

# Transcription factor E93 specifies adult metamorphosis in hemimetabolous and holometabolous insects

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All immature animals undergo remarkable morphological and physiological changes to become mature adults. In winged insects, metamorphic changes either are limited to a few tissues (hemimetaboly) or involve a complete reorganization of most tissues and organs (holometaboly). Despite the differences, the genetic switch between immature and adult forms in both types of insects relies on the disappearance of the antimetamorphic juvenile hormone (JH) and the transcription factors Krüppel-homolog 1 (Kr-h1) and Broad-Complex (BR-C) during the last juvenile instar. Here, we show that the transcription factor E93 is the key determinant that promotes adult metamorphosis in both hemimetabolous and holometabolous insects, thus acting as the universal adult specifier. In the hemimetabolous insect *Blattella germanica*, *BgE93* is highly expressed in metamorphic tissues, and RNA interference (RNAi)-mediated knockdown of *BgE93* in the nymphal stage prevented the nymphal–adult transition, inducing endless reiteration of nymphal development, even in the absence of JH. We also find that *BgE93* down-regulated *BgKr-h1* and *BgBR-C* expression during the last nymphal instar of *B. germanica*, a key step necessary for proper adult differentiation. This essential role of E93 is conserved in holometabolous insects as *TcE93* RNAi in *Tribolium castaneum* prevented pupal–adult transition and produced a supernumerary second pupa. In this beetle, *TcE93* also represses expression of *TcKr-h1* and *TcBR-C* during the pupal stage. Similar results were obtained in the more derived holometabolous insect *Drosophila melanogaster*, suggesting that winged insects use the same regulatory mechanism to promote adult metamorphosis. This study provides an important insight into the understanding of the molecular basis of adult metamorphosis.

insect metamorphosis | insect hormone | evolution of metamorphosis

The transformation from immature stages to a fully reproductive adult form is a fundamental hormonally dependent process during the development of higher organisms. Insects have been very useful model organisms to uncover the mechanisms underlying this complex transformation as all winged insects undergo metamorphosis to reach adulthood. Particularly interesting is that this transformation can be completed through two different metamorphic strategies, either directly (hemimetaboly) or through an intermediate metamorphic–pupal stage (holometaboly). Hemimetabolous insects, such as true bugs, cockroaches, or locusts, hatch from the egg resembling miniature adults, with external wing pads that encase the wing primordia, and metamorphose into adults with functional wings and genitalia during the last juvenile (also called nymphal) instar. In contrast, the larvae of holometabolous insects, like beetles, butterflies, or flies, undergo a complete morphological transformation to form the adult. The reorganization is so dramatic in these insects that a two-stage metamorphic process bridged by the intermediate pupal stage is required to fully transform the crawling larva into a winged adult (1).

Despite dramatic differences between hemimetaboly and holometaboly, both types of metamorphosis require a temporally regulated balance between cell death of nymphal/larval tissues and growth and morphogenesis of adult ones (2). Two hormones control this precise balance, the steroid 20-hydroxyecdysone (20E) and the sesquiterpenoid juvenile hormone (JH), produced

by the prothoracic glands and corpora allata glands, respectively (3–5). Whereas periodic pulses of 20E promote every developmental transition, the presence or absence of JH determines the nature of each transition. Thus, JH acting through its receptor Methoprene-tolerant prevents adult differentiation during the preultimate immature stages by inducing the expression of the antimetamorphic transcription factor gene *Krüppel-homolog 1* (*Kr-h1*) (6). Upon entering into the last juvenile stage, the last nymphal instar of hemimetabolous insects or the pupae of holometabolous insects, the disappearance of JH and the down-regulation of the *Kr-h1* gene expression permit the metamorphic transformations resulting in the adult (7, 8). In holometabolous insects, also required is the repression of the pupal-specifier factor *Broad-Complex* (*BR-C*) during the pupal stage to allow a proper pupal–adult transition (6).

The role and mode of action of JH has been extensively studied since V. B. Wigglesworth discovered its antimetamorphic function in the 1930s (6, 9, 10). However, the molecular mechanism underlying the switch from juvenile to adult genetic programs in holometabolous and hemimetabolous insects is not completely understood. In the present study, we identified the transcription factor E93 as the key factor that promotes adult metamorphosis in *Blattella germanica*, as a model of hemimetaboly, and the holometabolous *Tribolium castaneum* and *Drosophila melanogaster*. We show that, despite the different developmental strategies between hemimetabolous and holometabolous insects, *E93* is highly up-regulated during the metamorphic

## Significance

Transformation from immature to a fully reproductive adult form is an essential process during the development of higher organisms. In insects, transition from juvenile to adult stages is triggered by the decline of the juvenile hormone, but the molecular mechanisms underlying the dramatic morphological and physiological changes remain poorly understood. Here, we report that a single factor, E93, controls juvenile-to-adult transition in hemimetabolous and holometabolous insects, thus acting as the universal adult specifier in winged insects. Interestingly, we find that E93 not only promotes adult metamorphosis but also represses the expression of the antimetamorphic genes *Krüppel-homolog 1* and *Broad-Complex*, ensuring the proper juvenile–adult transition. This study represents a significant step toward defining the molecular mechanisms underlying insect metamorphosis.

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Data deposition: The sequence reported in this paper has been deposited in the GenBank database [accession no. [HF678196](https://www.ncbi.nlm.nih.gov/nuclseq/HF678196) (*BgE93*)].

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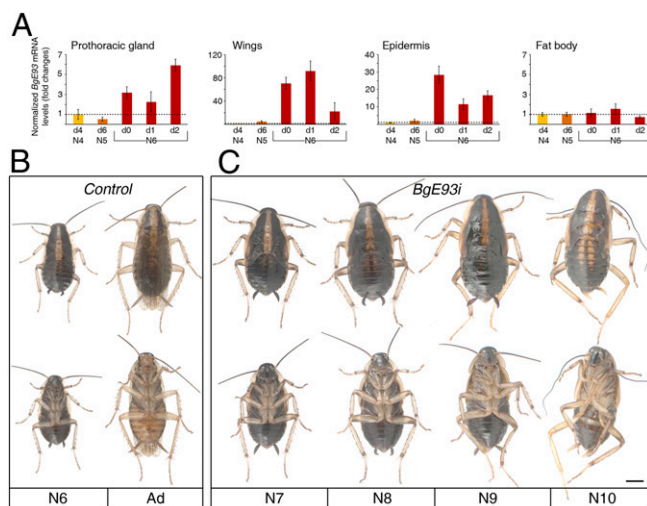
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stages in both types of insects and that this stage-specific activation is required for adult metamorphosis to occur. On the basis of our findings, we propose that *E93* functions as the universal adult specifier in insects.

## Results and Discussion

***E93* Is Up-Regulated in Metamorphic Tissues During the Nymphal-Adult Transition and Promotes Adult Differentiation in the Hemimetabolous *B. germanica*.** As a first step toward the characterization of the molecular mechanisms underlying adult differentiation, we studied the hemimetabolous insect *B. germanica* because hemimetabolous insects exhibit a simpler metamorphosis than holometabolous insects. This cockroach progresses through six nymphal instars (N1–N6) before molting into the adult. The metamorphic transition that occurs during the last nymphal instar (N6) is mainly characterized by changes in cuticle pigmentation and the appearance of genitalia and functional wings (Fig. S1 A–H). During this transition, the prothoracic gland, which is responsible for the synthesis of ecdysteroids, degenerates, and the corpora allata cells switch from the proliferative phase to the adult-specific nonproliferative state (11) (Fig. S1 I–N). Recently, we have shown that a complex 20E-triggered hierarchy of nuclear receptors controls these metamorphic transformations in *B. germanica* (11, 12). The same gene cascade, however, is also present during the previous nymphal–nymphal transitions (12), implying that stage-specific factors modify the responsiveness of target genes during the metamorphic transition. Because metamorphosis requires tissue death and remodeling, we speculated that both types of metamorphic changes could be regulated by the same factor(s) in a coordinated manner. We searched for such a determinant in *B. germanica* by looking for genes that control apoptosis in degenerating tissues (prothoracic gland) and that were also highly expressed in tissues undergoing morphogenetic transformations (wings and epidermis) during N6. Using this approach, we identified the *BgE93* gene (Fig. S2), the ortholog of *D. melanogaster* helix-turn-helix transcription factor *Eip93F* (13, 14), as it was strongly up-regulated in all metamorphic tissues during the N6 instar whereas no significant changes were observed in nonmetamorphic tissues, such as the fat body (Fig. 1A and Fig. S3 A and B).

To examine the role of *BgE93* in *B. germanica* metamorphosis, we used systemic RNA interference (RNAi) to knockdown *BgE93* during the last N6 instar. Injection of *BgE93* dsRNA into newly emerged penultimate N5 instar (*BgE93i*) caused a 95% reduction in the *BgE93* transcript level (Fig. S3 C–F). All *BgE93i* animals ( $n = 150$ ) molted to normal N6 nymphs but subsequently failed to metamorphose into adults and instead repeated the nymphal molt to a succeeding supernumerary N7 instar (Fig. 1B and C). Instead of the normal adult phenotype with brown cuticle and fully developed and articulated wings, supernumerary N7 *BgE93i* animals had all of the external characteristics of a nymph: namely, black cuticle, two thick stripes of black melanin in the pronotum, nymphal cerci, and, most notably, external wing pads with no evidence of morphological transformation of the wing epithelial cells (Fig. S4 A–A"). Consistently, the prothoracic gland of *BgE93i* nymphs did not show any sign of degeneration, and the corpora allata cells did not stop dividing (Fig. S4 B–C"). Furthermore, the duration of the N6 that is 48 h longer than previous stages in untreated insects was shortened in *BgE93i* ones. Unable to molt into adults, all of the supernumerary *BgE93i* nymphs continuously molted to new supernumerary nymphal instars to finally reach giant N10, when they terminally arrested development due to problems in the shedding of the exuvia (Fig. 1C). Overall, our data therefore identify *BgE93* as the critical stage-specific factor that promotes adult metamorphosis in the hemimetabolous *B. germanica*.

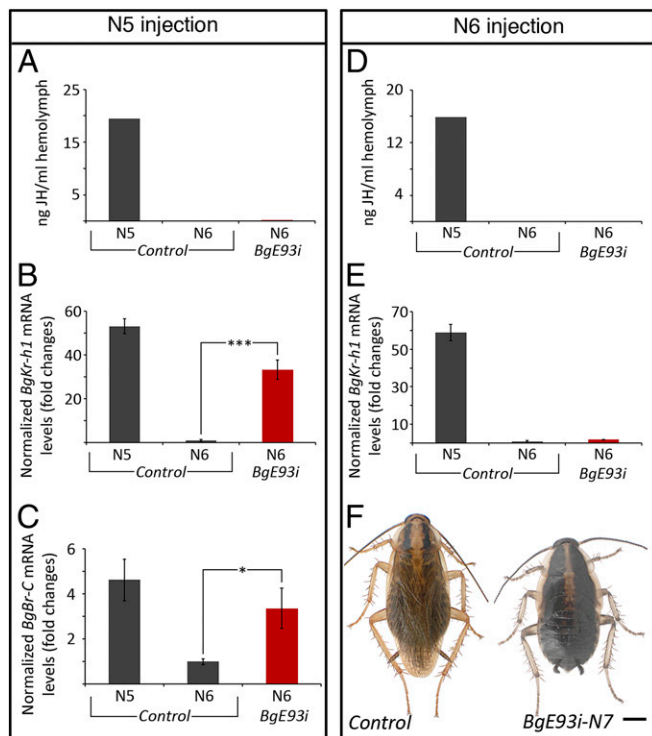


**Fig. 1.** *BgE93* is required for adult differentiation in the hemimetabolous insect *B. germanica*. (A) *BgE93* expression, normalized to *BgActin5C* (qRT-PCR), in metamorphic (prothoracic gland, wings, and epidermis) and non-metamorphic (fat body) tissues during the last three nymphal instars (N4, N5, and N6). Fold changes are relative to the expression of *BgE93* in N4 nymphs, arbitrarily set to 1. Error bars represent SEM ( $n = 3$ ). (B and C) Effect of RNAi of *BgE93* on adult metamorphosis. Newly emerged N5 nymphs were injected with *dsBgE93* (*BgE93i*) or with *dsMock* (Control) and left until the next molts. (B) Dorsal and ventral views of a N6 Control nymph and a winged adult (Ad). (C) Dorsal and ventral views of supernumerary *BgE93i* nymphs (from N7 to N10). (Scale bar: C, 2 mm.)

## *BgE93* Represses *BgKr-h1* and *BgBr-C* During the Last Nymphal Instar of *B. germanica*.

Adult differentiation requires the disappearance of JH and its target gene *Kr-h1* during the last nymphal instar of hemimetabolous insects (15, 16). To examine whether the failure of *BgE93i* nymphs to molt into adults is the result of elevated levels of JH and the *BgKr-h1* gene transcript during N6, we injected *BgE93* dsRNA into early penultimate N5 instar and measured JH titer and *BgKr-h1* expression in *BgE93i* nymphs during the following N6 stage. As shown in Fig. 2A, the disappearance of JH occurred normally in *BgE93i* nymphs, indicating that *BgE93* is not involved in the decline of the JH titer. However, *BgKr-h1* expression during N6 was not properly down-regulated in these animals (Fig. 2B), indicating that the decline of *BgKr-h1* transcription in the final nymphal instar is controlled by *BgE93* rather than from the disappearance of JH. The expression patterns of *BgE93* and *BgKr-h1* are consistent with the repressive role of *BgE93* as the up-regulation of *BgE93* at the onset of N6 precedes by 24 h the down-regulation of *BgKr-h1* (Fig. S5). This finding prompted us to explore the possibility that the *BgE93i* phenotypes might stem from the sustained expression of *BgKr-h1* in N6. If this hypothesis were correct, then depleting *BgE93* in 1-d-old N6 nymphs, when JH is absent and *BgKr-h1* expression has been already repressed by *BgE93*, would be sufficient to redirect the molt to adult differentiation. Consequently, we injected N6 nymphs with *BgE93* dsRNA 24 h after ecdysis (*N6-BgE93i*) and confirmed that, under this RNAi treatment, JH, *BgKr-h1*, and *BgE93* remained low during N6 in these animals (Fig. 2D and E and Fig. S3G). Interestingly, all of the *N6-BgE93i* nymphs also failed to metamorphose into adults and instead molted into perfect new supernumerary N7 nymphal instar (Fig. 2F), indicating that the repetition of the nymphal molt in the absence of *BgE93* was not due to the sustained *BgKr-h1* gene expression. Taken together, these results clearly demonstrate that *BgE93* is the crucial factor that controls the developmental switch from nymph to adult in the hemimetabolous *B. germanica* and that its metamorphosis-promoting effect is not





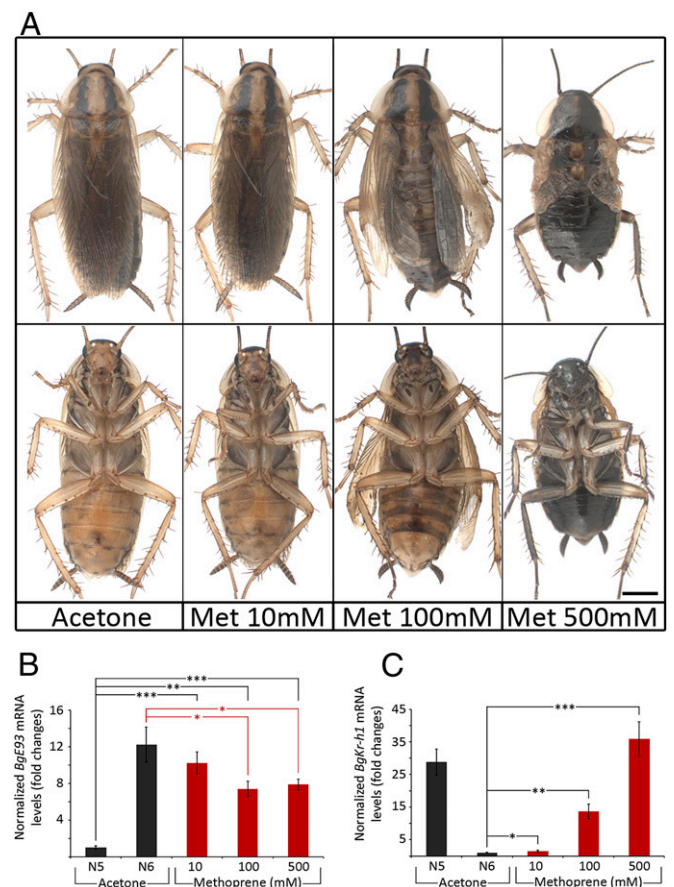
**Fig. 2.** *BgE93* represses *BgKr-h1* and *BgBr-C* expression during the last nymphal instar of *B. germanica*. (A–C) Newly emerged penultimate N5 nymphs were injected with *dsBgE93* (*BgE93i*) or with *dsMock* (Control) to ensure the reduction of *BgE93* at the onset of N6. (A) JH levels, (B) *BgKr-h1* expression, and (C) *BgBr-C* expression, relative to *BgActin5C*, measured by qRT-PCR in wings from 5-d-old N5 and 5-d-old N6 nymphs. Fold changes are relative to N6 control nymphs, arbitrarily set to 1. Error bars represent SEM ( $n = 5$ ). Asterisks indicate differences statistically significant as follows:  $*P \leq 0.05$ ;  $***P \leq 0.001$  (t test). (D–F) Twenty-four-hour-old N6 nymphs were injected with *dsBgE93* to knockdown *BgE93* expression after the natural decline of JH and *BgKr-h1* or with *dsMock* as control. (D) JH levels and (E) *BgKr-h1* expression relative to *BgActin5C* measured by qRT-PCR in wings from 3-d-old N5 and 3-d-old N6 nymphs. Fold changes are relative to N6 control nymphs, arbitrarily set to 1. Error bars represent SEM ( $n = 5$ ). (F) Dorsal view of adult control and a supernumerary *BgE93i* N7 nymph upon injection of *dsBgE93* in 1-d-old N6 nymphs. (Scale bar: F, 2 mm.)

exclusively channeled through the repression of *BgKr-h1* during the last nymphal instar, as adult metamorphosis is completely blocked in *BgE93i* nymphs irrespective of the presence or absence of *BgKr-h1*. Furthermore, we also show that the absence of JH and *BgKr-h1* during the final nymphal instar is not sufficient to cause the formation of the adult.

*Br-C* is the second transcription factor that is involved in regulation of metamorphosis of hemimetabolous insects. RNAi experiments showed that *B. germanica* *BgBr-C* is required for progressive growth of wings to reach the correct size and form at the end of the nymphal development, a function that is conserved in the hemimetabolous species *Oncopeltus fasciatus* and *Pyrhocoris apterus* (15, 17). *BgBr-C* mRNA is present in all nymphal stages, but its level declines in the final N6 nymphal instar paralleling *BgKr-h1* mRNA levels (16, 18), just after *BgE93* up-regulation (Fig. S5). Given this similarity, we therefore asked whether *BgE93* also controls *BgBr-C* repression during the last nymphal instar, and, for that, we measured *BgBr-C* levels in *BgE93i* animals in N6. Notably, as Fig. 2C shows, *BgE93* depletion prevented the down-regulation of *BgBr-C*, as was the case with *BgKr-h1*. So far, several studies show that *Kr-h1* and *Br-C* are induced by JH in hemimetabolous insects, leading to the assumption that the natural fall of JH at the onset of the final

nymphal instar is responsible for the down-regulation of *Kr-h1* and *Br-C* (15, 17, 19). Importantly, our results show that this idea is not correct as *BgKr-h1* and *BgBr-C* repression largely depends on *BgE93* rather than on the normal decline of JH levels observed in the wild-type shift from N5 to N6.

**The Antimetamorphic Effect of Methoprene Is Not Mediated by *BgE93* Repression.** The up-regulation of *BgE93* at the onset of the final nymphal instar of *B. germanica* coincides with the disappearance of JH. This inverse relationship, together with the antimetamorphic effect of ectopic JH during final nymphal instar in hemimetabolous insects (14, 15), suggests a possible effect of JH upon *BgE93* expression. To check this hypothesis, we first tested whether the JH-mimic methoprene would prevent adult metamorphosis in *B. germanica*. As Fig. 3A shows, when mid-penultimate N5 instar nymphs were treated with 10 nmol methoprene, all animals developed into normal adults after two molts. When 100 nmol methoprene were applied, slight nymphal characteristics such as patches of dark coloration were visible on the ventral abdomen of otherwise normal adults. In contrast, after exposure to 500 nmol, adult differentiation



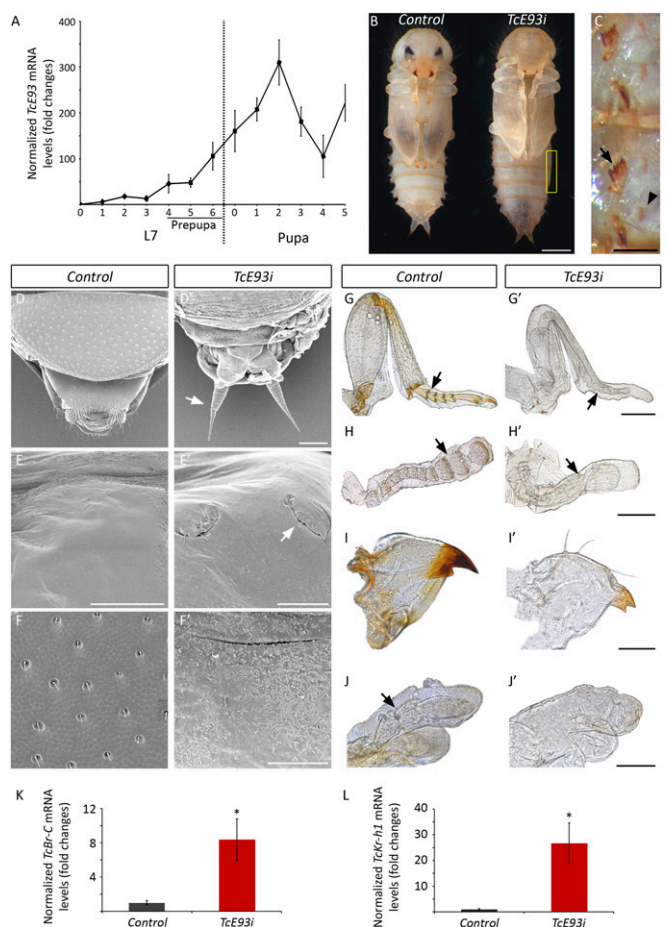
**Fig. 3.** The antimetamorphic effect of methoprene is not mediated by *BgE93* repression. (A) Dorsal (Upper) and ventral (Lower) views of adult animals resulting from acetone- and methoprene-treated mid-N5 nymphs showing that the severity of the juvenilization correlated with the levels of *BgKr-h1* expression during N6, irrespective of the levels of *BgE93*. (B and C) Expression levels of (B) *BgE93* and (C) *BgKr-h1*, relative to *BgActin5C* (qRT-PCR), were measured in wings of 3-d-old N5 (acetone-treated only) and 1-d-old N6 nymphs (acetone- and methoprene-treated). Fold changes are relative to acetone-treated N5 nymphs in B and acetone-treated N6 nymphs in C, arbitrarily set to 1. Error bars represent SEM ( $n = 5$ ). Asterisks indicate differences statistically significant as follows:  $*P \leq 0.05$ ;  $**P \leq 0.005$ , and  $***P \leq 0.001$  (t test). (Scale bar: A, 2 mm.)

was strongly prevented, and the animals molted into an almost perfect supernumerary N7 instar (Fig. 3A, Right). We next examined the expression of *BgE93* in these animals during the N6 stage and found that, although a significant reduction in *BgE93* mRNA levels was observed, *BgE93* was still strongly up-regulated regardless of the concentration of methoprene used (Fig. 3B). Therefore, these data show that the critical stage-specific induction of *BgE93* in N6 is controlled in a JH-independent manner although the disappearance of JH is also required for the full up-regulation of this gene.

The results above demonstrate that topical application of methoprene blocks metamorphosis despite an elevated level of *BgE93*. To address a possible mechanism of methoprene action, we examined its effect on the expression of the antimetamorphic *BgKr-h1* gene as this transcription factor is activated by JH in all insects analyzed to date (20, 21). We found a clear methoprene dose-dependent increase in *BgKr-h1* transcript levels (Fig. 3C). Comparing *BgKr-h1* and *BgE93* transcript levels with the phenotypes of the methoprene-treated animals, it is interesting to note that adult metamorphosis was blocked only when the increase of *BgKr-h1* transcript levels was comparable with that normally seen during the penultimate N5 nymphal instar of *B. germanica*, irrespective of *BgE93* transcript levels (Fig. 3C). These results suggest that the *BgE93*-dependent metamorphic differentiation is prevented in the presence of high levels of *BgKr-h1*, indicating that two requirements are essential to promote adult differentiation during the last nymphal instar, the up-regulation of the *BgE93* transcript level and the repression of the *BgKr-h1* gene expression. In this regard, our data suggest that the very low levels of *BgKr-h1* mRNA during N6 are due to the strong up-regulation of *BgE93* and to the critical decline of the JH titer. This JH decline, which is not sufficient for the down-regulation of *BgKr-h1* (Fig. 2 A and B), is presumably required to avoid the detrimental reinduction of *BgKr-h1* during the last nymphal instar.

Overall, our data demonstrate that *BgE93* acts as the adult specifier during the last nymphal instar of the hemimetabolous *B. germanica* by promoting the metamorphic transition to the adult and also by repressing *BgKr-h1* expression, thus eliminating the antimetamorphic effect of the latter and, in doing so, ensuring the proper nymphal–adult transition when the appropriate stage of development is achieved.

**The Role of E93 as the Adult Specifier Is Conserved in the Basal Holometabolous *T. castaneum*.** Next, we asked whether the essential role of E93 in adult differentiation might be common to holometabolous insects, which arose from hemimetabolous ancestors some 350 million years ago (1, 22) and turned to the coleopteran *T. castaneum* as a model of holometabolous development. In contrast to hemimetabolous insects, transformation from immature wingless larvae to winged adults in holometabolous insects requires a transitory stage known as the pupa, which can be considered as the last immature stage (Fig. S6). The genome of *T. castaneum* contains one *E93* homolog (*TcE93*) that is highly up-regulated during the end of the last larval stage (prepupal stage) and the entire pupal period (Fig. 4A). To assess the functional role of *TcE93*, we injected *TcE93* dsRNA into midfinal instar larvae, which resulted in 98% reduction of *TcE93* transcript abundance during the pupal period (Fig. S7A). The majority of the *TcE93i* larvae (84%;  $n = 69$ ) pupated normally, although with reduced elytra and wings (Fig. S7 C–F). Consistent with the function as the adult specifier, depletion of *TcE93* prevented adult morphogenesis as the *TcE93i* pupae produced a supernumerary second pupa instead of an adult at the end of the pupal stage (Fig. 4 B and C). A closer examination of the supernumerary pupae after removal of the apolysed pupal cuticle revealed a new cuticle with all of the pupal-specific structures, such as a second set of urogomphi,



**Fig. 4.** Loss of *TcE93* blocks adult differentiation and produces a supernumerary pupa in the holometabolous insect *T. castaneum*. (A) Developmental expression profile of *TcE93* relative to *TcRpl32* (qRT-PCR) from last larval stage (L7) and the pupal period. Fold changes are relative to *TcE93* expression in 0-d-old L7 larvae, arbitrarily set to 1. Error bars represent SEM ( $n = 3$ ). (B and C) Effect of RNAi of *TcE93* on adult metamorphosis. Mid-L7 larvae were injected with *dsTcE93* (*TcE93i*) or with *dsMock* (Control). (B) Ventral view of a 5-d-old control and *TcE93i* pupae. (C) High magnification of the abdomen of *TcE93i* pupa (rectangle in B) showing ectopic gin traps (arrowhead) underneath the ones (arrow) from the external cuticle. (D–J') Adult metamorphic transformations were blocked in *TcE93i* pupae. (D–F') Scanning electron microscopy photographs of (D', E', and F') 5-d-old *TcE93i* pupae compared with (D, E, and F) a 5-d-old control pupae, after removal of the apolysed first pupal cuticle in both cases, showing (arrow in D') supernumerary pupal urogomphi, (arrow in E') supernumerary pupal gin traps, and (F') the pupal-like cuticle surface with the absence of the typical adult microsculpture and rounded pits with sensillae (F). (G–J') Comparison of the external morphology of appendages in (G–J) pharate control adults and (G'–J') supernumerary *TcE93i* pupae 5 d after pupation. (G and G') Hind-legs, (H and H') antennae, (I and I') mandible, and (J and J') maxilla. Arrows indicate the degree of adult segmentation and pigmentation. (K and L) *TcE93* represses the expression of the pupal specifier *TcBr-C* and *TcKr-h1* during the pupal stage. (K) *TcBr-C* and (L) *TcKr-h1* mRNA levels were measured by qRT-PCR in control and *TcE93i* animals 2 d after pupation. Transcript abundance values are normalized against *TcRpl32* transcript. Fold changes are relative to *TcKr-h1* and *TcBr-C* expression in control pupae, arbitrarily set to 1. Error bars in K–L indicate the SEM ( $n = 5$ ). Asterisks indicate differences statistically significant as follows:  $*P \leq 0.05$  (t test). (Scale bars: B, 0.5 mm; C, D, D', E, I, I', J, and J', 100  $\mu$ m; E', F, and F', 50  $\mu$ m; G, G', H, and H', 200  $\mu$ m.)

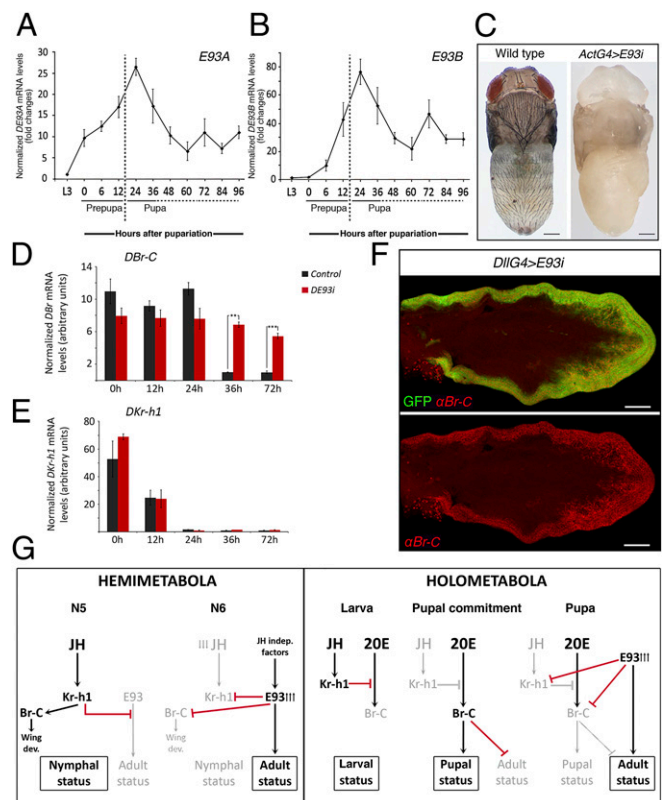
abdominal gin traps, and a surface with the characteristic pupal-like microsculpture (Fig. 4 D–F'). In addition, the appendage metamorphic changes normally occurring at the pupal stage, which include segmentation, pigmentation, and major changes in



shape, were also prevented (Fig. 4 *G–J*). Confirming that the adult genetic program has not been installed in the *TcE93i* pupae, the activation of the adult-specific cuticle gene *TcCPR27* normally occurring during the pupal–adult molt (23) was completely abrogated (Fig. S7B).

In *T. castaneum*, adult metamorphosis requires the disappearance of the *TcKr-h1* and *TcBr-C* transcripts during the pupal period (20, 21, 24). In a consistent manner, JH treatment at the onset of the pupal stage of *T. castaneum* causes the reexpression of *TcKr-h1* and *TcBr-C* and the block of adult morphogenesis (18, 20, 21). Having established that BgE93 represses *BgKr-h1* and *BgBr-C* expression in the final nymphal instar of *B. germanica*, we next tested whether a similar relation occurs in *T. castaneum*. We found that mRNA levels of *TcKr-h1* and *TcBr-C* were maintained at high levels in *TcE93i* pupae (Fig. 4 *K* and *L*), clearly indicating that TcE93 is necessary for the down-regulation of *TcKr-h1* and *TcBr-C* during the pupal stage of *T. castaneum*. Collectively, our findings indicate that the function and the regulatory mechanisms underlying E93-mediated adult differentiation are evolutionary conserved between hemimetabolous and holometabolous insects. In this regard, it is important to note that, unlike a highly conserved role of *Kr-h1* as the JH-dependent repressor of metamorphosis in hemimetabolous and holometabolous insects, the role of *Br-C* is dramatically different in these insect groups. Whereas *Br-C* does not play major metamorphic effects in hemimetabolous insects, being mainly limited to wing size control (15, 17, 19), it exerts a crucial stage-specific metamorphic role as the pupal specifier in all holometabolous insects studied so far (18, 20, 24). Previous work has shown that the evolution of the metamorphic functions of *Br-C* has been achieved, in part, through marked changes in its expression. Although *Br-C* is expressed during embryogenesis and throughout nymphal development in hemimetabolous insects, its expression is confined to the prepupal period of the final larval instar in holometabolous insects (15, 17, 19, 20). In contrast to these heterochronic changes of *Br-C* expression, its E93-dependent down-regulation during the metamorphic stage is a conserved trait of hemimetaboly and holometaboly. Based on this conservation, it is tempting to speculate that the cooption of *Br-C* for specifying the pupal state in holometabolous insects could have been favored, in addition to the heterochronic changes of *Br-C* expression, by the conserved transcriptional relationship between E93 and *Br-C* during the last metamorphic transition already present in hemimetabolous insects.

**E93 Is also Required for Adult Differentiation in the Derived Holometabolous *D. melanogaster*.** Finally, we have found that the role of E93 is also conserved in the highly derived holometabolous insect *D. melanogaster*. Although first identified as a specific regulator of cell death during the prepupal stage (13, 14), DE93 has recently been found to be a more general temporal regulator of gene expression during the metamorphosis of the fly (25, 26). Consistent with its role as adult specifier, we observed that *DE93* expression is restricted to the metamorphic prepupal and pupal stages (Fig. 5 *A* and *B*). Likewise, to confirm that silencing *DE93* blocks adult differentiation, we next assayed the effects of down-regulating *DE93* with an RNAi transgene driven by the ubiquitous *Actin-Gal4* driver. These flies with reduced levels of the *DE93* transcript (70% reduction in *DE93A* and 80% in *DE93B*) pupated normally but died at the end of the pupal stage with an overall impairment of adult differentiation (Fig. 5 *C* and Figs. S8 and S9 *E* and *F*). In accordance with this result, we found that the expression level of the pupal gene *DEdg78E* was unaffected whereas the induction of adult-specific *DACP65A* gene was abrogated in *DE93*-depleted flies (Fig. S9 *A–D*). Moreover, the absence of *DE93* during the pupal stage of *D. melanogaster* also prevented the critical down-regulation of *DBr-C* that is required for adult differentiation to occur (Fig. 5 *D* and *F*). In contrast to



**Fig. 5.** DE93 is necessary for proper differentiation of adult characters and for the repression of the pupal specifier *DBr-C* in the derived holometabolous insect *D. melanogaster*. (*A* and *B*) Developmental expression profile of the two *D. melanogaster* *DE93* isoforms, (*A*) *DE93A* and (*B*) *DE93B*, relative to *DRpL32* (qRT-PCR) from last larval stage (L3) and the prepupal and pupal periods. Fold changes are relative to *DE93* expression in L3, arbitrarily set to 1. Error bars represent SEM ( $n = 3$ ). (*C*) Dorsal views of wild-type and *ActG4>DE93i* animals 96-h after puparium formation (APF). (*D* and *E*) Expression levels of (*D*) *DBr-C* and (*E*) *DKr-h1* relative to *DRpL32* in wild-type (in black) and *ActG4>DE93i* pupae (in red) (qRT-PCR). Fold changes are relative to 72-h APF wild-type pupae, arbitrarily set to 1. Error bars represent SEM ( $n = 5$ ). Asterisks indicate differences statistically significant as follows:  $***P \leq 0.005$ ;  $****P \leq 0.0005$  (t test). (*F*) Wing from 30-h APF pupa expressing a *DE93*-RNAi construct and GFP (Upper) under the control of *Distal-less* (*Dll*)-*Gal4* driver. *DBr-C* expression is maintained in absence of *DE93* in the distal part of the wing (red, Lower). (Scale bars: *C*, 0.3 mm; *F*, 200  $\mu$ m.) (*G*) Model depicting the regulatory activity of E93 in controlling adult differentiation and repressing the expression of *Kr-h1* (except in *D. melanogaster*) and *Br-C* in hemimetabolous and holometabolous insects. Black arrows represent inductive effects, and red lines represent repressive effects. Gray colors denote genes and transcriptional regulatory events that are absent during each particular period.

*T. castaneum*, the absence of *DE93* did not prevent the decline of *DKr-h1* expression during the pupal stage, likely due to the uniqueness of *D. melanogaster* development (Fig. 5*E*). Indeed, it has been shown that the repression of *DKr-h1* that is required for the neural maturation and remodeling during the metamorphic stage of the fly is controlled by the conserved homeobox protein Orthodenticle acting together with the ecdysone receptor (27).

**Conserved Function of E93 in Hemimetabolous and Holometabolous Insects.** Our study has demonstrated that, despite the evolutionary distance and the differences in the developmental strategies of reaching adulthood, E93 is the universal adult specifier that acts as the “master factor” of adult metamorphosis in hemimetabolous and holometabolous insects. Based on our results, we thus propose the following model for adult metamorphosis in hemimetabolous

and holometabolous insects (scheme in Fig. 5G): (i) During the preterminal nymphal stages in hemimetabolous insects, the JH-effector Kr-h1 maintains the nymphal status of the animal by preventing the adult-promoting activity of E93. At the onset of the last nymphal instar, increasing levels of E93 down-regulate Kr-h1 expression and induce adult differentiation. Coupling the adult promoting activity with the repression of the antimetamorphic factor Kr-h1 in a single regulatory factor ensures that a proper nymphal–adult transition takes place during the last nymphal instar. E93 also represses transcription of Br-C during the last nymphal instar of hemimetabolous insects although Br-C has not been linked with metamorphosis in these insects. Concurrently with E93 up-regulation, the hemolymph JH titer drops to undetectable levels, preventing Kr-h1 reinduction. It has been proposed that the acquisition of a metamorphosing “critical weight” triggers the dramatic decline in the JH titer (28). Given that the fall of the JH titer occurs simultaneously with the strong up-regulation of E93, we hypothesize that a similar critical weight mechanism may control the latter event. (ii) In contrast to hemimetabolous insects, holometabolous metamorphosis takes place in two different stages, first to pupa and then to adult. The transition to pupal stage is mainly controlled by the pupal specifier Br-C after the decline of the JH hemolymph titer and the Kr-h1 transcript in the last larval instar (29). Upon pupation, and similar to the last nymphal instar in hemimetabolous insects, E93 induction promotes adult differentiation and also ensures the repression of the antimetamorphic factors Br-C and Kr-h1 (with the exception of *D. melanogaster*), thus allowing adult morphogenesis to take place.

Similar to the last juvenile stages in insects, mammalian puberty, including that of humans, is characterized by a number of hormonal events, resulting in the attainment of adult reproductive capacity. Two mammalian E93 orthologs, ligand-dependent corepressor (LCoR) and its paralog LCoR-like, have been identified

(30, 31). LCoR is widely expressed in fetal and adult tissues and has been reported to interact with a series of ligand-bound steroid nuclear receptors, including the thyroid hormone receptor, a protein that controls metamorphosis in amphibians (30, 32). Given the conservation of the E93/LCoR factors, the interesting possibility remains to be explored that such factors could be involved in the regulation of the adult genetic program during the passage of puberty in mammals or the amphibian metamorphosis.

In conclusion, our study has clearly demonstrated that E93 is the universal adult specifier in hemimetabolous and holometabolous insects. The present work thus represents a significant step toward defining the molecular mechanisms underlying insect metamorphosis. Furthermore, it contributes to our understanding of evolutionary conservation of the molecular program determining the developmental transition to adulthood in animals.

## Materials and Methods

Specimens of *B. germanica* were obtained from a colony reared in the dark at  $30 \pm 1^\circ\text{C}$  and 60–70% relative humidity. Wild-type *T. castaneum* strain and the enhancer-trap line *pu11* (obtained from Y. Tomoyasu, Miami University, Oxford, OH) were reared on organic wheat flour containing 5% nutritional yeast and maintained at  $29^\circ\text{C}$  in constant darkness. Flies were raised on standard *D. melanogaster* medium at  $25^\circ\text{C}$ , unless otherwise required. Details on the metamorphic characteristics of each insect strain, as well as other experimental methods, are provided in *SI Materials and Methods*.

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