A decade with the juvenile hormone receptor

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Abstract

Juvenile hormone (JH) and ecdysteroids represent equally important nonpeptide signals governing insect reproduction and development. For a considerable time, understanding of JH action lagged behind ecdysteroid research. Arriving with a 20-year delay, the intracellular receptor for JH was finally identified as a ligand-activated bHLH-PAS transcription factor, originally named Methoprene-tolerant (Met), by virtue of the resistance *Drosophila* mutants exhibited to morphogenetic and lethal actions of JH and its mimic methoprene. Systemic RNAi in suitable insect models revealed the anticipated function of Met in preventing metamorphosis and promoting reproduction, thus providing the missing link to the chief roles of JH in insects. That, along with defining the

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JH-binding pocket and the JH-response DNA elements of Met, established the JH receptor (JHR) 10 years ago. After reviewing the functional attributes of the JHR, this chapter will focus on advances in the genetics, cell biology, and biochemistry of the JHR achieved during this past decade. Although hormone receptor function of bHLH-PAS transcription factors is unprecedented, the well-studied mammalian aryl hydrocarbon receptor (AhR) belonging to the same protein family affords functional parallels with the JHR. We can now begin to understand the mechanisms of JHR interaction with the chaperone Hsp90/83, nucleocytoplasmic transport and post-translational regulation by phosphorylation, dimerization with bHLH-PAS partner proteins, and activation by agonist ligands binding to the PAS-B domain. A section is dedicated to current efforts exploiting the JHR as a basis of chemical high-throughput screening, aimed at discovery of novel compounds for environmentally friendly control of insect pests and disease vectors.

1. Introduction

Arthropods possess lipophilic hormones of two major types: steroids (ecdysteroids, mainly represented by ecdysone and its active metabolite 20-hydroxyecdysone; 20E), and the sesquiterpenoid juvenile hormones (JHs). In insects, ecdysteroids and JHs together coordinate the complex post-embryonic development that involves the periodic moulting of larvae and their metamorphosis to adults. While 20E promotes both larval and metamorphic moults, the presence of JH maintains the juvenile character of a moulting larva and prevents it from metamorphosing prematurely, before attaining a required stage and body size (Bellés, 2020; Jindra, 2019; Jindra et al., 2013; Nijhout et al., 2014; Riddiford, 1994; Truman, 2019; Tsang et al., 2020; Yamanaka et al., 2013). Both 20E and JH are also important for reproduction, particularly for vitellogenesis and ovarian maturation, with the exact role of either hormone depending on the insect species (Hansen et al., 2014; Roy et al., 2018; Santos et al., 2019; Swevers, 2019; Wu et al., 2021; Wyatt and Davey, 1996). Long before their chemical identification (Butenandt and Karlson, 1954; Röller et al., 1967), both hormones had been postulated as bloodborne factors emanating from localized organs based on their effects on moulting and metamorphosis of the kissing bug, *Rhodnius prolixus* (Wigglesworth, 1934, 1936, 1940). Besides their general biological importance, both ecdysteroid and JH signalling became targets of chemical intervention, aiming to disrupt the endocrine system in insect pests and disease vectors (Pener and Dhadialla, 2012). JH mimics such as methoprene (Henrick, 2007; Henrick et al., 1973) became the first insecticides of a new generation termed "insect growth regulators" (IGRs) or,

better, insect growth disruptors (Minakuchi and Riddiford, 2006; Parthasarathy et al., 2012; Pener and Dhadialla, 2012; Sláma et al., 1974).

Steroid hormones occur universally in arthropods and other animals. They regulate expression of target genes through intracellular receptors of the nuclear receptor (NR) family (Evans and Mangelsdorf, 2014; King-Jones and Thummel, 2005; Mangelsdorf et al., 1995). An active 20E receptor, initially identified in the fruit fly, *Drosophila melanogaster*, is a heterodimer of the ecdysone receptor (EcR) and ultraspiracle (Usp) NR proteins (Koelle et al., 1991; Thomas et al., 1993; Yao et al., 1993) acting on target genes through ecdysone response DNA elements (Cherbas et al., 1991). Work on *Drosophila* mutants revealed the essential requirement for *EcR* and *usp* genes in 20E-dependent processes including moulting, metamorphosis, and oogenesis (Bender et al., 1997; Carney and Bender, 2000; Hall and Thummel, 1998; Henrich et al., 1994; Oro et al., 1992). Three-dimensional structures of the EcR and Usp proteins from several insect species have been resolved through X-ray crystallography (Billas et al., 2003; Carmichael et al., 2005; Iwema et al., 2007; Ren et al., 2014), providing deep mechanistic insights into the hormone-receptor interaction (reviewed by Hill et al., 2012, 2013).

Despite equal importance of 20E and JH signalling in insect biology, the JH field lagged considerably behind the rapidly progressing ecdysteroid research. The molecular mode of JH action had for long remained a conundrum, particularly as the receptor mediating the anti-metamorphic effect of JH could not be identified (Riddiford, 2020). Sesquiterpenoids are structurally unique hormones. Their biosynthesis downstream of the mevalonate pathway involves several steps that are considered specific to arthropods (Goodman and Cusson, 2012; Hiruma and Kaneko, 2013; Qu et al., 2015; Tsang et al., 2020) [although a direct JH precursor with hormonal activity, methyl farnesoate, has been found in annelids (Schenk et al., 2016)]. Unlike steroid hormones with their common gene-regulatory mechanism, no precedent intracellular receptor system was available to guide the quest for JH receptors.

This chapter will briefly recapitulate the milestones en route to identifying JH receptors encoded by the *Methoprene-tolerant* (*Met*) genes and their *Drosophila* ortholog known as *germ cell-expressed* (*gce*) before explaining attributes that define their receptor function. Next, we will summarize what has been learned so far about the functional architecture and molecular interactions of these JH receptor proteins. Finally, we will mention recent attempts to exploit knowledge of the JH receptor

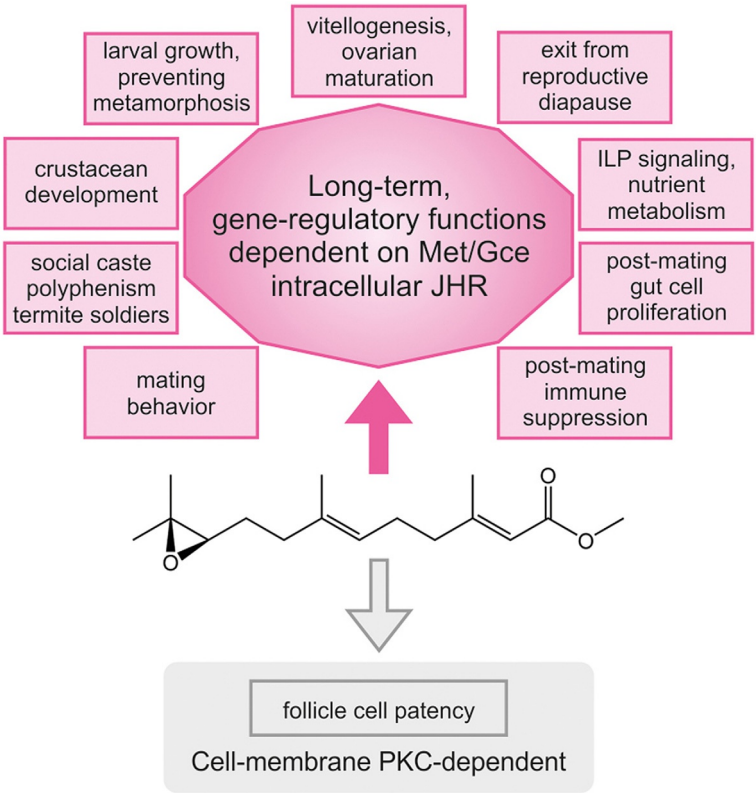

Fig. 1 Examples of processed known to be regulated by JH either through Met/Gce or at the cell-membrane level. A structure of JH III is shown. For citations see Section 3.

molecules towards developing new compounds for manipulating JH signalling and for chemical control of insects.

Throughout the text, Met/Gce will be explicitly or implicitly regarded as an intracellular JH receptor, abbreviated JHR where appropriate. This is to distinguish the gene-regulatory signalling mediated by the intracellular JHR (Fig. 1) from additional effects exerted by JH on as yet unidentified receptor(s) at the cell surface.

2. From JH effects to discovering a JH receptor

Some of the processes regulated by JH are reflected in Fig. 1. The history of research leading to discoveries of JH receptors has been eloquently captured in a recent review by Riddiford (2020), and previously detailed in

another chapter of this book series (Dubrovsky and Bernardo, 2014). In this section, we will highlight selected studies, which formed our perception of how an intracellular JHR should function, and those leading to its eventual identification.

2.1 Developmental action of JH: A take-home message from one paper

In an early experiment, Wigglesworth (1958) treated the cuticle of last-instar *Rhodnius* larvae with extracts from male *Hyalophora cecropia* silkmoths (Williams, 1956), a natural source of JH. Prior abrasion of the cuticle ensured that the hormone, applied in oil, would penetrate the cuticle and act on the larval epidermis locally. During the ensuing metamorphic moult, the epidermal cells produced the smooth adult cuticle except in the areas that were previously abraded and exposed to JH, and where the cuticle retained the rough and dark larval character. In this way, even the author's initials (V.B.W.) could be made to appear on the adult, literally printed in the dark larval cuticle type. Similarly, formation of adult structures such as the wings could be blocked by treating the wing pads (Wigglesworth, 1958). In all instances, the hormone had to be delivered early during the last larval instar before the epidermis became committed to metamorphosis.

Viewed from today's perspective of a developmental geneticist, this simple and elegant experiment has had important implications for understanding the mode of JH action. First, while being a systemic, bloodborne signal, JH acts in a cell-autonomous manner. In other words, the signal is not uniformly spread throughout the body. Rather, JH reception is an inherent property of each individual cell. Second, the timely presence of JH alone can block metamorphosis of tissues or body parts. Therefore, JH alters the outcome of a moult induced by ecdysteroids; its presence *vs.* absence, respectively, determines the larval *vs.* adult fate decision in target organs. By cell-autonomously perpetuating the larval developmental programme within an animal undergoing metamorphosis, JH can cause heterochronic phenotypes, wherein treated individuals are spatiotemporal mosaics bearing juvenile and adult structures.

Developmental effects such as those described above are best compatible with a gene-regulatory mechanism, suggesting that JH may alter the genetic programme of recipient cells at the transcriptional level. By analogy to other nonpeptide, lipophilic hormones and morphogens such as steroids or retinoic acid, one would expect JH action to be mediated by an intracellular receptor, likely a ligand-activated transcription factor functionally akin to

members of the NR family (King-Jones and Thummel, 2005). The pursuit of such a receptor would eventually occupy the research community for many decades following Wigglesworth's seminal discoveries.

2.2 Mutual benefits of *Drosophila* and complementary insect models

The thriving ecdysteroid field owed a great deal to the well-defined effects of 20E on *Drosophila* genes and development. Transcriptional activity triggered by the hormone could be directly observed *ex vivo* as "puffs" forming on giant salivary gland chromosomes (Ashburner et al., 1974). This facility aided identification and subsequent cloning of 20E-induced genes, including the canonical 20E receptor (EcR) itself (for reviews see Hill et al., 2013; Thummel, 1996). Unrivalled genetic tools have made *Drosophila* the perfect model to study steroid hormone signalling (Yamanaka et al., 2013).

By contrast, the lack of a robust effect of JH on *Drosophila* (Gilbert et al., 2000; Riddiford, 1994) hindered a straightforward approach to uncovering JH signalling effectors and target genes. Unlike in many other insects, ectopic JH treatment in *Drosophila* does not cause extra larval instars and does not prevent metamorphosis of most body parts of the adult fly. However, limited effects on *Drosophila* adult development do occur when exogenous JH is given during a sensitive period shortly after pupariation. These include disrupted abdominal bristle patterns, incomplete rotation of male genitalia, and failure to eclose (Madhavan, 1973). Similarly, ectopic JH application compromises formation of the adult abdominal epidermis. Some JH mimics such as methoprene and pyriproxyfen [now established JH receptor agonists (Jindra and Bittova, 2020)] induce these morphological defects and block adult eclosion more effectively than JH (Riddiford and Ashburner, 1991; Wilson and Fabian, 1986; Zhou and Riddiford, 2002).

The late-stage lethality caused by methoprene was exploited by Wilson and Fabian (1986), who took a forward-genetic approach to finding targets of JH action. Their mutagenesis screen uncovered a single, chromosome X-linked gene, *Methoprene-tolerant* (*Met*), whose loss mitigated the lethal and morphogenetic impact of treatments with methoprene or with native JH III. Curiously, flies hemi- or homozygous for mutant alleles of *Met* were viable and displayed subtle anomalies, namely of the thoracic muscles, nervous system, externally minor eye defects, and reduced oogenesis (Restifo and Wilson, 1998; Wilson and Ashok, 1998; Wilson and Fabian, 1986; Wilson et al., 2006). Later work revealed that the internal visual system

in *Met* mutants was disordered due to precocious development of certain photoreceptors (Riddiford et al., 2010) (for a review see Dubrovsky and Bernardo, 2014).

The absence of an obvious, JH-related phenotype discouraged implications that Met might be a JH receptor, even after the gene had been cloned and shown to encode a member of the basic helix-loop-helix (bHLH)/Per-Arnt-Sim (PAS) family of transcription factors (Ashok et al., 1998). There was no precedent of a bHLH-PAS protein acting as a receptor for any known animal hormone. The scepticism did not dramatically change even after an initial indication that the recombinant Met protein-bound JH III with a high affinity (Miura et al., 2005).

To explain the dispensability of *Met*, Wilson and Ashok (1998) speculated that (*A*) JH had no essential role in pre-adult *Drosophila* development, or (*B*) another gene provided a partial functional redundancy, making *Met* nonvital; (*B*) turned out to be correct. *Met* arose *via* a recent duplication from the ancestral gene *gce* (Baumann et al., 2010) in derived flies (two paralogs occur within the Schizophora). As later demonstrated by Abdou et al. (2011), *Met* only becomes vital when *gce* is deleted and *vice versa*. While the null *gce* mutants are also viable, loss of both *Met* and *gce* causes unconditional lethality at the time of pupation (Abdou et al., 2011). Indeed, both *Met* and *gce* encode JH receptors that are functional in live *Drosophila*, and either gene can substitute for the missing paralog when expressed in the *Met gce/Y* double-mutant males (Jindra et al., 2015b). The situation is easier in most other insect species possessing only single orthologs of *Met/gce*, one exception being lepidopterans where the gene has also duplicated (Kayukawa and Shinoda, 2015).

Unlike *Drosophila*, insects such as moths, beetles, or true bugs present "canonical" JH regulation. This means that supernumerary larval instars or extra pupal cuticles can be induced by ectopic treatment with JH agonists and, conversely, precocious metamorphosis ensues when JH signalling is blocked (Jindra et al., 2013). The arrival of RNA interference (RNAi), enabling systemic gene knockdown in whole larvae (Tomoyasu and Denell, 2004), turned the red flour beetle, *Tribolium castaneum*, into a superior model for JH studies. When the single *Tribolium Met* gene was knocked down just prior to pupation, pupae became refractory to the effects of JH agonist treatment and produced normal adults instead of repeating the pupal programme (Konopova and Jindra, 2007, 2008; Minakuchi et al., 2009). Besides their developmental implications, these data also confirmed Wilson's thesis that "methoprene tolerance results from an absence of target gene product" (Wilson and Ashok, 1998).

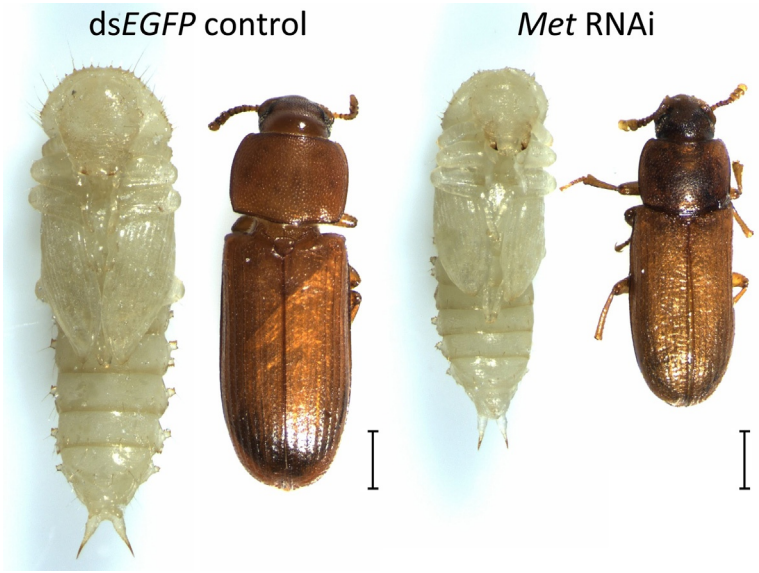

Fig. 2 Precocious metamorphosis in *T. castaneum*, induced by RNAi-mediated knockdown of *Met* during the fourth larval instar, manifested as pupation after the penultimate (sixth) larval instar and formation of an adult beetle (see also Konopova and Jindra, 2007). The same phenotype results from knocking down *JHAMT* (Minakuchi et al., 2008a). Scale bar, 0.5 mm.

More importantly, however, *Met* RNAi caused *Tribolium* larvae to pupate prematurely, before reaching their final (seventh) instar, and some of the miniature pupae even developed into pygmy adult beetles (Konopova and Jindra, 2007) (Fig. 2). It was this particular experiment, unachievable in *Drosophila*, which supplied the missing evidence that Met indeed executed the natural, developmental function of JH. The phenotype was a perfect copy of JH deficiency itself (Minakuchi et al., 2008a). In their next study on the linden bug, *Pyrrhocoris apterus*, Konopova et al. (2011) extended the anti-metamorphic role of Met to hemimetabolous insects. This finding, followed by work on additional holo- and hemimetabolous species, has indicated that a common molecular endocrine mechanism directs development in all metamorphosing insects (Bellés, 2020; Jindra, 2019; Truman, 2019).

Although the phenotypes produced by removal of Met in *Tribolium* or *Pyrrhocoris* were clearly linked to JH signalling, they did not prove that Met was a JH receptor. Yet these results gave a new impetus to JH research, and the long-sought receptor was soon to be confirmed. As highlighted in

the historical review (Riddiford, 2020), the winning combination enabling the progress was that of complementing *Drosophila* genetics with insect models better representing developmental regulation by JH.

3. Functional attributes defining Met/Gce as a JHR

The processes that depend on Met/Gce proteins as effectors of JH signalling are rather long-term and likely based at the genome level (Fig. 1). Before embarking on the discussion of those gene-regulatory functions of JH, let us briefly pause to consider a more instantaneous effect that relies on signalling pathways other than the intracellular JHR. A reduction of volume of ovarian follicle cells, known as patency, enables yolk precursors to pass through the follicle epithelium. Patency had long been known to depend on a Na^+/K^+-ATPase in *Rhodnius* and other insects (Davey, 1996; Sevala and Davey, 1989). A recent study on *Locusta migratoria* showed that JH induced patency by activating the Na^+/K^+ pump *via* protein kinase C (PKC)-dependent phosphorylation (Jing et al., 2018). The phosphorylation was sensitive to a number of chemical inhibitors, further implicating G protein-coupled receptor, receptor tyrosine kinase, inositol trisphosphate receptor, and phospholipase C signalling upstream of PKC. Nevertheless, a hypothetical cell-membrane receptor, through which JH sets in motion the phosphorylation cascade leading to patency, remains to be identified.

3.1 Loss-of-function phenotypes consistent with JH deficiency

3.1.1 Metamorphosis

As alluded to above, a match between phenotypes caused by hormone deficiency and loss of its candidate receptor is prerequisite to establishing an agonist-receptor relationship. For JH and Met, the initial evidence relied on systemic RNAi in *Tribolium*. The precocious metamorphosis seen in *Met* RNAi larvae (Konopova and Jindra, 2007) (Fig. 2) could be phenocopied by knocking down JH acid methyltransferase (JHAMT) (Minakuchi et al., 2008a). JHAMT catalyzes one of the two final steps in JH biosynthesis within the *corpora allata* glands and is necessary to produce an active JH (Shinoda and Itoyama, 2003; Tsang et al., 2020). The other of the two final steps (the order of which depends on insect species) is epoxidation either of farnesoic acid or of the direct JH III precursor, methyl farnesoate (MF) (Helvig et al., 2004; Tsang et al., 2020). Relative to *JHAMT* RNAi, knockdown of the responsible epoxidase CYP15A1 affected

Tribolium metamorphosis only mildly (Minakuchi et al., 2015), possibly because MF exerts a partial JH agonist activity (Bittova et al., 2019; Jindra et al., 2015b; Wen et al., 2015). Precocious adult development consistent with JH deficiency could be induced by RNAi-mediated knockdown of *Met* in hemimetabolans, including the true bugs *P. apterus* and *R. prolixus* (Konopova et al., 2011; Smykal et al., 2014b; Villalobos-Sambucaro et al., 2015), or the cockroach *Blattella germanica* (Lozano and Bellés, 2014).

Phenotypes resulting either from complete loss of *Met* function or from JH deficiency could be rigorously compared when Daimon et al. (2015) generated gene knockout lines in the silkworm, *Bombyx mori*. The silkworm normally undergoes five larval instars before pupating. Null mutants lacking JHAMT (and hence JH) developed through embryogenesis until they entered precocious metamorphosis as third- or fourth-instar larvae. The $JHAMT^{-/-}$ larval-pupal intermediates or abnormal pupae produced no adults (Daimon et al., 2015). A similar albeit milder phenotype occurred in the *dimolting* (mod) strain, which lacks the CYP15C1 epoxidase of farnesoic acid. At their third or fourth larval instar, the *Bombyx mod* mutants formed precocious miniature pupae and adults (Daimon et al., 2012). $JHAMT^{-/-}$ *mod* double mutants mostly began to metamorphose at the third instar (Daimon et al., 2015).

There are two *Met* paralogs in *Bombyx*, of which *Met1* is vital while *Met2* is dispensable (Daimon et al., 2015). Some of the $Met1^{-/-}$ or double-knockout $Met1^{-/-}$ $Met2^{-/-}$ larvae died during the second moult and none pupated. Importantly, those surviving to the third instar carried patches of pupal cuticle, a hallmark of precocious metamorphosis consistent with JH deficiency. A fusion of maternal genotypes in mosaic progeny led to fourth-instar larvae in which as much as half of the body, consisting of $Met1^{-/-}$ cells, displayed the pupal character (Daimon et al., 2015). These genetic data reinforce the notion that Met is a cell-autonomous JH receptor.

The knockout study in *Bombyx* has cemented previous indications that, in contrast to a traditional view, insect larvae of the earliest two instars are incompetent to enter metamorphosis and therefore JH is not required to maintain their juvenile status (Daimon et al., 2012; Feyereisen and Jindra, 2012; Smykal et al., 2014b; Tan et al., 2005). To be precise, at the tissue-autonomous level the absence of JH reception (Met1) is required, but not sufficient, to permit the pupal programme in the first- and second-instar *Bombyx* epidermis (Inui and Daimon, 2017). The dispensability of JH during the earliest larval instars has been further suggested in the *Aedes aegypti* mosquitoes (Zhu et al., 2019). CRISPR/Cas9-induced

mutagenesis of *Met* caused lethality of G0 larvae no earlier than before pupation at the final (fourth) instar.

The effects of *Met/gce* loss could be reconciled with JH deficiency also in *Drosophila*. The cell-death gene *grim* proved effective in removing the source of JH when misexpressed in the *corpora allata* cells, embedded within the *Drosophila* larval ring gland (Liu et al., 2009; Riddiford et al., 2010). Depletion of JH resulted in lethality at the time of pupation, thus matching the lethal phase of *Met gce* double mutants (Abdou et al., 2011). As one would expect, supplementing a JH agonist compensated for the genetic allatectomy, but not for loss of JHR genes (Abdou et al., 2011; Riddiford et al., 2010).

3.1.2 Female reproduction

Promoting vitellogenesis and ovarian maturation in insects is another major role for JH (Hansen et al., 2014; Roy et al., 2018; Santos et al., 2019; Wu et al., 2021; Wyatt and Davey, 1996) (Fig. 1). Reduced oviposition was noticeable in *Drosophila Met* or *gce* mutants (Abdou et al., 2011; Wilson and Ashok, 1998). In *Tribolium* adult females, systemic RNAi-mediated knockdown of either *JHAMT* or *Met* decreased expression of a yolk protein precursor vitellogenin (Vg) in the fat body (Sheng et al., 2011). This work has revealed that in the beetles, JH- and Met-dependent induction of the *Vg2* gene involved up-regulated expression of insulin-like peptide (ILP 2 and 3) genes, thus illustrating a synergy between JH and nutrient-dependent insulin signalling during insect female reproduction (for reviews see Hansen et al., 2014; Smykal and Raikhel, 2015; Wu et al., 2021).

Vitellogenesis strictly depends on JH in hemimetabolous insects such as orthopterans, cockroaches, and some hemipterans. Accordingly, the indispensable requirement of Met for vitellogenesis could be demonstrated in *P. apterus*, *R. prolixus*, the bed bug *Cimex lectularius* (all Heteroptera) (Gujar and Palli, 2016; Smykal et al., 2014a; Villalobos-Sambucaro et al., 2015), the locusts *Schistocerca gregaria* and *L. migratoria* (Gijbels et al., 2019; Guo et al., 2014), or the cockroach *Diploptera punctata* (Marchal et al., 2014). In all cases, RNA-mediated knockdown of *Met* partially or completely blocked *Vg* expression and/or ovarian maturation and oogenesis. In *L. migratoria*, whose *corpora allata* are sensitive to chemical inactivation with precocene, the effect of *Met* RNAi could also be matched by means of chemical allatectomy (Guo et al., 2014). Similarly, surgical allatectomy was performed to corroborate the data in adult females of *P. apterus* (Smykal et al., 2014a) (Fig. 3). As in genetically allatectomized *Drosophila*, chemical

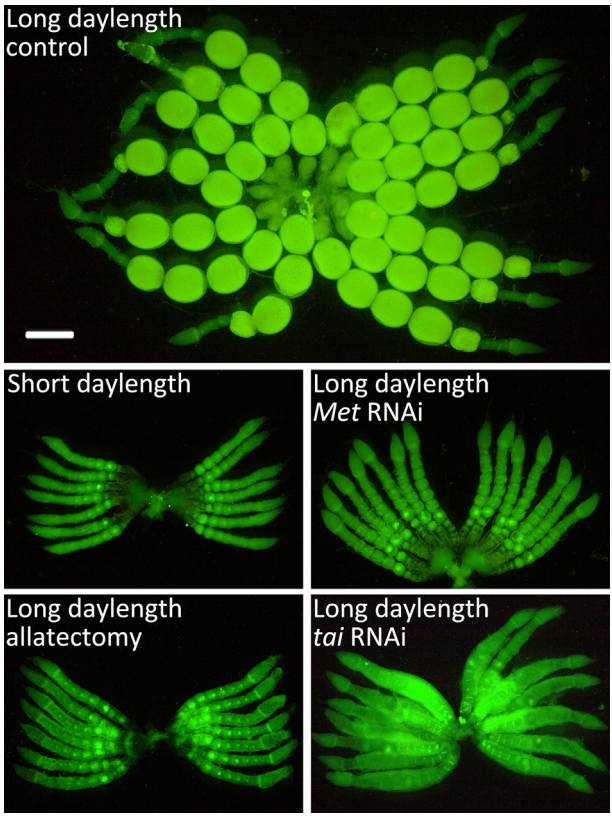

Fig. 3 Oogenesis in the linden bug, *P. apterus*, requires JH and the JHR proteins Met and Tai. Vitellogenic oocytes of reproductive females (top) disappear from the ovarioles during diapause (short daylength) or when the source of JH is removed (allatectomy). RNAi knockdown of *Met* or *tai* blocks oogenesis in formerly reproductive females (Smykal et al., 2014a). The ovaries were stained with Bodipy. Scale bar, 1 mm, applies to all panels. Photographs kindly provided by Vlastimil Smykal.

or surgical depletion of JH could be remedied with JH agonist treatments, but only as long as Met was present.

The *Pyrrhocoris* model offers a condition of natural JH absence during reproductive diapause in adult females (Dolezel, 2015). When their brain perceives shortening daylength, the *corpus allatum* shuts down, causing *Vg* expression in the fat body to cease in preparation for winter. The resulting arrest of oogenesis matches the effects of allatectomy or *Met* knockdown in reproductive females experiencing long daylight (Smykal et al., 2014a) (Fig. 3). Methoprene treatment can override the photoperiodic sensory input and induce *Vg* expression and oogenesis in formerly diapausing,

non-reproductive females, provided that *Met* had not been suppressed by prior RNAi treatment (Bajgar et al., 2013; Smykal et al., 2014a). Virtually the same regulation of vitellogenesis applies to a summer diapause in the cabbage beetle, *Colaphellus bowringi*, except in reverse: it is the long daylight associated with adverse summer conditions that informs the reproductive arrest (Liu et al., 2016). The function of Met in JH-mediated exit from a reproductive diapause is therefore conserved across insect orders and eco-physiological strategies.

Extensive evidence for JH- and Met-dependent regulation of genes associated with reproduction has been afforded by studies on *A. aegypti*. In these anautogenous mosquitoes, JH does not directly induce vitellogenesis but rather primes the female for reproduction enabled by the blood meal (Hansen et al., 2014; Roy et al., 2018). Transcriptome analyses of *Aedes* females during post-eclosion, pre-vitellogenic development uncovered large clusters of genes that are differentially regulated in response to the changing JH titre (Saha et al., 2016; Zou et al., 2013). Importantly, expression of many genes positively correlated with high JH titre also required Met, and upstream regions of these genes often contained sequences matching consensus elements capable of binding Met (Zou et al., 2013).

JH also supports reproduction indirectly, by balancing its trade-offs (Flatt et al., 2005). Met/Gce signalling suppresses immune defence in mated *Drosophila* (Schwenke and Lazzaro, 2017) or *A. aegypti* (Chang et al., 2021) females, maintains gut cell homeostasis necessary for increased metabolism in reproductive flies (Rahman et al., 2017; Reiff et al., 2015), adjusts carbohydrate and lipid metabolism (Wang et al., 2017a; Xu et al., 2013), and stimulates mating behaviour (Bilen et al., 2013) (Fig. 1). Some other JH functions such as differentiation of the termite soldier caste also require Met (Masuoka et al., 2015, 2018). Finally, it is interesting to note that the function of Met as a receptor for MF is conserved in crustaceans (Li et al., 2021a,b; Miyakawa et al., 2018; Qu et al., 2015).

3.2 Transcriptional regulation of JH-response genes

3.2.1 JHRs are bHLH-PAS protein complexes containing Met/Gce

Transcription factors of the bHLH-PAS family, to which Met belongs, form heterodimers of class I and class II members such as the circadian clock protein complex Clock-Bmal (the insect ortholog of Bmal is Cycle). The hypoxia inducible factor (HIF-α) or the aryl hydrocarbon receptor (AhR) dimerize with their common class II partner Arnt. The latter pair interacts when activated by low-molecular weight agonist ligands binding to the

PAS-B domain of AhR, upon which the complex recognizes specific DNA response elements to initiate transcription of target genes (Beischlag et al., 2008; Denison and Faber, 2017; Denison et al., 2011; Kewley et al., 2004).

By analogy to AhR, Met should also require another bHLH-PAS domain protein as a binding partner to form an active JHR complex. *Drosophila* Met was shown to interact with itself and with Gce in the absence of a JH agonist (Godlewski et al., 2006), but the exact nature of these homophilic complexes and their function remain unknown. Candidate members of the bHLH-PAS family from *A. aegypti* were tested for interaction with Met in yeast two-hybrid assays. This approach yielded two interesting candidates: the circadian clock protein Cycle (Shin et al., 2012) and an ortholog of *Drosophila* Taiman (Tai) (Li et al., 2011), which had been earlier described as a transcriptional coactivator of EcR (Bai et al., 2000; Zhu et al., 2006). Tai (also called FISC or SRC) is homologous to the vertebrate steroid receptor coactivator 1 (SRC-1). For clarity, and in keeping with a prevailing consensus in the current literature, we will further refer to this protein as Tai (Taiman) rather than SRC.

Reminiscent of AhR-Arnt dimer formation, presence of JH agonists induces association of Met with either Tai or Cycle. Mutations preventing Met from binding JH abolished the interaction with Tai but not formation of the homophilic Met-Met complex (Charles et al., 2011), indicating that the protein subunits of the active JHR assemble in an agonist-dependent manner. A similar effect of JH binding could be demonstrated for interaction between Gce/Met and Tai proteins from *Drosophila* and other insects using two-hybrid assays (Bittova et al., 2019; Miyakawa and Iguchi, 2017) (Fig. 4E and F).

The requirement for Tai in transducing the JH signal by Met/Gce has been corroborated by work on *A. aegypti* (Li et al., 2011, 2014; Liu et al., 2018), *T. castaneum* (Charles et al., 2011; Kayukawa et al., 2013; Zhang et al., 2011), *B. mori* (Kayukawa et al., 2012), *D. melanogaster* (Jindra et al., 2015b), *P. apterus* (Bajgar et al., 2013; Smykal et al., 2014a), *B. germanica* (Lozano et al., 2014), and *L. migratoria* (Guo et al., 2014; Wang et al., 2017b). Importantly, some of these studies have revealed the roles of Tai in insect metamorphosis and reproduction, both in holometabolous and hemimetabolous species (see also Jindra et al., 2015a; Roy et al., 2018, for reviews). Moreover, interactions between Tai and Met orthologs from cladoceran crustaceans, *Daphnia pulex* and *D. magna*, could be enhanced by JH agonists including MF, as shown using two-hybrid (Miyakawa and Iguchi, 2017; Miyakawa et al., 2013) and BRET (Kakaley et al., 2017) protein-protein interaction assays.

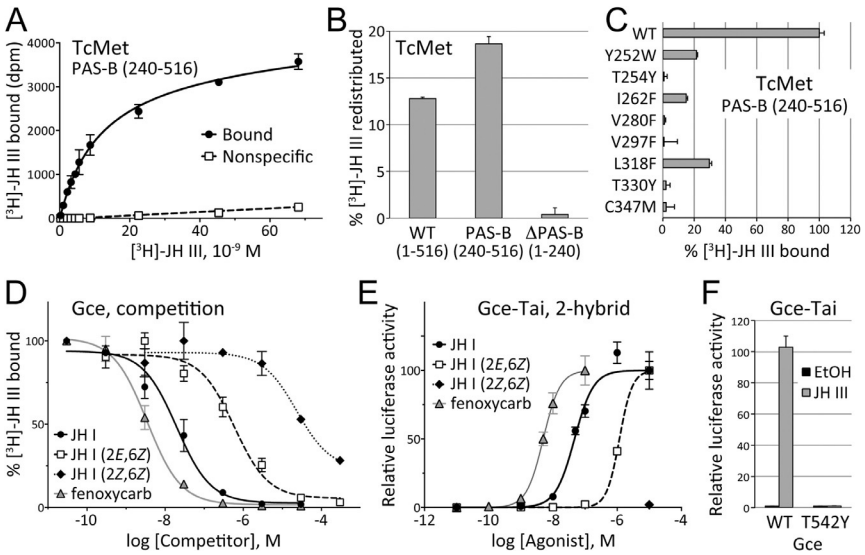

Fig. 4 Different methods demonstrate specific JH binding to the JHR proteins from *T. castaneum* (TcMet) and *D. melanogaster* (Gce). (A) Binding of [^{3}H]-JH III to the *in vitro* translated TcMet protein (the C-terminal half containing the PAS-B domain) as assessed using the dextran-coated charcoal method. A K_d value of 11.7 nM was determined in this experiment (performed by Jean-Philippe Charles). (B) Equilibrium dialysis with the entire (WT) or truncated *in vitro* translated TcMet led to a biased distribution of [^{3}H]-JH III towards those proteins containing the PAS-B domain. (C) Effects of mutations within the PAS-B domain of TcMet on the relative amount of [^{3}H]-JH III bound in the dextran-coated charcoal assay. (D) In the glass-fibre filter assay, JH I (but not its geometric isomers) efficiently competed ($K_i = 15$ nM) against [^{3}H]-JH III for binding to the *in vitro* translated Gce protein. Fenoxycarb outcompeted JH I. (E) A two-hybrid assay that measures the agonist binding-dependent interaction between VP16-Gce and Gal4-Tai fusion proteins expressed in the human HEK293 cells recapitulated the results shown in (D). (F) Mutated Gce (T542Y) that is incapable of binding [^{3}H]-JH III was inactive in the two-hybrid assay. T542 in Gce corresponds to T254 in TcMet (panel C). Data in (A–C) are from Charles et al. (2011); data in (D and E) are from Bittova et al. (2019). Data in all panels are mean values; error bars represent standard deviations.

Together, ample evidence establishes Tai as a broadly conserved, functional component of JHR complexes containing agonist-bound Met or Gce. At the same time, it should be kept in mind that Met/Gce likely interacts with additional bHLH-PAS partners, such as Cycle or the insect Arnt ortholog Tango (Tgo), as has been indicated by yeast two-hybrid assays with the *A. aegypti* proteins (Shin et al., 2012). The latter interaction did not require JH. Similarly, Met and Tgo from *T. castaneum* co-immunoprecipitated in a JH-independent manner (Jindra and Rynes, unpublished data).

3.2.2 Immediate target genes are driven by JH-response elements

As a JH receptor, Met should activate JH-inducible genes in an agonist-dependent manner. A number of JH-response genes became known through their involvement in processes regulated by JH. Ectopic induction of *Krüppel-homologue 1* (*Kr-h1*) by exogenous JH agonists in *Drosophila* and *Tribolium* pupae interfered with adult development (Minakuchi et al., 2008b, 2009), and JH-induced *Kr-h1* also blocked metamorphosis in last-instar hemimetabolous larvae (Konopova et al., 2011; Lozano and Bellés, 2011). These and additional studies in *Bombyx* (Kayukawa et al., 2012, 2014, 2017) have established Kr-h1 as a JH-inducible repressor of metamorphosis, universal to hemi- and holometabolous insects (Bellés, 2020; Jindra, 2019; Truman, 2019; Ureña et al., 2016). RNAi experiments initially revealed that Met was required for JH to induce *Kr-h1* mRNA expression (Konopova et al., 2011; Minakuchi et al., 2009; Zhu et al., 2010). Cell-based reporter studies complemented with chromatin immunoprecipitation or electrophoretic mobility shift assays (EMSA) confirmed that *Kr-h1* is indeed transcriptionally activated by binding of Met to its regulatory DNA elements (Cui et al., 2014; He et al., 2014; Kayukawa et al., 2012, 2013; Li et al., 2011, 2014; Zhang et al., 2011). Some of these studies have also implicated Tai as a component of the activating JHR complex. The *early trypsin* (*ET*) gene from *A. aegypti* (Noriega et al., 1997; Zhu et al., 2010) is another JH-inducible target, dependent on immediate transcriptional activation by the Met-Tai complex (Li et al., 2011, 2014).

The *ET* and *Kr-h1* genes provided material for detailed analyses of *cis*-acting JH-response elements (JHREs). A JHRE1 from the *A. aegypti ET* gene contained an imperfect palindrome (CACGCG) resembling *E*-box motifs bound by some bHLH-PAS proteins. JHRE1 was occupied by Met and Tai, which activated a JHRE1-driven luciferase reporter in response to JH III (Li et al., 2011). A similar reporter was induced by JH agonists through the endogenous Gce and Tai proteins in the *Drosophila* S2 cell line (Jindra et al., 2015b). JHREs from the *Kr-h1* genes (*k*JHREs) of several insects were described following systematic mutagenesis of regulatory sequences of the *Bombyx* and *Tribolium Kr-h1* orthologs (Kayukawa et al., 2012, 2013). These studies led to identification of a functional *k*JHRE with an *E*-box palindrome CACGTG. An unbiased selection of oligonucleotides bound by the complex of recombinant *Aedes* Met and Tai proteins essentially confirmed preference of the mosquito JHR for JHREs containing the core palindrome sequence (Li et al., 2014). A double-stranded DNA probe carrying this sequence bound the Met-Tai

complex with high affinity ($K_d = 5.8$ nM); K_d values of 13.8 nM and 103.0 nM were determined for the *Bombyx* *k*JHRE and the *Aedes* JHRE1, respectively (Li et al., 2014). By mutating N-terminal basic regions in Met and Tai, Li et al. (2014) have established that the bHLH domains of both interacting proteins are required for the DNA-binding capacity of the JHR complex. This situation resembles other bHLH-PAS transcription factors where each of the dimerizing partners contributes to form a bipartite DNA-binding domain (Kewley et al., 2004).

3.3 Binding JHs as agonist ligands

3.3.1 Validation of JH binding through independent assays

High-affinity, saturable and competitive binding of a cognate hormonal agonist is the key prerequisite for a receptor function. The hydrophobic, "sticky" nature and chemical instability of the native insect JHs posed many problems leading to false discoveries and artefacts (for a review see Riddiford, 2020). Therefore, having a reliable and properly controlled ligand-binding assay, validated by alternative methods based on different principles, was imperative. Tritium-labelled JH III ([10-^{3}H(N)]-JH III), formerly commercially available as a racemic mixture of 10*R* and 10*S* enantiomers, enabled several types of ligand-binding assays (Fig. 4).

A simple method, previously employed for hemolymph JH-binding proteins (JHBPs) (Touhara et al., 1993), relies on the capacity of dextran-coated charcoal to capture free, but not protein-bound [^{3}H]-JH III. Using this assay, Miura et al. (2005) initially reported a 5.3 nM K_d value for binding of [^{3}H]-JH III to the *Drosophila* Met protein, which was *in vitro* translated in rabbit reticulocyte lysates. Their experiment was difficult to repeat whether *Drosophila* Met was translated *in vitro* (Charles et al., 2011) or expressed using baculovirus in insect *Sf*9 cells (M. Jindra, unpublished data). In contrast, the *Tribolium* Met and *Drosophila* Gce proteins proved high-affinity JH III binders, with K_d of 2.9 nM and 19.3 nM, respectively (Charles et al., 2011; Jindra et al., 2015b) (Fig. 4A–D). The ortholog of Met from the primitive ametabolous firebrat, *Thermobia domestica* (Zygentoma), also bound JH III avidly (Charles et al., 2011), indicating that the JHR function of Met predates the origin of insect metamorphosis. High-affinity binding to JH III ($K_d = 4.4$ nM) was further reported for Met from *A. aegypti* (Li et al., 2014). While the above cited reports utilized proteins translated *in vitro*, specific JH III binding has been achieved with recombinant *T. castaneum* and *A. aegypti* JHR proteins expressed in and purified from the *Sf*9 cells (Jindra et al., 2021). It should be noted that obtaining

JH-binding data for Met proteins from some insect species was unsuccessful, possibly due to improper folding of the intricate bHLH-PAS domain structure.

Importantly, the JH-binding capacity of Met/Gce could be validated through additional, independent methods. During equilibrium dialysis, [^{3}H]-JH III accumulated in the compartment of the dialysis chamber which contained an intact *Tribolium* Met protein or its ligand-binding domain, but not a truncated Met lacking this domain (Charles et al., 2011) (Fig. 4B). Another method was adapted from an early protocol for ecdysteroid binding studies (Cherbas et al., 1988; Graham et al., 2007). In this assay, receptor protein is captured on a glass-fibre filter, while any unbound ligand is washed away. Like the charcoal method, this filter assay enabled verification of specific binding for Gce or Met proteins (Bittova et al., 2019) (Fig. 4D). Finally, the ligand-dependent interaction of Met/Gce with Tai in a two-hybrid assay (Miyakawa and Iguchi, 2017) could be used as a proxy to the direct ligand-binding assays (Fig. 4E). Critical controls in any of the above experiments included Met/Gce proteins where the ligand-binding PAS-B domain was removed or mutated (Fig. 4B, C and F).

3.3.2 Ligand selectivity and stereoselectivity

Besides JH III and its precursor MF, common to most species, some insects produce additional types of JHs that differ by ethyl *vs*. methyl branches and by the number and position of epoxide groups (Goodman and Cusson, 2012; Hiruma and Kaneko, 2013; Tsang et al., 2020). A cell-based, JHRE-luciferase reporter assay indicated a slight preference (a 3.6-fold difference in ED_{50} values) of *Bombyx* Met2 towards the lepidopteran, ethyl-branched JH I relative to JH III (Kayukawa and Shinoda, 2015). This difference was cancelled when the Met PAS-B domain from *Tribolium* (whose native hormone is JH III) was substituted for that in *Bombyx* Met2. In addition to JH III, MF and JH III bisepoxide (JHB3) are circulating JHs in *Drosophila* (Dubrovsky and Bernardo, 2014; Richard et al., 1989; Wen et al., 2015). *In vitro* binding assays, based on competition of an unlabelled compound against [^{3}H]-JH III, have revealed that the Gce protein binds all of the three native *Drosophila* JHs, while showing no appreciable preference for JH III over the lepidopteran JH I (Bittova et al., 2019). Relative to the epoxidated JHs, the affinity of MF was about 8-fold lower (K_i values 90 nM and 11 nM for MF and JH III, respectively), indicating the relevance of the epoxide moiety for the bond with Gce. These results were corroborated for endogenous Gce using a JHRE-luciferase reporter in

Drosophila S2 cells (Bittova et al., 2019). Complementary data for *Drosophila* Met were obtained in a similar assay based in the transfected human HEK293T cell line, where Met showed a minor preference for JH III over JH I, and was slightly less activated by MF (Yokoi et al., 2020).

The study by Bittova et al. (2019) has also shown that Gce prefers the native (10*R*) enantiomer of JH III over its unnatural (10*S*) antipode, and that ligand binding by Gce is highly sensitive to the correct geometric isomerism of the JH skeleton (Fig. 4D and E). Testing all possible JH I isomers demonstrated that any deviation from the native 2*E*,6*E* conformation dramatically reduces binding to Gce and its agonist-dependent interaction with Tai, the transcriptional activation of *Kr-h1*, and the effect on *Drosophila* adult development. The 2*Z*,6*Z* conformation renders JH I inactive (Bittova et al., 2019) (Fig. 4D and E). The stereoselectivity of Gce corresponds to early data on JH isomer activities in bioassays (Wigglesworth, 1969) and matches binding affinities towards hemolymph JHBPs (Goodman et al., 1978).

3.3.3 Delineating the JH-binding pocket

Ligand-binding assays with truncated versions of *Tribolium* Met have localized JH-binding activity to the PAS-B domain (Charles et al., 2011) (Fig. 4A–C), corresponding to the site of ligand interaction in AhR (Pandini et al., 2007, 2009; Soshilov and Denison, 2014). Within their respective receptor complexes, AhR and Met/Gce are the ligand-sensing subunits. Thus, for instance, the interaction of *Tribolium* Met with Tai is induced by JH agonist binding to the PAS-B domain of Met, whereas Tai itself neither binds JH nor is required for ligand binding by Met (Charles et al., 2011).

While the PAS-B domain of AhR has so far escaped structural resolution, models for PAS-B domains of Met (Charles et al., 2011; Li et al., 2014) and Gce (Bittova et al., 2019) could be developed based on partial homology to HIF-2α, whose NMR and crystal PAS-B structures are available (Erbel et al., 2003; Scheuermann et al., 2009). The modelling and ligand docking images highlighted amino acid residues lining a putative JH-binding pocket. When individually replaced with amino acids of similar physicochemical nature but bulkier side chains, these mutations either reduced or completely abolished the capacity of *Tribolium* Met to bind [^{3}H]-JH III (Charles et al., 2011) (Fig. 4C). Molecular modelling coupled with mutagenesis and ligand-binding assays were also performed with the *Aedes* Met protein (Li et al., 2014), essentially confirming a common set of about 10 highly conserved residues required for binding JH.

Corresponding mutations prevented both JH binding and transcriptional activation by *Drosophila* Gce (Jindra et al., 2015b). A docking simulation based on the model of Gce PAS-B predicted a hydrogen bond between the epoxide oxygen in JH III and the hydroxyl group of a conserved tyrosine (Bittova et al., 2019). Removal of this tyrosine hydroxyl by mutation to phenylalanine substantially reduced [^{3}H]-JH III binding by Gce.

Besides probing the JH-binding pocket, the mutagenesis of Met/Gce has provided essential experimental means to decide whether any tested effect depends on JH binding. For instance, Met or Gce variants incapable of binding JH could not engage in the JH-induced interaction with Tai, either in co-immunoprecipitation (Charles et al., 2011) or two-hybrid (Fig. 4F) assays, and mutated Gce or Met could not activate a JHRE-driven reporter in a cell-based assay (Jindra et al., 2015b).

3.3.4 JH binding by Met/Gce is required for insect development

While the studies cited thus far have established the necessity of the receptor-hormone interaction in cell-based assays, it remained to be seen whether the JH-binding capacity of Met/Gce was also relevant *in vivo*. This could be tested in *Drosophila* deficient for Met and Gce (Jindra et al., 2015b). *Met* mutant flies are viable but abnormally resistant to methoprene and other JH agonists (Wilson and Ashok, 1998; Wilson and Fabian, 1986), whereas hemizygous *Met gce* double-mutant males never survive past pupation (Abdou et al., 2011). Ubiquitous transgenic expression of either Met or Gce rendered the *Met* mutants naturally sensitive (or hypersensitive in the case of Gce) to methoprene or pyriproxyfen treatment as long as the transgenic Met/Gce retained the JH-binding capacity (Jindra et al., 2015b). Accordingly, expressing functional Met or Gce was sufficient to rescue full viability in the unconditionally lethal *Met gce* males (Fig. 5). Mutations of individual amino acids that are critical for binding JH rendered both Met and Gce incapable of restoring the sensitivity to JH agonists and of sustaining the development of the fly. This genetic evidence has unequivocally proven that JH-binding capacity is essential for both Met and Gce to function as JH receptors *in vivo*.

3.3.5 Insect Met and Tai confer JH responsiveness to mammalian cells

A number of cell-based reporter assays for monitoring JH activity have been devised in both insect and mammalian cell lines (reviewed by Ito-Harashima and Yagi, 2021). While activation of JHRE-luciferase reporters in insect cells can rely on endogenous Met/Gce and Tai proteins (Bittova et al., 2019;

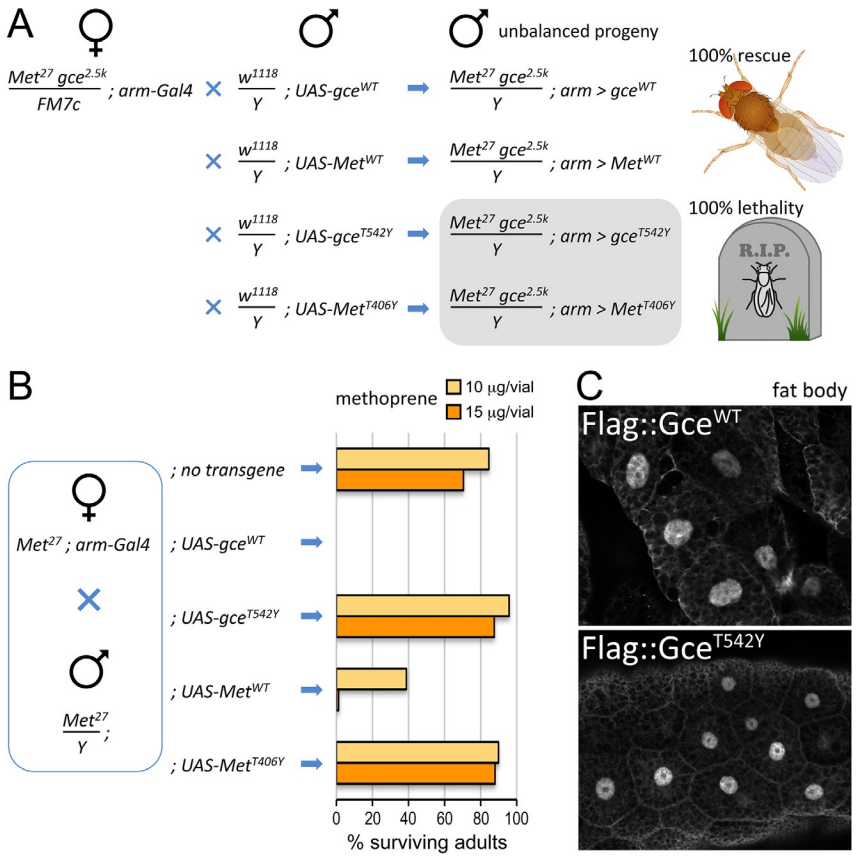

Fig. 5 Binding of JH is required for the function of the *Drosophila* Gce and Met JHR proteins *in vivo*. (A) The unconditionally lethal Met^{27} $gce^{2.5k}$ double-hemizygous males can be rescued to adulthood by ubiquitous (*armadillo*; *arm-Gal4*-driven) transgenic expression of either Gce or Met. Met^{27} $gce^{2.5k}$ males cannot complete development when the mutated Gce^{T542Y} or Met^{T406Y} variants incapable of binding JH are expressed instead. (B) Met^{27} flies are viable but resistant to methoprene. Transgenic expression of either Gce or Met but not of their mutated versions restore the natural methoprene sensitivity in the Met^{27} background. (C) While deficient in JH binding, the Flag epitope-tagged Gce^{T542Y} protein resides in the nuclei of the larval fat body as assessed by immunostaining (performed by Mirka Uhlirova). For details see Jindra et al. (2015b).

Jindra et al., 2015b; Kayukawa et al., 2012, 2013; Li et al., 2011), mammalian cells lack an ortholog of Met and thus are nonresponsive to JH. However, strong expression of JHRE-driven reporters could be induced by JH agonists in human (HEK293) cells transfected with constructs encoding *Bombyx* or *Drosophila* Met and Tai proteins (Kayukawa and Shinoda, 2015;

Kayukawa et al., 2012, 2021; Yokoi et al., 2020). Transfection with either Met or Tai alone was insufficient for the reporter activation. These studies have demonstrated that both Met and Tai are required and sufficient to constitute an active JHR complex in heterologous cells.

4. Molecular architecture and function of JHR

Intracellular receptors comprise ligand-binding and gene *trans*-activation functions. In order to regulate target genes, such receptors translocate to cell nuclei, recognize specific *cis*-regulatory DNA elements, and engage in contacts with transcriptional coactivators or corepressors. Moreover, their DNA-bound, active forms are typically dimers of the same or different members of the given protein family. These multiple functions are reflected in the modular architecture of the receptor molecules. In this regard, nuclear receptors of the NR family and the bHLH-PAS receptors such as AhR and Met/Gce are functionally analogous in spite of their unrelated molecular structures.

4.1 Functional parallels between Met/Gce and AhR

AhR exemplifies a bHLH-PAS transcription factor that is activated by agonist ligands. While AhR is neither a hormonal receptor nor a true ortholog of Met/Gce, it is the closest vertebrate homologue by sequence, and probably the best available proxy in terms of mode of action. Considering AhR function may thus be a useful guide to begin to understand how JHR works. As the Met and AhR proteins share a common functional domain architecture (Fig. 6A), some parallel mechanisms emerge.

4.1.1 The canonical AhR pathway

The mass of knowledge on AhR signalling has been covered in several comprehensive reviews (*e.g.*, Beischlag et al., 2008; Denison and Faber, 2017; Denison and Nagy, 2003; Denison et al., 2011; Jackson et al., 2015; Stejskalova et al., 2011; Stockinger et al., 2014). In the absence of an agonist [such as the environmental pollutant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)], inactive AhR resides in the cytoplasm, bound in a multiprotein complex including the chaperone heat-shock protein 90 (Hsp90), a co-chaperone p23, and an AhR interacting protein (AIP/XAP2). Exposure to TCDD and its high-affinity binding to the PAS-B domain of AhR triggers a conformational change that allows the AhR complex to translocate *via* β-importin to the cell nucleus, where AhR dissociates from Hsp90

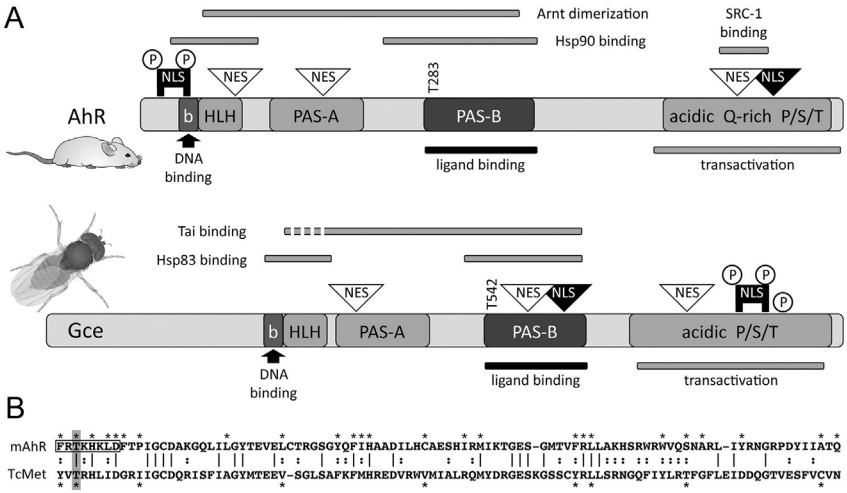

Fig. 6 Analogy of functional domains in mouse AhR and *Drosophila* Gce proteins. (A) The approximate positions of functional domains are denoted (b, basic; HLH, helix-loop-helix; PAS, Per-Arnt-Sim; P/S/T, proline/serine/threonine-rich). Interaction interfaces are indicated below and above the schemes of each protein. NES (nuclear export signal) and NLS (nuclear localization signal) are indicated by triangles or, for bipartite NLS, by black rectangles. Circled Ps denote areas of phosphorylation. Threonine residues critical for ligand binding are indicated. (B) The ligand-binding pockets of the mouse AhR (mAhR) and *T. castaneum* Met (TcMet) show similarity in some positions (asterisks) that are involved in binding of agonists (TCDD and JH, respectively). Strand A of the putative β-sheet in mAhR (open box) is critical for contacting Hsp90. The shaded box indicates the conserved residues T283 (mAhR) and T254 (TcMet, corresponding to T542 in Gce) that are required for ligand binding.

(Soshilov and Denison, 2011; Soshilov et al., 2020). The liganded AhR dimerizes with Arnt and the resulting complex binds enhancers such as the xenobiotic/dioxin response elements to induce transcription of detoxification genes including CYP1A1. There are numerous deviations from this canonical xenobiotic response. These entail diverse AhR ligands (including endogenous agonists) and different protein partners [*e.g.*, the oestrogen receptor (Ohtake et al., 2003, 2007)], enabling AhR to regulate alternative target genes and hence many relevant cellular responses (Denison and Faber, 2017; Denison and Nagy, 2003; Jackson et al., 2015; Stejskalova et al., 2011; Stockinger et al., 2014).

4.1.2 Interactions with bHLH-PAS partners

Crystal structures capturing the bHLH and PAS-A domains of the AhR-Arnt dimer in a complex with the dioxin response element have

determined the contact interfaces between the two proteins and DNA at atomic resolution (Schulte et al., 2017; Seok et al., 2017). This work has revealed the individual amino acids responsible for the AhR-Arnt dimerization and DNA binding within the bHLH and PAS-A domains. While the PAS-B structure of AhR has not yet been resolved, the amino acid residues effecting contacts between the PAS-B domains of AhR and Arnt could be identified through modelling and functional assays with the proteins mutated at specific sites (Corrada et al., 2016, 2017; Soshilov et al., 2020).

Similar to AhR, Met must interact with another bHLH-PAS protein, such as Tai, in order to activate transcription (Kayukawa et al., 2012; Li et al., 2011; Zhang et al., 2011), and the basic motifs within the bHLH of both Met and Tai are required for binding the JHRE DNA (Li et al., 2014). The regions of mutual interaction have been mapped, albeit only roughly, for the *Aedes* Met-Tai complex (Li et al., 2011) (Fig. 6A). While both PAS-A and PAS-B domains of Met were essential for JH-induced interaction with Tai in a two-hybrid assay, the bHLH domain of Met seemed dispensable. However, this does not rule out a participation of the bHLH domain in forming the protein complex in a manner similar to the bHLH domain of AhR. Conversely, deletion of the PAS-B and, to a lesser extent, of the PAS-A and bHLH domains from *Aedes* Tai reduced JH-dependent interaction with Met (Li et al., 2011). Co-immunoprecipitation of *Tribolium* Met and Tai demonstrated that the PAS-B domain of Met was both necessary and sufficient for JH-induced binding to Tai (Charles et al., 2011).

It is worth noting that Tai is neither an insect ortholog of Arnt nor a typical class II bHLH-PAS protein. SRC-1, the mammalian ortholog of Tai, directly contacts AhR to enhance its transcriptional activity in response to agonists such as TCDD (Beischlag et al., 2002; Zhang et al., 2008). However, rather than acting through the PAS domains, LXXLL motifs in the central region of SRC-1 interact with the glutamine-rich C-terminal transactivation part of AhR (Kumar and Perdew, 1999) (Fig. 6A). Therefore, the architecture of the JHR complex consisting of Met and Tai may differ from that of the AhR-SRC-1 and AhR-Arnt heterodimers.

4.1.3 The role of Hsp90/Hsp83

Interaction with the chaperone Hsp90 (orthologous to Hsp83 in insects) is a common feature of AhR and Met action (Fig. 6A). The role of Hsp90 is not only to keep the unliganded AhR inactive while in the cytoplasm, but also to protect AhR from proteasomal degradation. Furthermore, Hsp90 is critical for conferring competence of AhR to bind agonist ligands to activate

transcription (Beischlag et al., 2008; Lees and Whitelaw, 1999; Pongratz et al., 1992; Whitelaw et al., 1995). A similar requirement applies to the ligand-dependent activities of some NRs such as the glucocorticoid receptor (Picard, 2006); EcR requires Hsp83 to bind DNA (Arbeitman and Hogness, 2000). Moreover, the chaperone complex including Hsp90 facilitates nuclear import of AhR (Kazlauskas et al., 2001). Hsp90 contacts AhR at the bHLH and PAS-B domains, of which the latter encompasses the ligand-binding site and partly overlaps with the dimerization interface for Arnt (Soshilov and Denison, 2011) (Fig. 6A). Simultaneous interactions of liganded AhR with Hsp90 and Arnt therefore appear to be mutually exclusive or, rather, limited to a brief transitional state (Soshilov et al., 2020). Whilst the PAS-B domain mediates activation by agonist ligands, its interaction with Hsp90 also blocks AhR in the absence of an agonist. Therefore, under *in vitro* conditions, deletion of the PAS-B domain lifts inhibition by Hsp90 and turns the truncated AhR into a constitutive DNA binder independent of TCDD (Soshilov and Denison, 2008).

The Hsp90-AhR mechanism is at least partially conserved for Hsp83 and Met in insects (He et al., 2014; Liu et al., 2013). In *Drosophila*, Hsp83 has been implicated in JH agonist-stimulated nuclear import of Met and induction of the JH-response gene *Kr-h1*. Loss of Hsp83 function or its chemical inhibition with geldanamycin in *Drosophila* larvae blocked the JH response. Short deletions within the *Drosophila* Met protein revealed that, like in AhR, the bHLH and PAS-B domains of Met were involved in the interaction with Hsp83 (He et al., 2014) (Fig. 6A). Nuclear import of the Met-Hsp83 complex was further shown to depend on nucleoporin Nup358 (He et al., 2017), a nuclear pore protein associated with β-importin. Both Hsp83 and Nup358 were captured through affinity binding to a JH-responsive upstream DNA sequence of *Kr-h1* among proteins extracted from *Drosophila* cells that had been cultured with methoprene (He et al., 2014). This is somewhat surprising, given that agonist binding to the PAS-B domain displaces Hsp90 from the complex with AhR (Soshilov and Denison, 2011) and that Hsp90 is unable to bind AhR once engaged in the active AhR-Arnt heterodimer (Beischlag et al., 2008).

4.1.4 Ligand-binding pockets and rich agonist repertoires

The ability to bind a diverse range of activating ligands is perhaps the most intriguing and distinguishing common feature of AhR and Met/Gce. As the three-dimensional structure of the PAS-B in AhR has not yet been resolved, our understanding of the receptor-agonist interactions has thus far

relied on molecular modelling, supported by site-directed mutagenesis and testing of the mutated protein variants in ligand- and DNA-binding assays (Tagliabue et al., 2019; Motto et al., 2011; Pandini et al., 2007, 2009; Soshilov and Denison, 2014; Soshilov et al., 2020). Structures of HIF-α proteins in complexes with Arnt and/or artificial low-molecular ligands have been employed as the closest available homology templates for modelling AhR. Interestingly, some of the artificial ligands co-crystallized with HIF-2α proved to be competitive agonist ligands of AhR, further strengthening the relevance of the homology template (Motto et al., 2011; Tagliabue et al., 2019). Crystallographic and NMR data for HIF-2α have also been applied towards initial characterization of the JH-binding pocket in Met/Gce (Bittova et al., 2019; Charles et al., 2011; Li et al., 2014) (see Section 3.3.3) although the *Drosophila* circadian clock protein Period could serve as an alternative template (Bernardo and Dubrovsky, 2012; Yokoi et al., 2021).

Analyses of AhR have identified specific amino acid residues in the PAS-B domain whose mutations result in a partial or complete loss of TCDD- or other ligand-binding activity (Pandini et al., 2007, 2009; Soshilov and Denison, 2014; Soshilov et al., 2020; Tagliabue et al., 2019). These include a stretch of amino acids forming the A strand of the PAS-B β-sheet, known to mediate contact with Hsp90 (Soshilov and Denison, 2011; Soshilov et al., 2020). This short sequence is well conserved in insect Met/Gce proteins (Fig. 6B), including an invariable threonine that is required for [^{3}H]-JH III binding by *Drosophila* Gce and *Tribolium* and *Aedes* Met proteins (Charles et al., 2011; Jindra et al., 2015b; Li et al., 2014) (Fig. 4C). Additional point mutations within the PAS-B domain of Met/Gce that disable JH III binding occur at positions known to affect TCDD binding by AhR and often represented by amino acids with similar properties (Fig. 6B). These positions are also conserved in crustacean (*Daphnia*) Met orthologs and likewise predicted to contact JH agonists (Hirano et al., 2020).

Both AhR (Denison and Faber, 2017; Denison and Nagy, 2003; Denison et al., 2011; Soshilov and Denison, 2014; Stejskalova et al., 2011) and Met/Gce (Bittova et al., 2019; Charles et al., 2011; Jindra et al., 2015b) display a remarkable capacity to bind ligands of diverse chemistry. Examples for Met/Gce include insecticidal JH mimics (juvenoid IGRs) (Jindra and Bittova, 2020). It seems paradoxical that while Gce exhibits strict stereoselectivity towards the native hormones (Bittova et al., 2019) (Fig. 4D), it can accommodate many structurally unrelated agonists. Not surprisingly, disparate ligands could be docked to the computational models of Met/Gce PAS-B domains (Charles et al., 2011; Hirano

et al., 2020). However, being based on the rather distant homology to the *apo* structure of HIF-2α, those rigid models contained large pockets, allowing room for diverse potential ligands.

Advanced modelling of AhR builds on multiple HIF-2α structures crystallized alone or with various artificial ligands, thus affording flexibility in shape and size of the ligand-binding cavity (Tagliabue et al., 2019). Ten AhR agonist ligands representing diverse chemistries were docked to the different models and results were reconciled with their actual binding affinities. This approach has unveiled some ligand-specific interactions within the ligand-binding pocket of AhR, suggesting a mechanism underlying the broad agonist spectrum.

4.2 Nuclear import

When expressed in insect tissues or heterologous cell lines, the Met and Gce proteins are predominantly nuclear or, to a variable degree, dispersed through both nuclei and the cytoplasm (Greb-Markiewicz et al., 2011, 2015; He et al., 2014, 2017; Jindra et al., 2015b; Miura et al., 2005; Pursley et al., 2000) (Figs. 5C and 7). Nuclear localization is enhanced by JH agonist treatment or when JH is naturally abundant *in vivo*. As already explained above, ligand-stimulated nuclear enrichment of *Drosophila* Met requires Hsp83 and importin machinery (He et al., 2014, 2017) (Fig. 8), similar to nuclear import of AhR.

AhR is subject to nucleocytoplasmic shuttling dependent on importin and leptomycin B-sensitive exportin/CRM1 activities (Ikuta et al., 1998, 2000, 2004). Intracellular trafficking of AhR relies on nuclear localization and export signals (NLS and NES, respectively), which were primarily identified in the N-terminal sequence of AhR (Ikuta et al., 1998) (Fig. 6A). A bipartite NLS consisting of two short stretches of basic amino acids overlaps with the bHLH domain and is masked by Hsp90 in the unliganded state. Agonist binding to AhR exposes the NLS, permitting nuclear import of the AhR-Hsp90 complex. Phosphorylation or phosphomimetic mutation of two serine residues immediately abutting the basic NLS sequence inhibits nuclear import of AhR (Ikuta et al., 2004). The N-terminal NES overlaps with the Arnt-binding interface and its blocking by Arnt or by phosphorylation of a serine residue within the NES retains AhR in the nucleus. Additional sequences that regulate intracellular trafficking of AhR have been identified in the PAS-A domain (Berg and Pongratz, 2001) and in the C-terminal region rich in Gln (Q) and Pro/Ser/Thr (P/S/T) residues (Tkachenko et al., 2016) (Fig. 6A).

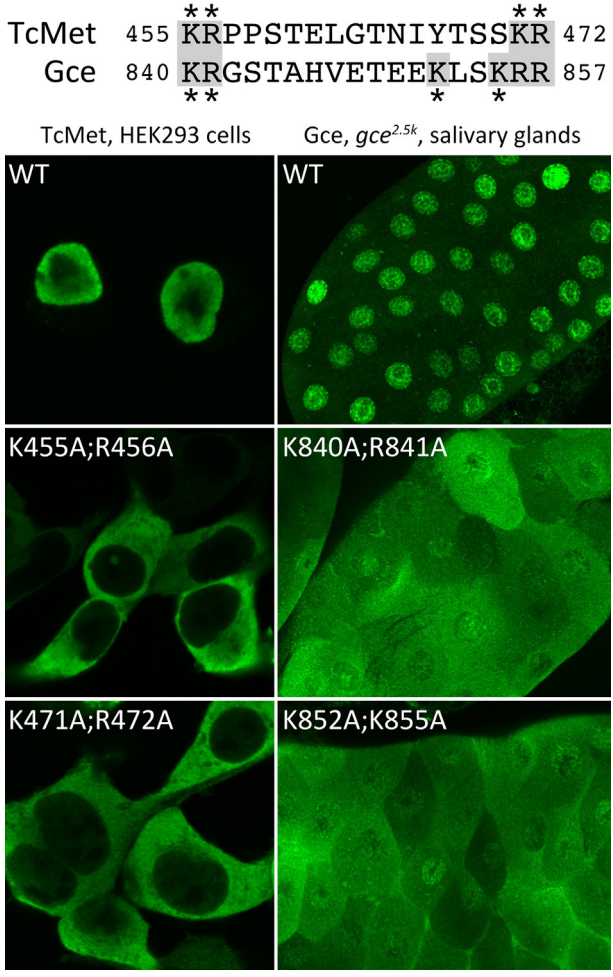

Fig. 7 Conservation of a bipartite NLS in the carboxyl termini of *T. castaneum* Met (TcMet) and *Drosophila* Gce proteins. The asterisks indicate basic amino acids in each protein that were mutated to alanine. The Myc epitope-tagged wild type (WT) and mutated TcMet proteins were expressed in human HEK293 cells; the variants of Gce were expressed from transgenic constructs in the salivary glands of homozygous $gce^{2.5k}$ wandering third-instar larvae. As detected by immunocytochemistry, the WT proteins are predominantly nuclear whereas the mutated versions occur in the cytoplasm. Images are single confocal slices.

Functional NLS and NES motifs have been found in the *Drosophila* Met and Gce JHR proteins, expressed with N-terminal YFP-tags in mammalian cell lines (Greb-Markiewicz et al., 2011, 2015). By means of systematic deletions, the authors delimited an NES in each of the PAS-A, PAS—B, and the

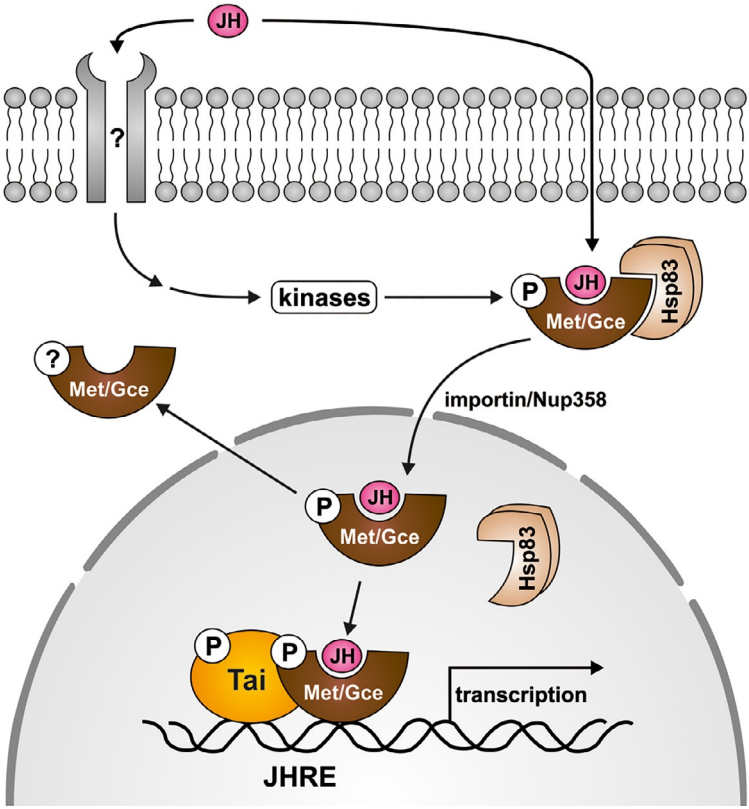

Fig. 8 A scheme of the gene-regulatory JHR action. In addition to regulation by JH and Hsp83, Met/Gce is also phosphorylated, at least partly as a result of JH acting through a hypothetical receptor at the cell membrane. Whether and how phosphorylation affects nucleocytoplasmic shuttling of Met/Gce and its interaction with Hsp83 is presently unknown.

C-terminal regions of either protein (Fig. 6A). Both Met and Gce also contain two NLS motifs, which reside either in each of the PAS domains of Met or in the PAS-B domain and near the carboxyl terminus of Gce. No NLS or NES have been predicted in N-terminal regions of *Drosophila* Met and Gce that would correspond to the positions of the signals in AhR (Greb-Markiewicz and Kolonko, 2019).

The NLS located in the PAS-A of *Drosophila* Met is a JH-independent signal for constitutive nuclear localization which, however, is not conserved in Gce or in Met proteins from other insects. In contrast, the C-terminal NLS in *Drosophila* Gce (Fig. 6A) contributes to the JH-induced nuclear

import (Greb-Markiewicz et al., 2015). While absent from *Drosophila* Met, this bipartite NLS is functionally conserved in the C-terminal region of *Tribolium* Met, with each cluster of basic residues being required for nuclear localization of the beetle protein (Jindra et al., 2021) (Fig. 7). The position of the bipartite NLS in carboxyl termini of Gce and *Tribolium* Met is intriguing, as these non-conserved regions are intrinsically disordered (Kolonko et al., 2016, 2020) and may thus be prone to post-translational modifications. Indeed, we have detected phosphorylation of serine and threonine residues straddling the bipartite NLS in the *Tribolium* Met protein (Jindra et al., 2021).

4.3 Phosphorylation of JHR proteins

As mentioned at the beginning of Section 3, JH exerts rapid effects initiated at the cell membrane in addition to its gene-regulatory function (Fig. 1). Besides inducing patency in the follicle epithelium (Jing et al., 2018), sub-nanomolar concentrations of JH III were shown to stimulate protein synthesis in the *Drosophila* male accessory glands in a manner dependent on Ca^{2+} and PKC (Yamamoto et al., 1988).

Working on the fat body and a cell line from *A. aegypti*, Liu et al. (2015) have shown that JH III triggers a Ca^{2+} wave, followed by a surge of the diacylglycerol (DAG) and inositol trisphosphate (IP_3) second messengers. These rapid signalling events lead to phosphorylation of intracellular JHR proteins Met and Tai, resulting in their increased capacity to occupy the JHREs of the *ET* and *Kr-h1* genes and to induce their transcription in response to JH. In other words, JH also appears to engage in alternative, membrane-initiated signalling pathways that potentiate gene-regulatory functions of its own intracellular receptor (Fig. 8). Based on treatments with chemical inhibitors and RNAi, this phosphorylation pathway may involve Ca^{2+}/calmodulin dependent kinase II (CaMKII) (Liu et al., 2015) and PKC (Ojani et al., 2016) acting downstream of phospholipase C (PLC). Inhibitor experiments have also led the authors to postulate a receptor tyrosine kinase (RTK) as a hypothetical cell-membrane receptor for JH (Liu et al., 2015). Although such a receptor is yet to be discovered, the work has added a potentially important rapid-response element to regulation of JH signalling (Fig. 8).

The data from Liu et al. (2015) suggest that several as yet unidentified positions in the *Aedes* Met and Tai proteins may be phosphorylated, some in a JH-dependent manner. Indeed, our phosphoproteomic analysis of recombinant *Tribolium* and *Aedes* JHR protein complexes, purified from the *Sf*9 cells, detected multiple phosphorylated amino acid residues, some enriched

following exposure of the cell culture to a JH agonist. Most of the phosphorylations occurred in the intrinsically disordered C-terminal regions of the Met proteins (Jindra et al., 2021) (Fig. 6A). A current study (Li et al., 2021a,b) has proposed that, surprisingly, a single threonine within the ligand-binding domain of the Met1 protein from a moth, *Helicoverpa armigera*, becomes phosphorylated in the presence of JH. This threonine phosphorylation prevents the homophilic Met1-Met1 association and instead promotes a productive interaction between Met1 and Tai, the binding of the complex to a JHRE DNA, and the transcriptional activation of the *Helicoverpa Kr-h1* gene (Li et al., 2021a, b). Whether this is a conserved mechanism remains to be investigated.

5. JHR as a target of insecticide action

JH mimics are attractive for insect control because they hijack the arthropod-specific endocrine system without affecting vertebrates or even non-target invertebrates. The latter possibility has been exemplified by bis-acylhydrazine ecdysone receptor agonists (Hill et al., 2012; Pener and Dhadialla, 2012). The potential of JH signalling for controlling insect pests and disease vectors was recognized very early (Williams, 1967). The idea was inspired by discovery of a terpene juvabione in paper produced from American fir trees. Juvabione proved extremely potent and selective towards *Pyrrhocoris* and its closest relatives (Sláma and Williams, 1965). The chemical structure of juvabione (Bowers et al., 1966) is quite distinct from structures of native insect JHs, the first of which (JH I) was reported soon thereafter (Röller et al., 1967). The first synthetic JH mimic methoprene (Henrick et al., 1973) was registered in 1975, almost four decades before its molecular target, Methoprene-tolerant (Wilson and Ashok, 1998; Wilson and Fabian, 1986), became established as a JHR (Charles et al., 2011). Methoprene and other aliphatic juvenoids (Henrick, 2007) were followed by carbamate and pyridine derivatives such as fenoxycarb and pyriproxyfen, respectively (Dorn et al., 1981; Hatakoshi et al., 1986), in which aromatic or heterocyclic rings replaced the isoprene units of JH (Fig. 9). These juvenoid insecticides of the IGR category are more stable than JH and are highly active against a broad spectrum of insects (Minakuchi and Riddiford, 2006; Parthasarathy et al., 2012; Pener and Dhadialla, 2012; Sláma et al., 1974).

5.1 Juvenoid IGRs are agonist ligands of JHR

Given the practical utility of the JH mimics as insecticides and as tool compounds substituting for native JHs in hundreds of research studies, it is critically important to know whether these compounds act through the same

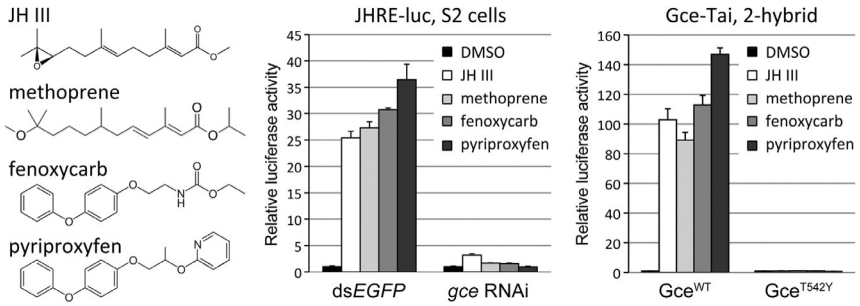

Fig. 9 JH and its synthetic agonists (juvenoid IGRs) act through the Gce protein in a ligand binding-dependent manner. All depicted compounds (left) activated a JHRE-luciferase reporter in the *Drosophila* S2 cells (centre). Activation was cancelled by knocking down *gce*, but not by treatment with a control (*EGFP*) dsRNA. When incapacitated for JH binding, Gce^{T542Y} could no longer engage in the agonist-dependent interaction with Tai. Data are mean values; error bars represent standard deviations.

molecular mechanism as the hormone itself. In spite of their diverse chemistries, the few synthetic JH mimics tested thus far competed against [^{3}H]-JH III for binding to the recombinant *Tribolium* and *Drosophila* Met and Gce proteins (Bittova et al., 2019; Charles et al., 2011; Jindra et al., 2015b). Moreover, as inferred from these competition assays, the binding affinities of the most potent IGRs such as pyriproxyfen or fenoxycarb were in the nanomolar range and surpassed those of JH III itself (Fig. 4D).

In vitro ligand-binding data could be complemented by two-hybrid assays wherein Met/Gce and Tai proteins, fused to the VP16 *trans*-activator and Gal4 DNA-binding domains, respectively, are expressed in mammalian cells (Miyakawa and Iguchi, 2017). As JH binding to VP16-Met/Gce stimulates its interaction with Gal4-Tai, the activity of a Gal4-driven luciferase reporter gauges the potency and efficacy of any tested agonist ligand. This two-hybrid assay clearly discriminated biologically active and inactive geometric isomers of JH I, and confirmed that relative to any native JH, fenoxycarb is a more potent agonist ligand of Gce (Bittova et al., 2019) (Fig. 4E). Importantly, when mutated at the threonine residue critical for binding JH III, VP16-Gce could no longer respond to synthetic JH mimics by dimerizing with Tai (Fig. 9). Fenoxycarb or pyriproxyfen also could not activate a JHRE-luciferase reporter in *Drosophila* S2 cells subjected to RNAi-mediated knockdown of either *gce* or *tai* (Bittova et al., 2019; Jindra et al., 2015b) (Fig. 9).

Prior knockdown of *Met* prevented the anti-metamorphic effects of JH mimics in *Tribolium* pupae (Konopova and Jindra, 2007, 2008; Minakuchi

et al., 2009) and in last-instar larvae of *Pyrrhocoris* (Konopova et al., 2011). Similarly, adverse effects of pyriproxyfen on mosquito female reproduction and metabolism required Met in *Aedes* (Ahmed et al., 2020). Finally, unlike functional Met or Gce, the mutated proteins incapable of binding JH failed to restore the natural methoprene or pyriproxyfen sensitivity when expressed in the *Methoprene-tolerant* mutant flies (Jindra et al., 2015b) (Fig. 5B). Therefore, the JH-binding function is required for Met/Gce to mediate the developmental effect of the juvenoid IGRs *in vivo*.

Based on the evidence summarized above, it can be inferred that at least some synthetic JH mimics activate the intracellular JHR as genuine agonist ligands (Jindra and Bittova, 2020). Nonetheless, one common molecular mechanism for all JH mimics cannot be taken for granted. The chemical diversity of natural and synthetic compounds exerting JH-like effects is extensive, perhaps comparable to that of natural and man-made agonists of AhR. It is thus likely that some JH agonists interact with the JHR in distinct modes, as has been predicted for binding of diverse agonists to the ligand-binding pocket of AhR (Tagliabue et al., 2019). Ligand-specific modes of interaction have been demonstrated rigorously for the 20E receptor EcR through resolving crystal structures of EcR bound by various steroid or nonsteroidal agonists (Billas et al., 2003; Browning et al., 2021; Carmichael et al., 2005; Hill et al., 2012; Iwema et al., 2007; Ren et al., 2014). Some nonsteroidal agonists induced major conformational changes to the ligand-binding pocket, illustrating its adaptability to the diverse ligand chemistries (Browning et al., 2021).

5.2 Search for novel agonists and antagonists of JHR

While just a handful of juvenoid IGRs have been commercialized, a large number of compounds that exert JH-like effects on insects, reportedly over six thousand, have been produced (Parthasarathy et al., 2012). It remains to be ascertained whether all these compounds might be JHR agonists as has been shown for methoprene, fenoxycarb, and pyriproxyfen (Jindra and Bittova, 2020). In striking contrast, no authentic JHR antagonists have been obtained to date.

The currently available juvenoid IGRs have disadvantages such as low selectivity to target insect species and toxicity to crustaceans, while resistance and cross-resistance have also arisen in mosquitoes, whiteflies, lepidopterans, and other insects (reviewed by Pener and Dhadialla, 2012). Moreover, juvenoids prevent metamorphosis and prolong the larval feeding period,

in some cases leading to giant supernumerary larvae and increased crop losses. A related problem is that JH agonists are often ineffective until late during development. In contrast, a JH antagonist would terminate the larval stage early and provoke a fatal precocious metamorphosis. Therefore, JH antagonists should in theory surpass JH mimics in insect control. They would also be very useful as tool compounds for research.

5.2.1 *Activators discovered* via *high-throughput and virtual screening*

Efforts are underway to discover novel compounds targeting the JH signalling pathway either positively or through specific inhibition. These efforts are greatly aided by the knowledge of the JHR. The structure of the JH-binding pocket in Met/Gce remains to be resolved before a rational design of novel compounds may be attempted. Meanwhile, cell-based platforms built on the agonist-dependent capacity of the JHR to induce JHRE-driven reporters can be exploited in high-throughput screening (HTS) of chemical libraries. This approach has been taken by Kayukawa et al. (2021). They selected a stable *Bombyx* cell line carrying a *k*JHRE-luciferase reporter to screen a diversity collection of 9600 compounds. The HTS yielded nine confirmed primary hits of which six proved dose-dependent activators of the *k*JHRE reporter. Compounds of analogous structures were then retrieved from a larger library, expanding the number to 10 validated activators, of which some shared structural features with existing IGRs such as pyriproxyfen, while others were distinct. Seven of the activators could delay metamorphosis of final-instar *Bombyx* larvae although none was able to block pupation and provoke an extra larval moult. Although it remains to be determined whether these JH signalling activators bind *Bombyx* Met as agonist ligands, they present lead compounds with a potential for further development (Kayukawa et al., 2021).

A chemical HTS is currently concluding in collaboration between our laboratory and Dr. Sedlák of the national core facility for chemical biology (CZ-OPENSCREEN). Starting from a large set of compounds, our campaign produced a great diversity of validated hits, corresponding to the structural repertoire of known JHR agonists. The outcome is somewhat reminiscent of the situation with AhR, for which an extensive chemical HTS has revealed an extreme ligand promiscuity (Denison, 2010).

Building pharmacophore models based on quantitative structure-activity relationship (QSAR) of juvenoid compounds active against *Drosophila* was initially attempted without considering a JHR protein (Liszekova et al., 2009; Parthasarathy et al., 2012). However, the first successful example of

a JH agonist discovered through an *in silico* approach was recently set by Yokoi et al. (2021). Their virtual screen of five million compounds relied on a structural model of *Drosophila* Met. Virtual compounds selected based on similarity to pyriproxyfen and fenoxycarb were computationally docked to the model, finally yielding 11 hits. Candidate compounds were validated in a JHRE-driven reporter assay in human cells expressing the *Drosophila* Met and Tai proteins (Yokoi et al., 2020). One piperazin-based hit displayed a sub-micromolar potency, thus providing a novel lead compound (Yokoi et al., 2021). Its JHR agonist activities need to be tested in additional assays and *in vivo*.

One goal of screening for new agonists is to find compounds selective against target species. Juvabione (Bowers et al., 1966; Sláma and Williams, 1965) and certain small peptidic derivatives, such as ethyl L-isoleucyl-L-alanyl-*p*-aminobenzoate (Sláma et al., 1974; Zaoral and Slama, 1970), exemplify JH mimics displaying an exquisite selectivity towards true bugs of the Pyrrhocoridae family. Whether and how the action of such compounds reflects differences within the ligand-binding pockets of JHR proteins from specific insects is currently under investigation.

5.2.2 Inhibitors

Relative to agonists, JHR antagonists appear to be more challenging to find. Besides the absence of precedent validated antagonists, the problem may be that there are many ways in which any reporter assay can be inhibited. Nevertheless, it is encouraging to know that both allosteric and competitive antagonists exist for AhR (Denison and Faber, 2017; Denison and Nagy, 2003; Denison et al., 2011; Stejskalova et al., 2011).

One approach to discovering JHR inhibitors stems from the idea that plants might produce anti-JH compounds as a natural defence against insect herbivores. Using the yeast two-hybrid assay for agonist-stimulated interaction between Met and its partner proteins, diterpenic and acyclic alkyl compounds were extracted from plants (Lee et al., 2015, 2018; Oh et al., 2017; Shin et al., 2018). At $>10\,\mu M$ doses, some of the diterpenes interfered with assembly of JHR complexes, while some appeared to affect JH-dependent processes in mosquitoes and other insects. However, it is unclear whether these natural products directly antagonize the JHR or whether they compromise interaction of its subunit proteins in other ways.

The same chemical HTS in the *Bombyx* cell line described above for JHR agonists (Kayukawa et al., 2021) was also performed in an antagonistic mode (Kayukawa et al., 2020). The library comprising 218 thousand items was

exploited to yield 69 compounds structurally related to primary hits. These compounds inhibited JH I-dependent activation of the *k*JHRE-luciferase reporter, albeit in distinctive, competitive or noncompetitive modes. Interestingly, relatively minor structural differences turned some potential antagonists to activators of the *k*JHRE reporter. One of the competitive JH signalling inhibitors (JHSI48) was active at sub-micromolar concentrations in the cell-based assay and also reduced *Kr-h1* mRNA levels in isolated *Bombyx* tissues. Notably, JHSI48 induced precocious pupation in penultimate-instar larvae, a phenotype marking JH deficiency (Daimon et al., 2012, 2015; Tan et al., 2005). Whether JHSI48 is a genuine antagonist of the *Bombyx* JHR and whether it might be active in other insects should be addressed by future studies.

6. Conclusion

Together, the data reviewed in this chapter establish several key attributes defining the bHLH-PAS proteins Met/Gce as *bona fide* intracellular receptors for juvenile hormones and their synthetic mimics. The evidence is based at molecular, genetic, cellular, and organismal levels.

(1) JHs competitively bind to Met/Gce proteins from diverse species with nanomolar affinities; ligand binding is stereoselective and reliant on specific, conserved amino acid residues within the PAS-B domain of Met/Gce. Mutations of these residues block Met/Gce functions. (2) Met/Gce constitute JHR complexes that bind to JH-response regulatory DNA elements of direct target genes and induce their transcription in a manner dependent on JH binding to Met/Gce. (3) Met/Gce and its partner protein Tai are sufficient to confer JH responsiveness to mammalian cells. (4) Aberrant phenotypes caused by loss of Met/Gce function match specific roles of JH in insect development and reproduction; defects such as precocious metamorphosis phenocopy the deficiency of JH itself. (5) The capacity of Met/Gce to bind JH *in vivo* is required to sustain insect development.

While the discovery of the intracellular JHR has enabled considerable progress in diverse areas of arthropod research, much still remains to be learned about the molecular mechanism of JHR action. For instance, the exact mode of interaction and function of Tai, Cycle, Tgo, and other, hitherto unknown protein partners of Met/Gce within JHR complexes requires detailed mechanistic studies. The same applies to post-translational modification of the JHR, primarily phosphorylation induced by JH acting upon a hypothetical cell-membrane receptor. The role of phosphorylation in the

nucleocytoplasmic transport of Met/Gce has not been addressed. Any ligand-induced conformational changes of Met/Gce leading to nuclear import and formation of an active JHR complex are completely unknown. At the moment, the nature of interactions of either native hormones or diverse synthetic JHR agonists with the ligand-binding pocket of JHR proteins is merely subject to computational modelling predictions. Clearly, the ultimate understanding of the architecture of the JHR complexes with their agonist ligands awaits structural resolution at the atomic level. Answering these questions will unleash the potential of the JHR as a target of novel compounds for selective and efficient intervention with insect development.

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