Received: 18 June 2019 | Revised: 18 July 2019 | Accepted: 23 July 2019

DOI: 10.1002/arch.21609

Archives of INSECT BIOCHEMISTRY AND PHYSIOLOGY WILEY

MICRO-REVIEW

Krüppel homolog 1 and E93: The doorkeeper and the key to insect metamorphosis

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Funding information
Ministerio de Economía y Competitividad, Grant/Award Numbers: CGL2015–64727-P, CGL2012–36251; Catalan Government, Grant/Award Number: 2017 SGR 1030; European Fund for Economic and Regional Development

Abstract

Insect metamorphosis is regulated by two main hormones: ecdysone (20E), which promotes molting, and juvenile hormone (JH), which inhibits adult morphogenesis. The transduction mechanisms for the respective hormonal signals include the transcription factors Krüppel homolog 1 (Kr-h1) and E93, which are JH- and 20E-dependent, respectively. Kr-h1 is the main effector of the antimetamorphic action of JH, while E93 is a key promoter of metamorphosis. The ancestral regulatory axis of metamorphosis, which operates in insects with hemimetabolan (gradual) metamorphosis and is known as the MEKRE93 pathway, is based on Kr-h1 repression of E93. In the last juvenile stage, when the production of JH dramatically decreases, Kr-h1 expression is almost completely interrupted, E93 becomes upregulated and metamorphosis proceeds. The holometabolan (complete) metamorphosis mode of development includes the peculiar pupal stage, a sort of intermediate between the final larval instar and the adult stage. In holometabolan species, Broad-Complex (BR-C) transcription factors determine the pupal stage and E93 stimulates the expression of BR-C in the prepupa. The MEKRE93 pathway is conserved in holometabolan insects, which have added the E93/BR-C interaction loop to the ancestral (hemimetabolan) pathway during the evolution from hemimetaboly to holometaboly.

KEYWORDS
broad-complex, E93, ecdysone, juvenile hormone, Krüppel homolog 1, metamorphosis

1 | INTRODUCTION

Metamorphosis is a key innovation in insect evolution, wherein the individual acquires characteristic adult features and stops molting during postembryonic development. The ancestral metamorphosis mode was hemimetaboly, in which the embryogenesis gives rise to a first-instar nymph that features the essential adult body structure. The nymphs grow gradually and the final molt to the adult stage completes the formation of functional wings and genitalia. Another metamorphosis mode known as holometaboly emerged from hemimetaboly; this is characterized by embryogenesis that produces a larva with a body form that may be substantially different from that of the adult. The larva grows through various stages until molting to the pupal stage, which bridges the gap between the morphologically divergent larva and that of the winged and reproductively competent adult (Belles, 2011).

In both modes, metamorphosis is regulated by two major hormones: the juvenile hormone (JH), chemically a terpenoid, and ecdysone plus its biologically active derivative, 20-hydroxyecdysone (20E), which are steroids (Nijhout, 1994). The function of these hormones is underpinned by the mechanisms that transduce the hormonal signal through a hierarchical pathway of gene activation. The 20E signaling pathway was first described in the 1990s, whereas the most important details of the JH pathway were only revealed recently. The present review updates the information on these pathways, focusing on the role of two emblematic factors, Krüppel homolog 1 (Kr-h1) and E93, and their key role in the regulation of insect metamorphosis.

2 | THE ECDYSONE AND JH SIGNALING PATHWAYS

Initial work that unveiled the main elements of the ecdysone signaling pathway was carried out in the fly *Drosophila melanogaster*, as described in comprehensive reviews (King-Jones & Thummel, 2005; Ou & King-Jones, 2013). Early studies by Clever, Karlson and Ashburner established a model wherein ecdysone would bind to its receptor and induce the expression of early genes, whose respective products (the early proteins) would induce the expression of late genes. At the same time, the ecdysone receptor complex would repress the expression of the late genes, and the early proteins would repress the expression of their own genes. The so-called Ashburner model was confirmed early in the 1990s by the identification of a number of genes corresponding to the ecdysone receptor (EcR) and its receptor partner, Ultraspiracle (USP), as well as a number of early and late genes encoding signal transducers (Hill, Billas, Bonneton, Graham, & Lawrence, 2013; King-Jones & Thummel, 2005; Ou & King-Jones, 2013). Among the early response genes discovered to date, *Broad-Complex* (*BR-C*) and *E93* are of particular interest for this review, as we shall see below.

The JH signaling pathway was reviewed recently (Jindra, Belles, & Shinoda, 2015). Relative to the discovery of the receptor for 20E, it took additional two decades before a JH receptor has been established. In vitro and in vivo genetic studies definitively demonstrated that Methoprene-tolerant (Met), a transcription factor belonging to the basic helix–loop–helix-Per-ARNT-Sim (bHLH-PAS) family, behaves as a genuine JH-specific receptor (Bittova et al., 2019; Charles et al., 2011; Jindra, Uhlirova, Charles, Smykal, & Hill, 2015). Previously, RNAi depletion of Met in the beetle *Tribolium castaneum* was shown to induce a precocious metamorphosis to pupa, which provided a direct relation between Met and JH signaling (Konopova & Jindra, 2007). RNAi experiments also demonstrated the role of Met as a transducer of the JH signal in hemimetabolan species, from cockroaches, such as *Blattella germanica* (Lozano & Belles, 2014) to bugs, such as *Pyrrhocoris apterus* (Konopova, Smykal, & Jindra, 2011). The absence of developmental phenotypes in the *Met* mutants of *D. melanogaster* was explained later, because in this species *Met* has a paralog gene, *germ cell-expressed* (*gce*), with partially redundant functions with respect to *Met*, while *T. castaneum* has only one *Met* gene (Jindra, Belles, et al., 2015). As in the case of 20E receptor, the JH receptor is not a single protein. JH binding stimulates Met (or gce) to form a complex with another bHLH-PAS protein called Taiman (Tai, also known as FISC or SRC; Jindra, Belles, et al., 2015). In the cockroach *B. germanica*, RNAi experiments specifically depleting different Tai isoforms have demonstrated that Tai mediates the inhibitory effects

of JH on metamorphosis (Lozano, Kayukawa, Shinoda, & Belles, 2014). The JH-Met + Tai complex, in turn, activates the transcription of the gene Krüppel homolog 1 (Kr-h1), the main effector of the antimetamorphic action of JH.

3 | KRÜPPEL HOMOLOG 1, THE METAMORPHOSIS DOORKEEPER

Kr-h1 was discovered in *D. melanogaster* as a gene with structural similarity to the segmentation gene *Krüppel*, with which it shares the zinc-finger motifs and amino acid spacers connecting them. In *D. melanogaster*, *Kr-h1* expresses two major isoforms—α and β. The α isoform predominates in postembryonic stages and was initially reported as mediating ecdysone signaling in the larval–pupal transition (Beck, Pecasse, & Richards, 2004; Pecasse, Beck, Ruiz, & Richards, 2000), whereas the β isoform is abundantly expressed in embryonic neuronal cells (Beck et al., 2004). The first evidence to connect Kr-h1 and JH was also obtained in *D. melanogaster*. In this fly, the adult epidermis of the abdomen derives from larval histoblasts, which start proliferating after puparium formation. Administration of JH before the prepupal stage prevents the normal differentiation of the abdominal epidermis and the bristles that should be formed in the adult are shorter or simply do not develop (Ashburner, 1970). Similarly, *Kr-h1* expressed ectopically in the abdominal epidermis during metamorphosis of *D. melanogaster* also resulted in missing or short bristles, thereby suggesting that Kr-h1 mediates the antimetamorphic action of JH (Minakuchi, Zhou, & Riddiford, 2008). In *T. castaneum*, RNAi depletion of Kr-h1 in young larvae caused a precocious larval–pupal transformation, providing clear evidence that Kr-h1 represses metamorphosis and works downstream from Met in the JH signaling pathway (Minakuchi, Namiki, & Shinoda, 2009). The antimetamorphic action of Kr-h1 was generalized to the hemimetabolan species in two parallel works conducted in the cockroach *B. germanica* (Lozano & Belles, 2011) and the bugs *P. apterus* and *Rhodnius prolixus* (Konopova et al., 2011), respectively. In these two studies, RNAi experiments showed that Kr-h1 depletion in nymphs in the penultimate or antepenultimate nymphal stage triggers precocious metamorphosis.

4 | E93, THE KEY TO METAMORPHOSIS

E93 is an early gene in the ecdysone signaling cascade that is specifically expressed in late prepupae of *D. melanogaster*. The gene encodes for a protein with RHF domains that are significantly similar to pipsqueak motifs, which was found to be a key player in the degeneration process of the salivary glands during *D. melanogaster* metamorphosis (Baehrecke & Thummel, 1995; Lee et al., 2000; Woodard, Baehrecke, & Thummel, 1994). However, the action of E93 in metamorphosis is not restricted to the regulation of degeneration processes, as it also plays morphogenetic roles. Mou, Duncan, Baehrecke, and Duncan (2012) observed that *E93* is widely expressed in adult cells of *D. melanogaster* pupa, as it is required for patterning processes. This suggested that E93 might play a general role in changing the responsiveness of target genes during metamorphosis. Studying the induction of the *Distal-less* (*Dll*) gene within bract cells of the pupal leg using epidermal growth factor (EGF) receptor signaling, Mou et al. (2012), found that E93 causes *Dll* to become responsive to EGF receptor signaling, indicating that E93 is both necessary and sufficient for directing this switch. These results suggested that E93 controls the responsiveness of many other target genes because it is generally required for patterning during metamorphosis (Mou et al., 2012). RNAi experiments carried out 1 year later (Ureña, Manjón, Franch-Marro, & Martín, 2014) showed that E93-depleted *D. melanogaster* larvae are able to pupate but die at the end of the pupal stage. In the beetle *T. castaneum*, E93 depletion by RNAi prevented the pupal–adult transition, resulting in the formation of a supernumerary second pupa. Similar results were obtained in the cockroach *B. germanica*, where E93 depletion in nymphs prevented the nymphal–adult transition, giving rise to reiterated supernumerary nymphal instars (Ureña et al., 2014). Subsequently, Belles and Santos (2014) showed that the expression of *E93* in juvenile nymphs of *B. germanica* is inhibited by the transcription factor Krüppel homolog 1 (Kr-h1), which uncovered the essential mechanism by which JH represses metamorphosis.

5 | BROAD-COMPLEX, THE THIRD PLAYER

BR-C is an early gene of the ecdysone signaling pathway that encodes for different protein isoforms of the BTB/POZ family of C2H2 zinc-finger transcription factors. In *D. melanogaster*, *BR-C* expresses four isoforms depending on which zinc finger module is incorporated into the protein. Mutation experiments that disrupted all *BR-C* isoforms resulted in late larval lethality, suggesting their essential role in pupal morphogenesis (Kiss, Beaton, Tardiff, Fristrom, & Fristrom, 1988). Further studies showed that BR-C determines the larval–pupal transformation in *D. melanogaster* and other holometabolan species such as the lepidopteran *Manduca sexta* (Karim, Guild, & Thummel, 1993; Zhou, Hiruma, Shinoda, & Riddiford, 1998). *BR-C* depletion using a recombinant Sindbis virus expressing a double-stranded RNA targeting *BR-C* in the silkworm *Bombyx mori* (Uhlirova et al., 2003), or using systemic RNAi in the beetle *T. castaneum* (Konopova & Jindra, 2008; Parthasarathy, Tan, Bai, & Palli, 2008; Suzuki, Truman, & Riddiford, 2008) and the neuropteran *Chrysopa perla* (Konopova & Jindra, 2008), further confirmed that BR-C is key for pupal differentiation. In hemimetabolan species, BR-C has been depleted with RNAi in the cockroach *B. germanica* (Huang, Lozano, & Belles, 2013) and the bugs *Oncopeltus fasciatus* (Erezyilmaz, Riddiford, & Truman, 2006) and *P. apterus* (Konopova & Jindra, 2008). In contrast with observations in holometabolan species, results showed that the main role of BR-C in the postembryonic development of hemimetabolan species is to regulate the growth of the wing primordia.

6 | THE MEKRE93 PATHWAY

The first indication that Kr-h1 represses *E93* expression was obtained in the cockroach *B. germanica*, upon observing that Kr-h1 depletion triggers a remarkable stimulation of *E93* (Belles & Santos, 2014). This led to propose the MEKRE93 pathway as the essential axis regulating insect metamorphosis. Accordingly, in nymph–nymph transitions, JH acts through its receptor Met-Tai to induce the *Kr-h1* expression, while Kr-h1 represses the expression of *E93*. In turn, the fall of JH production in the final juvenile stage interrupts *Kr-h1* expression, thus allowing a strong induction of *E93* through ecdysone signaling, which triggers adult morphogenesis (Figure 1). RNAi experiments in *B. germanica* also revealed that E93 depletion increases *Kr-h1* expression (Belles & Santos, 2014; Ureña et al., 2014), which indicates that *Kr-h1* and *E93* are reciprocally

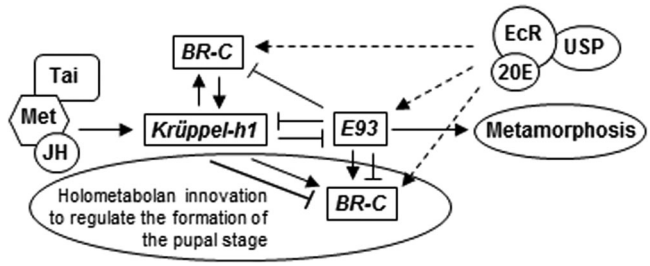

FIGURE 1 The MEKRE93 pathway in hemimetabolan and holometabolan metamorphoses. In the nymph-nymph transitions of the ancestral, hemimetabolan metamorphosis, the JH (through Met-Tai) induces the expression of *Kr-h1*, which, in turn, represses the expression of *E93*. In contrast, the fall of JH production in the final juvenile stage, interrupts the expression of *Kr-h1* and allows a strong induction of *E93* by the ecdysone signaling, which triggers metamorphosis. The main difference between the hemimetabolan and holometabolan modes is the regulation (and function) of BR-C: in the hemimetabolan mode it is mainly involved in promoting the growth of wing primordia, whereas in the holometabolan mode, BR-C triggers the formation of the pupa. For studies that have led to the model, please see citations in the text

repressed. The inhibitory action of Kr-h1 upon *E93* expression was later corroborated in the holometabolan *T. castaneum* (Chafino et al., 2019; Ureña, Chafino, Manjón, Franch-Marro, & Martín, 2016), which extended the MEKRE93 pathway to holometabolan metamorphosis.

The main difference between the hemimetabolan and holometabolan metamorphoses is the regulation and function of BR-C. As shown in *B. germanica*, BR-C is mainly involved in promoting the growth of wing primordia in the hemimetabolan mode. *BR-C* expression is induced by JH and Kr-h1 during juvenile stages (Huang et al., 2013), but it is repressed by E93 in the metamorphic transition (Ureña et al., 2014). RNAi studies in the cricket *Gryllus bimaculatus*, also a hemimetabolan species, confirmed the mentioned interactions and additionally revealed that BR-C and Kr-h1 are reciprocally activated (Ishimaru, Tomonari, Watanabe, Noji, & Mito, 2019). In contrast, BR-C triggers pupa formation in holometabolan species, where, intriguingly, JH inhibits the expression of *BR-C* during larval stages and stimulates *BR-C* expression after pupal commitment (Zhou et al., 1998). As shown in *T. castaneum*, E93 is also involved in triggering the pupal stage, as it promotes *BR-C* expression (Chafino et al., 2019). The MEKRE93 pathway is, therefore, conserved in the holometabolan species, which have added the E93/BR-C interaction loop to the ancestral (hemimetabolan) pathway during the evolutionary transition from hemimetaboly to holometaboly (Figure 1).

7 | MOLECULAR MECHANISMS

Regarding the mechanism by which JH stimulates the *Kr-h1* expression, Kayukawa et al. (2012) used Kr-h1 from *B. mori* and reporter assays and identified a JH response element (kJHRE) comprising 141 nucleotides and located ~2 kb upstream from the gene transcription start site. Remarkably, the core region of kJHRE (GGCCTCCACGTG) contains a canonical E-box sequence to which Met and other bHLH-PAS proteins can bind. Interestingly, the JHREs previously described for other JH-dependent genes (see Riddiford, 2008 for a review), do not contain this E-box, but it is present in the *Kr-h1* promoter of the mosquito *Aedes aegypti* (Cui, Sui, Xu, Zhu, & Palli, 2014; Shin, Zou, Saha, & Raikhel, 2012).

In holometabolan models, Kr-h1 prevents the larva–pupa transformation triggered by BR-C (Kayukawa et al., 2014; Minakuchi et al., 2009, 2008). Subsequent work in *B. mori* led to the identification of a Kr-h1 binding site (KBS) in the *BR-C* promoter (Kayukawa et al., 2016). As *BR-C* is activated by 20E in the absence of JH, the authors suggested Kr-h1 may bind in the vicinity of the ecdysone response elements (EcREs) of *BR-C*, so as to prevent 20E activation. In line with this idea, a 30-bp sequence required for Kr-h1 binding, called KBS core region (GACCTACGCTAACGCTAAATAGAGTTCCGA), was identified in the *BR-C* promoter and as conjectured is located between two EcREs. This location, and further analysis of the 20E and JH regulation of the *BR-C* promoter, led the authors to suggest that *Kr-h1* expression is induced by JH via Met/Tai and two Kr-h1 molecules bind to the KBS core region of *BR-C*, so that 20E cannot induce its expression (Kayukawa et al., 2016).

The same research group hypothesized that a similar mechanism may be involved when Kr-h1 represses *E93* (Kayukawa, Jouraku, Ito, & Shinoda, 2017). Thus, searching for sequences similar to the *BR-C* KBS core region in the promoter of *B. mori E93* led to the discovery of a KBS candidate located near a putative EcRE. Using a *B. mori* cell line and reporter assays, the researchers observed that the *E93* reporter is activated by 20E, whereas JH represses this activation. Mutations in the putative KBS region prevented this JHA-dependent repression and deletion of the putative EcRE abolished 20E-induced expression. These observations and additional RNAi experiments confirmed that *E93* is activated by 20E via the EcRE and 20E-induction is repressed by JH and Kr-h1 via the KBS (Kayukawa et al., 2017).

8 | DOWNSTREAM E93

The pathway regulating metamorphosis does not end with E93, as this factor activates the genes that contribute to the formation of the adult. The aforementioned Mou et al. (2012) study, which revealed that E93 makes *Dll* responsive to EGF receptor signaling, already suggested that E93 may regulate the responsiveness of other target genes. Another example of E93 downstream effects is its enhancing activity upon *decapentaplegic* (*dpp*) expression in the wing of *D. melanogaster* (Wang et al., 2019). *E93* knockdown in the wing and ChIP-seq analysis revealed that *dpp* is a downstream target of E93, while ChIP-PCR analysis and dual-luciferase reporter assays confirmed E93 can bind to the *dpp* promoter, enhancing its activity. Moreover, *E93* overexpression in *Drosophila* S2 cells increases *dpp* expression, whereas this expression decreases after *E93* knockdown in the wing. These results indicate that E93 modulates the dpp signaling pathway, thus regulating wing development during *D. melanogaster* metamorphosis (Wang et al., 2019). Finally, a noteworthy study by Uyehara et al. (2017) has shown that E93 acts as a chromatin modifier, enabling or preventing expression in given genetic regions. This is further evidence of the role of E93 as a master regulator driving metamorphosis forward and unveils a powerful mechanism for this general function based on the modulation of chromatin accessibility.

ACKNOWLEDGMENTS

Work supported by Spanish Ministry of Economy and Competitiveness Grants CGL2012–36251 and CGL2015–64727-P, by Catalan Government Grant 2017 SGR 1030, and by the European Fund for Economic and Regional Development (FEDER funds). This paper is the summary of a lecture given at the 1st International Molecular Plant Protection Congress, held in Adana (Turkey) in April 2019. I thank Dr. Umut Toprak for inviting me to the Congress to give the lecture.

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How to cite this article: Belles X. Krüppel homolog 1 and E93: The doorkeeper and the key to insect metamorphosis. *Arch. Insect Biochem. Physiol.* 2019;e21609. https://doi.org/10.1002/arch.21609