Stereotyped Terminal Axon Branching of Leg Motor Neurons Mediated by IgSF Proteins DIP-α and Dpr10

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ABSTRACT

For animals to perform coordinated movements requires the precise organization of neural circuits controlling motor function. Motor neurons (MNs), key components of these circuits, project their axons from the central nervous system and form precise terminal branching patterns at specific muscles. Focusing on the *Drosophila* leg neuromuscular system we show that the stereotyped terminal branching of a subset of MNs is mediated by interacting transmembrane Ig superfamily proteins DIP-α and Dpr10, present in MNs and target muscles, respectively. The DIP-α/Dpr10 interaction is needed only after MN axons reach the vicinity of their muscle targets. Live imaging suggests that precise terminal branching patterns are gradually established by DIP-α/Dpr10-dependent interactions between fine axon filopodia and developing muscles. Further, different leg MNs depend on the DIP-α and Dpr10 interaction to varying degrees that correlate with the morphological complexity of the MNs and their muscle targets.
**Introduction**

Animal behavior depends on the stereotyped morphologies of neurons and their assembly into complex neural circuits. Distinct neurons in many neural systems use combinations of effector molecules, such as cell-surface proteins, to form stereotyped connections with specific synaptic partners during circuit assembly (Catela et al., 2015; Hong and Luo, 2014; Hattori et al., 2008). Importantly, these effector molecules have specific roles in circuit assembly, ranging from pathfinding decisions to synapse formation, depending on the cellular context and developmental stage (Peek et al., 2017; Koropouli and Kolodkin, 2014; Christensen et al., 2013; Sanes and Yamagata, 2009).

The problem of circuit assembly is particularly important for motor circuits, where motor neurons (MNs) must form topographically organized connections between pre-motor interneurons in the central nervous system (CNS) and specific muscles in the periphery, thus establishing myotopic maps in both vertebrate and invertebrate systems (Kania, 2014; Brierley et al., 2012; Baek and Mann, 2009; Landgraf et al., 2003). Myotopic maps ensure that the correct inputs into MN dendrites are relayed through corresponding MN axons to the appropriate muscle groups (Clark et al., 2018; Baek et al., 2017; Syed et al., 2016). In order to assemble accurate myotopic maps, combinations of transcription factors specify distinct MN identities early during development, which in turn activate transcriptional programs to specify distinct MN morphologies during maturation, ranging from the specification of distinct MN pools in the vertebrate spinal cord to individual MNs targeting the legs of adult
Drosophila (Enriquez et al., 2015; Santiago and Bashaw, 2014; Philippidou and Dasen, 2013). Early work on MN axon pathfinding revealed that MN axons are capable of matching with their appropriate muscle targets even when their cell bodies are displaced along the A-P axis of the vertebrate spinal cord (Landmesser, 2001; Hollyday and Hamburger, 1977). Molecular evidence for synaptic matching between MNs and muscles was later identified in the form of attractive and repulsive receptor-ligand pairs expressed in subsets of MNs and muscles in both vertebrate and invertebrate systems (Luria et al., 2008; Huber et al., 2005; Winberg et al., 1998). Additionally there must be a balance between axon-axon and axon-muscle interactions to ensure the proper innervation and branching of MNs on their muscle targets (Yu et al., 2000; Tang et al., 1994; Landmesser et al., 1988). While much is known about the initial steps, in which MN axons navigate in response to guidance cues at several ‘choice’ points (Bonanomi and Pfaff, 2010; Vactor et al., 1993), less well understood is how MNs acquire and maintain their stereotyped terminal branching morphologies and thereby establish their synaptic connections known as neuromuscular junctions (NMJs).

The formation and maturation of NMJs is a highly precise process in which the terminal branches of each MN contain stereotyped numbers and sizes of synaptic connections (Ferraro et al., 2012; Collins and DiAntonio, 2007; Johansen et al., 1989). In vertebrates, differences in axon fasciculation and terminal branching morphologies are observed between MNs innervating ‘fast’ and ‘slow’ muscles, which have distinct physiological properties and functions.
Further, the precise location of NMJ formation along each muscle fiber, defined by MN branch innervation as well as pre-patterned sites along each fiber, might also require reproducible terminal branching patterns (Kummer et al., 2006). This precision is also observed in Drosophila MNs that target larval body-wall muscles, where there are stereotyped differences between synapse size, terminal branching morphologies and electrophysiological properties (Newman et al., 2017; Choi et al., 2004; Hoang and Chiba, 2001).

In adult Drosophila melanogaster, ~50 morphologically unique MNs innervate 14 muscles in each leg. Each MN has stereotyped terminal branches that are located at specific regions of their muscle targets (Brierley et al., 2012; Baek and Mann, 2009; Soler et al., 2004). The similarities in the anatomical organization between Drosophila leg MNs and muscle fibers with their counterparts in the vertebrate limb suggest that common mechanisms might be involved. In order to identify genes used by Drosophila leg MNs, we characterized the expression patterns of various Drosophila cell-surface proteins in the adult leg neuromusculature using the MiMIC gene trap library (Lee et al., 2018; Nagarkar-Jaiswal et al., 2015; Venken et al., 2011). We focused on two families of genes that encode Ig-domain transmembrane proteins, the Dprs (Defective proboscis retraction) and DIPs (Dpr interacting proteins), which were identified as heterophilic binding partners (Özkan et al., 2013). Subsequent studies have shown that the DIPs and Dprs are expressed in specific neurons in the adult visual system in patterns that suggest they may be involved in mediating synaptic connectivity between ‘partner’ neurons (Cosmanescu et al.,
Additional functions of the DIPs and Dprs in axon self-adhesion in the olfactory system and synaptic specificity and synapse formation in the adult optic lobe and larval body-wall MNs have also been identified (Xu et al., 2018; Barish et al., 2018; Carrillo et al., 2015). Here we find that while dprs are broadly expressed in Drosophila adult leg MNs, the expression of DIPs tends to be more restricted to specific cell types, including small subsets of adult leg MNs. Most notably, DIP-α is expressed in a small number of adult leg MNs and its binding partner, Dpr10, is expressed in target leg muscles. Using in vivo live imaging of the leg MNs during development, we describe the process by which Drosophila leg MNs attain their unique axon targeting and terminal branching morphologies. Our results suggest that binding of DIP-α in MNs with Dpr10 in muscles is necessary for the establishment and maintenance of MN terminal branches in the adult leg. Moreover, the accompanying paper (Ashley et al., 2018) shows that the DIP-α-Dpr10 interaction plays a similar role in the larval neuromuscular system, suggesting a remarkably conserved function for these IgSF proteins at two stages of Drosophila development.

RESULTS

Terminal Branching of Leg MNs Occurs Through Sequential Rounds of Branching and Defasciculation Followed by Synapse Formation.

To characterize the role of the DIP and Dpr proteins in MN development we first describe the process by which leg MN axons achieve their stereotyped
muscle targeting and terminal branching patterns. We used a gene-trap within the VGlut locus to genetically label the glutamatergic leg MNs (Figure 1A) (Diao et al., 2015) and either an antibody or enhancer-trap for Mef2 (Lin and Potter, 2016), a transcription factor necessary for muscle development in Drosophila (Lilly et al., 1995), to label muscle precursors. Although we focused on the development of leg MNs targeting the foreleg (T1), the developmental processes described here are consistent across all three pairs of legs.

By the late third larval (L3) stage, adult leg MN axons from each thoracic hemisegment have exited the VNC through a single primary axon bundle and have targeted and terminate at the segment-specific ipsilateral leg imaginal disc, the precursor to the adult appendage (Figure 1A, Figure 1- figure supplement 1A). Larval MNs occupying the same nerve bundle are also labeled by VGlut but extend beyond the leg discs to target body wall muscles (Figure 1- figure supplement 1B). At this stage stereotyped groups of leg muscle precursor cells are present at specific positions in the leg imaginal disc (Maqbool et al., 2006). Shortly thereafter, 5 to 10 hrs after puparium formation (APF), leg MN axon bundles begin to defasciculate and generate fine filopodia at their termini. By 20 hrs APF, MN axons are organized into secondary bundles that target nascent muscles within each of four leg segments (Coxa, Trochanter, Femur and Tibia) (Figure 1A). Filopodia at the distal tips of these secondary bundles form net-like structures that insert between Mef2-expressing leg muscle precursor cells, the first indication that MNs are associated with distinct muscles (Figure 1C). By performing in vivo live-imaging on pupal legs expressing myr::GFP in the lineage
that produces the largest number of leg MNs (LinA/15; 29 MNs) (Enriquez et al., 2018; Brierley et al., 2012; Baek and Mann, 2009), we observed that by 30 to 35 hrs APF leg MN axons appear to maintain their initial connections to the same groups of muscle precursors even as their axons elongate and the shape of the leg disc changes (Video 1, Figure 1A). During this extension phase, as the main axon bundle lengthens, the process of axon branching continues to fine tune the targeting to distinct muscle fibers (Video 2). For example, at 25 hrs APF the axons targeting the Tibia-long tendon muscle (Ti-ltm) remain fasciculated within the secondary bundle innervating the immature Ti-ltm (Figure 1C). Soon after leg extension is initiated, the Ti-ltm secondary bundle is sequentially split (Figure 1D-E) such that by 45 hrs APF it has resolved into three distinct tertiary bundles with stereotyped terminal branching morphologies that are associated with distinct fibers of the Ti-ltm (Figure 1E). Although filopodia are still observed, they remain confined to the regions contacted by distinct terminal branches on each muscle fiber. From 45 to 60 hrs APF, terminal branches maintain a similar branching pattern, while elaboration and pruning continues to establish finer branching, and finally each branch develops characteristic swellings known as synaptic boutons that ultimately mature into the NMJs present in the mature MNs of the adult (Figure 1- figure supplement 1C-E). Together, these observations indicate that MN axon bundles target distinct muscle groups as early as 20 hrs APF, but stereotyped terminal branching onto specific muscle fibers is established between 25 to 45 hrs APF.
Expression of DIPs and Dprs in Distinct Patterns in the *Drosophila* Leg Neuromuscular System.

Because the establishment of stereotyped MN terminal branching involves the close association of developing MN axon termini with their target muscles, we expected cell-surface molecules to be required for this process. We focused on the Ig superfamily, the *dprs* and their interacting partners, the *DIPs*, and mapped the expression patterns of 8 *DIPs* and 16 *dprs* (Carrillo et al., 2015; Özkan et al., 2013) in the adult leg using MiMIC insertions converted to *T2A-Gal4* lines (Lee et al., 2018) (Figure 2, Figure 2- figure supplement 1A, Supplementary File 1). In general, the *dprs* are more widely expressed than the *DIPs*, as all the *dpr-MiMIC-T2A-Gal4* lines labeled the majority of adult leg MNs and leg sensory neurons (SNs) (Figure 2A,E, Figure 2- figure supplement 1A-B). In contrast, the *DIPs* were either expressed in a specific subset of leg MNs (*DIP*-α, *DIP*-β, *DIP*-ζ) (Figure 2B), in many but not all leg MNs (*DIP*-γ) (Figure 2B), or in subsets of leg MNs, SNs and/or muscles (*DIP*-δ, *DIP*-ε, *DIP*-η, *DIP*-θ) (Figure 2C-E). This difference in expression between the *DIPs* and *dprs* is also observed in the *Drosophila* optic lobe, mushroom body and protocerebral bridge (Davis et al., 2018). Second, unlike the neurons projecting to the medulla neuropil in the visual system (Tan et al., 2015), *DIP* and *dpr* expression patterns in the leg were not selectively biased to either the pre/post-synaptic partner of the circuit as both *DIPs* and *dprs* are expressed in leg MNs, SNs and muscles (Figure 2E, Figure 2- figure supplement 1B). Interestingly, only *DIP*-ε and *dpr10* are expressed broadly
in adult leg muscles (dpr1 is expressed in a single fiber in the most proximal muscle of the Coxa), indicating that interactions between cognate DIP-Dpr pairs might function at multiple steps during the development of the adult leg, from axon fasciculation, synaptic specificity to proper synapse formation, consistent with their roles in the adult olfactory system, larval body-wall NMJ formation, as well as in the adult optic lobe (Xu et al., 2018, Xu et al., 2018; Barish et al., 2018; Carrillo et al., 2015).

**DIP-α is Necessary for the Terminal Branching of Three Leg MNs.**

Since dpr10 is strongly expressed in leg muscles, we initially focused on a potential role for its sole interactor, DIP-α, in axon targeting. To examine DIP-α-expressing neurons at high resolution, we identified an enhancer from the DIP-α locus (DIP-α-A8) that specifically labels three of four adult DIP-α expressing leg MNs (DIP-α-A8 also labels two rows of segmentally repeating larval MNs; Figures 3A,B, Figure 3- figure supplement 1A-B). Of the three adult leg MNs labeled by DIP-α-A8, two MNs target long tendon muscles (ltms), one in the Femur (αFe-ltm, which targets the Fe-ltm, also called ltm2) and one in the Tibia (αTi-ltm, which targets the Ti-ltm, also called ltm1) (Soler et al., 2004). The third MN labeled by DIP-α-A8, αTi-tadm, targets the tarsal depressor muscle (tadm) located in the Tibia (Figure 3A). Based on their expression of DIP-α, we collectively refer to these three MNs as α-leg MNs.
We noticed a striking absence of terminal branching in α-leg MNs in homozygous DIP-α mutant animals using multiple alleles and genetic backgrounds (null, chromosomal deficiency, and homozygous MiMIC-T2A-Gal4 – see Materials and Methods) (Figure 3B-D). Similar defects were not observed in mutants for other DIP-expressing leg MNs, e.g. DIP-β, DIP-γ mutant or DIP-ζ knock-down animals (Figure 3- figure supplement 1C). Interestingly, the terminal branching of αFe-ltm, αTi-ltm and αTi-tadm displayed different but consistent penetrance of the mutant phenotype. αFe-ltm lost all terminal axon branches in 80-100% of the mutant samples analyzed while αTi-ltm lost all terminal axon branching in 20-40% of mutant samples analyzed (Figure 3D). The remaining αTi-ltm samples had some terminal branches, which were highly reduced in length and/or number (Figure 3C,E). αTi-tadm rarely displayed a complete loss of terminal axon branching (in homozygous DIP-α-T2A-Gal4 animals), but showed a loss of two to three terminal branches in several samples (Figure 3C-E).

Strikingly, even when αTi-ltm and αFe-ltm have no terminal branches, their axons enter the leg and reach the vicinity of their muscle targets (Figure 3- figure supplement 1D). These results suggest that DIP-α is not required for these MNs to reach their respective muscle targets but is required to generate their stereotyped terminal branching morphologies. Importantly, terminal branching was fully restored in the α-MNs when DIP-α-A8-Gal4 was used to re-introduce DIP-α only in these leg MNs in a DIP-α mutant background (Figure 3B,D, Figure 3- figure supplement 1E).
In order to test whether DIP-α is sufficient to induce terminal branching at the ltms of a MN that normally does not target these muscles, we ectopically expressed DIP-α in LinB/24 leg MNs, which normally target muscles in the Coxa, Trochanter and distal Femur (Enriquez et al., 2015; Brierley et al., 2012; Baek and Mann, 2009), using MARCM (Lee and Luo, 2001) and the strong MN driver \textit{VGlut(OK371)}-\textit{Gal4} (Mahr and Aberle, 2006) (Figure 3F). We also performed this experiment in a \textit{DIP-α} mutant background using an enhancer-trap (\textit{hkb-Gal4}) expressed in LinB/24 MNs. In both cases normal targeting and terminal branching of Lin B/24 neurons was observed in nearly all samples; in only one of nine \textit{DIP-α} mutant samples we observed branching at the Fe-ltm (Figure 3F).

Because DIP-α was not able to efficiently target non-DIP-α-expressing leg MNs to the ltm, we hypothesize that rare ectopic branching events might be a consequence of stabilizing occasional ‘stray’ filopodia that come close to the ltm during pupal development. Additionally, we did not observe any obvious defects in dendritic arborization of the α-leg MNs in \textit{DIP-α} mutants (Figure 3- figure supplement 1F).

From our expression analysis of the DIPs in the leg MNs, we also identified the expression of \textit{DIP-β} in the α-ltm MNs (Figure 3- figure supplement 2A). In order to test for combinatorial DIP functions in leg MN targeting we assessed the function of DIP-β in these MNs. Loss of \textit{DIP-β} alone did not affect the terminal branching of either α-ltm MN. Further, removing \textit{DIP-β} in a \textit{DIP-α} mutant background did not increase the penetrance or frequency of the terminal branching defects of αTi-ltm (Figure 3- figure supplement 2B) and expressing
DIP-β in these neurons did not rescue the DIP-α mutant phenotype (Figure 3-figure supplement 2C). While these results do not rule out the possibility that DIP-β performs other functions in the α-ltm MNs, they confirm that DIP-α is primarily responsible for the terminal branching of the α-ltms described above.

**DIP-α Can Rescue Terminal Branching Defects Late in Development.**

To better assess the role of DIP-α in terminal branching we characterized the spatial and temporal expression of DIP-α during pupal development. Using MARCM we first assigned the α-leg MNs to the LinA/15 adult leg MN lineage (Baek and Mann, 2009; Brierley et al., 2012) (Figure 4A, Figure 4-figure supplement 1A). By mapping the expression of DIP-α over the course of metamorphosis, we noticed that DIP-α turns ‘ON’ sequentially in the three LinA/15 α-leg MNs between 10 and 25 hrs APF (Figure 4A). At 25 hrs APF, the immature axons of all three α-leg MNs can be identified in the developing leg, within their respective secondary axon bundles and associated with their respective muscle groups (Figure 4B). In parallel, we used the MiMIC-GFP protein fusion (DIP-α-GFSTF) (Nagarkar-Jaiswal et al., 2015; Tan et al., 2015; Carrillo et al., 2015) to characterize the sub-cellular localization of DIP-α protein in the leg MNs during development (Figure 4C, Figure 4-figure supplement 1B-D). From the onset of expression until the adult, DIP-α is continually observed in the entire axon terminal of all three α-leg MNs. By 45 hrs APF DIP-α localizes to the fine filopodial projections that are closely associated with the developing
muscles and in the adult DIP-\(\alpha\) is localized to the presynaptic sites of the mature synaptic boutons along the terminal branches (Figure 4- figure supplement 1D).

Although these results reveal the timing and location of DIP-\(\alpha\) expression, they do not tell us when DIP-\(\alpha\) is required during development. To address this question we conducted a temporal rescue of the terminal branching phenotype using a temperature-sensitive Gal80 to inhibit \textit{DIP-\(\alpha\)-T2A-Gal4} activation of DIP-\(\alpha\) in a \textit{DIP-\(\alpha\)} mutant background (Figure 4D-E, Figure 4- figure supplement 2A). In parallel, we examined positive (no \textit{tub-Gal80}^{ts}) and negative (no \textit{UAS-DIP-\(\alpha\)-V5}) control animals from the same cross to test for an effect of the temperature shift (Supplementary File 2). We focused specifically on the terminal branching of \(\alpha\)Fe-ltm since the targeting of \(\alpha\)Ti-ltm was partially affected by the temperature shift even in the positive control (Figure 4- figure supplement 2A). Surprisingly, DIP-\(\alpha\) is able to rescue the terminal branching in 90\% of mutant \(\alpha\)Fe-ltm samples when expressed as late as 75 hrs APF. Even when provided at 125 hrs APF, which coincides with eclosion, partial rescue was observed in 70\% of mutant samples, although rescue at this time point consisted of shorter terminal branches compared to the positive control (Figure 4D).

\textbf{Dpr10 Expression in Muscles is Necessary for Terminal Branching of the \(\alpha\)-Leg MNs.}

From the interactome measurements of the DIPs and Dprs, DIP-\(\alpha\) interacts exclusively with Dpr6 and Dpr10 (Carrillo et al., 2015; Özkan et al.,
Dpr10, in turn, binds only DIP-α while Dpr6 can interact with DIP-α, DIP-β, DIP-ε, and DIP-ζ. Using double and single mutants of dpr6 and dpr10 we found that dpr10 alone was necessary for the terminal branching of the α-leg MNs: dpr10 mutants reduced terminal branching of αFe-ltm and αTi-ltm from 100% in the control to ~ 9% and 36%, respectively (Figure 5A,D). Notably, the same trends in penetrance and frequency of the terminal branching phenotype were observed in αFe-ltm, αTi-ltm, and αTi-tadm for dpr10 and DIP-α mutants. Because dpr10 is also expressed in SNs and MNs we used RNAi to knockdown dpr10 specifically in muscles using Mef2-Gal4 and separately in MNs using OK371-Gal4, and only observed a terminal branching phenotype when dpr10 was reduced in muscles (Figure 5- figure supplement 1A-B). The dpr10 mutant phenotype was partially rescued by expressing Dpr10 in the muscles using Mef2-Gal4 but, curiously, this manipulation induced patchy expression of DIP-α-T2A-QF in additional leg cells (Figure 5B,D, Figure 5- figure supplement 1C). As an additional test, rescuing dpr10 expression using DIP-ε-T2A-Gal4, which is also expressed in leg muscles, in dpr10 mutants significantly rescued the terminal branching phenotype of αFe-ltm to 85.7% compared to controls (Figure 5D, Figure 5- figure supplement 1A).

Because DIP-α binds both Dpr6 and Dpr10 (Carrillo et al., 2015; Özkan et al., 2013), we next tested if expressing dpr6 in muscles could rescue the terminal branching phenotypes of α-ltm MNs in dpr10 mutants. Strikingly, using Mef2-Gal4 to express Dpr6 in a dpr10 mutant background we observed significant
rescue in both αFe-ltm (88.8% of samples with terminal branching) and αTi-ltm (100% of samples with terminal branching) (Figure 5B,D).

In parallel to the above experiments, we also conducted an expression analysis of Dpr10, using a MiMIC-GFP protein-trap (dpr10-GFSTF) (Nagarkar-Jaiswal et al., 2015; Tan et al., 2015), during pupal development and observed that Dpr10 expression is ‘ON’ in subsets of muscle precursors in the leg imaginal discs at late L3 (Figure 5- figure supplement 1D-E) and remains expressed in adult leg muscles throughout pupal development (Figure 5C, Figure 5- figure supplement 1F). While Dpr10 was broadly observed in early pupal leg muscle precursors at 25 hrs APF, we noticed higher levels in the ltms and depressor muscles in the Femur and Tibia at 45 hrs APF (Figure 5C).

Taken together, the above results suggest that Dpr10 expression in the muscles normally interacts with DIP-α in a subset of leg MNs to ensure proper terminal branching of the DIP-α expressing leg MNs. Since exchanging the DIP-α binding partners, Dpr10 with Dpr6, in the muscles is sufficient to rescue terminal branching, we further conclude that the physical interaction, possibly adhesion, between leg MN axon termini and muscles provided by the DIP-Dpr interaction may be sufficient for the stereotyped terminal branches of these α-leg MNs.

DIP-α is Specifically Required for Terminal Axon Branching Between 30 and 45 hrs APF.
Leg MN axons normally exhibit sequential rounds of defasciculation followed by dynamic branching during pupariation (Figure 1). The defects in terminal branching seen in DIP-α mutant leg MNs could potentially occur at any of the above stages. Since the MNs targeting the Ti-ltm show clear differences before and after defasciculation from secondary to tertiary bundles (Figure 1), we focused on characterizing terminal branching of the αTi-ltm in DIP-α mutants (DIP-α-T2A-Gal4/DIP-α−) compared to controls (DIP-α-T2A-Gal4>UAS-DIP-α) using a combination of immunostaining and confocal imaging along with in vivo live imaging (Supplementary File 2). Although we focused these experiments on αTi-ltm, because it was more accessible to image than αFe-ltm, both MNs appear to behave similarly. Since mutant α-ltm MNs reach the vicinity of their muscle targets when examined in the adult, we expected the terminal branching defects in DIP-α mutants to occur after leg MNs have sorted into their secondary axon bundles (20 to 25 hrs APF). Indeed, when we image mutant samples at 25 hrs APF along with a VGlut reporter to label all leg MNs, we see that mutant α-leg MNs, including αTi-ltm, properly sort into their secondary axon bundles (Figure 6- figure supplement 1B). Moreover, live in vivo imaging at 30 to 40 hrs APF show that mutant αTi-ltm axons are also able to generate dynamic filopodia during leg extension (Video 3). However, by 30 hrs APF branches innervating the developing Ti-ltm are shorter in length in mutant samples compared to the control (Figure 6A, Figure 6- figure supplement 1A). When we analyzed fixed samples between 30 to 50 hrs APF, we noticed a gradual decrease in terminal branching in both αTi-ltm and αTi-tadm such that by 50 hrs APF, mutant samples resemble
the final adult phenotype (Figure 6A, Figure 6- figure supplement 1B-C). At this stage mutant αTi-ltm axons lack a prominent terminal branch and mutant αTi-tadm axons lack the four more proximal terminal branches and retain only the distal-most branch.

We next compared mutant and control samples at a slightly later time window, between ~35 and 45 hrs APF, using live in vivo imaging (Figure 6B, Videos 4-5.). Control αTi-ltm samples initially generate several filopodial projections along the length of the axon terminal, which soon result in a stable terminal branch at the distal region of the main αTi-ltm axon. Although mutant αTi-ltms also generate filopodial projections, none of them result in the generation of a stable terminal branch. Instead, by ~45 hrs APF, mutant αTi-ltm axons accumulate globular, punctate looking structures at their termini (Figure 6- figure supplement 1B). Defects in overall axon lengthening between mutant and control samples are also observed, with mutant samples terminating more proximally compared to control samples. The gradual decline in filopodial branching of the α-leg MNs in DIP-α mutants suggests that DIP-α is needed continuously between 30 and 45 hrs APF to generate the correct length and number of terminal branches.

Dpr10 Protein is Gradually Restricted to Distal Fibers of the Ti-ltm 30 to 45 hrs APF.
From our live imaging analysis, we found that \textit{DIP-\textalpha} is necessary to generate a stable terminal branch in \textalpha Ti-ltm axons. However, \textit{DIP-\textalpha} protein, which is localized along the entire \textalpha Ti-ltm axon terminal during development (Figure 4C), cannot by itself explain the stereotyped terminal branch formation that occurs specifically at the distal region of the \textalpha Ti-ltm axon. Therefore we took a closer look at Dpr10 protein expression in the Ti-ltm during development using an antibody against Dpr10 while simultaneously labeling developing muscles with Mef2 and the \textalpha-leg MNs with a GFP reporter (Figure 7A-B). At 25 hrs APF Dpr10 is broadly observed in the entire immature Ti-ltm (Figure 7A) and does not specifically localize at positions of filopodial branch innervation (Figure 7A'). However, by 45 hrs APF (Figure 7B), Dpr10 is enriched in the subset of distal Ti-ltm fibers that are targeted by the terminal branches of \textalpha Ti-ltm MNs and is also highly concentrated at the precise locations of \textalpha Ti-ltm branches (Figure 7B'). We also analyzed Dpr10 protein localization in animals where \textit{DIP-\textalpha} was overexpressed (\textit{DIP-\textalpha-T2A-Gal4>UAS-DIP-\textalpha}) and observed a strong association between Dpr10 expression and MN axon innervation at the Ti-ltm as early as 25 hrs APF and up until 45 hrs APF (Figure 7- figure supplement 1B-C). These results support the idea that \textit{DIP-\textalpha} can physically interact with Dpr10 \textit{in vivo}.

Interestingly, up until 35 hrs APF, \textit{DIP-\textalpha} mutant \textalpha Ti-ltm axons still project filopodial branches towards Dpr10 expressing muscle precursor cells (Figure 7- figure supplement 1A). Since \textit{DIP-\textalpha} mutant \textalpha Ti-ltm axons display a gradual decline in terminal branching (Figure 6) these results suggest that while multiple mechanisms might be involved in directing filopodial branches towards their
muscle targets, maintaining filopodial branching through DIP-α/Dpr10 interactions is required to promote additional branching, which together gradually refines the stereotyped terminal branching pattern.

**DISCUSSION**

In this study we used *in vivo* live imaging to describe the steps by which adult *Drosophila* leg MNs achieve their stereotyped axon terminal branch patterns at their muscle targets. By observing the process of leg MN targeting during pupariation, we began to query the relationships between the various steps, such as targeting the correct muscle, sequential axon defasciculation, organization of dynamic filopodial branches into stable terminal branches, fusion of muscle precursors into muscle fibers, and how these steps are ultimately coordinated with the morphogenesis of the adult leg with its complete proximo-distal axis.

We focused here on a small number of leg MNs and the role of the IgSF proteins, the DIPs and Dprs. Although many DIPs and Dprs are expressed in the adult neuromuscular system, we found a definitive requirement for DIP-α in MNs and one of its two cognate partners, Dpr10, in muscles for establishing the terminal branch pattern for three leg MNs. An analogous conclusion was made by examining phenotypes of the ISN-1s MN of the larva, suggesting a remarkably conserved role for this DIP-Dpr interaction at multiple stages of *Drosophila* neuromuscular development (see accompanying paper by Ashley et al., 2018).
Moreover, we found that another DIP-α binding partner, Dpr6, which is not normally expressed in leg muscles, could functionally replace Dpr10 when expressed in muscles. As the amino acid residues in the interaction interface between DIP-α and Dpr6 are conserved in Dpr10 and are necessary for binding (Carrillo et al., 2015), these results suggest that binding between MN terminal branches and muscles, mediated by an extracellular protein-protein interaction, may be sufficient to establish the correct terminal branching pattern for these MNs. Additional evidence to support this idea comes from experiments in the *Drosophila* optic lobe where entirely heterologous interaction domains were used to replace extracellular DIP-α and Dpr10 interacting Ig domains and rescue a mutant phenotype (Xu et al., 2018).

Notably, we found that neither DIP-α nor dpr10 was required for MN axons to navigate to the correct muscle. However, the DIP-α–Dpr10 interaction appears to be critical to maintain the MN–muscle connection as the leg elongates and the muscles take their final shape. Based on these observations, we propose that α-leg MN axons target the correct cluster of muscle precursor cells during the first 20 hrs of pupal development in a DIP/Dpr-independent manner, but then require this molecular interaction for the fine terminal branching pattern and for maintaining the MN–muscle interaction as the leg elongates and muscles mature to their final shape (Figure 7C). Interestingly, the transsynaptic cell adhesion complex comprising of Neurexin and Neuroligin is required for a similar process of terminal axon arbor growth in the abdominal body wall MNs in adult *Drosophila* (Constance et al., 2018) suggesting that multiple cell surface molecules are
employed in different sub-cellular contexts to establish and maintain accurate terminal branching.

In general, the DIPs tend to be more restricted in their expression patterns compared to the Dprs in the leg neuromuscular system. The more limited expression patterns of DIPs has also been observed in other neural cell types (Davis et al., 2018; Cosmanescu et al., 2018), implying that differences in specificity and redundancy may be a general feature of these two Ig domain protein families. However, in contrast to DIP-α, we failed to observe obvious terminal branching or axon targeting defects for MNs that express other DIP genes, such as DIP-γ and DIP-ζ. One explanation for this observation is that dpr10, a strong binder of DIP-α, is unique among the dpr genes to be strongly expressed in leg muscles. Thus, it may be that other DIPs are playing roles in MN morphogenesis that are distinct from muscle targeting and terminal branching.

In addition to differences in how broadly the DIPs and Dprs are expressed, we also observed striking differences in the timing of their expression. Specifically, we found that Dpr10 begins to be expressed in leg muscle precursors as early as the late third instar larval stage (96 hrs AEL). In contrast, DIP-α expression initiates in three leg MNs only after they have sorted into secondary axon bundles that subsequently associate with distinct muscle groups (15 to 25 hrs APF). In DIP-α and dpr10 mutants, α-leg MNs still sort into their secondary bundles but fail to establish terminal branches. Further, misexpressing DIP-α in non-α-expressing leg MNs as early as the late third instar stage had...
virtually no affect on their axon trajectories, consistent with the idea that these
molecules are not involved in the initial steps of MN pathfinding. The initial broad
expression pattern of Dpr10 in muscles might help promote early filopodial
branching of the DIP-α expressing leg MNs while they are still fasciculated within
their secondary bundles, thereby ensuring selective adhesion between the α-leg
MN axons and their muscle partners during leg extension, a process that
includes the physical rearrangement of muscle precursor cells into fibers. This is
then followed by the gradual restriction of Dpr10 expression to specific muscle
fibers and/or subregions on muscle fibers, which might contribute to the
generation and stabilization of stereotyped terminal branching (Figure 7C). Both
DIP-α and Dpr10 expression persist into the adult, and DIP-α localizes to pre-
synaptic sites at mature NMJs (Figure 4C, Figure 4- figure supplement 1D),
suggesting that this interaction might also be necessary for maintaining functional
synapses. It is interesting to note, however, that muscle-specific rescue with
Dpr10 was unable to recover branching in the larval MNs compared what we
have observed in the adult (Ashley et al., 2018). We speculate that this might be
due to the difference in the amount of time MNs have to establish their
stereotyped branching in the larvae (several hours) and adult (several days),
during which other cell-surface molecules involved in the branching process may
have to be appropriately coordinated.

Interestingly, we observed consistent differences in the penetrance of the
DIP-α and Dpr10 mutant phenotypes in the three leg MNs analyzed here.
Terminal branching of αFe-ltm was lost in nearly every mutant sample. αTi-ltm,
on the other hand, lost all of its terminal branches in only one-third of the mutant samples, with the remaining samples showing a partial loss of terminal branches. Finally, αTi-tadm only lost proximal terminal branches but always retained its distal most branch. Analogous to this phenotype, the DIP-α–Dpr10 interaction is also required for one of two terminal branches in the larval MN ISN-1s (see accompanying paper by Ashley et al., 2018). The decreasing dependencies of αFe-ltm, αTi-ltm and αTi-tadm on the DIP-α/Dpr10 interaction suggest that this interaction is context dependent. Interestingly, the number of tertiary bundles that these terminal branches stem from may be a relevant difference. αFe-ltm generates its terminal branches from a single tertiary bundle, while αTi-ltm does so from two tertiary bundles, and the terminal branches of αTi-tadm stem from four distinct tertiary bundles (Figure 6- figure supplement 1C). Further, the targeted muscles also differ in their complexity: Fe-ltm comprises three muscle fibers, Ti-ltm comprises of six to seven fibers, and Ti-tadm is made up of twenty to twenty-four fibers in the foreleg (Soler et al., 2004). Therefore, as the morphological complexity of a MN and its muscle target increases, there may be a greater dependency on multiple molecular interactions, resulting in weaker phenotypes when only one interaction is removed. Consequently, we expect more combinations of interacting cell-surface proteins to function between leg MNs and muscles whose terminal branches stem from multiple tertiary bundles or have more complex muscle morphologies to navigate.
### Key Resources Table

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Gift from Gary Struhl
Bellen Lab, provided by R. Carrillo
Generated by B. Paterson; (1:500)
(1:500)
(1:1000)
Generated by the Zipursky Lab; (1:20)
Generated by the Zipursky Lab; (1:500)
Detailed fly genotypes are provided in Supplementary File 2.

**Temporal Rescue of DIP-α**

Two-day embryo collections were performed over a week at 18°C and since *Drosophila* develop at a slower rate at lower temperatures, external morphological features of the pupae were used to stage the flies (samples are referred to by their normal 25°C stage-time). Vials were then shifted together to 30°C for 5 days before dissection. Positive and negative controls were dissected along with experimental samples from each vial. Samples were included in the final analysis only when the positive controls displayed proper terminal branching.

**MARCM**

To generate MARCM clones, embryos were collected for 12 hrs at 25°C. First-instar larvae were heat shocked at 37°C for 25 mins. Adult progeny were screened under the fluorescent microscope for T1 clones.

**Adult Leg and VNC Dissection and Mounting**

Adult flies were first immersed in 80% Ethanol for ~1min and rinsed in 0.3% PBT for ~15mins. After removal of abdominal and head segments, adult legs attached to thoracic segments were fixed overnight at 4°C followed by at least five washes.
in 0.3% PBT for 20 mins at room temperature. VNC and legs were dissected and mounted onto glass slides using Vectashield mounting medium (Vector Labs).

Due to their large size, final leg images may be a composite of more than one image. Detailed protocol for leg dissection, mounting and imaging can be found in (Guan et al., 2018).

**Immunohistochemistry**

**Antibodies**

Rabbit Anti-Mef2 (1:500, Gift from B.Paterson), Sheep Anti-GFP (1:500, Biorad), Chicken Anti-GFP (1:1000, Abcam), Mouse Anti-DIP-α (1:20, Gift from S.L. Zipursky), Mouse Anti-Dpr10 (1:500, Gift from S.L. Zipursky), Rabbit Anti-Twist (1:300, Gift from K. Jagla), Mouse Anti-V5:549 (Biorad). Secondary antibodies used were Goat Anti-Rabbit Alexa 647 (Invitrogen); Goat Anti-Rabbit Alexa 555 (Invitrogen); Goat Anti-Guinea-pig Alexa 555 (Invitrogen); Goat Anti-Mouse Alexa 555 (Invitrogen); Donkey Anti-Mouse 647 (Jackson Immunolabs); Donkey Anti-Mouse 555 (Jackson Immunolabs, Gift from W.Grueber); Donkey Anti-Rabbit 555 (Jackson Immunolabs, Gift from W.Grueber); Donkey Anti-Sheep 488 (Jackson Immunolabs, Gift from C.Desplan); Goat Anti-Chicken Alexa 488 (Invitrogen)

**Dissections**

Larval CNS and leg discs – Larvae were inverted to expose the CNS and attached leg imaginal discs; Adult VNC – After removal of the head, abdomen and legs, the thoracic ventral cuticle was removed to expose the adult VNC; Pupal legs – Pupae were extracted from the pupal case and dissected open from
the dorsal surface along the A-P axis, followed by gentle washes with a 20ul pipette to flush out the fat cells; Adult legs – T1 legs were dissected from the thoracic segment and transverse cuts were made across the middle of the Femur and Tibia segments with micro-dissection scissors.

**Immunostaining**

Dissections were performed in 1XPBS, followed by fixation in 4% Formaldehyde (prepared with 1X PBS) for 25 mins or for 1hr (adult legs) at room temperature. Samples were blocked for 2hrs (~3-5 washes) or overnight (adult legs) at room temperature and incubated with primary antibodies for one to two days and secondary antibodies for one day at 4°C. Fresh PBT with BSA (1XPBS, 0.3% Triton X-100, 1%BSA) was used for blocking, incubation and washing after fixation and after primary/secondary antibodies (~3-5 washes, 20 mins each). Samples were stored in Vectashield mounting medium (Vector Labs) until mounting and imaging.

**Mounting**

Larval VNC and leg discs – Inverted larvae were cut along the body wall with micro-dissection scissors such that larval VNC and leg discs remained attached to each other and the body wall. Samples were mounted with VNCs oriented lateral side up; Adult VNC – VNC were dissected out from the thoracic segment and mounted ventral side up; Pupal legs – Pupae were mounted ventral side up; Adult legs – Adult leg segments were mounted lateral side up. Samples were mounted in Vectashield mounting medium (Vectorlabs) on glass slides using sticker wells (iSpacer, SunJin Lab Co.).
Microscopy

Multiple 0.5um-thick sections in the z-axis were imaged with a Leica TCS SP5 II. Binary images for z-stack images and 3D reconstructions were generated using Image J software (Schneider et al., 2012).

Quantification and Statistical Analysis

For the binary quantification of the presence of terminal branching, T1 legs of multiple F1 animals obtained from parallel genetic crosses for each genotype were pooled together and scored for the presence of any amount of terminal branching in the leg MNs (sample size (N) is directly reported on the graph). Statistical significance was determined using Fisher’s exact test and assigned using the following criteria: *p<0.05; **p<0.01; ***p<0.001.

For the quantification of branch number, automatic tracings of motor neurons from each genotype were obtained using Vaa3D (Peng et al., 2010, 2014) and the ‘total number of tips’ displayed in the ‘morphology info’ was used to calculate the branch number (sample size is reported on the graph). Data was assessed for normality using the Schapiro-Wilk normality test and statistical significance was determined using either a two-tailed unpaired t-test or a Mann-Whitney U test and assigned using the following criteria: *p<0.05; **p<0.01; ***p<0.001.

In vivo Live Imaging
Pupae were first staged and sorted for the correct genotype. A small window on either the left/right ventral side of the pupal case was made using forceps to expose just the T1 leg. Individual pupae were placed on a glass slide, surrounded by two layers of filter paper dampened with distilled water. A 5ul drop of distilled water was placed at the center of a glass coverslip (N-1.5) and placed exactly over the exposed T1 leg. Petroleum jelly surrounding the filter paper was used to seal the space between the coverslip and the glass slide to retain humidity. Samples were imaged on a Zeiss LSM700 microscope, 25X objective, with a 10 min interval between each z-stack series. Videos were generated using the FIJI software (Schindelin et al., 2012) at 5 frames per second.

**Plasmids and Transgenic Lines**

*MiMIC-T2A-QF2* – Donor plasmids were obtained from Addgene (#62944 and #62945) and injected into BDSC stocks (#32808 and #34458 respectively). Refer to Diao et al., 2015 for detailed protocol. Transformants were screened and verified by crossing to *10XQUAS-6XGFP* (attp2).

*DIP-α-A8-Gal4* – Intronic region in the *DIP-α* genomic locus was PCR amplified from genomic DNA and inserted into a Gal4 vector with the DSCP promoter, generated by R.Voutev, Mann Lab and inserted into attp2 and 86Fa.

**DIP-α-A8** Forward Primer Nhe1: `aattgctagccagtcgcaaactcgttacctc`  
Reverse Primer AgeI: `aattcaggttaagatattaaaaacatcaggaattttctctc`  

**UAS-DIP-β** - *DIP-β* cDNA (synthetically generated and provided by S.L.Zipursky) was PCR amplified and inserted into pJFRC28 (Addgene #36431) using Not1 and Xba1. Plasmid DNA was inserted into VK00027.
Hexameric Fluorescent reporters – Original plasmids were obtained from S. Stowers and inserted into VK00027 and 86fa.

Injection services were provided by BestGene Inc.

Acknowledgements

We thank S.L. Zipursky for generously sharing fly stocks and reagents; M. Courgeon and C. Desplan for sharing MiMIC-T2A-Gal4 lines, antibodies and other fly stocks and reagents; V. Fernandes for help with the in vivo live imaging; M. Eveland for identifying hkb-Gal4 expression in LinB/24 leg MNs; G. Struhl, W. Grueber and M. Kohwi and their lab members for sharing time on their confocal microscopes; Members of the Mann lab, W. Grueber, O. Hober, R. Carrillo, and K. Zinn for comments and suggestions. This work was funded by NIH grants R01NS070644 and U19NS104655 to R.S.M. and R01GM067858 to H. Bellen.

Declaration of Interests

The authors declare no competing interests.

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Role of ladybird, a Conserved Regulator of Appendicular Myogenesis. PLoS ONE 1, e122.


**FIGURE LEGENDS**

**Figure 1.** Sequential Defasciculation and Branching of Developing *Drosophila* Adult Leg Motor Neurons

**A.** Development of *Drosophila* adult leg motor neurons across six distinct time points during pupariation – Late L3 (96 hrs AEL/0 hrs APF), Early Pupa (10 hrs APF), Mid Pupa (20 hrs and 25 hrs APF), Late Pupa (45 hrs APF) and Adult (120 hrs APF). **Left Column:** Schematic representation of *Drosophila* larval to adult stages denoting the locations of adult leg MN cell bodies and dendrites (green) in the CNS (gray) along with axons (green) targeting ipsilateral legs (T1 - forelegs, T2 - midlegs and T3 - hindlegs). **Middle Column:** Schematic representation of the developing T1 leg denoting the locations of muscle precursors (magenta) and leg MN axons (green). Locations of muscles within the four leg segments (Coxa, Trochanter, Femur and Tibia) are denoted from 20 hrs APF onwards. **Right Column:** Leg MN axons in the developing T1 leg labeled by *VGlut-QF>10XUAS-6XGFP* (green) and stained for *Mef2* (magenta) to label muscle precursors. Mature MNs and muscles in the Adult T1 leg are labeled using *OK371-Gal4>20XUAS-6XGFP* and *Mef2-QF>10XQUAS-6XmCherry* respectively. (scale Bar: 50um)

**B.** Snapshots from a time-lapse series of developing LinA/15 leg MNs expressing myr::GFP at 25 hrs APF (**B**; before extension) and 35 hrs APF (**B’**; after extension) (see also Video 1). Arrows denote distinct axon bundles within the T1-ltm-targeting bundle. Axon bundles are labeled according to muscle targeting –
Ti-Itm: Tibia-long tendon muscle, Tadm: Tarsal depressor muscle, Talm: Tarsal levator muscle. (scale Bar: 50 um)

C-E. Confocal images of LinA/15 Ti-Itm-targeting leg MN axons expressing myr::GFP (green) and muscles stained for Mef2 (magenta) at 25 hrs APF (C-C'), 35hrs (D-D') and 45 hrs APF (E-E'). Arrows point to defasciculating tertiary bundles and arrowheads (E-E') point to terminal axon branches. (scale Bar: 50um)

Figure 1- figure supplement 1. Sequential Defasciculation and Branching of Developing Drosophila Adult Leg Motor Neurons

A, A'. Maximum projection confocal images of a T1 leg disc denoting the terminal position (white arrowhead) of LinA/15 leg MNs expressing myr::GFP (green; generated by lineage tracing) and all MNs labeled with VGlut-QF>10XQUAS-6XmCherry (red). Note that VGlut+ axons extend beyond the leg disc, while the LinA/15 MNs are within this same fascicle but stop in the middle of the leg disc (arrowhead). Muscle precursors in the leg disc are identified by Mef2 expression (blue).

B. Maximum projection confocal image of the glutamatergic axon bundle labeled with VGlut-QF>10XQUAS-6XGFP (red), comprising of adult leg and larval body wall MNs, passing through the T1 leg disc and innervating mature larval body wall muscles. Muscle precursors in the leg disc and mature body wall muscles are identified by Mef2 expression (blue). (scale bar: 50um).
Maximum projection confocal images of LinA/15 leg MNs expressing myr::GFP (green) targeting Tibia muscles, labeled by staining against Mef2 (magenta), at 45 hrs APF (C) and leg MNs alone at 45 hrs APF (C'), 60 hrs APF (D) and in the adult (E). White arrows denote distinct axon bundles within the Ti-Ltm-targeting bundle and colored arrowheads represent examples of secondary branches whose stereotyped morphologies are retained between 45-60 hrs APF. (scale Bar: 50 um).

Figure 2. Expression Patterns of DIPs and dprs in Drosophila T1 Adult Leg Neuro-musculature

A-B. dpr (A) and DIP (B) expression patterns in the Drosophila T1 adult leg for a subset of heterophilic binding partners identified by DIP-Dpr ‘interactome’ studies (Carrillo et al., 2015; Özkan et al., 2013): DIP-α (green) and dpr10 (black); DIP-β (red) and dpr8 (black); DIP-γ (magenta) and dpr11 (black); DIP-ζ (cyan) and dpr13 (black). These DIPs were selected because they are MN-specific. The expression patterns in this and other panels were generated with MiMIC Gal4 insertions (see Supplementary File 1).

C. Expression of four additional DIPs (DIP-δ, DIP-ε, DIP-η, and DIP-θ) in the T1 adult leg (yellow). In addition to MNs, these DIPs are expressed in leg sensory neurons (DIP-δ, DIP-η, and DIP-θ) or muscles (DIP-ε).
D. DIP-δ, DIP-ε, DIP-η, and DIP-θ expression restricted to glutamatergic MNs neurons in the T1 adult leg using a genetic intersectional approach (see Materials and Methods). (scale bar: 50um).

E. Heat-map summary of DIP-dpr expression patterns in the T1 leg. Each column represents a distinct DIP or dpr expression pattern and each row represents a specific component of the adult leg-neuro-musculature. MN expression is categorized according to their terminal branching in different segments of the leg: Co, Coxa; Tr, Trochanter; Pr Fe, Proximal Femur; Di Fe, Distal Femur; Pr Ti, Proximal Tibia; Di Ti, Distal Tibia. SN expression is categorized according to their expression in sub-types of SNs (Tuthill and Wilson, 2016): Fe Ch, Femur Chordotonal Organ; Br, Bristle SNs; Ta Br, Tarsal Bristle SNs (campaniform sensilla and hairplate SNs were not included in the expression analysis). Muscle expression is not categorized because two of the three lines were broadly expressed in most muscles. (*) dpr1 is expressed in a single muscle fiber entering the Coxa leg segment.

**Figure 2- figure supplement 1.** Expression Patterns of additional DIPs and dprs in the *Drosophila* T1 Adult Leg

A. dpr expression patterns in the T1 adult leg. Bottom row – dpr6 and dpr10 expression was restricted to glutamatergic MNs in the T1 leg using a genetic intersectional approach (see Materials and Methods). (scale bar: 50um).
**B.** Schematic representation of MNs, sensory neurons (SNs) and muscles in the T1 leg.

**Figure 3.** Effects of mutating DIP-α on the terminal branching of α-leg MNs.

**A. Left Column:** Proximal-Distal (P-D) oriented Fe and Ti T1 adult leg segments depicting axon muscle-targeting of three DIP-α expressing leg MNs labeled by *DIP-α-A8-Gal4(86Fa)>20XUAS-6XGFP* (Green) (Fig S3-1.A) (See Materials and Methods). Muscles are labeled using *Mef2-QF>10XQUAS-6XmCherry* (Red); Grey; cuticle. MNs are named according to the muscle target (αFe-ltm, αTi-ltm/, and αTi-tadm) (Soler et al., 2004). **Right Columns:** Transverse sections of Fe and Ti leg segments at specific locations along the P-D axis, corresponding to the numbered white dotted lines on the left, depicting terminal branching (Green arrows) on the Fe and Ti ltm (encircled by white dotted lines) and tadm. (scale bar: 50um).

**B.** Terminal branching of the T1 α-leg MNs labeled by *DIP-α-A8-Gal4(86Fa)>20XUAS-6XGFP* in wild type (WT), DIP-α mutant and rescue contexts. **Left;** T1 legs; **Right;** Fe and Ti leg segments (axons; green (WT/rescue) or white (mutant), cuticle; grey). Absence of terminal branching of the α-ltm MNs in the DIP-α mutant T1 leg is indicated by white dotted circles; White arrowheads demarcate axons reaching the vicinity of their muscle targets (refer to Figure 3- figure supplement 1D). (scale bar: 50um).
C. Intermediate terminal branching defects in T1 legs displayed by αTi-ltm and αTi-tadm in DIP-α mutants. Single cell labeling of αTi-ltm and αTi-tadm terminal branches in the T1 proximal Ti is shown in WT (green) and DIP-α mutant (white). (scale bar: 50um).

D. Quantification of mutant phenotypes (αFe-ltm, light green; αTi-ltm, medium green; and αTi-tadm, dark green) in WT (N=20), mutant (diagonal lines) and rescue contexts (N = 7 to 20) using a DIP-α null, chromosomal deficiency and MiMIC-T2A-Gal4/QF as indicated. Statistical significance was determined using Fisher’s exact test: *p<0.05 ; **p<0.01 ; ***p<0.001

E. Quantification of number of branches on αTi-ltm and αTi-tadm single-cell samples in WT and DIP-α mutant contexts using genotypes indicated in Figure 1C. Statistical significance was determined using a two-tailed unpaired t-test for αTi-ltm samples, where error bars represent mean ± SD and a Mann-Whitney U test for αTi-tadm samples, where error bars represent median ± interquartile ranges. ***p<0.001

F. Ectopic expression of DIP-α in LinB/24 leg MNs targeting the Coxa, Trochanter and Distal Fe using OK371-Gal4 MARCM (Top) or an enhancer trap hkb-Gal4 (Bottom) which also labels an additional leg MN targeting the distal Fe (white arrowhead). Normal axon targeting of LinB/24 leg MNs (white) is shown on the left without any terminal branching at the Fe-ltm. However, in a rare case (N=1/9), ectopic expression of DIP-α using hkb-Gal4 in a DIP-α mutant background caused ectopic branching at the Fe-ltm. (scale bar: 50um).
**Figure 3- figure supplement 1.**

**A.** Genomic locus of *DIP-α* showing introns (black line), non-coding exons (grey rectangles) and coding exons (orange rectangles). The location of the *DIP-α-A8* enhancer is denoted by a green bar (see Materials and Methods). The location of the MiMIC insertion (MI02031) in a coding intron is denoted by a grey arrow. Using recombination mediated cassette exchange (RMCE), MI02031 has been independently swapped into a T2A-binary activator (Gal4/QF) that acts as a ‘gene trap’ and a GFP protein fusion (GFSTF) that acts as a ‘protein trap’ (see Materials and Methods) (Venken et al., 2011).

**B.** Expression of *DIP-α-A8-Gal4>20XUAS-6XGFP* in the larval CNS and adult VNC (black, scale bar: 50um). *DIP-α-A8-Gal4* is expressed in two segmentally repeated rows of cells along the length of the larval VNC (ventral and dorsal) and in the adult VNC, *DIP-α-A8-Gal4* is expressed specifically in three leg MNs in each hemi-segment of the thoracic ganglia, in addition to 6-8 cells in the abdominal ganglia (in between the T3 hemi-segments). Co-expression of *DIP-α-A8-Gal4>20XUAS-6XmCherry* (Magenta) and *DIP-α-GFSTF* (detected by anti-GFP – see Materials and Methods) in the ventral and dorsal larval VNC as well as the T1 hemi-segment of the adult VNC, shows that the *DIP-α-A8-Gal4*+ cells (marked by magenta asterisks in the dorsal larval VNC and adult T1 VNC hemi-segment) express *DIP-α*. 
C. Comparisons of terminal branching of DIP-expressing T1 leg MNs (MiMIC-T2A-Gal4/+; Top Row) in WT (Enhancer-Gal4>20XUAS-6XGFP; Middle Row) and mutant contexts (bottom row); DIP-β (red), DIP-γ (magenta) or DIP-ζ (cyan) (grey, cuticle). 13C09-Gal4 labels a DIP-β-expressing leg MN in the distal Ti (red asterisk marks a leg MN in the distal Fe that is inconsistently labeled even in WT animals); OK371-Gal4 labels the DIP-γ-expressing leg MNs targeting the majority of adult leg muscles; hkb-Gal4 labels DIP-ζ-expressing leg MNs in the Tr and distal Fe. DIP-expressing leg MN terminal branches that are consistently labeled by the Enhancer-Gal4 (colored arrowheads) are maintained in the mutant contexts (see Materials and Methods). (scale bar: 50um).

D. Magnified images of T1 leg segments showing individual axons (white arrows) of the DIP-α mutant leg MNs from Fig 3.B. Three axons enter the Coxa (left) and proximal Fe (middle) and two axons enter the proximal Ti (right) (grey, cuticle).

E. Expression of DIP-α in the T1 adult VNC hemisegment of DIP-α-A8-Gal4>UAS-DIP-α-V5 animals detected by anti-V5 (red) and anti-DIP-α (green) (see Materials and Methods). Anti-DIP-α worked to detect DIP-α protein levels only in the cell bodies when overexpressed. White arrowheads denote DIP-α-A8+ leg MNs. (scale bar: 10um).

F. No major aberrations were detected in the leg MN cell bodies and dendritic projections (Black) of the α-leg MNs by comparing WT (Left) and mutant (Right) adult T1 VNC hemisegments. (scale bar: 10um).

Figure 3- figure supplement 2.
A. Expression of DIP-β in the α-ltm-leg MNs is confirmed by co-expression of DIP-β-T2A-QF>10XQUAS-6XmCherry (red) along with DIP-α-A8-Gal4>20XUAS-6XGFP (green). In the Fe (A, left), DIP-β is expressed in the DIP-α-expressing MN innervating the Fe-ltm (denoted as αβFe-ltm in yellow) while DIP-β alone is expressed in leg MNs innervating the distal Fe. In the Ti (right), DIP-β is expressed in the DIP-α-expressing MN innervating the Ti-ltm (denoted as αβTi-ltm in yellow) as well an additional proximal Ti-ltm innervating neuron that does not express DIP-α. Both DIP-α and DIP-β are independently expressed in two tadm-targeting leg MNs (denoted as αTi-tadm in green and βTi-tadm in red) whose terminal branches are closely associated with one another; Magnified inset; green arrowhead points to the αTi-tadm GFP+ axon that does not express mCherry. (grey; cuticle). In the adult VNC (A'), co-expression of DIP-β-T2A-QF>10XQUAS-6XmCherry (Red) with DIP-α-A8-Gal4>20XUAS-6XGFP (green) confirms that DIP-β is expressed in only two of three DIP-α-expressing leg MNs as seen in the magnified image of the T1 VNC hemisegment (green asterisks demarcate the locations of the DIP-α-A8-Gal4+ leg MN cell bodies). (scale bar: 50um).

B. Left: Terminal branching of the T1 α-leg MNs is unaffected in a DIP-β mutant (green, axons; grey, cuticle). Right: T1 Ti leg segments of a DIP-α homozygous and DIP-β heterozygous mutant (top) in comparison to a DIP-α and DIP-β homozygous double mutant (bottom) showing no increase in the severity of the
terminal branching defect seen in αβ-Ti-ltm (Figure 3.C). (white, axons; grey, cuticle). (scale bar: 50um).

C. DIP-β cannot rescue the DIP-α mutant branching defect when reintroduced in the mutant DIP-α leg MNs using DIP-α-T2A-Gal4>UAS-DIP-β. (red, axons; grey, cuticle). Note that mutant αTi-tadm branching was not affected by ectopically expressing UAS-DIP-β. (scale bar: 50um).

Figure 4. Spatial and Temporal Characterization of DIP-α Expression

A. T1 LinA/15 leg MN MARCM clones using OK371-Gal4>20XUAS-6XmCherry (magenta) and DIP-α-T2A-QF>10XQUAS-6XGFP (green) to label leg MN cell bodies in the VNC at multiple developmental time points. At late L3 stages DIP-α expression is not yet ‘ON’ in LinA/15 leg MNs although expression is observed in non-LinA/15 cells (asterisk). Between 10-25hrs APF, the three LinA/15 α-leg MNs (Fig S4.A) initiate DIP-α expression in a sequential manner, one after the other (arrowheads point to DIP-α+ cells in LinA/15 clones) (scale bar: 10um).

B. Pupal leg at 25hrs APF stained for all MNs (OK371-Gal4>20XUAS-6XmCherry; magenta), immature muscles (Mef2 expression; grey), and DIP-α-expressing MNs (green) . (scale bar: 25um).

C. Endogenous DIP-α expression in αTi-ltm and αTi-tadm axon termini using GFP-tagged DIP-α-GFSTF (green, detected by anti-GFP, see Materials and Methods) and labeled by DIP-α-T2A-QF>10XQUAS-6XmCherry magenta) at 35 hrs APF, 45 hrs APF and in 1 week old adults. White arrowheads point to
selected regions of mCherry and GFP co-expression. White-dotted boxes denote magnified insets in Figure 4- figure supplement 1D. (scale bar: 25um)

D. Temporal rescue at 125 hrs APF of axon terminal branching of αFe-ltm in the proximal Fe of T1 adult legs in samples mutant for DIP-α using DIP-α-T2A-Gal4×20X-6XGFP, UAS-DIP-α-V5 and tub-Gal80ts (see Materials and Methods).

Top row: Negative control (no UAS-DIP-α-V5) showing absence of αFe-ltm terminal branching in flies that were temperature shifted from 18°C to 30°C at 125 hrs APF (axons, white; cuticle, grey). Middle row: Positive control (no tub-Gal80ts) showing complete terminal branching of αFe-ltm in flies that were temperature shifted from 18°C to 30°C at 125 hrs APF (axons, green; cuticle, grey). Bottom row: Temporal rescue of terminal branching of αFe-ltm in a DIP-α mutant background in flies that were temperature shifted from 18°C to 30°C at 125 hrs APF; Terminal branches are shorter and/or fewer in number compared to the positive controls (axons, green; cuticle, grey).

E. Quantification of T1 leg samples with terminal branching of αFe-ltm in temporally rescued samples (N=8) (green circles) compared to positive controls (N=4-6) (no tub-Gal80ts, dark green squares) that were temperature-shifted together at 75 hrs, 100 hrs or 125 hrs APF. Terminal branching of αFe-ltm was seen in 87.5% of samples that were temperature-shifted at 75 hrs APF and in 71.42% of samples that were temperature-shifted at 100 hrs or 125 hrs APF. Terminal branching was always observed in 100% of samples of the positive control and always absent in the negative control (N=4-6, Figure 4C). Stages of leg MN axon development are indicated below the graph as defined in Figure 1.
Initiation of endogenous \textit{DIP-\(\alpha\)} expression in the three WT LinA/15 \(\alpha\)-leg MNs is indicated by a vertical green bar at 10 to 25 hrs APF. Time of eclosion is indicated by a vertical line at 120 hrs/5 days APF.

**Figure 4- figure supplement 1.** Spatial and Temporal Characterisation of \textit{DIP-\(\alpha\)} Expression

\textbf{A.} T1 LinA/15 leg MN cis\textsuperscript{2}MARCM clones (Enriquez et al., 2018) using \textit{OK371-Gal4>20XUAS-6XmCherry} (magenta) and \textit{DIP-\(\alpha\)-T2A-QF>10XQUAS-6XGFP} (green) to label leg MN axons in the T1 adult Fe and Ti leg segments. Green arrows indicate terminal branching of \(\alpha\)Fe-ltm, \(\alpha\)Ti-ltm, and \(\alpha\)Ti-tadm as belonging to the LinA/15 leg MN lineage in Fe and Ti segments respectively. (scale bar: 50um)

\textbf{B.} T1 \(\alpha\)-leg MNs labeled by \textit{DIP-\(\alpha\)-A8-Gal4>20XUAS-6XGFP} (green) showing normal terminal branching in \textit{DIP-\(\alpha\)-GFSTF/DIP-\(\alpha\)}– animals (\(N=7/7\)). (cuticle, grey). (scale bar: 50um)

\textbf{C.} Endogenous DIP-\(\alpha\) expression in P-D oriented \(\alpha\)Fe-ltm axon terminals using GFP-tagged \textit{DIP-\(\alpha\)-GFSTF} (green, detected by anti-GFP – see Materials and Methods) and labeled by \textit{DIP-\(\alpha\)-T2A-QF>10XQUAS-6XmCherry} (magenta) at 45hrs APF and in 1 week adults. White arrowheads point to selected regions of mCherry and GFP co-expression. (scale bar: 25um).

\textbf{D.} Magnified insets from Figure 4C showing localization of \textit{DIP-\(\alpha\)::GFP} protein expression using \textit{DIP-\(\alpha\)-GFSTF} (green, detected by anti-GFP; see Materials and
Methods) in fine filopodial projections of αTi-ltm and αTi-tadm labeled by $DIP-\alpha-T2A-QF>10XQUAS-6XmCherry$ (magenta) at 35 hrs and 45 hrs APF, and in pre-synaptic regions of the NMJ/synaptic boutons along the terminal branches in 1 week old adults (denoted by white arrowheads) (scale bar: 25 um).

E. Antibody staining against DIP-α (green) in an αTi-ltm axon labeled by $DIP-\alpha-T2A-QF>10XQUAS-6XGFP$ (magenta) at 45 hrs APF showing low levels of expression in specific axon branches (denoted by white arrowheads). (scale bar: 25 um).

F. Antibody staining against DIP-α (green) in α-leg MN axons (Left) overexpressing DIP-α and labeled by $DIP-\alpha-T2A-Gal4>20XUAS-6XGFP, UAS-DIP-\alpha$ (magenta) at 25 hrs APF showing high levels of expression in axon terminals including filopodial projections. White-dotted box denotes magnified inset of αTi-ltm (right) (scale bar: 25 um).

Figure 4- figure supplement 2. Temporal Rescue of DIP-α in a Mutant Background

A. Temporal rescue at 75 hrs APF of axon terminal branching of αFe-ltm (left column) and αTi-ltm (right column) in the proximal Fe of T1 adult legs in samples mutant for DIP-α using $DIP-\alpha-T2A-Gal4>20XUAS-6XGFP, UAS-DIP-\alpha-V5$ and $tub-Gal80^{ts}$ (see Materials and Methods). Top row: Negative control (no $UAS-DIP-\alpha-V5$) showing absence of αFe-ltm and αTi-ltm terminal branching in flies that were temperature shifted from 18°C to 30°C at 75 hrs APF (axons, white;
cuticle, grey). Middle row: Positive control (no tub-Gal80ts) showing complete terminal branching of αFe-ltm but incomplete terminal branching of αTi-ltm and αTi-tadm in flies that were temperature shifted from 18°C to 30°C at 75 hrs APF (refer to Fig 3. for WT terminal branching) (axons, green; cuticle, grey). Bottom row: Temporal rescue of terminal branching of αFe-ltm in a DIP-α mutant background in flies that were temperature shifted from 18°C to 30°C at 75 hrs APF; αTi-ltm and αTi-tadm were not compared to defective positive controls. (axons, green; cuticle, grey).

**B.** T1 VNC hemisegments expressing DIP-α-T2A-Gal4>20XUAS-6XGFP, UAS-DIP-α-V5 at 25°C (top) and with/without tub-Gal80ts at 18°C (bottom) stained for V5 expression (red). (scale bar: 25um)

**Figure 5.** dpr10 Expression in Muscles is Necessary for Terminal Branching of the α-Leg MNs.

**A.** Terminal branching of the T1 α-leg MNs labeled by DIP-α-T2A-QF>10XQUAS-6XGFP in dpr6 and dpr10 double and single mutants. **Left** – T1 legs; **Right** – Fe and Ti leg segments (axons, green; cuticle, grey). Terminal branching of αFe-ltm and αTi-ltm is absent only in the dpr6, dpr10 double mutant and dpr10 single mutant and phenocopies the DIP-α mutant phenotype (white dotted circles), while terminal branching of the α-leg MNs is intact in a dpr6 single mutant. (scale bar: 50um)
B. Muscle-specific expression of dpr10 (top) and dpr6 (middle) using Mef2-Gal4>UAS-dpr10/6-V5 in a dpr10 mutant background in Fe and Ti T1 leg segments showing rescue of terminal branching of αFe-ltm and αTi-ltm labeled by DIP-α-T2A-QF>10XQUAS-6XGFP. Expression of dpr10 in the muscles with the strong muscle driver, Mef2-Gal4, caused ectopic aberrant induction of DIP-α-T2A-QF>10XQUAS-6XGFP expression in the cuticle of the leg. Wild-type terminal branching of the α-leg MNs is displayed using DIP-α-T2A-QF>10XQUAS-6XGFP (bottom). (scale bar: 50um)

C. Endogenous dpr10 expression in the developing T1 leg (Left column) using a GFP protein-trap inserted into a coding intron of dpr10 (Figure 5- figure supplement 1D) (detected using a anti-GFP (green) – see Materials and Methods) at 15 hrs, 25 hrs and 45 hrs APF. Developing α-leg MNs are concurrently labeled by DIP-α-T2A-QF>10XQUAS-6XmCherry (magenta) (middle column, merge; right column, DIP-α-T2A-QF>10XQUAS-6XmCherry). At 15 hrs APF (top row), at most only two of three α-leg MNs express DIP-α (immature axon terminals are indicated by a white arrowhead) and dpr10 is broadly expressed in immature adult muscle precursors. By 25 hrs APF (middle row) when axons are normally associated with their muscle groups, immature axons of αTi-ltm and αTi-tadm form filopodia in the dpr10 expressing Ti-ltm and Tadm. At 45 hrs APF (bottom row) when leg MN axons are normally associated with distinct muscle fibers, αFe-ltm (Fig S5.F), αTi-ltm and αTi-tadm have generated their terminal branches in the dpr10 expressing Ti-ltm and Tadm. (scale bar: 100um)
D. Quantification of percentages of T1 leg samples with terminal branching of αFe-ltm (light green) and αTi-ltm (medium green) in WT (N=15), mutant (diagonal lines) and rescue contexts (N = 7 to 11) using *dpr6* and *dpr10* double and single null mutations as indicated (see Materials and Methods). Statistical significance was determined using Fisher’s exact test.

**Figure 5**- **figure supplement 1.** *dpr10* Expression in Muscles is Necessary for Terminal Branching of the α-Leg MNs.

A. Terminal branching of the T1 α-leg MNs in Fe and Ti segments labeled by *DIP-α-T2A-QF>10XQUAS-6XGFP* (axons, green; cuticle, grey). Top – Muscle-specific RNAi knockdown of *dpr10* using *Mef2-Gal4>UAS-dpr10 RNAi, dpr10-/+* resulted in the absence of terminal branching of αFe-ltm and αTi-ltm. Bottom – Inducing *dpr10* expression with *DIP-ε-T2A-Gal4>UAS-dpr10-V5* rescued the *dpr10* mutant terminal branching of αFe-ltm and αTi-ltm. (scale bar: 50um)

B. Quantification of percentages of T1 leg samples with terminal branching of αFe-ltm (light green) and αTi-ltm (medium green) in WT samples (N=15) as compared to negative controls, *dpr10* mutant (diagonal lines) and muscle-specific RNAi knockdown of *dpr10* (diagonal lines) as indicated. Muscle-specific RNAi knockdown of *dpr10* caused a significant reduction in the frequency of αFe-ltm terminal branching (N=7, ***p<0.001) but not that of αTi-ltm.

C. Muscle-specific expression of *dpr10* using *Mef2-Gal4>UAS-dpr10-V5* induces high levels of Dpr10 in the immature T1 adult leg muscles (Left, white curved...
lines outline the T1 leg and white rectangular box demarcates region of magnification; right, magnified image of the T1 distal Fe and proximal Ti segments) visualised here by staining against V5 (red) and Mef2 (blue) at 25 hrs APF (See Materials and Methods). (scale bar: 50um)

D. Genomic locus of dpr10 showing introns (black line), non-coding exons (grey rectangles) and coding exons (orange rectangles). The location of the MiMIC insertion (MI03557) in the coding intron is denoted by a grey arrow. Using recombination mediated cassette exchange (RMCE), MI02031 was swapped into a T2A-binary activator (Gal4) that acts as a ‘gene trap’ and a GFP protein fusion (GFSTF) that acts as a ‘protein trap’ (see Materials and Methods) (Venken et al., 2011).

E. Endogenous dpr10 expression in a subset of immature leg muscle precursors in a late L3 T1 leg imaginal disc using dpr10-GFSTF (Fig S5.D) (detected using a ch-anti-GFP (green) – see Materials and Methods) and stained for Twist (Twi) a muscle-precursor marker (red). (scale bar: 50um)

F. dpr10 expression in the developing T1 leg (left) at 45 hrs APF using dpr10-GFSTF (Fig S5.D) (detected using anti-GFP (green) – see Materials and Methods) at 45 hrs APF showing terminal branching of αFe-ltm labeled by DIP-α-T2A-QF>10XQUAS-6XmCherry (magenta) in dpr10 expressing Fe-ltm (green).

The tidm also expresses high levels of Dpr10 at this stage. (middle column – merge; right column, DIP-α-T2A-QF>10XQUAS-6XmCherry). (scale bar: 100um)
Figure 6. DIP-α is Required for Terminal Axon Lengthening and Branching 30 to 45 hrs APF.

A. Terminal axon branching of control (left) and DIP-α mutant (right) αTi-ltm and αTi-tadm leg MNs at 30 hrs (top), 40 hrs (middle) and 50 hrs (bottom) APF using DIP-α-T2A-Gal4>UAS-DIP-α and DIP-α-T2A-Gal4/DIP-α−, respectively. Axons are labeled using DIP-α-T2A-Gal4>20XUAS-6XGFP (green) and muscles are labeled with antibody against Mef2 (magenta). White arrowheads demarcate branch points along the axon terminal. At 50 hrs APF, mutant αTi-ltm axons lack a prominent contralateral branch and mutant αTi-tadm axons lack four contralateral branches and retain the distal-most branch. White-dotted box denotes magnified inset in Fig S6.A. (scale bar: 25um).

B. Snapshots from time-lapse videos (Video 4, Video 5) comparing control (top) and mutant (bottom) αTi-ltm and αTi-tadm axons between ~35 hrs and 45 hrs APF (time-stamp is located on the top-right corner of each snapshot). Axons are labeled using DIP-α-T2A-Gal4>20XUAS-6XGFP (yellow). White open arrowheads demarcate the contralateral branch point on the αTi-ltm axon in the control sample while filled white arrowheads demarcate assorted filopodial projections along the αTi-ltm axon in both control and mutant samples. The distal-most tip of the αTi-ltm axon is more proximally located in the mutant sample compared to the control at ~35 hrs APF (far left), as measured from the axon 'bend' at the joint between the distal Femur and proximal Tibia (denoted by white vertical bars) as well as at ~45hrs APF (far right), as measured from the
distal most branch of αTi-tadm (denoted by white vertical bars). White circles demarcate globular punctate structures that form on the mutant αTi-Ltm axon by ~45hrs APF (far right). (scale bar: 25um).

Figure 6- figure supplement 1. DIP-α is Required for Terminal Axon Lengthening and Branching 30 to 45 hrs APF.

A. Magnified inset from Figure 6A showing decreased αTi-Ltm terminal axon branching in a DIP-α mutant (right) compared to the control (left) at 30 hrs APF. Axons are labeled using DIP-α-T2A-Gal4>20XUAS-6XGFP (green) (scale bar: 25um).

B. 3D projections of axon terminals of Ti-Ltm targeting leg MNs, including αTi-Ltm, in a DIP-α mutant background at 25 hrs (top), 40 hrs (middle) and 45 hrs (bottom) APF. Leg MNs are labeled using VGlut-QF>10XQUAS-6xmCherry (magenta) and αTi-Ltm is labeled using DIP-α-T2A-Gal4>20XUAS-6XGFP (green) (left, merge; right, αTi-Ltm). At 25 hrs APF, DIP-α mutant αTi-Ltm axon terminals are located within their secondary axon bundles (2°) and generate filopodial projections. Between 40-45 hrs APF, although mutant αTi-Ltm axon sort into tertiary axon bundles (3°), they fail to generate terminal branches. White circles denote globular, punctate structures that form on the mutant αTi-Ltm axon by ~45 hrs APF. (scale bar: 25um).

C. 3D projections of axon terminals of Ti targeting leg MNs, including αTi-Ltm (lateral view) and αTi-tadm (medial view), in a control background at 50hrs APF.
Leg MNs are labeled using $\text{VGlut-QF}>10\times\text{QUAS-6xmCherry}$ (magenta) and α-leg MNs are labeled using $\text{DIP-α-T2A-Gal4}>20\times\text{UAS-6XGFP}$ (green) (left-unlabeled, merge; middle-labeled, $\text{VGlut}$; right-labeled, merge). White arrowheads point to tertiary bundles of MNs targeting the Ti-ltm and tadm and white arrows point to distinct terminal branches. In total there are four tertiary bundles targeting the Ti-ltm (one of which does not belong to the LinA/15 leg MN lineage – white asterix) composed of eight terminal branches, two of which belong to αTi-ltm. In the portion of the tadm pictured here, there are four tertiary bundles composed of approximately thirteen distinct terminal branches, of which nine belong to αTi-tadm. (scale bar: 25um)

Figure 7. Dpr10 Expression is Gradually Restricted to Distal Fibers of the Ti-ltm 30 to 45 hrs APF.

A-B. Dpr10 protein expression (grey) in the developing Ti-ltm and proximal tadm, labeled by Mef2 (magenta) along with αTi-ltm and αTi-tadm axons labeled by $\text{DIP-α-T2AQF}>10\times\text{QUAS-6XGFP}$ (green) at 25 hrs (A) and 45 hrs (B) APF. Left: GFP, Mef2 and Dpr10; middle: GFP and Mef2; right: Dpr10. A’,B’ show magnified single slice images of the dashed boxes indicated in A,B. While Dpr10 is widely expressed in the entire Ti-ltm at 25 hrs APF (A, dashed boundary demarcates immature Ti-ltm), it is later restricted to the distal muscle fibers of the Ti-ltm at 45 hrs APF (B, white arrowhead, dashed boundaries demarcate distinct groups of muscle fibers of the Ti-ltm). Lack of Dpr10 expression in the proximal
muscles fibers of the Ti-ltm at 45hrs APF (B) are denoted by white arrows. Dpr10
is continuously expressed in the tadm. (scale bar: 25um). At 25 hrs APF (A’)
there is no correlation between Dpr10 protein and αTi-ltm innervation, denoted by
white arrowheads. By 45 hrs APF (B’) higher levels of Dpr10 protein are seen in
regions innervated by the αTi-ltm terminal branches (white arrowheads).

C. Schematic representation of a DIP-α expressing axon (green) innervating
Dpr10 expressing muscle precursors (black outlined pink cells) during the course
of development between 25 to 45 hrs APF, depicting the restriction of Dpr10 to
specific muscle fibers targeted by the DIP-α expressing axon. Leg extension
occurs between ~30 to 40 hrs APF.

**Figure 7- figure supplement 1.**

A,A’. DIP-α mutant αTi-ltm axon labeled by *DIP-α-T2A-Gal4>20XUAS-6XGFP*
(green) retains its ability to target regions of high Dpr10 expression (grey) in the
developing Ti-ltm (labeled by Mef2, magenta) at 35 hrs APF. (Left: GFP, Mef2
and Dpr10; middle: GFP and Mef2; right: Dpr10). Numbered white-dotted boxes
demarcate locations of magnified single-slice insets (A’) (scale bar: 25um). White
arrowheads demarcate locations of axon innervation by the *DIP-α* mutant αTi-ltm.

B-C. Overexpression of *DIP-α* in α-leg MNs using *DIP-α-T2A-Gal4>UAS-DIP-α*
(labeled by 20XUAS-6XGFP, green), causes accumulation of Dpr10 protein
(grey) at sites of axon innervation in the Ti-ltm (labeled by Mef2, magenta), as
well as within the primary axon and filopodial projections of αTi-ltm at 25 hrs APF
(B-B') and 45 hrs APF (C-C'). Numbered white-dotted boxes demarcate locations of magnified single-slice insets (B',C'). Left: GFP, Mef2 and Dpr10; middle: GFP and Mef2; right: Dpr10). White asterix demarcates a filopodial projection of αTi-ltm that is not physically associated with the Ti-ltm but nevertheless expresses Dpr10 (B'.1). White arrowheads point to regions of axon innervation in the Ti-ltm or within the main axon of αTi-ltm that express high levels of Dpr10.

**Video 1.** WT, 25 to 37 hrs APF

Time lapse *in vivo* live imaging of developing T1 LinA/15 leg MNs expressing myr::GFP (yellow) between ~25 hrs APF (00:00) to ~37 hrs APF (12:20) (10 min interval, 5 fps). In the first frame the Ti-ltm targeting secondary axon bundle is labeled within the white-dotted box demarcating the entire Ti segment. Leg extension is initiated at ~30 hrs APF and axons within the secondary bundle begin to defasciculate while filopodial branches maintain physical contact with their muscle targets (Figure 1B-D, Figure 1- figure supplement 1C-E). (scale bar: 50um).

**Video 2.** WT, 40 to 50 hrs APF

Time lapse *in vivo* live imaging of developing T1 LinA/15 Ti-ltm targeting leg MNs expressing myr::GFP (yellow) between ~40 hrs APF (00:00) to ~50 hrs APF (12:20) (10 min interval, 5 fps). In the first frame the Ti-ltm targeting secondary axon bundle is labeled within the white-dotted box. The generation of stable
terminal branches occurs between ~43 to 45 hrs APF, at which point leg muscles are being reorganized into distinct muscle fibers (Figure 1B-D, Figure 1- figure supplement 1C-E). (scale bar: 50um).

**Video 3.** **DIP-α** mutant, 30 to 38 hrs APF
Time lapse *in vivo* live imaging of Ti-ltm targeting leg MNs, including αTi-ltm, in a DIP-α mutant animal between ~30 hrs APF (00:00) to ~38 hrs APF (08:30) (10 min interval, 5 fps). Leg MNs are labeled using VGlut-QF>10XQUAS-6xmCherry (magenta) and αTi-ltm is labeled using DIP-α-T2A-Gal4>20XUAS-6XGFP (green) (left: αTi-ltm; right: Merge). In the first frame, both, the Ti-ltm and Ti-tadm, lm (levator muscles), and rm (reductor muscles) targeting secondary axon bundles (magenta), as well as individual αTi-ltm and αTi-tadm axons (green) within these bundles are visible. **DIP-α** mutant αTi-ltm axons generate dynamic filopodia during leg extension, but show a gradual decline in branching between 30 to 45 hrs APF (Figure 6A, Figure 6- figure supplement 1A-B). (scale bar: 50um).

**Video 4-5.** WT (Video 4) and **DIP-α** mutant (Video 5), 35 to 45 hrs APF
Time lapse *in vivo* live imaging of αTi-ltm leg MNs in control (Video 4) and **DIP-α** mutant animal (Video 5), using DIP-α-T2A-Gal4>UAS-DIP-α and DIP-α-T2A-Gal4>/DIP-α− respectively, between ~35 hrs APF (00:00) to ~45 hrs APF (Control: 11:30; Mutant: 11:40) (10 min interval, 5 fps). α-leg MNs are labeled using DIP-α-T2A-Gal4>20XUAS-6XGFP (yellow). αTi-ltm and αTi-tadm axons are labeled in
the first frame. The DIP-α mutant αTl-Itm axon fails to generate stable terminal branches, while the control αTl-Itm axon begins to generate a collateral branch at ~38 hrs APF which stabilizes and extends in length by ~45 hrs APF (Figure 6B). (scale bar: 50um).
**Temporal Rescue - Post-Eclosion (125hrs APF)**

- **D** temporal rescue - post-eclosion (125hrs APF)
  - DIP α T2A-Gal4>GFP/DIP α -/–
  - tub-Gal80 ts UAS-DIP α

- **E**
  - Temporal Rescue (n=8)
  - (CTRL) No tub-Gal80 ts (n=4-6)

**Development**

- Mef2
- T1 Tibia 25hrs APF
- DIP α T2A-QF>GFP / DIP α-GFSTF

**Diagram**

- **C**
  - DIP α T2A-QF>mCh / DIP α-GFSTF
  - 25um

**Legend**

- **Proximal Distal**
- **Proximal**
  - DIP α T2A-QF>mCh / DIP α-GFSTF
  - 10um

- **T1 Leg 35hrs APF**
- **T1 Leg 1 Week Adult**
- **Proximal Fe**
- **Fe-ltm**
  - DIP α T2A-QF>GFP

**Graph**

- **% of Fe-ltm With Terminal Branching (T1 Leg)**
- **DIP α Turns 'ON'**
- **Adult Ecllosion**

**GFP**

- **GFP**
**Mef2 VGlut\>mCh LinA/15\>myr::GFP**

- **A**
  - T1 Leg Disc
  - Leg Fascicle

- **A'**
  - T1 Leg Disc

- **B**
  - Larval Body Wall

- **Mef2 LinA/15\>myr::GFP**

- **C**
  - 45hrs APF
  - Tarm
  - Talm
  - Tadm

- **C'**
  - 45hrs APF
  - Tarm
  - Talm
  - Tadm

- **D**
  - 60hrs APF
  - Tarm
  - Talm
  - Ti-ltm

- **E**
  - ADULT
  - Tarm
  - Talm
  - Ti-ltm
A

ADULT EXPRESSION - TI LEG

dpr1-Gal4

dpr2-Gal4

dpr3-Gal4

dpr4-Gal4

dpr5-Gal4

dpr6-Gal4

dpr7-Gal4

dpr9-Gal4

dpr12-Gal4

dpr15-Gal4

dpr16-Gal4

dpr17-Gal4

dpr10-Gal4 ∩ VGlut-QF

dpr6-Gal4 ∩ VGlut-QF

B

MNs

SnSs

Muscles

50um
A

Proximal Fe

DIP α T2A-Gal4>GFP/DIP α

DIP α T2A-Gal4>GFP/DIP α

DIP α T2A-Gal4>GFP/DIP α

αFe-ltm

αFe-ltm

αFe-ltm

Proximal Ti

DIP α T2A-Gal4>GFP/DIP α

DIP α T2A-Gal4>GFP/DIP α

DIP α T2A-Gal4>GFP/DIP α

αTi-ltm

αTi-tadm

αTi-ltm

B

DIP α-T2A Gal4>GFP, UAS-DIPα-V5 (25°C)

Merge

GFP V5

V5

T1 VNC Hemisegment

DIP α-T2A Gal4>GFP, UAS-DIPα-V5 (18°C)

tub-Gal80

tub-Gal80

25um
**A**

Proximal Fe Distal Fe

Mef2-Gal4>UAS-dpr10RNAi, dpr10 -/+ Proximal Ti Distal Ti

DIP α-T2A-Gal4>UAS-dpr10

**B**

% With Any Terminal Branching (T1 Leg)

Bar graph showing the percentage of T1 legs with any terminal branching for different genotypes.

**C**

Mef2-Gal4>UAS-dpr10V5

T1 Leg 25hrs APF

**D**

dpr10 38,655bp

**E**

dpr10-GFSTF Twi

Late L3 T1 Leg Disc

**F**

dpr10-GFSTF DIP α-T2A QF>QUAS-mCh

45hrs APF
(MUTANT) DIP α-T2A Gal4>GFP / DIP α−

A

35hrs APF (Max Projection)

DIP α-T2A Gal4>GFP, UAS-DIP α

25hrs APF (Max Projection)

A′

35hrs APF (Slices)

DIP α-T2A Gal4>GFP, UAS-DIP α

25hrs APF (Slices)

B

DIP α-T2A Gal4>GFP, UAS-DIP α

25hrs APF (Max Projection)

B′

35hrs APF (Max Projection)

C

35hrs APF (Max Projection)

C′

35hrs APF (Slices)