Epigenetic regulation of olfactory receptor gene expression by the Myb–MuvB/dREAM complex

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In both mammals and insects, an olfactory neuron will usually select a single olfactory receptor and repress remaining members of large receptor families. Here we show that a conserved multiprotein complex, Myb–MuvB (MMB)/dREAM, plays an important role in mediating neuron-specific expression of the carbon dioxide (CO_2) receptor genes (*Gr63a/Gr21a*) in *Drosophila*. Activity of Myb in the complex is required for expression of *Gr63a/Gr21a* and acts in opposition to the histone methyltransferase Su(var)3-9. Consistent with this, we observed repressive dimethylated H3K9 modifications at the receptor gene loci, suggesting a mechanism for silencing receptor gene expression. Conversely, other complex members, Mip120 (Myb-interacting protein 120) and E2F2, are required for repression of *Gr63a* in inappropriate neurons. Misexpression in mutants is accompanied by an increase in the H3K4me3 mark of active chromatin at the receptor gene locus. Nuclei of CO_2 receptor-expressing neurons contain reduced levels of the repressive subunit Mip120 compared with surrounding neurons and increased levels of Myb, suggesting that activity of the complex can be regulated in a cell-specific manner. Our evidence suggests a model in which olfactory receptors are regulated epigenetically and the MMB/dREAM complex plays a critical role in specifying, maintaining, and modulating the receptor-to-neuron map.

[*Keywords*: Myb; odorant receptor; Gr63a; olfaction; *Drosophila*; chromatin]

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The olfactory system detects a variety of volatile chemicals using a vastly differentiated array of sensory neurons that express transmembrane receptor proteins. Individual olfactory receptor (*Or*) genes are expressed in unique classes of olfactory receptor neurons (ORNs), forming a precise receptor-to-neuron map. Neurons expressing the same receptor send axonal projections to a specific area in the antennal lobe, a strategy conserved in insects and mammals (Couto et al. 2005; Mombaerts 2006).

A few principles are emerging to explain how a "one receptor per neuron" pattern is specified in sensory systems. In the *Drosophila* eye, interlocked feed-forward loops of transcriptional activation and repression can define expression of a rhodopsin gene in a cell type-specific manner (Johnston et al. 2011). Moreover, negative-feedback regulation from the expressed rhodopsin can provide additional exclusion mechanisms to ensure one rhodopsin per photoreceptor (Vasiliauskas et al. 2011). In the *Drosophila* olfactory system, receptor gene expression is driven by combinatorial codes of *cis*-acting sites that recruit transcriptional activators and repressors without negative feedback being involved (Ray et al. 2007, 2008; Tichy et al. 2008; Bai et al. 2009; Bai and Carlson 2010; Miller and Carlson 2010; Jafari et al. 2012). Based on these studies, transcription factors seem likely to act at two levels on olfactory receptor promoters: first to restrict expression in an organ-specific manner, and then within an organ to restrict expression to one class of neuron. By itself, this mechanism requires a transcription factor code exclusive to each of the 50 ORN classes. This would be biologically costly, and there are most likely additional mechanisms to ensure the precise expression patterns observed.

Recently, it has been shown that repressive chromatin plays an important role in olfactory receptor expression in mammals (McClintock 2010; Magklara et al. 2011). As olfactory neurons mature, entire blocks of *OR* genes are silenced by histone H3K9 di/trimethylation, and a single receptor allele per cell is thought to be activated by some

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unknown mechanism. The heterochromatin structure is proposed to maintain the remaining numerous *OR* genes in a stable "OFF" state for the remainder of the neuron's life. Active genes are accessible in an "ON" euchromatic state, accompanied by H3K4me3 histone modifications, but little is known about how chromatin in a specific receptor locus is selected to be turned ON. Such a bimodal chromatin structure can provide a restricted open template in a given cell type for subsequent selection of gene expression by combinatorial transcription factor codes. Although mature neurons are post-mitotic and lack the heritability aspect associated with classical epigenetic regulation, it has been argued that the stable chromatin status of a neuron could use the same epigenetic mechanisms seen in mitotic cells (Dulac 2010). We asked whether such a model of chromatin-mediated epigenetic gene regulation can also play a role in *Drosophila* in establishing the receptor-to-neuron map.

We focused on the *Drosophila* carbon dioxide (CO_2) receptor to understand regulatory mechanisms due to its central role in insect olfaction. The receptor consists of a heterodimer of two seven-transmembrane proteins encoded by *Gr21a* and *Gr63a* that are selectively expressed in the antennal ab1C ORNs. These neurons form a labeled line circuit responsible for innate avoidance of CO_2 in *Drosophila* (Suh et al. 2004; Jones et al. 2007; Kwon et al. 2007). The mosquito homologs *Gr1*, *Gr2*, *and Gr3* are expressed in the maxillary palp (Lu et al. 2007) and are used to detect CO_2 in exhaled air, the primary host-seeking cue for insects that transmit deadly human diseases (Gillies 1980).

The dREAM complex is composed of transcription factors and chromatin modifiers, including Myb, E2F2, DP, RBF1/2, Mip40 (Myb-interacting protein 40), Mip120, Mip130, and p55/Caf1, a histone chaperone. p55/Caf1 is also a member of nucleosome remodeling factor (NURF) and nucleosome remodeling and deacetylase (NuRD) complexes. In addition to these proteins, the larger Myb–MuvB (MMB) complex also contains Lin-52; Rpd3, a histone deacetylase; and L(3)mbt, a H4K20me1-binding protein (Korenjak et al. 2004; Lewis et al. 2004; Lipsick 2004; Trojer et al. 2007; Song et al. 2008; van den Heuvel and Dyson 2008; Clapier and Cairns 2009). The MMB/dREAM complex is thought to act as a molecular switch in certain aspects of development (Cayirlioglu et al. 2001; Beall et al. 2002) and could regulate expression and epigenetic control of cell cycle-specific and developmentally regulated genes (Dimova et al. 2003; Korenjak et al. 2004; Lewis et al. 2004; Georlette et al. 2007; Wen et al. 2008). We hypothesized that this complex may be part of the "elusive" mechanism that expresses a single receptor while excluding expression of all other members of the large gene family (McClintock 2010; Magklara et al. 2011).

Here we show that the MMB/dREAM protein complex plays a critical role in specifying the normal expression of *Gr63a* and *Gr21a*. Myb is required for expression in the antenna, and its absence substantially reduces activity of ab1C neurons and behavioral avoidance to low levels of CO_2. Interestingly, the DNA-binding domain of Myb is not required for receptor regulation, consistent with the previous observation that Myb can bind to chromatin indirectly via other members of the MMB/dREAM complex (Andrejka et al. 2011). Expression of the receptor genes tested in the antenna show a pattern of repressive histone methylation (H3K9me2), an enrichment that has been previously seen in developing olfactory neurons in mammals (Magklara et al. 2011). A role for heterochromatin in this process is implied, since Su(var)3-9, an H3K9 histone methyltransferase, acts in opposition to Myb. Remarkably, changing levels of Myb activity in the adult affect expression of *Gr63a*. These results suggest that chromatin structure plays a role in modulation of a receptor after the formation of the receptor-to-neuron map. Promoter constructs inserted randomly into the genome mimic *Gr63a* and *Gr21a* expression in response to Myb, indicating that the chromatin changes are primarily dependent on local *cis*-acting sequences, rather than the neighboring chromatin state.

Another member of the complex, Mip120, acts in opposition to Myb by preventing *Gr63a* from being misexpressed in neurons of the peripheral sensory system and the brain. Loss of E2F2, another complex member, causes derepression of *Gr63a* in the neurons of the antenna and palp. Changing the stochiometric doses of E2F2 and Myb modulates *Gr63a* receptor expression and CO_2 neuronal sensitivity in opposing manners. However, the role of the MMB/dREAM complex is not limited to the CO_2 receptors. Novel olfactory responses are observed in *mip130* mutant antenna, presumably caused by ectopic misexpression of another olfactory receptor gene. Together, our results provide evidence for combinatorial regulation of olfactory receptors by the multiprotein MMB/dREAM complex and chromatin, revealing mechanistic insights into how a sophisticated receptor-to-neuron map can be generated, maintained, and modulated.

Results

Expression of Gr63a *and* Gr21a *depends on Myb, a member of the MMB/dREAM complex*

We performed an in-depth analysis of the regulatory sequences of the CO_2 receptors *Gr21a* and *Gr63a* in search of predicted or known protein-binding sites. We identified an interesting multiprotein complex called MMB/dREAM, which binds strongly to the *Gr63a* promoter in chromatin immunoprecipitation (ChIP) combined with DNA microarray (ChIP–chip) experiments (Supplemental Fig. S1A; Georlette et al. 2007). The *myb*-null mutant is larval-lethal, so in order to test whether members of the MMB/dREAM complex have any effect on the expression of *Gr63a* in adults, we analyzed *mip130, myb* double mutants that are adult-viable (Manak et al. 2002; Beall et al. 2004). We compared these double-mutant animals with *mip130* single-mutant and wild-type animals. *Gr63a* expression was significantly reduced in *mip130,myb* mutant antennae but not in *mip130* antennae, as shown by whole-mount RNA in-situ hybridization (Fig. 1A) and quantitative RT–PCR (qRT–PCR) (Fig. 1B). A similar

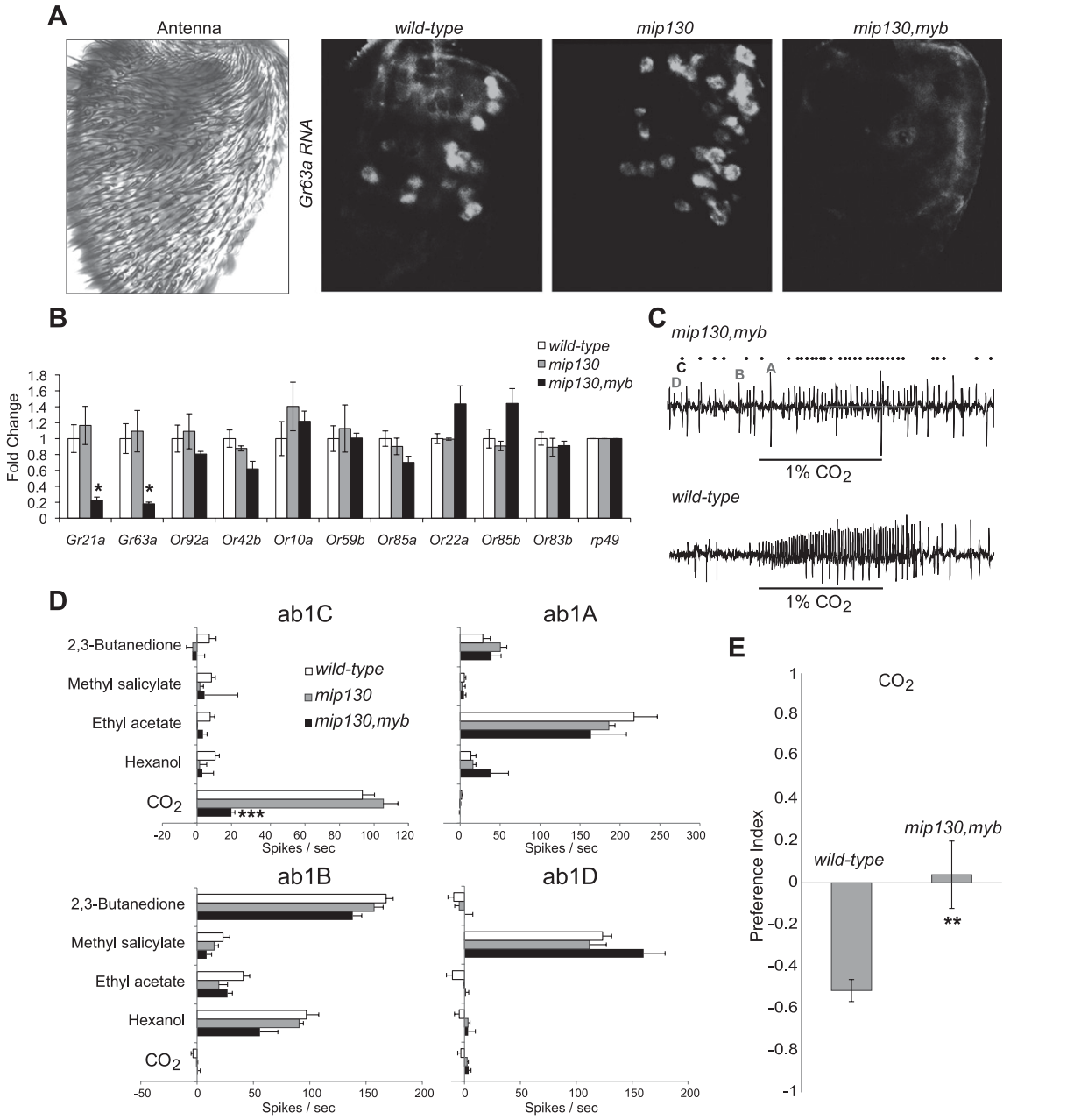

Figure 1. Myb is required for response to CO_2. (*A*) Whole-mount antennae showing a bright-field microscopy image (*left*) and *Gr63a* RNA in situ hybridization (*right*). Male flies of the following genotypes were used: wild-type ($w^{Canton\text{-}S}$), *mip130* mutant ($mip130^{1-36}$), and *mip130,myb* double mutant ($mip130^{1-36}$,myb^{MH107}). (*B*) qRT–PCR using antennal cDNA of indicated genotypes to measure expression of *Or* genes found in large basiconic sensilla using rp49 as a normalizer. $n = 3$ independent experiments per genotype. (*C*) Representative single-sensillum electrophysiological traces of the ab1 sensillum when wild-type or *mip130,myb* double-mutant flies were exposed to 1% CO_2. Characteristic spikes of the four neurons are indicated by their respective letters (A–D). Each spike belonging to the C neuron is denoted by a black dot in the mutant trace. (*D*) Electrophysiological responses of the four ab1 neurons to the indicated diagnostic odors. $n = 10$ recordings per genotype. (*E*) Preference index to measure behavioral responses of indicated flies to 0.33% CO_2 in a T-maze. $n = 10$ trials per genotype (~40 flies per trial). Data are presented as mean ± SEM. Two-tailed Student's *t*-test was used: (*) $P < 0.05$; (**) $P < 0.01$; (***) $P < 0.001$.

effect was observed for *Gr21a*, which is closely related and shares significant sequence similarity to *Gr63a* in the region bound by the MMB/dREAM complex (Fig. 1B; Supplemental Fig. S1A,B). Other large basiconic receptor genes and the broadly expressed *Or83b*/*Orco* coreceptor showed no significant change in expression (Fig. 1B). These experiments indicate that Myb is required for the normal expression of *Gr63a* and *Gr21a*.

Both *Gr63a* and *Gr21a* are necessary for formation of a functional CO_2 receptor and for neuronal response to

CO_2 (Jones et al. 2007; Kwon et al. 2007). As predicted by our studies of gene expression, the *mip130,myb* mutants showed a substantially reduced CO_2 response in the ab1C neurons (Fig. 1C,D). This phenotype is highly specific, and odorant responses of neurons in the other three classes (A, B, and D) housed within the ab1 sensillum were unaffected (Fig. 1D). Interestingly, the ab1C neuron itself was still present in the mutant, as shown by stereotypic action potentials of the appropriate amplitude (Fig. 1C). Together, these data imply that Myb is required specifically for expression of *Gr63a* and *Gr21a* in the ab1C neurons but not for the development of the neuron itself.

Drosophila melanogaster have an innate avoidance to CO_2, which is found in stress odor and unripe fruit (Suh et al. 2004; Faucher et al. 2006). The avoidance to 0.33% CO_2 was abolished in the *mip130,myb* mutant flies, indicating that the reduction of *Gr63a* and *Gr21a* expression affects behavior (Fig. 1E). However, at a higher dose of CO_2, the avoidance response was still observed (data not shown), as we would expect from the residual electrophysiological activity.

Pan-neuronal reintroduction of Myb in *mip130,myb* mutant animals using *elav-GAL4* to drive transgenic *UAS-RFP::myb*$^{+}$ cDNA rescued *Gr63a* expression, as observed by both RNA in-situ hybridization (Fig. 2A,B) and RT–PCR (Fig. 2C). Similar results were also observed for *Gr21a* (Supplemental Fig. S1B). The electrophysiological response to CO_2 was also partially rescued in the

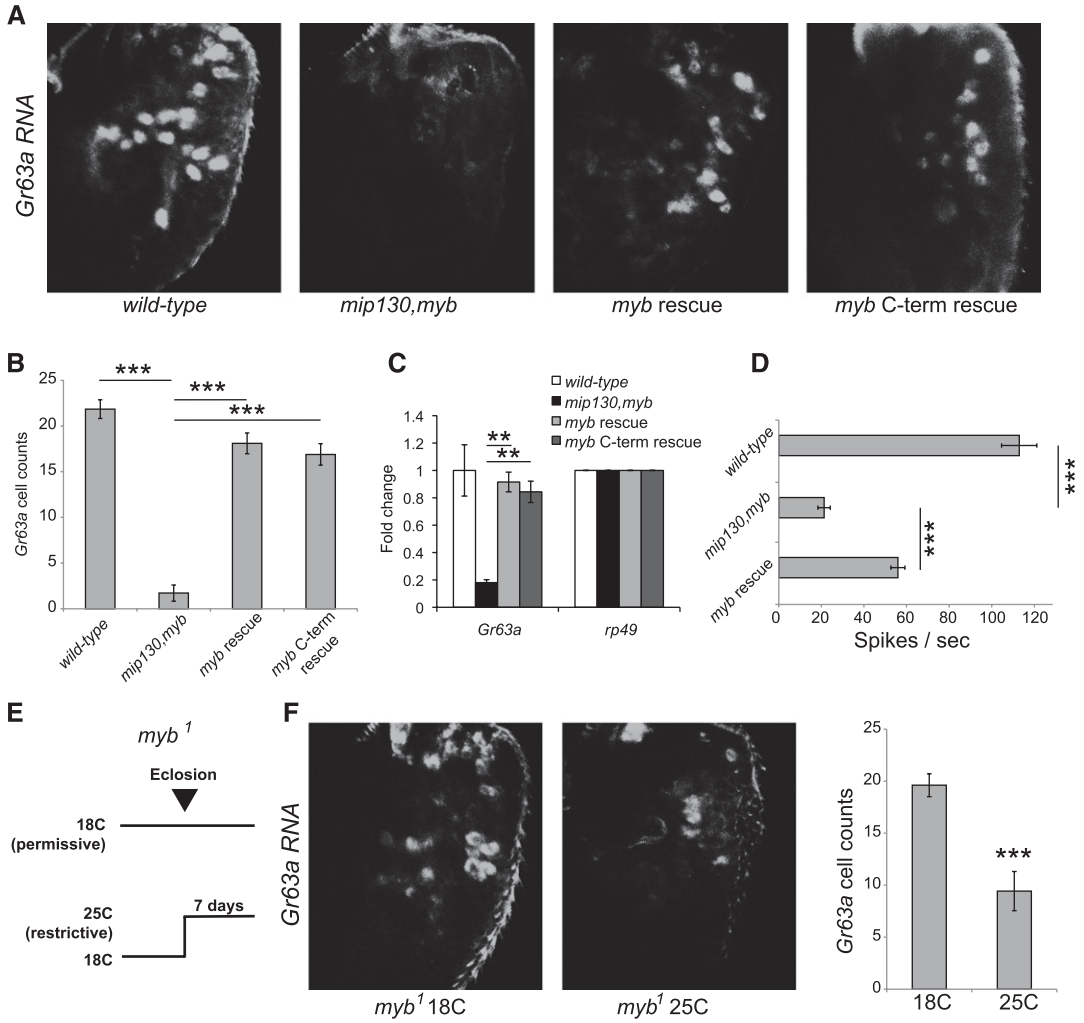

Figure 2. Myb activates *Gr63a* expression post-developmentally via the MMB/dREAM complex. (*A*) Whole-mount male antennae showing *Gr63a* RNA in situ hybridization on wild-type, *mip130,myb* double mutant, *myb* rescue (*mip130*$^{1-36}$,*myb*^{MH107}; *elav-GAL4/+; UAS-RFP::myb*$^{+}$/+), and *myb* C-term rescue (*mip130*$^{1-36}$,*myb*^{MH107}; *myb promoter-GFP::myb*$^{C\text{-}terminal}$/+). (*B*) Number of *Gr63a*-expressing cells in genotypes indicated in *A*. n = 9–13 antennae per genotype. (*C*) qRT–PCR using antennal cDNA to measure *Gr63a* expression in the indicated genotypes. n = 3 biological replicates. (*D*) Electrophysiological responses to 1% CO_2 in wild-type, double-mutant, and *myb* rescue genotypes. n = 8–24 per genotype. (*E*) Schematic of culturing temperature-sensitive *myb*^{1} males at 18°C (permissive) and shifting them to 25°C (restrictive) post-eclosion. (*Top* panel) Control *myb*^{1} flies were kept at 18°C throughout the entire period. (*F*) Whole-mount antenna *Gr63a* RNA in situ hybridization on *myb*^{1} flies. n = 23–31 per condition. Data are presented as mean ± SEM. Two-tailed Student's t-test was used: (**) $P < 0.01$; (***) $P < 0.001$.

ab1C sensillum (Fig. 2D). Ectopic expression of myb^+ did not drive additional ectopic *Gr63a* expression, suggesting that Myb alone is insufficient for receptor expression (Supplemental Fig. S4C).

Myb regulates CO_2 receptor expression via the MMB/dREAM complex

Interestingly, we found that a truncated version of Myb containing only the C-terminal domain also rescued *Gr63a* and *Gr21a* expression (Fig. 2A–C; Supplemental Fig. S1B). Despite lacking all three repeats of the highly conserved Myb DNA-binding domain (Peters et al. 1987), this Myb C-terminal domain is sufficient to assemble into the MMB/dREAM complex and localize to specific regions in the genome to regulate gene expression (Wen et al. 2008; Andrejka et al. 2011). Thus, the rescue of receptor expression in the absence of the highly conserved Myb DNA-binding domain suggests that Myb is acting via the complex at the receptor loci.

Since the *myb*-null mutant is adult-lethal (Manak et al. 2002), we initially investigated the *mip130,myb* double mutant. We chose not to analyze myb^- clones, since loss of *myb* during pupal development can cause defects such as incomplete cell division (Katzen and Bishop 1996), which could confound results in mature ORNs. To test the effect of loss of Myb alone on *Gr63a* expression, we used a temperature-sensitive myb^1 mutant that is viable if raised to adulthood at the permissive temperature (18°C) (Katzen and Bishop 1996). This myb^1 allele has a point mutation in the functionally important C-terminal domain, disrupting protein function at the restrictive temperature (25°C) (Katzen et al. 1998). Flies were cultured at the permissive temperature and, upon reaching adulthood, were transferred to the restrictive temperature (Fig. 2E). There was a significant reduction in *Gr63a* expression at the restrictive temperature (Fig. 2F; Supplemental Fig. S4A). Interestingly, at the restrictive temperature, the number of $Gr63a^+$ cells per antenna followed a bimodal distribution (Supplemental Fig. S4A), and we conjecture that this may suggest a stochastic process with two discrete chromatin states influenced in part by the dose of Myb protein. We cannot, however, rule out other possibilities. These results indicate that the complex is required post-developmentally to maintain receptor gene expression.

A heterochromatin signature in odorant receptor genes

Epigenetic control of odor receptor gene expression has recently been shown in mice where unexpressed *OR* genes are repressed by H3K9 dimethylation in olfactory stem cells and by trimethylation in mature olfactory neurons (Magklara et al. 2011). To test whether repressive chromatin is also found at *Drosophila* olfactory receptor genes, we performed ChIP-PCR on the adult antenna using antibodies specific for the H3K9me2 mark. Since individual odor receptor genes are expressed in a very small fraction of antennal olfactory neurons, we expect that >98% of ORNs will potentially display repressive marks in receptor gene loci. We observed enrichment of the H3K9me2 mark at *Gr21a*, *Gr63a*, and another receptor gene, *Or42b*, in the antenna compared with head tissue (Fig. 3A). Negative controls such as *β-tubulin* and *Gapdh1* did not show a high level, as would be expected for broadly expressed genes, while positive controls such as satellite repeats (Rudolph et al. 2007) and pericentric heterochromatin (Zhang et al. 2008) showed the expected high levels for H3K9me2 (Fig. 3A). A widely used antibody against H3K9me3 showed nonspecific binding when tested on a peptide array (data not shown), so we do not present data from those ChIP experiments. The H3K9me2-repressive chromatin structure at odor receptor genes is similar to that observed in mammalian olfactory stem cells and raises the interesting possibility that the MMB/dREAM complex may also play a conserved role in its regulation.

Epigenetic regulation of receptor expression by MMB/dREAM

Because ab1C neurons make up a small fraction of the total cell population (~20–30 cells per antenna), it is not feasible to measure H3K9 methylation status exclusively in these cells. However, in *Drosophila,* it is known that the majority of H3K9me2 methylation marks are catalyzed by the histone methyltransferase Su(var)3-9 (Rea et al. 2000; Ebert et al. 2004). To test whether regulation of *Gr63a* expression by Myb involves H3K9 methylation via Su(var)3-9, we tested for a genetic interaction between the two. Upon halving the gene dosage of *Su(var)3-9* in the *mip130,myb* mutant, we found a partial rescue of *Gr63a* expression (Fig. 3B). Halving the gene dosage of *Su(var)3-9* alone did not affect *Gr63a* expression (Fig. 3B). Thus, Myb opposes the repressive effects of H3K9 histone methyltransferase Su(var)3-9 in *Gr63a* regulation. Taken together, our data indicate that the MMB/dREAM complex is required to affect permissive chromatin at the *Gr63a* locus in the ab1C ORNs.

Myb also acts on transgenic receptor–promoters inserted randomly in the genome

In *Drosophila* and mammals, transgenes containing short *Or* gene promoter sequences can reproduce the spatial expression patterns of the endogenous receptor (Vassalli et al. 2002; Rothman et al. 2005; Ray et al. 2007, 2008). To test whether short promoters are sufficient to recapitulate regulation of the *Gr63a* locus by Myb, we used a 2.64-kb *Gr63a promoter-GAL4* to drive expression of a GFP reporter (*UAS-mCD8::GFP*) in wild-type and *mip130, myb* mutant flies. We found a significant decrease in the number of GFP-expressing neurons (Fig. 4A,C), consistent with the expression of endogenous *Gr63a* RNA. Likewise, a *Gr21a-GAL4* reporter labeled fewer neurons in the double mutant (Fig. 4A,C). In contrast, *Or42b-GAL4* and *Or22a-GAL4* showed no significant difference in GFP^+ cell numbers (Fig. 4B,C), again consistent with the expression of endogenous genes. A direct *promoter-GFP* fusion (*Gr21a promoter-mCD8::GFP*) was used to demonstrate that expression is rescued by introducing *elav-GAL4;*

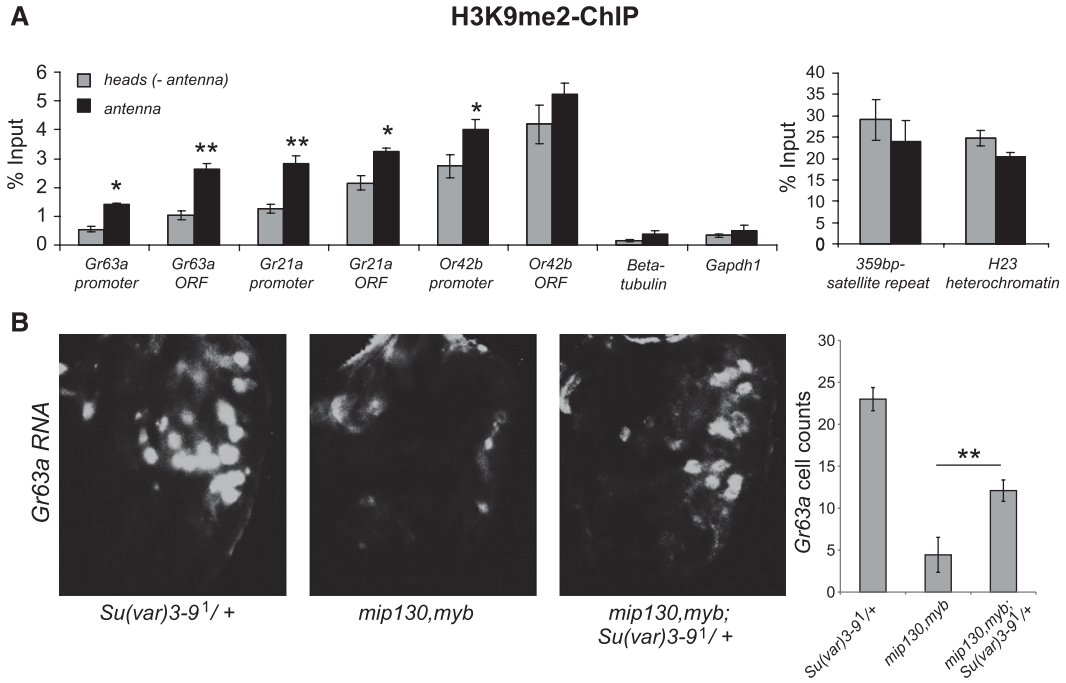

Figure 3. Myb opposes a repressive chromatin modifier. (*A*) ChIP using antibodies to H3K9me2 on wild-type antennae or heads without antennae. qRT–PCR was performed for indicated regions. $n = 3$–4 biological replicates. (*B*) Whole-mount antennae showing *Gr63a* RNA in situ hybridization on heterozygous *Su(var)3-9*^{1}/+, *mip130,myb* double mutant, and heterozygous *mip130,myb; Su(var)3-9*^{1}/+. $n = 9$–21 per genotype. Data are presented as mean ± SEM. Two-tailed Student's *t*-test was used: (*) $P < 0.05$; (**) $P < 0.01$; (***) $P < 0.001$.

UAS-RFP::myb$^+$ in the double-mutant flies (Fig. 4D,E). This reporter was also used to demonstrate genetic opposition between *mip130,myb* and *Su(var)3-9*, as seen with *Gr63a* RNA in situ hybridization (Fig. 4G,H). Thus, the MMB/dREAM complex affects the chromatin state of both the endogenous and transgenic promoters of *Gr63a* and *Gr21a* integrated randomly at other regions of the genome.

ORNs expressing a particular olfactory receptor project their axons to a distinct glomerulus in the antennal lobe (Couto et al. 2005), and transcription factors such as *pdm3* and *acj6* have dual roles in mediating both *Or* gene expression and ORN axonal projection (Clyne et al. 1999; Komiyama et al. 2004; Tichy et al. 2008). Using *Gr63a-GAL4, UAS-mCD8::GFP*, which still had some residual expression in the absence of both Myb and Mip130, we showed that the axonal targeting of ab1C neurons to the V glomerulus was unaffected (Fig. 4F).

We asked whether *myb* has genetic interactions with other genes known to affect *Gr63a* expression. The microRNA *mir-279* and its regulator, *prospero*, have been shown to play a developmental role in repressing the formation of ectopic CO_2 neurons in the maxillary palp (Cayirlioglu et al. 2008; Hartl et al. 2011). Using *Gr21a promoter-mCD8::GFP* to test for a genetic interaction between *myb* and *mir-279*, we found that the number of CO_2 ORNs was similar between *mip130,myb; mir-279* triple mutants and *mir-279* single mutants in the antenna and maxillary palp (Supplemental Fig. S2A,B), suggesting that ectopic cells in the maxillary palp in the *mir-279* mutant do not require Myb for expression. In addition, *myb* expression was normal in *mir-279* mutant maxillary palps (Supplemental Fig. S2C), while *mir-279* expression was normal in *mip130,myb* mutant antenna (Supplemental Fig. S2D), indicating lack of genetic interaction. *mir-279* appears to play a developmental role in preventing the birth of CO_2-like neurons in the maxillary palp (Cayirlioglu et al. 2008; Hartl et al. 2011), while Myb plays a role in the expression of *Gr63a* and *Gr21a* in the mature ORN.

Repression of receptor expression in inappropriate cells by Mip120

The specification of olfactory receptor gene expression requires activation in the correct neurons as well as silencing in other neuronal classes. The Mips of the MMB/dREAM complex have previously been shown to have a repressive role antagonistic to Myb (Korenjak et al. 2004; Lewis et al. 2004), raising the possibility that these proteins might mediate repression of *Gr63a* in inappropriate neurons. Wild-type flies express *Gr63a* only in the ab1C neurons of the third antennal segment. In contrast, we found that *mip120* mutant flies misexpressed *Gr63a promoter-GAL4* in other classes of sensory neurons, including the second antennal segment (hearing) (Fig. 5A), maxillary palp (olfactory) (Fig. 5B,D,E), and labellum (taste) (Fig. 5C). *Gr63a* was also overexpressed in approximately twice the number of ORNs in the large basiconic region of the third antennal segment (Fig. 5A,D).

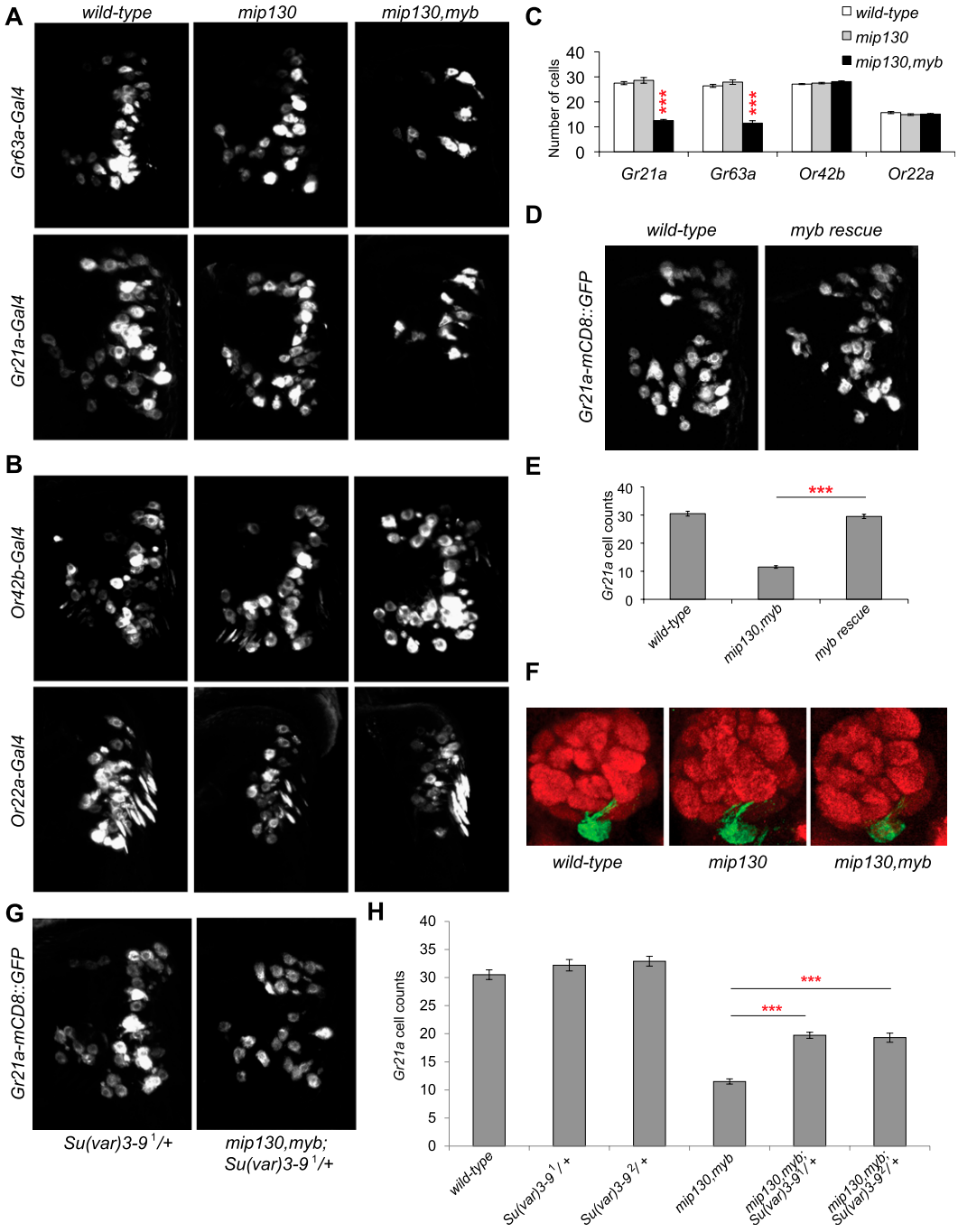

Figure 4. Promoters of *Gr63a* and *Gr21a* are important for Myb regulation (*A*,*B*) Whole-mount antennae showing neurons expressing *GAL4*-responsive membrane targeted GFP (*UAS-mCD8::GFP*) with *Gr63a* or *Gr21a promoter-GAL4s* (*A*) or controls, *Or42b* or *Or22a promoter-GAL4s* (*B*), in wild-type, *mip130* mutant, and *mip130,myb* double mutant. (*C*) Counts of GFP$^+$ cells for *A* and *B*. n = 10 per genotype. (*D*) Whole-mount antennae showing neurons expressing a different *Gr21a* promoter direct fusion transgene (*Gr21a promoter-mCD8::GFP*) and *myb* rescue (*mip130,myb; Gr21a promoter-mCD8::GFP/elav-GAL4; UAS-RFP::myb*$^+$). (*E*) Counts of GFP$^+$ cells for *D*. n = 10 per genotype. (*F*) Immunofluorescence using anti-GFP (green) and anti-nc82 (neuropil marker; red) antibodies on antennal lobes of wild-type, *mip130* mutant, and *mip130,myb* double mutant with *Gr63a-GAL4; UAS-mCD8::GFP*. (*G*) Whole-mount antennae showing neurons expressing *Gr21a promoter-mCD8::GFP* in *Su(var)3-9^1*/+ and *mip130,myb;Su(var)3-9^1*/+. (*H*) Counts of GFP$^+$ cells for *G* and *Su(var)3-9^2*, a second null allele. n = 10 per genotype. Data are presented as mean ± SEM. Two-tailed Student's t-test was used: (***) $P < 0.001$.

Consistent with these ectopic expression patterns, *mip120* mutant antennal lobes showed innervation by *Gr63a promoter*-expressing neurons of multiple glomeruli in addition to the V glomerulus (Fig. 5F; Supplemental Fig. S3A,B). *Gr63a* was also misexpressed in a large number of additional neurons in the brain (Fig. 5F). Using

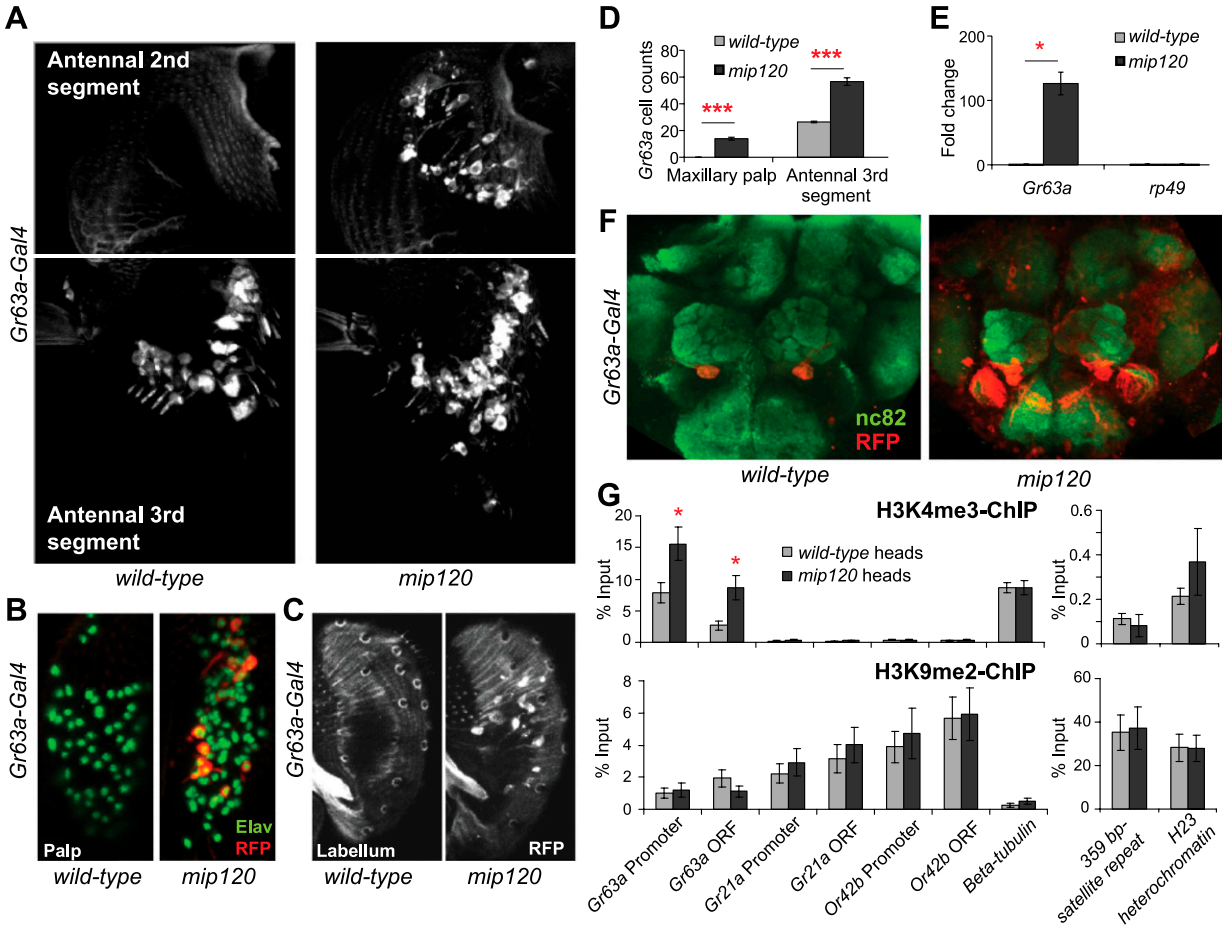

Figure 5. Mip120 is required to repress *Gr63a* misexpression. (*A*) Whole-mount antennae of wild-type and *mip120* mutant ($mip120^{67\text{-}21\text{-}6}$/$mip120^{67\text{-}9A\text{-}9}$) expressing *Gr63a-GAL4, UAS-mCD8::chRFP*. (*B*) Immunofluorescence using anti-Elav (pan-neuronal marker; green) and anti-RFP (red) of whole-mount maxillary palps of the genotypes indicated in *A*. (*C*) Whole-mount labellum of the indicated genotypes. (*D*) Number of *Gr63a*-expressing cells in *A* and *B*. $n = 10$ antennae or maxillary palps per genotype. (*E*) qRT–PCR using maxillary palp cDNA of indicated genotypes to measure *Gr63a* misexpression in the palp. $n = 3$ independent experiments per genotype. (*F*) Immunofluorescence using antibodies to nc82 (green) and RFP (red) on whole-mount brains of the genotypes indicated in *A*. (*G*) ChIP using antibodies to H3K4me3 or H3K9me2 on wild-type and *mip120* mutant heads. $n = 5$–6 biological replicates per genotype. Data are presented as mean ± SEM. Two-tailed Student's *t*-test was used: (*) $P < 0.05$; (***) $P < 0.001$.

single-sensillum electrophysiology, we tested whether the misexpression of *Gr63a* in ORNs of the maxillary palps can impart responsiveness to CO_2, and found that the neurons were not responsive to 1% CO_2 ($n = 6$ neurons) (data not shown), suggesting that other required components of the CO_2 detection machinery, such as Gr21a or Gαq, may not be misexpressed in the same neurons of the palps (Yao and Carlson 2010). Indeed, whole-mount in situ hybridizations showed, on average, only one cell positive for *Gr21a RNA* in the maxillary palps of *mip120* mutants ($n = 6$ palps). The olfactory responses to a diagnostic five-odor panel were normal from each of the six classes of ORNs ($n = 3$ for each class), suggesting that there is no obvious developmental defect in the maxillary palps of the *mip120* mutants. Taken together, these results indicate that the role of Mip120 in the MMB/dREAM complex is to restrict *Gr63a* expression to the correct antennal segment by mediating silencing in other sensory tissues.

Since misexpression of *Gr63a* is widespread across the head, it provides us with sufficient starting material to directly test by ChIP-PCR whether there are changes in the chromatin corresponding to derepression of *Gr63a* in the *mip120* mutant heads. We found a significant increase in H3K4me3 that marks active chromatin at the *Gr63a* locus, while the levels of H3K9me2 remained unaffected (Fig. 5G). These results indicate that in the absence of Mip120, ectopic expression of *Gr63a* is associated with enrichment of H3K4me3, further emphasizing the combinatorial role of the MMB/dREAM complex in epigenetic regulation of *Gr63a*.

Refinement of Or gene expression by Mip130

Mip130 is a generally repressive MMB/dREAM complex member (Wen et al. 2008). However, unlike *mip120* mutants, *mip130* flies did not show an obvious misexpression phenotype for *Gr63a* (Fig. 1A,B). One possibility

could be that *mip130* instead plays a role in refining expression of other receptor gene families, such as ionotropic or odorant receptors, which also require precise expression in a one receptor per neuron fashion. In order to test this, we used a diagnostic odor panel and screened eight classes of ORNs in the antennae of the *mip130* mutant flies using single-sensillum electrophysiology. Indeed, one of the eight classes, the ab3A neuron, had an unusual odor response profile in the *mip130* antenna (Fig. 6F; Supplemental Fig. S6). The ab3A neuron in the *mip130* mutant antenna detected diagnostic odors characteristic of its endogenous receptor, *Or22a* (such as pentyl acetate), but also showed strong responses to methyl salicylate, ethyl acetate, and anisole, odors that are normally not detected by this neuron (Fig. 6F). *Or22a* was still expressed in the mutant antenna (Figs. 4B, 6G). However, gained odor responses do not match that of any characterized odorant receptors (Hallem and Carlson 2006; Kreher et al. 2008). A possible interpretation of these results is that the novel odor responses are conferred by the misexpression of an uncharacterized *Or* gene specifically in the ab3A cells of the *mip130* mutant flies. These results suggest that the MMB/dREAM complex and its effect on chromatin structure may play an important role in the differential expression of receptor genes in a variety of olfactory neurons.

Modulation of CO_2 neuronal sensitivity by MMB/dREAM

Another member of the MMB/dREAM complex, E2F2, has also been shown to play a repressive role (Lewis et al. 2004). The *e2f2* mutant flies showed a significant increase in the levels of *Gr63a* in both the antenna and the maxillary palp (Fig. 6A), whereas *Or42b* showed no significant change in expression by RT–PCR (Fig. 6A). These observations were validated by RNA in situ hybridization experiments (Fig. 6B). *Gr21a* was also sporadically misexpressed in the maxillary palps of the *e2f2* mutants with detectable RNA in an average of two cells per palp ($n = 6$). However, a thorough survey using single-sensillum electrophysiology of the maxillary palps showed that neurons were unresponsive to CO_2 stimuli of up to 4.8% ($n = 25$ neurons) (data not shown). Characteristic olfactory responses to each of the six classes of ORNs were identifiable ($n = 3$ for each class), suggesting that there is no obvious developmental defect in the *e2f2* mutant maxillary palps.

We also found that halving the gene dosage of *e2f2* partially suppressed the *mip130,myb* phenotype in *Gr63a* expression (Fig. 6C). In addition, heterozygous *e2f2* flies in an otherwise wild-type genetic background also showed an increase of *Gr63a* expression compared with wild-type flies (Fig. 6C). The genetic opposition of *e2f2* to *myb* is consistent with our expectation that *e2f2* has a repressive regulatory role in the MMB/dREAM complex (Wen et al. 2008). Interestingly, heterozygous *mip130, myb* female flies also showed an intermediate *Gr63a* expression relative to wild-type and homozygous *mip130,myb* females (Supplemental Fig. S4B), suggesting that the stoichiometric dose of each MMB/dREAM component could play a critical role in gene regulation.

The olfactory response of a neuron may depend on not only the identity of the receptor being expressed, but also the level of receptor expression (Tanoue et al. 2008). Since little is known about mechanisms that ensure appropriate levels of receptor expression, we wanted to investigate whether the doses of individual MMB/dREAM members, some with opposing effects, could play a role in receptor modulation. Using single-sensillum electrophysiology to probe CO_2 response in the ab1C sensillum, we found that the electrophysiological response was affected in opposite directions by the dose of *myb* and *e2f2*. In Figure 2D, we show that the electrophysiological response to CO_2 was substantially lowered in the *myb, mip130* flies and partially rescued when *myb* was expressed using a pan-neuronal driver, *elav-GAL4*. Conversely, the dose response to CO_2 showed that the response was significantly increased in the *e2f2* mutant flies at the lower concentrations (Fig. 6D). These results demonstrate that the levels of CO_2 receptor expression affect the sensitivity of the neuron and indicate that the MMB/dREAM complex can act to mediate a gain-control mechanism for the olfactory neuron by integrating doses of positive (Myb) and negative (E2F2) subunits.

In the absence of E2F2, the misexpression of *Gr63a* is associated with a concomitant enrichment of the H3K4me3 mark in the heads (Fig. 6E). This pattern is similar to what we found in *mip120* mutants, suggesting a correlation between misexpression and chromatin changes.

MMB/dREAM complex members are present in ORN nuclei

The genetic analysis demonstrates roles for several members of the MMB/dREAM complex, such as Myb, Mip120, Mip130, and E2F2, in the ORN nuclei. We generated antibodies against the two repressive members, Mip120 and E2F2, to characterize their expression. The specificity of the antibodies was validated by the absence of staining in *mip120* or *e2f2* mutants (Supplemental Fig. S7B). We found that both proteins were detected inside the nuclei of a large fraction of olfactory neurons of the antenna and the maxillary palps, which were costained with anti-Elav, a pan-neuronal marker (Fig. 7A,B). To map expression of Myb, we used a genomic construct encoding a Myb-GFP fusion protein and found that this protein was also present in a large fraction of the olfactory neuron nuclei (Fig. 7A,B). Taken together, these experiments demonstrate that these nuclear proteins are present in ORNs and persist post-developmentally.

Since the members of the complex are present in several antennal neurons, we asked whether the MMB/dREAM complex could be differentially regulated to achieve neuron-specific selectivity. We performed antibody staining and demonstrated that Myb, the activator of the complex, was consistently higher in ab1C nuclei compared with surrounding neurons. Interestingly, a repressive subunit of the complex, Mip120, was present at

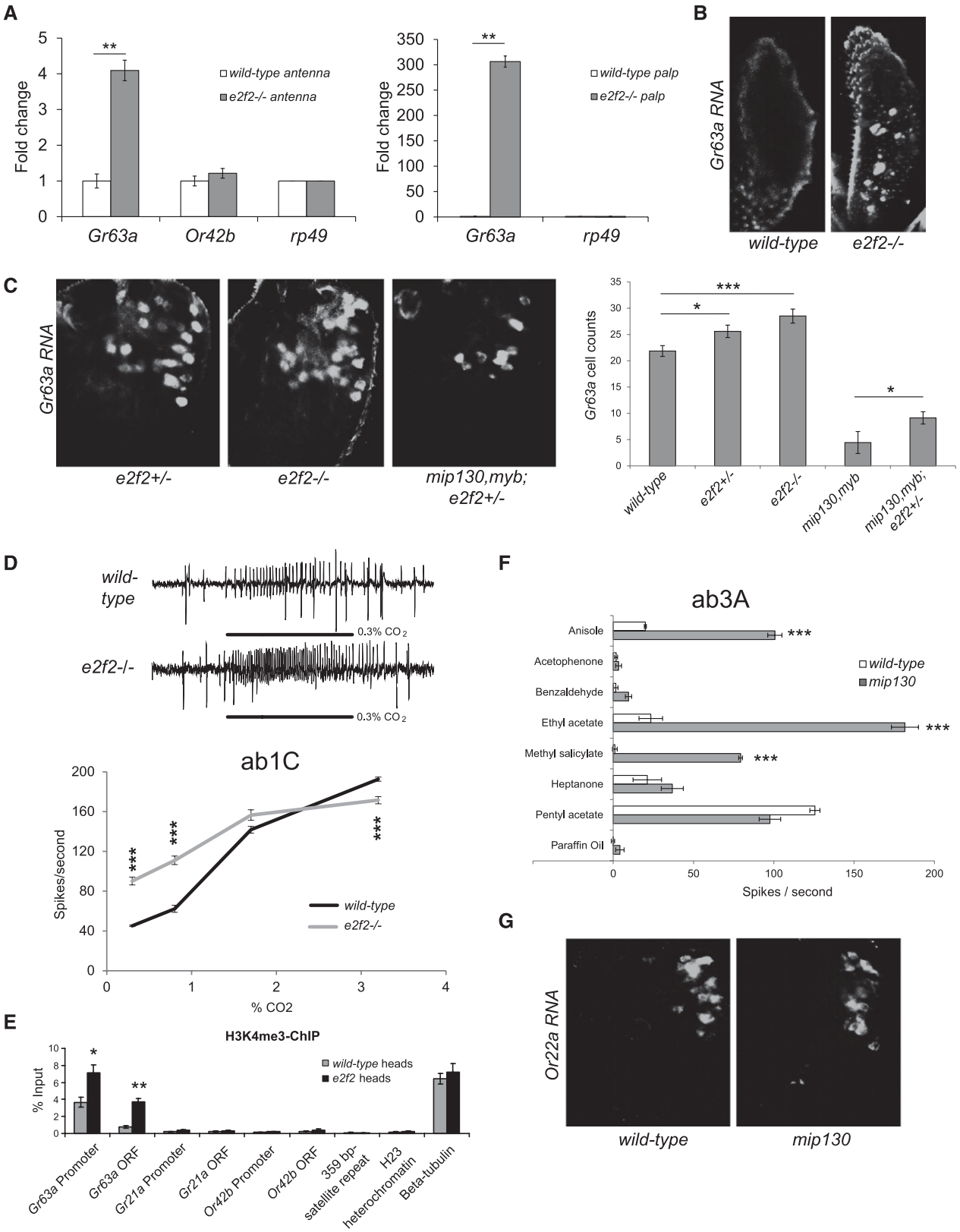

Figure 6. MMB/dREAM has broader roles in olfactory receptor regulation. (*A*) qRT–PCR using antennal (*left* panel) or maxillary palp (*right* panel) cDNA of wild-type or *e2f2* mutant ($e2f2^{1-188}/e2f2^{329}$) to measure expression of *Gr63a* or control *Or42b*. $n = 3$ independent experiments per genotype. (*B*) Whole-mount maxillary palp showing *Gr63a* RNA in situ hybridization in wild-type and *e2f2* mutant. (*C*) Whole-mount antennae showing *Gr63a* RNA in situ hybridization on heterozygous $e2f2^{329}/+$ and homozygous $e2f2^{1-188}/e2f2^{329}$ mutant flies and *mip130,myb*; $e2f2^{329}/+$ flies. $n = 9$–15 per genotype. Cell counts are shown in the *right* panel. (*D*, *top*) Representative electrophysiological traces of ab1 sensillum when e*2f2* mutant flies were exposed to 0.3% CO_2. (*Bottom*) Dose response of e*2f2* mutant ab1C neuron to CO_2. $n = 6$. (*E*) ChIP using antibodies to H3K4me3 on wild-type and *e2f2* mutant heads. $n = 3$ biological replicates per genotype. (*F*) Electrophysiological responses of *mip130* mutant ab3A neuron to diagnostic odors. $n = 5$ recordings per genotype. (*G*) Whole-mount antennae showing *Or22a* RNA in situ hybridization on wild-type and *mip130* mutant. Data are presented as mean ± SEM. Two-tailed Student's *t*-test was used: (*) $P < 0.05$; (**) $P < 0.01$; (***) $P < 0.001$.

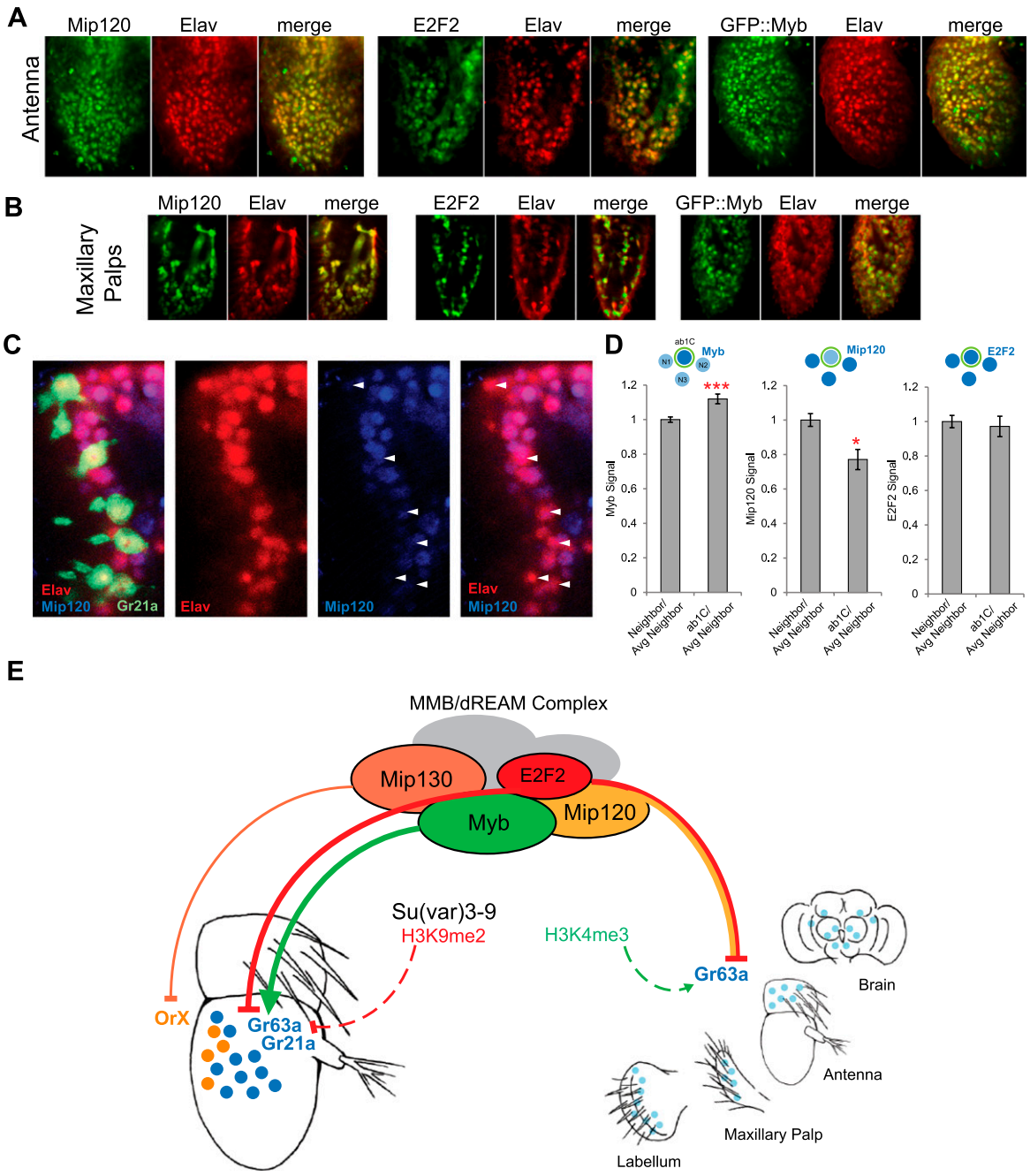

Figure 7. MMB/dREAM in olfactory organs and model for olfactory receptor expression. Confocal micrographs of antennae (*A*) and maxillary palp (*B*)s using anti-Mip120 and anti-Elav in wild-type flies (*left* panels), anti-E2F2 and anti-Elav in wild-type flies (*middle* panels), or anti-GFP and anti-Elav in *myb promoter-GFP::Myb* flies (*right* panels). (*C*) Representative confocal micrograph from a *Gr21a promoter-mCD8::GFP* antenna stained with anti-CD8, anti-Elav, and anti-Mip120 antibodies. Overlays of all three (*left*) and of anti-Elav with anti-Mip120 (*right*) are provided. Arrowheads indicate nuclei of $Gr21a^{+}$ ab1C neurons. (*D*) Schematic showing cells that were selected for analysis of nuclear stain signal intensity and mean Myb, Mip120, or E2F2 signal intensity in ab1C or a neighboring nucleus as a ratio of the mean signal in the neighboring three cells. For Myb signal, $n = 48$ for neighboring cells and $n = 16$ for ab1C cells. For Mip120 signal, $n = 70$ for neighboring cells and $n = 25$ for ab1C cells. For E2F2 signal, $n = 29$ for neighboring cells and $n = 13$ for ab1C cells. Error bars indicate SEM. (*) $P < 0.05$; (***) $P < 0.001$. (*E*) Olfactory receptors have a H3K9me2-repressive chromatin mark (red dashed line). Expression of *Gr63a/Gr21a* receptors in the ab1C neurons of antennae requires Myb (green arrow), and Myb opposes histone methyltransferase Su(var)3-9. Conversely, repression of *Gr63a* in other sensory organs and the brain requires Mip120 and E2F2 (combined red and orange line). The absence of Mip120 or E2F2 causes misexpression and increased H3K4me3 chromatin mark at *Gr63a* gene (green dashed arrow). E2F2 also has a repressive modulatory effect on *Gr63a* expression in ab1C cells (solo red line), while Mip130 represses expression of an unknown olfactory receptor in the antenna (dark-orange line).

lower levels in the ab1C neurons as compared with neighboring neurons. E2F2 levels appeared similar between ab1C and its neighbors (Fig. 7C,D). In total, we observed that the Myb:Mip120 ratio in ab1C nuclei is ~45% higher compared with neighboring nuclei. The simplest interpretation of these results is that the ab1C nuclei are able to express the CO_2 receptors by virtue of having higher levels of activating Myb and lower levels of the repressive Mip120 subunit of the dREAM complex, while the neighboring cells with lower Myb and higher Mip120 levels cannot.

Discussion

Heterochromatin-like repression of odor receptors in Drosophila

A central question in olfactory receptor regulation is how a neuron expresses a single member of a large gene family while excluding other members. One model emerging from mammals suggests that histone H3K9 di/trimethylation maintains the odor receptor family members in a generalized repressed state and that expression of a single receptor may be induced by removal of these repressive marks. Here we observed that a pattern of repressive H3K9me2 chromatin exists in the promoters and gene bodies of olfactory receptors of *Drosophila*, similar to the olfactory stem cells in mammals. In mammals, activating H3K4me3 is found at gene loci in the specific cells where a receptor is expressed (Magklara et al. 2011). Although a similar cell-sorting experiment is not technically feasible in *Drosophila*, we did find a similar H3K4me3-mediated derepression of the *Gr63a* gene when it is misexpressed in the *mip120* mutant brain. Although the olfactory receptor gene families in mammals and insects are not evolutionarily related, both systems appear to use repressive chromatin in solving part of the one receptor per neuron problem.

MMB/dREAM epigenetically regulates receptor gene expression

Establishing one receptor per neuron likely involves two processes: the initial selection of a receptor for expression while keeping all other receptors repressed, and the maintenance of this expression profile throughout the life of the neuron. Here we show that the multiprotein MMB/dREAM complex in flies has a role in this process. Myb, the permissive member of the complex, is required for maintaining *Gr63a/21a* expression in the antenna, genetically opposing the H3K9 methyltransferase Su(var)3-9. Repressive members Mip120 and E2F2 are required for maintaining repression of *Gr63a* in other sensory organs. This bears a resemblance to other epigenetic complexes, such as the Polycomb group (PcG) and Trithorax group (TrxG) proteins, which can stably maintain repressive and active chromatin, respectively, at *Hox* gene loci during embryonic development (Schuettengruber et al. 2011).

The roles of other members of the MMB/dREAM complex in receptor gene regulation will be very interesting to pursue, especially the roles of the chromatin-modifying proteins found associated with the complex: Rpd3 (a histone deacetylase), L(3)mbt (a H4K20me1-binding protein) (Trojer et al. 2007), and p55/Caf1 (a histone chaperone) (Song et al. 2008). It will be intriguing to study in depth how MMB/dREAM and chromatin remodeling can participate in epigenetically regulating olfactory receptor expression. Analysis of the *mip130* mutant raises the possibility that members of the complex may regulate the odorant receptor gene family members in the antenna as well.

Myb is required in the mature neuron

It has recently been demonstrated that chromatin modification at *Notch* target loci via Hamlet plays a role in cell fate determination in the developing sensillum (Endo et al. 2011). Our findings are distinct from this pathway, as Myb is required post-mitotically. Loss of Myb does not perturb formation of the C neuron in ab1 sensilla or axonal targeting to the V glomerulus. The only detectable defect is the loss of *Gr63a* and *Gr21a* expression, which is rescued when *myb* is expressed with *elav-GAL4*, a promoter only active in mature neurons. Furthermore, Myb is required to maintain expression of *Gr63a* in the adult, as demonstrated with the temperature shift experiments using the myb^1 allele. Taken together, our data suggest that Myb participates in receptor gene expression after the final neuronal cell division and is required continuously to maintain expression of the CO_2 receptors throughout the life of the neuron.

MMB/dREAM acts on Gr63a *and* Gr21a *promoters*

The presence of the MMB/dREAM complex at the *Gr63a* promoter was identified by ChIP–chip from a *Drosophila* cell line (Georlette et al. 2007), and this region of the promoter, including a consensus complex-binding sequence, is partly conserved upstream of *Gr21a* as well. Because the regulatory effect of the complex is reported reliably by both *Gr63a* and *Gr21a promoter–GAL4* constructs, this opens the possibility of more detailed characterization of predicted MMB/dREAM-binding sites through mutational analysis of the promoters. In mice, the property of singular expression is also retained by short transgenic promoters of *OR* genes inserted outside of their endogenous loci (Serizawa et al. 2003; Lewcock and Reed 2004). It is possible that these DNA sequences contain instructive cues that are responsible for enrichment of repressive H3K9 methylation, which can be conveniently examined using the fly model.

Evolutionary conservation and odor receptor gene expression

Unlike *D. melanogaster*, which avoid CO_2, mosquitoes are strongly attracted to it via orthologous receptors (*Gr1*, *Gr2*, and *Gr3*) in the maxillary palps. Compared with the 12 *Drosophila* species, Myb and Mip130 are well conserved in *Anopheles gambiae* (malaria), *Aedes aegypti* (dengue and yellow fever), and *Culex quinquefasciatus* (filariasis and West Nile), while Mip120 and E2F2 are

more poorly conserved (Supplemental Fig. S5A,B). We predict that differences in Mip120 and E2F2 activity in mosquitoes might provide a template for evolutionary differences in CO_2 receptor expression in the maxillary palps. Alternatively, changes in *cis*-regulatory sequences upstream of the CO_2 receptor gene orthologs could be responsible for differences in expression across species. Since the CO_2 receptor plays a central role in the ability of mosquitoes to find humans and transmit diseases, our findings could have a broader impact for human health. Knowledge about the mechanisms of CO_2 receptor gene expression and the evolution of its expression patterns could have important implications in controlling mosquito-borne diseases such as malaria and dengue fever that affect hundreds of millions of people.

The MMB/dREAM complex is also conserved in mammals as well (Litovchick et al. 2007; Pilkinton et al. 2007; Schmit et al. 2007). There are three mammalian homologs of the *Drosophila myb*: *A-Myb/MYBL1*, *B-Myb/MYBL2*, and *c-Myb/MYB* (Lipsick 2004). In fact, there are some indications that A-Myb and c-Myb may have a role in the mouse olfactory system. *A-myb* is expressed in the olfactory epithelium, while *c-myb* mutant mice have smaller olfactory bulbs (Trauth et al. 1994; Malaterre et al. 2008).

One receptor per neuron: combination of chromatin accessibility and transcription regulation

Previous studies investigating *Or* gene expression have reported that a combinatorial code of *cis*-regulatory elements and transcription factors can specify restricted expression domains in olfactory neuron classes. For example, organ-specific, region-specific, and neuron-specific regulatory elements have been identified for *Or* genes expressed in the maxillary palps (Ray et al. 2007, 2008). Given that a receptor gene is expressed in only one class of olfactory neurons (~20 cells) out of >100,000 neurons in the organism, it is very unlikely that each of ~50 ORN classes would possess a unique transcription factor code that is not recapitulated elsewhere in the nervous system. A recent study reported that seven transcription factors from different protein families could combinatorially regulate the expression of several olfactory receptors in the adult antenna (Jafari et al. 2012). The MMB/dREAM complex appears to function in a different fashion, as it integrates the effect of transcriptional activators, repressors, and chromatin-modifying proteins in a single complex and acts as an adaptable molecular switch for *Or* expression. A key question is how various regulatory mechanisms are able to act in a neuron-specific manner to achieve selective receptor expression. Most known factors that are required for olfactory receptor expression, like POU, HLH, HTH, and Zn finger transcription factors, are expressed far more broadly than the cells where they are required for receptor expression (Ray et al. 2007, 2008; Tichy et al. 2008; Bai et al. 2009; Bai and Carlson 2010; Miller and Carlson 2010; Jafari et al. 2012).

A possible model supported by our observations is that most receptor genes are in a repressed chromatin state in neurons, and selective opening of chromatin structure in certain cells at a specific receptor locus provides a permissive template for transcription factors to drive transcription of the receptor (Fig. 7E). After one set of factors initiates receptor gene choice, epigenetic complexes such as MMB/dREAM would be recruited to the locus in order to maintain stable expression. Two layers of regulatory mechanisms could restrict expression patterns in only those cells where both pathways are active. Complexes such as MMB/dREAM, which contain epigenetic modulators along with transcriptional activators and repressors, would be exceptionally well suited to initiate, maintain, and modulate expression. Other complexes, like MMB/dREAM, may also be involved in *Or* gene regulation. While Myb itself seems to be specific to the CO_2 receptors among the eight classes of neurons that we surveyed, there are ~40 additional ORN classes in the adult and ~21 in the larvae that we have yet to examine. We speculate that while repressive chromatin may be a general strategy for receptor regulation, the dREAM complex could be one of many factors that interact with it and influence the final outcome of receptor expression.

The selectivity question is particularly interesting for the MMB/dREAM complex, since it is the first multiprotein complex shown to be involved in regulation of receptor gene expression and contains both permissive and repressive subunits. We show that ab1C nuclei contain significantly higher Myb and lower Mip120 than neighboring neurons, implying that the stoichiometry of the complex can determine whether a gene will be active or not. The mechanism underlying this differential regulation of Myb and Mip120 may be the key to achieving neuron-specific expression of olfactory receptors.

We also demonstrate that expression of *Gr63a* can be modulated in the adult stage by the activity of Myb. The level of E2F2 affects *Gr63a* expression in the opposite direction of Myb activity. Changes in levels of *Gr63a* expression via the MMB/dREAM complex alter the sensitivity to a CO_2 stimulus significantly. This finding could provide a molecular template for modulation of specific chemosensory receptors in the differentiated olfactory system without perturbation of the precise receptor-to-neuron map. Such receptor-specific plasticity could be important for modulating receptor expression and neuronal sensitivity to various changes in the physiological state, such as feeding, mating, and circadian rhythms, or to external stimuli such as changes in environmental conditions.

Materials and methods

qRT–PCR

Total RNA was isolated from 100 antennae or maxillary palps by Trizol extraction (Invitrogen). cDNA was generated using SuperScript III reverse transcriptase (Invitrogen). One microliter of the resulting cDNA, fast SYBR Green PCR master mix (Applied Biosystems), and gene-specific primers were combined in a 20-μL reaction on 96-well plates. Primer sequences are provided (Supplemental Fig. S8). The SYBR Green reaction was then used for qPCR on the StepOnePlus real-time PCR system (Applied Biosystems). Also see the Supplemental Material.

ChIP

Ten fly heads or 100 antennae were used per ChIP sample. Chromatin was sonicated to obtain 300- to 500-base-pair (bp) DNA fragments and incubated with Dynabeads (Invitrogen) and 2 μg of antibodies to H3K9me2 (ab1220) or H3K4me3 (ab8580). Specificity of anti-H3K9me2 antibody was validated using a peptide array (Supplemental Fig. S7A). Immunoprecipitated chromatin was eluted off the beads and purified. One microliter of ChIP DNA was used for each qPCR reaction on the StepOnePlus real-time PCR system (Applied Biosystems). Primer sequences are provided (Supplemental Fig. S8). Also see the Supplemental Material.

Immunofluorescence

Immunostaining was performed on brains as described previously (Lee and Luo 1999) and on maxillary palps and antennae with 3% Triton X-100 permeabilization instead of 0.3%, as stated in the protocol. Primary antibodies were mouse anti-Elav (1:5 dilution; Developmental Studies Hybridoma Bank), mouse anti-nc82 (1:5; Developmental Studies Hybridoma Bank), chicken anti-GFP (1:2000; ab13970), rat anti-CD8 (1:100; Invitrogen), rabbit anti-Myb (1:200) (Manak et al. 2002), rabbit anti-RFP (1:500; gift from S. Heidmann), rabbit anti-Mip120 (1:500), and rabbit anti-E2F2 (1:100). Also see the Supplemental Material.

Single-sensillum electrophysiology

Recordings were obtained as described previously (Turner and Ray 2009).

RNA in situ hybridization

Procedures were performed as described previously (Goldman et al. 2005). Antenna whole-mount RNA in situs were performed using ~900-bp antisense RNA–digoxigenin probes in fixed tissue. Probes were detected using an anti-digoxigenin fragment conjugated to an alkaline phosphatase. Signal was developed using Fast Red and imaged using a Zeiss LSM 560 confocal microscope with a 543-nm laser and 560LP filter.

Behavior

T-maze assays were performed as described previously (Turner and Ray 2009), except that 0.33% CO_2 was used.

Statistics

Statistical analysis was done using unpaired two-tailed Student's *t*-test using R software.

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