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Molecular basis of juvenile hormone signaling

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Despite important roles played by juvenile hormone (JH) in insects, the mechanisms underlying its action were until recently unknown. A breakthrough has been the demonstration that the bHLH-PAS protein Met is an intracellular receptor for JH. Binding of JH to Met triggers dimerization of Met with its partner protein Tai, and the resulting complex induces transcription of target genes. In addition, JH can potentiate this response by phosphorylating Met and Tai via cell membrane, second-messenger signaling. An important gene induced by the JH–Met–Tai complex is *Kr-h1*, which inhibits metamorphosis. *Kr-h1* represses an 'adult specifier' gene *E93*. The action of this JH-activated pathway in maintaining the juvenile status is dispensable during early postembryonic development when larvae/nymphs lack competence to metamorphose.

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Introduction

JH was originally known for its capacity to maintain juvenile character of insect larvae and thus ensure proper timing of metamorphosis. However, JHs govern many aspects of development and reproduction, not only in insects but also in related crustaceans. Being essential to arthropods and absent from vertebrates, JH signaling offers a target to control insect pests and disease vectors. Insecticides that mimic the effect of JH on development have been employed for decades. Detailed knowledge of the mode of JH action is therefore of major interest, both from the biological and the practical perspectives. The purpose of this review is to highlight important new findings en route to understanding the molecular action of the recently characterized JH receptor, and the role JH signaling plays during insect development.

Establishing Met as a JH receptor

Sesquiterpenoids in structure, JHs differ from other animal non-peptide lipophilic hormones, which all activate proteins of the nuclear receptor family [1,2]. In contrast, the intracellular JH receptor, Methoprene-tolerant (Met), belongs to an ancient family of basic helix–loop–helix Per/Arnt/Sim (bHLH-PAS) transcription factors [3,4,5••] (reviewed in [6,7]).

Met was discovered in 1986 through a mutagenesis screen in *Drosophila melanogaster* as a genetic lesion that caused resistance to the JH mimic methoprene, but permitted the flies to survive [8]. Lethality resulted when loss of *Met* was combined with a deletion in a paralogous *D. melanogaster* gene, *germ cell-expressed* (*gce*) [9]. However, no developmental defect obviously linked to disrupted JH signaling (i.e. precocious metamorphosis), could be discerned in the mutants. The expected phenotype was revealed through RNAi knockdown of a single *Met/gce* ortholog in the red flour beetle, *Tribolium castaneum*, which triggered precocious pupation of larvae [10]. This phenotype matched the effect of JH depletion [11], thus establishing Met as a mediator of the capacity of JH to prevent metamorphosis.

Met/Gce proteins from several insect species have been shown to bind the natural JH (JH III) at physiological (nanomolar) range [4,5••,12,13•]. Synthetic JH mimics, including methoprene or pyriproxyfen, are also true agonists of *T. castaneum* Met and *D. melanogaster* Gce, as they compete with JH III for binding to the same receptor domain [4,5••]. Interestingly, Gce can bind the non-epoxidated JH precursor, methyl farnesoate (MF), albeit with an affinity nearly fivefold lower than that for JH III [5••]. Consistently, MF is less potent than epoxidated JHs in inducing transcription of Met/Gce-dependent genes [5••,14,15]. MF prevails over JH III and is considered a genuine circulating hormone in *D. melanogaster* larvae [15,16], but in live silkworms (*Bombyx mori*) MF cannot compensate for loss of epoxidated JH [17].

Although the crystal structure of the JH receptor is yet to be resolved, a model of the JH-binding domain of Met/Gce has been developed based on homology to related bHLH-PAS proteins of known structures [4,7,13•]. When individual amino acids forming the presumed ligand-binding pocket of *T. castaneum* Met were replaced with residues possessing bulkier side chains, binding of JH III was abolished or reduced [4]. These results have been

corroborated with Met/Gce from *D. melanogaster* and the mosquito *Aedes aegypti* [5••,13•]. A recent demonstration that Gce and Met indeed require the JH-binding capacity *in vivo* to sustain the normal development in *D. melanogaster* provides unequivocal genetic evidence for the JH receptor function of the two proteins [5••].

Biological roles and their partition between duplicated JH receptor genes

Although Met/Gce may not be the single universal JH receptor, it seems versatile enough to execute several major functions of JH in insects. A model in which JH-activated Met represses metamorphosis via regulating downstream genes has been validated across distant orders [18–22,23••] (for recent reviews see [6,24•]). A growth-promoting effect of JH in *D. melanogaster* larvae also relies on Met [25]. Recently, Met has been implicated in soldier caste formation in termites [26], where development of specific body parts into 'weapons' is a striking example of polyphenism induced by JH.

In addition to the roles JH plays in juveniles, Met is required for JH-dependent reproductive maturation in adult females across distant insect orders [8,27–34]. Met has been shown to act autonomously in the fat body to induce expression of yolk precursor proteins, vitellogenins, in response to JH [29,31,33]. While this induction appears to be indirect, a mechanism involving JH-dependent and Met-dependent polyploidization of fat body cells has been proposed [33]. Other adult roles of Met include stimulation of mating behavior [35] or termination of seasonal reproductive diapause [34]. In the viviparous tsetse fly, *Glossina morsitans*, knockdown of Met reduced lipid accumulation, which is required for sufficient lactation and hence for successful pregnancy [36•]. Finally, a recent study reveals that in preparation for the increased food intake that is needed for oogenesis, JH acts through Met and Gce to induce proliferation of gut progenitor cells in mated *D. melanogaster* females [37•].

One reason explaining why *D. melanogaster Met*-null mutants do not die is that the function of *Met* is partly redundant with *gce* [9]. *gce*-null flies are viable and tolerate high levels of JH mimics. Only loss of both *Met* and *gce* causes lethality at the onset of pupation, similarly to genetic elimination of the JH-producing gland, the corpus allatum [9,38]. Like Met, Gce is capable of binding JH [5••] and mediating its effect on target gene expression [5••,9,39•]. When expressed from transgenic constructs in *Met gce* double-mutant flies, either Met or Gce alone can compensate for the loss of both genes [5••,9], although Gce was not sufficient to restore the full rate of oogenesis in *Met* mutants [27]. Met has been shown to play other specific roles, such as in optic lobe development (*Met*-null mutants are blind due to precocious development of that part of the brain) [38], during female mating [35], or in JH-dependent growth [25]. Lipid accumulation for milk synthesis in the tsetse fly females also relies on Met rather than Gce [36•]. Conversely, Gce rather than Met seems to mediate JH effect on enterocyte proliferation in the gut of mated *D. melanogaster* females [37•]. Thus, while Met and Gce can mutually substitute for each other under experimental conditions, they are not fully redundant during normal development.

Met and *gce* arose via gene duplication during dipteran evolution, as both paralogs are present in *Drosophila* species and in the tsetse fly, but not in less modified dipterans such as mosquitoes [36•,40,41]. *gce* is more similar than *Met* to the single orthologs of other insects. Conserved positions of 10 introns in *gce* and presence of only two introns in *Met* suggest that *gce* is the ancestral gene. Two paralogs of *Met*/*gce*, called *Met1* and *Met2*, are also found in *B. mori* [14,42], and phylogenetic analyses show that the gene duplication occurred independently after the divergence of Diptera and Lepidoptera [43]. Similarly to *gce* in *D. melanogaster*, *Met2* contains 9 introns; *Met1* is intronless.

B. mori Met2 and Met1 both mediate JH signaling, albeit the latter was less potent in a cell-based assay [14,44]. It was initially reported that RNAi knockdown of either *Met1* or *Met2* prevented pupation or adult emergence and that both proteins were essential for normal transcriptional activation by the steroid hormone 20-hydroxyecdysone (20E) in *B. mori* [42]. However, in another lepidopteran, *Helicoverpa armigera*, *Met1* RNAi shortened the final larval instar but neither inhibited pupation nor affected the response to 20E [45]. These inconsistencies might be due to off-target effects or efficiency of RNAi, which varies greatly in lepidopterans [46]. The transcription activator-like effector nuclease (TALEN) technique that has been adapted for *B. mori* [47,48] is much more robust as it produces genetic mutants. TALEN-based knockout has recently revealed that loss of *Met1* in *B. mori* causes lethality at the second-to-third larval instar molt, with patches of prematurely forming pupal cuticle. In contrast, *Met2* knockout silkworms develop normally to adults and lay eggs [23••]. Thus, Met1 mediates the anti-metamorphic action of JH in *B. mori* larvae, whereas the role of Met2 remains unclear.

The intracellular JH receptor complex

Like other bHLH-PAS proteins, Met dimerizes with other members of the bHLH-PAS family to form a functional transcription factor. Thus far, two bHLH-PAS protein partners of Met have been identified. One is the circadian clock protein Cycle from *A. aegypti*. Met and Cycle dimerize in the presence of JH and activate circadian-rhythmic expression of JH-response genes in the mosquito females [49]. The other partner reported for Met is a homolog of the vertebrate steroid receptor coactivator (SRC-1/NCoA-1/p160), initially named

Taiman in *D. melanogaster* [50], FISC in *A. aegypti* [51] and SRC in other insects [14,33,52] or crustaceans [53]. According to recent phylogenetic analyses [54•], we will use the name Taiman (Tai) for all insect homologs.

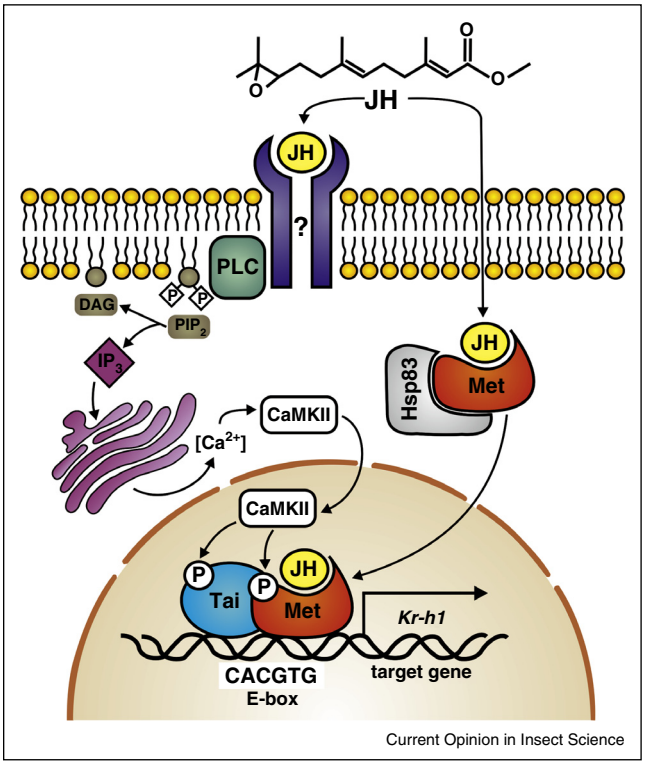
Tai has been shown to mediate some of the biological roles of JH that depend on Met, namely during vitellogenesis [31,33] and metamorphosis [54•]. In addition, Tai can physically and functionally interact with the ecdysone receptor (EcR) and other nuclear receptors (such as Ftz-F1) in the ecdysteroid pathway [50,51], thus suggesting a potential for Tai to modulate both JH and 20E signaling. A recent study in the cockroach *Blattella germanica* suggests that diverse functions might be achieved through alternatively spliced Tai isoforms, some of which have been ascribed a specific role in the anti-metamorphic action of JH [54•].

The direct interaction between Met and Tai is stimulated by JH [4,13•,14,52,53,55]. While Tai itself does not seem to bind JH, the ability of Met to bind JH is critical for the dimer formation [4]. Importantly, the basic regions of both Met and Tai that contribute to the bipartite DNA-binding domain are required for the ability of the receptor complex to bind DNA [13•]. When activated by JH, the Met–Tai complex associated with specific JH response elements (JHREs) on target genes induces transcription [13•,14,39•,52,55] (Figure 1).

JHREs were initially derived from regulatory regions of two JH-response genes, *Krüppel-homolog 1* (*Kr-h1*) in *B. mori* [14] and *early-trypsin* in *A. aegypti* [55], and inferred from analysis of upstream sequences of Met-regulated genes identified in *A. aegypti* through a whole-transcriptome approach [30•]. The JHRE sequences contained an E-box CACGTG or an E-box-like imperfect palindrome CACGCG; the latter is also recognized by the Met-Cycle complex [49]. Finally, unbiased selection of random oligonucleotides confirmed a strongly represented consensus GCACGTG, containing the canonical E-box [13•]. E-boxes are typical binding elements for bHLH-PAS proteins [56].

The JH receptor resembles in action the vertebrate bHLH-PAS protein Aryl hydrocarbon receptor (AhR), which responds to its ligands by dimerizing with ARNT and subsequently activating target genes [57]. Met/Gce also resembles AhR in its association with the chaperone heat shock protein Hsp90 (Hsp83 in *D. melanogaster*) and ligand-induced nuclear import [39•,58,59]. Met and Gce engage in a JH-dependent interaction with Hsp83, which facilitates both nuclear import of Met and expression of JH-response genes [39•] (Figure 1). Similarly, in *H. armigera* cells, JH stimulates nuclear translocation of Hsp90 and its phosphorylation through a phospholipase C (PLC)/protein kinase C (PKC) pathway [60]. In this context, Hsp90 has been proposed to interact with Met1

Figure 1



Two parallel branches of JH signaling converge at the intracellular JH receptor complex. A cell membrane-based pathway involves an unidentified JH receptor tyrosine kinase, which activates phospholipase C-dependent inositol trisphosphate (IP_3) signaling, leading to Met and Tai phosphorylation by a Ca^{2+}/calmodulin-dependent kinase II (CaMKII). JH presumably also enters the cell directly to bind Met and stimulate its Hsp83-dependent nuclear import.

and modulate expression of 20E-regulated and JH-regulated genes.

Cell-membrane and nuclear branches of JH signaling converge at Met

It has long been suspected that besides regulating gene expression in the nucleus, JH may exert effects at the cell membrane [61]. A recent study demonstrates a parallel JH signaling branch that operates through a hypothetical cell membrane-associated JH receptor via PLC signaling, mediated by inositol trisphosphate (IP_3) and intracellular calcium [62••] (Figure 1). This branch leads to Met and Tai phosphorylation by a Ca^{2+}/calmodulin-dependent kinase II (CaMKII). Using RNAi experiments and inhibitor treatments in *A. aegypti* cells and tissues, the authors have shown that the above signaling pathway is important for Met and Tai to induce gene expression in response to JH [62••]. Thus, the parallel membrane-based and intracellular branches of JH signaling converge at the Met–Tai receptor complex in the nucleus, leading to the previously known transcriptional activation (Figure 1).

No cell membrane-associated JH receptor has yet been identified, but effects of chemical inhibitors favor a receptor tyrosine kinase (RTK) rather than a G protein-coupled receptor [62••]. Interestingly, the calcium wave dynamics was more pronounced when elicited by a native mosquito JH (JH III) and its biosynthetic precursor MF than by the JH mimics methoprene or pyriproxyfen [62••]. Thus, the hypothetical RTK might discriminate between natural and synthetic JH agonists.

Phosphorylation of Met and Tai may be an operative mechanism to modulate the function of the intracellular JH receptor in a tissue-specific and context-specific manner, by the rapid mode of second-messenger signaling. However, the nature of Met and Tai phosphorylation and its impact on the functional performance of the receptor complex is yet to be investigated.

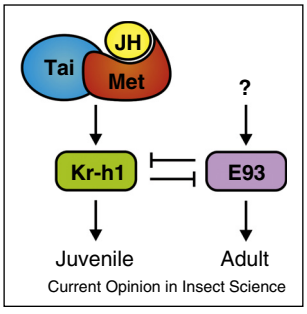
JH signaling during metamorphosis (the MEKRE93 pathway)

The *Kr-h1* gene, which is directly induced by the JH-activated Met/Gce–Tai receptor complex [5••,13•,14,15,39•,55,63], encodes a well-conserved transcription factor with eight zinc fingers in its DNA-binding domain [64]. JH-stimulated *Kr-h1* expression precludes metamorphosis [19,21,65,66], whereas the dramatic fall of *Kr-h1* expression following the natural drop in JH titer during the final juvenile stages (particularly last-instar nymphs in hemimetabolans and pupae in holometabolans) permits adult development [6,24•]. In *B. germanica*, and possibly in other species, the fall of *Kr-h1* mRNA at the last nymphal instar results not only from downregulation of *Kr-h1* transcription but also from the action of the microRNA *miR-2* [67•]. Depletion of *miR-2* at that stage prevents the normal elimination of *Kr-h1* mRNA and impairs adult formation, suggesting that this miRNA crucially contributes to the regulation of metamorphosis.

JH or JH mimic treatments, which induce ectopic *Kr-h1* expression during the final juvenile stage and the formation of supernumerary juvenile instars, suggest that the absence of Kr-h1 is prerequisite for metamorphosis. This hypothesis has been tested by constitutively expressing Kr-h1 in transgenic silkworms. As expected, the ectopic gain of Kr-h1 caused incomplete pupation [68•]. However, it neither led to an extra larval instar nor prevented expression of the pupal specifier gene *Broad-Complex* (*BR-C*). These results indicate that while Kr-h1 is essential for the maintenance of the larval status, it alone is insufficient to block metamorphosis [68•].

Recent studies have identified E93, a helix–turn–helix transcription factor containing a Pipsqueak (Psq) motif [69], as an important player downstream of Kr-h1 [70••] (Figure 2). Depletion of *E93* in final-instar nymphs of *B. germanica* or in pupae of *T. castaneum* and *D. melanogaster* prevents the transition to the adult stage. Thus, in contrast to Kr-h1 (the keeper of larval status), E93 has been proposed as an 'adult specifier' in both hemimetabolan and holometabolan species [70••]. The effects of *Kr-h1* and *E93* are to some extent antagonistic, and at least during the prefinal nymphal instars of *B. germanica*, *Kr-h1* and *E93* are mutual repressors [24•,70••]. Taken together, presently available data indicate that up until the final juvenile stage (the last-instar nymph or pupa), JH signals through Met and Tai to induce expression of *Kr-h1*, which in turn blocks adult development at least partly by repressing the *E93* gene (Figure 2).

Figure 2



A simplified model for the role of the MEKRE93 pathway in insect postembryonic development. During the penultimate juvenile instar, the JH-receptor complex maintains 'status quo' by activating expression of Kr-h1, which prevents metamorphosis by suppressing E93. Decline in JH signaling during the final juvenile instar together with presence of an unknown 'competence factor' permit expression of E93, which suppresses Kr-h1 and informs adult development.

JH signaling is dispensable for maintaining early juvenile status

Since the discovery of the 'status quo' action of JH by Wigglesworth [71], it is implicitly believed that JH is essential for maintaining a juvenile status throughout all larval or nymphal instars. However, there is evidence against this paradigm. For example, in the linden bug *Pyrrhocoris apterus*, which normally undergoes five nymphal instars, suppression of JH signaling induced precocious adult development when *Met* or *Kr-h1* RNAi was delivered during the third and fourth (penultimate) but not during the earliest two instars [72••]. In *B. mori*, also with five larval instars (L1–L5), depletion of JH achieved either through surgical allatectomy [73,74] or genetically [75] did not lead to precocious pupation before at least three larval instars have been completed. Similarly, *B. mori mod* mutants that lack epoxidized JHs due to deficiency in a JH-epoxidase (CYP15C1), pupate no earlier than after L3 [17].

On the basis of the above findings, it was proposed that insect postembryonic development is initially independent of JH and only later, when the juveniles gain

competence to metamorphose, JH becomes necessary to delay metamorphosis until stage is optimal [72••]. This 'competence theory' has been confirmed in *B. mori* through TALEN-mediated gene knockout of the essential JH-biosynthetic enzyme, JH acid methyltransferase (JHAMT), and the Met1 and Met2 JH receptors [23••]. Despite the complete blockade of either JH production or JH signaling, no pupal characters appeared in L1 or L2 larvae, and precocious metamorphosis occurred upon molt to L3 at the earliest. The simultaneous loss of JHAMT and CYP15C1 in *JHAMT*$^{-/-}$*; mod* double-mutant silkworms also resulted in pupal development at the third instar [23••], confirming that neither MF nor epoxidated JH or JH acid are required to maintain larval character at L1 and L2. Similar to the *mod* mutants [72••], *JHAMT*$^{-/-}$ and *Met1*$^{-/-}$ larvae did not express the pupal specifier gene *BR-C* during the L1 and L2 instars, even though *Kr-h1* mRNA was virtually absent due to the deficiency in JH production and reception, respectively [23••]. Thus, embryonic and early larval development of *B. mori* is largely independent of JH and the Met-Kr-h1 pathway is not essential to prevent metamorphosis or suppress *BR-C*. Nonetheless, the JH receptor might still play some roles at these stages, as both *D. melanogaster Met* and *B. mori Met1* mutant larvae suffer from growth retardation [23••,25].

These results anticipate existence of a 'competence factor' for insect metamorphosis, which would permit expression of the pro-metamorphic genes such as *BR-C* and *E93*. This factor might be absent until insect larvae attain a critical weight [76]. Although the nature of a 'competence factor' is yet unknown, classical experiments suggest that it might be blood-borne. In *Rhodnius prolixus*, precocious adults resulted from a decapitated first-instar nymph when joined to a molting final-instar nymph [71]. In the wax moth *Galleria mellonella*, the epidermis of L1 larvae pupated directly when implanted into a last-instar larva [77]. In both cases, the early-stage acceptor was not only depleted in JH but at the same time exposed to the hemolymph of the competent donor.

Questions for future

While our understanding of JH action has improved tremendously during the past several years, many questions remain to be answered and others emerge as the work progresses. Here are a few we consider most imminent:

- What is the molecular structure of the JH–Met–Tai receptor complex?
- How does this complex activate transcription; what is the repertoire of its direct target genes and what are their functions?
- What is the hypothetical membrane receptor for JH, and how does it convey the hormonal signal?
- What is the precise mechanism of Met and Tai regulation by phosphorylation?
- Is there a role for JH in the embryo and youngest nymphal or larval instars?
- Does the 'competence factor' to undergo metamorphosis derive from critical weight? How is it sensed and translated to *E93* expression? What is downstream of *E93*?

The discovery of the JH receptor has opened a wide avenue. Research in the field is now very dynamic, promising that the above questions may soon be answered.

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