

1 **Systematic expression profiling of *dprs* and *DIPs* reveals cell surface codes in**  
2 ***Drosophila* larval peripheral neurons**

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18

19 **Abstract**

20 In complex nervous systems, neurons must identify their correct partners to form synaptic  
21 connections. The prevailing model to ensure correct recognition posits that cell surface proteins  
22 (CSPs) in individual neurons act as identification tags. Thus, knowing what cells express which  
23 CSPs would provide insights into neural development, synaptic connectivity, and nervous  
24 system evolution. Here, we investigated expression of *dprs* and *DIPs*, two CSP subfamilies  
25 belonging to the immunoglobulin superfamily (IgSF), in *Drosophila* larval motor neurons (MNs),  
26 sensory neurons (SNs), peripheral glia and muscles using a collection of GAL4 driver lines. We  
27 found that *dprs* are more broadly expressed than *DIPs* in MNs and SNs, and each examined  
28 neuron expresses a unique combination of *dprs* and *DIPs*. Interestingly, many *dprs* and *DIPs*  
29 are not robustly expressed, but instead, are found in gradient and temporal expression patterns.  
30 Hierarchical clustering showed a similar expression pattern of *dprs* and *DIPs* in neurons from  
31 the same type and with shared synaptic partners, suggesting these CSPs may facilitate synaptic  
32 wiring. In addition, the unique expression patterns of *dprs* and *DIPs* revealed three  
33 uncharacterized MNs - MN23-Ib, MN6-Ib (A2) and MN7-Ib (A2). This study sets the stage for  
34 exploring the functions of *dprs* and *DIPs* in *Drosophila* MNs and SNs and provides genetic  
35 access to subsets of neurons.

## 36 **Introduction**

37 During nervous system development, neurons contact thousands of cells but only form  
38 synapses with a small subset. Precise neural wiring is achieved through a series of steps  
39 including axon pathfinding, partner recognition, and synaptic pruning (Sanes and Zipursky,  
40 2020; Zarin and Labrador, 2019). Although the mechanisms underlying these processes are not  
41 completely understood, one prevailing model proposes that cell surface proteins (CSPs) instruct  
42 chemo attraction and repulsion, self-avoidance, and synaptic partner recognition (Honig and  
43 Shapiro, 2020; Wit and Ghosh, 2016). CSPs fall into several protein families, including the  
44 immunoglobulin superfamily (IgSF), the Cadherin protein family (Cdhs), the leucine-rich repeat  
45 protein family (LRRs), the receptor tyrosine kinases (RTKs), and many more (Jontes, 2017;  
46 Kurusu et al., 2008; Sanes and Zipursky, 2020; Zinn and Özkan, 2017). In vitro biochemical  
47 studies showed that subsets of these CSPs interact homo- or heterophilically, and many of  
48 these interactions are implicated in synaptic connectivity in both vertebrates and invertebrates  
49 (Cheng et al., 2019; Honig and Shapiro, 2020; Özkan et al., 2013; Wit and Ghosh, 2016).

50 In the well-studied vertebrate retina, retinal ganglion cells require multiple CSPs,  
51 including Sidekicks (Sdks) 1 and 2 and Dscams, to form stereotyped connections and avoid  
52 self-synapses, respectively (Garrett et al., 2018; Krishnaswamy et al., 2015; Yamagata and  
53 Sanes, 2019). Similarly, ON-OFF direction-selective ganglion cells and ON-OFF bipolar  
54 interneurons establish correct partnership by homophilic interactions of classical Cdhs (Duan et  
55 al., 2018, 2014). In hard wired invertebrate nervous systems, such as *C. elegans*, the  
56 heterophilic interaction between two IgSF proteins, Syg1 and Syg2, is required for HSNL motor  
57 neuron synapse formation (Shen et al., 2004; Shen and Bargmann, 2003). The genetically  
58 tractable *Drosophila melanogaster* is also an excellent model to study CSP expression and  
59 function due to the stereotyped neurogenesis and circuit wiring as well as extensive genetic  
60 tools. Neurons in the fly brains assemble into highly complex circuits similar to those observed  
61 in vertebrates but are numerically less daunting. In the fly mushroom body, neurons rely on  
62 different isoforms of Dscam1 to discriminate self-/non-self (Hattori et al., 2009; Wang et al.,  
63 2004; Zhan et al., 2004). In the olfactory system, epidermal growth factor (EGF)-repeat  
64 containing transmembrane Teneurin proteins, Ten-m and Ten-a, are required for the one-to-one  
65 matching between olfactory receptor neurons and projection neurons (Hong et al., 2012).

66 Specific challenges are also encountered in the *Drosophila* larval neuromuscular system  
67 where 33 motor neurons (MNs) within each neuromere in the ventral nerve cord (VNC) send  
68 their projections to the periphery where they follow defined paths and ultimately choose specific  
69 muscle(s) to innervate among 30 potential targets (Grueber et al., 2007; Hoang and Chiba,

70 2001; Menon et al., 2013). Unlike the dense neuropil where synaptic connections are difficult to  
71 identify, the neuromuscular innervation patterns are genetically hard-wired and easily identified.  
72 Thus, each efferent motor neuron can be recognized by its stereotyped innervation pattern and  
73 morphology. Utilizing the neuromuscular system, many CSPs were identified as recognition  
74 cues between MNs and muscles, including Toll (Inaki et al., 2010; Rose et al., 1997), Connectin  
75 (Nose et al., 1997, 1992) and Capricious (Kurusu et al., 2008; Shishido et al., 1998) from the  
76 LRR family and Fasciclin 2 (Davis et al., 1997; Winberg et al., 1998) and Fasciclin 3 (Chiba et  
77 al., 1995; Kose et al., 1997) from the IgSF. In contrast, the afferent neurons of the sensory  
78 nervous system are localized in the periphery and send their projections to the VNC. Forty-two  
79 sensory neurons (SNs) are stereotypically distributed throughout each hemisegment of the  
80 larval body wall and establish synaptic connections with interneurons (Orgogozo and Grueber,  
81 2005). Studies from dendritic arborization (da) neurons identified several CSPs for self-  
82 avoidance, such as Dscam1 and Semaphorin (Meltzer et al., 2016; Miura et al., 2013; Soba et  
83 al., 2007). Thus, the unambiguous identification of cells in the motor and sensory circuits  
84 provide an ideal system to examine the genes and mechanisms that underlie synaptic specificity  
85 and development.

86 In a previous “interactome” screen, we and others identified two subfamilies of the  
87 *Drosophila* IgSF, the Defective proboscis response proteins (Dprs; 21 members) and the Dpr-  
88 interacting proteins (DIPs; 11 members) (Carrillo et al., 2015; Özkan et al., 2013). Dprs and  
89 DIPs are different from other CSPs found to wire the peripheral nervous system because they  
90 have more family members and can interact both homo- and heterophilically, providing a vast  
91 repertoire of unique combinations for synaptic specificity. Interactions between Dprs and DIPs  
92 have been implicated in synaptic connectivity, cell survival, and synaptic growth (Ashley et al.,  
93 2019; Bornstein et al., 2021; Carrillo et al., 2015; Courgeon and Desplan, 2019; Menon et al.,  
94 2019; Sanes and Zipursky, 2020; Venkatasubramanian et al., 2019; Xu et al., 2019, 2018).  
95 However, most studies focused on Dprs and DIPs have implicated only a small subset, likely  
96 due to low-penetrance targeting defects and molecular redundancy. For example, in the larval  
97 neuromuscular circuit, loss of *DIP-α* leads to complete loss of muscle 4 innervation by a specific  
98 motor neuron; however, neuromuscular junctions (NMJs) on other muscles formed by the same  
99 neuron are unaffected, suggesting different synaptic recognitions utilize different pairs of CSPs  
100 even within the same neuron (Ashley et al., 2019). Thus, obtaining a complete expression map  
101 of families of CSPs in individual neurons within specific circuits would facilitate subsequent  
102 functional studies.

103 Different approaches are available to map the expression patterns for genes of interest.  
104 Modern technologies like single cell RNA sequencing (scRNAseq) provide enormous  
105 information about gene expression in each cell type and has been successfully applied in the fly  
106 nervous system (Avalos et al., 2019; Li, 2020; Tang et al., 2009). However, most scRNAseq  
107 datasets do not capture the dynamic expression during development, and it is difficult to identify  
108 individual cell types from heterogenous clusters. Another approach, possibly more accurate for  
109 closely related cells, is to generate genetic reporter lines for genes of interest and directly  
110 visualize their expression. For example, in *Drosophila*, a collection of GAL4 drivers representing  
111 Gr taste receptors were used to map the projection of Gr expressing neurons (Kwon et al.,  
112 2014). These genetic reporters together with imaging allow unambiguous characterization of  
113 gene expression at a higher spatial and temporal resolution.

114 In this study, we interrogate the expression patterns of *dprs* and *DIPs*. These IgSF CSPs  
115 form extensive interactions and are highly enriched in the nervous system, suggesting important  
116 roles in circuit development. To access the expression of these genes, we and others generated  
117 a collection of GAL4 lines of 19 *dprs* and 11 *DIPs*. We utilized different UAS reporters to  
118 examine expression of *dprs* and *DIPs* in the *Drosophila* larval neuromuscular and sensory  
119 circuits. The distinct and stereotyped morphologies and positions of these cells allow us to  
120 unambiguously identify the reporter gene expression patterns. Here, we generated expression  
121 maps of *dprs* and *DIPs* in MNs, SNs, and muscles, and found that each MN and SN expresses  
122 a unique subset of *dprs* and *DIPs*. Utilizing hierarchical clustering, we found that the same class  
123 of SNs expresses similar *dprs* and *DIPs*, suggesting roles in identifying overlapping synaptic  
124 partners. Finally, the highly distinct expression patterns of *dprs* and *DIPs* in MNs revealed  
125 previously unidentified MNs. The expression analyses generated by this study will benefit future  
126 functional studies of Dprs and DIPs in the motor and sensory circuits. The genetic tools and  
127 pipeline provided here will facilitate expression studies of *dprs* and *DIPs*, and other CSPs, in  
128 other *Drosophila* neural circuits to promote the discovery of identification tags utilized for circuit  
129 assembly.

130

## 131 **Results**

### 132 **Generating a GAL4 collection of *dprs* and *DIPs***

133 Using *Drosophila* Minos-Mediated Integration Cassette (MiMIC) insertions followed by Trojan  
134 conversion, and CRISPR-Mediated Integration Cassette (CRIMIC) insertions, we and others  
135 generated a collection of GAL4 lines of all *DIPs* and *dpr1-dpr19* (Diao et al., 2015; Kanca et al.,  
136 2019; Lee et al., 2018; Nagarkar-Jaiswal et al., 2015b; Venken et al., 2011) (Figure 1A). For  
137 each *dpr*- and *DIP-GAL4*, the cassette is inserted into a common intron or the 5'UTR shared by  
138 all isoforms (Figure 1A and Table 1). Therefore, GAL4 expression should report the expression  
139 of all isoforms of each gene. Insertion of the *SA-T2A-GAL4-PolyA* tail should generate truncated  
140 transcripts because of the presence of the PolyA tail (Logan et al., 1987; Zhang et al., 2015). In  
141 addition, the presence of a T2A-*GAL4* leads to an arrest during translation at the T2A site  
142 followed by a reinitiation of translation at the GAL4 sequence (Diao et al., 2015; Szymczak-  
143 Workman et al., 2012). To confirm the disruption of the gene of interest, we measured transcript  
144 expression by qRT-PCR using primers downstream of the insertion site and confirmed that most  
145 GAL4 lines are loss-of-function alleles. For example, in homozygous viable GAL4 lines, *DIP- $\alpha$ -*  
146 *GAL4* and *DIP- $\zeta$ -GAL4* showed no detectable *DIP- $\alpha$*  and *DIP- $\zeta$*  mRNA, respectively (Figure 1 –  
147 figure supplement 1A) suggesting they are null alleles. Several GAL4 lines, like *DIP- $\beta$ -GAL4*  
148 and *dpr15-GAL4*, showed a reduction in mRNA levels, whereas some lines like *DIP- $\iota$ -GAL4* and  
149 *dpr16-GAL4* showed no change in mRNA expression. Although these GAL4 lines do not show a  
150 significant loss of transcription, the T2A sequence should still disrupt translation and generate  
151 mutant proteins. For homozygous lethal lines, we examined mRNA levels in heterozygous  
152 animals and found most GAL4 lines show expression near 50% (Figure 1 – figure  
153 supplementary 1B), suggesting these GAL4 lines are severe loss-of-function alleles. The qRT-  
154 PCR results are summarized in Table 2. In summary, approximately 70% of the insertions  
155 cause a severe disruption of transcription.

156 Because most GAL4 insertions are mutants, we used heterozygotes to map *dpr* and *DIP*  
157 expression. Loss of a single copy of any *dpr* or *DIP* did not affect gross viability, cell survival, or  
158 synaptic connectivity in heterozygotes as revealed by postsynaptic marker, Discs Large (DLG)  
159 and presynaptic marker, anti-horseradish peroxidase (HRP; a marker for all neuronal  
160 membranes (Jan and Jan, 1982)) (see Methods and Materials). Thus, the *dpr/DIP-GAL4* driver  
161 lines should faithfully report the cells that express *dprs* and *DIPs* (Lee et al., 2018; Nagarkar-  
162 Jaiswal et al., 2015a).

163

### 164 **Expression of *dprs* and *DIPs* in MNs**

165 The larval body wall is segmented, and each abdominal hemisegment consists of 30 muscles  
166 that are grouped into three major muscle groups – ventral, lateral, and dorsal (Figure 1B) (Bate,  
167 1990; Hooper, 1986; Zarin et al., 2019). Innervating those muscles are 33 MNs classified as  
168 type-I (29), type-II (3) and type-III (1) based on their terminal morphology and neurotransmitter  
169 type (Choi et al., 2004; Hoang and Chiba, 2001; Landgraf et al., 1997; Zarin et al., 2019). All MN  
170 axon terminals contain strings of bead-like structures called boutons which house the active  
171 zones. Type-I MNs are excitatory glutamatergic neurons, and they are further subdivided into  
172 type-I big (Ib) and type-I small (Is) due to their bouton size and innervation patterns: Ib MNs (in  
173 the larva named MN1-Ib to MN30-Ib corresponding to the muscle number) generally have larger  
174 boutons and innervate single muscle fibers whereas Is MNs have smaller boutons and innervate  
175 muscle groups (Choi et al., 2004; Lnenicka and Keshishian, 2000). The Is MN that innervates  
176 ventral muscles is referred to as the ventral common exciter (vCE), RP5, or MNISNb/d-Is, and  
177 the Is MN that innervates dorsal muscles is called the dorsal common exciter (dCE), RP2, or  
178 MNISN-Is (Broadus et al., 1995; Doe et al., 1988; Takizawa et al., 2007). Similarly, three  
179 neuromodulatory type-II MNs innervate the ventral, lateral, and dorsal muscle groups, and the  
180 single type-III MN primarily innervates m12 (Hoang and Chiba, 2001; Schmid et al., 1999).  
181 Based on these distinguishing features – terminal morphology and innervation patterns – we  
182 can unambiguously identify MNs that express each *dpr* and *DIP*.

183 To examine the expression of *dprs* and *DIPs* in MNs, we first crossed each GAL4 line to  
184 a fluorescent reporter line and monitored reporter expression at third instar NMJs (Figure 2A).  
185 GAL4 lines derived from MiMIC insertions were crossed to a GFP reporter, whereas CRIMIC  
186 GAL4 lines were crossed to an mCherry reporter as CRIMIC insertions carry a 3XP3-GFP  
187 marker that expresses in glial cells and the lateral bipolar dendrite (lbd) neuron (Figure 2 –  
188 figure supplement 1). To identify all NMJs, we labeled preparations with antibodies against DLG  
189 and HRP and confirmed that the gross muscle innervation was normal in *dpr/DIP-GAL4*  
190 heterozygous lines. GFP or RFP labeling of NMJs revealed the corresponding MNs that express  
191 each *dpr* and *DIP*. We followed this pipeline for each *dpr/DIP-GAL4* to record expression in all  
192 MNs.

193 We mapped the expression of *dprs* and *DIPs* in all larval MNs. The expression of GAL4  
194 and the fluorescent reporter should correlate with the endogenous gene expression. In prior  
195 work, we observed expression of *dpr6*, *dpr10*, *dpr11*, *DIP- $\alpha$* , and *DIP- $\gamma$*  in MNs (Ashley et al.,  
196 2019; Carrillo et al., 2015). Here, we confirmed these expression patterns; for example, *DIP- $\alpha$*   
197 was selectively expressed in Is MNs but not in Ib MNs (Figure 2 – figure supplement 2A). Our  
198 data also revealed that several *dprs* and *DIPs* are not always expressed at the same level in a

199 specific MN. For example, *DIP- $\delta$ -GAL4* only labeled 22% of abdominal MN12-Ib (Figure 2 –  
200 figure supplement 2B and 2C). Additionally, some *dprs* and *DIPs* are expressed in a gradient  
201 along the anterior to posterior axis. For example, *dpr2* showed high expression in MN1-Ib in the  
202 anterior but became undetectable from abdominal segment 4 (A4) to the posterior (Figure 2B).  
203 *DIP- $\zeta$ -GAL4*, on the other hand, labeled anterior MN16/17-Ib weakly (also known as  
204 MN15/16/17-Ib from (Hoang and Chiba, 2001; Kim et al., 2009)) but was much stronger in the  
205 posterior (Figure 2C). Note that *dpr2* also has a variable expression in MN9-Ib (Figure 2B).  
206 These complex expression patterns suggest intricate regulatory mechanisms of *dprs* and *DIPs*.

207 Work from our lab and others suggested that *Dprs* and *DIPs* are synaptic recognition  
208 molecules (Ashley et al., 2019; Bornstein et al., 2021; Carrillo et al., 2015; Courgeon and  
209 Desplan, 2019; Menon et al., 2019; Venkatasubramanian et al., 2019; Xu et al., 2021, 2018). In  
210 the fly neuromuscular circuit, MN axons explore the musculature field beginning in embryonic  
211 stage 14 and synaptic markers are observed in stage 16 (Yoshihara et al., 1997). A traditional  
212 UAS reporter expression in third instar larva will only report real-time expression and will not  
213 reveal if a *dpr* or *DIP* is temporally expressed earlier in development. To capture the temporal  
214 expression patterns of *dprs* and *DIPs*, we utilized a permanent labeling reporter to constantly  
215 label the GAL4-expressing neuron (Figure 2A). This method takes advantage of the FLP-out  
216 system to remove a stop codon within two FRT sites and activate an *actin-GAL4* to maintain  
217 GAL4 expression in any cells that expressed the gene of interest GAL4. Interestingly, we  
218 observed only a few *dprs* and *DIPs* that are temporally expressed in MNs. For example,  
219 MN21/22-Ib is not labeled when *dpr9-GAL4* is crossed to *UAS-GFP*, but with the permanent  
220 labeling reporter, the same neuron showed strong expression (Figure 2D). It is noteworthy that  
221 the CRIMIC cassettes are excisable by Flippase as well due to the presence of flanking FRT  
222 sites (Figure 1A). However, because of the activation of the permanent *actin-GAL4*, the excision  
223 of CRIMIC cassettes does not pose a technical issue.

224 We summarized the expression of *dprs* and *DIPs* in all MNs in Figure 3. Here, we  
225 included variable expression patterns (defined by how frequent a cell expresses the reporter)  
226 and gradient and temporal expression patterns. Criteria for each expression category is  
227 described in the Methods and in Figure 2 – figure supplement 3. In general, *dprs* are expressed  
228 in many MNs while *DIPs* are expressed much more selectively. Each MN expresses at least  
229 one *DIP*, and overall, each MN has a unique *dpr* and *DIP* expression signature. For example,  
230 we found that additional *DIPs* (*DIP- $\gamma$* , *- $\epsilon$* , *- $\zeta$* , *- $\eta$* , *- $\theta$* , *- $\kappa$* ) are also expressed in *Is* MNs.  
231 Interestingly, *DIP- $\epsilon$*  is expressed only in the ventral *Is* (vCE) whereas *DIP- $\eta$*  is expressed only in

232 the dorsal lS (dCE) (Figure 3). Taken together, we generated a *dpr/DIP* expression map in all  
233 larval MNs and found that each MN expresses a unique subset.

234

### 235 **Expression of *dprs* and *DIPs* in SNs**

236 Next, we examined expression of *dprs* and *DIPs* in larval SNs using similar approaches. Two  
237 morphologically distinct types of SNs can be classified in the larval body wall, and they project  
238 their axons to the VNC (Figure 1B) (Orgogozo and Grueber, 2005; Veling et al., 2019). Type-I  
239 SNs project a single dendrite that associates with chordotonal (ch) organs or external sensory  
240 (es) organs to detect mechanical and chemical stimuli. Type-II SNs are multidendritic neurons  
241 that transmit proprioceptive information. Type-II SNs can be further classified into bipolar  
242 dendrite (bd) neurons, tracheal dendrite (td) neurons, and dendritic arborization (da) neurons.  
243 The da neurons are then subdivided into four classes based on the complexity of their dendrite  
244 morphology (da-I, da-II, da-III and da-IV) (Figure 1B) (Grueber et al., 2002). SNs from the same  
245 class are uniformly distributed in the body wall into four regions: ventral, ventral', lateral, and  
246 dorsal. The distribution of SNs enables the larva to respond to different stimuli across its entire  
247 body.

248 To examine expression of *dprs* and *DIPs* in SNs, we labeled larvae with anti-HRP to  
249 locate the cell bodies of SNs. The *dpr/DIP* expression map in all SNs is shown in Figure 4.  
250 Similar to MNs, *DIPs* are more sparsely expressed in SNs compared to *dprs* which are broadly  
251 expressed. However, several *dprs* (*dpr14*, *dpr15*, and *dpr17*) are only expressed in a subset of  
252 SNs, unlike their broad expression pattern in MNs. We also observed that some *dprs* and *DIPs*  
253 are temporally expressed. For example, the dorsal da neurons (ddaA, C, F and D) are labeled  
254 when *dpr5-GAL4* is crossed to the permanent labeling reporter, but not in *dpr5-GAL4>UAS-*  
255 *GFP* animals (Figure 4 – figure supplement 1).

256 Taken together, we generated expression data for *dprs* and *DIPs* in SNs (Figure 4) and  
257 showed that each SN expresses a unique subset of *dprs* and *DIPs*, providing support for their  
258 roles as identification tags.

259

### 260 **SNs in the same class express similar subsets of *dprs* and *DIPs***

261 Larval SNs can be divided into types based on their morphology and function, including ch, es,  
262 bd, td and da neurons. Although SNs from the same type are distributed throughout the body  
263 wall and project their afferent axons through different trajectories, their axon terminals innervate  
264 the same region in the VNC and contact common interneuron partners (Grueber et al., 2007;  
265 Landgraf et al., 2003b; Merritt and Murphey, 1992; Murphey et al., 1989). For example, the



266 ventral, ventral', and lateral mechanosensory ch neurons project to the ventral medial region of  
267 the VNC and share synapses with several interneurons, including the Basin, Ladder, Griddle,  
268 Drunken and Even-skipped interneurons (Heckscher et al., 2015; Valdes-Aleman et al., 2021).  
269 Similarly, different classes of da neurons innervate unique sections of the VNC (Grueber et al.,  
270 2007; Merritt and Whittington, 1995; Schrader and Merritt, 2000). Overall, these innervation  
271 patterns suggest that some SNs share synaptic recognition cues while others have distinct  
272 cues.

273 Each SN projects a single axon into the VNC to synapse with postsynaptic targets but  
274 lacks a presynaptic neuron. Thus, SNs from the same class may share similar identification tags  
275 to wire with common interneurons. Dprs and DIPs have been implicated in synaptic partner  
276 recognition so we hypothesized that shared *dpr/DIP* expression may be utilized by the same  
277 type/class of neurons to instruct synaptic specificity. To test this model, we generated an  
278 unbiased hierarchical clustering of SNs based on their *dpr/DIP* expression map (Figure 5A).  
279 Surprisingly, we found a high correlation between SN types/classes and the expression of *dprs*  
280 and *DIPs*. For example, most es neurons are grouped together, as well as all ch neurons,  
281 indicating that these two subclasses of type-I SNs can be distinguished by their expression of  
282 *dprs* and *DIPs*. Similarly, subclasses of da neurons are clustered separately. We found that da-I  
283 neurons are identifiable by expression of *DIP- $\theta$*  and the lack of *dpr2*, *dpr6*, *dpr9*, *dpr11*, *dpr13*,  
284 and da-II/da-III neurons are grouped by expression of *dpr2* and *dpr18*, and the lack of *dpr9*  
285 (Figure 5A). These results suggest that SNs in the same type/class may utilize similar sets of  
286 *dprs* and *DIPs* to recognize their common interneuron targets.

287

### 288 **Expression of *dprs* and *DIPs* is more diversified in MNs**

289 Next, we examined if MNs that project to the same muscle groups also share the same  
290 expression patterns of *dprs* and *DIPs*. Muscles are grouped into three main spatial and  
291 functional groups – ventral, lateral, dorsal – and further divided into six subgroups based on  
292 their orientation – dorsal longitudinal (DL), dorsal oblique (DO), ventral longitudinal (VL), ventral  
293 oblique (DO) ventral acute (VA), and lateral transverse (LT) (Figure 1B) (Bate, 1990; Hooper,  
294 1986; Zarin et al., 2019). Each muscle is normally innervated by one Ib MN and previous  
295 studies showed that Ib MNs innervating a muscle group project their dendrites to the same  
296 region in the VNC neuropil where they receive input from common premotor interneurons  
297 (PMNs) (Kim et al., 2009; Landgraf et al., 2003a, 1997; Landgraf and Thor, 2006; Mauss et al.,  
298 2009; Zarin et al., 2019). These connectivity patterns enable coordinated contraction waves that  
299 underlie larval locomotion. Thus, if Ib MNs of the same muscle group share common PMN

300 partners, they may share similar wiring molecules. We generated an unbiased hierarchical  
301 clustering based on expression of *dprs* and *DIPs* for all MNs (Figure 5B). Type-I, type-II and  
302 type-III MNs form independent clusters and are distinct from Ib MNs. For example, *DIP- $\alpha$* , *DIP- $\zeta$* ,  
303 *dpr6* and *dpr16* are expressed in type-I MNs, and lateral and dorsal type-II MNs are identified  
304 by the lack of *DIP- $\kappa$* , *dpr15*, *dpr17* and the expression of *dpr3* and *dpr16* (Figure 5B). However,  
305 within Ib MNs, only the MNs innervating LT and DL muscles are clustered together, whereas the  
306 other MNs appear randomly distributed. These results suggest that based on the expression  
307 patterns of *dprs* and *DIPs*, MNs can be clustered by their type, but Ib MNs cannot be further  
308 clustered by the muscles they innervate. MNs must identify not only their presynaptic inputs, but  
309 also their distinct postsynaptic partners. The combination of pre- and postsynaptic partnerships  
310 may explain the inability to cluster Ib MNs based on their expression patterns of *dprs* and *DIPs*.  
311 Therefore, more complex identification codes may be necessary for MNs to distinguish both pre-  
312 and postsynaptic partners.

313

#### 314 ***dpr/DIP* expression maps reveal additional MNs**

##### 315 ***Alary muscle MN***

316 In addition to the muscles required for larval locomotion, larvae have another segmentally  
317 repeated muscle – the alary muscle – that attaches to the trachea along the larval heart tube  
318 (Bataillé et al., 2015). Although the morphological and functional properties of the alary muscle  
319 have been examined (Boukhatmi et al., 2014), the development, connectivity, and functional  
320 properties of the MN that innervates alary muscles are still lacking. The alary muscle MN axon  
321 resides in the transverse nerve (TN) and projects along m8 towards the alary muscle. Here, we  
322 mapped the expression of *dprs* and *DIPs* in the alary muscle MN. As previously observed, the  
323 dendrite of the lbd neuron travels in parallel with the alary muscle MN axon within the TN  
324 (Gorczyca et al., 1994; Macleod et al., 2003; Thor and Thomas, 1997). Thus, if a *dpr* or *DIP* is  
325 expressed in both the alary muscle MN and lbd, we would be unable to distinguish them in the  
326 nerve. Therefore, we monitored the co-localization of DLG and the fluorescent reporter on the  
327 alary muscle to unambiguously assign expression (Figure 3 – figure supplement 1). We  
328 observed that alary muscle MN NMJs share features of type-I boutons including the size and  
329 DLG labeling surrounding the boutons (type-I boutons are surrounded by significant DLG (Guan  
330 et al., 1996)). Using the same criteria described for MNs and SNs, we found one *DIP* and many  
331 *dprs* that are expressed in the alary muscle MN, including *DIP- $\kappa$* , *dpr4*, and *dprs7-19*. These  
332 expression data and driver lines will facilitate future characterization of this MN.

333

334 **MN23-Ib**

335 Most Ib MNs have a single muscle target. However, some Ib MNs innervate two muscles in  
336 close proximity, likely due to shared recognition cues. For example, a previous study found that  
337 Ib MNs innervating the lateral muscles can synapse with neighboring muscles and thus named  
338 these neurons MN21/22-Ib, MN22/23-Ib, and MN23/24-Ib (Figure 6A) (Hoang and Chiba, 2001).  
339 These innervation patterns were later confirmed by MARCM analysis (Kim et al., 2009).

340 In our analyses, we observed that m23 has several Ib NMJ branches and m24 has only  
341 one NMJ (Figure 6B). While a single MN can form several branches on a muscle, we found  
342 some *dpr/DIP-GAL4s* that only label one Ib branch on m23 and no other branches on lateral  
343 muscles (Figure 6C). These data suggest the existence of an additional MN that solely  
344 innervates m23, and we named it MN23-Ib. The bouton size and DLG labeling intensity of M23-  
345 Ib boutons indicates that it is a type-Ib NMJ. *DIP-β* and *DIP-κ* are expressed in MN23-Ib and not  
346 in the nearby MN22/23-Ib and MN23/24-Ib (Figure 6C). Note that MN23/24-Ib forms long, linear  
347 Ib NMJs on the underside of m23 before it reaches m24 (Figure 6A and 6C). We also found that  
348 *dpr5* was expressed in MN23/24-Ib and nearby MN22/23-Ib, but not in MN23-Ib (Figure 6D),  
349 providing further evidence for an additional Ib MN solely innervating m23. Additional *dprs* and  
350 *DIPs* are expressed in both MN23-Ib and MN23/24-Ib (Figure 6B). Thus, we describe a  
351 previously unidentified Ib MN that innervates m23.

352

353 **MN6-Ib and MN7-Ib in A2**

354 Another example of a dual-targeting Ib MN is MN6/7-Ib (also known as RP3 in the embryo)  
355 (Schmid et al., 1999; Sink and Whitington, 1991a, 1991b). The innervation pattern of MN6/7-Ib  
356 was initially identified by dye fill labeling and MARCM (Hoang and Chiba, 2001; Kim et al., 2009;  
357 Sink and Whitington, 1991b). Due to the ease of accessibility of m6 and m7, MN6/7-Ib is  
358 extensively used for studies of synaptic connectivity, synaptic growth, and synaptic  
359 homeostasis.

360 Based on these previous studies, we predicted that if a *dpr* or *DIP* were expressed in  
361 MN6/7-Ib, the Ib NMJs on both m6 and m7 would be completely fluorescently labeled (Figure  
362 7A, right). Surprisingly, in A2, we observed several *dpr/DIP-GAL4s* that are expressed in Ib MNs  
363 that have large NMJs on m6 and others that are expressed mainly in the Ib NMJs on m7 (Figure  
364 7A, left). For example, *DIP-β*, *DIP-γ* and *DIP-ε* were expressed in a MN that mainly innervates  
365 m6 (Figure 7B), whereas *dpr15* was expressed in a MN that mainly innervates m7 (Figure 7C).  
366 These expression patterns suggested that two Ib MNs innervate m6 and m7 in A2. Hereafter,  
367 we named these MNs as MN6-Ib and MN7-Ib. Prior studies hinted at the possibility of two MNs

368 based on the larger synaptic terminal area on m6/7 in A2 compared to A3-A6 (Lnenicka and  
369 Keshishian, 2000). However, it was thought that the larger synaptic area was due to a large  
370 NMJ from a single Ib MN. In the larval neuromuscular circuit, the number of boutons reflect the  
371 size of the NMJ. We quantified the m6 and m7 Ib NMJs and observed a significantly larger arbor  
372 in A2 compared to A3 (Ib NMJ on m6: 34.2 on A2 and 18.5 on A3; Ib on m7: 23.1 on A2 and  
373 11.7 on A3) (Figure 7 – figure supplement 1). Taken together, m6 and m7 in A2 are innervated  
374 by two Ib MNs.

375

### 376 **Characterization of MN6-Ib and MN7-Ib**

377 A recent study reported a GAL4 driver (*GMR79H07-GAL4*) that labels MN6-Ib in A2 (Aponte-  
378 Santiago et al., 2020). We tested this driver and confirmed MN6-Ib expression; however, it  
379 sometimes labels MN7-Ib NMJs or both MN6-Ib and MN7-Ib, suggesting that this reporter is not  
380 specific to MN6-Ib (Figure 8 – figure supplement 1). We examined MN6-Ib and MN7-Ib further to  
381 better understand their innervation patterns and dendritic projections. Interestingly, we found  
382 that MN6-Ib and MN7-Ib preferentially innervate their corresponding muscle, but sometimes,  
383 these MNs also form minor NMJs on the neighboring muscle (Figure 7A). Next, we monitored  
384 the frequency of dual innervation of each MN using *GMR79H07-GAL4* and found that 68.2% of  
385 MN6-Ib and 72.7% of MN7-Ib innervate both muscles (Figure 8A). We also determined the size  
386 of each NMJ by counting Ib boutons and found that on average MN6-Ib forms 48.6 boutons on  
387 m6 and 5.9 boutons on m7, while MN7-Ib forms 3.1 and 30.8 boutons on m6 and m7,  
388 respectively (Figure 8B).

389 MN6/7-Ib (RP3) is derived from neuroblast 3-1 (NB3-1) (Schmid et al., 1999; Sink and  
390 Whittington, 1991a, 1991b). To visualize the dendritic projections of MN6-Ib and MN7-Ib in A2,  
391 we examined their cell body position in the VNC and dendrite morphology using a pan MN  
392 driver, *OK6-GAL4*, with multi-color FLP-out (MCFO) (Nern et al., 2015). We found that the cell  
393 bodies of MN6-Ib and MN7-Ib are both localized at the dorsal neuropil and project axons to the  
394 contralateral hemisegment. They also extend a small dendritic arbor to the ipsilateral side  
395 (Figure 8C-F). These features are shared with RP3 (MN6/7-Ib) (Kim et al., 2009). These data  
396 suggest that these two MNs likely both originated from NB3-1. Overall, we identified and  
397 confirmed the presence of two Ib MNs in A2 that preferentially innervate m6 or m7.

398

### 399 **Expression of *dprs* and *DIPs* in the glial cells**

400 In the *Drosophila* larval peripheral nervous system, glia plays important roles in neuronal  
401 development, axon path finding, and synaptic homeostasis (Bittern et al., 2020; Yildirim et al.,

402 2019). In segmental nerves, sub-perineural glia and wrapping glia form extensive interactions  
403 with the axons (Kottmeier et al., 2020). Therefore, we examined the glial expression of *dprs* and  
404 *DIPs*.

405 In the previous analyses using UAS-GFP and the permanent labeling reporter, we were  
406 unable to unambiguously distinguish glial and neuronal expression due to labeling of the MN  
407 axons in each segmental nerve. Therefore, to probe glial expression, we crossed each *dpr/DIP-*  
408 *GAL4* line with a nuclear reporter line, G-TRACE (Evans et al., 2009). The G-TRACE system  
409 utilizes FLP-FRT and GAL4-UAS to report both temporal and real-time gene expression (Figure  
410 9A). If a GAL4 is transiently expressed, then cell nuclei will be GFP positive. However, if the cell  
411 nuclei are labeled by both GFP and RFP, this may suggest the GAL4 is consistently expressed.  
412 We crossed each *dpr/DIP-GAL4* line to the G-TRACE reporter and found that *dpr1* is expressed  
413 in glia (Figure 9-figure supplement 1). Additionally, *dpr1* expression is highly dynamic since  
414 some glia temporarily express *dpr1* while others maintain *dpr1* expression. Overall, *dpr1* was  
415 only expressed in a subset of glia, and the restricted expression of *dprs* and *DIPs* suggest that  
416 these CSP subfamilies have limited roles in glial cells.

417

#### 418 **Expression of *dprs* and *DIPs* in muscles**

419 In a previous study, we observed *dpr10* expression in ventral and dorsal muscles and its  
420 interacting partner, *DIP- $\alpha$* , in Is MNs (Ashley et al., 2019). Loss of Dpr10-DIP- $\alpha$  interactions  
421 cause a complete loss of Is MN innervation on m4 while other Is NMJs were unaffected,  
422 suggesting that other pairs of synaptic recognition molecules are involved in Is MN-muscle  
423 recognition (Ashley et al., 2019). To examine the role of other *dprs* and *DIPs* in this process, we  
424 mapped their expression in muscles utilizing the G-TRACE system to label muscle nuclei. We  
425 first confirmed expression of *dpr10* in all longitudinal muscles, but not in oblique or transverse  
426 muscles (Figure 9B,C). This expression pattern suggests distinct transcriptional regulation  
427 programs between muscle groups (Bate, 1990). In some hemisegments, a small subset of  
428 muscles, such as m5 and m8, showed inconsistent expression of *dpr10* (Figure 9B, arrow).  
429 Also, all muscle nuclei co-labeled with GFP and RFP in *dpr10-GAL4>G-TRACE* (Figure 9B),  
430 suggesting that *dpr10* expression is maintained throughout larval development.

431 We examined other *dprs* and *DIPs* and found that *dpr19* is expressed in all muscles  
432 (Figure 9D), including the oblique and transverse muscles (Figure 9E). Unlike *dpr10*, most  
433 muscle nuclei in *dpr19-GAL4>G-TRACE* are only GFP positive, suggesting that *dpr19* is  
434 temporally expressed and turned off in late larval stages. To support this temporal expression,  
435 we examined *dpr19-GAL4>mCherry* first instar larva and observed high level of muscle

436 expression, which we did not observe in third instar (Figure 9 – figure supplement 2). Taken  
437 together, we showed that muscles express many fewer *dprs* and *DIPs* compared to motor and  
438 sensory neurons (Figure 9F). These results suggest that a subset of *dprs* and *DIPs* may  
439 function in the MN-muscle recognition and others in PMN-MN recognition.

## 440 **Discussion**

441 Dprs and DIPs play important roles in nervous system development. To date, only a small  
442 subset of Dpr-DIP pairs has been examined, including Dpr11-DIP- $\gamma$ , Dpr6/10-DIP- $\alpha$ , and Dpr12-  
443 DIP- $\delta$ . These CSP pairs were implicated in synaptic recognition (Ashley et al., 2019; Bornstein  
444 et al., 2021; Venkatasubramanian et al., 2019; Xu et al., 2019, 2018), neuronal survival  
445 (Courgeon and Desplan, 2019; Menon et al., 2019; Xu et al., 2021), and synaptic growth  
446 (Carrillo et al., 2015). Furthermore, Dprs and DIPs are widely expressed across many neural  
447 circuits. Several labs have utilized the GAL4/UAS system to visualize expression of *dprs* and  
448 *DIPs* in olfactory neurons (Barish et al., 2018), adult leg MNs and SNs (Venkatasubramanian et  
449 al., 2019), optic lobe neurons (Cosmanescu et al., 2018), and fru P1 neurons (Brovero et al.,  
450 2021). While these studies revealed unique *dpr/DIP* expression in the respective neurons, the  
451 depth of the expression map was limited due to the less complete GAL4 collection at the time,  
452 and some studies only focused on a global expression pattern without characterization of  
453 individual cell types.

454 Here, we reported a collection of GAL4 enhancer trap lines for all *DIPs* and 19 *dprs*, and  
455 examined their expression in larval MNs, SNs, peripheral glia, and muscles. Interestingly, each  
456 neuron expresses a unique combination of *dprs* and *DIPs*. We also found that many *dprs* and  
457 *DIPs* are expressed in patterns including different expression levels, in anterior-posterior  
458 gradients, and temporal expression. Surprisingly, our expression analysis also revealed  
459 previously uncharacterized larval MNs that differentially express *dprs* and *DIPs*. Finally, we  
460 showed that *dpr10* and *dpr19* are expressed in muscles, suggesting that additional Dpr-DIP  
461 interactions may instruct MN-muscle recognition. The *dpr/DIP* expression map identified here,  
462 along with the GAL4 lines that are also hypomorphs or loss-of-function alleles, will facilitate  
463 examination of Dpr-DIP interactions in development of motor, sensory, and many other circuits.

464

### 465 **Using the *dpr/DIP* code to annotate single cell RNA sequencing data**

466 Recent advances in single cell RNA sequencing (scRNAseq) provide a powerful, high-  
467 throughput approach to identify large scale gene expression patterns. Various *Drosophila* neural  
468 tissues have been analyzed by scRNAseq, including the adult brain (Davie et al., 2018), optic  
469 lobe (Konstantinides et al., 2018), adult VNC (Allen et al., 2020; Genovese et al., 2019), larval  
470 brain (Avalos et al., 2019), eye disc (Ariss et al., 2018) and the larval VNC (Nguyen et al., 2021;  
471 Vicidomini et al., 2021). Most studies report the transcriptome of large cell clusters of MNs,  
472 ganglion cells, neuroblasts, and glial cells due to the difficulty of matching single cell reads to a  
473 specific cell type and identity, impeding detailed analyses from scRNAseq data.

474 One method to deconvolve these large cell clusters is to sort cells before performing  
475 scRNAseq. For example, many labs have used the GAL4/UAS system to label and sort  
476 olfactory neurons (Li et al., 2020; McLaughlin et al., 2021; Xie et al., 2021), T cells in the fly  
477 visual system (Hörmann et al., 2020; Kurmangaliyev et al., 2020), eye-antennal discs cells  
478 (González-Blas et al., 2020) or NBs (Michki et al., 2021). On the other hand, researchers may  
479 also use the scRNAseq data to identify specific drivers, and then identify which neuron  
480 expresses this driver (Li, 2020). However, this approach reduces the scale because only a few  
481 cell types can be identified in this manner. Utilizing the expression of a gene family known to be  
482 differentially expressed within a specific subset of cells can provide a more complete  
483 examination. This expression map would generate a cell-specific atlas to annotate clusters in  
484 scRNAseq data. Here, we utilized the GAL/UAS system and showed that each MN and SN has  
485 a unique expression pattern of *dprs* and *DIPs*. Because the GAL4 is inserted in coding regions,  
486 it should capture all regulatory mechanisms and faithfully report the expression of the  
487 corresponding endogenous mRNA (Nagarkar-Jaiswal et al., 2015a, 2015b). Thus, our *dpr/DIP*  
488 expression could serve as a map to identify individual MNs from a MN cluster in a larval VNC  
489 sample (Nguyen et al., 2021; Vicidomini et al., 2021). In addition to *dprs* and *DIPs*, other CSP  
490 subfamilies have been reported in several scRNAseq datasets, suggesting that expression  
491 maps of other subfamilies and even combinations of subfamilies can be utilized to refine cell  
492 types in datasets (Kurmangaliyev et al., 2020; Ma et al., 2021; Xie et al., 2021).

493

#### 494 **Insights from *dpr/DIP* expression maps to functional studies**

495 The goal of developing expression maps for *dprs* and *DIPs* in MNs and SNs is to instruct the  
496 functional study of Dpr-DIP interactions. Here, we discuss potential directions based on our  
497 expression map that may serve as an entry point for future research. First, for sparsely  
498 expressed *dprs* and *DIPs*, one can use reverse genetics to analyze loss-of-function phenotypes.  
499 Most sparsely expressing *dpr/DIP-GAL4* lines are homozygous viable and hypomorphs,  
500 facilitating their use in examining phenotypes. Some *dpr/DIP-GAL4* lines are embryonic lethal,  
501 suggesting that important developmental processes are perturbed upon loss of specific *dprs* or  
502 *DIPs*. However, in these lines, we cannot rule out second site mutations so that will need to be  
503 further explored.

504 Another way to approach the function of Dpr-DIP interaction is focusing on the  
505 commonly or differentially expressed *dprs* and *DIPs*. For example, hierarchical clustering  
506 analyses of SNs grouped SNs from the same class together based on the expression of *dprs*  
507 and *DIPs*, suggesting that similar SNs have common *dprs* and *DIPs*. Since SNs from the same



508 class share some downstream synaptic partners and expression of *dprs* and *DIPs*, these  
509 common CSPs may instruct recognition between SNs and interneurons. Future studies can  
510 determine the *dpr/DIP* expression maps in the downstream interneurons to identify synaptic  
511 partners that express cognate Dpr-DIP pairs. Instead of commonly expressed genes,  
512 differentially expressed *dprs* and *DIPs* in similar projecting neurons can shed light on  
513 connectivity mechanisms. For example, MN6-Ib and MN7-Ib identified in this study have similar  
514 morphology and innervation patterns, but with a preference for m6 and m7, respectively. One  
515 interesting question is how these neurons distinguish their muscle targets to generate such  
516 preference. Based on the expression map, MN6-Ib and MN7-Ib co-express a large subset of  
517 *dprs* and *DIPs*, but *DIP-β*, *DIP-γ*, *DIP-ε* and *dpr15* are selectively expressed. These differentially  
518 expressed genes are excellent candidates to explore the recognition mechanism of these MNs.  
519 Similar approaches can be adapted to other MNs that innervate neighboring muscles, including  
520 MN23-Ib and MN23/24-Ib or the dorsal MNs – MN9-Ib, MN10-Ib, MN1-Ib and MN2-Ib.

521 The Dpr-DIP interactome (Carrillo et al., 2015; Cosmanescu et al., 2018; Özkan et al.,  
522 2013) revealed promiscuity in the interactions and our expression maps showed that many cells  
523 co-express many *dprs* and *DIPs*, suggesting redundant mechanisms for synaptic recognition.  
524 Several subfamilies of CSPs are implicated in recognition, but loss-of-function mutants rarely  
525 are 100% penetrant. For example, loss of Teneurin signaling causes a 90% decrease of MN3-Ib  
526 innervation (Hong et al., 2012), and *Toll* null mutants revealed defects in 35% of MN6/7-Ib  
527 (Rose et al., 1997). These data suggested other CSPs are required in the recognition between  
528 MNs and their respective muscles. Similarly, *DIP-α* is expressed in the dorsal Is MN innervating  
529 multiple muscles, but *DIP-α* mutants only completely lose innervation of m4, suggesting that  
530 additional CSPs are required for recognition of other muscles (Ashley et al., 2019). Utilizing the  
531 *dpr/DIP* expression maps, co-expressed *dprs* and *DIPs* can be simultaneously knocked out to  
532 examine redundancy. Also, by combining the expression maps with scRNAseq data, additional  
533 CSPs can be identified to examine redundancy between CSP subfamilies.

534

### 535 **CSP expression patterns in the fly nervous system**

536 CSPs can serve several functions in nervous system development including molecular codes for  
537 partner recognition and self-avoidance. Based on these functions, the expression of CSPs could  
538 be deterministic to instruct stereotyped synaptic connectivity or stochastic to avoid dendritic  
539 overlap and self-synapses. Thus, CSP expression patterns can suggest function. In our study,  
540 we showed that many *dprs* and *DIPs* are robustly expressed in SNs and MNs. For example,  
541 *DIP-α* and *DIP-ζ* are expressed in Is MNs across all segments (Figure 3). Several studies have

542 implicated other CSPs in motor neuron-muscle specificity, and their expression patterns are  
543 also robust and limited to subsets of cells. For example, Capricious is expressed in MN12-Ib  
544 and some dorsal MNs (Nose, 2012; Shishido et al., 1998), and Connectin is expressed in  
545 MN27-Ib and MN29-Ib (Nose et al., 1997, 1992). Capricious and Connectin are also expressed  
546 in a unique subset of muscles. Loss-of-function and gain-of-function approaches revealed  
547 neuromuscular wiring defected, suggesting that the robust expression of Capricious and  
548 Connectin in corresponding MNs and muscles instruct synaptic partner recognition.

549 On the other hand, some CSPs are stochastically expressed in subsets of cells. For  
550 example, probabilistic splicing of *Dscam1* generates random isoform expression in SNs to  
551 mediate dendritic self-avoidance by inhibitory homophilic interactions (Miura et al., 2013).  
552 Interestingly, we found that many *dprs* and *DIPs* are also stochastically expressed in MNs and  
553 SNs. We showed that *DIP-β* is not always expressed in dorsal da neurons (*ddaC*, *ddaD*, *ddaE*  
554 and *ddaF*) (Figure 4). Such irregular expression patterns may suggest additional functions of  
555 *dprs* and *DIPs* in circuit formation.

556 In this study, we also uncovered some *dprs* and *DIPs* that are expressed in a gradient  
557 along the anterior to posterior axis. Such patterns are reminiscent of the expression of several  
558 Hox genes in the VNC. For example, *Ubx* and *Abd-A* are highly expressed in anterior segments  
559 whereas *Abd-B* is mainly in the posterior (Estacio-Gómez and Díaz-Benjumea, 2013; Meng and  
560 Heckscher, 2020). These similar expression gradients suggest that gradient transcriptional  
561 factors may set up segment cues through *dprs* and *DIPs*.

562

### 563 ***dpr/DIP-GAL4* collection to enable neuron identification and manipulation**

564 The map of *Drosophila* MNs and SNs was established decades ago using dye backfills  
565 (Broadus et al., 1995; Hoang and Chiba, 2001; Landgraf et al., 2003b). However, fluorescent  
566 dyes have some technical limitations since they do not always flow into every terminal structure,  
567 which may have resulted in some neurons being overlooked. In this study, we used a genetic  
568 approach to probe individual neurons and revealed three uncharacterized MNs – MN23-Ib,  
569 MN6-Ib (A2) and MN7-Ib (A2). Surprisingly, MN6-Ib and MN7-Ib have similar morphologies and  
570 dual innervation patterns with a preference for m6 or m7, respectively. These data suggest  
571 similar but distinct mechanisms that allow these neurons to recognize their synaptic partners.

572 In addition, the GAL4 lines in this study provide genetic access to manipulate subsets of  
573 neurons. In the *Drosophila* motor circuit, several studies have identified reporters that are  
574 expressed in subsets of motor neurons, muscles, and interneurons (Aponte-Santiago et al.,  
575 2020; Li et al., 2014; Pérez-Moreno and O’Kane, 2018; Wang et al., 2021). However, the

576 coverage of these reporters is very limited (i.e. only a small number of cells can be targeted).  
577 For example, most Ib MNs cannot be individually targeted. Also, emerging evidence suggests  
578 heterogeneity of function and plasticity between different MNs (Aponte-Santiago and Littleton,  
579 2020; Newman et al., 2017; Saunders et al., 2021), further highlighting the need for cell-specific  
580 genetic tools. To generate new genetic tools for targeting subsets of MNs, the *dpr/DIP*  
581 expression maps can be inspected for partially overlapping or non-overlapping *dpr/DIP-GAL4s*  
582 and converted to split-GAL4 or GAL80, respectively. For example, combining *dpr15-GAL80* and  
583 *dpr14-GAL4* should only label MN6-Ib and a few dorsal MNs; *dpr1-GAL4* and *DIP- $\alpha$ -GAL80*  
584 should label all Ib MNs, but not type-I<sub>s</sub>, -II, and -III MNs; using split-GAL4, a combination of *DIP-*  
585  *$\gamma$ -GAL4DBD* and *DIP- $\kappa$ -GAL4AD* should label MN6/7-Ib and some lateral MNs. Similar  
586 approaches can be applied to SNs. Thus, the expression data in the present study and the  
587 MiMIC/CRIMIC lines provide a pipeline to expand the genetic toolbox and to label and  
588 manipulate neurons in a highly specific manner.

589 **Material and Methods**

590

591 **Drosophila lines used in this study:**

592 All *dpr/DIP-GAL4* lines are listed in Table 1. Other lines used in this study are:

593 Driver lines:

594 *OK6-GAL4* (BL#64199)

595 *GMR79H07-GAL4* (gift from Troy Littleton, MIT)

596 *MHC-GAL80* (gift from Timothy Mosca, Thomas Jefferson University)

597 Reporter lines:

598 *10XUAS-mCD8::GFP* (BL#32184)

599 *20XUAS-mCherry* (BL# 52268)

600 *UAS-2XEGFP; actin-(FRT.STOP)-GAL4,UAS-FLP* (permanent reporter, gift from Ellie Heckscher, UChicago)

602 *UAS-nRedStinger, UAS-FLP, Ubi-p63E(FRT.STOP)-nStinger* (G-TRACE, BL#28280)

603 *R57C10-FLP;;UAS-MCFO* (BL#64089)

604 Lines used to generate Trojan-GAL4:

605 *yw; Sp/CyO; loxP(Trojan-GAL4)x3* (BL#60311)

606 *yw; loxP(Trojan-GAL4)x3; Dr/TM3,Sb,Ser* (BL#60310)

607 *yw,Cre,vas-phiC31:int* (BL#60299)

608

609 **Antibodies used in this study:**

610 Primary antibody:

611 Rabbit anti-GFP (1:40k, gift from Michael Glozter, University of Chicago)

612 Rabbit anti-HA (1:1000, Cell Signaling C29F4)

613 Mouse anti-DLG (1:100, Developmental Studies Hybridoma Bank 4F3)

614 Mouse anti-Repo (1:100, Developmental Studies Hybridoma Bank 8D12)

615 Mouse anti-Myosin (1:100, Invitrogen A31466)

616 Chicken anti-GFP (1:500, Invitrogen A10262)

617 Chicken anti-RFP (1:500, Novus Biologicals NBP2-25158)

618 Chicken anti-V5 (1:500, Bethyl Laboratories A190-118A)

619 Rat anti-Flag (1:200, Novus Biologicals NBP1-06712)

620 Secondary antibody:

621 Goat anti-Rabbit Alexa 488 (1:500, Invitrogen A11008)

622 Goat anti-Rabbit Alexa 568 (1:500, Invitrogen A11036)

623 Goat anti-Mouse Alexa 568 (1:500, Invitrogen A11031)  
624 Goat anti-Mouse Alexa 647 (1:500, Invitrogen A32728)  
625 Goat anti-Chicken Alexa 488 (1:500, Invitrogen A11039)  
626 Donkey anti-Chicken Cy3 (1:500, Jackson Immunological Research 703-165-155)  
627 Goat anti-Rat Alexa 647 (1:500, Invitrogen A21247)  
628 Goat anti-HRP Alexa 647 (1:100, Jackson Immunological Research 123-605-021)  
629 Goat anti-Phalloidin Alexa 405 (1:100, Invitrogen A30104)

630

### 631 **Fly genetics**

632 When examining available *dpr/DIP-GAL4* lines to confirm the GAL4 insertion sites and the  
633 version of GAL4 used, we found that the original *dpr13-GAL4* no longer contained the GAL4  
634 sequence (Barish et al., 2018; Brovero et al., 2021). Here, we generated new *dpr13-GAL4* and  
635 *dpr8-GAL4* from respective MiMIC insertion lines using Trojan exons (Diao et al., 2015). To  
636 generate *DIP-λ* CRIMIC insertions, gRNA (5'-AGCATCTATCGCTTGTGAAAGGG-3') was  
637 designed to target the coding intron. The insertion sites and GAL4 versions are indicated in  
638 Table 1.

639

### 640 **qRT-PCR**

641 Five larvae per genotype were collected and homogenized using pellet pestles (Fisher  
642 Scientific). All samples tested contained a mix of males and females, except for *dpr8-GAL4*,  
643 where only females were used due to its location on the X-chromosome and its inability to  
644 homozygous. RNA was extracted using RNAqueous Total RNA Isolation Kit (ThermoFisher  
645 AM1912) and subsequently treated with DNaseI for 30 minutes at 37°C to remove genomic  
646 DNA. cDNA was generated from 1 µg of RNA using random hexamers and SuperScript IV First-  
647 Strand Synthesis System (ThermoFisher 18091050) and remaining RNA was removed using  
648 RNase H at 37°C for 20 minutes. Primers were designed to be 18-23bp long, amplify 100-  
649 200bp, and have a melting temperature ~60°C (Table 2). All primer locations are downstream of  
650 mapped GAL4 insertion sites and were validated with control cDNA. qRT-PCR was performed  
651 with Power SYBR Green PCR Master Mix (Bio-Rad 4368577) and run on a QuantStudio 3  
652 (ThermoFisher). All reactions were normalized to the housekeeping gene Rpl32 and control  
653 flies, yielding  $\Delta\Delta C_t$  values (Ponton et al., 2011). Relative Fold Change was calculated as  $2^{-\Delta\Delta C_t}$ .  
654 Each reaction was run in technical and biological triplicates.

655

### 656 **Dissection and immunocytochemistry**

657 Larval dissections and immunostaining were performed as previously described (Ashley et al.,  
658 2019). Briefly, wandering third instar larvae were dissected along the dorsal midline in PBS on a  
659 Sylgard plate and stretched out with insect pins. To visualize alary muscles, larva was dissected  
660 from the ventral side. Dissected body walls were washed once with PBS and fixed for 30min  
661 with 4% paraformaldehyde. Samples were then washed three times with PBT (PBS+0.05%  
662 TritonX100). Samples were incubated with primary antibody at 4°C overnight, washed three  
663 times with PBT, and then incubated in secondary antibody at room temperature for 2 hours.  
664 Samples were finally mounted in 30µl vectashield (Vector Laboratories). Representative images  
665 were taken with a Zeiss LSM800 confocal microscope with a 40X plan-neofluar 1.3NA objective  
666 and processed with ImageJ.

667

### 668 **Examining expression of *dprs* and *DIPs* in MNs and SNs**

669 We dissected six third instar larvae from each cross and immunostained for GFP/RFP, DLG and  
670 HRP. Mounted slices were examined under Zeiss Axiolmager M2 with a Lumen light engine  
671 with a 20X plan-apo 0.8NA objective. Each sample was examined twice with the same criteria to  
672 reduce human error. To map the expression of *dprs* and *DIPs* in MNs, NMJs of each MN was  
673 identified by labeling for DLG or HRP, and then examined for GFP/RFP colocalization. For  
674 expression in SNs, SN cell bodies were located by HRP, and then examined for GFP/RFP  
675 colocalization. We counted all MNs and SNs from anterior to posterior hemisegments  
676 (abdominal segment A2-A7) to gain a full *dpr/DIP* expression map across the body wall. Note  
677 that we did not observe the third type-Is MN (MNSNa-Is) described by (Hoang and Chiba,  
678 2001). The pipeline and criteria of determining the expression level is below (Figure 2 – figure  
679 supplement 3):

680 1. In *dpr/DIP-GAL4>GFP/RFP* animals, if the reporter gene expressed constantly in a  
681 specific MN/SN in all hemisegments, then this GAL4 line is counted as “high expression  
682 level” in this MN/SN. If the fluorescent reporter is not expressed consistently in a specific  
683 MN/SN, then: (1) if the fluorescent reporter shows a gradient increase or decrease along  
684 the anterior to posterior axis, then the expression of this GAL4 line is reported as  
685 “gradient increase” or “gradient decrease”, respectively; (2) if the reporter gene does not  
686 express in a gradient, but randomly expresses in a specific MN/SN, then the expression  
687 is counted as “medium expression level” in this MN/SN. Note we did not record gradient  
688 expression for SNs, because the reporter expression had higher variation in SNs  
689 compare to MNs.

690 2. In the cross between *dpr/DIP-GAL4* and the permanent labeling reporter, we first  
691 confirmed the high, medium, and gradient expression level described above. Then, if a  
692 GAL4 line showed no expression in the cross to *UAS-GFP/RFP* but did show expression  
693 in the cross to the permanent labeling reporter, we counted how frequent this MN/SN is  
694 labeled: (1) if the labeling frequency is lower than 30% across all hemisegments, then  
695 this GAL4 is recorded as “low expression level” in this MN/SN because the expression  
696 could be too low to detect in the cross to *UAS-GFP/RFP* but sufficient to trigger some  
697 FLP-out; (2) if the labeling frequency is between 30%-60%, then this GAL4 expression is  
698 recorded as “medium expression level” in this MN/SN; (3) if the labeling frequency is  
699 higher than 60%, then this GAL4 expression is considered as “temporal expression” as it  
700 indicates a high GAL4 expression level temporally in early developmental stages  
701 because it triggers high frequency FLP-out. Finally, if a GAL4 is not expressed in both  
702 the cross to *UAS-GFP/RFP* or permanent reporter, it is recorded as “null expression”.

703 *dpr10-GAL4* was crossed to *UAS-GFP* together with *MHC-GAL80* to prevent muscle GFP  
704 expression, because high level of muscle GFP will mask NMJs and SN cell bodies. In addition,  
705 muscles expressing *dprs* (*dpr10* and *dpr19*) were not crossed to the permanent labeling  
706 reporter.

707

## 708 **Examining expression of *dprs* and *DIPs* in glial and muscles**

709 We examined expression of *dprs* and *DIPs* in glia and muscles with the G-TRACE reporter  
710 (Evans et al., 2009). We dissected 6 larvae from each cross and immunostained for GFP, RFP,  
711 HRP, and Repo. Glial expression was confirmed by GFP/RFP colocalization with Repo. Muscle  
712 expression was confirmed by GFP/RFP positive muscle nuclei. Although the cross to *UAS-*  
713 *GFP/RFP* and the permanent labeling line also showed muscle expression, the diffusible GFP  
714 signal impeded the clear distinction of muscle boundaries.

715

## 716 **Hierarchical clustering using *dpr/DIP* expression**

717 To perform hierarchical clustering, the expression of *dprs* and *DIPs* were first converted to  
718 binary values of “0” and “1”. Robust expression including high expression and temporal  
719 expression were considered as “1”, whereas medium and low expression, and gradient  
720 expression were considered as “0”. We reasoned that robust expression of *dprs* and *DIPs* may  
721 suggest more a significant role in the respective cell. Binary data was subjected to hierarchical  
722 analysis using Morpheus (Broad Institute) (Metric: Cosine Similarity; Method: Average). Figures  
723 were exported and color coded in Adobe Illustrator to indicate different types of MNs and SNs.

724

725 **Bouton number and dual innervation counting**

726 To quantify m6 and m7 NMJs in wild type animals, we located Ib NMJs by DLG labeling and

727 counted bouton number by HRP labeling. To measure the MN6-Ib or MN7-Ib NMJ sizes in

728 *GMR79H07-GAL4>GFP* animals, we first looked for GFP colocalization with DLG to distinguish

729 MN6-Ib and MN7-Ib. For example, if the major Ib arbor on m6 is GFP positive, then it is formed

730 by MN6-Ib, and the GFP negative boutons are formed by MN7-Ib. We then counted the bouton

731 numbers of each Ib arbor by HRP labeling. Statistical analyses were performed using Prism 8

732 software. Error bar indicates standard error of the mean (SEM).



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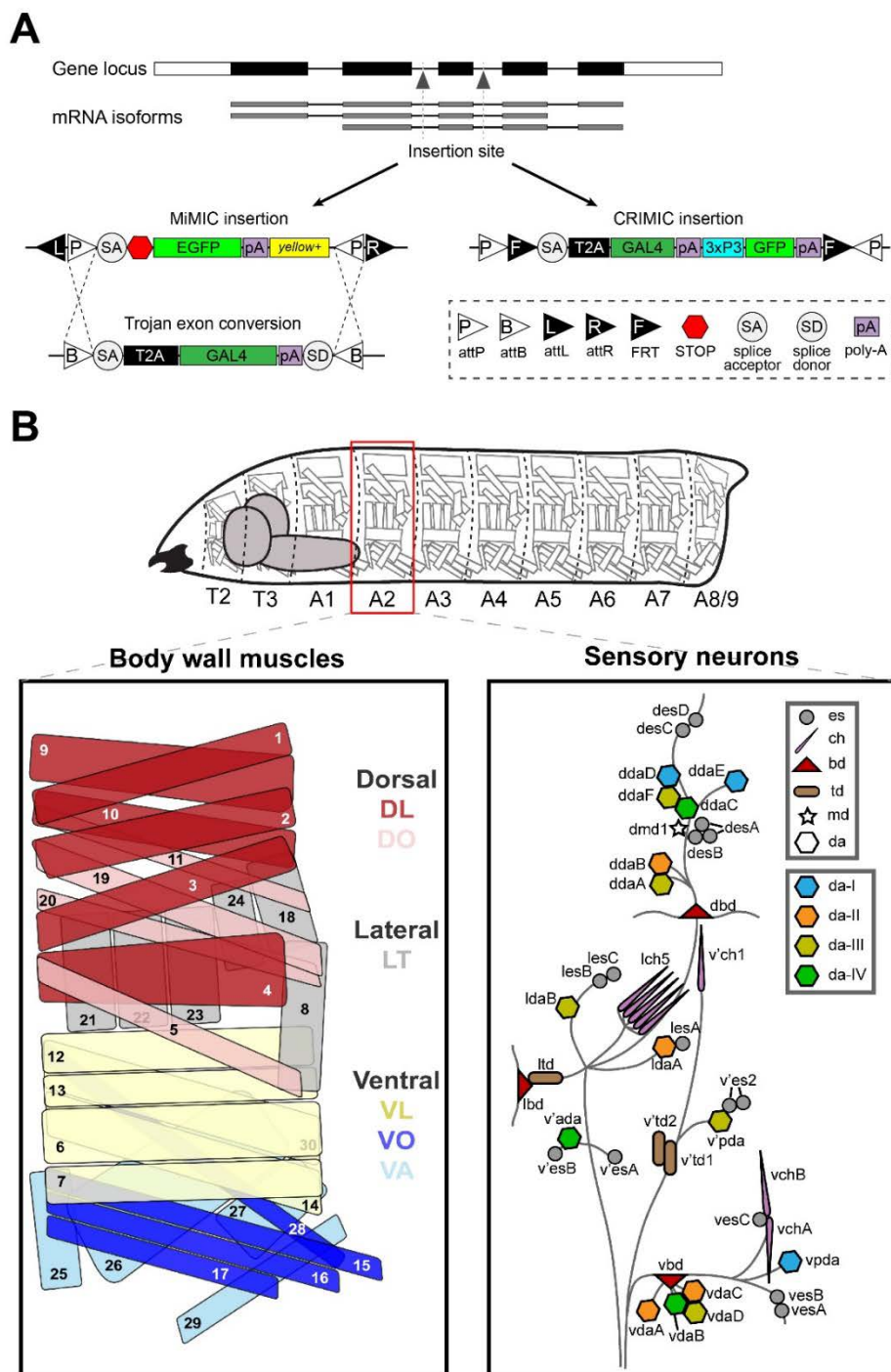
747

748 **Author contributions**

749 Y.W. and R.A.C designed research; Y.W., M.L.R., J.A., V.A. and P.C. performed experiments;  
750 Y.W., M.L.R., and R.A.C. analyzed data; Y.W. wrote the manuscript and J.A., M.L.R., H.J.B.,  
751 O.K., R.A.C. edited the manuscript.

752

## Figure 1



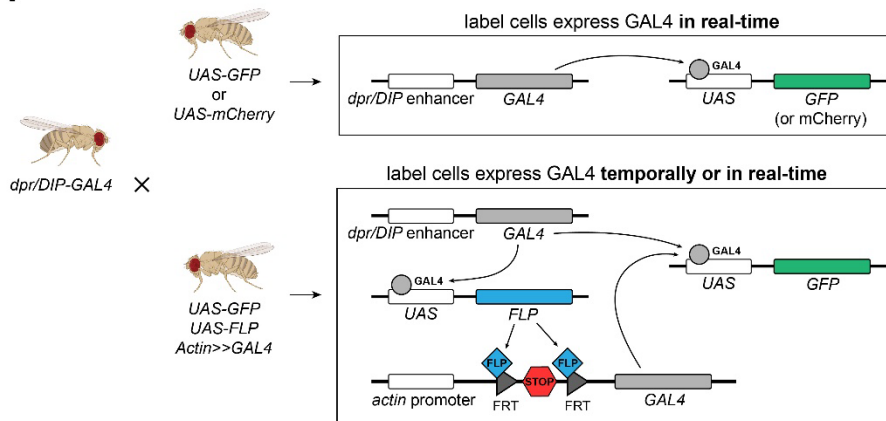
**Figure 1. Schematic of GAL4 insertion and larval body plan.**

A. MiMIC or CRIMIC cassettes were inserted into a common intron or 5'UTR to capture the expression of all isoforms for each *dpr* and *DIP*. MiMIC insertions were flanked by two attP sites which are later swapped by a GAL4 exon or T2A-GAL4 trojan exon. CRIMIC insertions already carry T2A-GAL4.

B. *Drosophila* larvae are divided into three thoracic segments and nine abdominal segments, with repeated muscles, MNs, and SNs. Muscles are divided into three main groups, the ventral, lateral and dorsal muscles. Ventral muscles include the ventral longitude (VL), ventral oblique (VO), ventral acute (VA) muscle groups. Dorsal muscles include the dorsal longitude (DL) and dorsal oblique (DO) muscle groups (Zarin et al., 2019). MNs innervating these muscles are not shown in this diagram. SNs are divided into six main classes: the es neurons, ch neurons, bd neurons, td neurons, md neuron, and da neurons (Orgogozo and Grueber, 2005). In addition, da neurons are further divided into da-I, da-II, da-III, and da-IV subclasses.

## Figure 2

A



**Figure 2. *dprs* and *DIPs* are expressed in various patterns in MNs.**

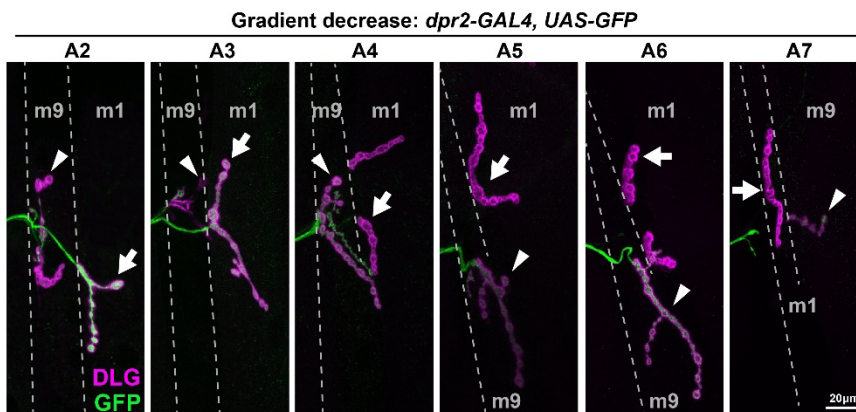
A. Schematic showing the experimental procedure. Each *dpr/DIP-GAL4* line was crossed to a real-time reporter (*UAS-GFP* or *UAS-mCherry*) and a permanent reporter (*UAS-GFP*, *UAS-FLP*, *actin-(FRT.STOP)-GAL4*) to reveal the dynamic expression of *dprs* and *DIPs*.

B. Representative images showing an example of a decrease in expression of *dpr2-GAL4* in MN1-lb (arrows) from anterior hemisegment A2 to posterior hemisegment A7. Note that the expression in nearby MN9-lb (arrowheads) is also not robust as it was not expressed in A2 and A3 but expressed in A4 to A7.

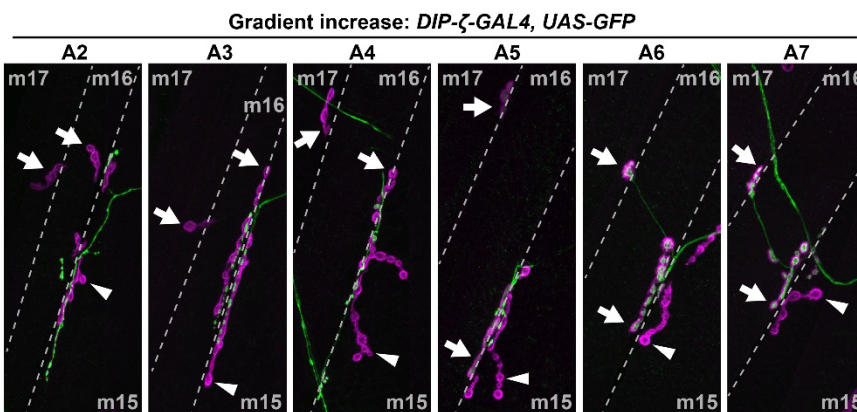
C. Representative images showing an example of an increase in expression of *DIP-ζ-GAL4* in MN16/17-lb (arrows) from anterior hemisegment A2 to posterior hemisegment A7. Note that the expression in nearby MN15/16-lb (arrowheads) was always absent.

D. Representative images showing an example of temporal expression of *dpr9-GAL4* in MN21-lb. MN21-lb was not labeled by *dpr9-GAL4*>GFP animals, but 50% of MN21-lb were labeled in the cross to the permanent reporter.

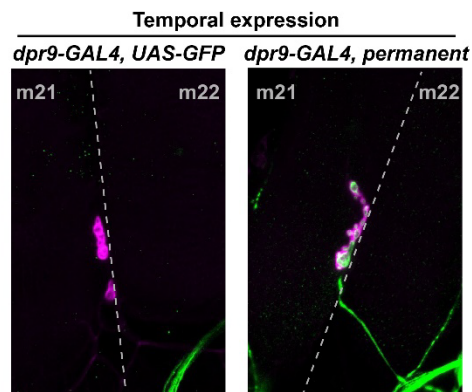
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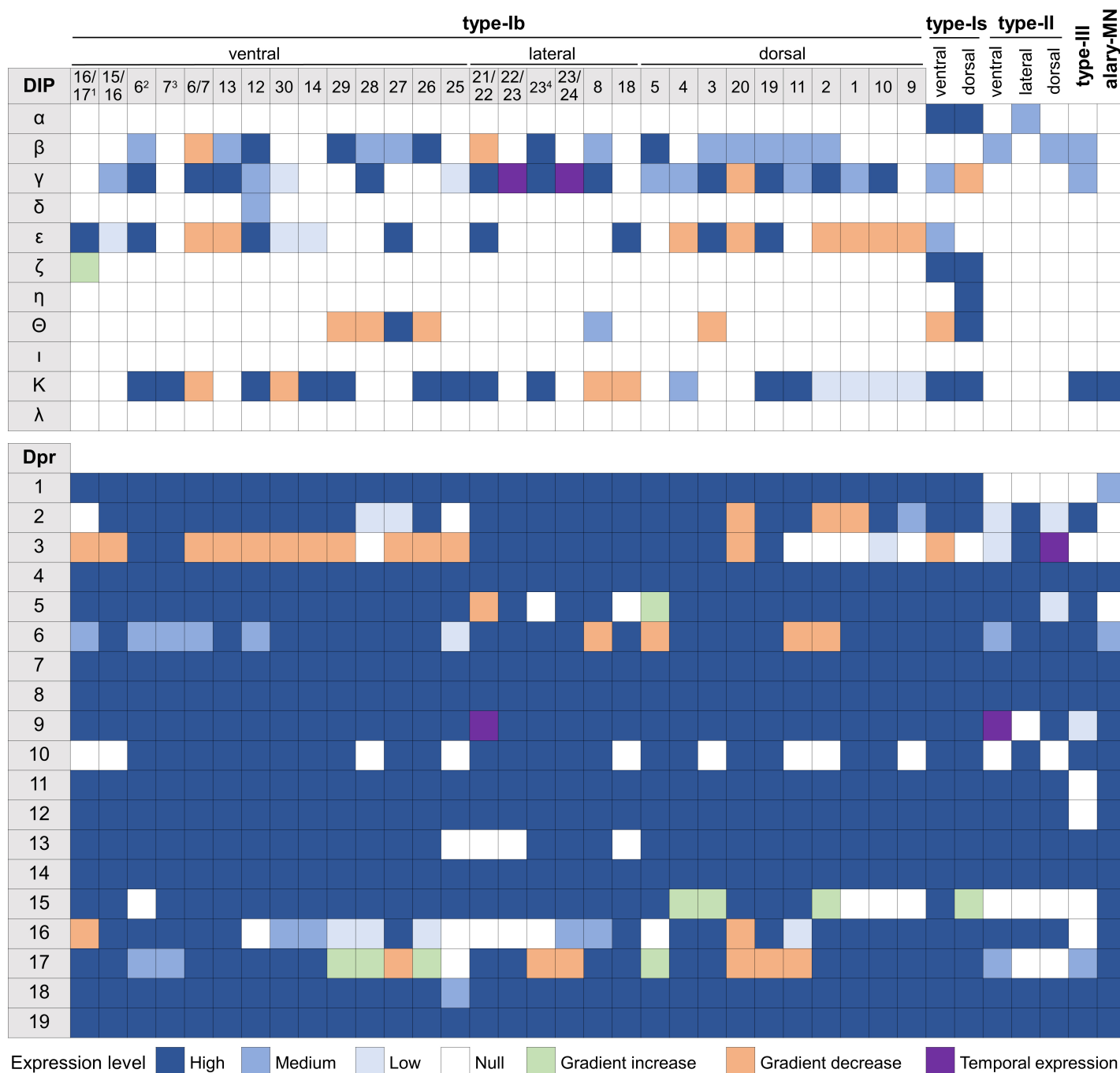
C



D

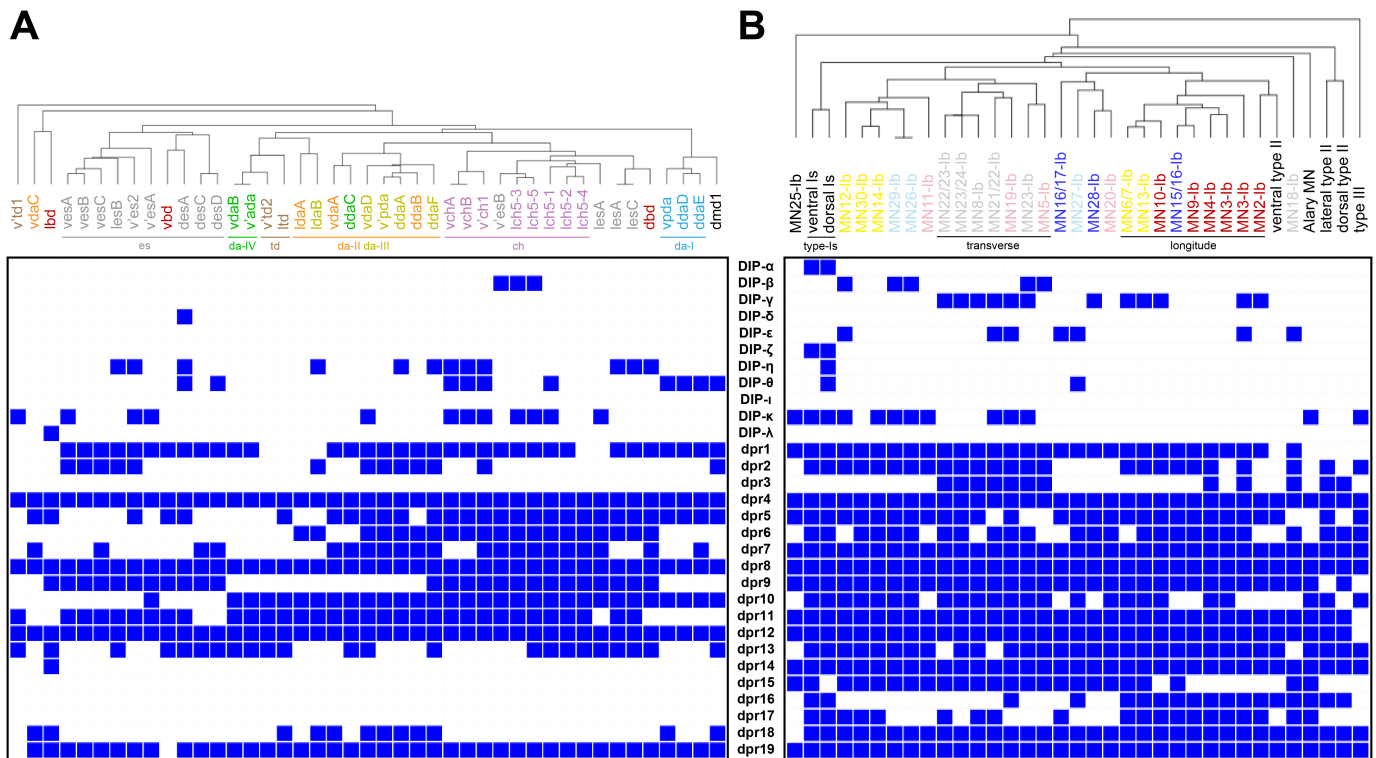


## Figure 3





## Figure 5

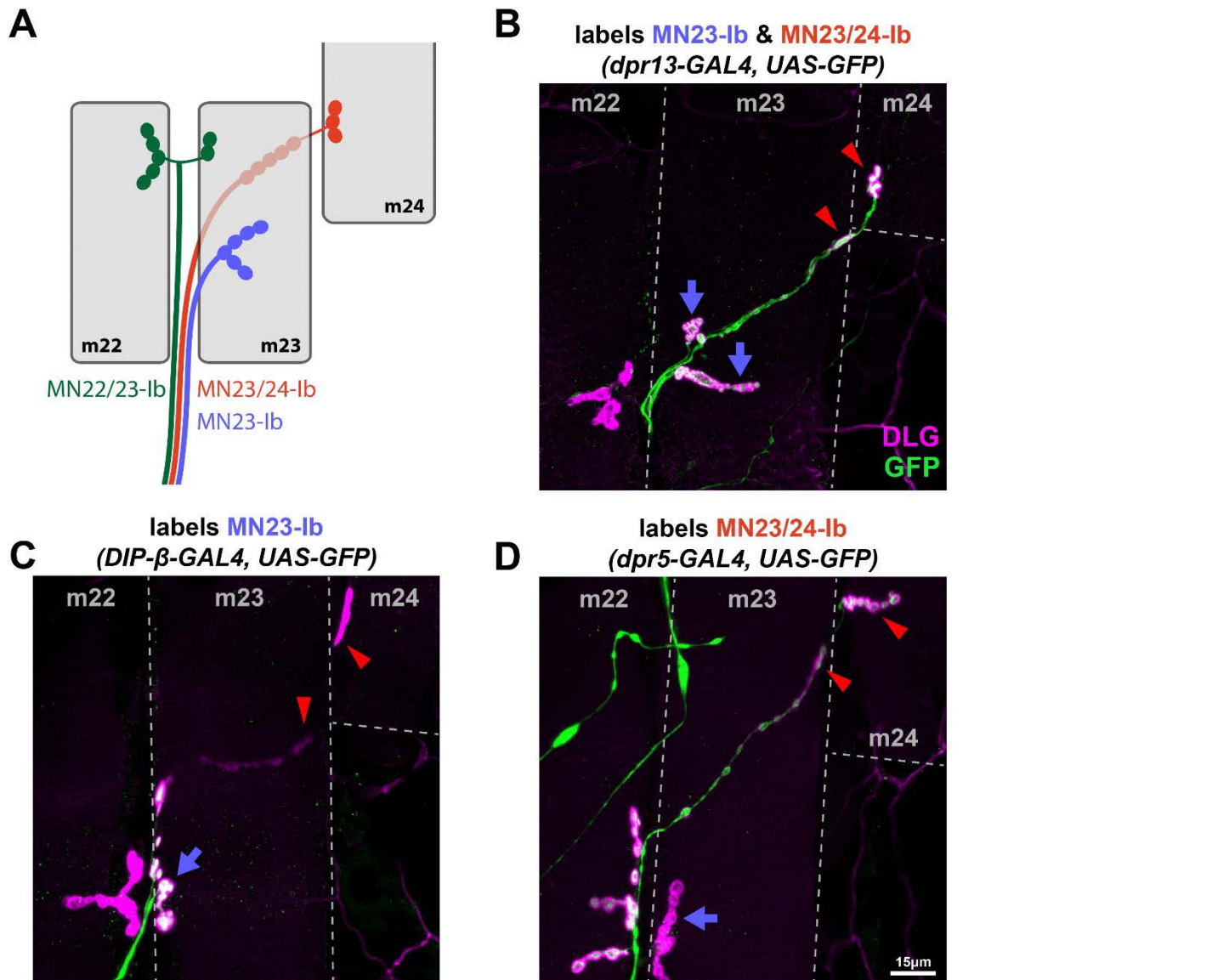


**Figure 5. Hierarchical clustering of SNs and MNs reveals shared expression patterns of *dprs* and *DIPs* in neurons from the same class.**

A. SNs from the same class are clustered together based on the expression pattern of *dprs* and *DIPs*. For example, most *es* neurons (grey), all chordotonal neurons (purple), and *da* neurons fall into distinct clusters.

B. Modulatory MNs (II and III) and type-IIs MNs are distinct from the main type-Ib cluster. However, individual type-Ib MNs are not easily distinguished based on their expression of *dprs* and *DIPs*, except the transverse MNs (grey) and dorsal longitude MNs (red).

## Figure 6



**Figure 6. Differentially expressed *dprs* and *DIPs* reveal a MN that solely innervates m23.**

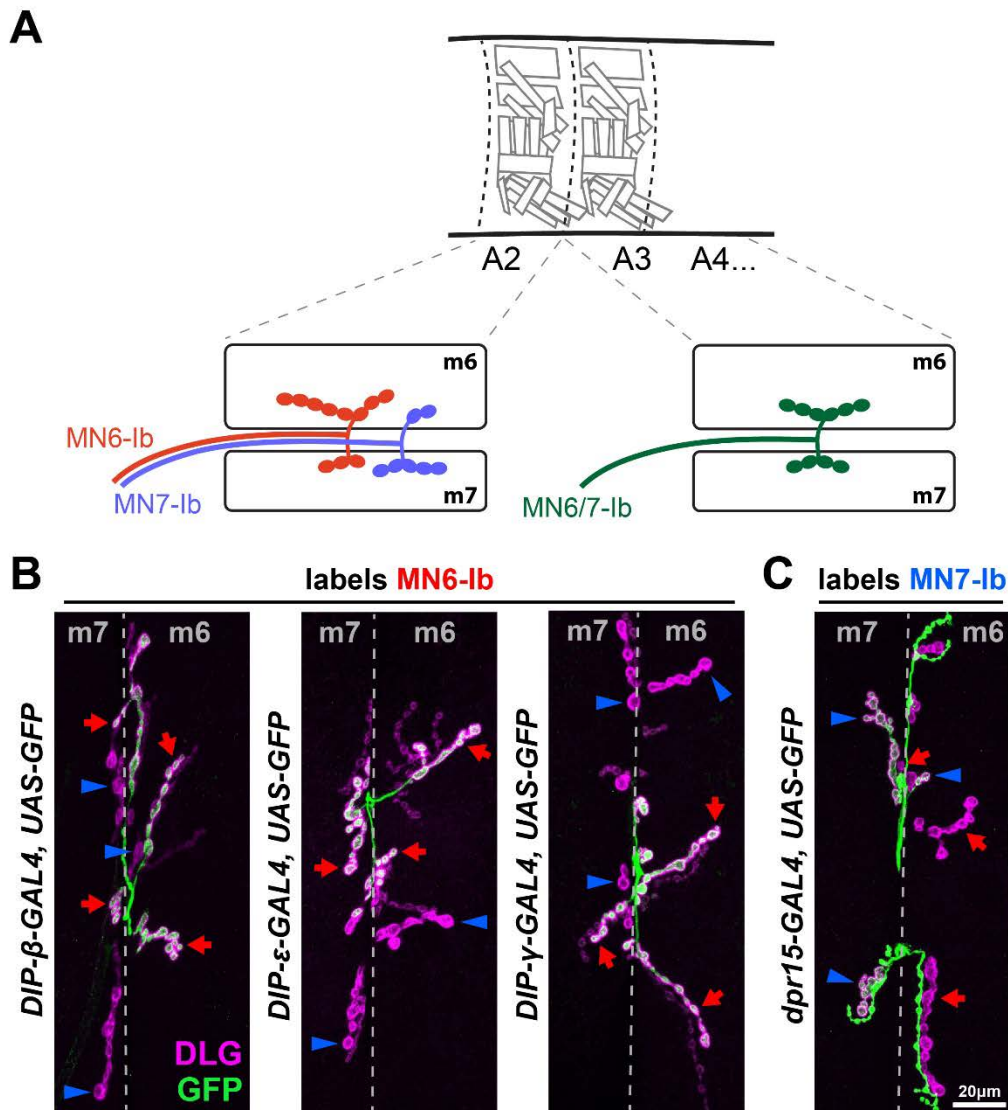
A. Schematic depiction of transverse muscles 22, 23 and 24 (grey) with previously identified MN22/23-Ib (green), MN23/24-Ib (red) and newly identified MN23-Ib (blue). MN22/23-Ib innervates the cleft between m22 and m23. MN23/24-Ib travels underneath m23 and forms boutons on m23 and m24. MN23-Ib only innervates m23.

B. Representative image showing *dpr13-GAL4* expression in both MN23/24-Ib (red arrowheads) and MN23-Ib (blue arrows). Thus, all boutons on m23 and m24 are labeled by GFP.

C. Representative image showing *DIP-β-GAL4* expression in MN23-Ib (blue arrow). Boutons underneath m23 and boutons from m22, m24 (red arrowheads) are not labeled by GFP, thus *DIP-β-GAL4* is not expressed in MN22/23-Ib and MN23/24-Ib.

D. Representative image showing *dpr5-GAL4* expression in MN22/23-Ib and MN23/24-Ib (red arrowheads), but not in MN23-Ib (blue arrow). The lack of GFP in the arbor on m23 indicated the existence of a MN that solely innervates m23.

## Figure 7



**Figure 7. Differentially expressed *dprs* and *DIPs* reveal MN6-Ib and MN7-Ib in segment A2.**

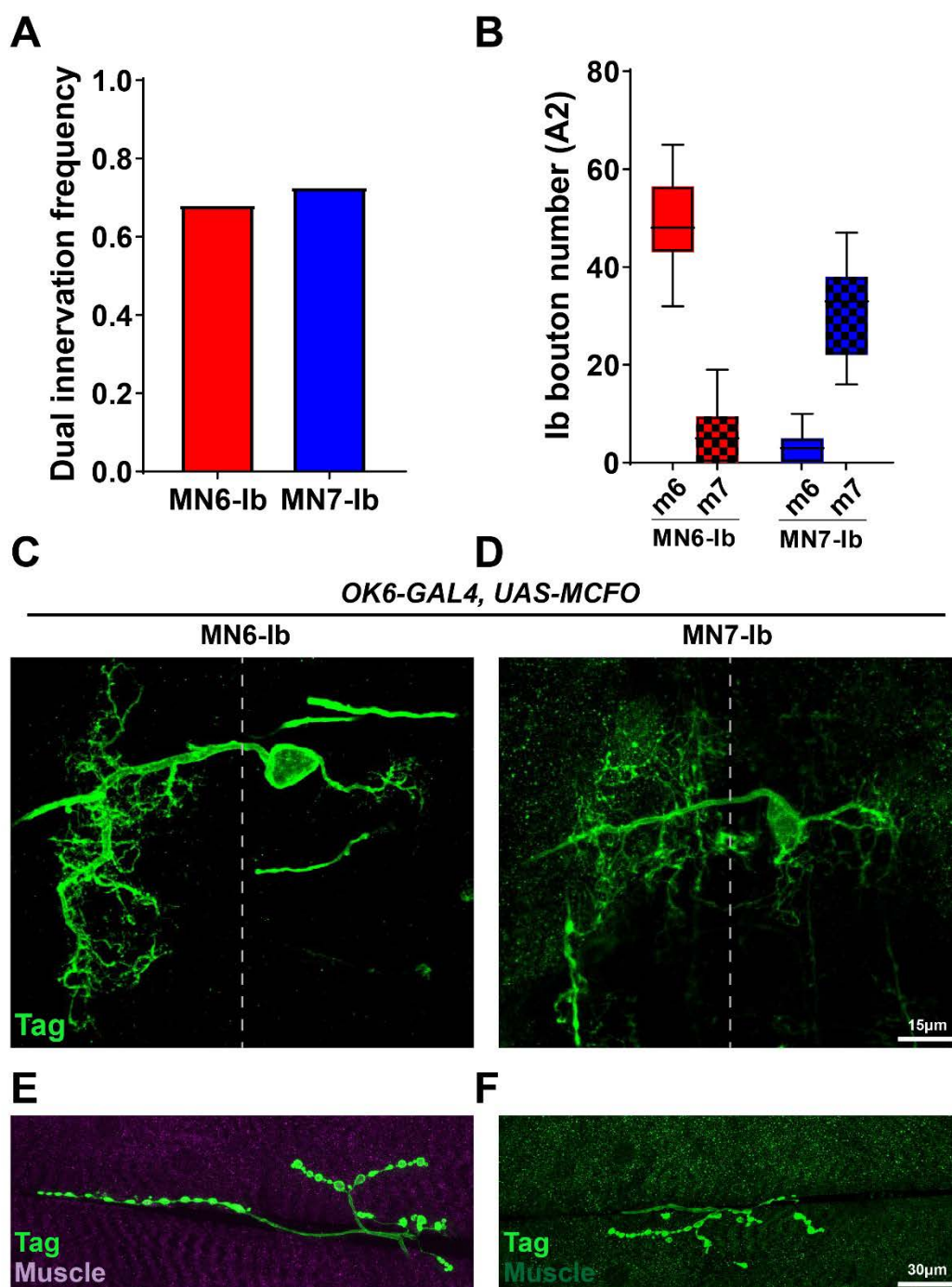
A. Schematic depiction of MN6-Ib (red) and MN7-Ib (blue) in segment A2, and MN6/7-Ib in A3-A7 (green). MN6-Ib preferentially innervates m6 but also forms a small NMJ on m7, whereas MN7-Ib prefers m7 but also forms a small NMJ on m6.

B. Representative images showing that *DIP-β*, *DIP-ε* and *DIP-γ* are specifically expressed in MN6-Ib (red arrows), but not in MN7-Ib (blue arrowheads). Note that MN6-Ib forms boutons with both m6 and m7, since there is a small GFP positive type-Ib NMJ on m7 (red arrows on m7). Conversely, the lack of GFP in most m7 type-Ib NMJ and the small m6 type-Ib NMJ (blue arrowheads) indicate MN7-Ib also dual innervates both muscles.

C. Representative image showing that *dpr15* is specifically expressed in MN7-Ib (blue arrows) but not in MN6-Ib (red arrowheads). MN6-Ib and MN7-Ib also show dual innervation patterns in this genetic background.



## Figure 8



### Figure 8. Further characterization of MN6-Ib and MN7-Ib.

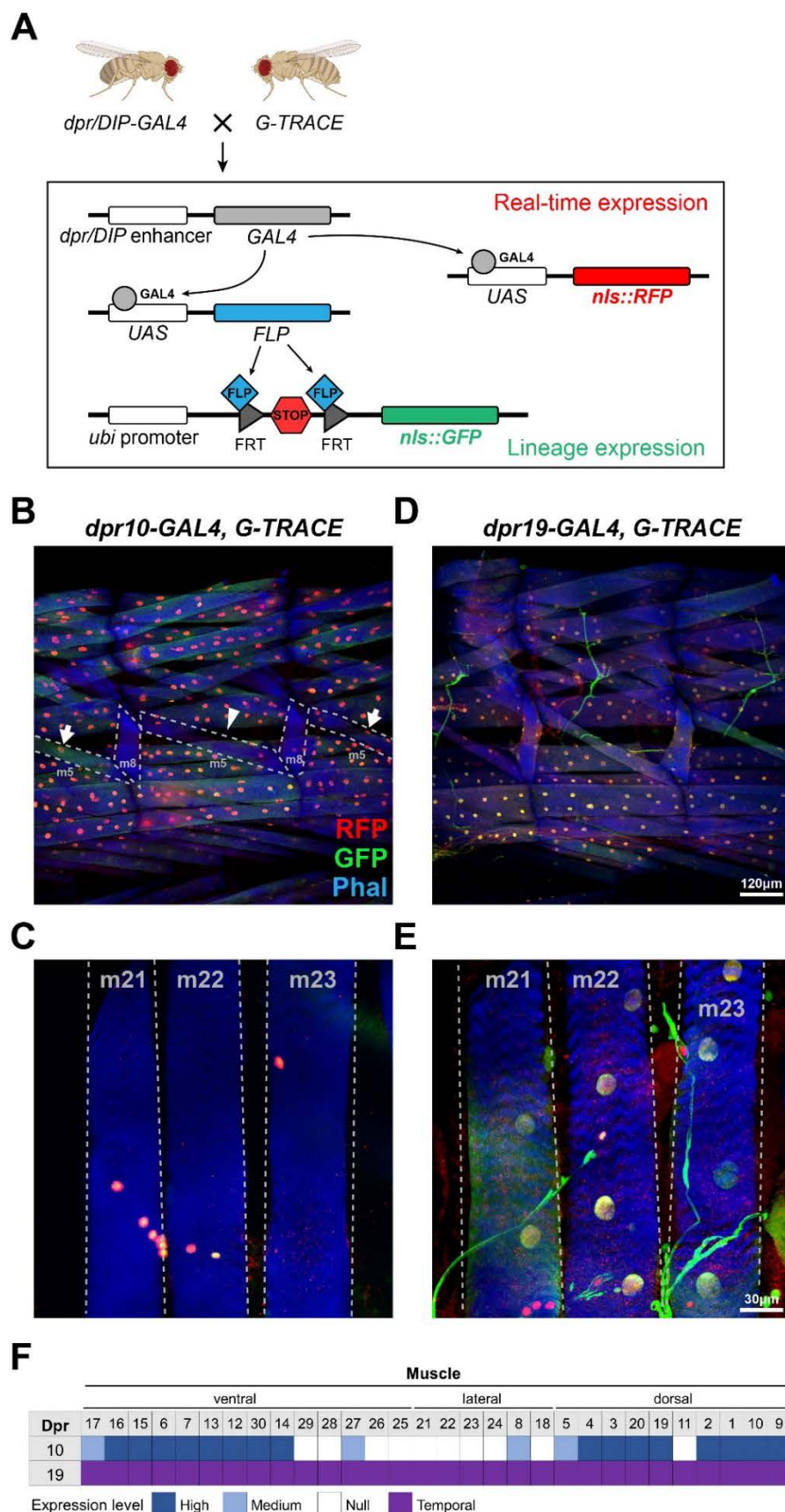
A. Quantification of the dual innervation frequencies of MN6-Ib and MN7-Ib. 68.2% of MN6-Ib also innervate m7 and 72.7% of MN7-Ib also innervate m6.

B. Quantification of MN6-Ib and MN7-Ib NMJ sizes on both muscles. On average, MN6-Ib forms ~49 boutons with m6 and ~6 boutons with m7, while MN7-Ib forms ~3 boutons with m6 and ~31 boutons with m7.

C-D. A pan MN driver *OK6-GAL4* driving MCFO revealed the dendritic morphology of MN6-Ib and MN7-Ib in the VNC. Both MNs have similar morphologies including large contralateral dendritic arbors and small ipsilateral arbors.

E-F. Corresponding NMJ images from the same neuron shown in C (MN6-Ib) and D (MN7-Ib)

## Figure 9



**Figure 9. Using the G-TRACE system to probe expression of *dprs* and *DIPs* in muscles and glial cells.**

A. Schematic depiction showing the cross between *dpr/DIP-GAL4* and the *G-TRACE* reporter. Red signal represents real-time GAL4 expression and green signal represents earlier GAL4 expression.

B-C. *dpr10* is consistently expressed in most muscles (B) but absent in transverse muscles (C) and some deeper ventral muscles. Expression in some muscles is not consistent. For example, in some hemisegments m5 nuclei are not labeled (arrowhead), but an adjacent hemisegment shows labeling of m5 nuclei (arrows). *dpr10* expression is maintained throughout development as revealed by co-labeling with GFP and RFP.

D-E. *dpr19* is expressed in all muscles (D) including transverse muscles (E). Compared to *dpr10*, these nuclei have less RFP intensity, which may indicate that *dpr19* is temporally expressed in early development and turned off later.

F. Expression map of *dpr10* and *dpr19* in muscles.

**Table 1**

GAL4 line	GAL4 derived from	Insertion site	Genotype	Sources
<i>DIP-α-GAL4</i>	MI10680 (T2A-GAL4)	6 <sup>th</sup> coding intron	<i>y' w' Mi{Trojan-GAL4.1}DIP-α<sup>MI10680-TG4.1</sup></i>	GDP*
<i>DIP-β-GAL4</i>	MI01971 (GT-GAL4)	5' UTR intron	<i>y' w' Mi{GT-GAL4}DIP-β<sup>MI01971-GAL4</sup></i>	RRID:BDSC_90316
<i>DIP-γ-GAL4</i>	MI03222 (GT-GAL4)	5' UTR intron	<i>Mi{GT-GAL4}DIP-γ<sup>MI03222-GAL4</sup></i>	RRID:BDSC_90315
<i>DIP-δ-GAL4</i>	MI08287 (T2A-GAL4)	4 <sup>th</sup> coding intron	<i>Mi{Trojan-GAL4.1}DIP-δ<sup>MI08287-TG4.1</sup></i>	RRID:BDSC_90320
<i>DIP-ε-GAL4</i>	MI11827 (T2A-GAL4)	1 <sup>st</sup> coding intron	<i>Mi{Trojan-GAL4.1}DIP-ε<sup>MI11827-TG4.1</sup>/CyO,Dfd-YFP</i>	RRID:BDSC_67502
<i>DIP-ζ-GAL4</i>	MI03838 (T2A-GAL4)	2 <sup>nd</sup> coding intron	<i>Mi{Trojan-GAL4.0}DIP-ζ<sup>MI03838-TG4.0</sup></i>	RRID:BDSC_90317
<i>DIP-η-GAL4</i>	MI07948 (T2A-GAL4)	4 <sup>th</sup> coding intron	<i>Mi{Trojan-GAL4.1}DIP-η<sup>MI07948-TG4.1</sup>/CyO,Dfd-YFP</i>	RRID:BDSC_90318
<i>DIP-θ-GAL4</i>	MI03191 (T2A-GAL4)	2 <sup>nd</sup> coding intron	<i>Mi{Trojan-GAL4.1}DIP-θ<sup>MI03191-TG4.1</sup>/CyO,Dfd-YFP</i>	GDP*
<i>DIP-i-GAL4</i>	CR00997 (T2A-GAL4)	1 <sup>st</sup> coding intron	<i>TI{CRIMIC-TG4.1}DIP-i<sup>CR00997-TG4.1</sup></i>	RRID:BDSC_83243
<i>DIP-k-GAL4</i>	CR01146 (T2A-GAL4)	1 <sup>st</sup> coding intron	<i>TI{CRIMIC-TG4.1}DIP-k<sup>CR01146-TG4.1</sup>Gpo3<sup>CR01114-TG4.1X</sup>/CyO,Dfd-YFP</i>	RRID:BDSC_83252
<i>DIP-λ-GAL4</i>	CR70096 (T2A-GAL4)	2 <sup>nd</sup> coding intron	<i>TI{CRIMIC-TG4.0}DIP-λ<sup>CR70096-TG4.0</sup></i>	This study
<i>dpr1-GAL4</i>	MI12729 (T2A-GAL4)	1 <sup>st</sup> coding intron	<i>Mi{Trojan-GAL4.1}dpr1<sup>MI12729-TG4.1</sup>/CyO,Dfd-YFP</i>	GDP*
<i>dpr2-GAL4</i>	MI05656 (T2A-GAL4)	4 <sup>th</sup> coding intron	<i>Mi{Trojan-GAL4.1}dpr2<sup>MI05656-TG4.1</sup>/CyO,Dfd-YFP</i>	GDP*
<i>dpr3-GAL4</i>	MI05963 (T2A-GAL4)	1 <sup>st</sup> coding intron	<i>Mi{Trojan-GAL4.1}dpr3<sup>MI05963-TG4.1</sup>/CyO,Dfd-YFP</i>	GDP*
<i>dpr4-GAL4</i>	CR00485 (T2A-GAL4)	1 <sup>st</sup> coding intron	<i>TI{CRIMIC-TG4.1}dpr4<sup>CR00485-TG4.1</sup>/TM6,Sb,Hu,Dfd-YFP</i>	RRID:BDSC_79271
<i>dpr5-GAL4</i>	MI11085 (T2A-GAL4)	2 <sup>nd</sup> coding intron	<i>Mi{Trojan-GAL4.1}dpr5<sup>MI11085-TG4.1</sup></i>	GDP*
<i>dpr6-GAL4</i>	MI01358 (T2A-GAL4)	1 <sup>st</sup> coding intron	<i>Mi{Trojan-GAL4.1}dpr6<sup>MI01358-TG4.1</sup></i>	GDP*
<i>dpr7-GAL4</i>	MI05719 (T2A-GAL4)	1 <sup>st</sup> coding intron	<i>Mi{Trojan-GAL4.1}dpr7<sup>MI05719-TG4.1</sup></i>	RRID:BDSC_78385
<i>dpr8-GAL4</i>	MI11830 (T2A-GAL4)	1 <sup>st</sup> coding intron	<i>Mi{Trojan-GAL4.1}dpr8<sup>MI11830-TG4.1</sup></i>	This study
<i>dpr9-GAL4</i>	MI03594 (T2A-GAL4)	3 <sup>rd</sup> coding intron	<i>Mi{Trojan-GAL4.1}dpr9<sup>MI03594-TG4.1</sup></i>	GDP*
<i>dpr10-GAL4</i>	MI03557 (T2A-GAL4)	1 <sup>st</sup> coding intron	<i>Mi{Trojan-GAL4.1}dpr10<sup>MI03557-TG4.1</sup></i>	GDP*
<i>dpr11-GAL4</i>	MI01743 (T2A-GAL4)	1 <sup>st</sup> coding intron	<i>Mi{Trojan-GAL4.1}dpr11<sup>MI01743-TG4.1</sup></i>	GDP*
<i>dpr12-GAL4</i>	MI01695 (T2A-GAL4)	1 <sup>st</sup> coding intron	<i>Mi{Trojan-GAL4.1}dpr12<sup>MI01695-TG4.1</sup>/CyO,Dfd-YFP</i>	GDP*
<i>dpr13-GAL4</i>	MI05577 (T2A-GAL4)	2 <sup>nd</sup> coding intron	<i>Mi{Trojan-GAL4.0}dpr13<sup>MI05577-TG4.0</sup>/CyO,actin-GFP</i>	This study
<i>dpr14-GAL4</i>	CR00516 (T2A-GAL4)	1 <sup>st</sup> coding intron	<i>TI{CRIMIC-TG4.1}dpr14<sup>CR00516-TG4.1</sup></i>	RRID:BDSC_80586
<i>dpr15-GAL4</i>	MI01408 (T2A-GAL4)	3 <sup>rd</sup> coding intron	<i>Mi{Trojan-GAL4.1}dpr15<sup>MI01408-TG4.1</sup>/TM6,Sb,Hu,Dfd-YFP</i>	RRID:BDSC_66827
<i>dpr16-GAL4</i>	MI05173 (T2A-GAL4)	1 <sup>st</sup> coding intron	<i>Mi{Trojan-GAL4.1}dpr16<sup>MI05173-TG4.1</sup></i>	GDP*
<i>dpr17-GAL4</i>	MI08707 (T2A-GAL4)	1 <sup>st</sup> coding intron	<i>Mi{Trojan-GAL4.1}dpr17<sup>MI08707-TG4.1</sup>/TM6,Sb,Hu,Dfd-YFP</i>	RRID:BDSC_76200
<i>dpr18-GAL4</i>	CR01009 (T2A-GAL4)	1 <sup>st</sup> coding intron	<i>TI{CRIMIC-TG4.1}dpr18<sup>CR01009-TG4.1</sup></i>	RRID:BDSC_83245
<i>dpr19-GAL4</i>	CR00996 (T2A-GAL4)	1 <sup>st</sup> coding intron	<i>TI{CRIMIC-TG4.1}dpr19<sup>CR00996-TG4.1</sup>/CyO,Dfd-YFP</i>	RRID:BDSC_83242

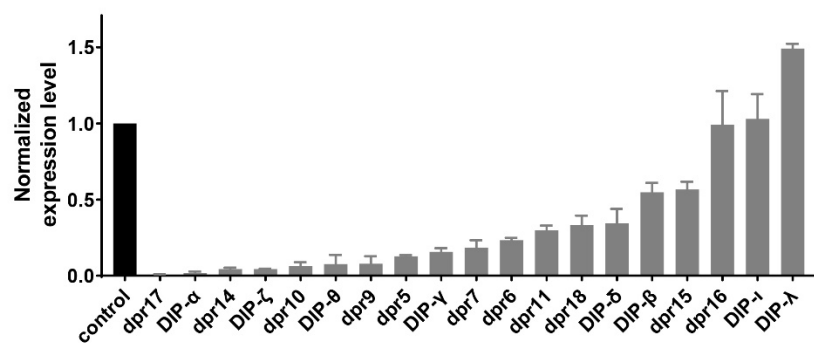
\* GDP: Gene Disruption Project

**Table 2**

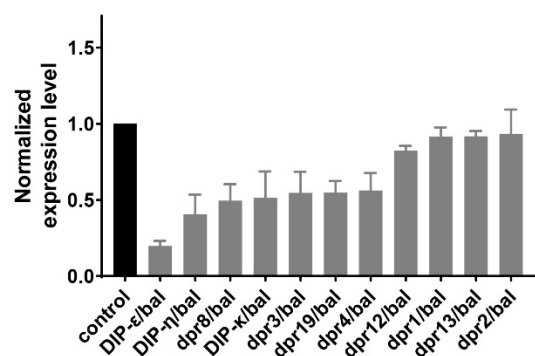
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DIP- $\alpha$	DIP- $\alpha$ <sup>MI10680-GAL4</sup>	0.02±0.02	DIP- $\alpha$ -qPCR-F	GGAGTACCGCCATCGGTCTC
			DIP- $\alpha$ -qPCR-R	GGCTTCGACGTGACACTCG
DIP- $\beta$	DIP- $\beta$ <sup>MI01971-GAL4</sup>	0.55±0.11	DIP- $\beta$ -qPCR-F	GAGCACGTGGTCATCCGAAG
			DIP- $\beta$ -qPCR-R	GGAGGCAATGCACATGTAGGC
DIP- $\gamma$	DIP- $\gamma$ <sup>MI03222-GAL4</sup>	0.16±0.04	DIP- $\gamma$ -qPCR-F	GAGAGCCTCCGATCAGACCG
			DIP- $\gamma$ -qPCR-R	CCTGCACATCGATAACAACCC
DIP- $\delta$	DIP- $\delta$ <sup>MI08287-GAL4</sup>	0.35±0.16	DIP- $\delta$ -qPCR-F	GGCAACTATCGATGCATCTCG
			DIP- $\delta$ -qPCR-R	GTCGTTGCGTGATGAGGGTATG
DIP- $\epsilon$	DIP- $\epsilon$ <sup>MI11827-GAL4</sup> /CyO,Dfd-YFP	0.20±0.06	DIP- $\epsilon$ -qPCR-F	CGGCCAAGACCCAGTATGG
			DIP- $\epsilon$ -qPCR-R	GATTTTGTTCGGTCATCGCG
DIP- $\zeta$	DIP- $\zeta$ <sup>MI03838-GAL4</sup>	0.04±0.004	DIP- $\zeta$ -qPCR-F	GTGGAAGCCACAGTCGGATTG
			DIP- $\zeta$ -qPCR-R	GAGGCAGTGGTTGGAGGATATG
DIP- $\eta$	DIP- $\eta$ <sup>MI07948-GAL4</sup> /CyO,Dfd-YFP	0.41±0.23	DIP- $\eta$ -qPCR-F	GGCAGTAACGTGACGCTCAAATG
			DIP- $\eta$ -qPCR-R	CGAGGGAGGGACTCCATTGG
DIP- $\theta$	FRT40A, DIP- $\theta$ <sup>MI03191-GAL4</sup>	0.08±0.10	DIP- $\theta$ -qPCR-F	CTCCTGCAGAACGTAACGGTG
			DIP- $\theta$ -qPCR-R	GCGTGAGTTATGCTCATGCG
DIP- $\iota$	DIP- $\iota$ <sup>CR00997-GAL4</sup>	0.89±0.20	DIP- $\iota$ -qPCR-F	GGGATGCCCTACTCACGTGTG
			DIP- $\iota$ -qPCR-R	CATCCGCGATCCGATTCCTG
DIP- $\kappa$	DIP- $\kappa$ <sup>CR011146-GAL4</sup> /CyO,Dfd-YFP	0.51±0.30	DIP- $\kappa$ -qPCR-F	GACAGCGACAGATCCTGTG
			DIP- $\kappa$ -qPCR-R	CGTGTTCACTTGGCACATGTAC
DIP- $\lambda$	DIP- $\lambda$ <sup>CR70096-GAL4</sup>	1.50±0.05	DIP- $\lambda$ -qPCR-F	CACCCACATGGTCTCACTAAATCC
			DIP- $\lambda$ -qPCR-R	CTCTGGATTATGTTCTGGGTGG
dpr1	dpr1 <sup>MI12729-GAL4</sup> /CyO,Dfd-YFP	0.92±0.10	dpr1-qPCR-F	GAGCCCAAGATGTCCCTGTG
			dpr1-qPCR-R	GAATATGTTGCCAGCTCGTGG
dpr2	dpr2 <sup>MI05656-GAL4</sup> /CyO,Dfd-YFP	0.93±0.27	dpr2-qPCR-F	CGTCGGCGCAGGATATTGG
			dpr2-qPCR-R	GATGACATTGACCACCAGCTG
dpr3	dpr3 <sup>MI05963-GAL4</sup> /CyO,Dfd-YFP	0.55±0.24	dpr3-qPCR-F	GGACGCTGCATGTGAAGGC
			dpr3-qPCR-R	GCCTTGAAGTGCAGATCGGG
dpr4	dpr4 <sup>CR00485-GAL4</sup> /TM6,Sb,Hu,Dfd-YFP	0.56±0.20	dpr4-qPCR-F	CGGATTCGGGCACTACACG
			dpr4-qPCR-R	GCACGGACGTGGATGAAAGG
dpr5	dpr5 <sup>MI11085-GAL4</sup>	0.13±0.02	dpr5-qPCR-F	CACGGAGCCCAAGATCAGTC
			dpr5-qPCR-R	CGTGCTCTGTGCCACAGC
dpr6	dpr6 <sup>MI01358-GAL4</sup>	0.23±0.03	dpr6-qPCR-F	CCATCACCAGGACACGGAGG
			dpr6-qPCR-R	GACACGCCGCTCTTGATG
dpr7	dpr7 <sup>MI05719-GAL4</sup>	0.18±0.10	dpr7-qPCR-F	GCACAGCCAAGAGACAGTGG
			dpr7-qPCR-R	GCCAGGGCAATAGTGCTATCCC
dpr8	dpr8 <sup>MI11830-GAL4</sup> /FM7,Dfd-YFP	0.49±0.19	dpr8-qPCR-F	GAATTTGGCAATCGCACG
			dpr8-qPCR-R	CGCAATGTCCAATCCTCGG
dpr9	dpr9 <sup>MI03594-GAL4</sup>	0.08±0.09	dpr9-qPCR-F	GGGCGATACGACCACATCG
			dpr9-qPCR-R	CTCCCTGGAAACGGAATGG
dpr10	dpr10 <sup>MI03557-GAL4</sup>	0.06±0.05	dpr10-qPCR-F	GGGCTGTCGTGCAAGATC
			dpr10-qPCR-R	GCCCACTTGATCTGCAGGG
dpr11	dpr11 <sup>MI01743-GAL4</sup>	0.30±0.06	dpr11-qPCR-F	GTGTCCAGCTGCAAGTTGTGG
			dpr11-qPCR-R	GGTTGGATCCAGTTGCGTG
dpr12	dpr12 <sup>MI01695-GAL4</sup> /CyO,Dfd-YFP	0.82±0.05	dpr12-qPCR-F	CGGCATGTACGAGTGCCAG
			dpr12-qPCR-R	CGTACTGCGGTGGTGTAGGAC
dpr13	dpr13 <sup>MI05577-GAL4</sup> /CyO,actin-GFP	0.92±0.06	dpr13-qPCR-F	GTTCACTGCCACGCACTTGAAG
			dpr13-qPCR-R	CAGCGTTGAACCTGGGGTTA
dpr14	dpr14 <sup>CR00516-GAL4</sup>	0.04±0.02	dpr14-qPCR-F	GGTGTGCATGTGCTGAACG
			dpr14-qPCR-R	CACTTGTCCTCAAGCCCAATCC
dpr15	FRT82B, dpr15 <sup>MI01408-GAL4</sup>	0.57±0.09	dpr15-qPCR-F	CCATTGTCTCCACGTGCTG
			dpr15-qPCR-R	CTTGTGGTGGCTTTGCTGGTG
dpr16	dpr16 <sup>MI05173-GAL4</sup>	0.99±0.38	dpr16-qPCR-F	CACTCCGGCAACTACAGTG
			dpr16-qPCR-R	CCCGTGACCCAGTCTGTGG
dpr17	FRT82B, dpr17 <sup>MI08707-GAL4</sup>	0.01±0.01	dpr17-qPCR-F	CGGCGCAACCTGACAATGC
			dpr17-qPCR-R	GCATGCGCACCCATGAAACG
dpr18	dpr18 <sup>CR01009-GAL4</sup>	0.33±0.11	dpr18-qPCR-F	GCCGTGTCGGTATGCTCAAG
			dpr18-qPCR-R	CGCCAATTGTTCCGGTACTGG
dpr19	dpr19 <sup>CR00996-GAL4</sup> /CyO,Dfd-YFP	0.55±0.13	dpr19-qPCR-F	CCTGAATCCCTCGGTCAAGTG
			dpr19-qPCR-R	CATGGCCGCCGTTTTCTCAC
Rpl32	ALL		Rpl32-qPCR-F	ATGCTAAGCTGTCGCACAAATG
			Rpl32-qPCR-R	GTTGATCCGTAACCGATGT

## Figure 1 – figure supplement 1

A



B

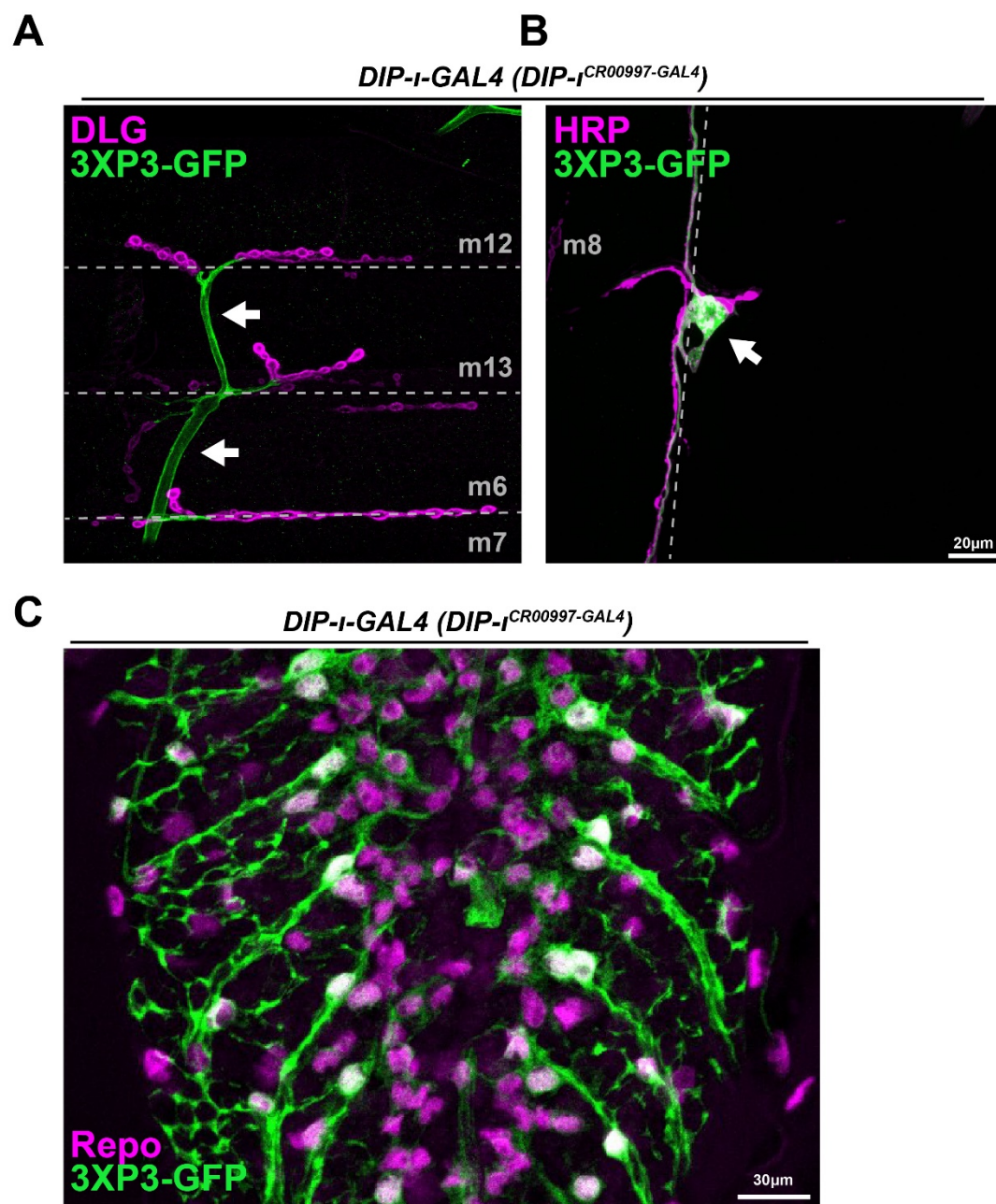


**Figure 1 – figure supplement 1. Respective mRNA level in *dpr/DIP-GAL4* lines.**

A. qRT-PCR results of homozygous viable *dpr/DIP-GAL4* lines. mRNA levels were double normalized to control animal and Rpl32 internal control. We found that most GAL4 lines are hypomorphs since the mRNA levels decrease below 50%.

B. qRT-PCR results of homozygous lethal *dpr/DIP-GAL4* lines. mRNA levels were double normalized to control animal and Rpl32 internal control. We found that most GAL4 lines have an expression level near 50%, indicating that they are hypomorphs.

## Figure 2 – figure supplement 1



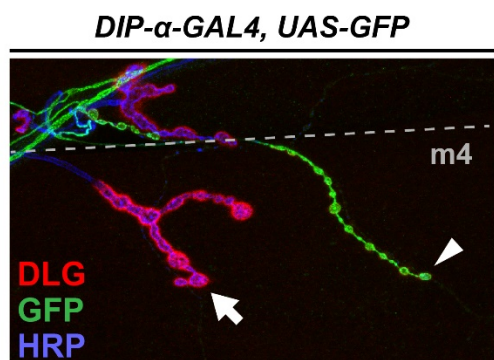
**Figure 2 – figure supplement 1. Expression of CRIMIC 3XP3-GFP in nervous system.**

A-B. Representative images labeled with DLG (magenta) and GFP (green) showing the expression of 3XP3-GFP in peripheral glial cells (arrow) and the lbd SN (arrow).

C. Representative image labeled with the glial cell marker, Repo (magenta), and GFP (green). Note the expression of 3XP3-GFP in some glial cells in the VNC.

## Figure 2 – figure supplement 2

A



B

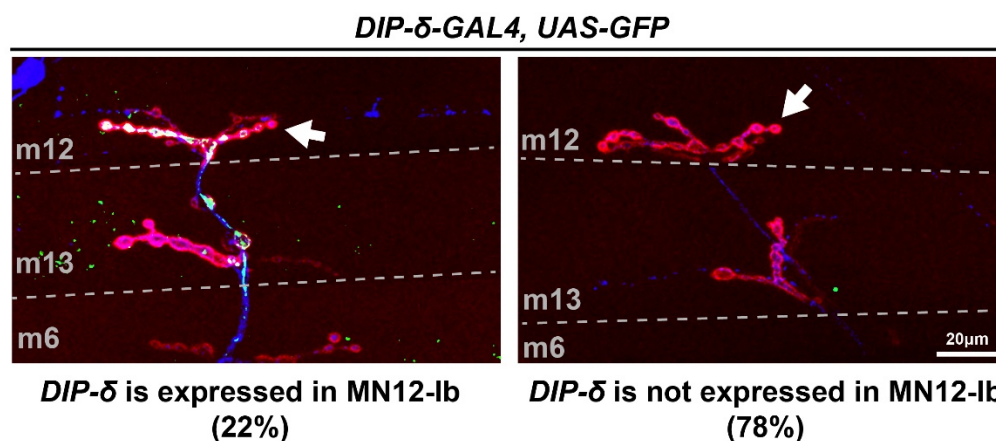
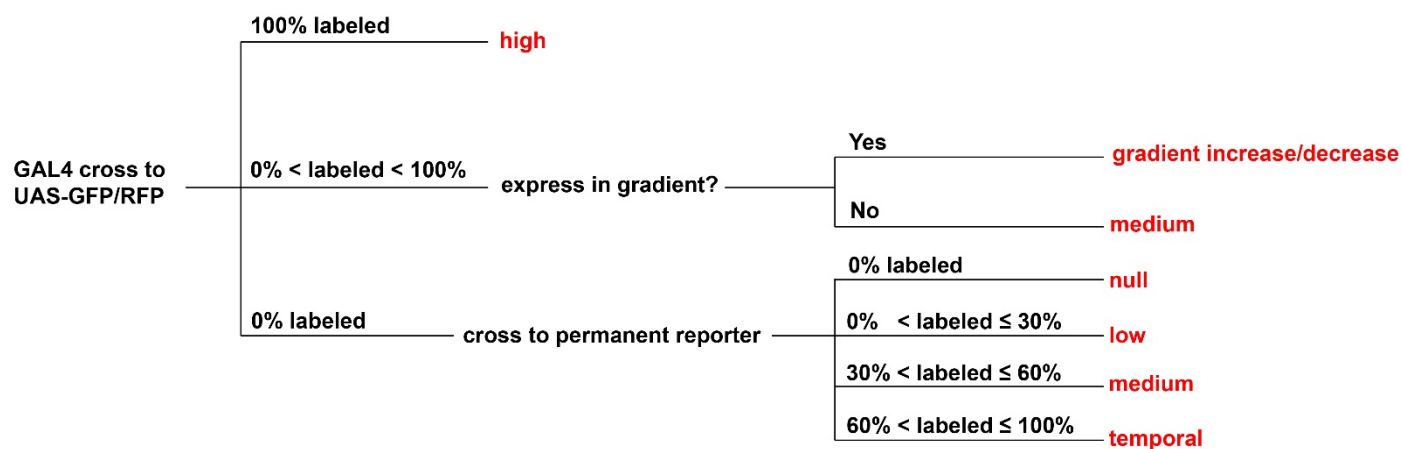


Figure 2 – figure supplement 2. Expression of *DIP- $\alpha$*  and *DIP- $\delta$*  in MNs.

A. Representative image labeled for HRP (blue), DLG (red) and GFP (green) showing the expression of *DIP- $\alpha$*  in Is MNJ (arrowhead) but not in adjacent Ib NMJ (arrow).

B. Representative images labeled with HRP (blue), DLG (red) and GFP (green) showing the varied expression of *DIP- $\delta$*  in MN12-lb (arrow). Note that 22% MN12-lb express *DIP- $\delta$*  (left) whereas 78% do not express (right).

## Figure 2 – figure supplement 3

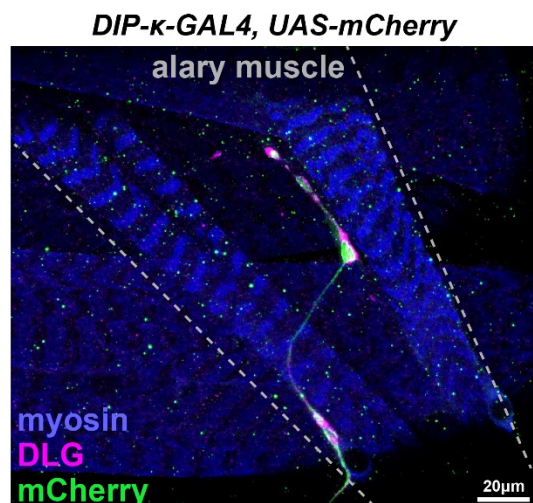


**Figure 2 – figure supplement 3. Criteria to determine the expression of GAL4 in a certain MN/SN.**

A graphical flow chart depicting how we scored the *dpr/DIP* expression data into categories.



## Figure 3 – figure supplement 1



**Figure 3 – figure supplement 1. The alary MN also expresses *dprs* and *DIPs*.**

Representative image labeled for myosin (blue), DLG (magenta) and mCherry (green) showing *DIP-κ* expression in the alary MN. Alary MNs display features of excitatory MNs as they have DLG accumulation around boutons.

## Figure 4 – figure supplement 1

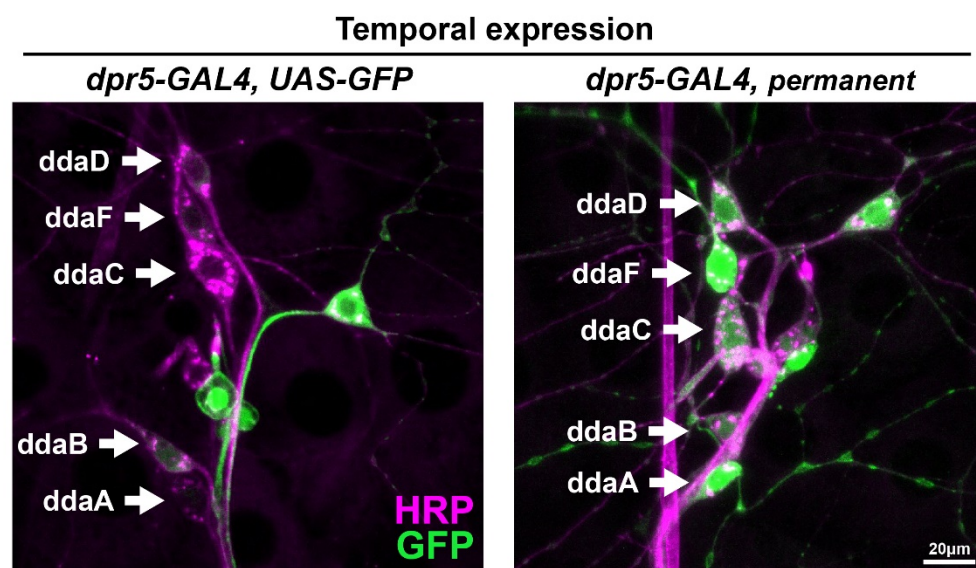


Figure 4 – figure supplement 1. Temporal expression of *dpr5* in the dorsal da neuron cluster.

*ddaA*, *ddaC*, *ddaF* and *ddaD* are not labeled in *dpr5-GAL4>GFP* larvae but are robustly labeled in the cross to the permanent reporter. Therefore, *dpr5* is temporally expressed in these SNs. *ddaB* is labeled in the cross to the real-time reporter with a low frequency, thus *dpr5* is considered as low expression in *ddaB*.

## Figure 7 – figure supplement 1

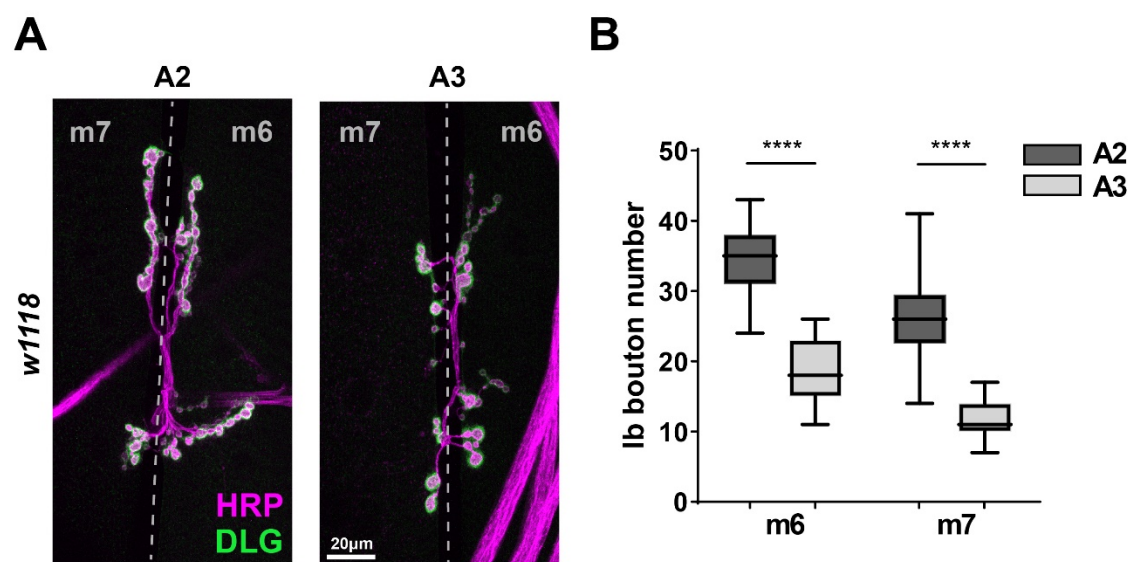
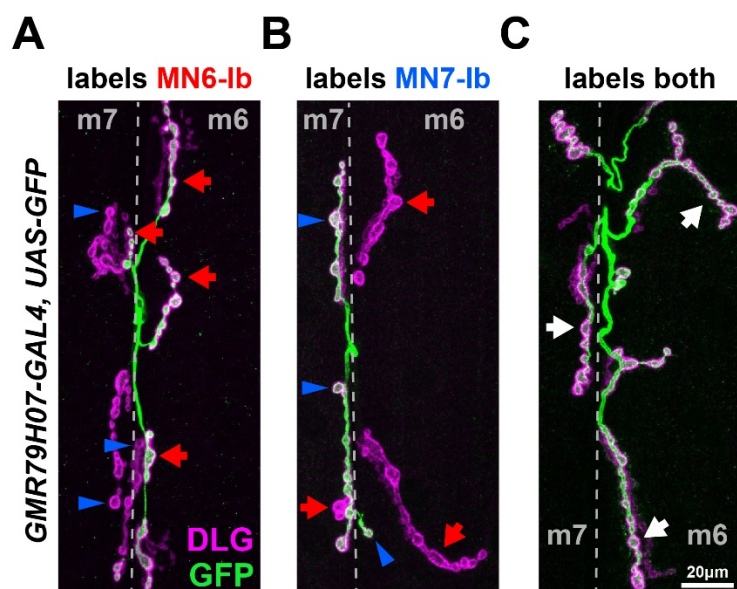


Figure 7 – figure supplement 1. Larger NMJs on m6 and m7 in A2.

A. Representative images showing larger type-Ib NMJs on m6 and m7 in A2 compared to A3.

B. Bouton number counts from m6 and m7 in A2 and A3 confirmed that A2 NMJs are double the size of A3.

## Figure 8 – figure supplement 1

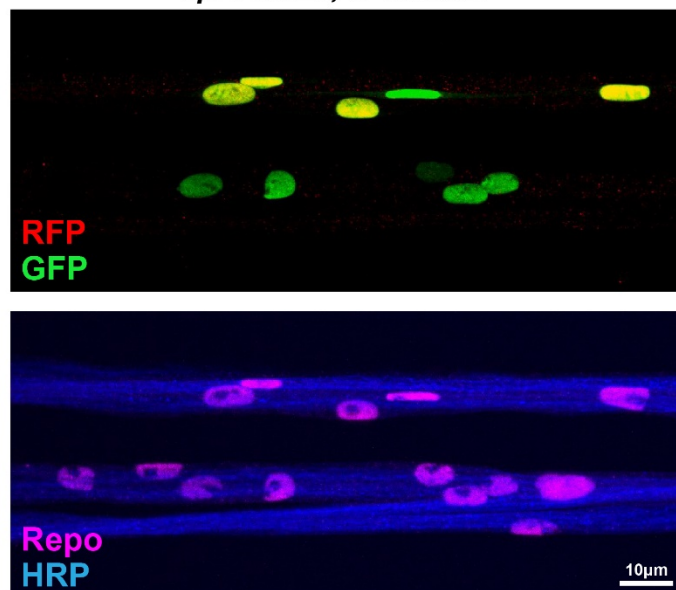


**Figure 8 – figure supplement 1. *GMR79H07-GAL4* randomly labels MN6-Ib and MN7-Ib.**

A previous study reported that *GMR79H07-GAL4* labels type-Ib NMJs on m6 in A2 (Aponte-Santiago et al., 2020). We crossed this driver to *UAS-GFP* and found inconsistent expression patterns since it (A) sometimes only labels MN6-Ib (red arrows), (B) sometimes only labels MN7-Ib (blue arrowheads), or (C) sometimes labels both MNs (white arrows). These expression patterns support the existence of MN6-Ib and MN7-Ib and their dual innervation properties.

## Figure 9 – figure supplement 1

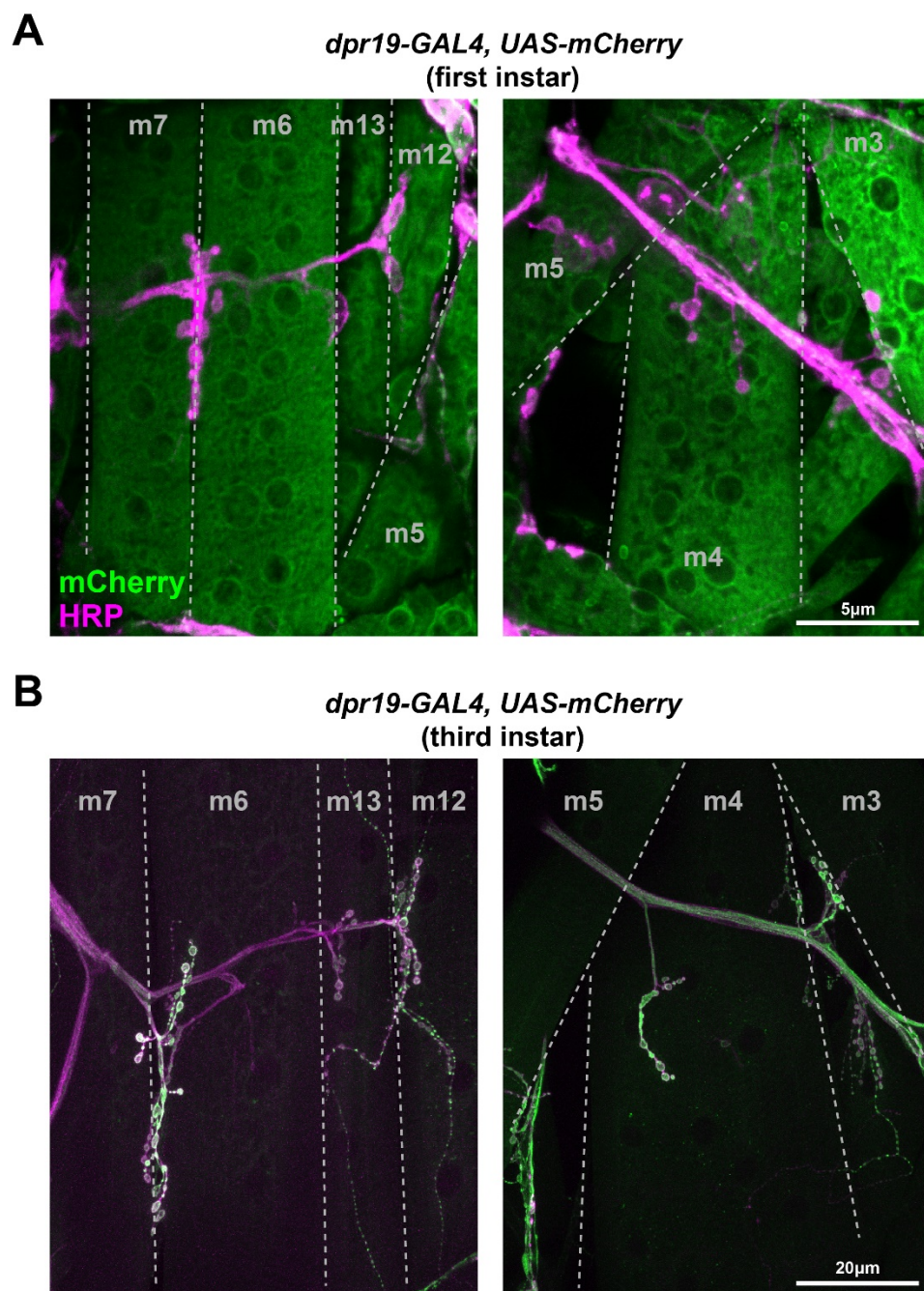
*dpr1-GAL4, G-TRACE*



**Figure 9 – figure supplement 1. The G-TRACE system revealed *dpr1* expression in peripheral glial cells.**

*dpr1* is the only *dpr/DIP* expressed in peripheral glial cells. *dpr1-GAL4* is expressed in subsets of peripheral glial cells as indicated by some glial nuclei labeled by both GFP and RFP, some only by GFP, and some lacking both GFP and RFP.

## Figure 9 – figure supplement 2



**Figure 9 – figure supplement 2. *dpr19* is expressed in muscles in early larval development.**

A. Representative images of *dpr19-GAL4>mCherry* in first instar larvae. *dpr19* is expressed in ventral (left) and dorsal (right) muscles.

B. Representative images of *dpr19-GAL4>mCherry* in third instar larvae. *dpr19* is not expressed in ventral (left) or dorsal (right) muscles.

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