Supporting Information for

Male cuticular pheromones stimulate removal of the mating plug and promote re-mating through pC1 neurons in *Drosophila* females

Minsik Yun[1], Do-Hyoung Kim[1], Tal Soo Ha[2], Kang-Min Lee[1], Eungyu Park[1], Hany K.M. Dweck[3,5], Markus Knaden[3,4], Bill S. Hansson[3,4], Young-Joon Kim[1]*

[1]School of Life Sciences, Gwangju Institute of Science and Technology (GIST), Cheomdangwagi-ro 123, Buk-gu, Gwangju, 61005, Republic of Korea.
[2]Department of Biomedical Science, College of Natural Science, Daegu University, Gyeongsan 38453, Gyeongsangbuk-do, Korea.
[3]Department of Evolutionary Neuroethology, Max Planck Institute for Chemical Ecology, Hans-Knöll-Str. 8, 07745 Jena, Germany.
[4]Next Generation Insect Chemical Ecology, Max Planck Centre, Max Planck Institute for Chemical Ecology, Hans-Knöll-Straße 8, D-07745, Jena, Germany.
[5]Department of Entomology, The Connecticut Agricultural Experiment Station, New Haven, CT 06511, USA.

* Corresponding author: Young-Joon Kim

Email: kimyj@gist.ac.kr

This PDF file includes:

Figures S1 to S10
Table S1

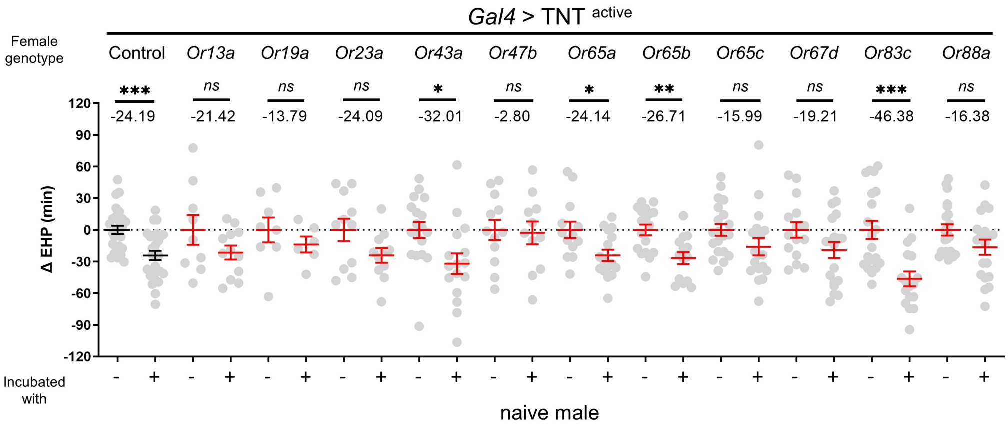


Fig. S1. Identification of ORNs in trichoid and intermediate sensilla required for MIES

Δ EHP of females of the indicated genotypes, incubated with or without naive males. Female genotypes are as follows from left to right: *+>TNT*^{active}, *Or13a>TNT*^{active}, *Or19a>TNT*^{active}, *Or23a>TNT*^{active}, *Or43a>TNT*^{active}, *Or47b>TNT*^{active}, *Or65a>TNT*^{active}, *Or65b>TNT*^{active}, *Or65c>TNT*^{active}, *Or67d >TNT*^{active}, *Or83c >TNT*^{active}, *Or88a>TNT*^{active}.

Mann-Whitney Test (n.s. $p > 0.05$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). Gray circles indicate the ΔEHP of individual females, and the mean ± SEM of data is presented. Normalized EHP (ΔEHP) is calculated by subtracting the mean of reference EHP of females kept alone after mating (left most column) from the EHP of females incubated with naive males. Numbers below the horizontal bar represent the mean of EHP differences between treatments.

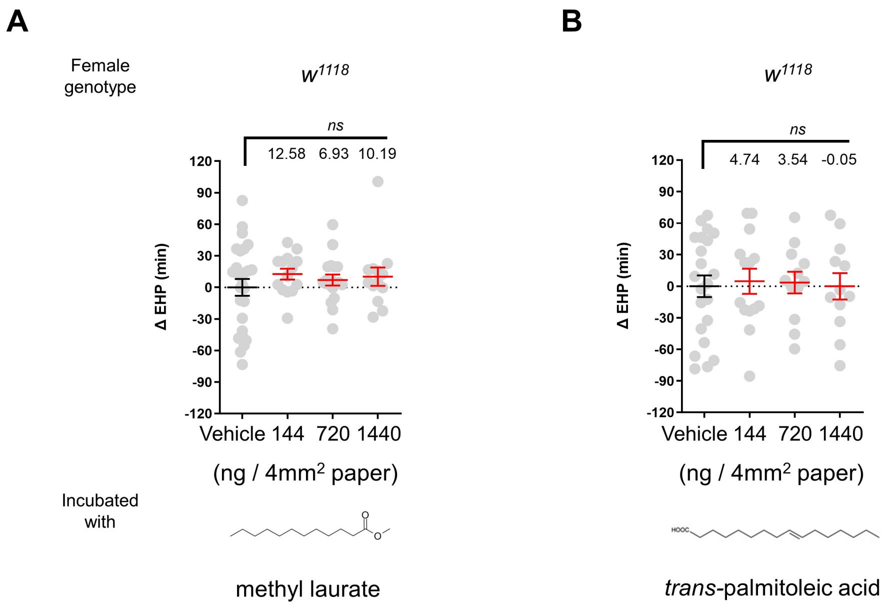


Fig. S2. Absence of EHP shortening when females are incubated with known odor ligands for *Or47b*

ΔEHP of w^{1118} females incubated with a piece of filter paper perfumed with solvent vehicle or with the indicated amounts of two known *Or47b* odorant ligands, methyl laurate (A) and trans-palmitoleic acid (B). Mann-Whitney Test (n.s. $p > 0.05$). Gray circles indicate the ΔEHP of individual females, and the mean ± SEM of data is presented. Numbers below the horizontal bar represent the mean of EHP differences between vehicle and odorant treatments.

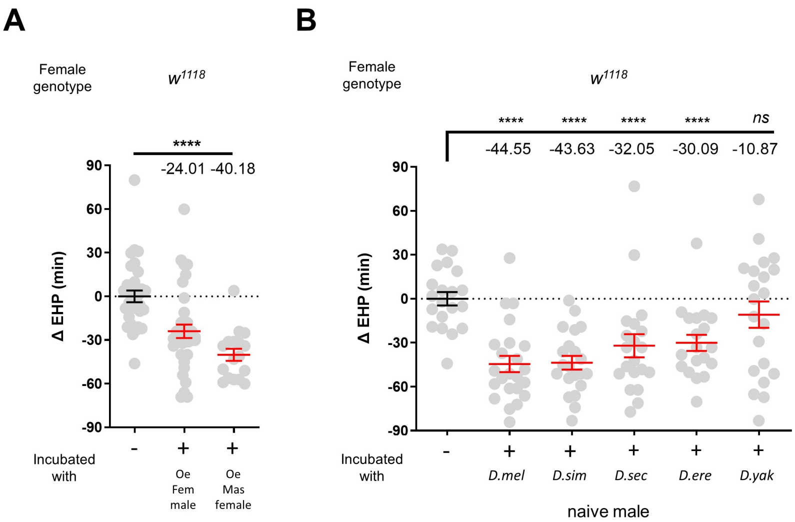
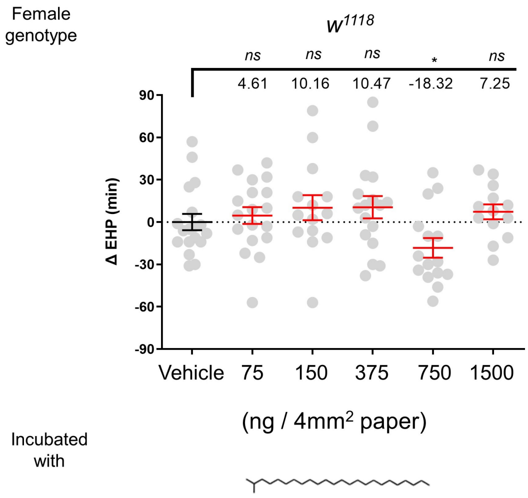


Fig. S3. EHP shortening by males with feminized oenocytes, females with masculinized oenocytes, and males of other closely related *Drosophila* species

A, ΔEHP of w^{1118} females incubated with males with feminized oenocytes (Oe Fem male; *PromE(800)-Gal4/UAS-Tra*) or virgin females with masculinized oenocytes (Oe Mas Female; *PromE(800)-Gal4/UAS-Tra-RNAi*).

B, Δ EHP of w^{1118} females incubated with naive males of the indicated *Drosophila* species. *D. mel* (*D. melanogaster*), *D. sim* (*D. simulans*), *D. sec* (*D. sechellia*), *D. ere* (*D. erecta*), *D. yak* (*D. yakuba*).

A, One-way ANOVA test (****$p < 0.0001$), B, Mann-Whitney Test (n.s. $p > 0.05$; ****$p < 0.0001$). Gray circles indicate the ΔEHP of individual females, and the mean ± SEM of data is presented. Numbers below the horizontal bar represent the mean of EHP differences between treatments.



2-methyltetracosane, 2MC

Fig. S4. 2MC shortens EHP at a specific concentration

ΔEHP of mated *w*[1118] females incubated with a piece of filter paper perfumed with solvent vehicle or the indicated amounts of 2MC.

Mann-Whitney Test (n.s. $p > 0.05$; $^*p < 0.05$). Gray circles indicate the ΔEHP of individual females, and the mean ± SEM of data is presented. Numbers below the horizontal bar represent the mean of EHP differences between vehicle and odorant treatments.

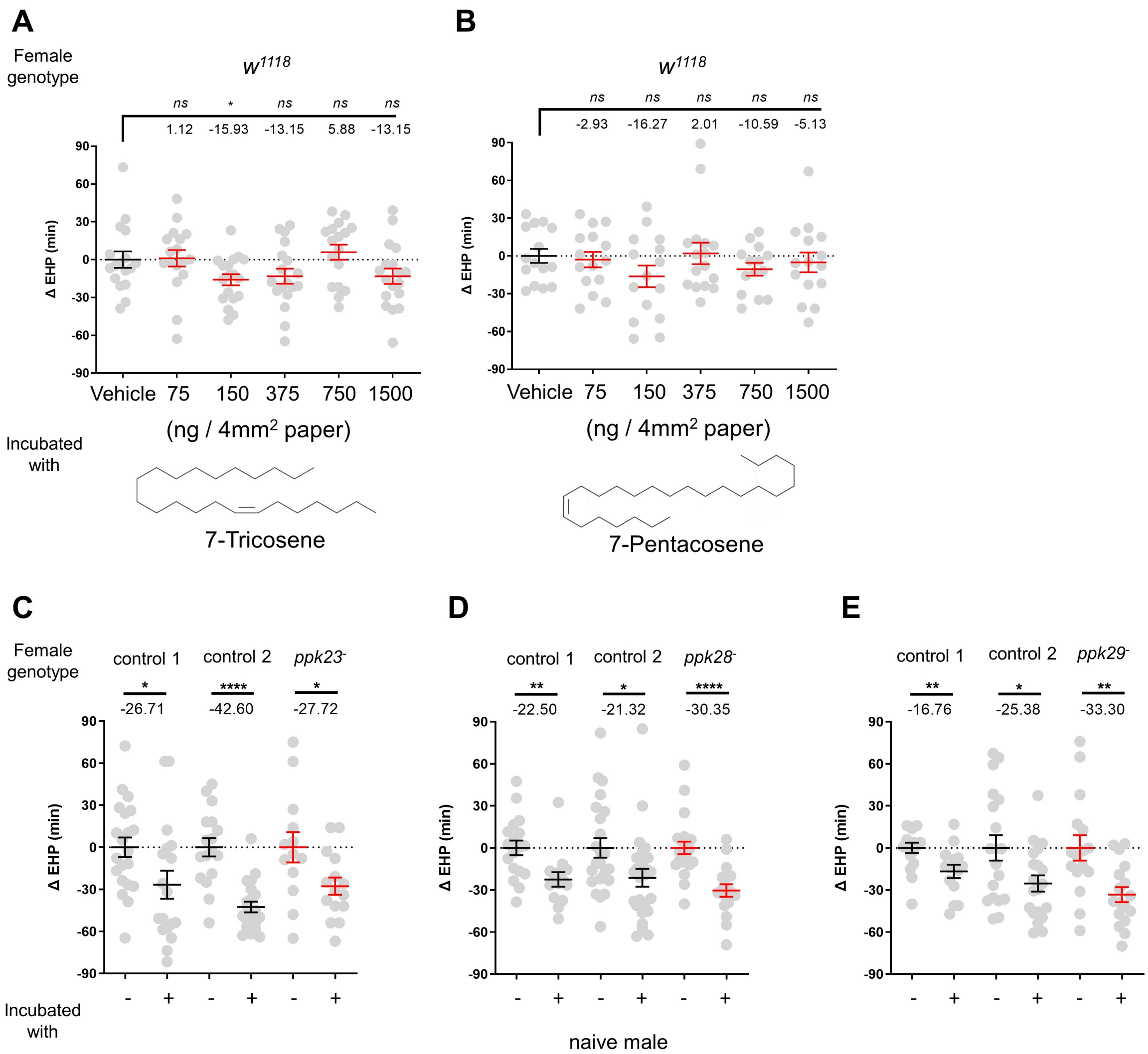


Fig. S5. 7-T induces EHP shortening at physiological concentrations, but DEG/ENaC channels expressed in *ppk23* neurons are not required for MIES

A-B, ΔEHP of mated w^{1118} females incubated with a piece of filter paper perfumed with solvent vehicle or the indicated amounts of 7-T (A), or 7-Pentacosene (B). Incubation with specific concentrations of 7-T significantly shorten EHP, but 7-Pentacosene does not.

C-E, ΔEHP of mated females of the indicated genotypes, incubated with or without naive males. Female genotypes are as follows from left to right:
(D) control 1 (w^{1118}), control 2 (*ppk23*$^-$/+), and *ppk23*$^-$ (*ppk23*$^-$/*ppk23*$^-$); (E) control 1 (w^{1118}), control 2 (*ppk28*$^-$/+), and *ppk28*$^-$ (*ppk28*$^-$/*ppk28*$^-$); (F) control 1 (w^{1118}), control 2 (*ppk29*$^-$/+), and *ppk29*$^-$ (*ppk29*$^-$ /*ppk29*$^-$).

A-B, Unpaired *t*-Test (n.s. $p > 0.05$; * $p < 0.05$), C-E, Mann-Whitney Test (n.s. $p > 0.05$; * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$). Gray circles indicate the ΔEHP of individual females, and the mean ± SEM of data is presented. Numbers below the horizontal bar represent the mean of EHP differences between treatments.

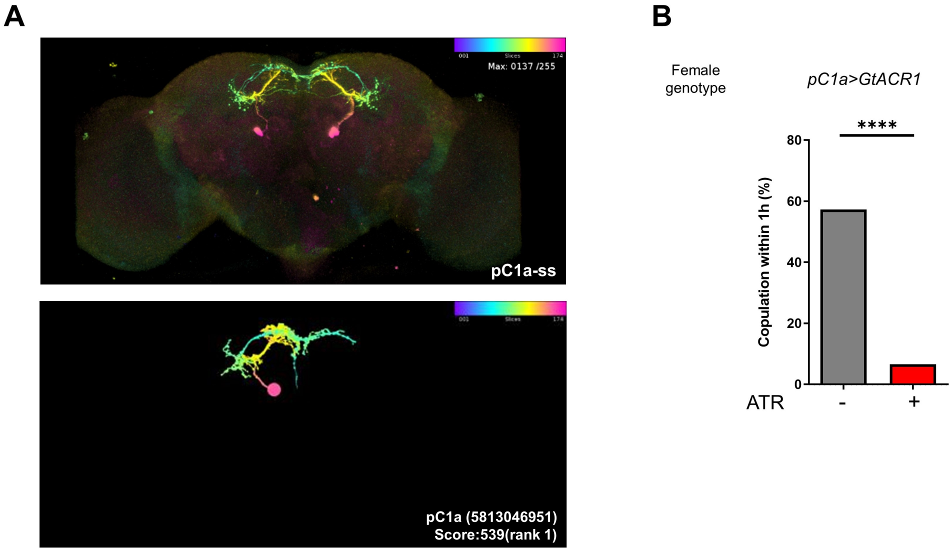


Fig. S6. Characterization of *pC1a-split-Gal4*

A, An anatomical comparison between *pC1a-split-GAL4* neurons (above; pC1a-ss) and a pC1a neuron (below; neuprint body ID, 5813046951). The panel above shows the maximum intensity projection image (MIP) of an aligned confocal image of the brain from a female carrying *pC1a-split-GAL4* and *UAS-myrEGFP* stained with anti-EGFP and anti-nc82.

B, Mating frequencies of *pC1a>GtACR1* (*pC1a-split-Gal4/UAS-GtACR1*) females during optogenetic silencing, scored as the percentage of females that copulate within 1 h. Females were cultured on food with or without all-*trans*-retinal (ATR) prior to the mating assay. Chi-square test (**** $p < 0.0001$).

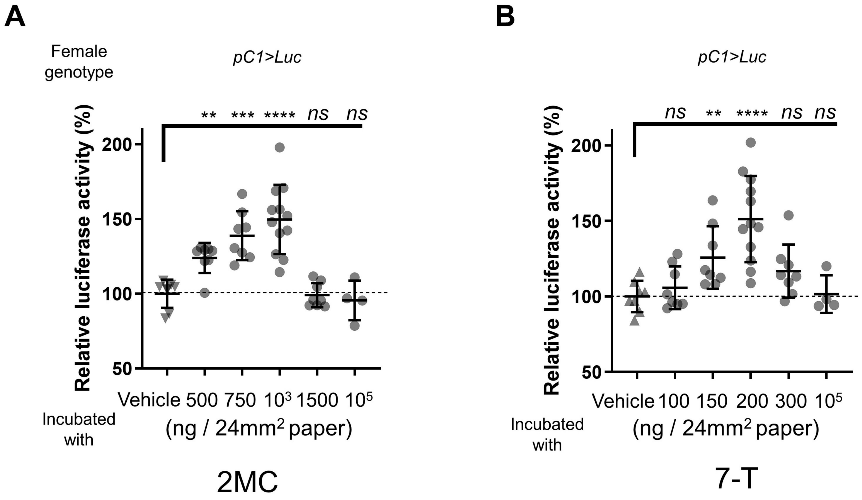


Fig. S7. 2MC and 7-T induces cAMP activity in pC1 neurons within a narrow concentration range
The relative *CRE-Luciferase* reporter activity of pC1 neurons in females incubated with a piece of filter paper perfumed with the indicated amounts of odorants, 2MC (A) and 7-T (B). To calculate the relative luciferase activity, we set the average luminescence unit values of female incubated with the vehicle to 100%. Gray circles indicate the relative luciferase activity (%) of individual females, and the mean ± SEM of data is presented. Mann-Whitney Test (n.s. $p > 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$).

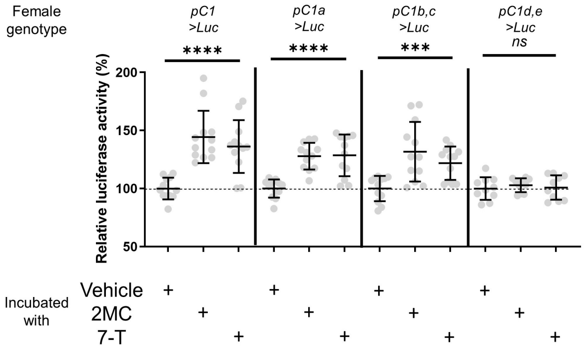


Fig. S8. Incubation with 2MC or 7-T increases cAMP activity in pC1a as well as pC1b and c subtypes in virgin females

The relative *CRE-Luciferase* reporter activity of pC1 neurons in virgin females of the indicated genotypes, incubated with a piece of filter paper perfumed with the indicated odorants. To calculate the relative luciferase activity, we set the average luminescence unit values of female incubated with the vehicle to 100%. One-way ANOVA test (n.s. $p > 0.05$; *** $p < 0.001$; **** $p < 0.0001$). Gray circles indicate the relative luciferase activity (%) of individual females, and the mean ± SEM of data is presented.

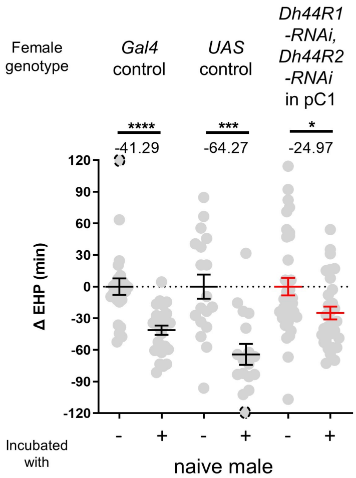


Fig. S9. Knockdown of Dh44R1 and Dh44R2 in pC1 neurons has limited impacts on MIES
ΔEHP of females of the indicated genotypes, incubated with or without naive males immediately after mating. Female genotypes are as follows from left to right: *Gal4* control (*UAS-Dcr2/+;GMR71G01-Gal4/+*), *UAS* control (*UAS-Dh44R1-RNAi/+; UAS-Dh44R2-RNAi/+*), *Dh44R1-RNAi, Dh44R2-RNAi* in pC1 (*UAS-Dcr2/+;GMR71G01-Gal4/Dh44R1-RNAi; Dh44R2-RNAi/+*). Mann-Whitney Test (*p <0.05; ***p < 0.001; ****p < 0.0001). Gray circles indicate the ΔEHP of individual females, and the mean ± SEM of data is presented. Gray circles with dashed borders indicate ΔEHP values beyond the axis limits (>120 or <-120 min). Numbers below the horizontal bar represent the mean of EHP differences between treatments.

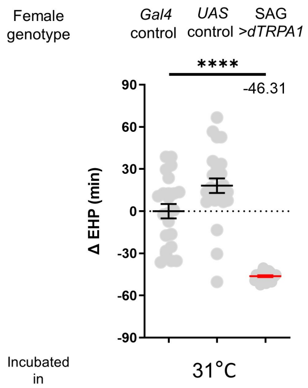


Fig. S10. Induced activation of SAG neurons shortens EHP in the absence of males

ΔEHP of females of the indicated genotypes, incubated at 31°C immediately after mating. Female genotypes are as follows from left to right: *Gal4* control (*SAG-Gal4/+), UAS* control (*UAS-dTRPA1/+*), SAG>*dTRPA1* (*SAG-Gal4/UAS-dTRPA1*). One-way ANOVA test (n.s. $p > 0.05$; ****$p < 0.0001$). Gray circles indicate the ΔEHP of individual females, and the mean ± SEM of data is presented. A number below the horizontal bar represents the mean of EHP difference between *Gal4* control and *SAG>dTRPA1*.

Figure	Genotype of			N number
	Ejection female	Mating partner	Incubation partner	
Fig.1.				
Fig.1B	w[1118]	Canton-S	Canton-S	59, 69
Fig.1C	w[1118]	Canton-S	Canton-S	18, 15
Fig.1D	w[1118]	Canton-S	Canton-S	12, 12
Fig.1E	w[1118]	Canton-S	Canton-S	20, 18, 23
Fig.1F	w[1118]; TI{w[+mW.hs]=TI}Orco[1]	Canton-S	Canton-S	55, 47
Fig.2.				
Fig.2A	w[1118];Or47b-Gal4/UAS-TNTinactive(P{UAS-TeTxLC.(-)Q}A2)	Canton-S	Canton-S	14, 14
	w[1118];Or47b-Gal4/UAS-TNTactive (P{w[+mC]=UAS-TeTxLC.tnt}E2)	Canton-S	Canton-S	16, 18
Fig.2B	w[1118];Or47b-Gal4/+	Canton-S	Canton-S	28, 31
	w[1118];;UAS-dTRPA1/+	Canton-S	Canton-S	21, 16
	w[1118];Or47b/+;UAS-dTRPA1/+	Canton-S	Canton-S	11, 15
Fig.2C	w[1118]; TI{w[+mW.hs]=TI}Orco[1]	Canton-S	Canton-S	12, 12
	w[1118]; Or47b-Gal4>UAS-EGFP-Orco; Orco[1]/Orco[1]	Canton-S	Canton-S	13, 14
Fig.2D	w[1118]; Or47b[2]/+	Canton-S	Canton-S	12, 15
	w[1118]; Or47b[3]/+	Canton-S	Canton-S	13, 14
	w[1118]; Or47b[2]/Or47b[3]	Canton-S	Canton-S	13, 12
Fig.2E	w[1118]; Or47b[2]/Or47b[2]	Canton-S	Canton-S	14, 15
	w[1118]; Or47b-Gal4>P{w[+mC]=UAS-Or47b.MYC}2; Or47b[2]/Or47b[2]	Canton-S	Canton-S	11, 11
Fig.3.				
Fig.3A	w[1118]	Canton-S		13, 16
Fig.3B	w[1118]; TI{w[+mW.hs]=TI}Orco[1]	Canton-S		11, 12
Fig.3C	w[1118]; TI{w[+mW.hs]=TI}Or47b[2]	Canton-S		14, 17
Fig.3D	w[1118];Or47b-Gal4/+;Orco[1]/Orco[1]	Canton-S		22, 22
	w[1118];UAS-Orco/+; Orco[1]/Orco[1]	Canton-S		15, 14
	w[1118];Or47b-Gal4/UAS-Orco;Orco[1]/Orco[1]	Canton-S		18, 19
Fig.4.				
Fig.4A	w[1118]	Canton-S	Canton-S	18, 22
Fig.4B	w[1118]	Canton-S		17, 18
Fig.4C	w[1118]	Canton-S		16, 17

Fig.4D	*w[1118];ppk23-Gal4/UAS-TNTinactive(P{UAS-TeTxLC.(-)Q}A2)*	*Canton-S*	*Canton-S*	18, 13
	w[1118];ppk23-Gal4/UAS-TNTactive (P{w[+mC]=UAS-TeTxLC.tnt}E2)	*Canton-S*	*Canton-S*	17, 17

Fig.5.				
Fig.5A	*w[1118];pC1(R71G01)-AD/+;Dsx-DBD/UAS-GtACR1*	*Canton-S*		22, 21
Fig.5B	*w[1118];VT25602-AD/+;UAS-GtACR1/VT2064-DBD*	*Canton-S*		18, 18
Fig.5C	*w[1118];R52G04-AD/+;UAS-GtACR1/Dsx-DBD*	*Canton-S*		15, 14
Fig.5D	*w[1118];;Dh44-pC1 (Dsx-DBD, Dh44A-AD)-GAL4/UAS-GtACR1*	*Canton-S*		17, 20
Fig.5E	*w[1118];UAS-FLP/+; GMR71G01-Gal4, CRE-F-Luc/+*	*Canton-S*		12, 12, 12
	w[1118];R52G04-AD/+;UAS-FLP, CRE-F-Luc/Dsx-DBD	*Canton-S*		12, 12, 12
	w[1118];;UAS-FLP, CRE-F-Luc/Dh44-pC1 (Dsx-DBD, Dh44A-AD)-GAL4	*Canton-S*		16, 16, 16
	w[1118];VT25602-AD/+;UAS-FLP, CRE-F-Luc/VT2064-DBD	*Canton-S*		12, 12, 12
Fig.5F	*w[1118]; R52G04-AD/+;UAS-PhotoAC/Dsx-DBD*	*Canton-S*		18, 22
	w[1118];;UAS-PhotoAC/Dh44-pC1 (Dsx-DBD, Dh44A-AD)-GAL4	*Canton-S*		22, 28
	w[1118]; VT25602-AD/+;UAS-PhotoAC/VT2064-DBD	*Canton-S*		21, 20
Fig.5G	*w[1118];UAS-GCaMP6m/+; pC1(GMR71G01)-GAL4/UAS-PhotoAC*	*Canton-S*		9, 9, 9
Fig.5H	*w[1118];;+/UAS-PhotoAC*	*Canton-S* (1st, 2nd)		60
	w[1118];;Dh44-pC1 (Dsx-DBD, Dh44A-AD)-GAL4/UAS-PhotoAC	*Canton-S* (1st, 2nd)		18

Fig. S1.	*w[1118];+/P{w[+mC]=UAS-TeTxLC.tnt}E2*	*Canton-S*	*Canton-S*	27, 27
	w[1118];Or13a-Gal4/P{w[+mC]=UAS-TeTxLC.tnt}E2	*Canton-S*	*Canton-S*	9, 12
	w[1118];+/P{w[+mC]=UAS-TeTxLC.tnt}E2;+/Or19a-Gal4	*Canton-S*	*Canton-S*	8, 6
	w[1118];+/P{w[+mC]=UAS-TeTxLC.tnt}E2;+/Or23a-Gal4	*Canton-S*	*Canton-S*	11, 11
	w[1118];+/P{w[+mC]=UAS-TeTxLC.tnt}E2;+/Or43a-Gal4	*Canton-S*	*Canton-S*	18, 16
	w[1118];+/P{w[+mC]=UAS-TeTxLC.tnt}E2;+/Or47b-Gal4	*Canton-S*	*Canton-S*	12, 11
	w[1118];Or65a-Gal4/P{w[+mC]=UAS-TeTxLC.tnt}E2	*Canton-S*	*Canton-S*	13, 16
	w[1118];Or65b-Gal4/P{w[+mC]=UAS-TeTxLC.tnt}E2	*Canton-S*	*Canton-S*	18, 14
	w[1118];+/P{w[+mC]=UAS-TeTxLC.tnt}E2;+/Or65c-Gal4	*Canton-S*	*Canton-S*	20, 18
	w[1118];Or67d-Gal4/P{w[+mC]=UAS-TeTxLC.tnt}E2	*Canton-S*	*Canton-S*	15, 19

	w[1118];Or83c-Gal4/P{w[+mC]=UAS-TeTxLC.tnt}E2	*Canton-S*	*Canton-S*	20, 17
	w[1118];Or88a-Gal4/P{w[+mC]=UAS-TeTxLC.tnt}E2	*Canton-S*	*Canton-S*	21, 19

Fig. S2.				
Fig. S2A	*w[1118]*	*Canton-S*		26, 14, 18, 13
Fig. S2B	*w[1118]*	*Canton-S*		22, 14, 12, 12

Fig. S3.				
Fig. S3A	*w[1118]*	*Canton-S*		33
	w[1118]	*Canton-S*	*+;PromE(800)-Gal4/UAS-Tra*	36
	w[1118]	*Canton-S*	*+;PromE(800)-Gal4/UAS-Tra-RNAi*	17
Fig. S3B	*w[1118]*	*Canton-S*		20
	w[1118]	*Canton-S*	*Drosophila melanogaster*	23
	w[1118]	*Canton-S*	*Drosophila simulans*	21
	w[1118]	*Canton-S*	*Drosophila sechellia*	20
	w[1118]	*Canton-S*	*Drosophila erecta*	19
	w[1118]	*Canton-S*	*Drosophila yakuba*	21

Fig. S4.	*w[1118]*	*Canton-S*		18, 18, 14, 17, 15, 13

Fig. S5.				
Fig. S5A	*w[1118]*	*Canton-S*		17, 17, 18, 18, 17, 18
Fig. S5B	*w[1118]*	*Canton-S*		15, 15, 15, 16, 14, 15
Fig. S5C	*w[1118]*	*Canton-S*	*Canton-S*	21, 18
	w[1118]/ppk23-	*Canton-S*	*Canton-S*	17, 21
	ppk23-	*Canton-S*	*Canton-S*	13, 15
Fig. S5D	*w[1118]*	*Canton-S*	*Canton-S*	18, 14
	w[1118]/ppk28-	*Canton-S*	*Canton-S*	23, 25
	ppk28-	*Canton-S*	*Canton-S*	22, 17
Fig. S5E	*w[1118]*	*Canton-S*	*Canton-S*	17, 14
	w[1118];ppk29-/+	*Canton-S*	*Canton-S*	19, 20
	ppk29-	*Canton-S*	*Canton-S*	16, 17

Fig. S6A.	*w[1118];R52G04-AD/UAS-myrGFP;Dsx-DBD/UAS-myrGFP*			
Fig. S6B.	*w[1118];R52G04-AD/+;Dsx-DBD/UAS-GtACR1*	*Canton-S*		82, 60

Fig. S7.	*w[1118];UAS-FLP/+; GMR71G01-Gal4, CRE-F-Luc/+*			8, 8, 8, 12, 8, 4
	w[1118];UAS-FLP/+; GMR71G01-Gal4, CRE-F-Luc/+			8, 8, 8, 12, 8, 4

Fig. S8.	*w[1118];UAS-FLP/+; GMR71G01-Gal4, CRE-F-Luc/+*			12, 12, 12
	w[1118]; R52G04-AD/+;UAS-FLP, CRE-F-Luc/Dsx-DBD			12, 12, 12
	w[1118];;UAS-FLP, CRE-F-Luc/Dh44-pC1 (Dsx-DBD, Dh44A-AD)-GAL4			12, 12, 12
	w[1118]; VT25602-AD/+;UAS-FLP, CRE-F-Luc/VT2064-DBD			10, 10, 10

Fig. S9.	*w[1118]/UAS-Dcr2;;GMR71G01-Gal4/+*	*Canton-S*	*Canton-S*	27, 26
	w[1118];UAS-Dh44R1-RNAi/+; UAS-Dh44R2-RNAi/+	*Canton-S*	*Canton-S*	18, 17
	w[1118]/UAS-Dicer2;UAS-Dh44R1-RNAi1/+; GMR71G01-GAL4/UAS-Dh44R2-RNAi2	*Canton-S*	*Canton-S*	35, 30

Fig. S10.	*w[1118];;SAG(VT50405)-Gal4/+*	*Canton-S*		23
	w[1118];;UAS-dTRPA1/+	*Canton-S*		25
	w[1118];;SAG(VT50405)-Gal4/UAS-dTRPA1	*Canton-S*		24

Table. S1. Fly genotypes and N number in this paper