Ligand Binding Pocket Function of *Drosophila* USP is Necessary for Metamorphosis

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Abstract:

The widely accepted paradigm that epoxidized methyl farnesoates ("juvenile hormones," JHs) are the principal sesquiterpenoid hormones regulating insect metamorphosis was assessed in *Drosophila melanogaster*. GC–MS analysis of circulating methyl farnesoids during the mid to late 3rd instar showed that methyl farnesoate is predominant over methyl epoxyfarnesoate (=JH III). The circulating concentration of methyl farnesoate (reaching nearly 500 nM), was easily high enough on a kinetic basis to load the *Drosophila*ortholog of the nuclear hormone receptor RXR (also known as "ultraspiracle," USP), whereas the circulating concentrations of JH III and methyl bisepoxyfarnesoate (bisepoxyJH III) were not. The hypothesis that the ligand pocket of USP necessarily binds an endogenous ligand for differentiation of the immature to the adult was tested with USP mutated at residue that normally extends a side chain into the ligand binding pocket. An equilibrium binding assay confirmed that the mutation (Q288A) strongly altered methyl farnesoate interaction with USP, while a heterologous cell-line transfection assay confirmed that the mutation did not allosterically alter the transcriptional response of the ultraspiracle/ecdysone receptor heterodimer to ecdysteroid signaling. Transgenic wildtype USP driven by the cognate natural promoter rescued null animals to develop to the adult inside a normally formed puparium, while in contrast animals transgenically expressing instead the ligand pocket mutant exhibited developmental derangement at the larval to pupal transition, including failure to form a properly shaped or sclerotized puparium. Other point mutations to the pocket strongly reducing affinity for methyl farnesoate similarly disrupted the larval to pupal

metamorphosis. These results suggest that normal larval to pupal maturation in this mecopteran model insect requires the involvement of a distinct endocrine axis of USP binding to its own endogenous terpenoid ligand.

Abbreviations

JH, juvenile hormone; USP, ultraspiracle; EcR, ecdysone receptor; 20E, 20-OH ecdysone; RXR, retinoid X receptor

Keywords: Methyl farnesoate | Ultraspiracle | Retinoid X receptor | Juvenile hormone

Article:

1. Introduction

An important nuclear hormone receptor that is necessary for cell differentiation in both vertebrates and invertebrates is the retinoid-X-receptor (RXR; known as “ultraspiracle” or USP in higher insects). RXR or USP is an obligate heterodimer partner for many other nuclear hormone receptors (e.g., for the farnesoid X receptor in vertebrates and for the ecdysone receptor (EcR) in invertebrates [15], [22] and [37]).

There is uncertainty on whether a nanomolar affinity endogenous ligand exists for RXR in either the vertebrates or invertebrates [9], [44], [63] and [69]. In some invertebrates, studies using different methods report various outcomes as to whether RXR binds 9-*cis* RA with high affinity or can be transcriptionally activated by 9-*cis* RA [7], [40] and [68]. While some insect RXRs are reported not to bind or not to be transcriptionally activated by 9-*cis* RA [5] and [28], in contrast is the report of the nanomolar affinity of retinoic acid for locust RXR [45].

Our recent data suggest that a nanomolar affinity sesquiterpenoid ligand exists for mecopteran RXR (USP). Of the compounds biosynthesized in culture by ring glands of *Drosophila melanogaster* (hereafter,*Drosophila*), two are unique to insects in the animal kingdom (methyl epoxyfarnesoate = JH III, and methyl bisepoxyfarnesoate = bisepoxyJH III), while the third, methyl farnesoate, is a circulating compound in Crustacea [30] and [41]. In an equilibrium binding assay screen of natural products of the insect farnesoid biosynthesis pathway, the insect farnesoid with strongest affinity for *Drosophila* USP was methyl farnesoate (K_d = 40 nM [34]), similar to the affinity of vertebrate RXR for 9-*cis* RA [11]. In addition, in some synthetic reporter contexts, methyl farnesoate potentiates the transcriptional response of the RXR or USP heterodimer partner, the ecdysone receptor (EcR), to its own ligand, 20-OH ecdysone (20E; [4] and [66], consistent with the hypothesis that the USP ligand binding pocket has a structural capacity to respond to an intrinsic ligand. In contrast, while JH III can be made to load USP at micromolar concentration [34],[35] and [36] and also at that concentration enhance 20E-induced reporter activity [18], JH III has been recently reported to have low nanomolar affinity instead for the germ cell expressed (GCE) protein and perhaps the methoprene tolerant (MET) protein [6] and [10].

However, there have not yet been reports providing a functional demonstration that the RXR or USP molecule requires a ligand binding capability to its ligand pocket in order to function *in vivo*. Nor has a measured circulating titer of methyl farnesoate been reported in insects that is at a level corresponding to theK_d of the cognate species' RXR or USP. Hence, in the present study, we have directly addressed these issues. We demonstrate that methyl farnesoate circulates in *Drosophila* at a level that corresponds favorably to its K_d for USP, and that USP ligand pocket debilitated for methyl farnesoate binding cannot sustain normal larval development through metamorphosis to the pupa.

2. Materials and methods

2.1. Physiochemical detection of circulating methyl farnesoids

The methods used here are the procedures for GC–MS–CI after micro-solid phase extraction, described previously for the determination of circulating methyl farnesoids in *D. melanogaster* [31] and [33].

2.2. Mutagenesis of ligand binding pocket residues

We identified a specific USP amino acid residue, Q288, with a side chain (a) that points into the *Drosophila*USP (dUSP) ligand binding pocket [12] and [57], (b) that also contacts terpenoid ligand in the respective vertebrate and mollusk RXR/ligand complexes [17] and [20], and (c) which was unlikely to be involved in allosteric effects to disrupt DNA binding, or heterodimerization with EcR, or ligand binding by EcR. For example, mutation of hRXR residue Q275 (=dUSP Q288) decreased transcriptional activation by 9-*cis* RA [56] and [73], but did not affect heterodimerization [56]. By a similar approach, we also mutated to alanine a residue at the deep end of the pocket (N325) that has been modeled as potentially interacting with the methyl ester of a terpenoid ligand [56], and that does not make contact with a phospholipid that occludes the pocket opening in crystal structure preparations [12] and [57].

The CaSpeR-based pMVZ15 vector [26] for transgenic expression of a wild type or mutant USP gene (mutation confirmed by sequencing) was used to prepare fly lines reported here. When the wild type USP coding sequence is driven by the natural USP promoter in this construct, in a null (usp^2) background, it can rescue larval development to the adult [26].

2.3. Ligand binding assay

We used an equilibrium binding assay, based on quenching of intrinsic receptor fluorescence, that has been widely used in the field of vertebrate nuclear hormone receptors to evaluate ligand affinity, e.g., RXR [11], farnesoid X-receptor (FXR, [23]), androgen receptor (AR, [29]), hepatocyte nuclear factor (HNF, [48]), peroxisome proliferator activated receptor (PPAR, [58]), and vitamin D_3 receptor (VDR, [61]), and to study ligand binding by other kinds of proteins (see Ref. in [70]). Advantages of this approach are that not only does it provide a means to measure ligand affinity

(i.e., K_d), but the differential effect of alternative ligands on the conformation of the receptor can possibly be also detected by the nature of the fluorescent response. E.g., the change in fluorescence report of the receptor population under saturating conditions may be a net fluorescence suppression for one ligand, little net change for another ligand, or an increase for another. When the ligands themselves do not absorb at the fluorescing wavelength of the receptor (as is the case with methyl farnesoids), differences in final changes in fluorescence of the receptor indicate differences in conformation of the receptor (differences in local tryptophan environments) induced upon binding of the respective ligand (discussed previously in further detail by [34] and [70]). There is the additional utility that a ligand which upon binding causes a receptor conformation that imparts little or no net change in fluorescence can still be kinetically measured as binding to the receptor by its concentration-dependent, competitive displacement of a ligand that does cause a strong net fluorescence change. For example, farnesol and JH acid, which do not themselves cause much net change in receptor fluorescence at their respective (very high) saturation concentrations, can by mass action at a sufficiently excess concentration finally displace the much higher affinity and more strongly fluorescence-suppressing methyl farnesoate [34] and [70]. The particular range of concentrations of the respective ligand that leads up to its saturation of the receptor population, measured either directly or by competitive displacement, then yields by conventional bimolecular binding analyses [62] the calculation of the respective affinity constant. For example, two compounds, such as methyl farnesoate and JH III, that cause different final protein conformations at their respective saturating concentrations [70], may at those respective saturation concentrations cause similar final net suppression of USP fluorescence [35], but their very different affinity for USP is shown by the very different range in respective concentration (nanomolar for MF; micromolar for JH III) over which the binding curve leads to saturation [34].

Recombinant wild type and mutant USP were bacterially expressed, and purified over, first, a nickel resin column and, second, a Superdex 200 size exclusion column, similar to that described previously [34], Fig. S1). Purified receptor was adjusted to 0.7–1 μM, allowed to equilibrate overnight, and then subjected to a fluorescence-based ligand binding assay as described previously [34]. In this assay, the receptor itself is stable, and its binding of ligand is measured by a change in fluorescence to a new stabilized level (Fig. S2). Because the concentration of receptor that was used either approached or was higher than the K_d for the tested ligand, the kinetic binding curves were analyzed by a nonlinear method [62]. Three independent preparations each of wild type and mutant receptor were used to estimate the K_d of the ligand methyl farnesoate (Echelon Inc.) for each receptor type. For certain controls, juvenile hormone III (prepared by P. Teal) or tributyltin (Sigma–Aldrich) were also used. The latter, an environmental endocrine disrupter [21], has been demonstrated to physically bind to both vertebrate and invertebrate RXR, and has been co-crystalized with RXR [43], in a near covalent association with a ligand pocket cysteine residue [43] that is conserved in *Drosophila* USP (C472).

2.4. Intracellular reporter assay of receptor function

The Chinese hamster ovary (CHO) cell transfection functional assay for response to ecdysteroid signaling, described previously [4] and [25], was used to assess the performance of wild type USP and Q288A mutant USP as the heterodimeric partner of the *Drosophila* ecdysone receptor (EcR). Cells were cotransfected with plasmids expressing each receptor under a constitutive actin promoter. Also cotransfected was a transcription reporter plasmid expressing firefly luciferase, and containing five copies of a USP/EcR heterodimer binding site. Finally, also cotransfected was a control reporter plasmid, constitutively expressing a different reporter, used for normalization.

2.5. *D. melanogaster* handling and transgenic lines

Wild type (*yw*) larvae were reared at 25 °C on standard diet and hemolymph collected as described elsewhere [31] and [33].

Wild type (w^{1118}) transgenic lines expressing transgenic wild type or mutant *ultraspiracle* in the w^{1118} wild type background were prepared by Rainbow Transgenic Flies Inc., Newberry Park, CA. Multiple independent lines were prepared and tested for each USP construct. For tests that challenged the particular form of USP to perform in a null (usp^2) background, females of the null line [usp^2/FM7i, P{ActGFP}JMR3] were crossed with males homozygous on either chromosome 2 or 3 for the test USP (the FM7 balancer, that is marked with *bar* has a wild type *usp* allele). The male usp^2 (i.e., null) progeny could be identified from the FM7 progeny by the lack of the GFP marker, and from usp^2/+ female progeny by the testes (late 3rd instar larvae) and in the adult by the *forked* marker on the usp^2 chromosome.

2.6. qPCR analysis of expression of selected genes

Null usp^2 larvae expressing transgenic Q288A L366A mutant USP identified as exhibiting stages of wandering behavior (‘can walk slowly,’ ‘can barely walk,’ ‘can sway and writhe but not walk’; four larvae per stage) were homogenized in Trizol reagent. Sibling normal larvae in comparable stages (‘can walk slowly,’ ‘can sway and writhe,’ ‘can sway with anterior spiracle beginning to protrude’; four larvae per stage) were similarly collected. The ‘can walk slowly’ stage is at the very end of gut clearance as assessed by inclusion of bromophenol blue in the food. The Trizol extracts were processed through chloroform, isopropanol precipitation, 70% EtOH washing, and then resuspended in DEPC-treated water. RNA concentrations were quantified and purity assessed by Nanodrop. Trace genomic DNA was digested using Ambion’s Turbo DNA-free kit and accompanying protocol. RNA was reverse transcribed using Invitrogen’s SuperScript III kit, oligo dT, and accompanying protocol. cDNA samples were appropriately diluted to be used in fluorescein-normalized/SYBR green-detected RT PCR assays using Fermentas’ Maxima reagent and BioRad’s iCycler system. Primers were designed to have a 20 bp length, 60 °C Tm, and 70–150 bp product size. Duplicate 25 μl reactions using 5 μl of diluted cDNA were used to quantify transcript for each sample.

No-template controls were used to assess spurious amplification or aberrant primer activity and melt curves were performed to assess product specificity. Data were normalized to expression of the indicated ribosomal protein gene.

3. Results

3.1. Physiochemical detection of circulating methyl farnesoids

Using our previously established GC–MS–CI methods [31] and [33], we carefully examined the hemolymph for the presence of three methyl farnesoids previously shown to be secreted in culture by the larval*Drosophila* ring gland [50]. During the period from the mid-3rd larval instar to puparium formation (that encompasses the start of larval to pupal metamorphosis), methyl farnesoate, JH III, and bisepoxyJH III were all physiochemically established to be present (Fig. 1). Example chromatograms for the single ion monitoring used to detect and quantify the amounts of the three methyl farnesoids in samples are shown in Fig. S3.

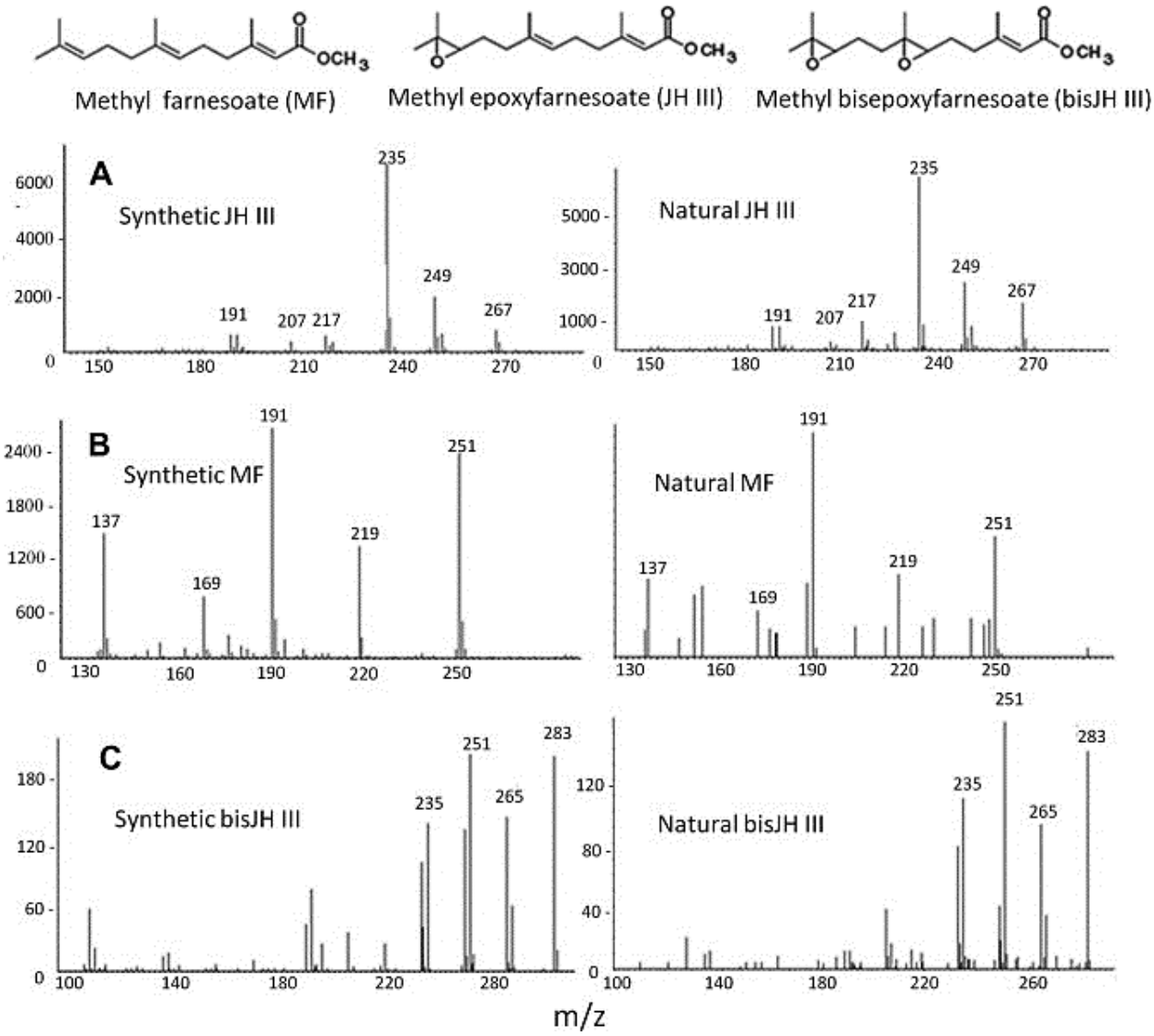


Fig. 1. GC–mass spectrometry analysis of the three natural methyl farnesoates isolated from the hemolymph of 3rd instar feeding stage of *Drosophila melanogaster*. Panels A–C show the fragments obtained from synthetic and natural methyl epoxyfarnesoate (JH III, juvenile hormone III), methyl farnesoate (MF) and methyl bisepoxyfarnesoate (bisJHIII).

3.2. Concentrations of circulating methyl farnesoids

In confirmation with previous reports of the presence of JH III in whole body extracts of *Drosophila* [8] and [59], an increase in the circulating JH III titer during the wandering stage was observed (up to ca. 100 nM) followed by a decline toward pupariation (Fig. 2). However, at all the time points sampled, methyl farnesoate was present in greater concentration than JH III (t, $p < 0.05$), ranging from 75 to 500 nM, and up to 50 times the concentration of classical JH III. BisepoxyJH III was significantly lower than methyl farnesoate at both wandering and puparium formation (t, $p < 0.05$), However, both were similar during last 24 h of the feeding period.

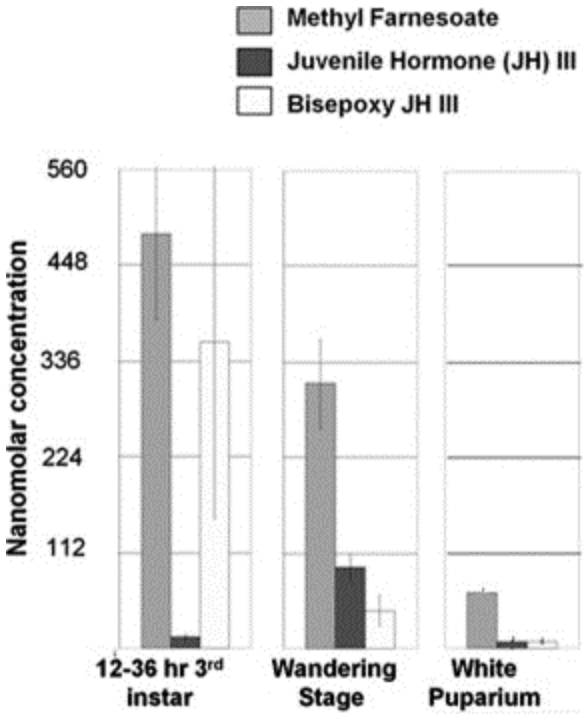


Fig. 2. Titer of circulating methyl farnesoids in *Drosophila melanogaster* during the 3rd larva instar prior to the onset of metamorphosis. At each stage, methyl farnesoate is the hormone in dominant concentration in the hemolymph. Values shown are the mean ± SEM of at least three independent samples, of at least 10 animals each.

The nuclear concentration in various tissues of most lipophilic hormones that bind vertebrate or invertebrate nuclear hormone receptors is unknown. Hence, inferences that specific hormones (e.g., 20E, JH III or methyl farnesoate) are binding intranuclearly with the respective receptors at particular developmental times is necessarily tentative. However, we noted that even if the nuclear concentration of methyl farnesoate is one tenth that of the circulating titer reached in Fig. 2, it would still be at the physical K_d of methyl farnesoate for USP [34], while in contrast, even if the nuclear concentration of either JH III or bisepoxyJH III were 10 times the circulating concentration shown in Fig. 2, it would still be at least 5–10 times lower than their respective K_d for USP [34]. These titer and binding affinity data further prompted our experimental consideration of whether the ligand binding pocket of USP is functionally necessary for successful larval to pupal metamorphosis.

3.3. Functional necessity of USP ligand binding pocket

Developing animals that are null for *usp* die at the first larval molt [47]. These null larvae are rescued to develop to the adult stage by transgenic insertion of a wild type *usp* that is under the control of a natural *usp* promoter [26]. Using this functional rescue assay, we tested whether the wild type function of specifically the ligand binding pocket of USP is necessary for larval–pupal metamorphosis.

3.4. High affinity equilibrium binding of methyl farnesoate

First, we reconfirmed the physical affinity of the ligand binding pocket of *Drosophila* USP for methyl farnesoate, using a fluorescence equilibrium binding assay that has been well established for a wide variety of nuclear hormone receptors. As shown in Fig. 3A, methyl farnesoate binding to USP (1) distinctly suppresses fluorescence of the receptor, (2) exhibits saturable binding kinetics, and (3) yields a K_d of ca. 40 nM (similar to the specific binding of 9-*cis* retinoic acid to vertebrate RXR), as we previously reported [34]. The equilibrium interaction of methyl farnesoate with the ligand binding pocket was confirmed by a competition assay using tributyltin (TBT), a ligand that forms a semi-covalent bond with a highly conserved cysteine residue near the opening of the ligand pocket of vertebrate RXR (residue C372 in *Drosophila* USP). Such properties confer to TBT the kinetic behavior of a 'slow, tight-binding' ligand. This cysteine residue to which TBT binds is on the opposite side of the ligand binding pocket from the fluorescent tryptophan residue W318 (Fig. S4B) that inserts its indole side chain into the pocket. The binding of TBT to USP hence has little effect on fluorescence of the receptor (Fig. 3B). However, when both the high-affinity, but equilibrium ligand methyl farnesoate and the slow, tight binding TBT are added together, the fluorescence is initially suppressed by the rapidly binding methyl farnesoate (Fig. 3B, inset). Then, as would be predicted by equilibrium kinetics, the equilibrium-binding ligand methyl farnesoate is slowly displaced from the USP pocket by the semi-covalent binding by TBT, so that finally the fluorescence of the USP protein is similar to that observed in the presence of TBT alone, i.e., little suppression (Fig. 3B, inset). These kinetics exhibit the properties that would be predicted by (1) the high affinity, equilibrium binding of methyl farnesoate to the established ligand pocket of USP, and (2) its competitive displacement from the ligand pocket by the established pocket ligand, TBT. These results confirm that *Drosophila* USP has a ligand pocket that is structurally competent to bind ligand.

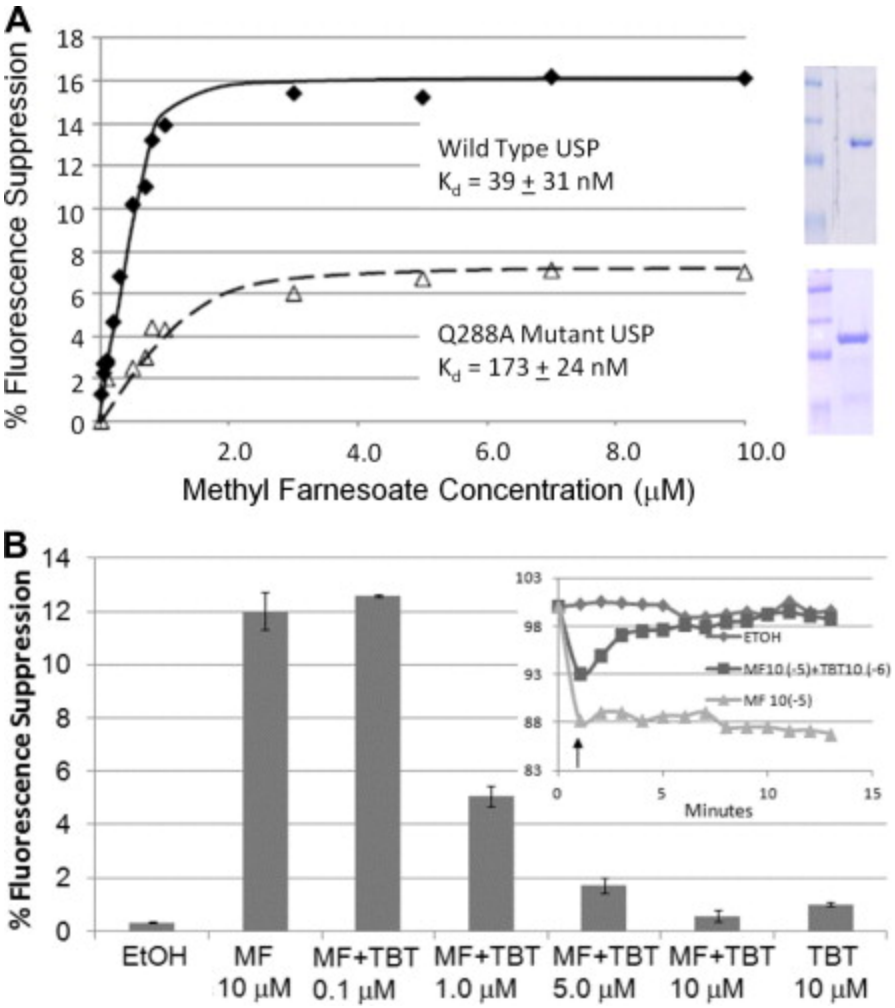


Fig. 3. Binding of methyl farnesoate to purified, recombinant wild type and mutant (Q288A) USP. (A) Panels at right show example preparations of purified wild type (above) and mutant (below) USP, following SDS–PAGE. At left, saturation equilibrium binding curves of methyl farnesoate with wild type (above) and mutant (below) USP. The average ± SEM K_d of methyl farnesoate calculated for wild type USP was 39 ± 31 nM, while the affinity constant calculated for Q288A USP was significantly reduced, 173 ± 42 nM ($p < 0.05$; $n = 3$ each). The % suppression of receptor fluorescence for wild type USP at saturating ligand concentration was 14.7% ± 1.3. (B) Tributyltin (TBT), is an established RXR ligand [21] and [63] that forms a near covalent association with a conserved cysteine (C472) in the ligand pocket [40]; this residue is on the other side of the pocket from the fluorescing tryptophan (W305) and TBT binding has little net effect on receptor fluorescence. In a concentration dependent manner, TBT displaces the fluorescence-suppressing methyl farnesoate from the ligand binding pocket. As predicted for a slow, tight-binding ligand, TBT in a time course slowly displaces the higher affinity, fluorescence-suppressing methyl farnesoate from the ligand binding pocket (inset).

3.5. Mutational weakening of methyl farnesoate binding (Q288A)

In the human RXRα-ligand crystal structure, the glutamine residue Q275 interacts with one of the two carboxylate oxygens of 9-*cis* RA [17]; Fig. S4A). This residue is conserved in *Drosophila* USP as Q288 (Fig. S4B). When the residue Q288 was mutated to alanine, the K_d for methyl farnesoate was reduced (K_d = 170 nM, $p < 0.05$; Fig. 3A). We considered previous reports that some point mutations to vertebrate RXR binding pocket can cause both reduced affinity to normal ligand and increased affinity to a nonnormal ligand [16], and that whereas wild type *Aedes aegypti* EcR is activated by both ecdysone and 20E (which differ by a single hydroxyl), a particular pocket point mutation dramatically changes affinity for ecdysone but not for 20E [65]. In the present study, we found that the micromolar affinity of USP to JH III [34] was not changed by the Q288 mutation (Fig. S5). Hence, misfunction of the USP ligand pocket due to mutation of Q288A is not likely to be due to 'new' nanomolar affinity binding to JH III.

3.6. Q288A USP mutation does not allosterically disrupt EcR performance

USP has been shown to function as an indispensible heterodimer partner of EcR in the nuclear transduction of ecdysteroid signaling [22] and [72]. We functionally confirmed that the Q288A mutation does not have an allosteric effect on the ability of the USP heterodimer partner, the ecdysone receptor (EcR), to transduce ecdysteroid signaling into a transcriptional response. In CHO cells treated with a physiological level of the ecdysone analog muristerone, the heterologously coexpressed wild type EcR/USP transduced the transcriptional report of a target plasmid containing an EcR/USP heterodimer binding site (Fig. 4; [25]). The same level of transcriptional report, as was seen with the presence of the wild type USP, was obtained when instead the mutant Q288A was used (Fig. 4, $p > 0.05$). The similar transcription report in the presence of the mutant USP ligand pocket as compared to the wild type USP was also observed when either the EcR isoform B1 or B2 was cotransfected.

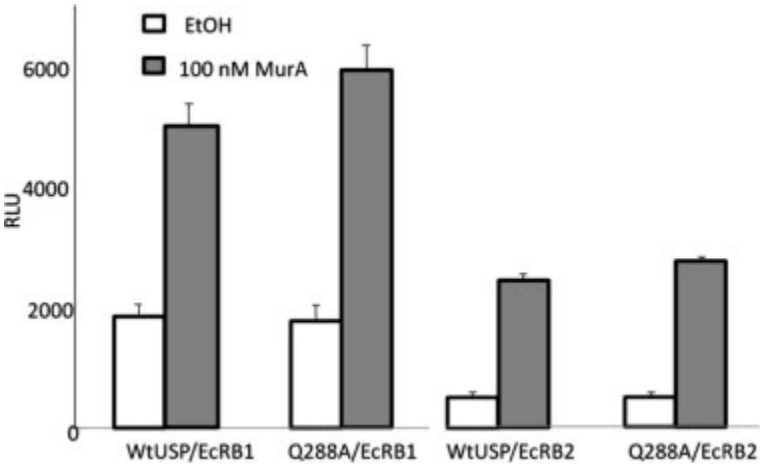
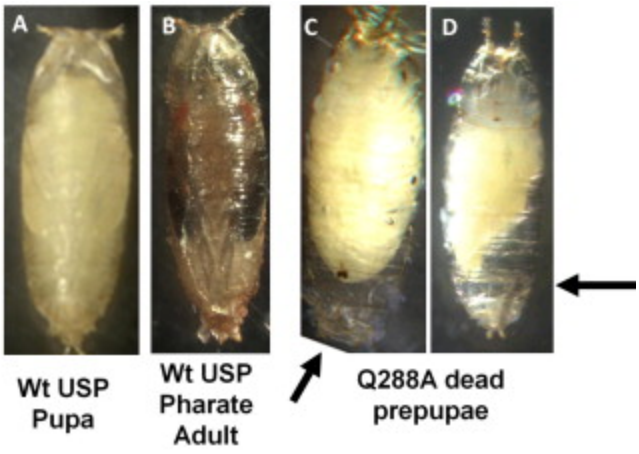


Fig. 4. CHO cell transfection assay of the response of USP/EcR heterodimer to 100 nM muristerone A (the approximate concentration 20-OH ecdysone prior to pupariation [67]). Cells were cotransfected with plasmids expressing USP and EcR, as well as a reporter plasmid

containing five tandem USP/EcR heterodimer binding sites, and a constitutively expressed reporter used for normalization. As indicated, cells were transfected with plasmid expressing EcR isoforms B1 or B2, each of which was paired with either wild type USP or the Q288A mutant USP. For every combination, the responsiveness of the heterodimer to the muristerone A was the same ($p > 0.05$), showing that the Q288A mutation did not allosterically affect the ability of the heterodimers to respond to ecdysteroid signaling. Values are average ± SEM, three independent replications each.

3.7. Q288A mutant cannot perform as wild type USP *in vivo*

The Q288A mutant USP was then challenged to functionally replace the missing wild type USP in the null*Drosophila* background. In *Drosophila*, development through the first two larval molts is driven by pulses of 20E that are widely understood to be transduced by 20E binding to the EcR partner of the USP/EcR heterodimer [51]. Null *usp* larvae, containing only transgenic Q288A USP (i.e., no wild type USP), progressed through the first two larval molts in synchrony with siblings that instead possess an endogenous wild type USP (confirmed in multiple independent lines). This result confirms that the Q288A mutation to USP does not in and of itself allosterically prevent the USP/EcR heterodimer from transducing 20E signaling*in vivo* (this result is concordant with the above CHO cell transfection results). Once the Q288A larvae attained the metamorphic 3rd instar (with a briefly longer 3rd instar feeding stage compared to normal larvae), larvae stopped feeding, came to the surface of the food, exhibited wandering behavior and then became quiescent, these all being normal stages signaling the onset of metamorphosis [52]. The larvae then exhibited apolysis of the epidermis away from the cuticle, to form the body of the cryptocephalic pupa ([2];Fig. 5, arrows), again as occurs in the normal metamorphic larval to pupal metamorphic process. However, most larvae did not form a normal puparium, i.e., did not form a structure that was barrel-shaped (with anterior spiracles everted at an angle), hardened (sclerotized), and brown (tanned). Rather, the puparium was dorso-ventrally flattened, the partially everted anterior spiracles pointed directly forward, the cuticle only faintly sclerotized, and the ‘puparial’ cuticle transparent rather than brown (Fig. 5). Most animals stopped development during the early pupal stage, and eventually died after dessication (though in some animals the compound eyes of the cryptocephalic head partially pigmented).



	Number of Emerging Adults			
	♀FM7	♂FM7	♀w+	♂null
1. Wt USP	103	99	107	58
2. Wt USP	95	77	98	40
1. Q288A USP	67	63	65	0
2. Q288A USP	49	46	50	0

Fig. 5. Transgenic USP mutated for decreased ligand binding cannot sustain normal development through metamorphosis by null (*usp*^{2}) animals. Above left two panels are wild type pupa and pharate adult stages. Above right two panels, mutant Q288A animals that developmentally arrested shortly after apolysis to the pupa (shown by retraction of the abdomen from the abnormally formed puparium, arrows), with incomplete head eversion. This was the predominant phenotype observed. Shown below are data for two independent transgenic USP fly lines each. When males of the given line are crossed to females that are heterozygous on chromosome 1 as *usp*^{2}/FM7i, P{ActGFP}JMR3, one fourth of the resulting progeny are expected to be male larvae that are null for endogenous *usp*, but which possess a single copy of the test transgenic 'rescue' *usp*.

3.8. Additional ligand pocket mutants cannot perform as wild type USP *in vivo*

We examined the effect on ligand binding and *in vivo* performance two more mutant USPs: (1) a double mutant (Q288A L366A; the L366 residue is highly conserved in vertebrate and invertebrate RXR, and contacts the carboxyl group of 9-*cis* RA [17] and [57]), and (2) the mutant N325A (this residue, that is at the deep end of the pocket, has been modeled as potentially interacting with the methyl farnesoid carboxyl group[57]; Fig. S4B and S4C)). Both mutant USPs possessed a significantly reduced affinity for methyl farnesoate (Q288A L366A, K_d = 1.3 ± 0.14 μM; *t*, $p < 0.05$; N325A, K_d = 2.3 ± 0.72 μM; *t*, $p < 0.05$). Both mutant receptors were able to sustain development through the 20E-driven first and second larval molts, supporting that the mutations to USP did not allosterically cause the EcR heterodimer partner to be unable to respond to 20E-signaling. However, neither the Q288A L365A nor the N325A mutant USP were able to sustain null *usp* larval development through the larval to pupal

transformation. For both, most larvae ceased feeding and came to the surface of the food, exhibited wandering behavior, became quiescent, and apolysed to the cryptocephalic pupal body (Fig. 6, arrows). However, most of the morphogenetic events of puparium formation were omitted by the Q288A L366A and N325A animals. The anterior spiracles did not evert to their full extension or proper angle; the cuticle did not become barrel-shaped, and remained soft and did not tan. These animals were never seen to exhibit pigmentation in the cryptocephalic eyes, prior to their desiccation and death within a day or two.

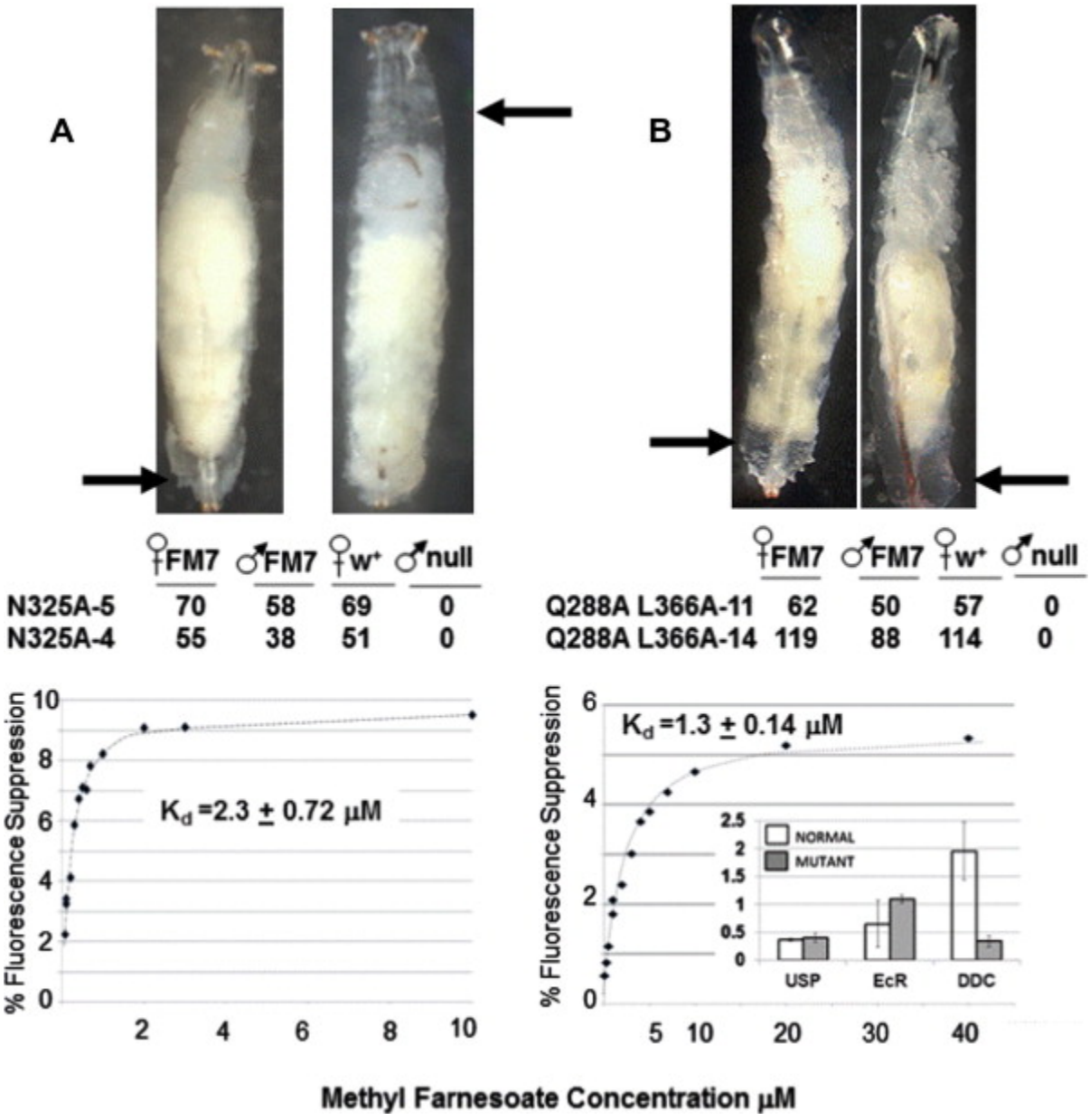


Fig. 6. Development of null *usp* male larvae cannot be sustained past the larval–pupal transformation by expression of either a transgenic N325A mutant or a Q288A L366A mutant USP. Shown in the upper panels of (A) and (B) is the respective predominant phenotype

observed (arrows show area of apolysis). The independent lines for each were generated by the methods described for Fig. 5. The data shown below the photographs in (A) and (B) confirm that neither USP mutant was functionally capable of sustaining normal development in the null *usp* males (i.e., no null males were rescued to attain puparium formation). By comparison, all null males were rescued to form a normal puparium by provision of a wild type *usp* transgene (as reported previously [22]). The lower panels of (A) and (B) show respective physical binding curves of methyl farnesoate to a preparation of purified N325A or Q288A L366A mutant USP. The calculated K_d is for the respective mutant USP is shown with each curve (avg ± SEM; $n = 3$ each). For each mutant USP, the K_d is significantly lower than the K_d for wild type USP (Fig. 3) ($p < 0.01$). Inset: qPCR analysis of mRNA levels for USP, EcR and dopa decarboxylase (DDC), during 3rd instar wandering stages (normalized to RP49), of null (usp^2) larvae expressing a transgenic mutant USP (Q288A L366A), and of normal siblings. There is no significant difference in relative levels (*y*-axis) of mRNA for USP or EcR ($p > 0.05$); however the level of DDC expression in null larvae expressing the mutant USP is significantly lower than that of normal larvae ($p < 0.05$).

qPCR analysis of Q288A L366A animals confirmed that during the period on the food prior to becoming motionless, the levels of expression of (the transgenic) USP and of the endogenous EcR isoform B1 in null*usp* larvae were not significantly different from normal sibling larvae (Fig. 6B, inset graph; *t*, $p > 0.05$). Relevantly, the level of expression of dopa decarboxylase (necessary for puparium hardening [71]) was significantly lower than in normal larvae (Fig. 6B, inset graph, *t*, $p < 0.05$). Analysis of the expression of a cuticle-specific protein gene that is specific for the pupal stage, *Edg84*, showed that the gene is not significantly expressed during the first several hours after puparium formation in normal larvae, or at the corresponding stage (by time post quiescence) of Q288A L366A larvae. However, for both the normal animals and correspondingly staged (by time) double mutant animals, *Edg84* becomes highly expressed in the several hours after pupal head eversion (>100×, about fivefold lower in the mutant larvae). Hence, the mutant USP animals have not simply arrested at a larval stage prior to the onset of metamorphosis; rather, specifically the formation of a normal puparium was not exhibited as the animal proceeded to a larval-to-pupal metamorphic program.

4. Discussion

4.1. Physical measurement of circulating methyl farnesoids

Until recently, physiochemical methods have suffered from a lack of sensitivity due to instrument limitations, which required large amounts of sample [8], [19] and [59]. However, advances in instrumentation over the past decade have resulted in both increases in sensitivity and reductions in signal to noise ratios (Fig. S3), which have reduced by orders of magnitude the amounts of analytes needed [64]. Thus, using the techniques we employed in this work, we were routinely able to effectively analyze and quantify samples containing as little as 2 nM of the given methyl farnesoid.

Although previous studies have reported that methyl farnesoate, JH III and JH III bisepoxide are released by cultured dipteran ring glands [50], there are no reports thus far the use of physicochemical approaches to detect methyl farnesoate in *Drosophila* larvae. (JH III has been reported from *Drosophila* whole body extracts [8] and [59]. It is crucial to identify the major methyl farnesoids that are in circulation following secretion by the ring gland, so as to enable defining their respective metamorphic roles [14] and [53]. Here, we have determined that all three methyl farnesoids are in larval circulation, with methyl farnesoate (reaching several hundred nanomolar) being more abundant than JH III at all sampled stages, and being the most abundant of all three at wandering and white puparium stages. However, the 3rd feeding stage period sampled across (12–36 h into the 3rd instar), is a long period during which time there could be differing changes in the concentrations of the three hormones.

4.2. Implications for functionally circulating methyl farnesoids

The ‘classical model’ of endocrine coordination of metamorphic transformation of insect cells specifies the interaction of two lipophillic ligands with their respective receptors: the steroid 20E and the terpenoid JH III. It is well established that the ecdysone receptor (EcR) is required in transducing 20E signaling, and there is increasing evidence that JH III signaling is transduced in insects through a MET paralog (GCE), or in *Drosophila*, both GCE and MET (both being bHLH-PAS transcription factors [3] and [38].

However, this 2-axis model (20E/EcR and JH/GCE(MET)) for metamorphic development appears incomplete. We have shown here that methyl farnesoate exists in circulation in significantly higher concentration than JH III. In addition to the 100× greater affinity of USP for methyl farnesoate than JH III[34], two independent recent studies have observed exogenous methyl farnesoate to be as active or more active than JH III in exerting some biological activities prior to pupariation [24] and [33]. These circumstances warrant the consideration of an alternative model in which methyl farnesoate is a *bona fide* circulating hormone contributing to the regulation of *Drosophila* larval development.

4.3. Implications for functional RXR/USP and ligand integration

The evolution of RXR and USP in the invertebrates in relation to ligand binding is an active area of *in silico*analysis. Attempts have been made to relate functional significance to patterns of evolutionary change in ligand binding residues to crystallographic structures or mass spectrometric fragments of the ligand binding domain, when it is expressed in isolation of the remainder of the receptor protein sequence. Interpretations have been complicated by what the investigators characterize as “fortuitous” insertion of various phospholipids at the opening of the ligand binding pocket under conditions necessary for crystallization or mass spectrometry [12] and [49], including that phospholipid becomes inserted in a manner that distortionally widens that opening of the pocket [60]. Recently, Hult et al. [27] performed a computational analysis that led the authors to confirm positively-selected evolutionary change in

the ligand binding domain of dipteran and other mecopteran USP, and agreed that phospholipid is not the natural endogenous ligand. Those authors urged that site-directed mutagenesis be conducted in order to better understand the physiological implications of the evolution of mecopteran USP.

In previous studies using a chimeric GAL4-USP ligand binding domain and a synthetic UAS reporter promoter, several researchers did not detect a direct activation response of the chimeric USP construct to methyl farnesoids in transgenic fly larvae [5], [39] and [46]. However, in a cell culture transfection system a similarly constructed USP ligand binding domain transduced potentiation of 20E activation of the ecdysone receptor, by either methyl farnesoate or JH III [4], consistent with the hypothesis that the USP ligand pocket has the structural capacity to bind and respond to an intrinsic ligand. In a transfection assay, an intact mollusc RXR shown to bind 9-*cis* retinoic acid did not activate transcription of a synthetic reporter from a heterologous RXR binding site [7]. Yet, transcriptional activation was observed in a cell transfection system that utilized full length USP, a natural *Drosophila* USP binding site (DR12), micromolar concentrations of an epoxidized analog of methyl farnesoate (i.e. JH III), and a JH-sensitive promoter from a lepidopteran species that also possesses a USP form of RXR [18], again consistent with *Drosophila* USP possessing a structurally competent ligand binding pocket. Recently, Jones et al. [32] have shown that in cell transfection reporter assays, selection of the appropriate reporter core promoter is crucial: even closely related core promoters have vastly different response (or none) to JH and trimming even a small region of natural sequence 5′ to the core promoter can greatly alter the level and even direction of reporting behavior. Hence, we consider that the differences in outcomes of the above reporter-based studies may have arisen from the presumptions as to how ligand binding affects USP function that are implicit in the particular synthetic features of the various reporter assay systems.

4.4. Implications for *in vivo* function of USP ligand binding pocket

The present study has used the different approach of directly assessing whether there is a necessary *in vivo*function of the ligand binding pocket of *Drosophila* USP. In the whole animal, a receptor expressed under its natural promoter is exposed to its putative natural endogenous ligands and its putative natural array of genomic targets. Our present report is the first to mutationally test specifically the ligand binding pocket of any full length RXR or USP protein in the whole animal environment. Our functional approach makes no *a priori* assumptions as to the identity of potential endogenous ligands and allows the test *Drosophila* USP to act upon the natural repertoire of putative USP target promoters in their normal tissues at normal developmental times. The outcome reported here of this functional approach, that yielded both morphogenetic and molecular marker phenotypes, supports an hypothesis that the USP ligand binding pocket has both a competence and a necessary function to bind ligand in order for normal morphogenetic larval to pupal development to proceed.

The phenotypes of different point mutational disruptions of the USP ligand binding pocket all converged on severely suppressing the formation of a normal puparium. These finer mutations thus yielded a similar phenotype to previously reported conditional removal of the entire USP molecule during the 3rd instar [22]. This phenotype is different from that observed under conditions of either complete loss of JH reception during all three larval instars (i.e., null for both MET and GCE [1]; or of JH overreception (high concentration of methoprene or pyriproxifen in the diet [54]). Under the conditions of loss of JH reception (loss of JH receptor) for the entire period of larval development the larvae nevertheless reached attainment of a normal puparium. Under conditions of extreme JH overreception, those larvae that reached the 3rd instar also proceeded on to form a normal puparium [54]. Other than conditional removal of the entire USP molecule[22], i.e., removed all USP functions, there have been published little experimental data upon which to postulate what periods of larval development need specifically the function of USP ligand reception. Hence, we also used an experimental design that forced the test animals to rely upon the mutant USP ligand binding pocket from the outset of larval development, i.e., the design enabled detection of acute or cumulative effects of underreception of ligand binding during the entire period of larval development. Under these conditions, the mutational interference with normal reception of ligand by USP resulted in the failure to form a normal puparium at the end of the 3rd instar.

The approach of disrupting a hormone/receptor axis by mutational interference with function of the specific receptor utilizes different tools than approaching it from the direction of interference with the production of ligand. From the direction of the receptor, the effect of ligand pocket mutation on the affinity for a specific postulated ligand can be carefully measured by kinetic techniques (e.g., Fig. 3, Fig. 5 and Fig. 6 here for methyl farnesoate and USP; Charles et al. [10] for JH III and GCE/MET). Approaches from the direction of disrupting ligand biosynthesis by corpora allatal cells of the ring gland have not yet reported what was resulting circulating titer of each of the three normally secreted methyl farnesoids during the three larval instars [42] and [55]. Also, suppression of HMGCR by RNAi driven in the corpora allatal cells yielded many larvae dying prior to the onset of metamorphosis [31], whereas in apoptotic ablation approaches, the death of the corpora allatal cells was not complete until sometime during the 3rd instar and the animals survived to the early pupal stage [42] and [55]. Hence, a more nuanced interpretation is required at this time on the phenotypic effects of suppressed methyl farnesoate production. In addition, it is also possible that a component of methyl farnesoate signaling during earlier instars becomes manifest at metamorphosis (e.g., as exogenous JH analog action during the 2nd instar becomes manifest at the pupal stage [54]).

Upon then reaching the 3rd instar wandering stage, which itself is triggered by a pulse of 20E [67], the mutant USP-expressing null larvae exhibited a normal level of transcript of both USP and EcR. We have shown that the mutational suppression in function of the USP ligand binding pocket did not allosterically cause the USP heterodimer partner EcR to become reduced in transducing ecdysteroid signaling, either (1) in a cultured cell assay, or (2) *in vivo* where the

mutant USP-expressing null larvae underwent the first two larval molts in synchrony with normal siblings. Hence, the USP mutation itself did misconform USP in a way that in turn misconformed EcR into not responding to 20E. Remaining to be determined is whether endogenous ligand signaling through the USP pocket acts independent of 20E signaling. Alternatively, USP ligand may either modulate the 20E signaling coming through its partner, the EcR, or modulate particular interactions of 20E and JH III (or bisepoxyJH III).

We have shown here that residues highly conserved in the ligand binding pocket of RXR and USP (Q288, N325) are necessary for high affinity binding of methyl farnesoate and enable a life-necessary function of*Drosophila* USP for metamorphic development. Q288 is conserved with a glutamine residue in vertebrate RXR that in cocrystal structure makes contact with the carboxylate end of the terpenoid 9-*cis* RA (Fig. S3A and S3B). It has been proposed that the methyl ester of a terpenoid ligand could interact with Q288 in*Drosophila* USP [57]. Evolutionary considerations have generated the hypothesis that residues unique to dipteran USP that are even deeper into the pocket, such as N325 (Fig. S3B), contribute to a hydrogen-bonding network that could interact with that methyl ester [57]. Supporting that proposition, we find here that disruption of larval to pupal metamorphosis occurs with mutation of either Q288 (Fig. 5) or N325 (Fig. 6A). This metamorphosis-necessary N325 residue contributing to the ligand binding environment does not make contact with the “fortuitous” phospholipid that inserts at the pocket opening under crystal-formation conditions, further supporting that phospholipid is not the endogenous ligand whose signaling was disrupted by the mutation. The ability of TBT to exchange in equilibrium with methyl farnesoate, when the TBT semicovalent attachment site is a cysteine near to the pocket opening [43], further confirms the functional performance of both the proximal and distal ends of the *Drosophila* USP ligand binding pocket under our conditions here.

4.5. Evolutionary implications

Methyl farnesoate is considered as the primary circulating hormone regulating a variety of morphogenetic events in crustacean development [41]. It has been suggested that in the evolution of Insecta, the insects have retained biosynthetic secretion of methyl farnesoate in addition to acquisition of a new hormone axis centered on the epoxidation product of methyl farnesoate, i.e., juvenile hormone [13] and [30]. We have reported here that methyl farnesoate is in circulation in a mecopteran (*Drosophila*) at a level favorably comparable with its binding affinity to USP, and that *in vivo* function of USP is disrupted by mutations to the ligand binding pocket that weaken the pocket’s affinity for methyl farnesoate. Our detection of circulating methyl farnesoate, in addition to JH III, not just in *Drosophila*, but also in late final instar mosquito larvae (Fig. S6), indicates that the findings reported here may also relate to dipteran vectors of disease.

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Appendix A. Supplementary data

Supplementary data 1. Available online at http://dx.doi.org/10.1016/j.ygcen.2012.11.009

Purification of recombinant, Q288A mutant ultraspiracle of *Drosophila melanogaster*. The receptor coding sequence in a pET32EK (Novagen) was induced to be expressed in AD494 bacteria by addition of IPTG and grown for several hours at 30 °C (Panel A, "IPTG"). After sonication and centrifugal precipitation of the insoluble bacterial debris, the supernatant was added to a nickel resin column. The recombinant protein, that includes an N-terminal His-STAG fusion segment, was retained on the column and did not appear either in the portion of the sample that flowed through ("Flow"), nor was it washed from the column by 10 mM imidazole ("Wash"). The retained protein was then eluted and concentrated to ca. 1 ml volume under nitrogen pressure with through an Amicon 30 kDa pore size membrane ("Elute Conc."). The concentrated sample was then passed through a Superdex 200 size exclusion column and fractions obtained, each containing the purified protein (Panel B). The purified protein migrates on SDS–PAGE slightly slower than pre-stained bovine serum albumin. The fractions containing the purified protein were combined and diluted to a final receptor concentration of 0.7–1 μM (SDS–PAGE of the combined fractions protein is shown in Fig. 3 and S2). Purification of the wild type USP behaved in the same way as shown here for the Q288A mutant.

Supplementary data 2. Available online at http://dx.doi.org/10.1016/j.ygcen.2012.11.009

Stability of wild type and mutant Q288A USP during the fluorescence binding assay. The purified receptor was diluted to 1 μM final concentration and held overnight at 4 °C. Then, 1.7 ml of that receptor preparation was dispensed into a quartz cuvette, and 1.7 ml of methyl farnesoate (in ethanol) was added to a final concentration (in this example, wt USP has 0.8 μM ligand; Q288A has 5 μM ligand). Shown is the % suppression in fluorescence at 5 min intervals after the addition (immediately after time 0) of methyl farnesoate. After 45 min, the average of the nine measured values for each concentration was determined and used as a datum in the calculation of K_d values shown in Fig. 3. Shown on the right in this figure is an aliquot of the given receptor preparation that was taken at the completion of the binding assay, and it shows no significant degradation for either wt USP or Q288A USP. Hence, the abrupt decrease in fluorescence seen upon ligand addition, and then the stable maintenance of suppressed fluorescence, is not due to degradation of the receptor during the 45 min course of the binding assay. Similar stability was demonstrated for the N325A and Q288 L366A mutants (not shown).

Supplementary data 3. Available online at http://dx.doi.org/10.1016/j.ygcen.2012.11.009

Comparison of ion chromatograms of base peaks (diagnostic ions used for quantification) of an extract of 1.5 μl of hemolymph obtained from 3rd instar larvae, with the ion chromatograms of

an equal mixture (25 pg each) of synthetic MF (*m*/*z* = 251 (m+1); 19–19.5 min), JH III (*m*/*z* = 235 (m+1−CH_3OH); 19.5–20.5 min), and JH IIIB (*m*/*z* = 283 (m + 1); 20.5–22.0 min).

Supplementary data 4. Available online at http://dx.doi.org/10.1016/j.ygcen.2012.11.009

Comparison of residues in the ligand binding pocket of *Drosophila melanogaster* ultraspiracle (USP), with those residues of the human retinoid acid receptor (hRXR) that make contact with the carboxyl end of 9-*cis* retinoic acid in crystal structure (USP, [9]; PDB #1hg4; hRXR, [14], PDB #1fby). On the left in Panels A and B, are global views of the respective ligand binding domains, as viewed with Cn3D software. On the right in Panels A and B are close-up views of the residues in the 'deep end' of the ligand binding pocket. In the hRXR structure, the side chain of Q288 makes a hydrogen bond with a carboxylate oxygen of ligand 9-*cis* RA (ligand terpenoid backbone in grey; oxygen atoms in red). The corresponding pocket space (dashed orange circle) is shown for in Panel B for USP. In that space, the glutamine residue is conserved as Q288, and there is also present N325 that is located even further back into the pocket. A phospholipid (shown in grey, with grey arrow) that is seen in crystal structure preparations of USP does not extend sufficiently into that space to make contact with N325. In a theoretical docking study Sasorith et al. [52] inferred that Q288 and N325 contribute to an intricate hydrogen bonding network that would preferably bind a methyl ester over a charged free carboxylate, approximately in the position diagrammed in Panel C (adapted from Sasorith et al. [52]). In the present study, we have performed an alanine mutational analysis of Q288 and N325 in *D. melanogaster* USP. Also shown here are W318 (USP) and W305 (hRXR). In crystal structure, W305 of hRXR is close to the bound 9-*cis* RA, which is a basis of an equilibrium fluorescence quenching assay for binding of 9-*cis* RA [14]. We have shown that this fluorescence binding assay can also be used to measure the binding affinity of methyl farnesoate and related structures to USP [31]. The red arrow in the global USP view in B is the position of the cysteine residue at which TBT binds in vertebrate RXR [40].

Supplementary data 5. Available online at http://dx.doi.org/10.1016/j.ygcen.2012.11.009

Fluorescence exhibited by the wild type and two mutants of USP in the presence of the indicated ligand, relative to the fluorescence in the presence of just the EtOH carrier. The binding of methyl farnesoate (MF) to wild type USP (fluorescence suppressed to 90%) is significantly weakened by the mutations to the ligand binding pocket (*t*, $p < 0.01$ each). At the same concentration of ligand, there is significantly less binding of juvenile hormone III (JH III) with the wild type USP (*t*, $p < 0.01$). The mutations of the ligand binding pocket did not cause the mutant receptor to exhibit with JH III the same fluorescence effect that occurs with MF binding to wild type USP (*t*, $p < 0.01$ each). At this ligand concentration, farnesol (FO) was indistinguishable from EtOH (*t*, $p > 0.05$).

Supplementary data 6. Available online at http://dx.doi.org/10.1016/j.ygcen.2012.11.009

qPCR expression analysis of the pupal cuticle gene Edg84 in normal vs. mutant USP, relative to the gene for ribosomal protein RPl32. Early Puparia = 0–3 h after white puparium formation for normal animals; Near Pupation = at or within several hours of pupal head eversion for normal animals. The stages for larvae expressing only mutant USP were selected by the corresponding time since the wandering larva became quiescent. For both normal and mutant USP animals, the expression increased more than 100× at the latter stage over the earlier stage (t, $p < 0.01$ each).

Supplementary data 7. Available online at http://dx.doi.org/10.1016/j.ygcen.2012.11.009

Chemical ionization (isobutane) GC–MS analysis , using limited ion monitoring, of synthetic and natural MF from late third instar *A. aegypti* larvae just prior to pupation. Panel A: ion chromatogram (m/z = 251, m+1) of synthetic MF. Panel B: ion chromatogram (m/z = 251, m+1) of natural MF; Panel C: mass spectrum of diagnostic ions used to document and quantify MF (m/z = 251, m+1;m/z = 219, m+1−CH_3OH; m/z = 191, m+1−CH_3OH–CO).

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