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Evolution of Developmental Control Mechanism

The hormonal pathway controlling cell death during metamorphosis in a hemimetabolous insect

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ABSTRACT

Metamorphosis in holometabolous insects is mainly based on the destruction of larval tissues. Intensive research in *Drosophila melanogaster*, a model of holometabolan metamorphosis, has shown that the steroid hormone 20-hydroxyecdysone (20E) signals cell death of larval tissues during metamorphosis. However, *D. melanogaster* shows a highly derived type of development and the mechanisms regulating apoptosis may not be representative in the insect class context. Unfortunately, no functional studies have been carried out to address whether the mechanisms controlling cell death are present in more basal hemimetabolous species. To address this, we have analyzed the apoptosis of the prothoracic gland of the cockroach *Blattella germanica*, which undergoes stage-specific degeneration just after the imaginal molt. Here, we first show that *B. germanica* has two inhibitor of apoptosis (IAP) proteins and that one of them, BgIAP1, is continuously required to ensure tissue viability, including that of the prothoracic gland, during nymphal development. Moreover, we demonstrate that the degeneration of the prothoracic gland is controlled by a complex 20E-triggered hierarchy of nuclear receptors converging in the strong activation of the death-inducer Fushi tarazu-factor 1 (BgFTZ-F1) during the nymphal–adult transition. Finally, we have also shown that prothoracic gland degeneration is effectively prevented by the presence of juvenile hormone (JH). Given the relevance of cell death in the metamorphic process, the characterization of the molecular mechanisms regulating apoptosis in hemimetabolous insects would allow to help elucidate how metamorphosis has evolved from less to more derived insect species.

Introduction

Holometabolan metamorphosis is a key innovation in insect development, which involves the stage-specific destruction of a number of tissues at the end of the larval growth and the formation of new adult structures. In this context, the selection of highly regulated mechanisms to control developmentally induced cell death has been a key associated event. In *Drosophila melanogaster*, a model of holometabolan metamorphosis, intensive research has revealed the steroid hormone 20-hydroxyecdysone (20E) as the main regulator of the destruction and elimination of larval tissues. In fact, two pulses of 20E, one at the end of the larval instar and the second approximately 10 h after the formation of the puparium, are responsible of the destruction of the larval midgut and the salivary glands, respectively (Yin and Thummel, 2005).

From a molecular point of view, a genetic cascade of transcription factors mediates the apoptotic effect of 20E. Upon binding to its heterodimeric receptor composed by the two nuclear receptors Ecdysone receptor (EcR) and Ultraspiracle (Usp), 20E activates the transcription of a set of genes including *broad* (zinc finger transcription factor), *E74* (ETS-domain transcription factor), *E93* (helix-turn-helix DNA binding protein) and a number of nuclear hormone receptors, such as E75, HR3 and FTZ-F1 which act as a competence factor for genetic responses to the prepupal–pupal ecdysteroid pulse (Baehrecke and Thummel, 1995; Bialecki et al., 2002; Broadus et al., 1999; Burtis et al., 1990; DiBello et al., 1991; Lam et al., 1999). In turn, these genes induce the expression of several secondary late genes including death inducers *reaper* (*rpr*) and *head involution defective* (*hid*), as well as the main executers of apoptotic death, the initiator caspase *Dronc*, the effector caspase *Drice* and the adaptor protein *Dark* (Thummel, 1996; Lee et al., 2000; Jiang et al., 2000; Cakouros et al., 2002). Interestingly, before the pupal stage, these caspases are kept as inactive proteins by the activity of the *Drosophila* inhibitor of apoptosis 1 (DIAP1). DIAP1 is defined by the presence of two Baculovirus IAP Repeat (BIR) domains that function as protein interaction modules that bind to caspases and prevent their activity (Orme and Meier, 2009). Rpr and Hid proteins induce cell death by

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interacting with DIAP1, thus impairing its inhibitory activity (Martin, 2002). In addition to 20E, another hormone involved in cell death is the sesquiterpenoid juvenile hormone (JH). Although the molecular mechanism by which JH operates remains basically unknown (Riddiford, 2008), it has been shown that this hormone prevents degeneration of the midgut in the dipteran *Aedes aegypti* (Wu et al., 2006; Parthasarathy and Palli, 2007a) and the lepidopteran *Heliothis virescens* (Parthasarathy and Palli, 2007b), and also antagonizes the 20E-induced apoptosis of the larval fat body of *D. melanogaster* by counteracting the activity of two transcription factors of the bHLH-PAS family that play crucial roles in JH action, Methoprene-tolerant and its paralog Germ-cell expressed (Liu et al., 2009).

It is worth noting, however, that *D. melanogaster* shows a highly derived type of development, so the mechanisms regulating apoptosis may be poorly representative in the insect class context. Unfortunately, no functional studies have been carried out to address whether these mechanisms are present in more basal hemimetabolous species. Unlike the holometabolous, hemimetabolous species hatch from the egg as first instar nymphs that resemble miniature adults. In these insects, growth and maturation occur simultaneously throughout sequential nymphal stages until the imaginal molt. Nevertheless, the absence of an intermediate pupal stage in hemimetabolous insects does not imply the lack of stage-specific cell death. In this sense, using the German cockroach, *Blattella germanica*, as a hemimetabolan model, we have shown that the prothoracic gland, which is responsible for the synthesis of ecdysteroids, undergoes a precise developmentally controlled degeneration just after the imaginal molt (Romañá et al., 1995; Cruz et al., 2006; Mané-Padrós et al., 2008). Since winged adult insects, as *B. germanica*, do not undergo further molting, the elimination of the ecdysteroid source at the onset of the adult stage is crucial to allow normal adult development. Thus, the degeneration of the prothoracic gland in a hemimetabolous species is a good model to analyze a less derived developmentally regulated apoptotic event during the post-embryonic development of an insect. Importantly, the characterization of the molecular mechanisms that regulate cell death in hemimetabolous insects would allow to understand how metamorphosis has evolved from less to more derived insect species.

In this study, by using an RNAi in vivo approach, we have functionally characterized activator as well as inhibitor proteins that coordinate the degeneration of the prothoracic gland during the onset of the adult stage of *B. germanica*. First, we have shown that this insect has two IAP proteins and that one of them, BgIAP1, is constantly required to block premature apoptosis during nymphal development. We have also demonstrated that the degeneration of the prothoracic gland depends on a complex 20E-triggered hierarchy of nuclear receptors that converges on the precise activation of the orphan nuclear receptor Fushi tarazu-factor 1 (BgFTZ-F1), which is responsible of the degeneration of the prothoracic gland at the onset of adult development. Finally, we have shown that JH plays a critical role in preventing prothoracic gland degeneration during nymphal development of *B. germanica*.

Materials and methods

Insects

Specimens of *B. germanica* were obtained from a colony reared in the dark at 30 ± 1 °C and 60–70% r.h. All dissections and tissue sampling were carried out on carbon dioxide-anaesthetized specimens.

Cloning of BgIAP1 and BgIAP2

A 765 bp clone corresponding to BgIAP1 was obtained from a suppression subtractive hybridization library that was carried out using the PCR-selected cDNA Subtraction Kit (Clontech), following the manufacturer's protocols. The tester library was prepared with 1 μg of polyA$^+$ mRNA from UM-BGE-1 embryonic cells from *B. germanica* treated with 20E during 10 h. The driver library was prepared with the same amount of polyA$^+$ mRNA from untreated UM-BGE-1 cells. To complete the sequence of the BIR domains, 5′RACE (5′RACE System Version 2.0; Invitrogen) was carried out using as reverse primer (BgIAP1R1), 5′-AACGTTGATGATCCCTGAATG-3′ and a nested reverse primer (BgIAP1R2), 5′-GGACATCTAACTACATCACTTCGA-3′.

B. germanica IAP2 cDNA sequence was isolated from a cDNA library prepared by M.D. Piulachs with an ovary pool of adult *B. germanica* including samples of each day of the first gonadotrophic cycle. To construct this library, total RNA was extracted using GenElute Mammalian Total RNA kit (Sigma). Subsequently, polyA$^+$ mRNA was obtained taking advantage of the Dynabeads Oligo (dT) technology (Dynal Biotech ASA). cDNA synthesis was performed with the lambda ZAPII cDNA Synthesis Kit (Stratagene) according to the manufacturer's protocol. Among the sequences obtained after massive sequencing, a fragment of 1056 bp, corresponding to the putative IAP2, was obtained (M.D. Piulachs and P. Irles, unpublished results). This fragment was extended to the 3′ end by a 3′RACE approach (Invitrogen) using the forward primer: 5′-CAGAGCTC-GACTGTGAGAGACTTA-3′. The product obtained was subcloned into the pSTBlue-1 vector (Novagen) and sequenced. Amino acid sequence comparisons indicated that the protein showed the characteristic domains of this family, precisely 3 BIR domains and 1 RING finger.

RNA interference

RNAi in vivo in nymphs of *B. germanica* was performed as described in Martín et al. (2006) and Cruz et al. (2007). The primers used to generate templates via PCR for transcription of the dsRNAs are described in Supplemental Table 1. A volume of 1 μl of each dsRNA solution (1 μg/μl) was injected into the abdomen of newly emerged female nymphs. In case of coinjection of two dsRNAs, 1 μl of each solution was applied in a single injection of 2 μl.

Treatments with methoprene in vivo

Newly ecdysed last instar nymphs were topically treated with 1 μg methoprene (isopropyl (*E,E*)-(*RS*)-11-methoxy-3,7,11-trimethyldodeca-2,4-dienoate) per specimen in 1 μl of acetone. Controls received the same volume of solvent.

Quantification of haemolymph ecdysteroids

Haemolymph ecdysteroids were quantified by ELISA following the procedure described by Porcheron et al. (1989), and adapted to *B. germanica* by Pascual et al. (1992) and Romañá et al. (1995). 20E (Sigma) and 20E-acetylcholinesterase (Cayman) were used as standard and enzymatic tracer, respectively. The antiserum (Cayman Chemical) was used at a dilution of 1:50,000. Absorbances were read at 450 nm, using a Multiscan Plus II Spectrophotometer (Labsystems). The ecdysteroid antiserum used has the same affinity for ecdysone and 20E (Porcheron et al., 1989), but since the standard curve was obtained with the later compound, results are expressed as 20E equivalents.

Microscopy, histological analysis and detection of cell death

All dissections of larval and adult tissues were carried out in Ringer's saline. Prothoracic glands were fixed in 4% paraformaldehyde and permeabilised in PBS–0.2% tween (PBT), then incubated for 10 min in 1 μg/ml DAPI in PBT. After two washes with PBT, the tissues were mounted in Mowiol 4-88 (Calbiochem). All samples were examined with a Zeiss Axiophot microscope, and images were subsequently processed using Adobe Photoshop. To detect cell death, TUNEL assays were

performed using the In Situ Cell Death Detection Kit, Fluorescein (Roche), following the manufacturer's instructions. Staged prothoracic glands, as well as other tissues, were fixed in 4% paraformaldehyde in PBS for 30 min, washed in PBST (0.1% Triton-PBS), and permeabilised by incubation in 0.1% Sodium Citrate–0.1% Triton-PBS for 30 min. Samples were rinsed in PBS and incubated in TUNEL reaction mixture for 1 h at 37 °C. Finally they were mounted in Mowiol 4-88 and examined with a Leica confocal microscope.

Prothoracic gland extracts and EMSA

Preparations of cell extracts from *B. germanica* prothoracic glands were carried out according to the method described by Miura et al. (1999) and Martín et al. (2001). Twenty prothoracic glands were used for each time-point extraction. Binding reactions were carried out in a 20 µl volume containing the prothoracic gland nuclear extract, 10 mM Tris–HCl (pH 7.5), 50 mM NaCl, 1 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM EDTA, 4% (v/v) glycerol, 1 µg poly(dI.dC), 1 µg of a single-stranded DNA (5′-TAATACGACTCACTATA-3′) and the indicated amount of competitor DNA or antibody when appropriate. After 15 min of incubation at 4 °C, 0.05 pmol of ^{32}P-labelled DNA probe was added, and the incubation was continued for another 45 min at the same temperature. The reaction was resolved on 5% non-denaturing polyacrylamide gel run at 4 °C and a constant voltage of 150 V in 0.5× TBE. The gel was then dried and autoradiographed. Oligonucleotides (only sense strands are shown) used to generate the DNA probe for EMSA were: (F1RE) 5′-GCAGCACCGTCTCAAGGTCGCCGAGTAGGAGAACC-3′.

Semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR)

For the analysis of the tissue distribution of BgIAP1 and BgIAP2 mRNAs, semiquantitative RT-PCR was used. Total RNA was extracted from different tissues using the GenElute Mammalian Total RNA Kit (Sigma). A sample of 0.3–1 µg of each RNA preparation was used for cDNA synthesis, as previously described (Maestro et al., 2005). Primers used for BgIAP1 detection were as follows: forward, 5′-AGACCGAATGCCATTCCTGAAAGA-3′ and reverse, 5′-TTGAAGTTGATGCTGCAGATGATG-3′; and for BgIAP2: forward, 5′-GTAGAGCCTCAACGCATAGCCAAA-3′ and reverse, 5′-CACTATCCCAGAGCAGAATGCAC-3′. As a reference, the actin5C transcript of *B. germanica* was also amplified using the primers: forward, 5′-TCGTGACATCAAGGAGAAGCT-3′_ and reverse, 5′-TGTCGGCAATTCCAGGGTACATGGTGGT-3′. RT-PCR without reverse transcription did not result in amplification, indicating that there was no genomic contamination.

Quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR)

Relative transcript levels were determined by real-time PCR (qPCR), using iQ SYBR Green Supermix (Bio-Rad). To standardize quantitative PCR (qPCR) inputs, a master mix that contained iQ SYBR Green Supermix and forward and reverse primers was prepared (final concentration = 100 nM/qPCR; see Supplemental Table 2 for primer sequences). The qPCR experiments were conducted with the same quantity of organ equivalent input for all treatments and each sample was run in duplicate using 2 µl of cDNA per reaction. All the samples were analyzed on the iCycler iQ Real Time PCR Detection System (Bio-Rad). For each standard curve, one reference DNA sample was diluted serially.

Results

The prothoracic gland degeneration of B. germanica is developmentally controlled

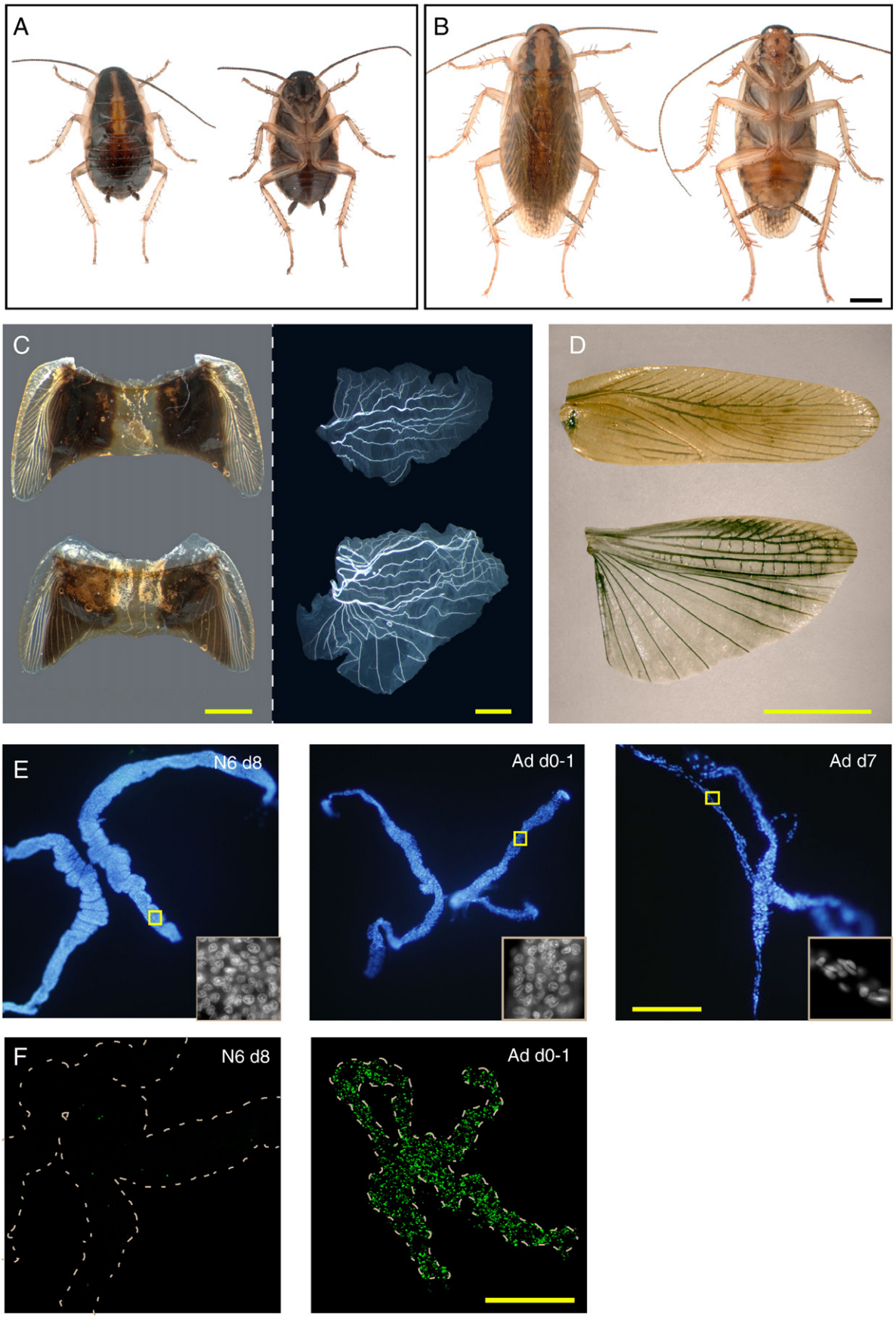
In hemimetabolous insects, growth and maturation take place gradually and simultaneously during a series of nymphal instars. *B. germanica*, our insect model, undergoes six nymphal instars until the imaginal molt. This last metamorphic transition is characterized by marked changes in cuticle pigmentation (Figs. 1A,B), appearance of functional wings which had been grown gradually with each molt as wing pads on the outside of the body (Figs. 1C,D) and functional genitalia (data not shown). Moreover, the imaginal molt also marks the onset of degeneration of the prothoracic gland, the X-shaped tissue located in the prothorax that is responsible for the synthesis of ecdysteroids during nymphal stages. As a first step towards the characterization of the mechanisms that control cell death in hemimetabolous insects, we examined the precise timing of degeneration of the prothoracic gland using TUNEL labelling, which determines DNA fragmentation associated to cell death. Whereas the prothoracic glands of late last instar nymphs showed no TUNEL labelling, those just after the imaginal molt presented strong and uniform labelling as the gland underwent total degeneration by day 1. After 7 days, the gland became reduced to a central muscular axis surrounded by few cells (Figs. 1E,F).

BgIAP1 inhibits premature tissue degeneration during nymphal development

The activation of a cell death response depends on the balance between survival and death regulators. Regarding anti-apoptotic proteins, we started the analysis of prothoracic gland degeneration by identifying two IAP cDNAs in *B. germanica*. Based on the similarity of their sequences with those of *D. melanogaster* IAPs, they were named BgIAP1 (GenBank accession no. FN668727), which contains two Baculovirus IAP Repeat (BIR) domains, and BgIAP2 (GenBank accession no. FN668728), which presents three (Fig. S1). During nymphal development, both mRNAs were present in all tissues analyzed, namely the fat body, epidermis, ovary, brain, *corpora allata*, digestive tract and prothoracic gland (Fig. 2A). Furthermore, we analyzed the mRNA levels of BgIAP1 and BgIAP2 in the prothoracic gland during nymphal–nymphal and nymphal–adult transitions (Fig. 2B). BgIAP1 mRNA level showed a cyclic pattern with its levels declining during each transition. Interestingly, BgIAP1 mRNA became almost undetectable 24 h after the imaginal molt, thus correlating with the onset of prothoracic gland degeneration. Conversely, the levels of BgIAP2 mRNA did not show significant changes during both stages, being present at high levels even 24 h after the adult molt.

We, next, determined the functional consequences of depleting both proteins by using RNAi in vivo. To lower BgIAP1 expression we injected 1 µg of dsBgIAP1 in the abdomen of freshly ecdysed last instar nymphs. As a consequence, the mRNA levels of BgIAP1 were dramatically reduced as early as 48 h after the treatment, either in

Fig. 1. Metamorphic changes in *Blattella germanica*. (A) Dorsal (left) and ventral (right) view of a last instar nymph characterized by black cuticle. (B) Dorsal (left) and ventral (right) view of a winged adult with brown cuticle. (C) During nymphal development wings (right) grow gradually as wing pads (left). (D) Fully developed and extended adult wings. Forewings are in the upper part and hindwings are in the lower part in C and D. (E–F) Degeneration of *B. germanica* prothoracic glands is also developmentally regulated. Staged prothoracic glands from sixth instar nymphs (N6) and adults (Ad) were stained with DAPI (E) or labelled with TUNEL (F) to determine DNA fragmentation associated to cell death. Prothoracic gland degeneration takes place after the imaginal molt as revealed by TUNEL positive nuclei in 0–2-day-old adult prothoracic gland, and correlates with the reduction in cell mass observed by DAPI staining. At the end of the first gonadotrophic cycle (day 7), the prothoracic gland is reduced to a central muscular axis surrounded by few cells. To improve TUNEL interpretation prothoracic glands are silhouetted. Scale bars: 2 mm in A, B; 0.5 mm in C; 5 mm in D; 200 µm in E; 100 µm in F.


A
B
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E
N6 d8
Ad d0-1
Ad d7
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N6 d8
Ad d0-1

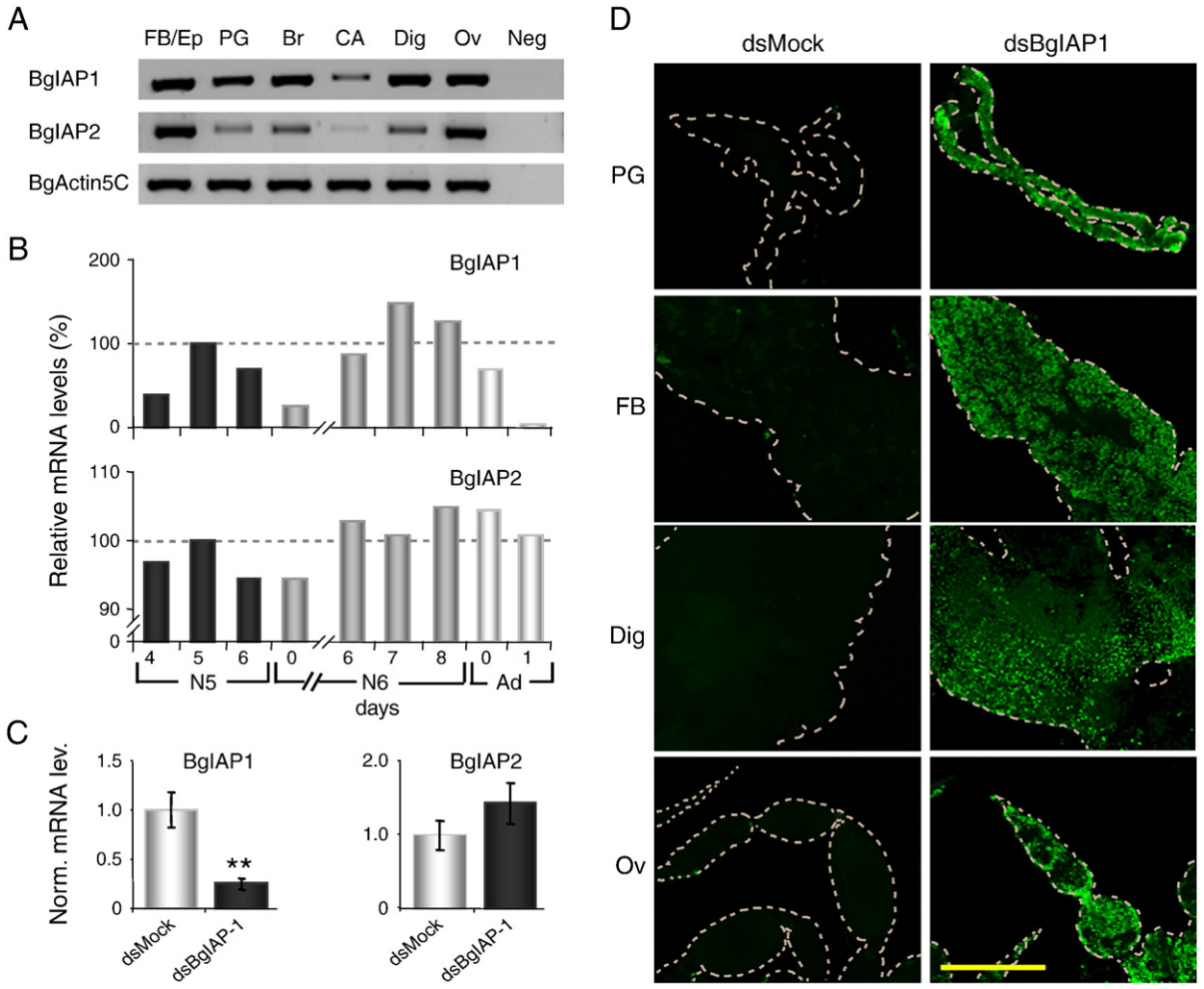

Fig. 2. BgIAP1 is crucial for survival during post-embryonic development of *Blattella germanica*. (A) BgIAP1 and BgIAP2 expression in fat body with adhered epidermis (FB/Ep), prothoracic gland (PG), brain (Br), *corpora allata* (CA), digestive tract (Dig) and ovaries (ov). RT-PCR without reverse transcription was performed as a negative control (Neg). BgActin5C was used to confirm cDNA integrity. (B) Expression patterns of BgIAP1 and BgIAP2 in the prothoracic gland during nymphal–nymphal and nymphal–adult transitions. Equal tissue equivalents were analyzed by qRT-PCR. mRNA profiles are representative of three independent replicates. (C–D) RNAi in vivo of BgIAP1 in last instar nymphs of *B. germanica*. (C) Effect of dsBgIAP1 on mRNA levels of BgIAP1 and BgIAP2. A dose of 1 μg of dsBgIAP1 was injected in newly emerged sixth instar nymphs, and mRNA levels in the prothoracic gland were measured 48 h later by qRT-PCR. Equivalent experiments injecting a non-specific sequence (dsMock) served as negative control. Transcript abundance values for BgIAP1 and BgIAP2 are normalized against BgActin-5C transcript. Vertical bars indicate the SEM. Asterisks indicate differences statistically significant at $p \leq 0.005$ (*t*-test) (n = 10). (D) Effect of RNAi-mediated knockdown of BgIAP1 on tissue degeneration. A dose of 1 μg of dsBgIAP1 was injected in newly emerged sixth instar nymphs, and PG, FB, Dig and Ov were dissected 48 h later and labelled with TUNEL. Strong and uniform TUNEL positive nuclei were detected in all tissues in dsBgIAP1-treated nymphs. To improve TUNEL interpretation all tissues are silhouetted. Scale bar: 400 μm in PG, FB and Dig and 200 μm in Ov.

the prothoracic gland (Fig. 2C) or in all other tissues analyzed, namely fat body, epidermis, ovary and digestive tract (data not shown). The levels of BgIAP2 were not affected by the treatment, thus indicating that the RNAi was BgIAP1-specific (Fig. 2C). Importantly, as a result of reduced BgIAP1 levels, all knockdown nymphs (n = 26) stopped moving and died 48–72 h after the treatment. Upon dissection of the knockdown nymphs before dying, tissues showed clear signs of massive degeneration as revealed by strong TUNEL labelling (Fig. 2D). The same results were obtained when the treatment was carried out in penultimate (fifth) instar nymphs (100% of specimens died 48 h after the treatment; n = 12), and when it was carried out in fourth instar nymphs (100% death specimens; n = 31), clearly indicating that BgIAP1 is constantly necessary during the post-embryonic development of *B. germanica* to prevent tissue degeneration.

Conversely, although there was a clear reduction in BgIAP2 mRNA levels in last instar nymphs treated with dsBgIAP2 (data not shown), they showed normal appearance and behaviour during nymphal development and molted into adults properly (n = 30). Furthermore, the organs and tissues of dsBgIAP2-treated animals had a normal aspect and the prothoracic gland degenerated after the imaginal molt as in dsMock-treated animals. This result demonstrates that, in contrast to BgIAP1, BgIAP2 does not play any important role in controlling apoptosis in *B. germanica*.

Prothoracic gland degeneration is mediated by the nuclear receptor BgFTZ-F1

Once demonstrated that BgIAP1 is a key survival factor in *B. germanica*, we wanted to identify genes that would mediate the tissue- and stage-specific degeneration of the prothoracic gland. In this context, we had previously shown that prothoracic gland death requires the presence of BgEcR-A, the 20E receptor (Cruz et al., 2006). In our present experiments, we further confirmed this result by reducing the levels of BgRXR, the heterodimeric partner of BgEcR-A, in last instar nymphs of *B. germanica*. Thus, when BgRXR levels were lowered by injecting 1 μg of dsBgRXR to newly emerged last instar nymphs, those specimens that were able to molt into adults (33%; n = 42) did not show TUNEL labelling (data not shown) and their prothoracic glands did not present

any sign of degeneration even 7 days after the imaginal molt (Fig. 3A). Given that BgEcR-A and BgRXR are the heterodimeric receptor of 20E, we analyzed the expression of 20E-responsive genes that had been previously shown to play key roles in 20E action in *B. germanica*, namely the nuclear receptors *BgE75*, *BgHR3* and *BgFTZ-F1* (Cruz et al., 2007, 2008; Mané-Padrós et al., 2008) to check a possible relation between their expression profiles in the prothoracic gland and the degeneration of this tissue. Whereas BgEcR-A and BgRXR were expressed without significant changes (data not shown), the mRNAs of BgE75-A and BgHR3-A presented cyclic patterns of expression that correlate with the levels of circulating ecdysteroids (Cruz et al., 2003). Furthermore, the level of activation of these genes was similar during both developmental transitions (Fig. 3B). Similar results were obtained with BgE75-B, BgE75-E and BgHR3-B (data not shown). Finally, BgFTZ-F1 mRNA levels increased at the end of each instar, showing a maximum before molting, when the circulating ecdysteroids declined. Two features of BgFTZ-F1 expression were particularly interesting for our study. First, significantly large amounts of BgFTZ-F1 mRNA were detected during the nymphal–adult transition compared to nymphal–nymphal one (12 fold higher), and second, BgFTZ-F1 mRNA levels were maintained remarkably high even 24 h after the imaginal molt (Fig. 3B). Interestingly, the stage-specific up-regulation of BgFTZ-F1 in the prothoracic gland during the imaginal molt was also tissue-specific, since it was not observed in other tissues that do not underwent degeneration, such as the fat body (Fig. 3C) or the epidermis (data not shown).

The occurrence of high mRNA levels of BgFTZ-F1 in the prothoracic gland foreshadowed the onset of its degeneration, raising the interesting possibility that BgFTZ-F1 would trigger the death of this

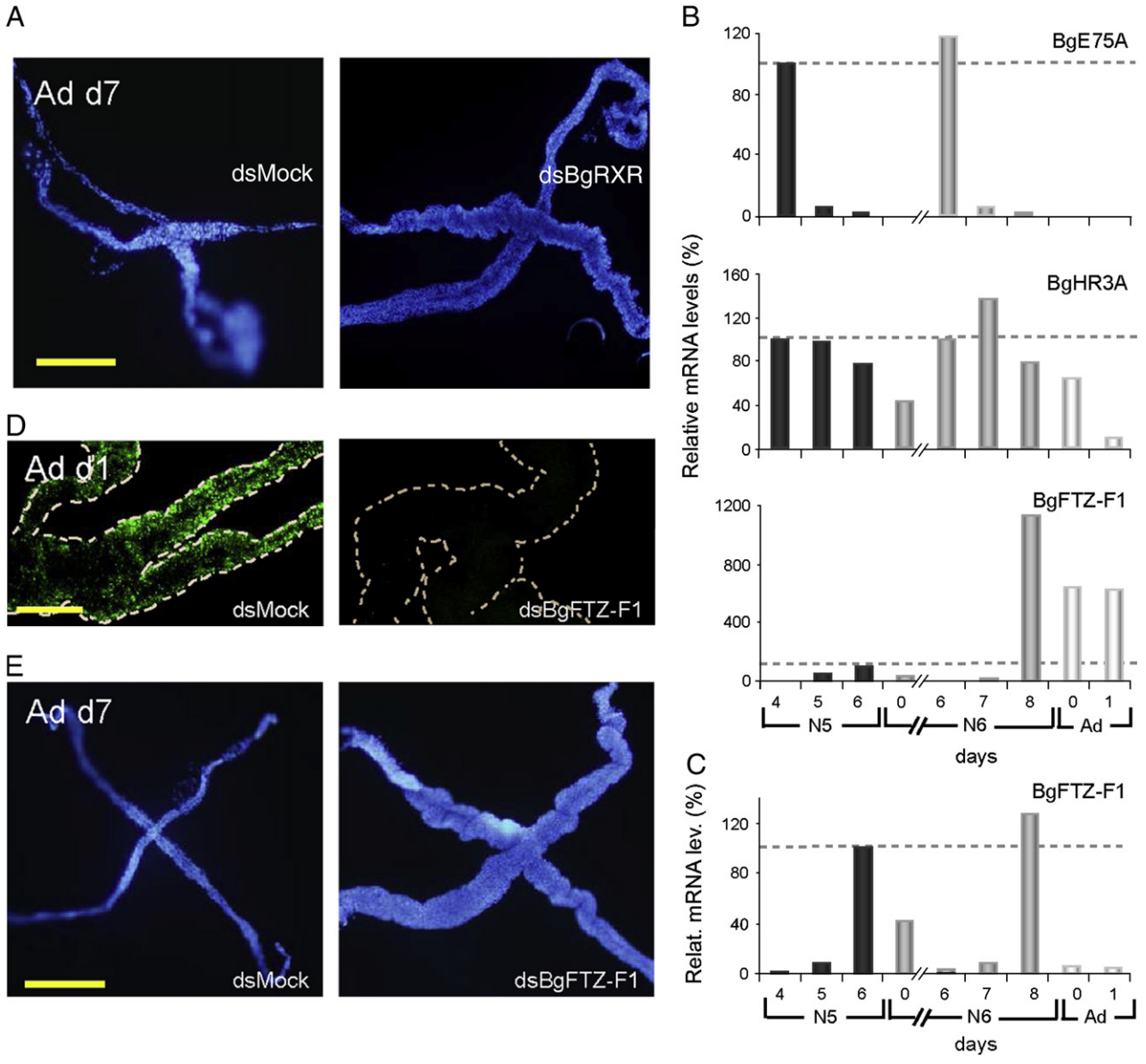

Fig. 3. BgFTZ-F1 is required for prothoracic gland death in *Blattella germanica*. (A) Effect of RNAi-mediated knockdown of BgRXR on the degeneration of the prothoracic gland. Prothoracic glands of dsMock-treated sixth instar nymphs 7 days after the imaginal molt, showing a dramatic reduction of cell mass (left panel). Prothoracic glands of dsBgRXR-treated nymphs showing no signs of degeneration 7 days after the imaginal molt (right panel). (B) Expression patterns in the prothoracic gland of several 20E-dependent nuclear receptors during nymphal–nymphal and nymphal–adult transitions. Equal tissue equivalents from staged prothoracic glands were analyzed by qRT-PCR. (C) Expression pattern of BgFTZ-F1 mRNA in the fat body of *B. germanica* during the same time points. mRNA profiles in B and C are representative of three independent replicates, and the relative expression of each gene was calculated based on their maximal expression during the fifth nymphal instar. (D–E) BgFTZ-F1 is necessary for prothoracic gland death. Newly emerged sixth nymphal instar females were injected with 1 μg of dsBgFTZ-F1 or dsMock, and the prothoracic glands of animals that molted into adults were labelled by TUNEL one day after the imaginal molt (D) or by DAPI staining 7 days later (E). Prothoracic gland of dsBgFTZ-F1-treated specimens did not show any sign of degeneration. To improve TUNEL interpretation prothoracic glands are silhouetted. Scale bars: 100 μm in A, E and 200 μm in D.

gland. To test this possibility, we reduced the levels of BgFTZ-F1 before the imaginal molt by injecting 1 μg of dsBgFTZ-F1 into newly ecdysed last nymphal instar. Although most of the BgFTZ-F1 knockdowns arrested development at the molting process (75%; see also Cruz et al., 2008), 25% of these animals ecdysed into the adult stage properly (n = 72). Confirming our hypothesis, the prothoracic gland of all the specimens that molted into adults did not show any sign of degeneration during the first days of adult development (Fig. 3D), and even after 7 days, the glands presented a normal shape and were well developed (Fig. 3E). Taken together, we concluded that BgFTZ-F1 is a critical factor in controlling prothoracic gland degeneration at the onset of adult development in *B. germanica*.

The nuclear hormone receptor BgE75 acts as a suppressor of PCD in B. germanica

We had previously shown that silencing the 20E-dependent nuclear receptor *BgE75* in last instar nymphs resulted in premature prothoracic gland degeneration (Mané-Padrós et al., 2008). To further understand the role of BgE75 in prothoracic gland death, we have analyzed in detail the effect of reducing BgE75 levels during the last nymphal instar on the viability of the gland. Confirming our previous results, the injection of 1 μg of dsBgE75 into newly ecdysed last instar nymphs elicited a premature degeneration of the prothoracic gland in mid-instar, that is, 3 days before the natural timing of degeneration (Fig. 4A). As a result, prothoracic glands of BgE75 knockdowns did not synthesize ecdysteroids (Fig. 4B). We, then, analyzed the outcome of reducing BgE75 levels on the expression of other 20E-dependent nuclear receptors as well as in BgIAP1 in staged prothoracic glands of dsMock- and dsBgE75-treated newly ecdysed last nymphal instar (Fig. 4C). Interestingly, the expression of BgHR3-A was strongly and prematurely up-regulated, indicating that BgE75 suppresses the 20E-dependent activation of BgHR3 during the first days of the nymphal stage. Correlating with the increase of BgHR3, the expression of BgFTZ-F1 was also significantly induced in the BgE75 knockdown nymphs (Fig. 4C), suggesting that BgHR3 would be responsible of the premature activation of BgFTZ-F1. We corroborate that by performing double RNAi of BgE75 and BgHR3

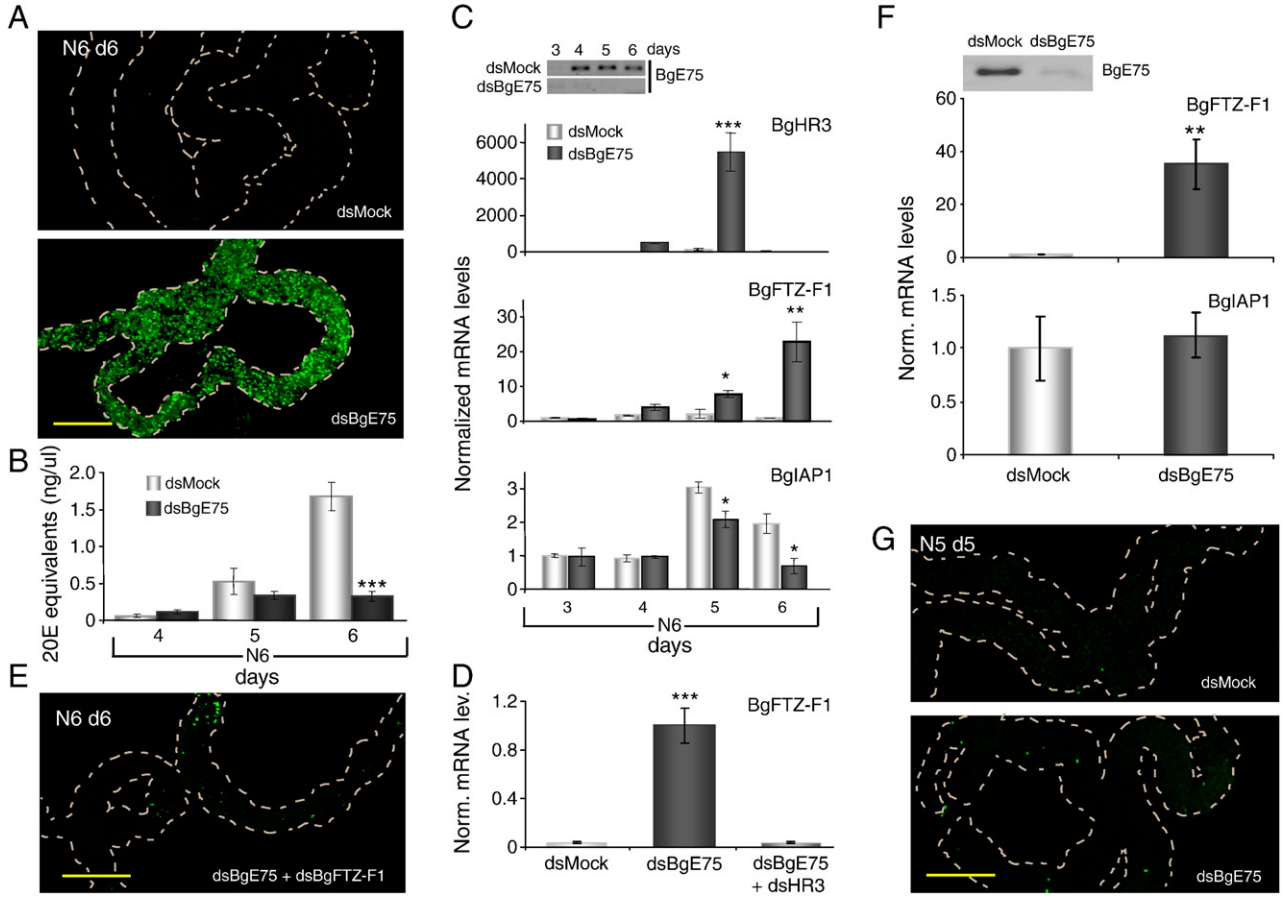

Fig. 4. RNAi-mediated knockdown of BgE75 during the last nymphal instar leads to premature prothoracic gland degeneration in *Blattella germanica*. (A) Newly emerged last instar nymphs were injected with 1 μg of dsBgE75 or with dsMock. After 6 days, prothoracic glands were labelled with TUNEL. Prothoracic glands from BgE75 knockdown nymphs showed strong TUNEL positive labelling. (B) Effect of RNAi of BgE75 on ecdysteroid levels. Hemolymph was collected at days indicated of the sixth nymphal instar, and ecdysteroid levels were determined by ELISA. Results are expressed as ng of 20E equivalents. Vertical bars indicate the SEM (n = 4–12). (C) Effect of RNAi of BgE75 on the expression of BgHR3, BgFTZ-F1 and BgIAP1. mRNA levels were analyzed in staged prothoracic glands of 3- to 6-day-old last instar nymphs by qRT-PCR and normalized against BgActin-5C transcript. Vertical bars indicate the SEM (n = 3). The inset panel shows the RT-PCR analysis of BgE75 transcript reduction. (D) Effect of BgE75 or combined BgE75 plus BgHR3 RNAi on the expression of BgFTZ-F1. mRNA levels were analyzed in 6-day-old last instar nymphs by qRT-PCR and normalized against BgActin-5C transcript. Vertical bars indicate the SEM (n = 10). (E) Effect of BgE75 or combined BgE75 plus BgFTZ-F1 RNAi on prothoracic gland degeneration. Newly emerged last instar nymphs were injected with 1 μg of dsBgE75 or with dsBgE75 plus dsBgFTZ-F1 simultaneously (1 μg each). After 6 days, prothoracic glands were collected and labelled with TUNEL. (F–G) RNAi-mediated knockdown of BgE75 in penultimate instar nymphs does not induce premature prothoracic gland degeneration in *Blattella germanica*. (F) Effect of BgE75 RNAi on the expression of BgFTZ-F1 and BgIAP1. A dose of 1 μg of dsBgE75 or dsMock was injected in newly emerged fifth instar nymphs, and mRNA levels were analyzed in the prothoracic gland of 4-day-old nymphs by qRT-PCR and normalized against BgActin-5C transcript. Vertical bars indicate the SEM (n = 4–7). The inset panel shows the RT-PCR analysis of BgE75 transcript reduction. (G) Effect of BgE75 RNAi on prothoracic gland degeneration. Newly emerged penultimate instar nymphs were injected with 1 μg of dsBgE75 or with dsMock. After 5 days, prothoracic glands were collected and labelled with TUNEL. To improve TUNEL interpretation prothoracic glands are silhouetted. Scale bars: 200 μm. Asterisks indicate differences statistically significant at $p \leq 0.05$(*); $p \leq 0.005$(**); $p \leq 0.0005$(***) (*t*-test).

simultaneously and showing that under these conditions, the premature expression of BgFTZ-F1 was totally abolished (Fig. 4D). Finally, the levels of BgIAP1 were significantly lower in the BgE75 knockdowns than in the dsMock-treated specimens. Again, the strong up-regulation of BgFTZ-F1 as well as the down-regulation of BgIAP1 in mid-instar BgE75 knockdown nymphs foreshadowed the onset of premature prothoracic gland degeneration.

To confirm that the premature expression of BgFTZ-F1 was responsible of the prothoracic gland degeneration in BgE75 knockdown nymphs, we reduced BgE75 and BgFTZ-F1 levels simultaneously by injecting 1 μg of the respective dsRNAs in freshly ecdysed last instar nymphs, and prothoracic glands were examined for signs of cell death by TUNEL labelling 6 days later. Importantly, under these conditions (n = 15), prothoracic glands did not degenerate (Fig. 4E).

Collectively, these results demonstrate that BgFTZ-F1 is a key regulator of the developmentally induced degeneration of the prothoracic gland, and that BgE75 acts as a suppressor of apoptosis by restricting the expression of BgFTZ-F1 to the end of the last nymphal instar and the onset of adult development.

JH prevents prothoracic gland degeneration

Once the role of BgIAP1, BgE75 and BgFTZ-F1 in the control of apoptosis in *B. germanica* prothoracic gland is established, and since this process occurs exclusively after the imaginal molt, we wondered whether the BgFTZ-F1-triggered degeneration of the prothoracic gland could be provoked during nymphal–nymphal transitions. With this aim, we ectopically induced BgFTZ-F1 expression by injecting 1 μg of dsBgE75 in freshly ecdysed penultimate instar nymphs. Whereas the levels of BgIAP1 were not affected, the treatment produced a strong up-regulation of BgFTZ-F1 (Fig. 4F). However, the prothoracic gland of these knockdown nymphs did not degenerate prematurely as shown by TUNEL labelling (Fig. 4G).

The main difference between the penultimate and the last nymphal instar is the absence of JH during the later (Cruz et al., 2003; Treiblmayr et al., 2006). This raised the possibility that JH might prevent prothoracic gland degeneration during the penultimate nymphal instar, even in the presence of ectopic high levels of BgFTZ-F1. We tested this hypothesis with two different experiments. First, newly emerged last instar nymphs were treated with methoprene, a potent JH analogue (Wu et

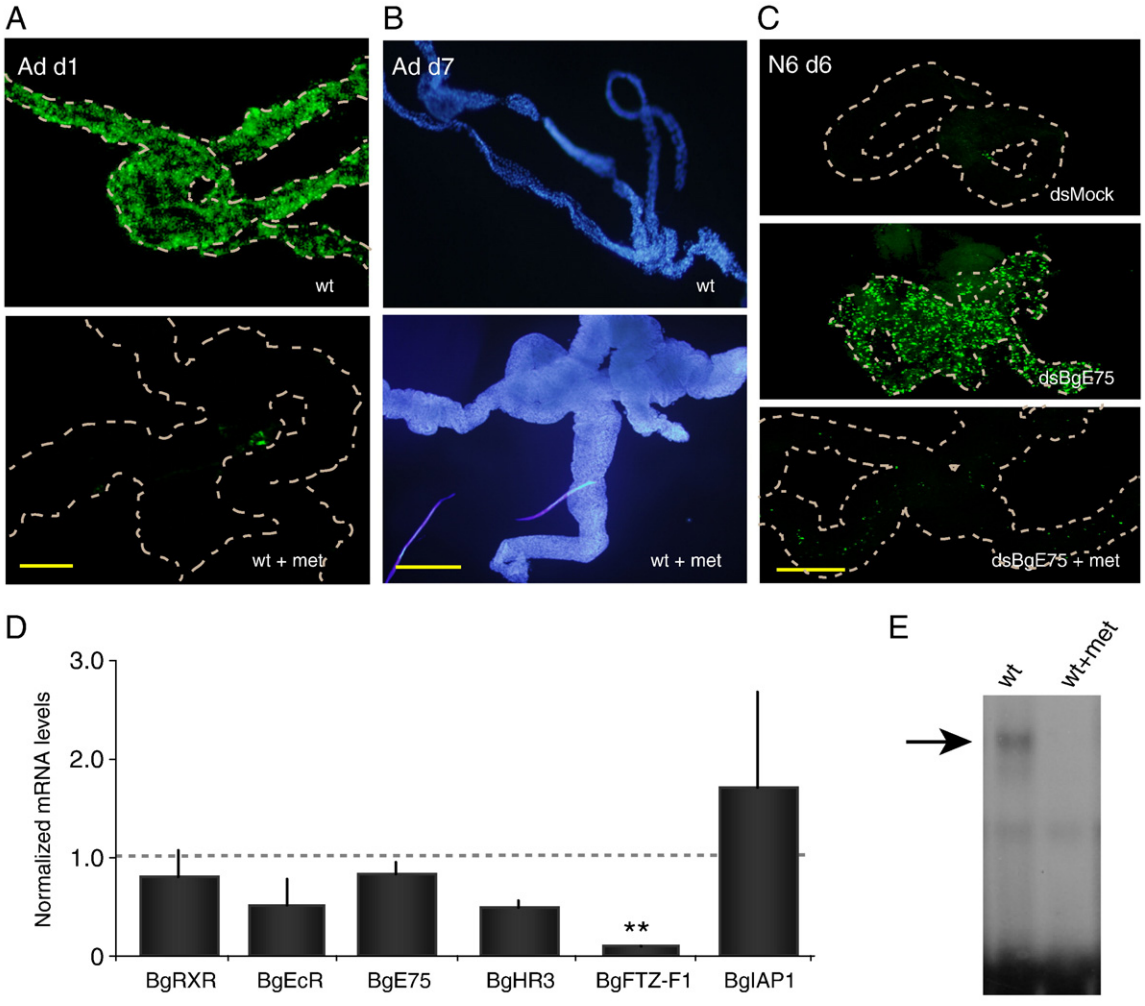

Fig. 5. The juvenile hormone analogue methoprene prevents prothoracic gland degeneration in *Blattella germanica*. (A) The anti-apoptotic effect of methoprene was demonstrated with TUNEL labelling of prothoracic glands from wild type newly emerged last instar nymphs (wt) or treated with methoprene (wt+met), 1 day after molting into adults. (B) The prothoracic glands of methoprene-treated animals presented a normal shape and were well developed even after 7 days of adult development, as revealed by DAPI staining. (C) A dose of 1 μg of dsBgE75 or dsMock was injected in newly emerged last instar nymphs. A fraction of dsBgE75-treated nymphs was also treated with 1 μg of methoprene (dsBgE75+met). After 6 days, staged prothoracic glands were collected and labelled with TUNEL. To improve TUNEL interpretation prothoracic glands are silhouetted. Scale bars: 200 μm. (D–E) Methoprene reduces BgFTZ-F1 levels in the prothoracic gland of *B. germanica*. (D) Newly emerged last instar nymphs were treated with 1 μg of methoprene (met) or with acetone (acet), and 1 day after the imaginal molt, mRNA levels of BgRXR, BgEcR, BgE75B, BgHR3A, BgFTZ-F1 and BgIAP1 were measured in the prothoracic gland by qRT-PCR. Transcript abundance values are normalized against BgActin-5C transcript. The relative expression for each gene was calculated based on their respective expression in wild type animals. Vertical bars indicate the SEM (n = 3–4). Asterisks indicate differences statistically significant at $p \leq 0.005$ (*t*-test). (E) EMSA analysis of nuclear extracts of prothoracic glands from wt and wt+met animals. Twenty prothoracic gland equivalents were used in each binding reaction. The protein samples were incubated with ^{32}P-labelled FTZ-F1 response element.

al., 2006; Liu et al., 2009), and scored for persistent prothoracic gland after the imaginal molt. Whereas prothoracic gland from untreated specimens degenerated 24 h after the molt, those from methoprene-treated animals showed no sign of degeneration and continued growing during the next 7 days (Figs. 5A,B). In the second experiment, freshly ecdysed last instar nymphs were simultaneously treated with dsBgE75 (to induce the premature expression of BgFTZ-F1) and with methoprene, and the degeneration of the prothoracic gland was studied 6 days later. Results (Fig. 5C) showed that whereas prothoracic glands of BgE75 knockdown nymphs presented a clear premature degeneration, the presence of methoprene rendered the prothoracic gland totally resistant to degeneration. These observations clearly confirm the critical role of JH in preventing the degeneration of the prothoracic gland during nymphal development.

In order to understand the molecular mechanism by which JH exert its anti-apoptotic effect, we wondered whether this hormone might act by modifying the activation of 20E-dependent genes, especially BgFTZ-F1. To address this question, we compared the mRNA levels of BgFTZ-F1 and other factors involved in this study in the prothoracic gland of untreated and methoprene-treated nymphs, just after the imaginal molt. As Fig. 5D shows, whereas the expression of BgEcR, BgRXR, BgE75-B, BgHR3-A and BgIAP1 was not significantly modified by the application of methoprene, the levels of BgFTZ-F1 were dramatically reduced (11-fold less than in untreated). Importantly, the same clear reduction was also observed in BgFTZ-F1 protein levels in the prothoracic gland of methoprene-treated adults (Fig. 5E).

Taken together, these results not only show that JH exerts an anti-apoptotic effect on the prothoracic gland, but also suggest that the singularly high expression of BgFTZ-F1 in the prothoracic gland during the nymphal–adult transition is due to the disappearance of JH in the last nymphal instar.

Discussion

During animal development, a critical balance between pro-apoptotic and anti-apoptotic factors controls cell viability. The regulation of this interplay results in highly regulated stage- and tissue-apoptotic responses, which are responsible of removing damaged and unwanted cells and tissues. One clear example of this is the metamorphosis in holometabolous insects, which entails on the destruction of larval tissues to accommodate the growth of new adult structures. The larval salivary gland and the midgut of *D. melanogaster* have been used to exemplify the mechanisms of developmentally regulated cell death in holometabolous insects (Yin and Thummel, 2005). However, given that metamorphosis arose from a hemimetabolous ancestor (Sehnal et al., 1996; Truman and Riddiford, 1999), it would be interesting to study whether the mechanisms that coordinate stage-specific apoptosis were already present in more primitive hemimetabolous insects or they are a novelty of holometabolous species. Unfortunately, up to now, no studies have been carried out to address the conservation of such mechanisms. Here, using reverse genetic studies, we report for the first time, a detailed functional analysis of the mechanisms that regulate cell death in the hemimetabolous insect *B. germanica*, using the adult-specific degeneration of the prothoracic gland as the system model.

BgIAP1 is a key survival factor in B. germanica

We started our work by characterizing BgIAP1 as the key death inhibitor in *B. germanica*, which in contrast to BgIAP2, exerts an essential role of repressing the premature death of all organs and tissues during nymphal development. The rapid degeneration observed in BgIAP1 knockdown nymph tissues suggests that active caspases are already present in all cells and that BgIAP1 is continuously counteracting them. The fact that DIAP1 in *D. melanogaster* is also required to impair inappropriate caspase activation during larval development (Wang et al., 1999; Yin and Thummel, 2004) indicates that the function of this protein is highly conserved in insects. Indeed, cell competence for apoptosis is achieved in some *D. melanogaster* tissues, such as the salivary glands, by down-regulating DIAP1 levels. Thus, transcriptional inhibition of DIAP1 expression is controlled by different mechanisms during *D. melanogaster* development, such as the *hippo* pathway (Huang et al., 2005), the presence of the transcriptional cofactor CREB binding protein (CBP) in mid-third instar salivary glands (Yin et al., 2007) or through HOW(L)-mediated inhibition of DIAP1 protein translation in mid-line glia cells (Reuveny et al., 2009). Interestingly, down-regulation of BgIAP1, in *B. germanica*, is only observed in adult prothoracic glands, and foreshadows the onset of destruction of this tissue. The correlation between the disappearance of BgIAP1 and the high levels of BgFTZ-F1 in the prothoracic gland after the imaginal molt, makes this nuclear receptor a good candidate to mediate the inhibition of BgIAP1 expression. However, we have not found functional evidence of such regulatory effect since the levels of BgIAP1 were unaffected in BgFTZ-F1 knockdown animals (unpublished results). Work directed to characterize the down-regulation of BgIAP1 expression in the adult prothoracic gland is currently in progress in our laboratory.

A 20E-dependent transcriptional hierarchy controls prothoracic gland degeneration

Our studies have demonstrated that a 20E-triggered transcriptional cascade of nuclear receptors that culminates with the proper activation of BgFTZ-F1 towards the end of the last nymphal instar and the onset of adult development tightly controls the stage-specific degeneration of the prothoracic gland of *B. germanica* (Fig. 6). During the cockroach nymphal development, this cascade is not only present in the prothoracic gland but also in other tissues, where it plays critical roles in molting, cell proliferation and ecdysteroid production (Cruz et al., 2006, 2007, 2008; Martín et al., 2006; Mané-Padrós et al., 2008). This complex hierarchy is initiated by the increase of ecdysteroid levels at mid-nymphal instar, which induces BgE75 expression (Mané-Padrós et al., 2008). Importantly, here we have demonstrated that the presence of BgE75 during the early increase in the ecdysteroid titer represses the activation of the 20E-dependent gene BgHR3 (Fig. 4). Given that BgHR3 is necessary for the activation of BgFTZ-F1 (Fig. 4D), the repressive effect of BgE75 on the expression of BgHR3 constrains the activation of the death-inducer factor BgFTZ-F1 to the nymphal–adult transition. The role of BgE75 as suppressor of cell death has not been observed during the larval development of *D. melanogaster*. Although salivary glands of late prepupae E75A and E75B repress *diap2* expression (Jiang et al., 2000), the mutation of *diap2* has revealed that this protein did not play any role in regulating apoptosis during *D. melanogaster* development (Huh et al., 2007; Yin and Thummel, 2005).

In *D. melanogaster*, a similar ecdysteroid-dependent cascade directs the stage-specific destruction of the salivary glands. In this insect, however, the cascade converges in the induction of *rpr* and *hid*, and the consequent massive caspase activation and cell death (Jiang et al., 2000). Interestingly, RPR and HID homologous proteins have not been reported in any insect outside *D. melanogaster* and closely related species, suggesting that although the role of 20E as inducer of cell death is conserved, the mechanisms by which the hormone controls such process would be different in hemimetabolous insects.

Prothoracic gland degeneration is mediated by the nuclear receptor BgFTZ-F1 and prevented by JH

Here, we have demonstrated that BgFTZ-F1 is a critical factor to specifically induce the degeneration of the prothoracic gland of *B. germanica* after the imaginal molt. Several observations support this:

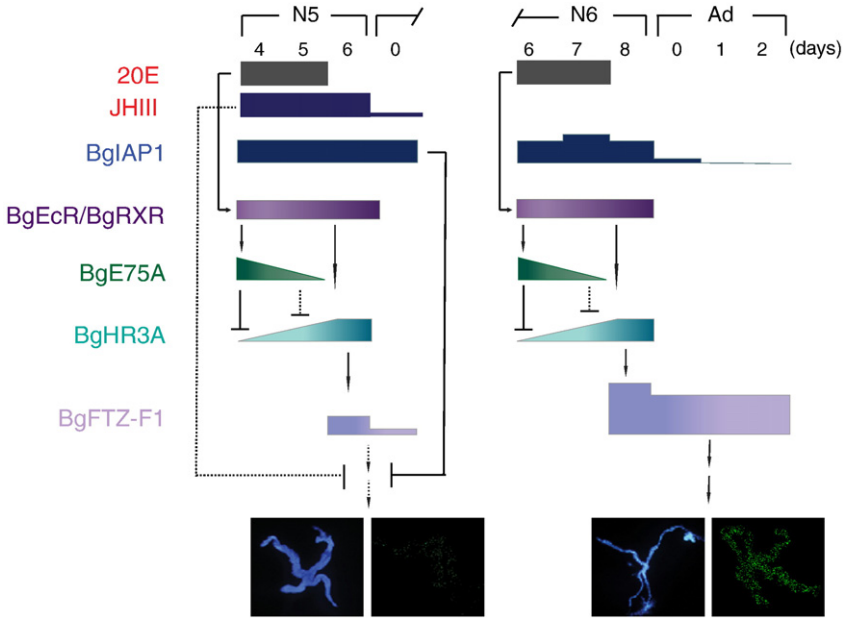

Fig. 6. A model for the ecdysteroid-mediated stage-specific cell death of the prothoracic gland in the hemimetabolous insect *Blattella germanica*. During ecdysteroid pulses that occur at each nymphal instar, the ecdysone receptor (BgEcR/BgRXR) binds to 20E and initiates a transcriptional cascade of nuclear receptors that includes the sequential activation of the early gene *BgE75* and the early-late gene *BgHR3*, and culminates with the up-regulation of the critical death-inducer BgFTZ-F1 at each developmental transition. Until the penultimate nymphal instar, the presence of BgIAP1 and the occurrence of low levels of BgFTZ-F1 maintain the viability of the gland. Conversely, in last nymphal instar, the absence of JH and the remarkable up-regulation of BgFTZ-F1, together with the decrease in BgIAP1 levels, make the prothoracic gland competent to execute a cell death program.

(1) the prothoracic gland of BgFTZ-F1 knockdown adults failed to degenerate; (2) ectopic expression of BgFTZ-F1 in mid-last nymphal instar, mediated by the reduction of BgE75, prematurely induced the degeneration of the prothoracic gland; (3) the prothoracic gland of last instar nymphs that had been simultaneously knockdown for BgE75 and BgFTZ-F1, thus preventing the ectopic expression of BgFTZ-F1 during mid-nymphal instar, also failed to degenerate; (4) the expression of BgFTZ-F1 in the prothoracic gland during the imaginal molt is significantly higher (12-fold) than during nymphal transitions, and its expression is maintained during the first days of the adult stage until the onset of prothoracic gland degeneration.

The nuclear receptor FTZ-F1 has also been implicated in the apoptotic response in *D. melanogaster*. In this insect, βFTZ-F1 acts as a transcriptional competence factor that provides the stage-specificity for salivary gland degeneration at the onset of the pupal development (Broadus et al., 1999). Moreover, its activity is required for the destruction of the cytoplasm (Lee and Baehrecke, 2001), nuclear DNA fragmentation (Lee et al., 2002) and controlling caspase levels in salivary gland cells (Martin and Baehrecke, 2004).

In addition, we have also demonstrated that the pro-apoptotic effect of BgFTZ-F1 is restricted to the last nymphal instar due to the anti-apoptotic effect exerted by JH before the last nymphal instar. Although JH regulates a number of developmental events in insects, its molecular mechanism of action still remains a matter of debate (Riddiford, 2008). The death inhibitor effect of JH has been also described in relation to the midgut remodelling that occurs during metamorphosis of the mosquito *A. aegypti* (Wu et al., 2006; Parthasarathy and Palli, 2007a) and the moth *H. virescens* (Parthasarathy and Palli, 2007b). Recently, it has been also shown that the larval fat body of JH-deficient *D. melanogaster* undergoes premature degeneration and that this response can be prevented by the application of methoprene. Interestingly, however, the anti-apoptotic effect of methoprene in *D. melanogaster* was based on the inhibition of two bHLH-PAS transcription factors involved in JH action, Methoprene-tolerant and Germ-cell expressed, but was not related, in contrast to *B. germanica*, to the suppression of the ecdysteroid-dependent transcriptional cascade (Liu et al., 2009).

In summary, our results led us to propose a model for developmentally regulated PCD in the hemimetabolous *B. germanica* (Fig. 6). In this model, JH is responsible to hold back the degeneration of the prothoracic gland during nymphal–nymphal transitions. The low levels of BgFTZ-F1 observed at the end of the penultimate nymphal instar would be below a critical threshold necessary to overcome the BgIAP1-mediated inhibition of prothoracic gland degeneration. During the last nymphal instar, however, the specific and dramatic up-regulation of BgFTZ-F1 in the prothoracic gland at the end of the instar (12 fold higher than in the penultimate instar) would overcome, in the absence of JH, the inhibitory effect of BgIAP1 making the prothoracic gland competent to execute the cell death program.

Our study shows that prothoracic gland degeneration in the nymphal–adult transition of *B. germanica* show conserved but also divergent features with respect to the highly derived and thoroughly studied species *D. melanogaster*. This underlines the importance of investigating basal, less modified species, in order to understand the evolutionary trends and mechanisms that led to the highly sophisticated holometabolan mode of metamorphosis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2010.07.012.

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