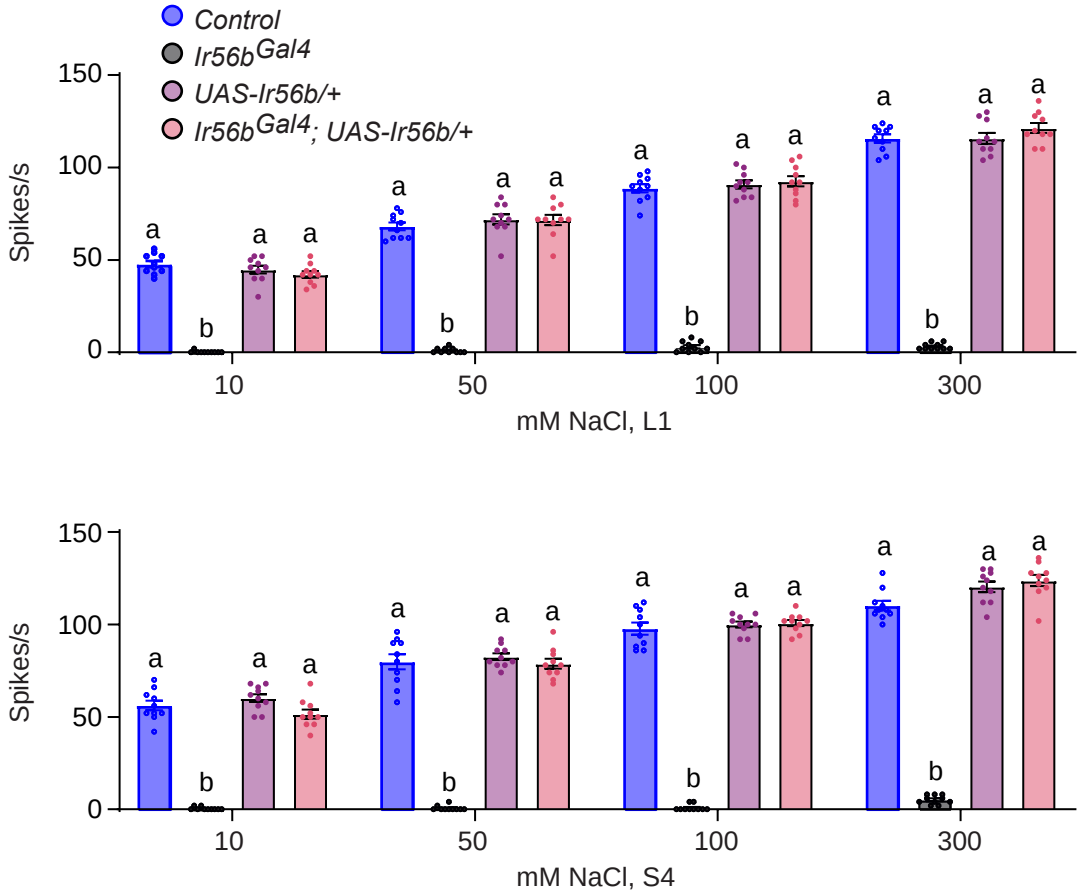

Figure S1. Rescue of the salt response phenotype of *Ir56b*^{Gal4} by *Ir56b*. Related to Figure 2. When an *Ir56b*^{Gal4} driver was used to drive a *UAS-Ir56b* transgene, it rescued the *Ir56b*^{Gal4} mutant phenotype, in both L1 and S4 sensilla. One-way ANOVA followed by Tukey's multiple comparison test; n = 10. Values indicated with different letters are significantly different.

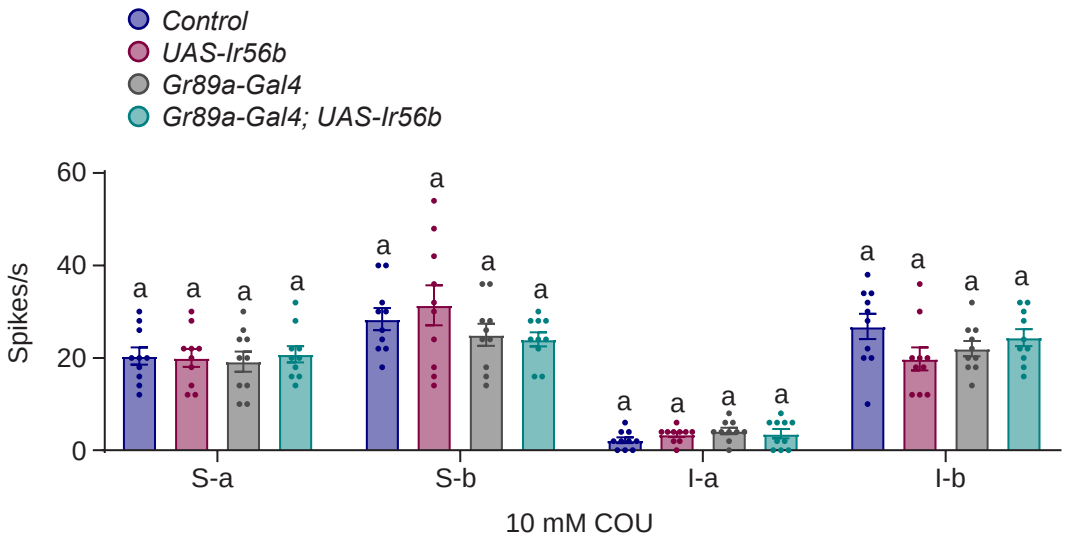

Figure S2. Normal responses to a bitter compound in neurons ectopically expressing *Ir56b*. Related to Figure 3.

Responses of the indicated classes of bitter neurons in the indicated genotypes to 10 mM coumarin, a bitter compound. One-way ANOVA followed by Tukey's multiple comparison test; n = 5.

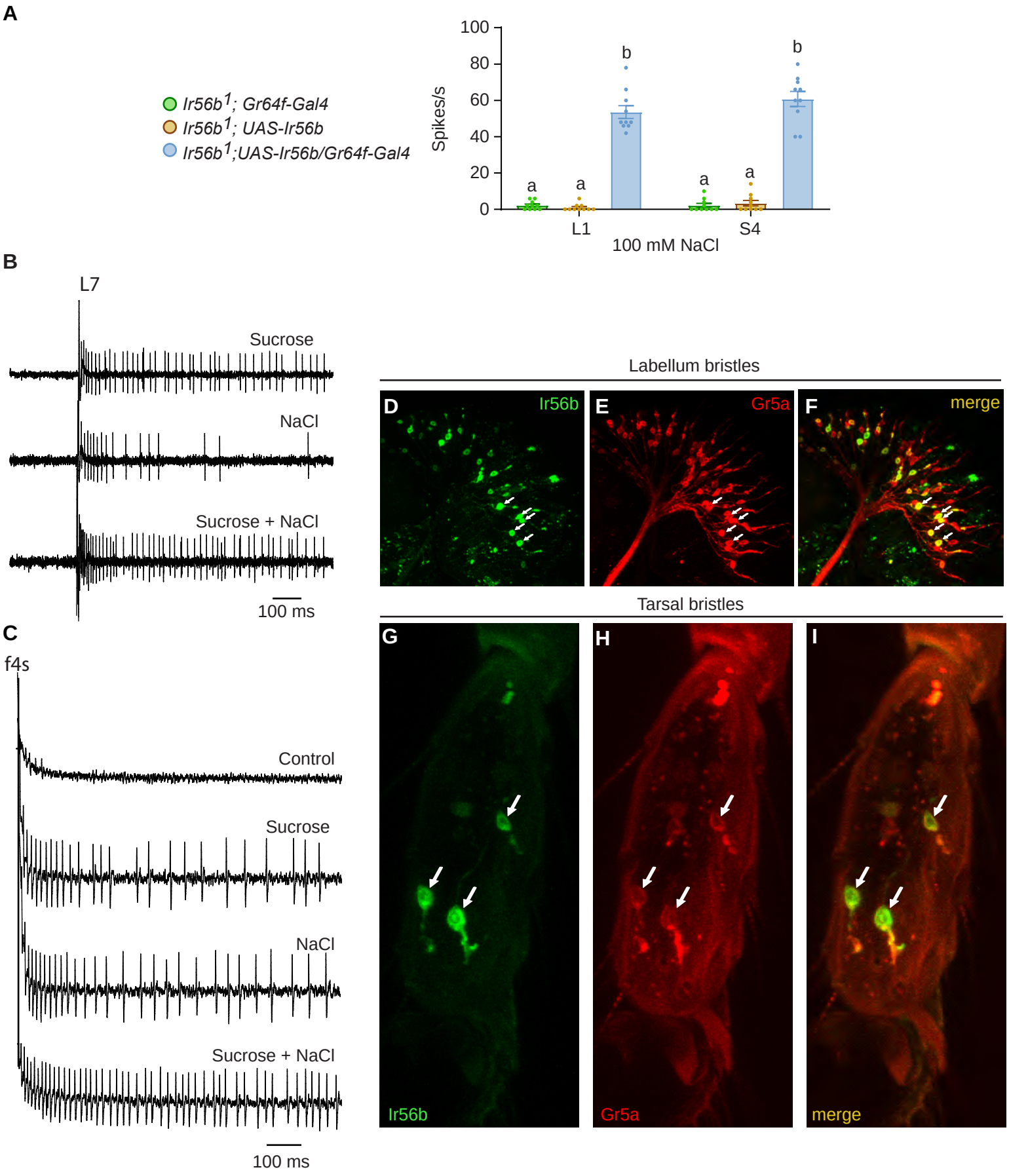

Figure S3. IR56b is expressed in sugar neurons. Related to Figure 4.
(A) Responses of L1 and S4 in the indicated genotypes to 100 mM NaCl. In this experiment a *Gr64f-Gal4* driver is used to express *UAS-Ir56b* in *IR56b* mutant sugar neurons. One-way ANOVA followed by Tukey's multiple comparison test; n = 6-11. Values indicated with different letters are significantly different.
(B) Recordings from L7 sensilla of the labellum in control flies presented with 10 mM sucrose, 10 mM NaCl, and a mixture of 10 mM sucrose and 10 mM NaCl. Sucrose, NaCl, and the mixture were all dissolved in 30 mM TCC.
(C) Sample traces of electrophysiological recordings from f4s sensilla of legs in control flies presented with diluent control (30 mM TCC), 50 mM sucrose, 50 mM NaCl, and a mixture of 50 mM NaCl and 50 mM sucrose.
(D-F) *Ir56a-GAL4* (green, GFP) is expressed in a subset of *Gr5a-LexA* (red, tandem-Tomato) neurons in the labellum.
(G-I) *Ir56a-GAL4* is expressed in a subset of *Gr5a-LexA*-expressing neurons in the tarsal segments. Arrows indicate labelled neurons.

Figure S4. All Ir56b orthologs have an unusually short N-terminal region. Related to figure 6.
All IR56b orthologs identified have a short NTR. Alignment of full-length NTR and S1 domains of Ir56b orthologs and alignment of partial length NTR and full length S1 domains of all other tuning IRs found in D. melanogaster. PID=percentage of identity relative to D. melanogaster sequence. Inclusion of all tuning IRs led to a slight shift in the alignment relative to Figure S6B.

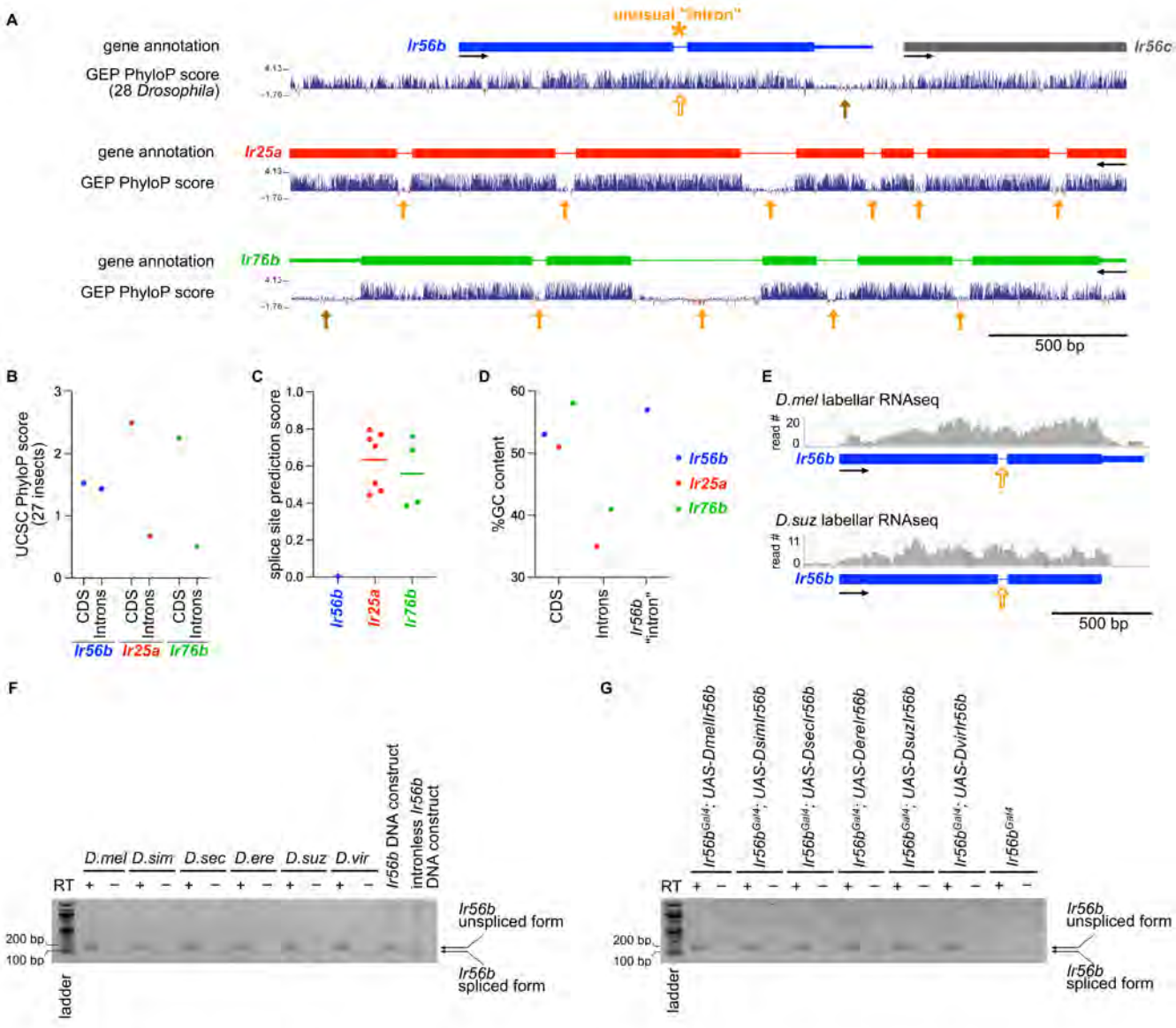

Figure S5. The Ir56b gene encodes a single-exon transcript. Related to figure 6.

(A) Genome annotation of Ir56b, from release 6.42. PhyloP score per nucleotide for Ir56b, Ir25a, and Ir76b genes; calculated from the alignment of 28 different Drosophila species. Plot obtained from GEP USCS genome browser Mirror at WUSTL (gander2.wustl.edu). Thick lines represent exons, lines of medium thickness represent untranslated regions, and thin lines represent introns. The phyloP score measures evolutionary conservation at individual alignment sites. It declines at introns (orange arrows) and untranslated regions (brown arrows), evidence of lower conservation relative to the coding regions. The annotated intron of Ir56b is indicated by an open orange arrow and an asterisk. In the case of Ir56b, the annotated intronic and exonic regions cannot be distinguished by the phyloP score. The scale is displayed on the bottom right and gene orientation is noted by a black arrow.

(B) Average phyloP score for Ir56b, Ir25a, and Ir76b genes; calculated from the alignment of 27 different insects. Data downloaded from USCS genome browser (https://hgdownload.soe.ucsc.edu/downloads.html). Unlike for Ir25a and Ir76b, the average phyloP score of the annotated Ir56b "intron" (1.43) is similar to the rest of the CDS (1.53).

(C) Average of 5' and 3' splice site prediction scores, as determined by NNSPLICE v0.9. Each dot represents the individual introns analyzed and the bar represents the average score. The Ir56b "intron" displays an unusually low splice site prediction score.

(D) GC content of Ir56b, Ir25a, and Ir76b exons and introns. Unlike other introns, the annotated Ir56b intron has a similar GC content to all exonic regions analyzed.

(E) D. melanogaster and D. suzukii labellar RNAseq coverage of Ir56b. No reads derived from a splicing event were detected in either species. In D. melanogaster, one synonymous substitution was detected at position 915, C>T, relative to the reference genome, and is indicated by a red vertical line.

(F) RT-PCR analysis of Ir56b transcripts from labellar preparations of different species. As controls, we used DNA constructs that included or excluded the "intronic" region as a template for PCR. The "unspliced" amplicon is ~160bp long and the "spliced" amplicon is 110bp long.

(G) RT-PCR analysis of Ir56b transcripts from labella of D. melanogaster expressing various UAS-constructs.

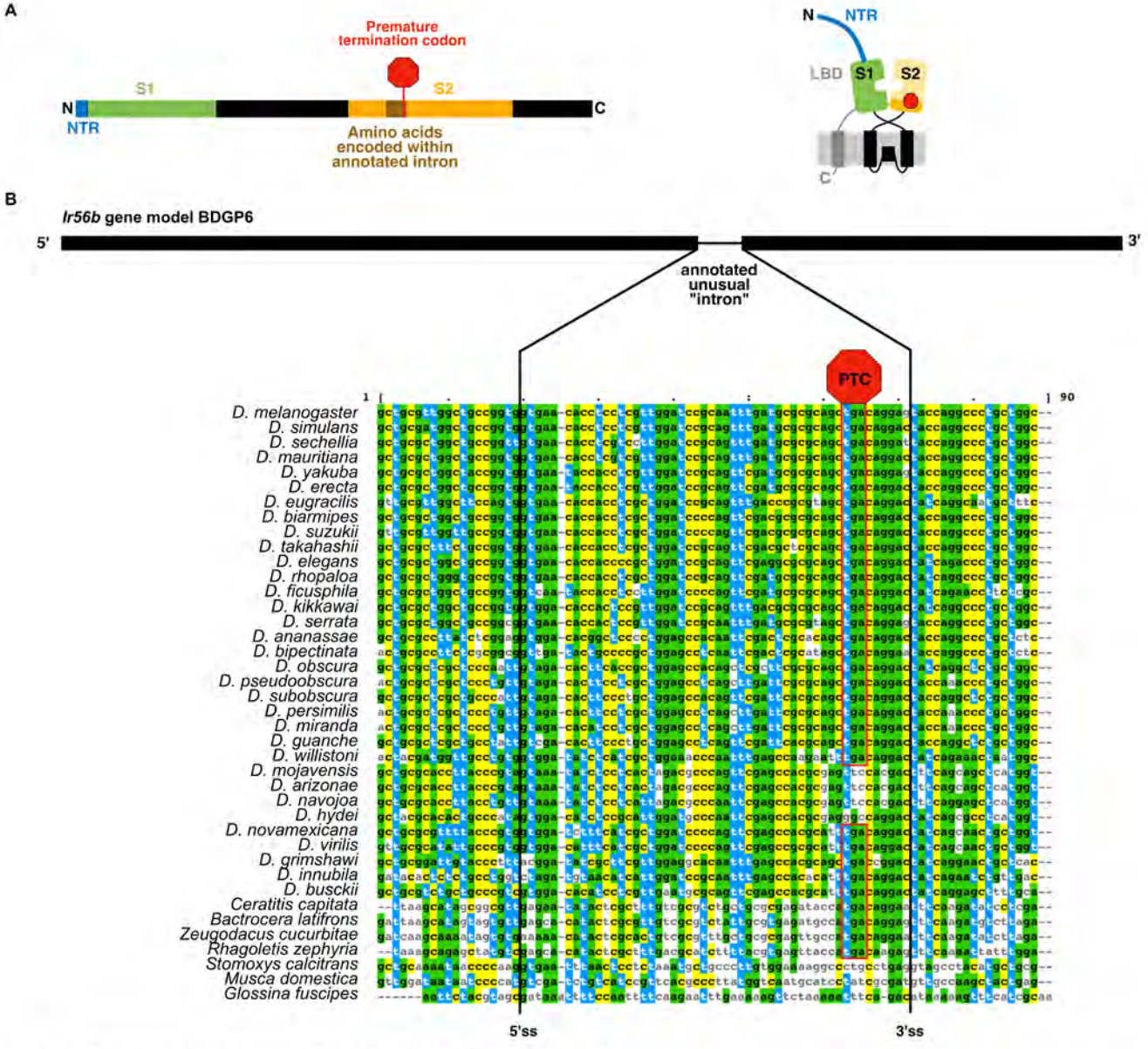

Figure S6. Conserved premature termination codon in Ir56b. Related to figure 6.
(A) Diagram of Ir56b protein in its linear form (left) and predicted structure (right) showing N-terminal region (NTR, blue), S1 domain (green), and S2 domain (orange). Part of the S2 domain is encoded within the annotated "intron" (brown), the region that contains the PTC. Without readthrough, a large fraction of the protein would be absent. Right panel adapted from Abuin et al. (2019).
(B) Gene model of D. melanogaster Ir56b (release 6.40) (top) and alignment of partial sequences of the Ir56b orthologous genes. This region includes the intron, delimited by black lines indicating the 5' and 3' splice sites (ss). The PTC is boxed in red.

Ir	Full length	NTR	S1 domain
25a	947	439	107
8a	936	376	126
93a	868	433	125
64a	859	309	113
21a	842	281	107
87a	796	331	151
40a	732	216	131
60a	716	243	126
68a	704	223	125
92a	678	245	120
75d	675	262	108
7b	655	211	105
41a	648	210	123
31a	648	206	115
76a	646	225	117
84a	644	224	113
11a	642	239	115
76b	636	81	109
68b	635	192	107
56d	632	215	104
75a	629	204	113
7c	625	214	110
75c	623	195	114
7f	621	227	110
7a	614	216	115
94e	611	199	101
10a	609	188	113
7e	608	211	99
62a	606	203	103
7g	606	201	116
85a	605	201	104
75b	605	179	123
94c	604	195	90
56a	603	202	102
100a	603	178	108
48b	600	191	96
52a	599	186	105
52c	599	184	104
51b	597	194	104
52b	596	184	99
94a	595	201	82
7d	594	210	111
52d	594	184	104
47a	593	185	101
94d	593	181	100
94b	592	192	85
54a	586	185	101
67a	584	173	102
60b	577	178	101
60d	574	175	100
67b	574	172	100
67c	573	164	106
60e	572	185	100
56c	567	178	101
20a	563	187	105
48c	561	176	100
94h	559	183	104
94f	558	187	104
94g	545	177	103
56b	408	11	104

Table S1. Length of all *D. melanogaster* Irs, related to Figure 6. Total length is provided in the 2nd column; the estimated N-terminal region (NTR) length in the 3rd column; the estimated S1 domain length in the 4th column.

Ir47a left Homology Arm	fwd	GGCTTCTGCAACTGATCTATTC
Ir47a left Homology Arm	rev	TCTAGATTTTTATGGCCTTTTGAAACTGAA
Ir47a right Homology Arm	fwd	ATGCGCTCTTCATATTCGTGGAAGTTGTCGTGG
Ir47a right Homology Arm	rev	CTAAGCTCTTCAGACATTATGGGCCAGGGTTATGG
Ir56b left Homology Arm	fwd	GTGCCGTCGAAACCCTAAA
Ir56b left Homology Arm	rev	TCTAGATTTATGGCCGACTACGGATTG
Ir56b right Homology Arm	fwd	ATGCGCTCTTCATATCAAGTGGAAATATCATTGTTCTC
Ir56b right Homology Arm	rev	CTAAGCTCTTCAGACGGTCAGAAGGTCGTAGATTG
Ir47a guide RNA	fwd	GCGGCCCGGGTTCGATTCCCGGCCGATGCATAGCCT TTTGTGTATTCATCGGTTTTAGAGCTAGAAATAGCAAG
Ir47a guide RNA	rev	ATTTTAACTTGCTATTTCTAGCTCTAAAACCATTTTTTA TGGCCTTTTGAATGCACCAGCCGGGAATCGAACCC
Ir56b guide RNA	fwd	GCGGCCCGGGTTCGATTCCCGGCCGATGCAGCCATA AAATGCTGCTTGACAGTTTTAGAGCTAGAAATAGCAAG
Ir56b guide RNA	rev	ATTTTAACTTGCTATTTCTAGCTCTAAAACCTTGTTAGT AATCGTAGCACTTGCACCAGCCGGGAATCGAACCC
Gal4 core promoter	fwd	cggccataaaTCGTTCAGCTTTCTTGTAC
Gal4 core promoter	rev	atgttgtggaTCTAGAAGCCAACGTGTATC
Ir56b donor plasmid	fwd	ggcttctagaTCCACAACATCCCGTTTAC
Ir56b donor plasmid	rev	agctgaacgaTTTATGGCCGACTACGGATTG
Ir56b UAS *D. mel*	fwd	actctgaatagggaattgggGTAGTCGGCCATAAAATG
Ir56b UAS *D. mel*	rev	ggttccttcacaaagatcctGTAAGCCAGGTTAATTTTTATG
Ir56b UAS *D. sim* / *D. sec*	fwd	actctgaatagggaattgggGTAGTCGGCCCTAAAATG
Ir56b UAS *D. sim* / *D. sec*	rev	ggttccttcacaaagatcctCCACGATTTGTAAGCCAG
Ir56b UAS *D. ere*	fwd	actctgaatagggaattgggGTAGTCGGCCCTAAAATG
Ir56b UAS *D. ere*	rev	ggttccttcacaaagatcctCTGAGACCTATTCAAAGTTATTA AAATC
Ir56b UAS *D. suz*	fwd	actctgaatagggaattgggATAGTCGACCCCAAAATG
Ir56b UAS *D. suz*	rev	ggttccttcacaaagatcctTGCTAAAACAATTCCTTGAATTC
Ir56b UAS *D. vir*	fwd	actctgaatagggaattgggACTTGCAGCACGAAATGG
Ir56b UAS *D. vir*	rev	ggttccttcacaaagatcctTAATTTGACTGATATTTATACTC ATAGTGAAG
UAS Ir56b *Δ51*	fwd	TACCAGGCCCTGCTGGCG
UAS Ir56b *Δ51*	rev	CACCGGCAGCCAACGCAG
UAS Ir56b *Δ462*	fwd	TAACAAGTGGAAATATCATTGTTCTCTATTATC
UAS Ir56b *Δ462*	rev	TCAGCTGCGCGCATCAAA

UAS Ir56b TTC	fwd	GCGCGCAGCTtcCAGGAGTACC
UAS Ir56b TTC	rev	ATCAAATTGCGGATCCAACG
Ir56b *D. mel – D.ere* RT-PCR	fwd	TCGAGACTGCGCATCGTTATC
Ir56b *D. mel – D.ere* RT-PCR	rev	GTGACCACATAGGCGTAGGAAC
Ir56b *D. suz* RT-PCR	fwd	AAAGCTGCGAATCGTCATCCA
Ir56b *D. suz* RT-PCR	rev	GCATCCTGGGTCACCACATAG
Ir56b *D. vir* RT-PCR	fwd	TATACCGGATACGCTGCTGGA
Ir56b *D. vir* RT-PCR	rev	CACGCATCCTGTGTAACCACATA

Table S2. List of primers. Related to Figures 2-6.