Supplemental Figures

Fig.S1

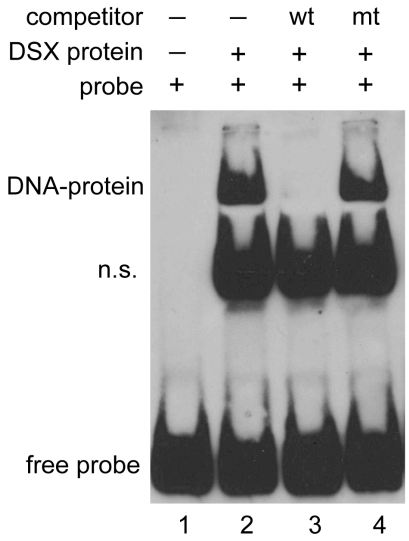

Figure S1. Related to Figure 1. DSX binds to a 13-bp consensus DSX-binding site in the Fmo-2 regulatory sequence in vitro. DSX^F protein expressed from bacteria was used in EMSA using the sequence ACTGCGGACTGCAACATTGTTGCCATCGACAGA from the 5' of Fmo-2 gene as a probe (lane 2). The binding was competed by unlabeled probe (wt, lane 3), but not competed by unlabeled sequence with mutations at the DSX binding site ACTGCGGACTGgggggggGggGCCATCGACAGA (mt, lane 4). Non-specific binding of the probe by some bacterial proteins was indicated (n.s.).

Fig.S2

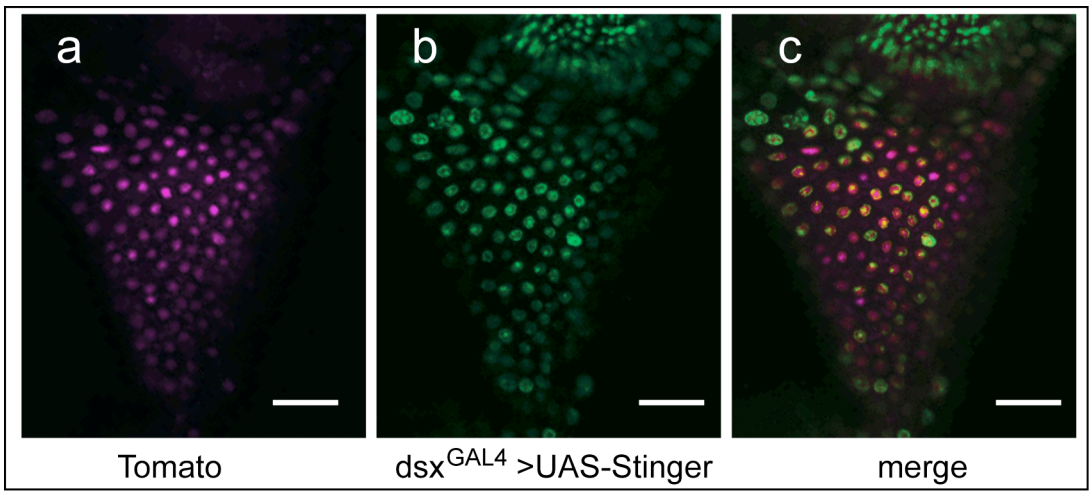

Figure S2. Related to Figure 2. The *Fmo-2* expressing cells at the CVJ also express *dsx*. Images of a single confocal section of the anterior midgut for a female fly carrying the *7k-tdT* reporter (a) and a nuclear GFP reporter (UAS-Stinger) driven by dsx^{GAL4} (b), and their merge (c). Scale bars, 25um.

Fig.S3

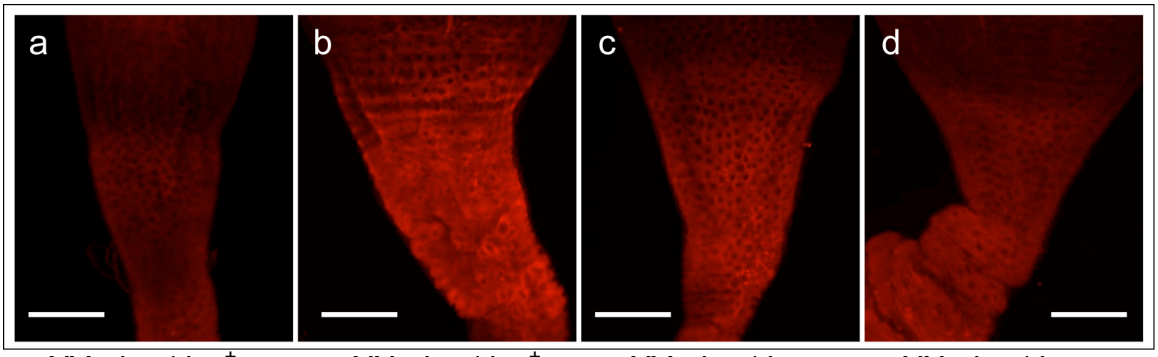

Figure S3. Related to Figure 2. FMO-2 protein level at the CVJ is regulated by *dsx*. The FMO-2 expression was examined by staining with an anti-FMO-2 in dsx^{-}/dsx^{+} male (a) and female (b) flies, as well as in the XY, dsx^{-}/dsx^{-} (c) and XX, dsx^{-}/dsx^{-} (d) flies. All images were taken with the same confocal settings. Scale bars, 50 um. The dsx^{-}/dsx^{+} here is dsx^{1}/dsx^{+} or dsx^{M+R13}/dsx^{+}, and dsx^{-}/dsx^{-} is dsx^{1}/dsx^{M+R13}.

Fig.S4

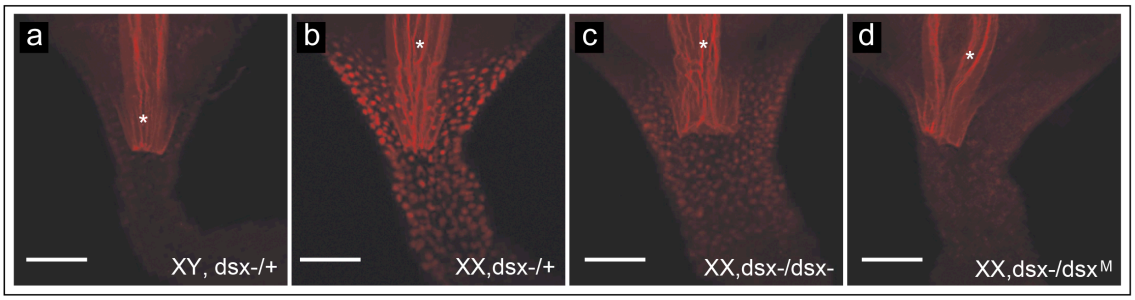

Figure S4. Related to Figure 2. *Fmo-2* transcription is repressed by DSX^{M}. The *7k-tdT* reporter expression was examined by confocal microscopy in dsx^{-}/dsx^{+} male (A) and female (B) flies, as well as in the *XX*, dsx^{-}/dsx^{-} (C) and *XX*, dsx^{-}/dsx^{M} (D) flies. Scale bars, 50 um. The dsx^{-}/dsx^{-} genotype is $dsx1/dsx^{1649\text{-}9625}$. The dsx^{-} allele is dsx^{1} or $dsx^{1649\text{-}9625}$ in other combinations. Autofluorescence is indicated by the asterisk.

Fig.S5

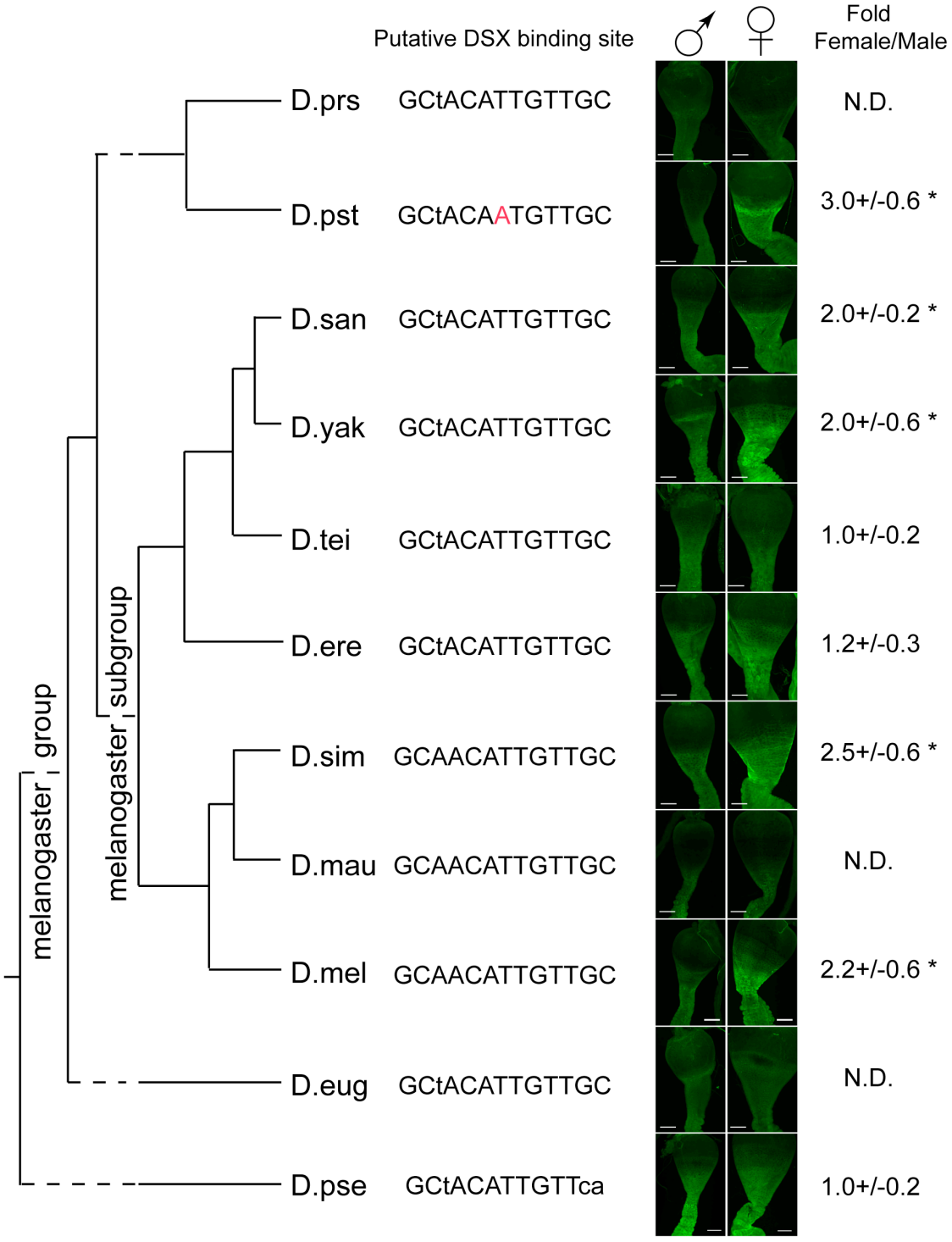

Figure S5. Related to Figure 4. The divergence of *Fmo-2* expression pattern at the midgut in closely related Drosophila species.
Left panel, the phylogenetic tree of the species whose *Fmo-2* expression was surveyed (drawing not to scale with respect to time). D.prs for *D. prostipennis,*

D.pst for *D.pseudotakahashii*, D.san for *D.santomea*, D.yak for *D.yakuba*, D.tei for *D.teissieri*, D.ere for *D.erecta*, D.sim for *D.simulans*, D.mau for *D.mauritiana*, D.mel for *D.melanogaster*, D.eug for *D.eugracilis* and D.pse for *D. pseudoobscura.*
Center panel, the putative DSX-binding site in the *Fmo-2* gene of each species is listed. Mismatched base pairs to the 13-bp optimal DSX bindings site are in lower case. Note that the center base for the *D.pst* sequence is an "A". For those species lacking a sequenced genome, the sequence of the *Fmo-2* gene 5' region was determined by sequencing the PCR products of this region.
Right panel, anti-FMO-2 staining (green) in males and females of each species. Males and females were imaged with the same confocal settings for each species (n=5~8 for each sample). Scale bars, 50 um. The immuno-fluorescence intensity for each sample was quantified using ImageJ, and the fold of the mean intensities for females vs males (+/- standard deviation) was calculated for each species. The asterisk indicates that the difference between the male and the female is significant for the species ($p<0.05$ by two-sided Student's t-test).

Fig.S6

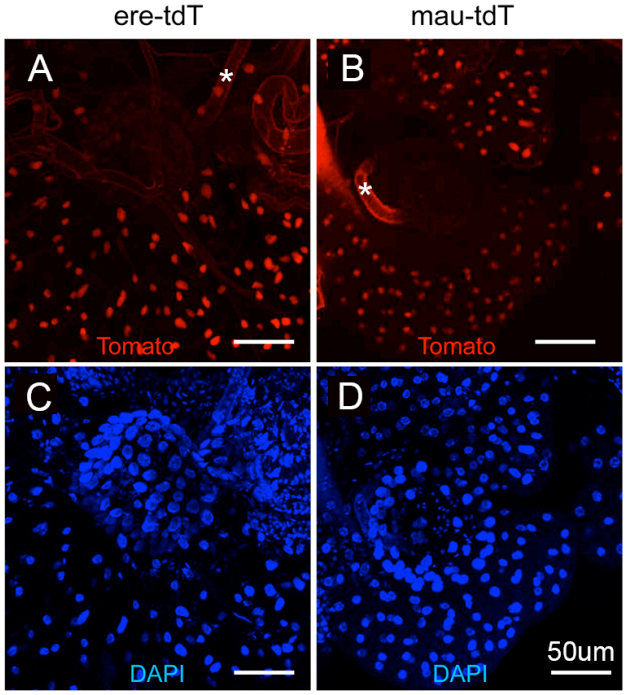

Figure S6. Related to Figure 4. The *Fmo-2* regulatory sequences from species of the melanogaster group drive conserved transcriptional activation in the fat body cells of the spermatheca. The expression of two reporters *ere-tdT* (A) and *mau-tdT* (B) in the spermatheca were examined in *D.melanogaster* transgenic flies. Autofluorescence (not from the Tomato reporter) is indicated by the asterisk. The DAPI staining showed the nuclei of these samples (C, D), respectively.

Fig.S7

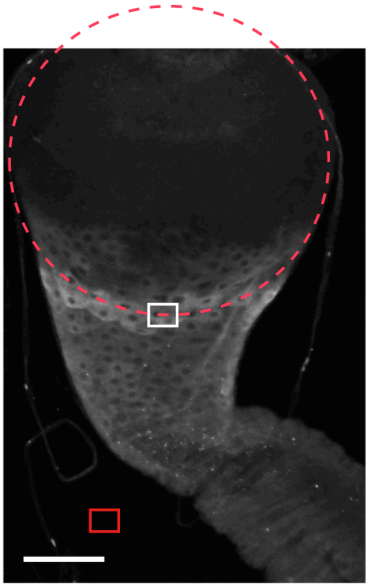

Figure S7. Related to Experimental Procedures. The schematic drawing of how to quantify the intensity of anti-FMO-2 immunofluorescence in the midgut samples. The full stack (with the maximal intensity option) of confocal images for each sample was converted to 8-bit grey scale format. A circle was fit to the outline of the cardia for the sample. An area (~400 square pixels) at the bottom of the circle (white box) was taken for the average intensity. The average intensity for an identical area outside of the sample (red box) served as background. The difference of average intensity between the two boxed-areas was registered as the anti-FMO-2 intensity at the CVJ for the sample.

Supplemental Experimental Procedure

Plasmids construction

We first constructed a nuclear Tomato-nls [1] reporter plasmid backbone (named *pMUHtdT*). For this aim, the coding sequence for Tomato-nls was cloned into *pJFRC-MUH* [2] between BglII and XhoI sites, resulting *pMUHtdT*.

To construct the *Fmo-2* reporter *7k-tdT*, a 7kb genomic region 5' of the *Fmo-2* gene (from -6994 to -61, in respective to the translation initiation site of

the Fmo-2 gene) was amplified from the genomic DNA of the wild type Cantonese A flies by PCR using *pfu* polymerase (Thermo Scientific) and two primers (see table S1). The PCR product was cloned into the *pCR-bluntII-TOPO* vector (Invitrogen) and sequenced. The 7kb *Fmo-2* sequence (excised by NotI and BamHI) and the Tomato-nls coding region together with SV40 terminator (excised from pMUHTdt by BglII and EcoRI) were cloned together into pBDP (the backbone for pJFRC-MUH [3]) between the sites NotI and EcoRI, resulted in *7k-tdT*.

To construct *2k-tdT* (Fig 4, construct #1), the 2kb fragment at the proximal end (from -2061 to -61, relative to the translation initiation site of the *Fmo-2* gene) of the 7kb Fmo-2 genomic sequence in the pCR-bluntII-TOPO vector was excised out by HindIII and BamHI and cloned into pMUHtdT between the HindIII and BglII sites.

To construct *2k(dsx_site_mut)-tdT*, site-directed-mutagenesis PCR was performed on the 2kb Fmo fragment cloned into pBluescript II between the HindIII and BamHI sites. The BglII/XhoI fragment containing the mutated DSX binding site from the mutated plasmid was used to replace the original BglII/XhoI fragment in *2k-tdT* to make *2k(dsx_site_mut)-tdT*. The plasmid was sequenced to confirm free of undesired mutations.

To create tdTomato-nls reporters driven by *Fmo-2* genomic sequences from different species, the genomic sequences were amplified by PCR using *pfu* polymerase (Thermo Scientific), genomic DNA from each species and primers specific for the respective species (For details of the primers, see table S1). The *Fmo-2* sequences were then cloned into the *pMUHTdt* plasmid between the HindIII and BglII sites. The plasmids were sequenced to confirm free of mutations.

To make the reporter constructs with sequences swapped between *D.melanogaster* and *D.erecta* (Fig.4, #3 to #7). The sequences were swapped using the restriction enzymes indicated in figure 4 (some of them by partial digestion).

To create construct #8, the proximal part of the *D.ere Fmo-2* sequence was amplified by PCR (Table S1 for detail of primers) and cloned into *pMUHtdT* between the BglII site and the HindIII site.

To construct *1k-tdT* (Fig.4, construct #9), the *7k-tdT* plasmid was digested with SphI, followed by partial digestion of NotI. The desired DNA fragment was purified, blunt-ended with Klenow, and ligated back to form the *1k-tdT* plasmid.

To make construct #10 (Fig.4), *1k-tdT* was digested with BbvCI, followed by partial digestion of SmaI. The desired DNA band was purified and treated with Klenow to blunt the ends, and ligated back to form construct #10.

To create the *CRM-d(m->e)* reporter, the *D.ere* sequence from -1004 to -772 was amplified by PCR (Table S1 for detail of primers) and used to replaced the orthologous sequence in the *2k-tdT* reporter between SphI site and AseI site.

To create the *dsxsite(m->e)* reporter, the DSX-binding site and the immediately flanking sequence were mutated to the *D.ere* sequence PCR based mutagenesis (Table S1 for detail of primers). The sequence between SphI and BbvCI in the *2k-tdT* was then replaced with the mutated sequence.

To create the reporters *1k(d1)-tdT*, *1k(d2)-tdT* and *1k(d3)-tdT*, the respective *Fmo-2* fragments were amplified from *2k-tdT* by PCR (Table S1 for detail of primers). The PCR products were digested with HindIII/BamHI, and cloned into *pMUHtdT* between the HindIII and BglII sites.

To create *delta-mel-ere-tdT*, the shortened chimeric *Fmo-2* regulatory sequence was amplified by PCR from *mel-ere-tdT* (Table S1 for detail of primers) and cloned into *pMUHtdT* between the HindIII and BglII sites.

All reporter constructs were sequenced to confirm free of undesired mutations, and integrated at the attP40 site as this site was shown to have very little positional effect on transgene expression [2].

Table S1. List of primers used for the reporter constructs.

Construct	Primers (forward + reverse)
7k-tdT	5'GTCGCGGCCGCGCTCTGATGTACTGACCACACA3' 5'ATGGATCCTCGCCTGACCAACGACTAACTGA3'

2k(dsx_site_mut)-tdT	5’ACTGCGGACTGGGGGGGGGGGGCCATCGACAGAGTTTTTGTAAT3’ 5’CTCTGTCGATGGCCCCCCCCCCCCAGTCCGCAGTGAAGCCTACGGA3’
ere-tdT	5’ATTTAGAACCAAACAAGCTTCCGCCTA3’ 5’CGAGATCTTGGGATTTCCGATTGGTAACGAAT3’
mau-tdT	5’ATGTAAGCTTTTTTAAATGAATCTTACCGA3’ 5’ATGGATCCTCGCCTGACCAACGACTAACTGA3’
pse-tdT	5’ATAAGCTTTAGACTGCAGAATCTGAATTGTTTCGT3’ 5’TTGGATCCTTTTGACGAGCAGTGTTTCTCTCTA3’
pst-tdT	5’CCAAGCTTGAACCATCAGCACTTTCTAATTGA3’ 5’CCAGATCTCATTTTGGGATATTCCTAAACGGT3’
sim-tdT	5’CTTATGTAAGCTTTTTTAAATGAATCTTACCGATCT3’ 5’ACTGGATCCGCGCCTGACCAACGACTAACTGA3’
Construct#8	5’TCAAGCTTCGGCAGAGTTGTTGTAATTGAATGA3’ 5’CGAGATCTTGGGATTTCCGATTGGTAACGAAT3’
CRM-d(m->e)	5’GGCTGTCACAGCATGCTTATGTTCAATTGTATTCCGGA3’ 5’ATGGATCCTCGCCTGACCAACGACTAACTGA3’
dsxsite(m->e)	5’ACTGCGGGCGCAACATTGTTGCGCTCGACAGAGTTTTTGT3’ 5’CTCTGTCGAGCGCAACAATGTTGCGCCCGCAGTGAAGCCTACGGA3’
delta-mel-ere-tdT	5’TCAAGCTTCCACCCCCAGCAATGACTTTTCCGTA3’ 5’CGAGATCTTGGGATTTCCGATTGGTAACGAAT3’
1k(d1)-tdT	5’TCAAGCTTCCCCACACCCGTTTCTAAGA3’ 5’ATGGATCCTCGCCTGACCAACGACTAACTGA3’
1k(d2)-tdT	5’TCAAGCTTCCACCCCCAGCAATGACTTTTCCGTA3’ 5’ATGGATCCTCGCCTGACCAACGACTAACTGA3’
1k(d3)-tdT	5’TCAAGCTTCACTGCGGACTGCAACATTGT3’ 5’ATGGATCCTCGCCTGACCAACGACTAACTGA3’

Table S2. Other Drosophila species

Species	**UCSD stock center number**
D. simulans	Florida city
D. mauritiana	D. maur^{01}
D. yakuba	14021-0261.01
D. santomea	STO.4
D. teissieri	14021-0257.00
D. erecta	14021-0224.01
D. prostipennis	14022-0291.00
D. pseudotakahashii	14022-0301.01
D. eugracilis	14026-0451.05
D. pseudoobscura	14011-0121.87

Fly stocks for the above species were obtained from the University of California, San Diego (UCSD) stock center and raised at RT (22ºC) on regular fly food [4].

Whole mount antibody staining

The midgut samples were dissected in PBS from age-matched male and female flies (~5 days old, unless otherwise specified) and fixed in 4% paraformaldehyde for 40 min at RT. The samples were washed in PBST (PBS with 0.3% Triton-X100) for 3 times at RT, 10 min each. After incubating in blocking buffer (PBS with PBS with 0.3% Triton-X100, 5% normal goat serum, 5% BSA), the samples were incubated with anti-FMO-2 antibody [5] diluted 1:300 in blocking buffer at 4ºC over night. The samples were washed in PBST for 3 times at 4ºC, 1 hour each, followed by incubation with Alexa Fluor 488 (or 568) goat anti-rabbit IgG (Invitrogen) diluted 1:1000 in blocking buffer at 4ºC over night. The samples were washed in PBST for 3 times at 4ºC, 1 hour each time, before they were mounted onto slides in VECTASHIELD mounting medium with DAPI (Vector Laboratories, Burlingame, CA). To reduce the variation of staining, samples from both male and female flies were stained in the same tube and mounted onto the same slide. To distinguish the two sexes, the crops were left attached to the male samples and removed from the female samples (or *vise versa* in another replicate). We found that this practice did not affect the staining

results. The samples on the same slide were subjected to confocal imaging (Zeiss) with the same settings.

Conditions for semi-quantitative RT-PCR

PCR reactions: 1cycle of 94C for 3 min; 3 cycles of 94C for 30s, 60C for 30s and 68C for 1min; 22 cycles of 94C for 30s, 60C for 30s and 72C for 30s; 1 cycle of 72C 1min. The PCR products were analyzed on a 1.5% agarose gel. Primers used were
5'- CCCCGACGATATCACCGAGTCTTTCA-3' and
5'- CACGCGCGTCTTTAAACTTATCTGCCT-3' (for Fmo-2);
5'-GCCACCCTGATACAAAGAAGCCTT-3' and
5'- TCGGCGGTTATAGAACTTAAGT-3' (for L10 control).

Examination of the reporter expression

The midgut or spermatheca (only in females) samples were dissected in PBS from age-matched male or female flies carrying the tdTomato reporter transgenes (~5 days old, unless otherwise specified) and fixed in 4% paraformaldehyde for 20 min at RT. The samples were washed in PBST (PBS with 0.1% Triton-X100) for 3 times at RT, 10 min each, before they were mounted onto slides in VECTASHIELD mounting medium with DAPI. The samples were then subjected to confocal imaging (Zeiss, with the same settings if samples were to be compared for expression level).

Identification of orthologous Fmo-2 regulatory sequences in other species

For those species whose genome has been sequenced, the 5' upstream sequences of the *Fmo-2* gene were downloaded from Flybase (www.flybase.org). For those species whose genome has not been sequenced, the 5' upstream sequences were obtained by sequencing the inverted PCR

products using primer resided in the first exon of the *Fmo-2* gene. These sequences were aligned with the 2kb sequence immediately 5' of the *Fmo-2* gene from *D.mel* using the NCBI alignment tool (Blastn, align two sequences). The similar sequences with an e-value of 0.1 or smaller were considered as homologous. The whole region that contains multiple such homologous sequences was taken as orthologous region of the *D.mel Fmo-2* regulatory region.

Supplemental References

1. Mellert DJ, Knapp JM, Manoli DS, Meissner GW, Baker BS (2010). Midline crossing by gustatory receptor neuron axons is regulated by fruitless, doublesex and the Roundabout receptors. Development. 137(2): 323-32.
2. Pfeiffer BD, Ngo TT, Hibbard KL, Murphy C, Jenett A, Truman JW, Rubin GM (2010). Refinement of tools for targeted gene expression in Drosophila. Genetics. 186 (2): 735-55.
3. Pfeiffer BD, Jenett A, Hammonds AS, Ngo TT, Misra S, Murphy C, Scully A, Carlson JW, Wan KH, Laverty TR, Mungall C, Svirskas R, Kadonaga JT, Doe CQ, Eisen MB, Celniker SE, Rubin GM (2008). Tools for neuroanatomy and neurogenetics in Drosophila. Proc Natl Acad Sci U S A.105(28): 9715-20.
4. Wirtz RA and Semey HG (1982). The Drosophila kitchen - equipment, media preparation, and supplies. Dros. Inf. Serv. 58: 176-180.
5. Scharf ME, Scharf DW, Bennett GW, Pittendrigh BR (2004). Catalytic activity and expression of two flavin-containing monooxygenases from Drosophila melanogaster. Arch Insect Biochem Physiol. 57(1): 28-39.