

Positive and Negative Regulation of Odor Receptor Gene Choice in *Drosophila* by Acj6

Lei Bai, Aaron L. Goldman, and John R. Carlson

Department of Molecular, Cellular, and Developmental Biology, Yale University, New Haven, Connecticut 06520

Little is known about how individual olfactory receptor neurons (ORNs) select, from among many odor receptor genes, which genes to express. Abnormal chemosensory jump 6 (Acj6) is a POU domain transcription factor essential for the specification of ORN identity and odor receptor (*Or*) gene expression in the *Drosophila* maxillary palp, one of the two adult olfactory organs. However, the mechanism by which Acj6 functions in this process has not been investigated. Here, we systematically examine the role of Acj6 in the maxillary palp and in a major subset of antennal ORNs. We define an Acj6 binding site by a reiterative *in vitro* selection process. The site is found upstream of *Or* genes regulated by Acj6, and Acj6 binds to the site in *Or* promoters. Mutational analysis shows that the site is essential for regulation *in vivo*. Surprisingly, a novel ORN class in *acj6* adults is found to arise from ectopic expression of a larval *Or* gene, which is repressed in wild type via an Acj6 binding site. Thus, Acj6 acts directly in the process of receptor gene choice; it plays a dual role, positive and negative, in the logic of the process, and acts in partitioning the larval and adult receptor repertoires.

Introduction

Animals possess many odor receptor genes and many functionally distinct olfactory receptor neurons (ORNs). Yet in animals ranging from mammals to fruit flies, individual ORNs express only one or a few odor receptor genes (Ache and Young, 2005). How do ORNs select which receptors to express?

Drosophila contains two olfactory organs, the antenna and the maxillary palp. Each contains sensilla that house the dendrites of ORNs. The most numerous antennal sensilla are the basiconic sensilla, which subdivide into large and small subtypes (Shanbhag et al., 1999). Physiological analysis of the antenna and maxillary palp has identified 35 functional ORN classes, housed in 17 sensillum types in stereotyped combinations (Clyne et al., 1997; de Bruyne et al., 1999, 2001; Elmore et al., 2003). The *Drosophila* larva contains a distinct olfactory organ with 21 ORNs (Stocker, 2008).

The fly genome contains 60 odorant receptor (*Or*) genes (Clyne et al., 1999a; Vosshall et al., 1999). Receptor-to-neuron maps were established by physiological and molecular analysis (Hallem et al., 2004; Couto et al., 2005; Fishilevich and Vosshall, 2005; Goldman et al., 2005). The maxillary palp contains three types of sensilla, pb1, pb2, and pb3, each of which contains two ORNs (i.e., pb1A, pb1B, pb2A, pb2B, pb3A, and pb3B) (see Fig. 1A). A single receptor gene has been mapped to each of these six ORN classes, except that two have been mapped to pb2A (Goldman

et al., 2005). The larval olfactory organ expresses a subset of *Or* genes that overlaps partially with the adult *Or* repertoire.

The first gene shown to be required for proper *Or* expression was *abnormal chemosensory jump 6* (*acj6*), identified in a behavioral screen (McKenna et al., 1989). In *acj6*⁶, the maxillary palp does not contain ORNs with the response spectra of pb1A, pb2A, pb2B, or pb3A; in contrast, pb1B and pb3B appear normal (Clyne et al., 1999b). Surprisingly, a novel ORN class, pb2C, was observed. Additional analysis showed that *acj6* encodes a POU domain transcription factor (Clyne et al., 1999b) and that some *Or* genes depend on *acj6* for their expression (Clyne et al., 1999a; Goldman et al., 2005). These results did not reveal whether Acj6 acts directly on *Or* genes or indirectly.

Here, we extend our analysis of Acj6 regulation to include all *Or* genes of the maxillary palp and all ORNs of the large antennal basiconic sensilla. Through an *in vitro* selection procedure, we define an Acj6 binding site, find that it lies upstream of Acj6-sensitive *Or* genes, and determine by mutational analysis that it is required for Acj6-mediated regulation. pb2C is found to derive its response spectrum from the ectopic expression of a larval *Or* gene, revealing an unexpected role for Acj6 in repression of larval odor receptors in the adult. The negative regulation is mediated by an Acj6 binding site. Thus, Acj6 exerts a dual role in receptor gene choice, positive and negative, adding a degree of freedom to the remarkable process by which each of 60 odor receptors is constrained to a discrete subset of neurons.

Materials and Methods

***Drosophila* stocks and transformation.** *Drosophila* stocks were cultured at 25°C. Wild-type flies were Canton-S unless otherwise stated. All DNA constructs were sequenced and then injected into *w*¹¹¹⁸ embryos. More than three transgenic lines were obtained and analyzed for each transgene. Each green fluorescent protein (GFP) reporter line was doubly heterozygous for *UAS-mCD8-GFP* unless otherwise indicated. In all experiments with *acj6*, the *acj6*⁶ allele, a null allele, was used.

Received July 21, 2009; revised Aug. 27, 2009; accepted Sept. 2, 2009.

This work was supported by grants from the National Institutes of Health and a McKnight Investigator Award (J.R.C.). We thank Sarah Certel for GST-Acj6 fusion protein and its expression constructs, Scott Kreher for *UAS-Or45b* transgenic flies, W. van der Goes van Naters for help with electrophysiology, Chih-Ying Su for critical reading of this manuscript, and Anandasankar Ray for discussion.

Correspondence should be addressed to Dr. John R. Carlson, Department of Molecular, Cellular, and Developmental Biology, Yale University, P.O. Box 208103, New Haven, CT 06520-8103. E-mail: john.carlson@yale.edu.

DOI:10.1523/JNEUROSCI.3525-09.2009

Copyright © 2009 Society for Neuroscience 0270-6474/09/2912940-08\$15.00/0

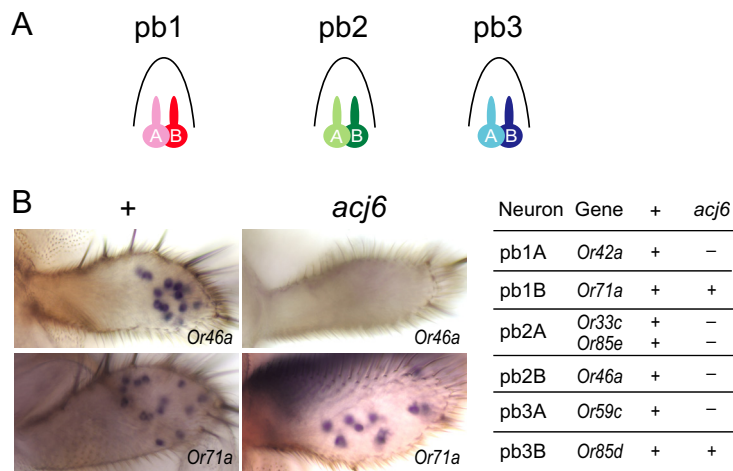


Figure 1. Role of *acj6* in the maxillary palp. **A**, The wild-type maxillary palp contains three sensillum types, each housing a pair of ORNs. **B**, Expression of a subset of *Or* genes depends on *acj6*. *In situ* hybridization of *Or* genes to wild-type and *acj6* maxillary palps is shown.

Identification of an *Acj6* binding site by systematic evolution of ligands by exponential

enrichment. The glutathione S-transferase (GST)-*Acj6* expression construct was a gift from Dr. S. Certel (Harvard Medical School, Boston, MA) and contains the coding region (nucleotides 131–1577) of *acj6* isoform (1, 4) (Certel et al., 2000). GST::*Acj6* fusion protein expression was induced in the *Escherichia coli* strain BL21 at room temperature, and the fusion protein was purified using glutathione-Sepharose 4B following the manufacturer's instructions (GE Life Sciences).

Systematic evolution of ligands by exponential enrichment (SELEX) was performed using methods previously described (Wey and Schäfer, 1996) with some modifications (supplemental Fig. S1, available at www.jneurosci.org as supplemental material). Double-stranded oligonucleotides were generated by annealing the template random oligonucleotides CAGGTCAGTTCAGCGGATCCTGTGCG(N)₁₆ GAGGCGAATTCAGTGCAAC-TGCAGC, to a 25 bp primer complementary to the 3'-invariant sequence (GCTGCAGTTGCACTGAATTCGCCTC) and then filling in with Klenow DNA polymerase. Unannealed oligonucleotides were removed using Microspin G-25 columns (GE Healthcare).

For the initial round of selection, 2 ng of random oligonucleotides was combined with 0.2 μg of GST::*Acj6* fusion protein in a 50 μl binding system that contained the following components: 0.5% Nonidet P-40, 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 4 μg of poly(dI-dC). The binding reaction proceeded at 4°C for 30 min with gentle rocking. Protein-oligonucleotide complexes were precipitated with 10 μl of glutathione-Sepharose beads prewashed and equilibrated in the binding buffer with 0.5% (w/v) skim milk. After three washes with the binding buffer, the beads were resuspended in 100 μl of elution buffer (10 mM Tris-Cl, pH 8.5), and heated at 85°C for 10 min to release bound oligonucleotides, a fraction (10 μl) of which was then amplified by PCR for 20 cycles. The PCR product was column-purified and a fraction (2 ng) was used for the next round of selection. Control binding reactions without GST::*Acj6* protein were performed with each selection to ensure that nonspecific binding of oligonucleotides to the Sepharose beads did not yield a visible band after PCR amplification. For the fifth and sixth rounds, oligonucleotides were incubated with GST::*Acj6* protein bound to glutathione-Sepharose beads; ~1 μg of *Acj6* protein was used. After six rounds of selection and amplification, PCR products were cloned and 26 clones were randomly selected and sequenced.

Electrophoretic mobility shift assay. The GST moiety of the GST-*Acj6* fusion protein was cleaved with PreScission protease (GE) in a buffer containing 50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, and 0.01% NP-40 "substitute" (Sigma-Aldrich) according to the manufacturer's instructions. 5'-end biotin-labeled complementary oligonucleotides and nonlabeled competitor sequences (W. M. Keck facility at Yale) were annealed in a buffer containing 10 mM Tris-Cl, pH 8.0, 1 mM EDTA, and 50 mM NaCl. The annealed double-stranded oligonucleotide

probes were purified from a native polyacrylamide gel and run through a Microspin G-50 column (GE). The sequence of the *Or85e* probe and its cold competitor was 5'-TTTTAAAACT-CATTAATGCATAAAATCAT-3'; the *Or85e* mutated competitor sequence was 5'-TTTTAA-AACTTCTAGAAAAGTTAAAATCAT-3' (mutated nucleotides are underlined). Binding sites in other *Or* genes were also tested in competition assays. The *Or46a* sequence was 5'-ATCGTCG-GCTAAAATAATTCAGTATTTTTGTC-3'; the mutant *Or46a* sequence was 5'-ATCGTCG-GCATCTCTAGACCGTATTTTTGTC-3'. The *Or45b* sequence was 5'-AACTAGTACGCAT-ACITGATCATATATATA-3'; the mutant *Or45b* sequence was 5'-AACTAGTACGTCGCCGATC-CCATATATATA-3'; the "switched binding site" sequence *Or45bMOr46a* was 5'-AACTAGTACTAAATAATTCAGATATATATA-3'. The amounts of protein, probes, and competing sequences are indicated in the figure legend. Protein was diluted in 2.5 mM HEPES, pH 7.6, buffer containing 0.1 M KCl, 1.25 mM MgCl₂, 0.01 mM EDTA, 1% glycerol, and 1 mg/ml BSA, when needed. The binding reactions included 20 mM Tris-Cl, 100 mM KCl, 2 mM DTT, 1 μg of poly(dG-dC) (Sigma-Aldrich), 5% glycerol, 0.05% NP-40, 1.25 mM MgCl₂, and 1% (w/v) skim milk, and were incubated at room temperature for 15 min. The resulting complexes were then resolved on a 5% native polyacrylamide gel in 0.5× TBE, 2.5% glycerol at 4°C and detected with Pierce chemiluminescent nucleic acid detection module according to the manufacturer's protocol.

Promoter mutagenesis. Transgenic flies carrying wild-type *Or85e-GAL4* (3.1 kb of upstream sequences), *Or42a-GAL4* (4.1 kb), and *Or46a-GAL4* (1.9 kb) were described previously (Goldman et al., 2005). For *Or85eM-GAL4*, the *Or85e* promoter regions upstream and downstream of an *Acj6* site (at -85 bp) were cloned separately with primers containing an XbaI site in place of the *Acj6* site. The upstream KpnI-XbaI fragment was ligated with the downstream XbaI-NotI fragment and then inserted into pG4PN (Dobritsa et al., 2003), resulting in an 8 nt substitution within the *Acj6* binding site (aaaaaCTCATTAATGCAtaaaa was mutated to aaaaaCTTCTAGAAAAGTtaaaa). Similar strategies were used to construct *Or46aM-GAL4* (tcggcTA-AAATAATTCAGgtatt was mutated to tcggcATCTCTAGACCGgtatt) and *Or42aM310-GAL4* (gaatcATCATTTATTAAtggaa was mutated to gaatcG-GATCCTTATAAtggaa). Overlap extension PCR (Higuchi et al., 1988) was used to introduce additional mutations in the *Acj6* sites at -249 and -102 bp of *Or42a*. In *Or42aM310-249-GAL4*, (gggctTTCATICTTTAGgtcat) at -249 bp was mutated to (gggctTTCACCGTTTAGgtcat). In *Or42aM310-102-GAL4*, (cgcctATGAATTATCTAattga) at -102 bp was mutated to (cgcctATGACCGATCTAattga). *Or42aM310-249-102-GAL4* bears mutations in all three *Acj6* sites.

Wild type *Or45b-GAL4* was described by Kreher et al. (2005). Overlap extension PCR was used to mutate the *Acj6* site at -392 bp (tagtaCG-CATACTTGATcatat) to (tagtaGCTGCCGGATCCcatat) in *Or45b (M)-GAL4*. In *Or45b (MOr46a)-GAL4*, this site was replaced by (tagtacTAAATAATTCAGatata), the *Acj6* site in the *Or46a* promoter.

In situ hybridization. Digoxigenin-labeled RNA probes were hybridized to whole maxillary palps attached to the proboscis, or to antennal sections, and were detected with alkaline phosphatase-conjugated anti-digoxigenin antibody and its substrates BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium) or Fast Red tablets (Roche) as previously described (Clyne et al., 1999a; Goldman et al., 2005).

Electrophysiology. Odor stimuli were presented, and action potentials were recorded as described previously (de Bruyne et al., 1999, 2001; Dobritsa et al., 2003).

Results

Expression of some but not all *Or* genes in the maxillary palp depends on *Acj6*

We extended our analysis of *Or* genes in an *acj6*⁶ background to include the entire repertoire of maxillary palp receptor genes. Of

To test the possibility of a functional role for these sites, we mutated them upstream of three genes, *Or85e*, *Or46a*, and *Or42a*. We examined the effects of these mutations on the expression of *Or* promoter-*GAL4* drivers that have previously been shown to drive faithful expression of a *UAS-GFP* reporter gene (Goldman et al., 2005).

Upstream of *Or85e* we mutated the site at -85 bp (Fig. 3A). We note that positions are determined with respect to translational start sites, but the transcriptional start sites as determined by 5'-rapid amplification of cDNA ends are within 50 bp of the predicted translational start sites in all cases examined, including *Or85e* and *Or46a* (Ray et al., 2007). We found that mutation of this -85 bp site in the *Or85e-GAL4* driver abolished expression, as determined by loss of GFP expression (Fig. 3A) (≥ 20 maxillary palps examined for each of four independent transgenic mutant lines).

Upstream of *Or46a* lie two *Acj6* sites that overlap. We mutated both simultaneously and saw no remaining expression of the GFP reporter in most maxillary palps (Fig. 3B). A small fraction ($\sim 20\%$) of maxillary palps showed staining of a few cells ($n = 2.3 + 0.7$ cells; $n = 13$ maxillary palps), a much smaller number than is observed when a wild-type promoter is used to drive expression ($n = 15.1 + 0.6$ cells; $n = 8$ maxillary palps).

Upstream of *Or42a* lie three *Acj6* sites, at positions -310 , -249 , and -102 . When we mutated the site at -310 , there was a small reduction ($p < 0.0001$, ANOVA) in the number of GFP-labeled cells in the maxillary palp compared with the control (Fig. 3C). When we mutated both the site at -310 and the site at -102 , there was a major reduction in labeling. Mutation of the sites at both -310 and -249 also led to a strong reduction in labeling. Mutation of all three sites virtually eliminated labeling.

These results show that *Acj6* sites are essential for the normal expression of each of three *Or* genes tested. The results also show that multiple *Acj6* sites are functional upstream of *Or42a*.

A subset of antennal ORNs depends on *Acj6* to acquire their identities

Although the ORNs of the maxillary palp are particularly convenient to study, the great majority of ORNs in the fly's olfactory system are located in the antenna. We wanted to examine the extent to which *acj6* is required for the specification of ORN identity and receptor gene expression in the antenna.

We performed an analysis of ORN responses in the antenna of *acj6⁶*. We systematically measured the responses of all ORNs in the large basiconic sensilla using a panel of diagnostic odorants. There are three types of large basiconic sensilla: ab1 contains four ORNs, ab1A, ab1B, ab1C, and ab1D; ab2 contains two ORNs, ab2A and ab2B; ab3 contains two ORNs, ab3A and ab3B.

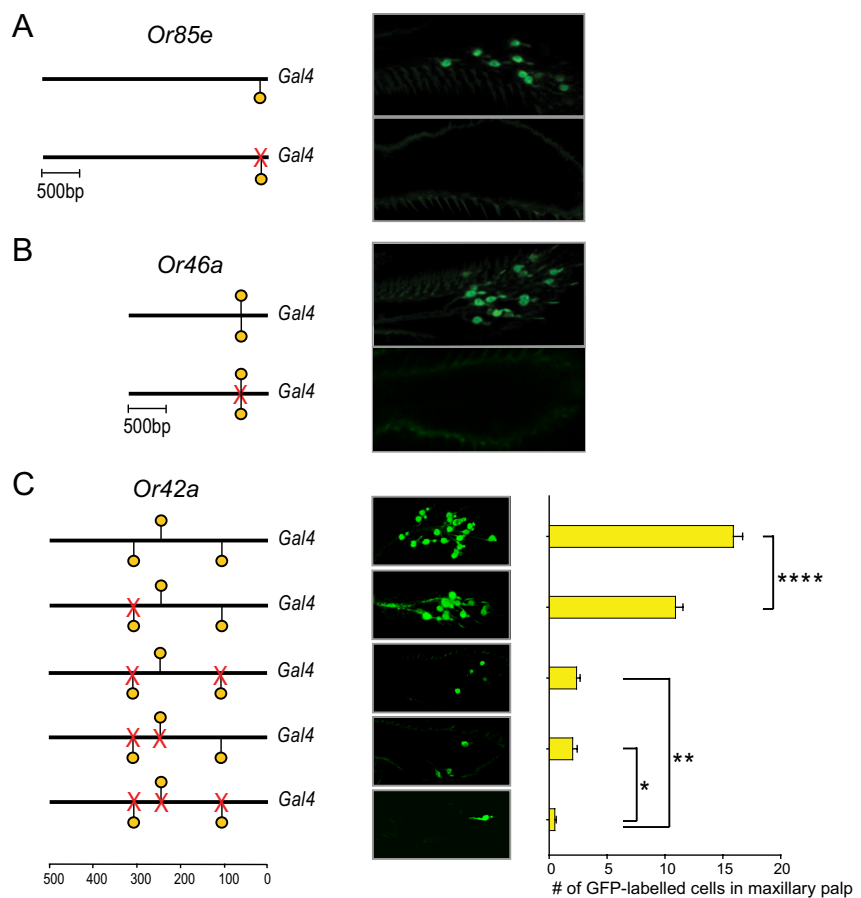


Figure 3. *Acj6* sites are required for expression of *Or* genes. **A, B**, GFP expression driven by wild-type *Or85e*- and *Or46a*-*GAL4* constructs (top panels) and constructs with mutations in predicted *Acj6* sites (bottom panels). The proximal *Acj6* site in *Or85e*, which is likely to lie in the 5'-untranslated region of the gene, is not shown. Data were from four independent transgenic lines for the mutant *Or85e* construct, and three lines for the mutant *Or46a* construct. **C**, The effects of mutations in *Acj6* sites in *Or42a*. Data were pooled from three independent transgenic lines for each mutated construct. $n = 11$ for wild type; $30 < n < 37$ for mutated constructs. * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$ (one-way ANOVA and Holm–Sidak test).

In *acj6⁶*, ab1A and ab1B were severely affected (Fig. 4A). We found no ORNs with the response spectra of these ORN classes. We did, however, observe sensilla containing ORNs with response spectra very similar to those of ab1C and ab1D: one ORN responded only to CO₂, as expected of ab1C, and another responded only to methyl salicylate, as expected of ab1D (de Bruyne et al., 2001). In these sensilla, we did not observe the spontaneous action potentials of large amplitudes that are characteristic of ab1A and ab1B, suggesting that these ORNs are either absent or electrically silent.

We observed a second type of sensillum in the *acj6⁶* antenna that contained an ORN with a response spectrum very similar to that of ab2A (Fig. 4A). In this sensillum type, we observed a second ORN that exhibited spontaneous action potentials but that did not respond to any tested odorant. Likewise, we observed a third type of sensillum containing an ORN similar to ab3A, paired with an ORN that showed spontaneous action potentials but no odor-evoked activity. We note that, among the four classes of odor-responding ORNs in the *acj6⁶* antenna, some yielded responses of smaller magnitude than in the wild-type control, but the overall response profiles of these four ORN classes appeared very similar to those of wild type in each case.

The simplest interpretation of these results is that ab1A, ab1B, ab2B, and ab3B all depend critically on *acj6* for the acquisition of

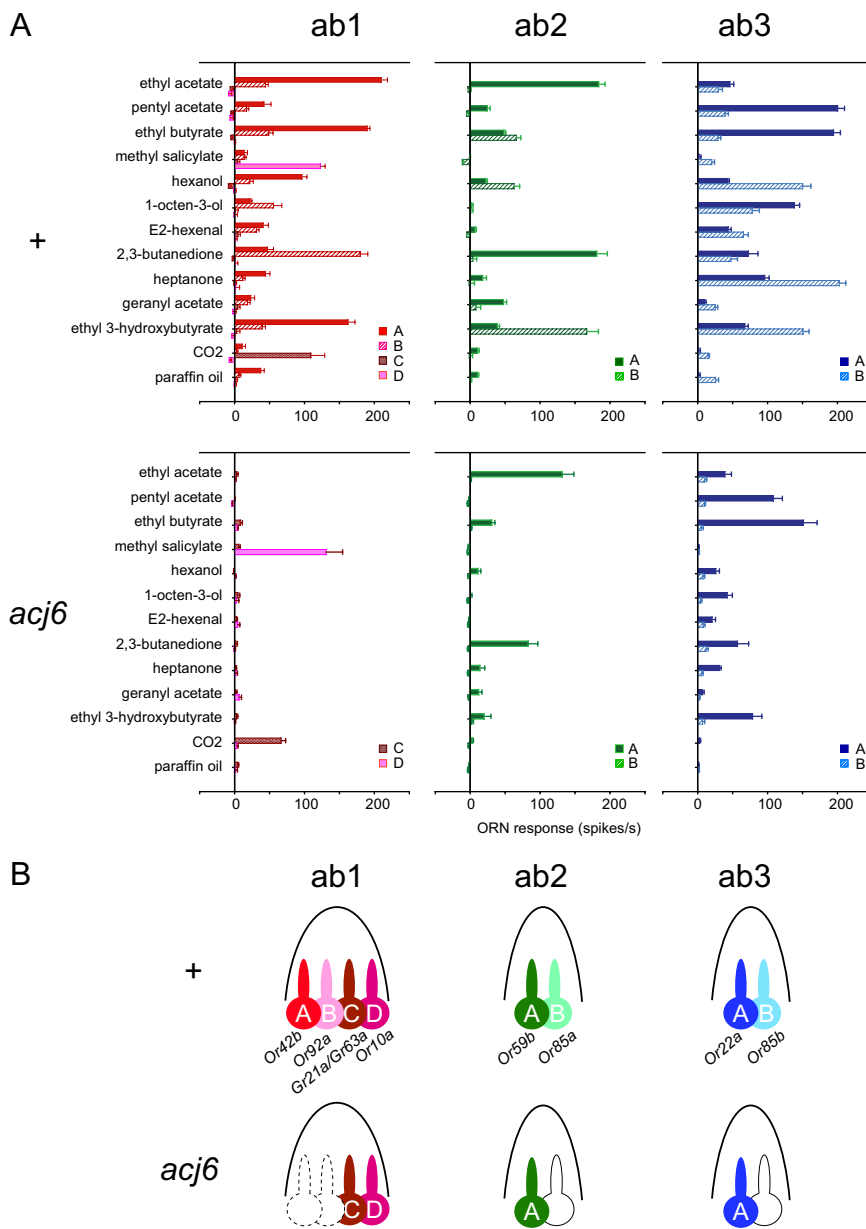


Figure 4. Odor responses of a subset of antennal ORNs are lost in *acj6*. **A**, Odor response profiles of large basiconic sensilla of wild-type and *acj6* antennae. $6 \leq n \leq 12$. Error bars indicate SEM. Odorants were applied as vapors of a 10^{-2} dilution. **B**, Fate changes of antennal large basiconic neuron classes in *acj6*. ab1A and ab1B are absent or electrically silent in *acj6* and are depicted by dashed lines. Uncolored neurons depicted by solid lines yield spontaneous action potentials but show no response to tested odors.

their odor sensitivities (Fig. 4B). It is possible that *acj6* plays a subtle role in the differentiation of the other ORN classes, but *acj6* does not seem to be required by these other ORNs in the process by which they select a receptor gene.

The *Or* genes of *Acj6*-dependent antennal ORNs depend on *Acj6*

We next tested the expression of *Or* genes in the *acj6* antenna (Fig. 5A) (data not shown). We found by *in situ* hybridization that the *Or* genes that are expressed in the ab1A, ab1B, ab2B, and ab3B ORNs, *Or42b*, *Or92a*, *Or85a*, and *Or85b*, respectively, were not detectable in *acj6*. As a control, we examined the wild-type antenna and detected expression of all four genes. As an additional control, we tested the expression of *Or59b* and *Or22a*,

which are expressed in ab2A and ab3A, respectively. Both of these *Or* genes were found to be expressed in both *acj6* and wild type. In summary, the lack of detectable odor responses from four ORN classes of the *acj6* antenna correlates with a lack of expression of the receptor genes that these ORNs express in wild type.

Antennal genes that depend on *Acj6* contain *Acj6* sites

Having identified four antennal *Or* genes that depend on *Acj6* for their expression, we asked whether they contained *Acj6* sites. All four contain *Acj6* sites within 1 kb of the predicted ATG translation start site (Fig. 5B); all of the *Acj6* sites are within or very near the 500 bp region upstream of this ATG. We also considered the receptor genes of ORNs that are present and show approximately normal response spectra in *acj6* (ab1C, ab1D, ab2A, and ab3A). ab1C expresses two gustatory receptor (*Gr*) genes in wild type, *Gr21a* and *Gr63a* (Jones et al., 2007; Kwon et al., 2007), neither of which contains an *Acj6* site within 1 kb of the translation start site. ab1D and ab3A express *Or10a* and *Or22a*, respectively, which do not contain *Acj6* sites. ab2A expresses *Or59b*, which contains an *Acj6* site despite its independence of *Acj6*. In summary, in large basiconic sensilla, all *Acj6*-dependent receptor genes contain *Acj6* sites and most *Acj6*-independent receptor genes lack them.

Acj6 represses a larval *Or* gene in the adult

We previously reported the surprising finding of a novel ORN class in the *acj6* maxillary palp (Clyne et al., 1999b). Sensilla containing either pb2A or pb2B were absent in the mutant, but sensilla containing a novel ORN class, pb2C, were detected. The response spectrum of pb2C could not be explained in terms of a linear amplification or summation of the response spectra of other maxillary palp ORN classes, and the simplest interpretation was that either pb2A or pb2B was transformed into pb2C.

We recently noticed that one receptor, *Or45b*, which had been characterized during the course of a systematic functional analysis of larval odor receptors (Kreher et al., 2005, 2008), exhibited a response spectrum similar to that observed for pb2C. We therefore compared directly the responses of pb2C to the responses conferred by *Or45b* in the "empty neuron" system, an *in vivo* expression system (Dobritsa et al., 2003; Hallem et al., 2004). We found the odor response profiles to be very similar (Fig. 6A), suggesting that *Or45b*, a larval *Or* gene, might be ectopically expressed in the maxillary palp of *acj6*.

We performed an *in situ* hybridization with an *Or45b* probe to the maxillary palp and found expression in *acj6*⁶, but not wild type (Fig. 6B). The simplest interpretation of these results is that *Acj6* represses *Or45b* in the adult maxillary palp.

To investigate the mechanism of *Acj6*-mediated repression, we first examined the upstream DNA sequences of *Or45b* and found two *Acj6* sites, at -499 and -392 (Fig. 6C, top left). The gene also contains two other regulatory sequences that are found upstream of *Or* genes expressed in the maxillary palp: there are two Dyad-1 elements, CTA(N)₉TAA, at -301 and -55 , and an Oligo-1 element, CTTATAA, at -193 . Dyad-1 is required for *Or* expression in the maxillary palp, and Oligo-1 both enhances expression of *Or* genes in the maxillary palp and represses their expression in the antenna (Ray et al., 2007).

The *Acj6* site at -392 is in fact capable of binding *Acj6*: it competed with the positively acting site in *Or85e* for binding to *Acj6* in gel shift analysis (Fig. 2C). To test the hypothesis that *Acj6* represses *Or45b* in the maxillary palp via the *Acj6* site, we mutated the site at -392 . (Numerous efforts to ablate the site at -499 were unsuccessful.) The loss of the site at -392 resulted in expression in maxillary palps in a wild-type (*acj6*⁺) background, consistent with a role for this site in repression of *Or45b* [Fig. 6C, *Or45b* (M)]. Thus, loss of either *Acj6* or an *Acj6* site allowed expression of *Or45b* in the maxillary palp. We conclude that *Acj6* acts negatively on *Or45b* via the *Acj6* site at -392 .

Finally, since other *Acj6* sites act positively, we wondered whether the negative effect of the *Acj6* site at -392 upstream of *Or45b* was attributable to its context (e.g., on other proteins that are bound in the vicinity). To test this possibility, we replaced the *Acj6* site at -392 with the *Acj6* site that acts positively at -346 upstream of *Or46a* [Fig. 6C, *Or45b* (MOr46a)]. We found that the *Acj6* site that promotes expression upstream of *Or46a* did not promote expression upstream of *Or45b* in the maxillary palp of wild type. The lack of *Or45b* expression depended on repression by *Acj6*: *Or45b* was expressed in the maxillary palp of *acj6*⁶ animals carrying the *Or46a* site upstream of *Or45b* (Fig. 6C). One possible interpretation of these results is that *Acj6* binds to the *Or46a* site when it is upstream of either *Or46a* or *Or45b*, but the mode of regulation that it exerts depends on other proteins that are bound in the vicinity (Fig. 6D).

Discussion

Acj6 and the logic of receptor gene choice

How individual ORNs select individual odor receptors to express is a remarkable problem in molecular neurobiology. Most ORN classes in the fly select one or a small number of receptor genes from a large family of 60 *Or* genes. The choice dictates the odor response spectrum of the ORN and, consequently, its contribution to the coding of olfactory information.

The simplest model for receptor gene choice in the fly is that it is governed by a combinatorial code of transcription factors (Ray et al., 2007, 2008) (for review, see Fuss and Ray, 2009). Although there is evidence to support this model, much remains to be learned about the elements of the code and the logic by which it operates. Distinct combinations of six transcription factors could

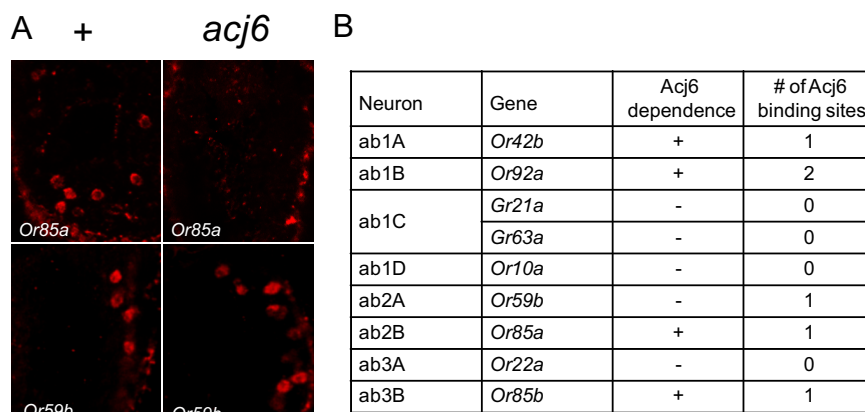


Figure 5. A subset of antennal *Or* genes require *acj6* for expression. *A*, *In situ* hybridizations of *Or* genes to wild-type and *acj6*⁶ antennae. Cells are labeled in red. *B*, Effects of *acj6*⁶ on receptor expression shown by *in situ* hybridization (*Or42b*, *Or92a*, *Or59b*, *Or85a*, *Or22a*, *Or85b*) or inferred by response properties of antennal neurons (ab1C, ab1D). The number of *Acj6* sites within 1 kb upstream of these genes is indicated.

in principle dictate the specification of $2^6 = 64$ distinguishable ORN classes, a number comparable with the number of *Or* genes and of the same order as the number of ORN classes. Thus for factors A–F, one ORN class might require factors A, B, and D and be specified by the combination ($A^+B^+C^-D^+E^-F^-$), whereas another class might be specified by the combination ($A^-B^-C^+D^+E^-F^+$). In this simple model, each factor would be required for the specification of one-half of the 64 ORN classes. Alternatively, a much larger number of such factors could act, each in a small number of classes. In the 14 ORN classes for which data are now available, 6 in the maxillary palp and 8 in the antenna, *Acj6* is required for the specification of 8, approximately one-half. Thus, we found evidence that at least one transcription factor acts broadly across the ORN repertoire.

One aspect of the problem of receptor gene choice is that each ORN must express one or a small number of receptor genes; another aspect is that each ORN must not express all the other receptor genes. In principle, each aspect of the problem could be governed by separate sets of factors. We found here that *Acj6* plays a role in both positive and negative regulation of *Or* genes. This duality of *Acj6* function imparts economy to the process, an economy that may be critical in a process that controls such a large number of *Or* genes in a large number of ORN classes. Allowing individual factors to act in two aspects of the problem reduces the number of required factors.

Another example of dual regulation by a single transcription factor was found in the differentiation of photoreceptor subtypes in the *Drosophila* visual system (Tahayato et al., 2003). Otd, a homeodomain transcription factor, activates the rhodopsin genes *rh3* and *rh5* but represses *rh6* in other photoreceptors, through a common binding site. The modes of regulation are likely to depend on the availability of a coactivator and a corepressor of Otd. An example of dual regulation in a chemosensory system is provided in *Caenorhabditis elegans* by the UNC-3 Olf/EBF (early B-cell factor) protein (Kim et al., 2005). This protein represses a number of genes, including the *odr-10* olfactory receptor gene, in the ASI chemosensory neuron, and it activates other chemoreceptor genes. UNC-3 was shown to repress multiple genes by direct binding to sites in the promoters, but it may activate the expression of ASI-specific genes through an indirect mechanism.

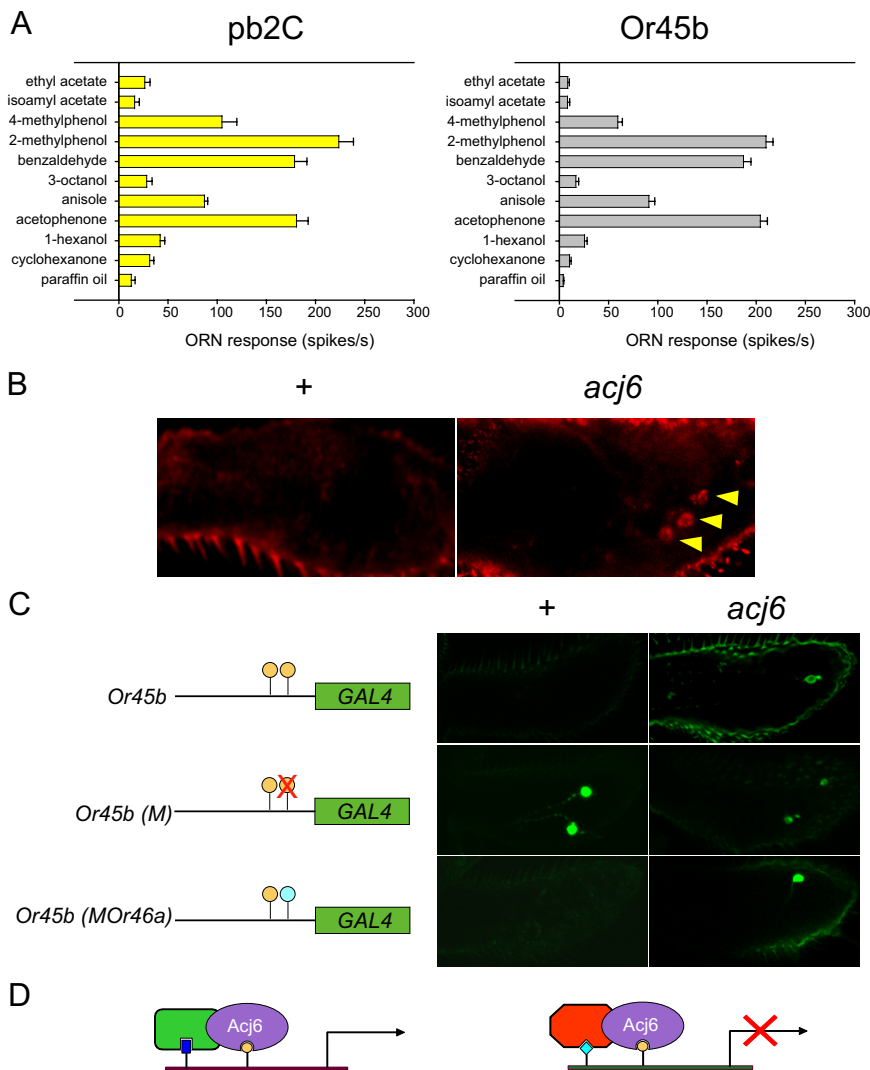


Figure 6. Acj6 represses the larval receptor gene *Or45b* in the adult maxillary palp. **A**, Response profiles of pb2C (in *acj6*⁶) and *Or45b* (in the empty neuron system). $n = 6$ for pb2C; $n = 19$ for *Or45b*. Error bars indicate SEM. **B**, *In situ* hybridization to *Or45b* mRNA in wild-type and *acj6* maxillary palps. The arrowheads indicate labeled cell bodies. **C**, Effects of altering an Acj6 site on *Or45b*-GAL4-driven GFP expression. *Or45b*-GAL4 is not expressed in the wild-type maxillary palp but is ectopically expressed in the *acj6*⁶ maxillary palp. Mutation of the Acj6 site at -392 caused expression in the wild-type adult, mimicking the effects of the *acj6*⁶ mutation. Replacement of the Acj6 site at -392 with another Acj6 site, one that directs activation of *Or46a*, yielded the same expression pattern as that driven by *Or45b*-GAL4. Two independent lines were examined for each genotype. The *Or46a* site contains two Acj6 consensus sequences that overlap by 1 bp and are on opposite strands of the DNA. **D**, Model for dual mode of regulation. Acj6 activates transcription (left) or represses transcription (right) depending on the presence of other proteins that bind in the vicinity.

One gene family, two olfactory systems

Drosophila contains two distinct olfactory systems, one in the larva and one in the adult. The two systems differ in morphology and developmental origins, and they operate under different conditions: the larva burrows in semisolid medium, whereas the adult walks and flies through air. Both olfactory systems, however, depend on members of the *Or* gene family. Some members of the *Or* family are larval specific, others are adult specific, and some are expressed in both (Couto et al., 2005; Fishilevich et al., 2005; Kreher et al., 2005). By what mechanism are members of the same gene family partitioned into two different systems?

We found that Acj6 acts in the partitioning mechanism. It is required to restrict at least one member, *Or45b*, to the larval system: when *acj6* is mutated, *Or45b* is ectopically expressed in the adult. *Or45b* contains in its upstream region two regulatory

elements, Dyad-1 and Oligo-1, which are found adjacent to all or most maxillary palp *Or* genes and are required for their expression in the maxillary palp. We do not know whether the Dyad-1 and Oligo-1 elements play a role in the expression of *Or45b* in larvae. These observations, together, suggest the possibility that *Or45b* may have shifted over evolutionary time from a maxillary palp gene to a larval gene. If so, the shift is likely to have occurred before the divergence of *Drosophila melanogaster* and *Drosophila pseudoobscura* tens of millions of years ago, since the odor response spectra of the maxillary palp ORNs in these two species are similar (Ray et al., 2008).

The Acj6 site and its context: odor receptors and beyond

Using an *in vitro* DNA binding assay, we defined a positional weight matrix and a consensus Acj6 binding site. Only one Acj6 site has been experimentally identified and tested previously, in a study of sequences upstream of the gene encoding choline acetyltransferase, *Cha* (Lee and Salvaterra, 2002). These *Cha* sequences were scanned for motifs similar to those found for the mammalian POU-IV transcription factors, and of seven identified, one was found to bind Acj6 protein in an EMSA. It does not show close similarity to our site (matrix score, 3.4), and its activity *in vivo* was not evaluated.

We found a strong correlation between the presence of the Acj6 site that we have identified and Acj6 dependence *in vivo*. Moreover, we have shown directly, *in vivo*, that these sites are required for normal expression of flanking *Or* genes. These results are consistent with a direct role for Acj6 in mediating *Or* expression, as opposed to an indirect role via another transcription factor.

We have shown that one of the Acj6 sites, the *Or45b* site, acts in negative regulation. Its score based on the positional

weight matrix is intermediate among the sites that act in positive regulation, which is consistent with our finding from a binding-site-swap experiment that repression of *Or45b* depends not on the specific sequence of an Acj6 site but on its flanking sequences—the *Or46a* site also mediated repression when placed upstream of *Or45b*.

In several cases, Acj6 sites lie within 8 bp of conserved sequence elements that are required for appropriate *Or* expression (Ray et al., 2007, 2008) (supplemental Fig. S4, available at www.jneurosci.org as supplemental material). An Acj6 site also lies very near a putative binding site for Pdm3, another POU domain transcription factor that regulates ORN development and that interacts genetically with Acj6 (Tichy et al., 2008). The proximity of these regulatory elements is likely to reflect the interaction of Acj6 and its cofactors. Identification of the cofactors of Acj6 will

further contribute to our understanding of the combinatorial regulation of specific *Or* expression.

Acj6 is likely to have a number of interesting transcriptional regulatory targets in addition to odor receptors and choline acetyltransferase. It may regulate other molecules that are essential for neuronal signaling: whereas some ORNs in *acj6⁶* exhibited spontaneous action potentials but did not respond to odorants, other ORNs showed no electrical activity, consistent with a loss of both odor receptors and other components.

Acj6 is also likely to regulate molecules required for synaptic connectivity. Acj6 is required for axon targeting specificity of a subset of ORN classes, some of which require Acj6 autonomously and some nonautonomously (Komiyama et al., 2004). The second-order neurons of the *Drosophila* olfactory system, the projection neurons, also require Acj6 for both normal dendritic targeting and axon terminal arborization (Komiyama et al., 2003). Moreover, the role of Acj6 extends beyond the olfactory system: *acj6* mutants have defects in retinal axon targeting and synapse selection as well (Certel et al., 2000).

Our definition of an Acj6 binding site may now facilitate the identification of some of its transcriptional targets. There are many sites in the genome with matrix scores >7, and analysis of the adjacent coding regions may be useful in identifying novel components required for synaptic connectivity and other processes.

References

- Ache BW, Young JM (2005) Olfaction: diverse species, conserved principles. *Neuron* 48:417–430.
- Certel SJ, Clyne PJ, Carlson JR, Johnson WA (2000) Regulation of central neuron synaptic targeting by the *Drosophila* POU protein, Acj6. *Development* 127:2395–2405.
- Clyne P, Grant A, O'Connell R, Carlson JR (1997) Odorant response of individual sensilla on the *Drosophila* antenna. *Invert Neurosci* 3:127–135.
- Clyne PJ, Warr CG, Freeman MR, Lessing D, Kim J, Carlson JR (1999a) A novel family of divergent seven-transmembrane proteins: candidate odorant receptors in *Drosophila*. *Neuron* 22:327–338.
- Clyne PJ, Certel SJ, de Bruyne M, Zaslavsky L, Johnson WA, Carlson JR (1999b) The odor specificities of a subset of olfactory receptor neurons are governed by Acj6, a POU-domain transcription factor. *Neuron* 22:339–347.
- Couto A, Alenius M, Dickson BJ (2005) Molecular, anatomical, and functional organization of the *Drosophila* olfactory system. *Curr Biol* 15:1535–1547.
- Crooks GE, Hon G, Chandonia JM, Brenner SE (2004) WebLogo: a sequence logo generator. *Genome Res* 14:1188–1190.
- de Bruyne M, Clyne PJ, Carlson JR (1999) Odor coding in a model olfactory organ: the *Drosophila* maxillary palp. *J Neurosci* 19:4520–4532.
- de Bruyne M, Foster K, Carlson JR (2001) Odor coding in the *Drosophila* antenna. *Neuron* 30:537–552.
- Dobritsa AA, van der Goes van Naters W, Warr CG, Steinbrecht RA, Carlson JR (2003) Integrating the molecular and cellular basis of odor coding in the *Drosophila* antenna. *Neuron* 37:827–841.
- Elmore T, Ignell R, Carlson JR, Smith DP (2003) Targeted mutation of a *Drosophila* odor receptor defines receptor requirement in a novel class of sensillum. *J Neurosci* 23:9906–9912.
- Fishilevich E, Vosshall LB (2005) Genetic and functional subdivision of the *Drosophila* antennal lobe. *Curr Biol* 15:1548–1553.
- Fishilevich E, Domingos AI, Asahina K, Naef F, Vosshall LB, Louis M (2005) Chemotaxis behavior mediated by single larval olfactory neurons in *Drosophila*. *Curr Biol* 15:2086–2096.
- Fuss SH, Ray A (2009) Mechanisms of odorant receptor gene choice in *Drosophila* and vertebrates. *Mol Cell Neurosci* 41:101–112.
- Goldman AL, Van der Goes van Naters W, Lessing D, Warr CG, Carlson JR (2005) Coexpression of two functional odor receptors in one neuron. *Neuron* 45:661–666.
- Hallem EA, Ho MG, Carlson JR (2004) The molecular basis of odor coding in the *Drosophila* antenna. *Cell* 117:965–979.
- Higuchi R, Krummel B, Saiki RK (1988) A general method of in vitro preparation and specific mutagenesis of DNA fragments: study of protein and DNA interactions. *Nucleic Acids Res* 16:7351–7367.
- Jones WD, Cayirlioglu P, Kadow IG, Vosshall LB (2007) Two chemosensory receptors together mediate carbon dioxide detection in *Drosophila*. *Nature* 445:86–90.
- Kim K, Colosimo ME, Yeung H, Sengupta P (2005) The UNC-3 Olf/EBF protein represses alternate neuronal programs to specify chemosensory neuron identity. *Dev Biol* 286:136–148.
- Komiyama T, Johnson WA, Luo L, Jefferis GS (2003) From lineage to wiring specificity. POU domain transcription factors control precise connections of *Drosophila* olfactory projection neurons. *Cell* 112:157–167.
- Komiyama T, Carlson JR, Luo LQ (2004) Olfactory receptor neuron axon targeting: intrinsic transcriptional control and hierarchical interactions. *Nat Neurosci* 7:819–825.
- Kreher SA, Kwon JY, Carlson JR (2005) The molecular basis of odor coding in the *Drosophila* larva. *Neuron* 46:445–456.
- Kreher SA, Mathew D, Kim J, Carlson JR (2008) Translation of sensory input into behavioral output via an olfactory system. *Neuron* 59:110–124.
- Kwon JY, Dahanukar A, Weiss LA, Carlson JR (2007) The molecular basis of CO₂ reception in *Drosophila*. *Proc Natl Acad Sci U S A* 104:3574–3578.
- Lee MH, Salvaterra PM (2002) Abnormal chemosensory jump 6 is a positive transcriptional regulator of the cholinergic gene locus in *Drosophila* olfactory neurons. *J Neurosci* 22:5291–5299.
- McKenna M, Monte P, Helfand SL, Woodard C, Carlson J (1989) A simple chemosensory response in *Drosophila* and the isolation of *acj* mutants in which it is affected. *Proc Natl Acad Sci U S A* 86:8118–8122.
- Ray A, van Naters WG, Shiraiwa T, Carlson JR (2007) Mechanisms of odor receptor gene choice in *Drosophila*. *Neuron* 53:353–369.
- Ray A, van der Goes van Naters W, Carlson JR (2008) A regulatory code for neuron-specific odor receptor expression. *PLoS Biol* 6:e125.
- Rhee JM, Gruber CA, Brodie TB, Trieu M, Turner EE (1998) Highly cooperative homodimerization is a conserved property of neural POU proteins. *J Biol Chem* 273:34196–34205.
- Shanbhag SR, Muller B, Steinbrech RA (1999) Atlas of olfactory organs of *Drosophila melanogaster*. 1. Types, external organization, innervations and distribution of olfactory sensilla. *Int J Insect Morphol Embryol* 28:377–397.
- Stocker RF (2008) Design of the larval chemosensory system. *Adv Exp Med Biol* 628:69–81.
- Tahayato A, Sonnevill R, Pichaud F, Wernet MF, Papatsenko D, Beaufile P, Cook T, Desplan C (2003) Otd/Crx, a dual regulator for the specification of ommatidia subtypes in the *Drosophila* retina. *Dev Cell* 5:391–402.
- Tichy AL, Ray A, Carlson JR (2008) A new *Drosophila* POU gene, *pdm3*, acts in odor receptor expression and axon targeting of olfactory neurons. *J Neurosci* 28:7121–7129.
- Vosshall LB, Amrein H, Morozov PS, Rzhetsky A, Axel R (1999) A spatial map of olfactory receptor expression in the *Drosophila* antenna. *Cell* 96:725–736.
- Wey E, Schäfer BW (1996) Identification of novel DNA binding sites recognized by the transcription factor mPOU (POU6F1). *Biochem Biophys Res Commun* 220:274–279.