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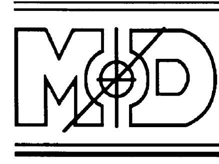

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A tissue specific cytochrome P450 required for the structure and function of *Drosophila* sensory organs☆

Aarron T. Willingham[a,*], Thomas Keil[b]

[a]*Division of Biology, University of California, San Diego, La Jolla, CA 92093-0649, USA*
[b]*Max-Planck-Institut fuer Verhaltensphysiologie, Arbeitsgruppe Kaissling, D-82319 Seewiesen, Germany*

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Abstract

Cytochrome P450s have generally been acknowledged as broadly tuned detoxifying enzymes. However, emerging evidence argues P450s have an integral role in cell signaling and developmental processes, via their metabolism of retinoic acid, arachidonic acid, steroids, and other cellular ligands. To study the morphogenesis of *Drosophila* sensory organs, we examined mutants with impaired mechanosensation and discovered one, *nompH*, encodes the cytochrome P450 CYP303a1. We now report the characterization of *nompH*, a mutant defective in the function of peripheral chemo- and mechanoreceptor cells, and demonstrate CYP303a1 is essential for the development and structure of external sensory organs which mediate the reception of vital mechanosensory and chemosensory stimuli. Notably this P450 is expressed only in sensory bristles, localizing in the apical region of the socket cell. The wide diversity of the P450 family and the growing number of P450s with developmental phenotypes suggests the exquisite tissue and subcellular specificity of CYP303a1 illustrates an important aspect of P450 function; namely, a strategy to process critical developmental signals in a tissue- and cell-specific manner.
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1. Introduction

In *Drosophila*, external sensory bristles mediate responses to chemical and mechanical stimuli, while internal chordotonal organs (e.g. stretch receptors) are primarily responsible for proprioception and hearing. Mechano- and chemosensory organs are derived from a sensory organ precursor cells that undergo several rounds of asymmetric cell divisions (Gho et al., 1999; Jarman and Ahmed, 1998; Posakony, 1994) and differentiate into the four cells that make-up the mature sensory organ: bristle shaft, socket, sheath and neuron for external bristles; cap, scolopale, ligament and neuron for chordotonal organs (Eberl, 1999; Jarman and Ahmed, 1998; Keil, 1997; Merritt, 1997). An earlier screen of *Drosophila* uncoordinated mutants with defects in mechanotransduction (Kernan et al., 1994) isolated two populations: mutants which specifically affected the function of mechanosensory neurons and those with both mechano- and chemotransduction defects suggesting disruption of a common process. We have been interested in the morphogenesis of sensory organs and therefore focused on this second class of mutants. We were surprised to discover one mutant, *nompH*, encodes a cytochrome P450.

P450s are an ancient and conserved family of proteins found in fungi, plants, and animals. P450s function primarily as oxygenases, catalyzing chemicals by a variety of oxidative mechanisms. They are most often implicated in detoxification; metabolizing natural products, drugs, pesticides, and pollutants (Nebert, 1991; Nelson; Tijet et al., 2001). P450 family members are abundant with the human genome containing approximately 50 P450s, while *Drosophila* has more than 80 distinct P450 genes (Feyereisen, 1999). Interestingly, some 60% of the human P450 genes identified contain polymorphisms that affect the protein

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* Corresponding author. Present address: Department of Chemistry, The Scripps Research Institute, Mail Stop #SR202, 10550 N. Torrey Pines Road, La Jolla, CA 92093, USA. Tel.: +1-858-812-1892; fax: +1-858-812-1746.

E-mail address: aarronw@scripps.edu (A.T. Willingham).

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sequence, potentially explaining the marked inter-individual differences in the extent of metabolism of xenobiotics such as drugs, carcinogens, and toxins (Ingelman-Sundberg et al., 1999). Such P450 polymorphisms are now central to studies of inherited differences that influence drug metabolism (i.e. pharmacogenomics).

Although P450s have traditionally been appreciated for their detoxification function, they have also been implicated in a variety of cellular functions including metabolism of hormone precursors, pheromones, and lipids (Ortiz de Montellano, 1995). In vertebrates, P450 enzymes generate arachidonic acid metabolites which have been implicated in diverse processes such as diabetes, cancer, hypertension, and atherosclerosis (reviewed in Capdevila and Falck (2001)). A subgroup of P450 steroid hydroxylases known as aromatases are expressed in regions of the gonads and brain important for the neuroendocrine regulation of reproduction and behavior. Furthermore these P450s have been shown to be essential for reproductive development, fertility, and normal sexual behavior (reviewed in Conley and Hinshelwood (2001)). In *Drosophila*, metamorphosis is coordinated by the hormone ecdysone and two developmentally regulated P450s have a demonstrated role in the biosynthesis of ecdysone (Chavez et al., 2000; Warren et al., 2002). Mutations in either of these P450s results in striking embryonic defects; including disruptions of dorsal closure, cuticle production, and other gross morphogenic defects.

We now report that loss of function of the *Drosophila* P450 CYP303a1 results in an uncoordinated mutant, *nompH*, with specific defects in mechano- and chemosensory perception. We demonstrate that *nompH* mutant sensory bristles, while having normal cuticular structures, are otherwise grossly disorganized in their internal structures. Notably, CYP303a1 is selectively expressed in sensory bristles. Our results support emerging evidence that P450s play a critical role in the regulation of morphogenesis and cell differentiation (see Section 3). We suggest this represents an important, perhaps common, functional role for this family in modulating development or cell function in a tissue specific manner. Furthermore, the large number of uncharacterized P450 genes present in any given genome may represent a vast reservoir of functional diversity which could be differentially expressed in various organs, tissues, or cell types.

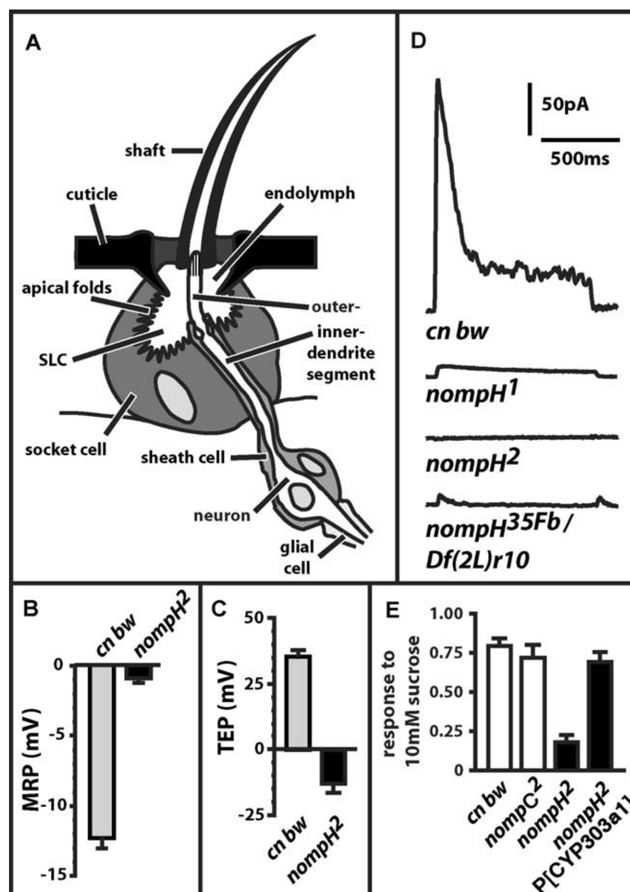

Fig. 1. *NompH* mutants have mechanosensory and chemosensory defects. (A) Schematic representation of an adult mechanosensory bristle organ highlights the morphology of the cells and cuticular structures including the tubular body at the dendritic tip (hash marks) and the *s*ensillum *l*ymph *c*avity (SLC) above the socket cell (modified from Walker et al., 2000). (B) *NompH* mutants have no *m*echano*r*eceptor *p*otential (MRP): wild type (*cn bw*) $= -12.25 \pm 0.73$ mV ($n = 37$), $nompH^2 = -0.91 \pm 0.26$ mV ($n = 33$) [mean ± SE]. (C) *NompH* mutants have no *t*rans-*e*pithelial *p*otential (TEP): *cn bw* $= 35.31 \pm 2.44$ mV ($n = 37$), $nompH^2 = -12.80 \pm 3.41$ mV ($n = 33$) [mean ± SE]. (D) Voltage-clamping at +40 mV TEP does not rescue the *nompH* mutant phenotype. Individual bristle recordings with a 35 μm stimulus show the three alleles of *nompH* have defective *m*echano*r*eceptor *c*urrents (MRC) compared to wild type animals (*cn bw*). $NompH^{35Fb\text{-}AS96}$ homozygotes have near normal MRC (data not shown) and were therefore tested as a more severe allele combination: $nompH^{35Fb\text{-}AS96}$/*Df(2L)r10* (see Section 4). Multiple voltage-clamp recordings (>10) were performed for each genotype and representative single traces were chosen for depiction. (E) *P*roboscis *e*xtension *r*eflex (PER) assay shows a *nompH* chemosensory defect which was rescued by the *P[CYP303a1]* transgene (see Fig. 2). The PER from five tests per individual fly were averaged and graphed as a PER index where 1.0 = 100%: *cn bw* $= 0.795 \pm 0.048$ ($n = 39$), $nompH^2 = 0.181 \pm 0.046$ ($n = 21$), $nompH^2$; *P[CYP303a1]* $= 0.693 \pm 0.062$ ($n = 30$), and $nompC^2 = 0.730 \pm 0.084$ ($n = 23$) [mean ± SE].

2. Results

2.1. NompH mutants have defective mechanotransduction

In *Drosophila*, each external sensory bristle is composed of four cells (see Fig. 1A): (1) a shaft cell that produces the bristle shaft and then dies upon completing this task; (2) a mechanosensory neuron; (3) a sheath cell that surrounds the dendrite of the neuron and attaches it to the base of the bristle shaft, and finally, (4) a socket cell responsible for maintaining K^+ potentials in the bristle (reviewed in Gho et al. (1999), Keil (1997), Posakony (1994)). The mechanosensory dendrite develops a ciliated outer segment containing at its distal tip a microtubule-based tubular body that is the putative site of mechanotransduction. The socket cell

forms a large number of membrane folds on its apical surface, greatly increasing surface area and thereby enhancing the amount of membrane associated machinery available for transport of K^+ ions. The pumping of K^+ against the concentration gradient from the hemolymph into the sensillum lymph cavity creates the *t*rans*e*pithelial *p*otential (TEP) critical for mechanosensation.

Previously, *Drosophila* uncoordinated mutants were screened for defects in mechanotransduction, isolating a large collection of complementation groups with *no mechanoreceptor potentials* (*nomp*) (Kernan et al., 1994). *Drosophila nomp* mutants exhibit behavioral defects characteristic of mechano-insensitive animals, including difficulty in walking, inability to fly, held-up wings, and a general uncoordination that makes survival in standard culture vials very difficult (Kernan et al., 1994). Three alleles of the mechanosensory mutant *nompH* ($nompH^{1}$, $nompH^{2}$, and $nompH^{35Fb\text{-}AS96}$) exhibited these distinctive phenotypes with varying degrees of severity (see Section 4). To confirm that the behavioral phenotype correlates with a disruption of mechanotransduction, we recorded electrophysiological responses from mechanosensory bristles. Mechanical deflection of the bristle shaft directly results in depolarization of the neuron: measured as a reduction in the TEP and quantified as the *m*echano*r*eceptor *p*otential (MRP). The loss of the MRP is a hallmark of the *nomp* class of mutants (Kernan et al., 1994). Fig. 1 shows that *nompH* mutants have a dramatic loss of MRP, with responses less than 8% of wild type bristles (-0.91 ± 0.26 mV compared to -12.25 ± 0.73 mV). However, *nompH* was distinguished from other *nomp* mutants by its striking loss of the transepithelial potential (TEP: -12.80 ± 3.41 versus 35.31 ± 2.44 mV for wild type animals).

The TEP is a measure of presence of the K^+-rich endolymph which drives a normal MRP (see Fig. 1A–C) and indicative of the integrity of the sensillum lymph cavity. Therefore, we considered two possible causes for the *nompH* mutant defect: (1) a disruption of K^+ pumping into the endolymph or (2) *nompH* is needed for bristle organ structure. To address the first possibility, we artificially supplied an exogenous TEP by voltage-clamping the bristle organ which should theoretically rescue basic TEP deficiencies. However, mutant bristles still failed to elicit a mechanosensory response (Fig. 1D). This result argues against a simple loss of the K^+ driving force underlying the loss of mechanoreceptor potentials, and instead suggests a disruption of bristle organ structure/function.

2.2. NompH is required for chemosensation

Mechano- and chemosensory bristles share a common developmental program, sensory organ structure, and reliance on a TEP for signaling (Morita, 1992). Therefore, we hypothesized that *nompH* may also be required for chemosensory bristle function. In *Drosophila*, chemosensory signaling can be readily examined using a simple behavioral assay that measures the *p*roboscis *e*xtension *r*eflex (PER) following stimulation of leg chemoreceptors with sugars or other attractive tastants (Kimura et al., 1986). Fig. 1E shows that *nompH* mutants have significant defects in their chemosensory responses (PER index of 0.18 ± 0.05 versus 0.79 ± 0.05 for wild type animals). This deficiency is not a non-specific consequence of defects in mechanotransduction since mechanosensory mutants with defective MRPs but normal TEPs (e.g. *nompC* (Walker et al., 2000)) show normal chemosensory responses. The combined mechanosensory and chemosensory defects indicate *nompH* function is essential for general sensory bristle activity.

2.3. NompH encodes a cytochrome P450

We mapped *nompH* to position 35F6-12 on the second chromosome and showed it was allelic to $l(2)35Fb^{AS96}$, a previously uncharacterized complementation group (Ashburner et al., 1990) which we have renamed $nompH^{35Fb\text{-}AS96}$ (Ashburner et al., 1999). These results placed *nompH* within a 20-kb interval containing nine candidate genes (Fig. 2A). Three overlapping genomic clones covering this interval were introduced into flies by P-element mediated germ line transformation vector and tested for rescue of the *nompH* phenotype. A 12 kb *Eco* RI genomic clone rescued the behavioral and physiological

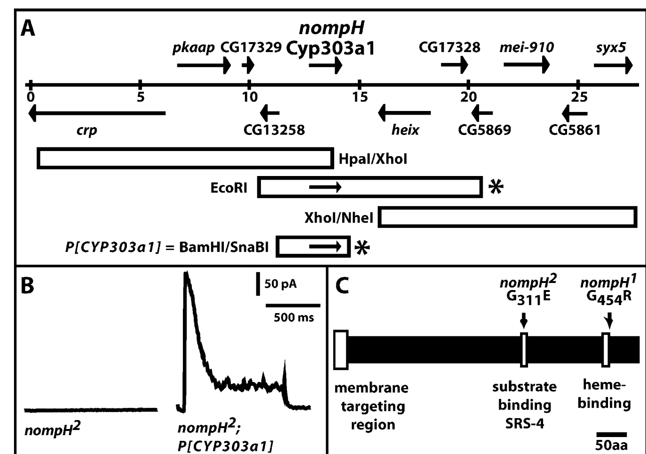

Fig. 2. *NompH* encodes cytochrome P450 CYP303a1. (A) Physical map of the genomic region containing *nompH* with coordinates in kb. Size and orientation of genes are indicated by black arrows (5′–3′). *NompH* was recombination mapped between *crp* and *syx5*, leaving nine candidate genes. Genomic rescue transgenes (created by indicated restriction enzymes) are shown as open boxes with the contained *nompH* gene indicated by a black arrow. Transgenes which rescue *nompH* mutants are indicated by asterisks. (B) Voltage clamp recordings show rescue of the MRC defect in homozygous $nompH^{2}$ mutants with the *P[CYP303a1]* transgene. (C) Map of the 503-aa CYP303a1 protein. $NompH^{1}$ has a G → A mutation at nt-1504 resulting in a G → R substitution at aa-454 and $nompH^{2}$ has a G → A mutation at nt-1076 resulting in a G → E substitution at aa-311. These mutations occur near key functional components of the protein indicated by white boxes (see Section 2.3).

defects (data not shown), and the four genes contained in this fragment were sequenced from wild type and homozygous mutant animals. CYP303a1, a cytochrome P450, was found to contain missense mutations in two *nompH* alleles (Fig. 2C). To demonstrate that *nompH* encoded this P450, a genomic clone containing only CYP303a1 (*P[CYP303a1]*) was tested for rescue. *P[CYP303a1]* restored wild type behavior and physiology, including coordination, MRC (Fig. 2B), TEP (39.58 ± 5.64 mV), and taste-induced proboscis extension (Fig. 1E).

Eukaryotic P450s share several structural hallmarks including a hydrophobic N-terminal membrane anchor, a catalytic pocket which binds the target substrate, and a heme cofactor binding domain (Bernhardt, 1996; Falquet et al., 2002; Gotoh, 1992; Poulos, 1995; Werck-Reichhart and Feyereisen, 2000; Williams et al., 2000). Notably, the *nompH^1* mutation replaces a conserved residue near the heme-binding domain ($G_{454}R$), while *nompH^2* substitutes glutamate for an evolutionarily conserved glycine ($G_{311}E$) within a substrate binding site of the enzyme considered the P450 signature sequence (Fig. 2C). No open reading frame mutations were identified in *nompH$^{35Fb\text{-}AS96}$*, a finding consistent with its hypomorphic phenotype (see Section 4). The mutations observed in *nompH^1* and *nompH^2* and their proximity to regions critical for P450 function suggests a link between the *nompH* mutant phenotype and a disruption of CYP303a1 function.

2.4. CYP303a1 is expressed in the socket cells of sensory bristles

The specificity of the *nompH* mutant phenotype suggested strong tissue selectivity in CYP303a1 expression. We generated an antibody directed against the carboxy-terminus of CYP303a1 and examined larvae, pupae and adult animals for *nompH* expression. CYP303a1 selectively localizes to the base of chemo- and mechanoreceptor external sensory organs, concentrating in a ring-shaped pattern (Figs. 3,5, and data not shown). In these preparations, the cuticular components of the bristle shaft and socket have a red and green autofluorescence (Fig. 3A–C) which sums to give a yellow color that aids in orientation of the sample. CYP303a1 was found concentrated in a ring-shaped pattern at the base of the bristle shaft (Fig. 3D,G). The stereotypical morphology of the four primary cells constituting the sensory bristle (Fig. 1A) suggested CYP303a1 expression most likely correlates with the socket cell which has a large, elaborate apical region surrounding the comparatively narrow sensory neuron and sheath cells. In fact, double-labeling with a neuron-specific marker (Grieder et al., 2000; Lin and Goodman, 1994) supports this, showing the ring of concentrated CYP303a1 expression surrounding the tip of the sensory dendrite (Fig. 3D–F). Co-labeling with the socket cell-specific marker (Barolo et al., 2000) demonstrates CYP303a1 corresponds to the apical area of the socket cell (Fig. 3G–I). The restricted expression of CYP303a1 in the socket cell correlates nicely with the TEP electrophysiological defects found in the *nompH* mutants. Homozygous *nompH* mutants (*nomp^{H2}* and *nompH$^{35Fb\text{-}AS96}$*) were also stained and showed the same CYP303a1 localization and expression level as wild type (data not shown). However, these mutations are not protein nulls likely to eliminate the protein; therefore, there is no reason to expect a strong reduction in CYP303a1 levels.

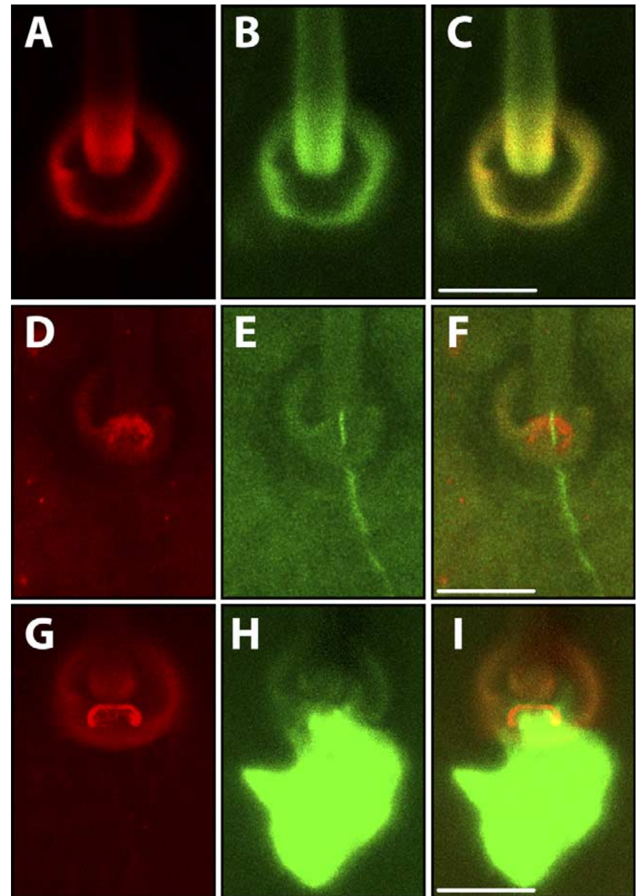

Fig. 3. CYP303a1 is expressed in the socket cell of external sensory bristles. Illumination of bristles with blue light causes the cuticle of the bristle shaft and socket to autofluoresce in the red (A) and green (B) channels. The merge of this autofluorescence results in a yellow color (C) which is seen in the subsequent antibody stains. (D,G) Antibody directed against CYP303a1 labels a ring-like structure at the base of the bristle socket (red). (E) The neuron specific *elav* promoter fused to GAL4 drives expression of a UAS tubulin–GFP fusion which effectively marks mechanosensory neurons (green) (Grieder et al., 2000; Lin and Goodman, 1994)). (F) Merge of images from (D,E) shows CYP303a1 does not co-localize with the mechanosensory neuron. (H) The Su(H) regulatory element ASE5 specifically expresses GFP in the socket cell (green) (Barolo et al., 2000). The extreme amount of apical membrane folding in the socket cell likely prevents the GFP from fully marking the apical-most regions of this cell. (I) Merge of images from (G,H) confirm that CYP303a1 is expressed in the apical region of the socket cell. Staining was performed on abdominal mechanosensory bristles from late pupae. Scale bars are 10 μm.

2.5. Bristle morphological defects underlie the electrophysiological deficiencies of nompH

To examine the structure of *nompH* mechanosensory organs, we undertook a systematic EM analysis of mutant and wild type bristles. In wild type bristle organs the mechanosensory neuron projects its dendrite to the base of the bristle shaft where deflections of the shaft compress the neuron's dendritic tip and gate mechanically sensitive channels. The socket cell is characterized by the presence of distinctive apical membrane folds needed for establishing and maintaining the K^+-rich endolymph. In *nompH* mutants, these apical folds are dramatically disrupted and largely absent (compare Figs. 4A,B). In addition, mutant organs have severe morphological defects in the shaft cell and neuron. The shaft cell normally degenerates after forming the bristle shaft and is seen as a dark mass (Fig. 4C, arrow) engulfed by the socket cell (black dotted line). In *nompH* mutants, while the cuticular shaft structures are normal, the shaft cell has failed to degenerate (Fig. 4D, white dotted line). The dendrite tip of the mechanosensory neuron contains a prominent microtubule-rich organelle known as the tubular body which is essential for mechanotransduction (Fig. 4E). The sheath cell produces the electron-dense dendritic sheath seen surrounding the dendrite (arrow). Though the soma and inner segment are present in *nompH* mutants (data not shown), the sensory neuron's outer segment is missing (Fig. 4F) with only the crumpled remains of the dendritic sheath visible (arrow). Collectively, these structural deficiencies can explain the loss of the TEP and MRP in *nompH* mutants and correlate with the specific localization of CYP303a1 within the apical region of the socket cell.

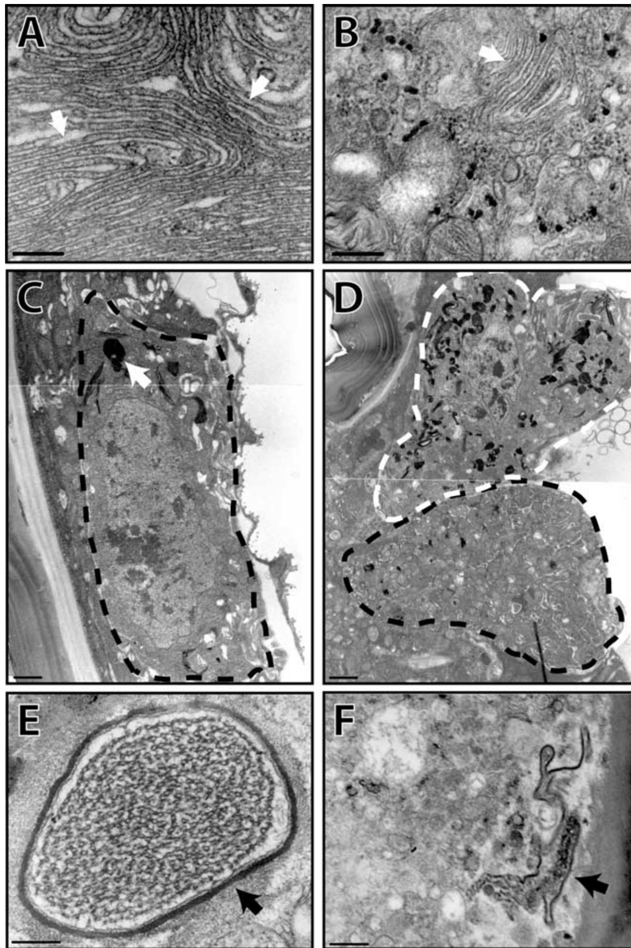

Fig. 4. Bristle organs of *nompH* mutants have morphological defects: a comparison of electron micrographs sections from wild type (*cn bw*) and *nompH* mechanosensory bristles. (A) Apical membrane folds of the socket cell (indicated by white arrows) in wild type bristles. (B) In *nompH*^{2} the membrane organization is disrupted and many of the membrane folds are lost. Other differentiated components of the socket cell appear intact, including the basal labyrinth and large number of mitochondria (data not shown). (C) In wild type the shaft cell degenerates to a small dark mass (indicated by white arrow) engulfed by the socket cell. (D) In *nompH*^{2} the shaft cell does not degenerate but rather is still intact (circled in white). The socket cell is present in both mutant and wild type animals (circled in black). The visible seam results from the overlap of two adjacent photographs. (E) Sensory neuron outer segment in wild type bristles, with tubular body surrounded by electron-dense dendritic sheath (arrow). (F) The neuron outer segment is missing in *nompH*^{2} and only the crumpled remnants of the dendritic sheath are present (arrow). Sections from a more basal region show the inner segment of the neuron is intact in *nompH*^{2} mutants (data not shown). Scale bars represent 1.0 μm (C,D) and 0.2 μm in all others. Similar structural defects were found for *nompH*^{1} (data not shown).

2.6. CYP303a1 function and expression are specific to external sensory organs

Developmentally related to external sensory bristles but markedly different in their morphology, chordotonal organs are composed of clusters of individual sensilla, each having a mechanosensory neuron surrounded by support cells (Eberl, 1999; Jarman and Ahmed, 1998; Merritt, 1997). These support cells attach to the cuticle and form a luminal space around the outer segment of the neuron which is thought to contain a K^+-rich endolymph similar to that of external sensory bristles (Corfas and Dudai, 1990; Eberl, 1999). Larval lateral chordotonal organs consist of an array of five mechanosensory neurons juxtaposed next to each other with their dendrites projecting towards the upper edge of the figure panel (Fig. 5B). Despite the concentration of chordotonal sensilla, no CYP303a1 expression was detected in these organs (Fig. 5A).

As well as internal chordotonal organs, the larval body wall contains external sensory structures related to adult sensory bristles. CYP303a1 expression was observed in a ring-like concentration at the base of external sensory organs, just beneath the cuticle (Fig. 5D). Also double labeling with a neuron marker (Fig. 5E) shows CYP303a1 surrounding the dendritic tip of a sensory neuron (Fig. 5F), a localization reminiscent of the CYP303a1 staining seen in adult sensory bristles. Therefore, Fig. 5 demonstrates that chordotonal organs do not express CYP303a1, even though the same animals express robust levels of CYP303a1 in their external sensory structures.

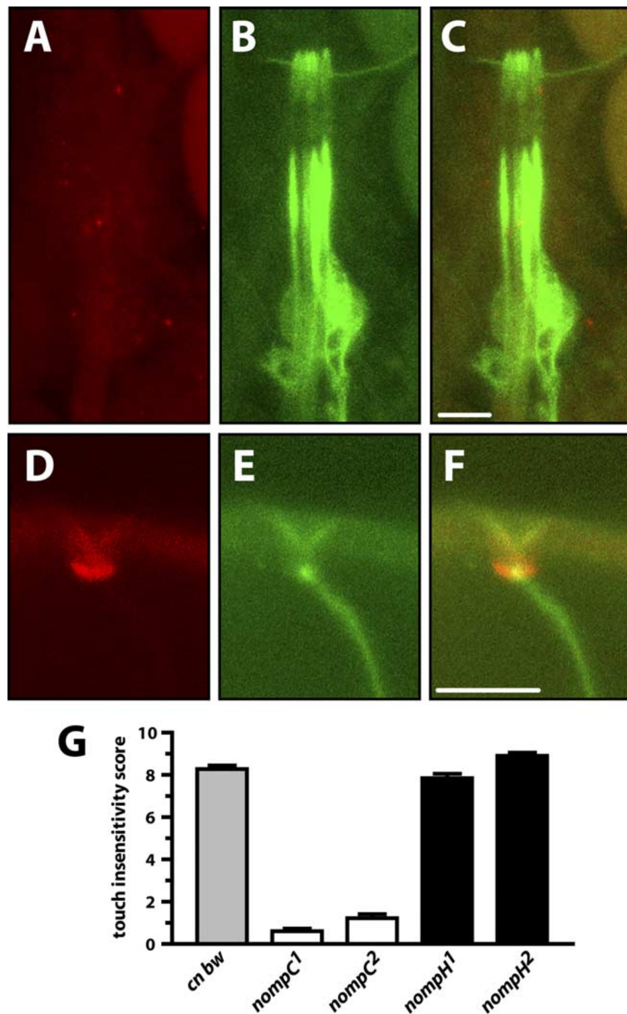

Fig. 5. CYP303a1 is not detected in chordotonal organs nor is it required for larval touch sensitivity. As in Fig. 3, cuticular autofluorescence results in a yellow color in the merged images. (A) CYP303a1 antibody staining of larval internal chordotonal organ reveals no concentrated CYP303a1 expression (red). (B) Double labeling with a neuron marker (see Fig. 3) marks a cluster of mechanosensory neurons (green) with their sensory dendrites at the top of the panel. (C) Merge of (A,B) shows no discernable CYP303a1 staining associated with chordotonal neurons. (D) Larval external sensory organ stained with anti-CYP303a1 shows robust expression at the base of the cuticle (red). (E) The GFP-labeled sensory dendrite (see Fig. 3) extends to the base of the cuticle. (F) Merge of (D,E) shows a concentration of CYP303a1 surrounding the sensory process of the neuron. Staining performed on the body wall from third instar larvae. Scale bars are 10 μm. (G) *NompH* mutants are touch sensitive. Touch sensitivity scores for wild type (*cn bw*), touch insensitive *nompC*, and *nompH* larvae (see Section 4): *cn bw* = 8.28 ± 0.16 ($n = 50$), $nompH^1 = 7.85 \pm 0.21$ ($n = 59$), $nompH^2 = 8.90 \pm 0.15$ ($n = 62$), $nompC^1 = 0.60 \pm 0.13$ ($n = 58$), $nompC^2 = 1.22 \pm 0.19$ ($n = 49$), [mean ± SE].

A prediction of these findings is that touch sensitivity, mediated primarily by internal chordotonal organs (Kernan et al., 1994), should be normal in *nompH* mutants. To test this hypothesis, we examined larval responses to gentle touch stimulus (Fig. 5G). Indeed, *nompH* mutants had responses that were indistinguishable from wild type animals (7.85 ± 0.21 versus 8.28 ± 0.16). In contrast, larvae carrying a null mutation in a mechanosensory channel essential for mechanotransduction, including touch reception (Walker et al., 2000), have a dramatic loss of touch sensitivity ($nompC^1 = 0.60 \pm 0.13$). Furthermore, *nompH* mutant adults were shown to have normal chordotonal-mediated auditory responses (M. Kernan, personal communication). Thus, the lack of detectable CYP303a1 expression in chordotonal organs, together with normal touch and auditory responses in *nompH* mutant larvae, support the postulate that CYP303a1 is specific for external sensory organs and is not required for chordotonal function.

3. Discussion

In this study we report the cloning and characterization of *nompH*. *NompH* mutants were isolated based on their TEP and MRC deficits. We cloned *nompH* and showed it encodes CYP303a1, a cytochrome P450 specifically expressed at the apical region of the socket cell. Two of the severe *nompH* alleles ($nompH^1$ and $nompH^2$) contain missense mutations that introduce non-conservative substitutions in domains critical for P450 function. $NompH^1$ substitutes an Arg for Gly within the L-helix which faces the heme-binding site; just six amino acids from the absolutely conserved Cys required to bind the heme cofactor. Whereas $nompH^2$ replaces an evolutionarily conserved Gly with Glu within the *s*ubstrate *r*ecognition *s*ite SRS-4 (Gotoh, 1992) which represents the proton transfer groove distal to the heme. Furthermore, this is the only SRS whose structural arrangement is highly conserved amongst the entire P450 family (Williams et al., 2000). Detailed inspection of bristle structure by EM analysis revealed a near total absence of the elaborate apical folds of the socket cell and the loss of the outer-segment of the mechanosensory dendrite. These structural defects, together with the nature of mutations in CYP303a1, substantiate a critical requirement for *nompH* in bristle morphogenesis and explain the TEP and MRC phenotypes.

What sensory organ-specific cellular function could CYP303a1 be regulating? Given its expression pattern in the socket cell and the absence of the TEP in mutants, CYP303a1 could have a direct role in K^+ transport within the mature socket cell. Cytochrome P450s have demonstrated roles in K^+ homeostasis, such as modulating K^+ channel activity in rats (Evans and Turner, 1997), and blockers of P450s disrupt K^+ dependant vasodilation (Busse and Fleming, 1996). The morphological defects of the neuron and shaft cell would then be a secondary consequence of the TEP loss, akin to the retinal degeneration phenotypes observed in vision mutants. However, the structural defects seen in *nompH* sensory bristles, particularly the shaft cell which fails to complete its developmental program and die, suggest a developmental deficit rather than a degenerative one.

As oxygenases, cytochrome P450s can affect a wide spectrum of processes by controlling the steady state of native cellular signaling ligands such as steroids, retinoic acid, and arachidonic acid (Makita et al., 1996; Nebert, 1991). Therefore, restricting expression of particular P450s to certain organs or cells would allow tissue specific processing of a more widely distributed ligand. In *Drosophila*, a P450 hypothesized to function in odorant clearance was found preferentially expressed in antennae (Wang et al., 1999). Humans with mutations in CYP1b1 develop primary congenital glaucoma resulting from defective development of the anterior chamber angle of the eye (Stoilov et al., 1997), possibly due to a loss of CYP1b1 mediated retinoic acid synthesis (Chen et al., 2000). Mouse CYP26b1 is expressed at the distal end of the limb bud and knocking it out results in major developmental defects in the outgrowing limb (Yashiro et al., 2004). Furthermore, retinoic acid treatments suggest CYP26b1 is responsible for generating a graded distribution of retinoic acid along the limb proximodistal axis. Human CYP19, which is preferentially expressed in brain and gonads, is integral to estrogen production with its loss resulting in abnormal female development and its overactivity resulting in male feminization (Stratakis et al., 1998). Other examples of P450s modulating development include CYP26a1's role in establishing the anterior–posterior axis in the mouse (Sakai et al., 2001), CYP90c1's regulation of leaf morphogenesis in *Arabidopsis* (Kim et al., 1999), and the role of CYP302a1 and CYP315a1 in ecdysone mediated development in *Drosophila* (Chavez et al., 2000; Warren et al., 2002).

In the case of *nompH*, mutant bristles exhibit a partial developmental non-autonomy, where disruption of a socket cell specific gene product results in structural defects in the adjacent neuron and shaft cell. CYP303a1 may indirectly play a role in the interaction between these cells, possibly required for a more specific signal from the socket cell to either the neuron and/or the shaft cell. Interestingly, phylogenetic tree prediction programs group CYP303a1 with *Drosophila* P450s that either play a role in ecdysone synthesis (CYP302a1 and CYP315a1) or are induced by ecdysone (CYP18a1) (Bassett et al., 1997; Warren et al., 2002) (see supplemental figures). Of the approximately 80 cytochrome P450 enzymes in *Drosophila*, nine have been associated with pesticide metabolism or resistance and five have been linked to ecdysone signaling, leaving a large number with unassigned function (www.flybase.org). Additionally, of the more than 15 mutants described in *Drosophila*, *nompH* is the sole mutant with such a late-stage and tissue specific developmental phenotype. Given the abundance and diversity of P450s in eukaryotic genomes, we suggest that targeting expression of selective P450s to subsets of cells may be a common biological strategy to produce a controlled and localized signal from a widely distributed non-active precursor (or alternatively, selectively degrade an active morphogen).

4. Experimental procedures

4.1. Fly stocks

NompH^{1}, *nompH^{2}*, *nompC^{1}*, and *nompC^{2}* were produced in the laboratory of C. Zuker, UCSD. The *cn bw* strain was the parental line used to produce the *nomp* mutants. *l(2)35Fb^{AS96}* (renamed *nompH$^{35Fb\text{-}AS96}$*), *Df(2L)r10*, and the *elav* promoter fused to GAL4 (C155 stock) were obtained from the Bloomington Stock Center. Compared to the other *nompH* alleles, *nompH$^{35Fb\text{-}AS96}$* has a higher number of viable homozygotes therefore we combined *nompH$^{35Fb\text{-}AS96}$* with the non-complementing deletion *Df(2R)r10*; creating hemizygous flies which eliminated a copy of the *nompH* gene. These flies exhibited a more severe phenotype with adult lethality and vastly reduced MRC similar to the other two *nompH* alleles (Fig. 1D). The stock expressing ASE5 fused to GFP was provided by J. Posakony, UCSD. The stock containing UAS-alpha-tubulin fused to GFP was provided by A. Spradling, Carnegie Institution of Washington.

4.2. Electrophysiology, PER, and larval touch sensitivity

Electrophysiological analysis of notoplural mechanosensory bristles on the thorax were performed as described (Kernan et al., 1994; Walker et al., 2000). To standardize recordings the TEP was voltage clamped at a physiological voltage corresponding to the average resting TEP (+40 mV). While the two other *nompH* alleles also had TEP and MRP defects (data not shown), *nompH^{2}* was chosen for systematic analysis. TEP recordings were gathered from adults within 24 h of eclosion; however the TEP defects were consistent in 2–3-day-old animals (data not shown). PER assay was performed as described (Galindo and Smith, 2001). Following the published protocol (Kernan et al., 1994), larval touch responses were gathered from third instar homozygotes which were selected using a GFP marked balancer chromosome (Casso et al., 2000).

4.3. Electron microscopy

After determining that *nompH* mutants had no visible socket or shaft cuticular defects indicative of gross developmental abnormalities, two *nompH^{1}* and two *nompH^{2}* homozygous mutant flies (altogether about 10 bristles) were sectioned One *cn bw* control animal was also examined. The rear half of the thorax was taken from flies shortly before or after eclosion. Samples were fixed for at least 2 h in ice-cold 2.5% glutaraldehyde in 0.1 M Na-cacodylate buffer, pH 7.2, containing 5% sucrose. Specimens were then rinsed or stored overnight in the same buffer, postfixed in 1–2% osmium tetroxide for 1–2 h at 4 °C, rinsed, and then stained in 1% aqueous uranyl acetate at 60 °C overnight. Samples were dehydrated in an

ascending ethanol series and then embedded in Spurr's medium. Specimens were sectioned with a diamond knife on a Reichert Ultracut, collected on Formvar-coated single slot grids, and investigated without poststaining in a Zeiss EM 10.

4.4. Molecular biology and generation of transgenic lines

DNA cloning, sequencing, mapping of mutant alleles, and *Drosophila* transformations were performed as described (Wu et al., 1995). A *nompH* cDNA 1791-bp in length with a single exon and a 1509-bp open reading frame was identified from a *Drosophila* head library (Wu et al., 1995). Genomic clone DS02740 mapped to the interval containing *nompH* and was digested with restriction enzymes to create the following fragments: *Hpa* I/*Xho* I (16.6 kb), *Xho* I/*Nhe* I (14.2 kb), *Eco* RI (12.1 kb), and *Sna* BI/*Bam* HI (4.0 kb and named P[CYP303a1]). These were cloned into pCasper4 and tested for ability to rescue *nompH* mutants. Rescue flies exhibited normal coordination and mechanosensation. Protein and cDNA sequences have been deposited in Genbank under these accession numbers (protein: AAF53514; cDNA: AY138853).

4.5. Antibody staining

Antibody against a carboxy-terminal epitope from CYP303a1 (TAAVKPYDIMLVAREQ) were generated in rabbits as described (Cassill et al., 1991). Abdomen were dissected from adults or 70–80-h-old pupa. Body walls were dissected from third instar larvae. Tissue was fixed in 4% paraformaldehyde for 0.5 h then washed in PBS with 0.3% Triton X-100 (PT) for 2 h. Tissue was blocked in PBS with 0.3% Triton X-100 and 2.0% BSA (PBT) for 2 h then incubated with primary antibody (1:50 dilution) overnight at 4 °C. After washing with PBT, sample was incubated for 2 h with a 1:100 dilution of secondary antibody conjugated with Fluorescein or Texas Red (Jackson Labs). Samples were mounted on slides with Gelmount (Biomeda) then examined by laser scanning confocal microscopy using a Biorad MRC 1024.

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Fig. S1. An unrooted dendrogram depicting the phylogenetic relationship of 85 Drosophila P450 proteins. CYP303a1 is marked with a yellow box. Three P450s (marked in blue) known to be involved in ecdysone synthesis are: *shadow* (*sad*) CYP315a1 (Warren et al., 2002); *shade* (*shd*) CYP314a1 (Petryk et al., 2003); and *disembodied* (*dib*) CYP302a1 (Chavez et al., 2000). Red asterisks mark CYP6a1 (Spiegelman et al., 1997) and CYP18a1 (Bassett et al., 1997) whose expression is induced by the molting hormone ecdysone. Nine P450s implicated in pesticide metabolism or resistance are marked by a green "R": CYP4e2, CYP4e3, CYP6a2, CYP6a8, CYP6a9, CYP404a1, CYP306a1, CYP308a1, and CYP313a1. Sequence and functional data was taken from Flybase (www.flybase.org) unless noted otherwise. Clustalw v1.8 was used for multiple sequence alignments and unrooted dendrogram generation. These analyses were performed with web-based software tools from Kyoto University (http://clustalw.genome.ad.jp/).

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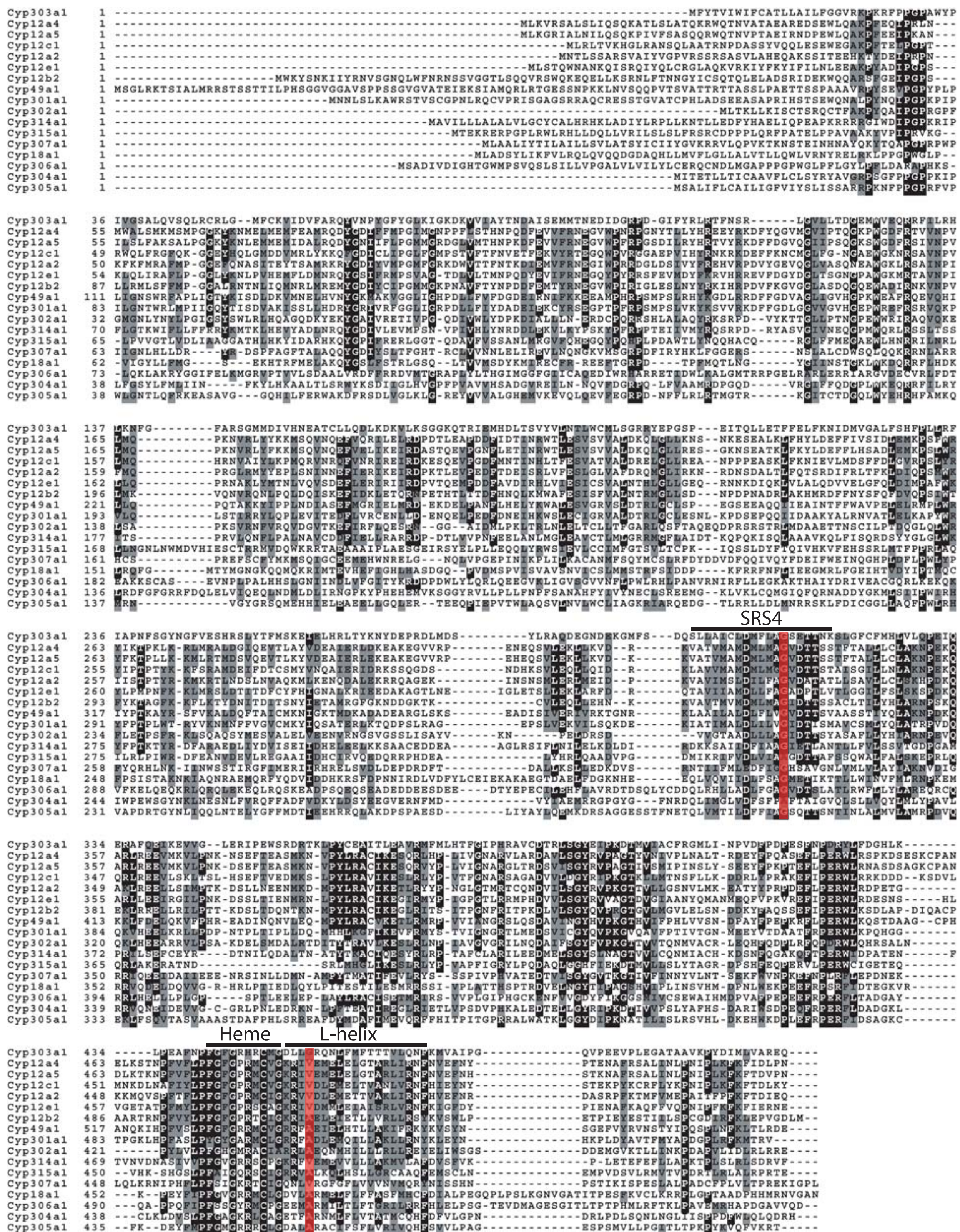

Fig. S2. Multiple sequence alignment of CYP303a1 with sixteen *Drosophila* P450 members which were grouped together in the unrooted dendrogram shown in supplemental figure 1. The sequence extents of the substrate recognition site SRS4, L-helix, and heme-binding region are marked (see Section 2.3). Residues mutated in nompH are marked with red bars: *nompH*[1] has a G to R substitution at aa-454 and *nompH*[2] has a G to E substitution at aa-311. Sequence data was taken from Flybase (www.flybase.org). Multiple sequence alignment was performed with Clustalw v1.8 (www.ebi.ac.uk/clustalw) and then formatted with Boxshade v3.21 (www.ch.embnet.org/software/BOX_form.html).