified sterols [37], compared to control (Figure 5D), TnBVank1 PGs (n = 60) showed a marked cholesterol accumulation in discrete vesicular drops (Figure 5E,F). These data suggest that TnBVANK1 does not affect lipid uptake, but that the endocytic pathway is in some way disrupted.

The endocytic pathway is organized into three major compartments, each characterized by specific Rab GTPase proteins that can be used as tags for the different endosomes [38]. Early endosomes are enriched in Rab5, late endosomes are associated with Rab7, and Rab11 marks the recycling endosomes. We used antibodies directed against these Rab proteins to investigate the endocytic pathway in PG cells (n = 60 PGs for each experiment) [19]. The cellular distribution of the early endosomes (Figure 6A,B) and recycling endosomes (Figure 6C,D) appeared to be comparable between PGs of control (Figure 6A,C) and of TnBVank1 larvae (Figure 6B,D). Whereas, compared to control (Figure 6E), in TnBVank1 PGs few Rab7 positive vesicles were detected.

Figure 4. TnBVank1 PG cells have an altered cytoskeleton. Phalloidin staining in control (A,B) and in TnBVank1 (C,D) PG cells. F-actin shows an altered distribution, characterized by thick masses of filaments in TnBVank1 PG cells. (E–H) α-tubulin-GFP fusion protein was expressed in yw;phm-Gal4 and TnBVank1 PG to investigate the microtubule network. Compared to control (E,F), in TnBVank1 the microtubule cytoskeleton is strongly affected and forms bundles (G,H). (I–L) Immunostaining with anti-Dynein heavy chain shows that, compared to control (I,J), in TnBVank1 PG cells the cortical localization of this protein is reduced and characterized by an evident dotted distribution (K,L). For each immunostaining we analyzed 60 PGs of five days AED larvae. PGs in panels A,C,E,G,I,K are at the same magnification and the reference scale bar is 25 μm and showed in A. Boxed regions are magnified in B,D,F,H,J,L and the reference scale bar 5 μm is indicated in B.

doi:10.1371/journal.pone.0095104.g004

Figure 5. TnBVank1 PG cells show lipids accumulation. (A) In the control yw;phm-Gal4 there are few lipid droplets stained with Oil Red O, while in TnBVank1 cells several lipid droplets are detected (B,C). (E,F) In TnBVank1 there is also a sterol accumulation, shown by filipin staining, which is absent in control PG (D). 60 PGs were stained for each experiment. PGs in panels A,B,D,E are at the same magnification and the reference scale bar 50 μm is showed in A. Boxed regions are magnified in C,F and the reference scale bar is 5 μm indicated in C.

doi:10.1371/journal.pone.0095104.g005
(Figure 6F). This suggests that TnBVANK1 may somehow affect the endocytic pathway.

We then analyzed the PG distribution of endosomes carrying the Hepatocyte growth factor-regulated tyrosine substrate (Hrs) (Figure 7A). This protein regulates inward budding of endosome membrane and multivesicular bodies (MVBs)/late endosome formation [22]. Interestingly, quite a few Hrs marked vesicles in the TnBVank1 PG cells showed the stroke-shaped form associated with TnBVANK1 signals (Figure 7D,E). In addition, most of the immunodetection signals of TnBVANK1 (Figure 7E) colocalized with the Hrs marked vesicles (Figure 7F; Pearson’s coefficient = 0.96±0.06). In contrast, most of these vesicles showing a normal round shape did not colocalize with TnBVANK1. This finding suggests an interaction of TnBVANK1 with endosome associated proteins, which may partly account for the observed alterations of the endocytic trafficking routes.

MVBs formation is controlled by a set of proteins, the endosomal sorting complex required for transport, ESCRT-0 to III, which sequentially associate on the cytosolic surface of endosomes [39]. A partner of the ESCRT proteins, which also regulates the making of MVBs, is the ALG-2-interacting protein X (Alix), first characterized as an interactor of apoptosis-linked gene protein 2 (ALG-2) [40]. It has been reported that the late endosomal lipid lysobisphosphatidic acid (LBPA) and its partner protein Alix play a direct role in cholesterol export [41]. Therefore, by using an antibody directed against Alix, we analyzed the distribution of this protein in control and TnBVank1 PGs (Figure 7B,G). According to its multifunctional activity [42], Alix was found widely distributed in the cytoplasm of wild type cells (Figure 7B), and, as expected, marked some Hrs positive vesicles (Figure 7C). Interestingly, in the TnBVank1 PG cells the TnBVANK1 positive stroke-shaped structures showed a strong colocalization with Alix (Figure 7H; Pearson’s coefficient = 0.99±0.07). In addition, several of these Alix positive stroke-shaped structures colocalized with Hrs (Figure 7I; Pearson’s coefficient = 0.95±0.16), indicating that these are modified endocytic vesicles. This strong interaction of TnBVANK1 with Alix containing vesicles and the altered cholesterol distribution observed in PG are concurrent evidences indicating that the cholesterol route was altered. Therefore, the interaction between TnBVANK1 and endosomes specifically affects the endosomal trafficking of sterols, likely limiting their supply to subcellular compartments where ecdysteroid biosynthesis takes place [35].

**Discussion**

PDVs are among the major host regulation factors used by parasitic wasps to subdue their hosts, which show immunosuppression and a number of developmental and reproductive alterations associated with disruption of their endocrine balance.
and late endosome formation is accompanied by an evident positive vesicle morphology. This defective mechanism in MVB endosomes, MVB: multivesicular bodies.

Relatively more studies have addressed the host immunosuppression mechanisms, focusing on virulence factors in the ank gene family largely shared among different taxa [43]. While an immunosuppressive function has demonstrated for the PDV ank gene family, if and how these viral genes impact endocrine pathways or other targets has not yet been addressed [11]. Here we report experimental evidence demonstrating the role of a TnBV ank gene in the disruption of E biosynthesis and the induction of developmental arrest.

The proteins encoded by PDV ank genes show significant sequence similarity with members of the IxB protein family involved in the control of NF-κB signaling pathways in insects and vertebrates [44]. Because they lack the N- and C-terminal domains controlling their signal-induced and basal degradation, they are able to bind NF-κB and prevent its entry into the nucleus to activate the transcription of genes under κB promoters [15,45,46]. The ank gene family is one of the most widely distributed in PDVs and contains members which are rather conserved across viral isolates associated with different wasp species [10,15,46–48]. These genes likely originate from horizontal gene transfer from a eukaryote, which could be the wasp, the host or another organism. Indeed, the nudiviruses, ancestors of bracoviruses [7], do not encode any gene showing similarity with ank family members. Their multiple acquisition and stabilization in different evolutionary lineages are clearly indicative of the key role they play in successful parasitism. This also suggests that ank genes may be involved in multiple tasks on host parasitization, by influencing different physiological pathways.

Here, we provide experimental data that corroborate this hypothesis for TnBVANK1, a gene of the bracovirus associated with the wasp T. nigriceps (TnBV), which parasitizes the larval stages of the tobacco budworm, H. virescens. Using Drosophila as a model system, we show that the TnBVANK1 protein acts as a virulence factor disrupting E biosynthesis (Figure 8) and causes developmental arrest of the larvae, which fail to pupariate. The number of late endosomes is reduced in the TnBVANK1 expressing cells and this is concurrent with an interesting change of Hrs-TnBVANK1 positive vesicle morphology. This defective mechanism in MVB and late endosome formation is accompanied by an evident alteration of sterol trafficking as indicated by the accumulation of lipid and sterol-rich vesicles. Cholesterol is processed to free cholesterol by lipase in the endosomal compartment and after that it moves to other compartments entering the ecdysone biosynthesis machinery [34]. Recent evidences from mammalian cell studies indicate that the late endosomal lipid LBPA and its partner Alix play a role in controlling the cholesterol export from endosomes [41]. Our finding that TnBVANK1 interacts with Alix positive vesicles and affects the sterol delivery suggests that Alix function in cholesterol export is conserved between Drosophila and mammals.

Our data let us to hypothesize that in TnBVANK1 expressing PG cells cholesterol may be trapped into the MVBs. This block leads to insufficient sterol supply to reach the ecdysone level necessary to complete development. Interestingly, the fact that TnBVANK1 expression in other tissues did not alter development suggests that TnBVANK1 impact on cholesterol trafficking may deeply affect the PG cells engaged in an intense steroidogenic activity. We show that TnBVANK1 disrupts the cytoskeletal structure of PG cells, and this appears to be a PG specific alteration. Indeed, in our previous work we demonstrated that the targeted expression of this ank gene in Drosophila germ cells alters microtubule network function in the oocyte, as shown by the mislocalization of several maternal clues, without affecting the cytoskeletal structure [16]. Therefore, we cannot exclude that the specific targeted effect of TnBVANK1 on the cytoskeleton function of PG cells may have a negative impact on ecdysteroidogenesis. However, it can also be true that the disruption of the cytoskeletal structure of these cells could be a downstream consequence of the impaired steroidogenic activity. The altered cell physiology and the consequent accumulation of lipids and steroids may have wide-ranging and more generalized effects on cell architecture/dynamics and survival. In fact, the prolonged expression of TnBVANK1 by phm-Gal4 during larval development causes cytoskeleton alteration and also apoptosis of a few cells, which may partly account for the observed reduction of the PG size.

It is interesting to note that the developmental arrest at L3 larval stage induced by TnBVANK1 expression in the PG perfectly mimics the developmental alteration of parasitized tobacco budworm larvae, which can regularly undergo larval molting but ultimately

---

[Image 8. Schematic overview showing the step in which TnBVANK1 affects the ecdysone biosynthesis in the PG cells. EE: early endosomes, MVB: multivesicular bodies. doi:10.1371/journal.pone.0095104.g008]
fail to pupate [13,49]. The reduced gland size observed in parasitized larvae and the low basal production of ecdysteroids [14,50] are fully compatible with a general reduction of the biosynthetic activity likely induced by ank genes. However, in naturally parasitized larvae these symptoms are also associated with a disruption of PTTH signaling, which requires active TnBV infection of PG, where different viral genes are expressed [13,51]. The high similarity of the recorded phenotypes represents a solid background on which to design specific experiments on the natural host. Indeed, the results reported here set the stage for specific in vivo studies in parasitized host larvae, that will have to address the respective roles of different TkBV genes in the suppression of ecdysteroidogenesis.

Supporting Information

Figure S1 Prolonged expression of TnBVank1 in PG cells during development alters cytoskeleton structure. Phalloidin staining in PGs from Gal80T-phan-Gal4 and Gal80T-TnBV ank1 larvae raised at 21°C (cyan) for different time intervals, then shifted at 31°C (red) and dissected at 120 h AED. PG cell cytoskeleton from Gal80T-TnBV ank1 larvae incubated at 21°C until 96 h AED (D) or until 72 h AED (E) shows no significant differences from Gal80T-phan-Gal4 (A, B). F-actin cytoskeleton is completely altered in PG cells of Gal80T-TnBV ank1 larvae incubated at 21°C until 48 h AED (F) compared to the control treated in the same condition (C). PG cells in all panels are at the same magnification and the reference scale bar 5 μm is indicated in A. (TIF)

References


Figure S2 Expression of TnBVank1 in fat bodies does not affect cell morphology. Phalloloid staining in fat bodies from the control yw; UAS-mCD8::GFP (A) and from fat bodies expressing TnBVank1 yw; UAS-mCD8::GFP/TnBVank1 (C, D). Fat bodies are at the same magnification in all panels and the scale bar is indicated in A. (TIF)

Table S1 Effects of TnBV ank1 expression using different Gal4 drivers.

(PDF)

Acknowledgments

This paper is dedicated to the memory of Franco Graziani, a wonderful person, a great friend and a brilliant scientist who has illumined our lives. A special thanks goes to Serena Duchi for her invaluable help on filipin experiment. We thank Silvia Gigliotti, Davide Andrenacci and Marilena Ignesti for critical reading of the manuscript and helpful suggestions. We thank Angela Algeri and Margherita Giacobazzi for proofreading the text. We thank Christen Mirth, Marek Jindra, Thomas Hays, Hugo Bellen, Michael O’Connor, Akira Nakamura, Toshiro Aiakgi and the Bloomington Stock Center for flies and reagents.

Author Contributions

Conceived and designed the experiments: LV PR VC FP GG. Performed the experiments: LV PR LT SL VC. Analyzed the data: LV PR LT SL VC FP GG. Wrote the paper: LV PR VC FP GG.