

1 **Distinct combinations of variant ionotropic glutamate receptors mediate**
2 **thermosensation and hygrosensation in *Drosophila***

3

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31 data analysis, Z.A.K., R. Benton and P.A.G. wrote the paper with contributions from all
32 authors.
33

34 **Abstract:** Ionotropic Receptors (IRs) are a large subfamily of variant ionotropic
35 glutamate receptors present across Protostomia. While these receptors are most
36 extensively studied for their roles in chemosensory detection in insects, recent work has
37 implicated two family members, IR21a and IR25a, in thermosensation in *Drosophila*.
38 Here we characterize one of the most deeply conserved receptors, IR93a, and show
39 that it is co-expressed and functions with IR21a and IR25a to mediate physiological and
40 behavioral responses to cool temperatures. IR93a is also co-expressed with IR25a and
41 a distinct receptor, IR40a, in a discrete population of sensory neurons in the sacculus, a
42 multi-chambered pocket within the antenna. We demonstrate that this combination of
43 receptors is important for neuronal responses to dry air and behavioral discrimination of
44 humidity differences. Our results identify IR93a as a common component of molecularly
45 and cellularly distinct IR pathways underlying thermosensation and hygrosensation in
46 insects.

47

48

49 **INTRODUCTION:**

50 Ionotropic Receptors (IRs) are a large subfamily of ionotropic glutamate
51 receptors (iGluRs) that evolved in the last common protostome ancestor (Benton et al.,
52 2009; Croset et al., 2010; Rytz et al., 2013). In contrast to the critical role of iGluRs in
53 synaptic communication, IRs have diverse roles in chemosensory detection (Koh et al.,
54 2014; Rytz et al., 2013). The best-defined functions of IRs are in olfaction, where they
55 mediate odor-evoked sensory neuron responses to diverse chemicals, including many
56 acids and amines (Rytz et al., 2013; Silbering et al., 2011). Most IRs are thought to form
57 heteromeric ligand-gated ion channels, in which broadly expressed co-receptor subunits
58 (e.g., IR8a, IR25a and IR76b) combine with more selectively expressed IR subunits that
59 confer stimulus specificity (Abuin et al., 2011; Rytz et al., 2013). Many of these IRs are
60 deeply conserved in insects, indicating that they define sensory pathways common to a
61 wide range of species (Croset et al., 2010; Rytz et al., 2013).

62 Although most conserved IRs have been assigned chemosensory roles, we
63 recently reported that one of these receptors, IR21a, mediates cool sensing (together
64 with IR25a), in a population of neurons in the *Drosophila melanogaster* larva, the dorsal
65 organ cool cells (DOCCs) (Ni et al., 2016). This finding raised the possibility that other
66 IRs serve non-chemosensory functions. In this work we characterize one of the most
67 deeply conserved “orphan” receptors, IR93a, which has orthologous genes across
68 arthropods (Corey et al., 2013; Groh-Lunow et al., 2014; Rytz et al., 2013). RNA
69 expression analysis in several insects and crustaceans indicate that this receptor gene
70 is transcribed in peripheral sensory organs (Benton et al., 2009; Corey et al., 2013;
71 Groh-Lunow et al., 2014; Rytz et al., 2013), but its role(s) are unknown. Using

72 *Drosophila* as a model, we find that IR93a acts in combination with distinct sets of IRs in
73 different populations of neurons to mediate physiological and behavioral responses to
74 both thermosensory and hygrosensory cues.

75

76 **RESULTS**

77 **IR93a is expressed in larval thermosensory neurons and is essential for cool** 78 **avoidance**

79 To investigate the expression and function of IR93a, we generated antibodies against a
80 C-terminal peptide sequence of this receptor, and obtained two *Ir93a* mutant alleles:
81 *Ir93a*^{M105555}, which contains a transposon insertion in the fifth coding exon, and *Ir93a*¹²²,
82 which we generated using CRISPR/Cas9 to delete 22 bases within sequences encoding
83 the first transmembrane domain (Figure 1a).

84 In larvae, IR93a protein is expressed in several neurons in the dorsal organ
85 ganglion, one of the main sensory organs in the larval head (Stocker, 1994) (Figure 1b-
86 c). These neurons encompass the DOCCs (labeled by an *Ir21a promoter-Gal4*-driven
87 GFP reporter), and the protein localizes prominently to the dendritic bulb at the tip of the
88 sensory processes of these cells (Figure 1c). All expression was absent in *Ir93a*
89 mutants, confirming antiserum specificity (Figure 1c).

90 These observations indicated that IR93a might function in cool sensing. Indeed,
91 when larval thermotaxis was assessed on a thermal gradient (Klein et al., 2015), we
92 found both *Ir93a* mutant alleles exhibit strong defects in cool avoidance (Figure 1d).
93 Cell-specific expression of an *Ir93a* cDNA in the DOCCs under *Ir21a-Gal4* control fully

94 rescued this mutant phenotype (Figure 1d). These data demonstrate an essential role
95 for IR93a in DOCCs in larval thermotaxis.

96

97 **IR93a is required, together with IR21a and IR25a, for cool-dependent**
98 **physiological responses of DOCCs**

99 We next assessed whether IR93a is required for the physiological responses of DOCCs
100 to cooling by optical imaging of these neurons using the genetically encoded calcium
101 indicator, GCaMP6m (Chen et al., 2013). As previously reported (Klein et al., 2015; Ni
102 et al., 2016), wild-type DOCCs exhibit robust increases in intracellular calcium in
103 response to cooling (Figure 2a). These responses were dramatically reduced in *Ir93a*
104 mutants, and could be rescued by cell-specific expression of an *Ir93a* cDNA (using the
105 *R11F02-Gal4* DOCC driver (Klein et al., 2015)) (Figure 2a). This dramatic loss of
106 DOCC temperature sensitivity resembles that observed in both *Ir21a* and *Ir25a* mutants
107 (Ni et al., 2016), and is consistent with IR21a, IR25a and IR93a functioning together to
108 mediate cool activation of the DOCCs.

109 To provide a more direct readout of thermotransduction in these neurons than
110 soma calcium measurements, we tested the requirement for IR93a, IR21a and IR25a in
111 cool-evoked membrane voltage changes using the genetically encoded voltage sensor,
112 Arclight (Jin et al., 2012). In wild-type animals, cool-dependent voltage changes were
113 observed in the DOCC sensory dendritic bulbs (Figure 2c-d), where IRs are localized
114 (Figure 1c). This response was completely eliminated in *Ir21a*, *Ir25a* and *Ir93a* mutants
115 (Figure 2c-e), indicating that each of these IRs is required for temperature-dependent
116 voltage changes in this sensory compartment.

117

118 **IR93a is co-expressed with IR25a and IR40a in the antennal sacculus**

119 In adults, *Ir93a* transcripts were previously weakly detected in a set of neurons in the
120 third antennal segment surrounding the sacculus, a three-chambered pouch whose
121 opening lies on the posterior surface of the antenna (Benton et al., 2009) (Figure 3a).
122 With our IR93a antibody, we detected IR93a expression in neurons innervating
123 sacculus chamber I (11.0 ± 0.5 neurons, $n=48$ animals; mean \pm SEM) and chamber II
124 (13.9 ± 0.7 , $n=23$), with signal detected both in the soma and in the sensory cilia that
125 project into cuticular sensory hairs (sensilla) (Figure 3b). By contrast, while IR25a is
126 expressed in IR93a-expressing cells in the sacculus (Figure 3c), neither *Ir21a*
127 transcripts nor *Ir21a* promoter drivers were detected in these cells ((Benton et al., 2009)
128 and data not shown). The IR93a sacculus neurons express instead a distinct receptor,
129 IR40a (Figure 3d) (Benton et al., 2009; Silbering et al., 2016). IR40a does not appear to
130 be expressed in the larval DOCCs (data not shown), suggesting that these sacculus
131 cells have another sensory function.

132

133 **IR93a, IR25a and IR40a are necessary for hygrosensory behavior**

134 Morphological studies have suggested that neurons in sacculus chambers I and II are
135 hygrosensitive (Shanbhag et al., 1995), raising the possibility that IR93a, IR25a, and
136 IR40a are required for hygrosensory behaviors. To test this hypothesis, we adapted an
137 experimental paradigm (Perttunen and Salmi, 1956) in which flies choose between
138 regions of differing humidity generated by two underlying chambers: one containing
139 deionized water and the other containing water saturated with a non-volatile solute

140 (ammonium nitrate) to lower its vapor pressure (Figure 4a). This assay design created a
141 humidity gradient of ~96% relative humidity (RH) to ~67% RH, with negligible variation
142 in temperature (Figure 4b). Consistent with previous observations (Perttunen and Salmi,
143 1956), wild-type flies exhibited a strong preferences for lower humidity (Figure 4c). This
144 preference was completely eliminated in *Ir93a* and *Ir25a* mutant flies, and significantly
145 reduced, but not abolished, in *Ir40a* mutants (Figure 4c). All of these behavioral defects
146 were robustly rescued by the corresponding cDNAs, confirming the specificity of the
147 mutant defects (Figure 4c). Importantly, the loss of *Ir21a* (or other antennal-expressed
148 IR co-receptors, *Ir8a* and *Ir76b*) did not disrupt dry preference. To exclude any potential
149 contribution of the non-volatile solute to the behavior observed, we also tested flies in a
150 humidity gradient (~89% to ~96% RH) generated using underlying chambers of
151 deionized water alone and air (Figure 4a). Even in this very shallow gradient, wild type
152 flies displayed a strong preference for lower humidity (Figure 4d), which was dependent
153 IR93a, IR25a and IR40a, but independent of IR21a (Figure 4d). This distinction between
154 the functions of IR21a and IR40a extended to thermotaxis, as *Ir40a* mutants exhibited
155 no defects in this IR21a-dependent behavior (Figure 4 – supplement 1b).

156

157 **IRs mediate dry detection by sacculus neurons**

158 To test whether the IR40a/IR93a/IR25a-expressing sacculus neurons are physiological
159 hygrosensors, we monitored their calcium responses to changes in the RH of an
160 airstream (of constant temperature) directed towards the antenna. We used *Ir40a-Gal4*
161 to express *UAS-GCaMP6m* selectively in these neurons, and measured GCaMP6m

162 fluorescence in their axon termini, which innervate two regions of the antennal lobe, the
163 “arm” and the “column” (Silbering et al., 2016; Silbering et al., 2011) (Figure 5a-b).

164 We observed that these sacculus neurons behave as dry-activated
165 hygrosensors: decreasing the RH from ~90% to ~7% RH elicited an increase in
166 GCaMP6m fluorescence, while increasing RH from ~7% to ~90% elicited a decrease
167 (Figure 5c-g). Calcium changes were most apparent in the “arm” (Figure 5c).
168 Importantly, these physiological responses are IR-dependent: mutations in either *Ir93a*
169 or *Ir40a* eliminated the dry response (*Ir25a* mutants were not tested), and these defects
170 were restored with corresponding cDNA rescue transgenes (Figure 5d-g). These data
171 corroborate the requirement for IRs in behavioral preference for lower humidity.

172

173 **DISCUSSION:**

174 From their ancestral origins within the synaptic iGluR family, IRs are widely appreciated
175 to have evolved functionally diverse roles in environmental chemosensory detection
176 (Croset et al., 2010; Rytz et al., 2013). Here we provide evidence that a previously
177 uncharacterized member of this repertoire, IR93a, functions in two critical non-
178 chemosensory modalities, thermosensation and hygrosensation. In both of these roles,
179 IR93a acts with the broadly expressed co-receptor IR25a. However, IR93a mediates
180 these two modalities in different populations of neurons in conjunction with a third,
181 distinct IR: with IR21a in cool sensation, but not dry sensation, and with IR40a in dry
182 sensation, but not cool sensation. All of these receptors are deeply conserved in
183 insects, indicating that these sensory pathways likely underlie behavioral responses of
184 diverse species to these important environmental stimuli. This conservation also

185 suggests that the non-chemosensory roles of IRs could be as ancient as the receptor
186 family itself.

187 The identification of an IR21a/IR25a/IR93a-dependent cool-sensing system
188 provides a molecular counterpart to the well-established Transient Receptor Potential
189 (TRP) (Barbagallo and Garrity, 2015), and Gustatory Receptor GR28B(D) (Ni et al.,
190 2013) warmth-sensing systems. By contrast, despite the importance of hygrosensation
191 in helping insects to avoid desiccation or inundation (Chown et al., 2011) and – in blood-
192 feeding disease vectors such as mosquitoes – to locate mammalian hosts (Brown,
193 1966; Olanga et al., 2010), the neuronal and molecular basis of this sensory modality
194 has been largely mysterious. Hygrosensitive neurons have been identified
195 electrophysiologically in large insects (Tichy and Gingl, 2001; Tichy and Kallina, 2010),
196 but their behavioral relevance has been hard to determine. In *Drosophila*, the antenna
197 has long been suspected to be an important hygrosensory organ (Perttunen and
198 Syrjamaki, 1958; Sayeed and Benzer, 1996), but there has been little consensus on the
199 relevant populations of neurons and sensory receptors (Ji and Zhu, 2015; Liu et al.,
200 2007; Yao et al., 2005). The TRP channels Nanchung and Waterwitch have been
201 suggested to contribute to hygrosensory behavior in *Drosophila* (Liu et al., 2007), but
202 these appear to be broadly expressed in the antenna, and there has been no direct
203 physiological analysis of the cells expressing these channels. Our characterization of
204 IR40a/IR93a/IR25a-expressing sacculus neurons, together with data from an
205 independent study (Enjin et al., 2016), provide physiological and behavioral evidence
206 supporting these as one pathway that enables flies to distinguish external humidity
207 levels.

208 In addition to the roles of IR93a in cool and dry sensing, it is very likely that this
209 receptor defines additional sensory pathways. Our expression analysis has identified
210 IR93a-positive cells that do not express IR21a or IR40a, such as non-DOCCs in the
211 larval dorsal organ (Figure 1c). Moreover, the milder hygrosensory behavior phenotype
212 of our protein null *Ir40a* mutants compared to *Ir93a* (or *Ir25a*) mutants hints that IR93a
213 may have broader roles in this sensory modality than acting exclusively with IR40a.
214 Finally, we suspect that the populations of IR93a-expressing neurons characterized in
215 this study are themselves functionally heterogeneous. For example, IR40a/IR93a-
216 expressing sacculus neurons appear to belong to two morphologically and
217 physiologically distinct subpopulations: “arm” neurons respond preferentially to low
218 humidity, while “column” neurons display preferential response to chemical stimuli
219 (Silbering et al., 2016). The molecular and/or cellular basis for this heterogeneity is not
220 yet known, but is reminiscent of the distinct subpopulations of IR64a-expressing
221 neurons in sacculus chamber III (Ai et al., 2013; Ai et al., 2010).

222 A key future challenge will be to determine the mechanism by which IRs sense
223 thermal and humidity cues. As IR21a and IR40a may be specificity determinants, it will
224 be of particular interest to determine the contribution (if any) of their Venus flytrap-like
225 “ligand-binding” domain (which recognizes glutamate in iGluRs, and diverse organic
226 molecules in chemosensory IRs). The requirement for IR93a (and IR25a) in these
227 distinct sensory modalities also indicates that they might share common mechanisms of
228 sensory detection. For example, hygrosensation could involve a thermosensory
229 component, based on evaporative cooling. Alternatively, both temperature and moisture
230 detection could involve mechanosensation, based on swelling or shrinkage of sensory

231 structures. Such mechanisms have been suggested to underlie hygrosensation in
232 mammals and *C. elegans* (Filingeri, 2015; Russell et al., 2014), and may explain why
233 hygrosensors in *Drosophila* are located in the morphologically highly-specialized
234 sacculus. Further characterization of how IRs mediate temperature and moisture
235 detection is currently hampered by our inability to reconstitute IR-based thermosensory
236 or hygrosensory responses in heterologous expression systems through expression of
237 the known combinations of IRs (G.B., L.N., A.F.S., R.B. and P.G., unpublished data).
238 These observations imply the existence of additional molecules and/or specialized
239 cellular structures that permit sensory detection of these ubiquitous and ever-changing
240 environmental stimuli.

241

242 **Material and Methods:**

243 **Fly strains.** *Ir25a*² (Benton et al., 2009), *UAS-Ir25a* (Abuin et al., 2011), *Ir8a*¹ (Abuin et
244 al., 2011), *Ir21a*¹²³ (Ni et al., 2016), *Ir76b*² (Zhang et al., 2013), *R11F02-Gal4* (Klein et
245 al., 2015), *Ir40a-Gal4* (Silbering et al., 2011), *Ir40a*¹ (Silbering et al., 2016), *UAS-Ir40a*
246 (Silbering et al., 2016), *Ir93a*^{M105555} (Venken et al., 2011), *UAS-GCaMP6m (P[20XUAS-*
247 *IVS-GCaMP6m]attP2* and *P[20XUAS-IVS-GCaMP6m]attP2attP40* (Chen et al., 2013)),
248 *UAS-Arclight* (Cao et al., 2013), *UAS-GFP (P[10XUAS-IVS-Syn21-GFP-p10]attP2*
249 (Pfeiffer et al., 2012)), and *y1 P(act5c-cas9, w+) M(3xP3-RFP.attP)ZH-2A w** (Port et
250 al., 2014).

251 *Ir40a*¹³⁴ (Figure 4 supplement 1a) and *Ir93a*¹²² (Figure 1a) were generated by
252 transgene-based CRISPR/Cas9-mediated genome engineering as described (Port et
253 al., 2014), using either an *Ir40a*-targeting gRNA (5'-GCCCGTTTAAGCAAGACATC) or

254 an *Ir93a*-targeting gRNA (5'-TCAGCAGAATGATGCCATT) expressed under U6-3
255 promoter control (dU6-3:gRNA) in the presence of *act-cas9* (Port et al., 2014). *UAS-*
256 *mCherry:Ir93a* contains codons 29-869 of the *Ir93a* ORF (corresponding to *Ir93a-PD*
257 [flybase.org], without the sequence encoding the predicted endogenous signal peptide),
258 which were PCR amplified from Oregon R antennal cDNA and subcloned into *pUAST-*
259 *mCherry attB* (Abuin et al., 2011) (which encodes the calreticulin signal sequence
260 upstream of the *mCherry* ORF). This construct was integrated into VK00027 by phiC31-
261 mediated transgenesis (Genetic Services, Inc.).

262

263 **Behavior.** Thermotaxis of early second instar larvae was assessed over a 15 min
264 period on a temperature gradient extending from 13.5 to 21.5°C over 22 cm (~0.36
265 °C/cm) as described (Klein et al., 2015). As thermotaxis data were normally distributed
266 (as assessed by Shapiro-Wilk test), statistical comparisons were performed by Tukey
267 HSD test, which corrects for multiple comparisons.

268 To assay hygrosensory behavior, 8 well rectangular dishes (12.8 x 8.55 x 1.5 cm;
269 ThermoFisher #267060) were modified to serve as humidity preference chambers. The
270 lids of two 8 well plates were used. A heated razor blade was used to cut out the middle
271 of one lid, and a nylon mesh was glued into place around the edges, providing a surface
272 for the animals to walk on which separated them from contact with any liquid. A
273 soldering iron was used to melt a small hole in a second culture plate lid, which could
274 then be placed over the screen, creating a chamber ~0.7 cm in height in which the flies
275 could move freely. To monitor the gradients formed, an additional chamber was
276 constructed with four holes equally spaced along its length to allow the insertion of

277 humidity sensors (Sensirion EK-H4 evaluation kit) for monitoring the humidity and
278 temperature.

279 Prior to the start of each experiment, 4 wells on one side of the culture dish were
280 filled with purified water, while the opposite 4 were filled with ~4 ml water and sufficient
281 ammonium nitrate to obtain a saturated solution (~3 g). The gradient was assembled
282 with the screen and lid piece, and the whole apparatus wrapped in food service film to
283 avoid any transfer of air between the inside and outside of the device. Gradients were
284 transferred to an environmental room that maintained at constant external temperature
285 and humidity (25°C and 70%RH). Ammonium nitrate gradients were permitted to
286 equilibrate for approximately 1 hour and were stable over many hours. For the water
287 and air only gradients, the air only side humidified over time. These gradients were
288 incubated for 25 minutes prior to use to allow the temperature to equilibrate; the
289 humidity of the dry side typically rose by ~2% RH during the 30 minute assay (values
290 shown are at the 30 minute time point). A small hole was poked through the food
291 service film covering the device to allow animals to be transferred to the gradient. This
292 hole was sealed using transparent scotch tape once the animals were inside.
293 Experiments used 1-4 day old adult flies that had been sorted under light CO₂
294 anesthesia into groups of 30 (15 male and 15 female) animals 24 hours before testing,
295 and transferred to fresh tubes. Flies were allowed 30 minutes to settle on the gradient,
296 at which point a photograph was taken of their position, and the number of animals on
297 each side counted, allowing calculation of a dry preference index as follows:

$$\text{Dry Preference} = \frac{\# \text{ animals on dry side} - \# \text{ animals on moist side}}{\text{total \# of animals}}$$

298 As moisture preference data did not conform to normal distributions (as assessed by
299 Shapiro-Wilk test, $p < 0.01$), statistical comparisons to wild-type control were performed
300 by Steel test, a non-parametric test that corrects for multiple comparisons, using JMP11
301 (SAS).

302

303 **Calcium and Arclight imaging.** Calcium and Arclight imaging of larval thermosensors
304 was performed as previously described (Klein et al., 2015). Pseudocolor images were
305 created using the 16_colors lookup table in ImageJ 1.43r. Adult antennal lobe calcium
306 imaging was performed as described for olfactory imaging (Silbering et al., 2012), with
307 slight modifications to sample preparation and stimulation. Briefly, 3-7 day old flies were
308 fixed to a Plexiglas stage using UV-glue (A1 Tetric Evoflow, Ivoclar Vivadent), the
309 antennae were pulled forward and a small opening was made in the head capsule to
310 allow visual access to the antennal lobes. For the stimulation compressed air from a
311 tank was passed through activated charcoal and then either through an empty gas
312 washing bottle or a gas washing bottle filled with distilled water producing either a dry
313 airstream of ~7%RH or a humid airstream of ~90%RH. A computer controlled solenoid
314 valve (The Lee Company, Westbrook, CT, USA) was used to switch the airflow between
315 the two gas washing bottles. The flow was kept constant at 1 l/min with a parallel
316 arrangement of two 500ml/min Mass Flow controllers (PKM SA, www.pkmsa.ch) placed
317 before the gas washing bottles. Activating the solenoid valve resulted in a complete
318 reversal of RH from low to high or high to low within less than 10 seconds. For each
319 animal tested, both high to low and low to high RH transitions were applied in random
320 order. Following humidity stimulation, a final pulse of 10% ammonia was applied as a

321 control to confirm cellular activity (Silbering et al., 2016) (animals showing no response
322 to this positive control were excluded from the analysis). Data were processed using
323 Stackreg (ImageJ) (Thevenaz et al., 1998) to correct for movement artifacts (animals
324 with movement artifacts that could not be corrected with Stackreg were excluded from
325 the analysis) and custom scripts in Matlab and R as previously described (Silbering et
326 al., 2011). As quantified imaging data did not conform to normal distributions (as
327 assessed by Shapiro-Wilk test, $p < 0.01$), statistical comparisons were performed by
328 Steel-Dwass test, a non-parametric test that corrects for multiple comparisons, using
329 JMP11 (SAS).

330

331 **Immunohistochemistry.** Larval immunostaining was performed as described (Kang et
332 al., 2012). Immunofluorescence on antennal cryosections or whole-mount antennae
333 was performed essentially as described (Saina and Benton, 2013), except that whole-
334 mount antennae were placed in Vectashield immediately after the final washes without
335 dehydration. The following antibodies were used: rabbit anti-IR25a (1:1000; (Benton et
336 al., 2009)), guinea pig anti-IR40a (1:200, (Silbering et al., 2016)), rabbit anti-IR93a
337 (peptide immunogen CGEFWYRRFRASRKRRQFTN, Proteintech, Rosemont, IL, USA,
338 1:4000 for tissue sections and 1:500 for whole-mount tissue), guinea pig anti-IR25a
339 (peptide immunogen SKAALRPRFNQYPATFKPRF, Proteintech, Rosemont, IL, USA,
340 1:200), mouse anti-GFP (1:200; Roche), goat anti-rabbit Cy3 (1:100 larva, 1:1000
341 sections; Jackson ImmunoResearch), goat anti-rabbit Alexa488 (1:100 antenna whole-
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345

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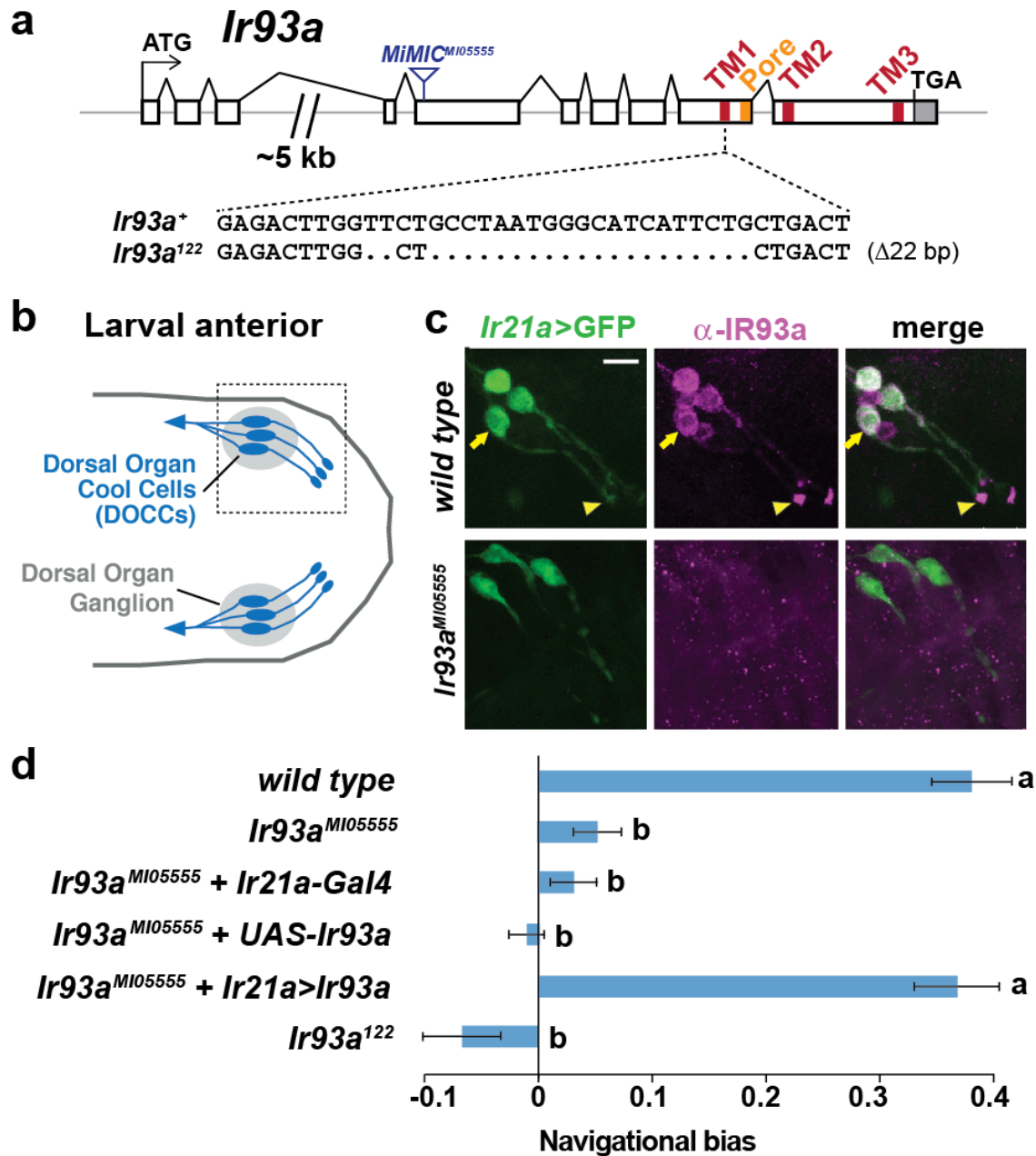
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Figure 1

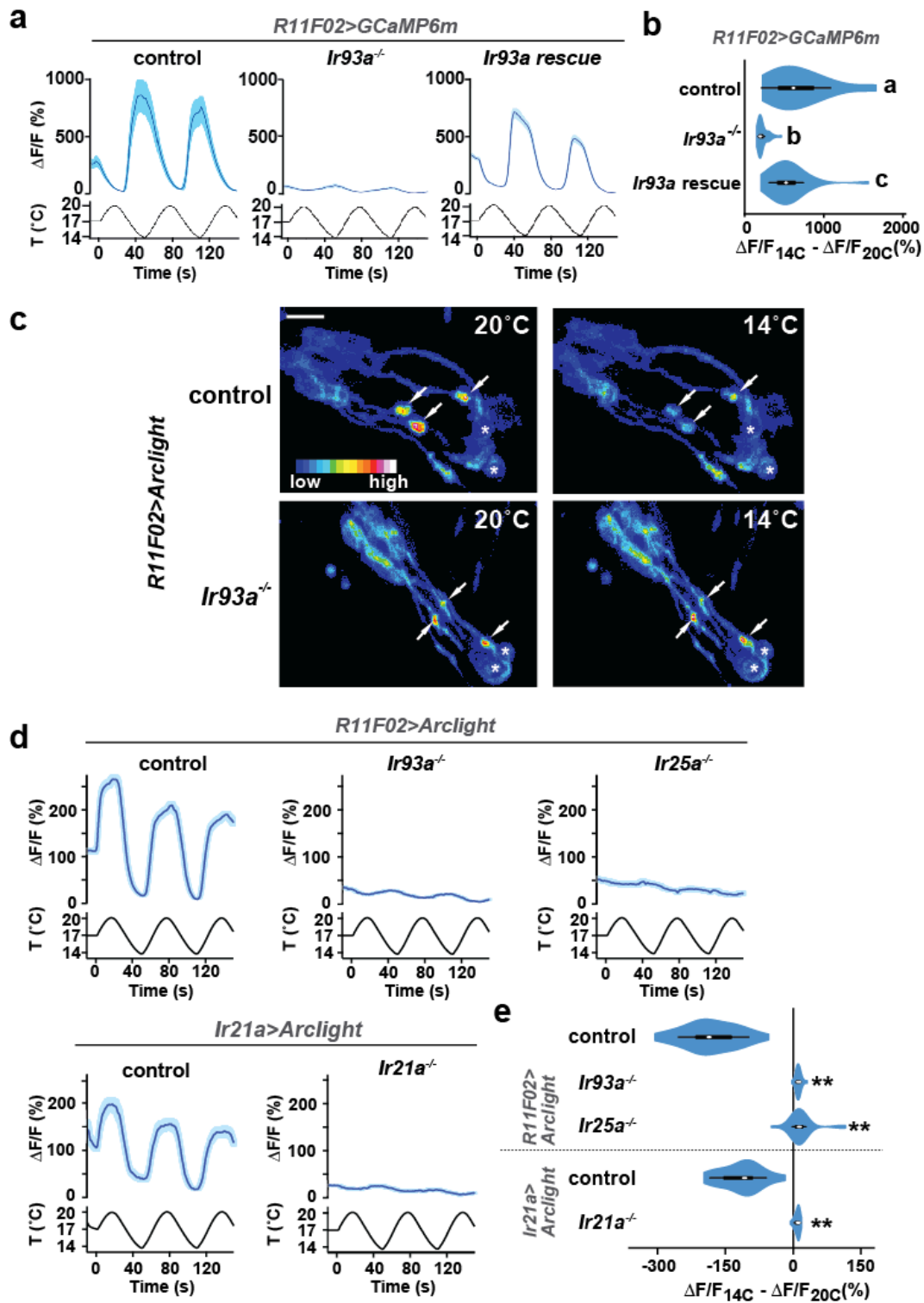


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499 **Figure 1. IR93a is expressed in Dorsal Organ Cool Cells (DOCCs) and is required**
500 **for cool avoidance.** (a) Gene structure of the *Ir93a* locus; sequences encoding the
501 transmembrane (TM) domains and channel pore are colored. The blue triangle denotes
502 site of *MiMIC* insertion in *Ir93a*^{MI05555}, and the CRISPR/Cas9-generated deletion in the
503 *Ir93a*¹³⁴ allele is shown below. (b) Schematic of the larval anterior showing the
504 bilaterally symmetric Dorsal Organ Ganglia (grey) within which three Dorsal Organ Cool
505 Cells (DOCCs) are located. (c) Immunofluorescence of the larval anterior
506 (corresponding to the boxed region in the schematic) showing expression of IR93a
507 protein (magenta) in DOCCs (*Ir21a-Gal4;UAS-GFP (Ir21a>GFP)*) (green), as well as
508 additional sensory neurons. *Ir93a*^{MI05555} mutants lack IR93a immunostaining. The arrow
509 and arrowhead label the soma and dendritic bulb of one of the DOCCs. Scale bar is 10
510 μm . (d) Cool avoidance behavior assessed as navigational bias (movement toward
511 warmth / total path length) of individual larval trajectories on an $\sim 0.36^\circ\text{C}/\text{cm}$ gradient
512 extending from $\sim 13.5^\circ\text{C}$ to $\sim 21.5^\circ\text{C}$, with a midpoint of $\sim 17.5^\circ\text{C}$. Letters denote
513 statistically distinct categories ($\alpha=0.05$; Tukey HSD). *wild type (Canton-S)*, n=37
514 animals. *Ir93a*^{MI05555}, n=132. *Ir21a-Gal4/+; Ir93a*^{MI05555}, n=72. *Ir93a*^{MI05555}, *UAS-Ir93a/*
515 *Ir93a*^{MI05555}, +, n=80. *Ir21a-Gal4/+; Ir93a*^{MI05555}, *UAS-Ir93a/ Ir93a*^{MI05555}, +, n=45. *Ir93a*¹²²,
516 n=101.
517

Figure 2



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519

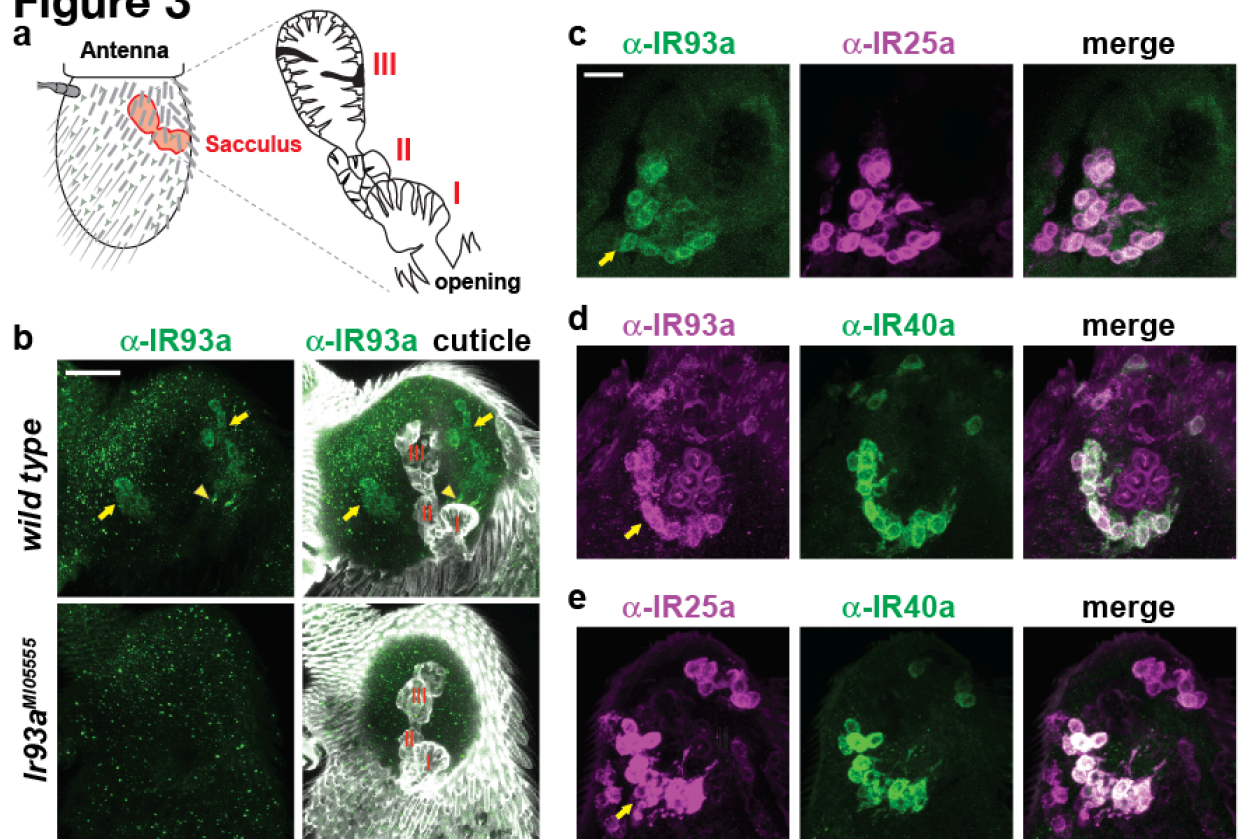
520 **Figure 2: Cool-responsive calcium and voltage changes in DOCCs require IR93a.**

521 (a) Left: DOCC responses monitored using *R11F02>GCaMP6m*. DOCC cool-
522 responsive increases in fluorescence are dramatically reduced in *Ir93a^{MI05555}*, and
523 responses are rescued by expression of a wild-type *Ir93a* cDNA under *R11F02-Gal4*
524 control. Traces, average \pm SEM. Right: Ratio of fluorescence at 14°C versus 20°C
525 depicted using a violin plot (internal white circles show median; black boxes denote 25th
526 to 75th percentiles; whiskers extend 1.5 times interquartile range). Letters denote
527 statistically distinct categories, $p < 0.01$, Steel-Dwass test. *wild type*, $n = 12$ cells.
528 *Ir93a^{MI05555}*, $n = 44$. *Ir93a^{MI05555}; R11F02>Ir93a*, $n = 46$. (b) Temperature-dependent DOCC
529 voltage responses in the sensory endings of *wild-type* (upper panels) or *Ir93a^{MI05555}*
530 mutant (lower panels) larvae monitored using *R11F02>Arclight*. Arrowheads denote
531 DOCC dendritic bulbs. Note that Arclight fluorescence decreases upon depolarization.
532 Asterisks denote cuticular autofluorescence from adjacent sensory structures. (c)
533 Robust cool-responsive depolarization of DOCC sensory endings is observed in
534 otherwise *wild-type* animals using either *R11F02>Arclight* or *Ir21a>Arclight*.
535 Depolarization response is eliminated in *Ir93a^{MI05555}*, *Ir25a²*, and *Ir21a^{Δ1}* mutants.
536 Traces, average \pm SEM. Violin plot depicts ratio of fluorescence at 14°C versus 20°C. **
537 denotes distinct from wild-type control, $p < 0.01$ compared to control, Steel-Dwass test.
538 *R11F02-Gal4;UAS-Arclight*, $n = 57$ cells. *R11F02-Gal4;UAS-Arclight;Ir93a^{MI05555}*, $n = 24$.
539 *R11F02-Gal4;UAS-Arclight; Ir25a²*, $n = 30$. *Ir21a-Gal4;UAS-Arclight*, $n = 18$. *Ir21a-*
540 *Gal4;UAS-Arclight; Ir21a^{Δ1}*, $n = 23$.

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Figure 3

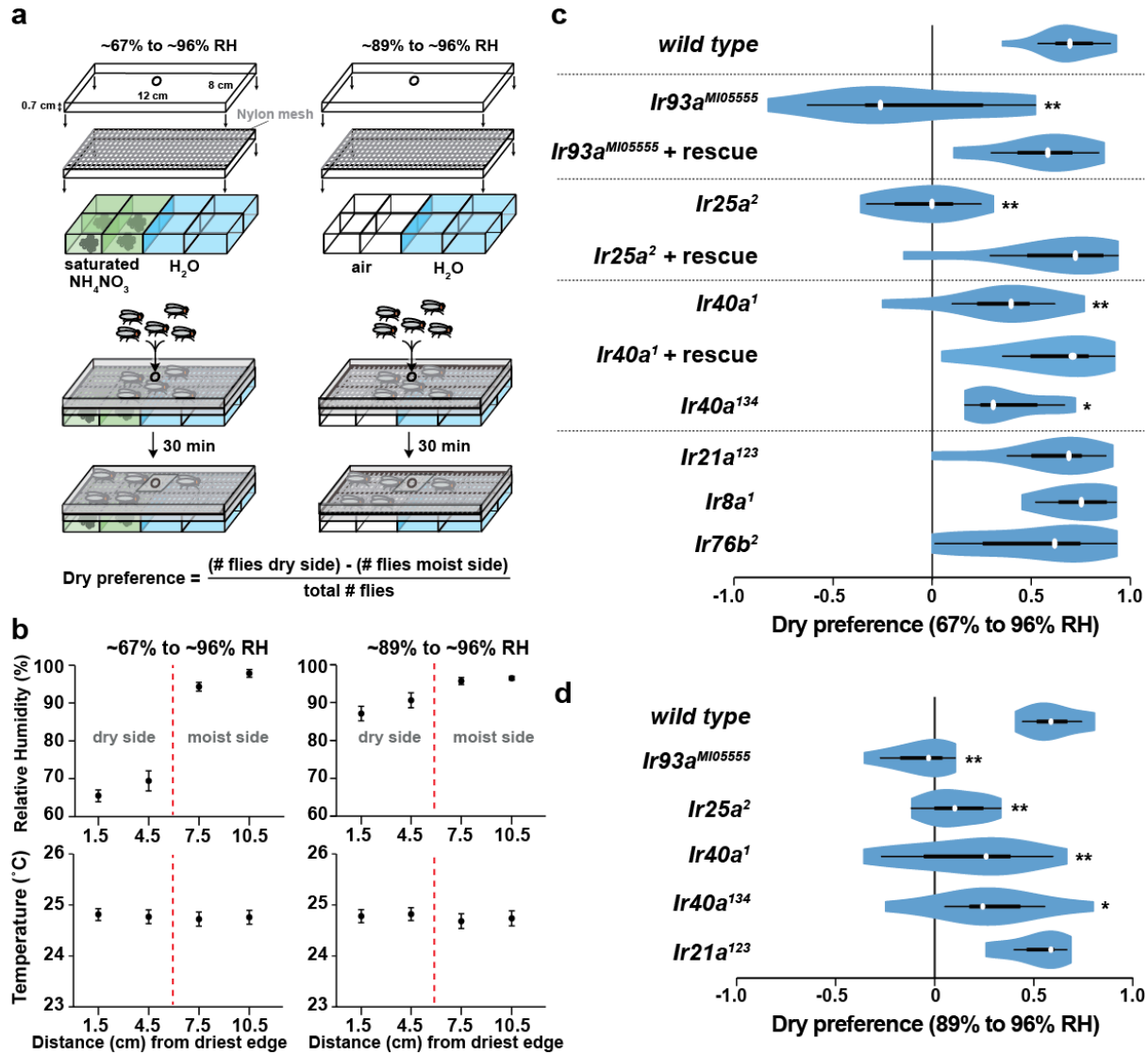


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545 **Figure 3: IR93a is co-expressed with IR25a and IR40a in sacculus neurons. (a)**
546 Left: schematic of the adult *Drosophila* antenna, illustrating the location of the sacculus
547 (red) in the interior of this appendage. Right: the sacculus is composed of three main
548 chambers (I, II, III), which are lined with sensilla of various morphologies (cartoon
549 adapted from (Shanbhag et al., 1995)). (b) Top: immunofluorescence on a whole-mount
550 wild-type antenna showing expression of IR93a protein (green) in two groups of soma
551 (arrows) around sacculus chambers I and II; these chambers are visualized by cuticle
552 autofluorescence shown in the images on the right. The arrowhead marks the
553 concentration of IR93a in the dendritic endings that innervate the sensilla in chamber I.
554 Note that the dendrites of chamber II neurons are no visible in this image; sensilla
555 localization of IR93a is more easily detected in these cells in antennal sections; see
556 panel (d). Bottom: *Ir93a*^{MI05555} mutants lack detectable IR93a protein. Scale bar is 20
557 μm . (c-e) Double immunofluorescence with the indicated antibodies on antennal
558 cryosections revealing co-expression of these IRs in sacculus neurons; the arrows point
559 to the cluster of neurons innervating chamber II. Scale bar is 10 μm . IR25a is expressed
560 in additional neurons that do not express IR93a or IR40a because of IR25a's broader
561 role as an olfactory IR co-receptor (Abuin et al., 2011).
562

Figure 4

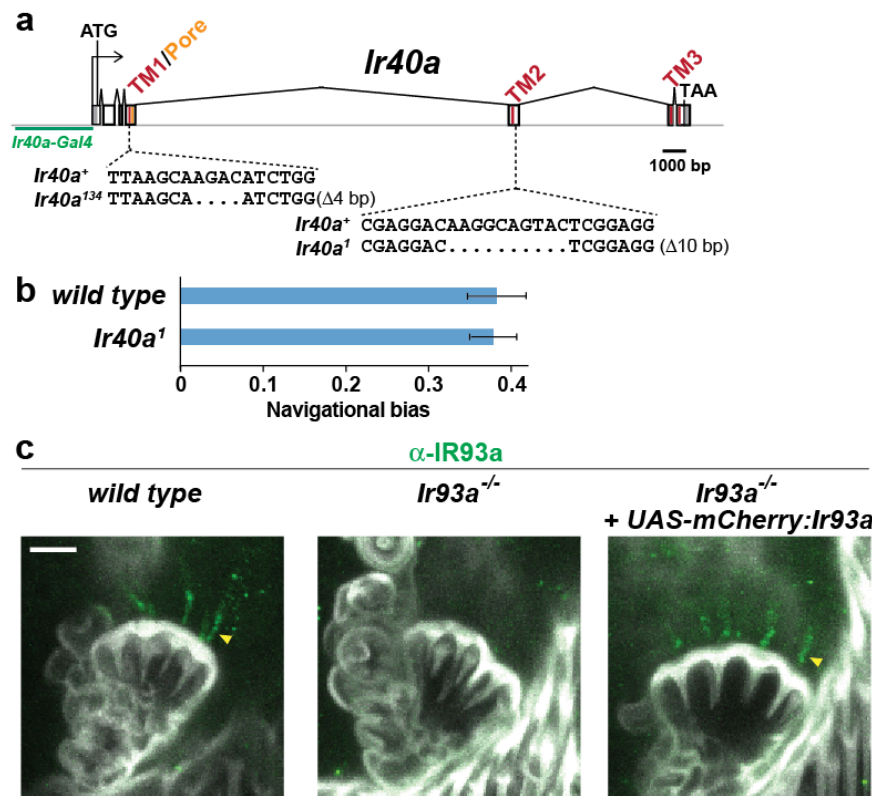


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565 **Figure 4: Hygrosensory behavior requires IR93a, IR25a and IR40a.** (a) Schematic of
566 the hygrosensory behavior assays. ~67% to ~96% RH gradients were generated by
567 filling wells with either a saturated solution of ammonium nitrate in water and or pure
568 water. ~89% to ~96% RH gradients were generated by pairing empty wells with wells
569 filled with pure water. Nylon mesh prevented fly contact with solutions. Dry preference
570 was quantified by counting flies on either side of chamber midline. 25-35 flies were used
571 per assay. (b) Mean \pm SD of RH and temperature measured at indicated gradient
572 positions. ~67% to ~96% RH, n=58 gradients. ~89% to ~96% RH, n=28. (c,d) Dry
573 preference assessed on ~67% vs. ~96% (c) and ~89% vs. ~96% (d) gradients.
574 Asterisks denote statistically distinct from *wild type* (** p<0.01; * p<0.05, Steel with
575 control). *wild type*, n=16 assays. *Ir8a* mutant (*Ir8a*¹), n=8. *Ir76b* mutant (*Ir76b*²), n=14.
576 *Ir21a* mutant (*Ir21a*¹²³), n=14. *Ir25a* mutant (*Ir26a*²), n=11. *Ir25a* rescue (*Ir25a*²; *UAS-*
577 *Ir25a*⁺), n=15. *Ir40a* mutant (*Ir40a*¹), n=15. *Ir40a* rescue (*Ir40a*¹; *UAS-Ir40a*⁺), n=9. *Ir40a*
578 CRISPR mutant (*Ir40a*¹³⁴), n=10. *Ir93a* mutant (*Ir93a*^{MI05555}), n=11. *Ir93a* rescue
579 (*Ir93a*^{MI05555}, *UAS-Ir93a*⁺), n=14. *Ir40a* mutant alleles and thermosensory behavior are
580 shown in Figure 4 supplement 1a-b. Note that *UAS-cDNA* rescues were observed in the
581 absence of *Gal4* drivers, reflecting *Gal4*-independent expression of *UAS* transgenes in
582 the sacculus (Figure 4 supplement 1c).
583

Figure 4 - supplement 1



584

585 **Figure 4– Figure supplement 1:** (a) Gene structure and sequence alterations in *Ir40a*

586 alleles. Regions encoding transmembrane domains (TMs) and pore region are in red.

587 The *Ir40a* promoter region present in *Ir40a-Gal4* is indicated in green. (b) Larval cool

588 avoidance behavior (assayed as in Figure 1d) is unaffected by mutation of *Ir40a*. *wild*

589 *type*, n=37 animals. *Ir40a*¹, n=55. (c) IR93a protein expression in the sacculus of *wild-*

590 *type*, *Ir93a*^{M105555} and *Ir93a*^{M105555};UAS-*IR93a* animals. The UAS-*mCherry:IR93a*

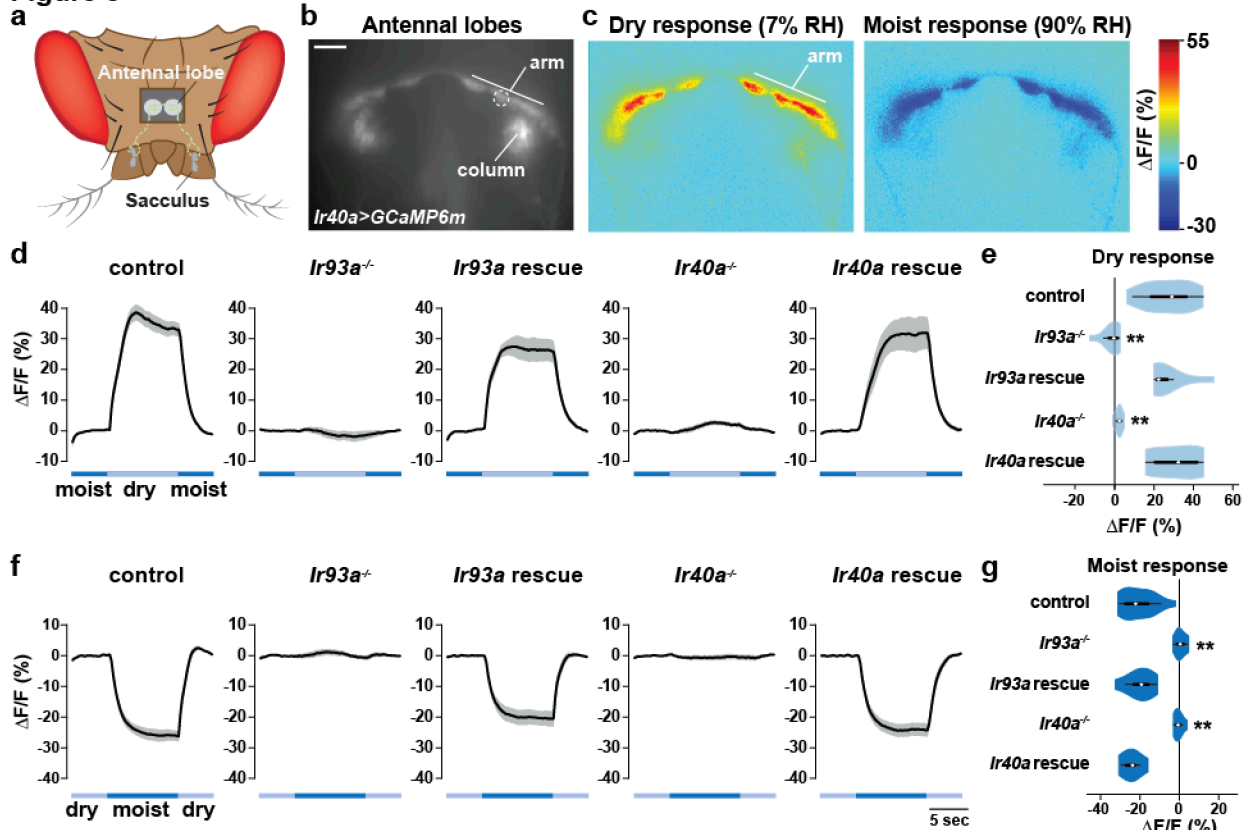
591 transgene restores expression of IR93a protein in the dendrites of sacculus neurons

592 (arrowhead) without the need for a Gal4 driver. The reason for the Gal4-independent

593 expression in these neurons is unknown, but Gal4-independent expression of UAS

594 transgenes in specific cellular contexts has been previously reported (Mao et al., 2014).

Figure 5



595

596 **Figure 5: IR-dependent physiological responses to dry air.** (a) Schematic of the
 597 *Drosophila* head (viewed from above) illustrating the projection of IR40a/IR93a/IR25a-
 598 expressing neurons (green) (labeled using *Ir40a-Gal4* (Silbering et al., 2011)) from the
 599 sacculus to the antennal lobes in the brain, visualized through a hole in the head cuticle.
 600 (b) Raw fluorescence image of *Ir40a* axons (in *Ir40a-Gal4;UAS-GCaMP6m* animals)
 601 innervating the arm and column in the antennal lobe. The dashed circle indicates the
 602 position of the ROI used for quantification in panels (d-g). (c) Color-coded images
 603 (reflecting GCaMP6m fluorescence intensity changes) of IR40a neuron responses to a
 604 switch from 90% to 7% RH (“Dry response”) and to a switch from 7% to 90% RH (“Moist
 605 response”). (d, f) Moisture-responsive fluorescence changes in the arm (moist=90% RH,
 606 dry=7% RH). Traces represent average \pm SEM. (e, g) Quantification of changes in $\Delta F/F$

607 (mean fluorescence change in the ROI shown in (b)) upon shift from moist to dry (e) or
608 dry to moist (g). Dry responses were quantified as [$\Delta F/F$ at 7% RH (average from 4.5 to
609 6.5 s after shift to 7% RH)] - [$\Delta F/F$ at 90% RH (average from 3.5 to 1 s prior to shift to
610 7% RH)], and moist responses quantified by performing the converse calculation.
611 Genotypes: control: n=17 animals (pooled data from *Ir40a-Gal4,Ir40a¹/IR40a-*
612 *Gal4,+;UAS-GCaMP6m/+*, n=9; *IR40a-Gal4;UAS-GCaMP6m,Ir93a^{MI05555}/+*, n=8). *Ir93a*
613 mutant (*Ir40a-Gal4;UAS-GCaMP6m,Ir93a^{MI05555}/Ir93a^{MI05555}*), n=10. *Ir93a* rescue (*Ir40a-*
614 *Gal4;UAS-GCaMP6m,Ir93a^{MI05555}/UAS-mcherry:Ir93a,Ir93a^{MI05555}*), n=8. *Ir40a* mutant
615 (*Ir40a-Gal4,Ir40a¹;UAS-GCaMP6m/+*), n=8. *Ir40a* rescue (*Ir40a-Gal4,Ir40a¹;UAS-*
616 *GCaMP6m/UAS-Ir40a*), n=6. **p<0.01, distinct from controls and rescues, Steel-Dwass
617 test.