The *Drosophila* Hox gene *Ultrabithorax* acts both in muscles and motoneurons to orchestrate formation of specific neuromuscular connections

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**Key words**

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**Summary statement**

The *Drosophila* Hox gene *Ultrabithorax* controls correct innervation of ventro-lateral muscles by coordinating Wnt4 ligand expression in muscles with the signalling pathway response in motoneurons
Abstract

Hox genes are known to specify motoneuron pools in the developing vertebrate spinal cord and to control motoneuronal targeting in several species. However, the mechanisms controlling axial diversification of muscle innervation patterns are still largely unknown. We present data showing that the *Drosophila* Hox gene *Ultrabithorax (Ubx)* acts in the late embryo to establish target specificity of ventrally projecting RP motoneurons. In abdominal segments A2 to A7, RP motoneurons innervate the ventro-lateral muscles VL1-4, with VL1 and VL2 being innervated in a *Wnt4*-dependent manner. In *Ubx* mutants, these motoneurons fail to make correct contacts with muscle VL1, a phenotype partially resembling that of the *Wnt4* mutant. We show that *Ubx* regulates expression of *Wnt4* in muscle VL2 and that it interacts with the Wnt4 response pathway in the respective motoneurons. *Ubx* thus orchestrates the interaction between two cell types, muscles and motoneurons, to regulate establishment of the ventro-lateral neuromuscular network.
Introduction

Establishment of region-specific muscle innervation patterns in animals is crucial for fulfilling functions specific to various body segments along the antero-posterior axis, such as locomotion or mating. How motoneuronal networks are regionally adjusted is a long-standing question in developmental neurobiology. The highly conserved Hox transcription factors are known to govern decisive aspects of motoneuronal network formation in flies and vertebrates (Philippidou and Dasen, 2013). In mice they regulate specification of motoneuron (MN) pools and control their projection patterns (Bell et al., 1999; Catela et al., 2016; Dasen et al., 2005; Lacombe et al., 2013; Philippidou et al., 2012; Studer et al., 1996). In *Drosophila* they regulate, among other processes, survival and identity of leg MNs (Baek and Mann, 2009; Baek et al., 2013). Whereas most studies investigated the role of Hox-mediated regulatory networks during specification and differentiation of neurons, the molecular mechanisms underlying these actions are just beginning to be discovered. Recent studies in flies provided first hints of a combined regulation of Hox-controlled targets in MNs and muscles. For example, the Hox gene *Deformed* (*Dfd*) is required in both establishment of the larval neuromuscular feeding unit and later in regulation of specific motoneuronal effector genes such as Ankyrin2-XL (Friedrich et al., 2016). Another study on larval crawling showed that this behaviour requires characteristic peristaltic movements of abdominal muscles, while movement of thoracic and head segments follows a rather different pattern (Dixit et al., 2008). These distinct patterns of movement rely on defined muscle architecture and precise motoneuronal innervation thereof. Regionally distinct muscle patterns are established early in development under the influence of Hox genes (Michelson, 1994) and ubiquitous expression of *Ultrabithorax* (*Ubx*), which specifies the first abdominal segment, results in thoracic segments exhibiting an abdominal peristaltic pattern (Dixit et al., 2008). Interestingly, providing *Ubx* either in neurons or in muscles alone does not produce the same effect. Although Dixit et al. showed a requirement for *Ubx* in both tissues, it remains unclear how this Hox gene establishes a functional neuromuscular system in the abdomen (Dixit et al., 2008). In particular, how expression of various factors involved in motoneuronal targeting of specific muscles is coordinated between these two tissues is still largely unknown.

In order to find and connect to their target muscles, MNs, upon being individually specified, need to extend their axons in a spatially and temporally highly regulated manner and navigate through a complex environment of different signals (Prokop,
1999). Several families of guidance molecules have been identified in various model organisms (Dickson, 2002; Nose, 2012). More recently, involvement of factors classically characterized as morphogens belonging to the Wnt, Hedgehog and TGF-β superfamilies have been shown to provide positional information and interact with pathfinding processes in different species (Charron et al., 2003; Inaki et al., 2007; Klassen and Shen, 2007; Lyuksyutova et al., 2003; Parker et al., 2006; Serpe and O’Connor, 2006). These factors provide signals to growth cones that guide them to the correct target or help in the decision where to make synapses (Marqués, 2005; Schnorrer and Dickson, 2004). Although the components, either expressed at the cell surface or secreted, have been characterised to a large extent (Kurusu et al., 2008; Nose, 2012) the underlying transcriptional programs to orchestrate the expression of these guidance factors and the corresponding neuronal responses remain less well understood (Santiago and Bashaw, 2014; Zarin et al., 2014).

Here we present data showing that the Drosophila Hox gene Ubx orchestrates the formation of a specific neuromuscular connection in abdominal segments of the Drosophila embryo. It does so by regulating Wnt4 expression in the muscle and by interacting with the Wnt4 signalling pathway in the corresponding MNs. Our data demonstrate that, through its dual function, Ubx coordinates communication between muscles and motoneurons to establish correct neuromuscular connections.
Results

Segment-specific differences in innervation of ventro-lateral muscles

The ventro-lateral (VL) muscles of embryonic abdominal segments A2 to A7 as well as their innervation pattern are well-described (Bate, 1993; Choi et al., 2004; Hoang and Chiba, 2001; Hooper, 1986; Landgraf et al., 1997; Mauss et al., 2009; Michelson, 1994). To explore whether this group of muscles provides a good model for investigating mechanisms leading to regional differences in motoneuronal innervation, we analysed the VL neuromuscular system in the currently less well-characterized thoracic segments T2 and T3, and in A1 of early stage 17 embryos. Muscles VL1, 2, 3 and 4 show only minor morphological changes between segments along the antero-posterior axis (Fig. 1A). They extend parallel to one another and are morphologically similar. In segments A1 to A7, they insert at adjacent muscle insertion sites. In T3 we find VL1 to extend dorsally inserting together with the A1 lateral longitudinal muscle 1 (LL1) into the T3/A1 apodeme (Fig. 1C). Segment T2 exhibits only three VL muscles, VL1-3 (Bate, 1993). These muscles lie somewhat diagonal compared to their counterparts in more posterior segments.

VL muscles are innervated, among other MNs, by RP1, 3, 4 and 5 that derive from the neuroblast NB3-1 (Bossing et al., 1996; Landgraf et al., 1997). We used the dHb9-Gal4 line, which is active in all postmitotic RP MNs and a limited set of interneurons and dorsal MNs (Broihier et al., 2002), in combination with CD4::tdGFP to visualize the axonal projections of Hb9-positive MNs. These MNs cross the posterior segment border and project into the intersegmental nerve branch b (ISNb) of the next segment, so that the MNs of segment A1 innervate ventro-lateral muscles in segment A2 (Broihier et al., 2002; Landgraf et al., 1997; Matthes et al., 1995). In segments A2 to A7, muscle VL1 receives a stereotypical “T”-shaped contact by RP5. It is also innervated by the so-called V neuron, and possibly a further MN (Choi et al., 2004; Hoang and Chiba, 2001; Inaki et al., 2007; Inaki et al., 2010; Landgraf et al., 1997; Mauss et al., 2009). From here on, we refer to this group of VL1-innervating MNs as VL1-MNs. In segments T3 and A1, one or more of the VL1-MNs extend an additional dorsal projection that contacts the lateral bipolar dendritic neuron (LBD) (Fig. 1B,C). In contrast, in A2-A7 the VL1-MNs do not make this contact and the LBD fuses with the transverse nerve (TN), which is not present in thoracic segments and A1 (Gorczyca et al., 1994). In T3 to A7, RP1 and RP4 innervate the ventral oblique muscles 2 (V02) and 1 (V01), respectively (Choi et al., 2004; Mauss et al., 2009). VL3 and VL4 are innervated by RP3 which forms a fine contact
in the cleft between these two muscles (Landgraf et al., 1997; Mitchell et al., 1996). This contact is also similar in T2 to A7. In T2 the ISNb innervates VL1-3 similarly as in abdominal segments (Fig. 1C). Furthermore, the A1-specific ventral internal muscle 1 (VI1) is innervated by the DC1 MN (Matthes et al., 1995), which projects through the ISNb and is also labelled in dHb9>CD4::tdGFP embryos (Fig. 1B,C).

Thus, in segments T3 and A1, muscle VL1 and the MNs that innervate it show deviations from the typical abdominal pattern. These segments are characterized by rather high overall expression levels of the Hox gene *Ubx*, especially in the ventral nerve cord (VNC).

Since Hox genes have generally been shown to be involved in late events of CNS maturation (Friedrich et al., 2016; Miguel-Aliaga and Thor, 2004; Rogulja-Ortmann et al., 2008), we wondered whether *Ubx* influences axonal projections of and target muscle selection by VL1-MNs.

A requirement for *Ubx* in establishment of correct motoneuron contacts on ventro-lateral muscles

We first examined Ubx expression in the VL muscles and RP MNs in more detail, since the RP MNs were the only VL1-MNs that we could address unambiguously by using the *dHb9*-Gal4 line. At late stage 14, when motoneuronal axons enter the muscle field, Ubx is not expressed in the thoracic VL muscles (Fig. 2A). In A1, these muscles show low Ubx levels (Fig. 2B). Strong expression extends from A2 to A4, while it gets progressively weaker in the posterior abdominal segments A5 to A7. Interestingly, Ubx expression levels in the VNC showed a shift of one segment to the anterior compared to the muscles: we observed high levels in A1 and these were progressively reduced towards posterior segments (Fig. 2A). Thus, Ubx expression levels in a particular segment of the VNC appear to correlate with the levels in the muscles of the next posterior segment. We show this in more detail for the thorax and anterior abdomen: RP MNs show low Ubx levels in segments T2 and T3, whereas Ubx levels are high in these neurons in A1 (Fig. 2C, compare to Fig. 2B). This Ubx expression pattern showed an intriguing correlation to the RP MN axonal projection patterns described above, where RP axons cross the posterior segment border and innervate VL muscles of the adjacent posterior segment.

To test whether *Ubx* plays a role in regionalising VL muscle innervation, we examined *Ubx* null mutants. The terminal differentiation of MN contacts on VL muscles indeed showed segment-specific defects. VL innervation in T3 and A1 did not seem to be
affected, possibly due to low Ubx expression levels in VL muscles and the RP MNs of segments T2 and T3 that innervate them (Fig. S1). In abdominal segments A2 to A7 however, we found that innervation of VL1 was either lost or strongly reduced (Fig. 3B). We assigned different categories to characterize the phenotype in more detail. The “T”-shaped connection normally seen on VL1 in wildtype embryos was classified as “correct contact on VL1” (Fig. 3A). In Ubx1 mutants, only 10.3% of analysed hemisegments (n=97) fall into this category (Fig. 3C, Table S1). The other connections were either reduced (category “reduced contact with VL1”; 25.8%; only thin Fas2 signal on VL1 that does not bifurcate in a “T” shape, see inset Fig. 3B,D) or completely lost (category “no contact on VL1”; 53.6%; Fig. 3B,D). The category “misrouting” comprises innervations, which could not be attributed to any of the above categories and often involved ectopic contacts with other nerves, most often with the TN. It also included completely aberrant projections, which spread over the ventro-lateral muscle field. The above effects in the Ubx1 null mutants were also seen in transheterozygote Ubx1/Ubx6.28 animals (Fig. 3C, Table S1), implying a specific and novel role for Ubx in this process.

Ubx and the abdominal Hox gene abdominal A (abdA) have been shown to function redundantly in several contexts (Dixit et al., 2008; Michelson, 1994). Although Ubx mutants alone show a strong VL1 innervation phenotype, we wanted to test whether abdA makes any contribution to this developmental event. As anticipated, VL1 innervation showed no significant changes in abdA MX1 mutants (Fig. S2, Table S1).

To exclude that the VL1 innervation phenotype observed in Ubx mutants is due to loss or temporal misspecification of RP MNs, we performed anti-dHb9 staining (Broihier et al., 2002) (Fig. S3). All RP MNs were present from A1 to A7 in Ubx1 mutants. Specifically RP5, which co-innervates VL1, could clearly be addressed with anti-dHb9 and anti-Cut double staining (Tran and Doe, 2008). Taken together, these data show a to-date unknown role of Ubx in regulating innervation of ventro-lateral muscles.

**Ubx regulates muscle-specific expression of factors required for proper VL1 innervation**

We next asked through which mechanism Ubx might regulate VL muscle innervation. The reduced innervation of VL1, with VL1-MNs stalling on VL2, that we observed in Ubx mutants was also reported for embryos mutant for Wnt4, a member of the Wnt family of signalling molecules (Inaki et al., 2007) (Fig. S2, Table S1). Furthermore, the same study
revealed differential Wnt4 expression between VL1 and VL2, with higher expression levels in VL2. Dsulf1, a sulfatase implicated in the regulation of Wnt and BMP gradients at the neuromuscular junction (NMJ) (Dani et al., 2012; Inaki et al., 2007), was also found to be expressed at higher levels in VL2 than in VL1 (Inaki et al., 2007). As the Ubx and Wnt4 mutant phenotypes are remarkably alike, and since Hox genes have been shown to regulate Wnt4 in the visceral mesoderm of Drosophila embryos (Graba et al., 1995), we wondered whether Ubx might be regulating Wnt4 and Dsulf1 expression in the VL muscles.

In wildtype embryos, Wnt4 shows a graded expression within the VNC, having the highest levels in the most posterior segments (Fig. 4A). In late stage 14, Wnt4 shows strongest expression in muscles VL2 and VA1 (Ventral Acute muscle 1) (Fig. 4A) (Inaki et al., 2007; Nose, 2012) and is subsequently downregulated. From stage 13 on, Dsulf1 also shows higher expression levels in VL2 than in VL1, as already reported (Inaki et al., 2007; Nose, 2012) (Fig. 4C). In Ubx mutants Wnt4 expression in muscles VL2 and VA1 is lost (Fig. 4B). In addition, Dsulf1 is strongly reduced in a graded manner, with a complete loss in segments A1 and A2 (Fig. 4D), showing that expression of both factors requires Ubx. We also examined Wnt4 and Dsulf1 expression when Ubx is provided in VL muscles of more anterior segments, where it is normally not expressed. Ectopic Ubx, driven by 24B-Gal4, is sufficient to induce Wnt4 and Dsulf1 expression in thoracic segments and in A1 (Fig. 4E-H). Thus, Ubx is necessary and sufficient to activate expression of the Wnt4 morphogen and of a potential Wnt4 modifier, Dsulf1, in VL muscles, and may thereby control innervation of VL1.

**Wnt4 can activate the canonical Wnt-signalling pathway in VL1-innervating motoneurons**

The aforementioned study on control of VL1 innervation showed that Wnt4 requires two Wnt receptors in this context, Frizzled-2 (Fz-2) and Derailed-2 (Drl-2). Both receptors exhibit mutant phenotypes similar to Wnt4 (Inaki et al., 2007). However, it remained unclear whether Wnt4 secreted from muscles activates the canonical signalling pathway in the MNs to ensure correct VL1 innervation. To address this question, we examined embryos mutant for the Drosophila β-catenn homolog armadillo (arm), a key transducer of Wnt signalling. We used arm8, a weak temperature-sensitive
allele that lacks arm function in epidermal and neuronal Wingless (Wg) signalling but retains it in adherens junctions (Jones et al., 2010; Loureiro and Peifer, 1998; Peifer and Wieschaus, 1990). Because of the high load of maternal arm product, these embryos develop until the end of embryogenesis with only mild patterning defects compared to null alleles which eliminate the zygotic gene product (Jones et al., 2010; Loureiro and Peifer, 1998; Peifer and Wieschaus, 1990). The observed effects on epidermal patterning in arm8 mutants can therefore be attributed to very late defects caused by disruption of canonical Wnt signalling. Thus, if the effect on VL muscle innervation is due to late canonical signalling and not to defects in cell adhesion, we would expect to observe a phenotype in arm8 mutants. Indeed, arm8 mutants exhibited defects in VL1 innervation at restrictive temperatures (Fig. 5B). MN contacts with VL1 were often reduced or not present, and correct contacts were found only in 34.3 % (n=143) of analysed hemisegments (Fig. 5D, Table S1). Instead, the contacts on VL2 were often strongly expanded. These defects were highly specific to VL1-MNs, as we were unable to find obvious phenotypes in the rest of the motoneuronal system or in muscle morphology. Loss of arm function in the canonical Wnt signalling pathway thus causes phenotypes similar to those of Ubx and Wnt4 mutants, suggesting that Wnt4 secreted from muscles activates the canonical Wnt signalling pathway in the VL1-MNs. To address more precisely whether activation of Wnt4 signalling is in fact required in these MNs, we performed an arm RNAi knock-down using dHb9-Gal4. Correct VL1 innervation was significantly reduced to 29.6 % (n=142) in these embryos (Fig. 5C,D, Table S1), whereas projections of other MNs were not affected. We also analysed the role of further canonical Wnt signalling pathway components in VL1-MNs by overexpressing the negative regulator Glycogen Synthase Kinase-3 (GSK3) and a dominant negative construct of the Wnt effector TCF (dTCF.DN) (Fig. 5D, Table S1). Indeed, with only 20.2 % (n=94) and 59 % (n=105) of correct contacts on VL1, GSK3 and TCF.DN, respectively, both induced significant defects specifically in targeting of VL1 (Fig. 5D, Table S1). Together these results indicate that canonical Wnt signalling is likely required cell autonomously in VL1-MNs for correct targeting this muscle.
The *Wnt4* signalling pathway and *Ubx* are required in postmitotic neurons for correct VL1 innervation

We then tested whether *Ubx* interacts with *Wnt4* and *arm* at the genetic level in this specific developmental context. Heterozygotes of *Ubx*¹, *Wnt4EMS²³*, *arm⁸* and the null mutant *arm⁴* showed only a slight decrease in correct innervation of VL1 compared to WT (Fig. 6A,B,D, Table S1). This changed considerably in *Wnt4EMS²³/+; Ubx¹/+*, *arm⁴/X; Ubx¹/+* or *arm⁸/X; Ubx¹/+* double heterozygotes where correct contacts on VL1 were significantly decreased to 29,1 % (n= 103), 38,3 % (n= 107) and 21,4 % (n=70), respectively (Fig. 6D, Table S1). Taken together, these data suggest that *Ubx* interacts with the canonical *Wnt4* signalling pathway for correct innervation of VL1 by VL1-MNs.

As *arm* is clearly required in the MNs (Fig. 5) and it has been shown previously that *Ubx* and Arm can interact both physically and genetically in other contexts (Bondos et al., 2006; Hsiao et al., 2014), we wondered whether *Ubx*, apart from being required in the muscles, is also required in the MNs to establish correct VL1 innervation. To this end we performed an RNAi knockdown of *Ubx* specifically in postmitotic *dHb9*-positive MNs. Indeed, a reduction of correct contacts on VL1 to 28 % (n= 107) resembles the *Ubx* mutant phenotype and can be rescued by providing *Ubx* in the same cells (Fig. 7, Table S1). Moreover, a simultaneous RNAi knock-down of *Ubx* and *arm* enhances the effect of single knock-downs: only 10,5 % (n= 95) of correct contacts compared to 27,7 % (UAS-*Ubx* RNAiII n=83) and 29,6 % (UAS-*arm* RNAi, n=142), further supporting the notion that *Ubx* and the *Wnt4* signalling component Arm interact in VL1-MNs to ensure proper VL1 innervation. Together, our results show that *Ubx* is necessary both in muscles (to activate *Wnt4* ligand expression) and in MNs (to interact with a crucial component of the signalling pathway) and therefore strongly suggest that it coordinates *Wnt4* signalling between these two cell types to establish proper neuromuscular connections.

*Ubx* plays a dual role in muscles and neurons to assure proper innervation of ventro-lateral muscles

To test whether *Ubx* function is indeed required in both muscles and neurons, we performed rescue experiments, restoring *Ubx* expression in a tissue-specific manner in the *Ubx* mutant background. We first asked whether expression in muscles alone would be enough to rescue the VL1 innervation phenotype. Neither of the two mesodermal drivers we used, 24B-Gal4 and *Mef2*-Gal4, was able to restore normal VL1 innervation
Expression of Ubx only in postmitotic MNs using the dHb9-Gal4 driver was also not sufficient for correct VL1 innervation (Fig. 8B,D, Table S1). To examine whether Ubx may be needed earlier during the specification process of NB3-1, we used the scabrous-Gal4 (sca-Gal4) driver, which drives in the early neuroectoderm and remains active in most cells of the VNC until late developmental stages. However, this driver line could also not rescue aberrant VL1-MN projections in the Ubx mutant (Fig. 8D, Table S1).

Finally, we employed the ubiquitous Actin5C-Gal4 driver line (Act5C-Gal4) to restore Ubx expression in both tissues. Here we observed a significantly higher rate of correct contacts on VL1 (38.7 %, n=168) compared to Ubx1 mutants (10.3 %, n= 97) (Fig. 8C,D, Table S1). The relatively low rescue rate is likely due to Act5C-Gal4 not being active at exactly the required developmental time-points in muscles and MNs. In addition, the levels of Ubx expressed under its control, especially in the CNS, were lower than the endogenous ones (Fig. S4) and were thus likely not sufficient for a more pronounced rescue of the Ubx phenotype. Nevertheless, partially restoring Ubx expression in both tissues shows significant rescue rates, highlighting a role for Ubx in coordination of the ligand trigger from muscles and the response in MNs to ensure proper innervation of ventro-lateral muscles.

**Discussion**

In this report, we address the question of how region-specific neuromuscular connections are established along the A/P body axis during development. Previous studies on *Drosophila* embryos reported segment-specific differences in the morphology of ventro-lateral (VL) muscles (Bate, 1993; Hooper, 1986), thus providing a good model for our investigations. Here we provide a more detailed characterisation of this muscle group in the thorax and anterior abdomen, including segment-specific variations in the patterns of VL innervation. VL muscles show the same morphological pattern in abdominal segments A1 to A7, but diverge from it in the thorax, with each thoracic segment exhibiting a distinct VL morphology. VL innervation follows a similar pattern distribution, with projections of the T2 and T3 VL1-MNs, which innervate VL1 muscles in T3 and A1, being alike. VL1-MNs from abdominal segments A1 to A6 innervate segments A2 to A7 and have similar projections in these segments.
**Ubx regulates region-specific Wnt4 expression in ventro-lateral muscles**

We find these segment-specific morphological characteristics to coincide closely with the expression pattern of the Hox gene *Ubx* (Fig. 2). In VL muscles, Ubx expression is excluded from thoracic segments, is low in A1, high in A2 and declines gradually towards A7. In the RP MNs, Ubx levels are low in T2 and T3, high in A1, and are reduced gradually until A6. These patterns correlate well with previous studies which found general Hox expression boundaries to be segmental in the muscles (Bate, 1993) and parasegmental in the nervous system (Hirth et al., 1998). In *Drosophila*, Hox genes are known to act early in the mesoderm and neuroectoderm to establish region-specific patterns of muscles and neurons, respectively (Hooper, 1986; Michelson, 1994; Technau et al., 2014). Their expression continues until late embryonic stages where they play more direct roles in later developmental events in both invertebrates and vertebrates, such as neuronal survival, migration and connectivity (Philippidou and Dasen, 2013).

We show that, in segments A2 to A7, *Ubx* controls the expression of *Wnt4* in muscle VL2. *Wnt4* is secreted to provide a repulsive signal to the VL1-MNs, forcing them to extend their growth cones further and synapse onto the more dorsal muscle VL1 (Inaki et al., 2007). Interestingly, VL1-MNs in segments T3 and A1 are not depending on the Wnt4 signal. Factors such as Toll, Beat-IIIc or Glutactin have been shown to have redundant functions with Wnt4 in the abdomen (Inaki et al., 2007; Inaki et al., 2010). We suspect that they could represent the repulsive signals in T3 and A1. *Ubx* additionally controls the expression of *Dsulf1*, which was shown to play a role in axonal targeting (Inaki et al., 2007). *Dsulf1* expression is entirely dependent on *Ubx* only in segments A1 and A2. Since expression in abdominal segments A3 to A7 is weaker in the *Ubx* mutant, but is not completely lost, it is reasonable to assume that *Dsulf1* may be co-regulated by *Ubx* and the more posterior Hox genes *abdominal-A* and *Abdominal-B* in these segments.

Expression of *Wnt4* and *Dsulf1* at late stage 14 correlates well with the time point at which the growth cones enter the muscle field to find their targets (Prokop, 1999), supporting their role in axonal targeting. Evidence from several species shows that Sulf1 regulates the secretion, stability and the diffusion range of different Wnt morphogens during canonical and non-canonical Wnt-signalling (Ai et al., 2003; Dhoot et al., 2001; Fellgett et al., 2015; Kleinschmit et al., 2010; Tran et al., 2012) which suggests that similar mechanisms may be at work in other organisms.
Wnt4 signalling in neural network formation

Once secreted, Wnt4 binds and activates receptors of the frizzled family (Frizzled-2) and of the RYK family (Derailed-2) on the VL1-MNs (Inaki et al., 2007). However, further details on the mechanisms involved were not reported. Functions for Wnt signalling in neural development, including early specification of neural stem and progenitor cells (Deshpande et al., 2001; Prokop and Technau, 1994), axonal pathfinding and synapse formation (Inaki et al., 2007; Klassen and Shen, 2007; Lyuksyutova et al., 2003; Maro et al., 2009; Reynaud et al., 2015; Yoshikawa et al., 2003; Zheng et al., 2015) are well-documented in multiple species. Wnts also exhibit late effects during physiologic regulation of the Drosophila NMJ (Kerr et al., 2014; Packard et al., 2002) and during long-term memory formation (Chen et al., 2006; Tan et al., 2013). However, activation of the canonical Wnt signalling pathway in MNs has, to our knowledge, only been described in C. elegans, where the β-catenin homolog Bar-1 is required in a specific type of MN for Wnt-regulated axonal guidance (Maro et al., 2009). We now show a MN-specific requirement for Arm and dTCF, a further downstream component of the canonical Wnt signalling pathway, in correct targeting of VL muscles.

Furthermore, our results suggest that Ubx itself interacts with the Wnt4 signaling pathway in MNs to ensure correct targeting of muscle VL1. A recent study unveiled dTCF to specifically interact with Ubx but not AbdA in an in vivo Bimolecular Fluorescent Complementation assay (Baëza et al., 2015). Assuming that motoneuronal targeting and synapse formation are, at least in part, transcriptionally regulated under influence of Wnt4, this difference in interaction capability might account for the different effects on VL1 innervation seen between Ubx and abdA mutants. As Ubx and Arm have been demonstrated to interact physically and genetically (Bondos et al., 2006; Hsiao et al., 2014), it is tempting to speculate that Ubx, dTCF and Arm might control target genes by forming a Wnt4-induced transcriptional complex (Fig. 9). This remains to be confirmed since techniques for visualizing such complexes in situ are currently not available. Alternatively, the genetic interaction may be based on Ubx and Arm/dTCF acting in parallel on target genes to regulate axonal targeting.

A coordinating role for Ubx in muscle innervation

Interestingly, expressing Ubx either in muscles or in MNs was not able to rescue correct VL1-MN contact formation on VL1 in Ubx mutants. Only a ubiquitous Ubx expression resulted in significant rescue of the phenotype, identifying Ubx as the key factor that
coordinates production of the Wnt4 ligand in, and, through regulation of Dsulf1 expression, possibly also its secretion from, muscle VL2 with the signalling pathway response in the corresponding MNs. Both of these events are required to direct proper innervation of VL1 by VL1-MNs in abdominal segments A2 to A7. This finding corroborates a previous report on larval crawling behaviour which showed that ectopic expression of Ubx, when restricted only to muscles or to neurons, did not alter thoracic peristaltic movements (Dixit et al., 2008). In contrast, ubiquitous ectopic expression of Ubx resulted in anterior thoracic segments showing peristaltic patterns characteristic of the abdomen. These observations strongly suggested a function for Ubx in both tissues, and our work now provides a possible mechanism to at least partially explain them. The finding that Ubx coordinates expression of muscle-specific axon guidance factors on the one side, and activation of the motoneuronal response on the other, reveals a novel function for Hox genes in nervous system development. In addition, our results provide new insights into the regulation of interactions between different cell types during development, suggesting that the same transcription factor can coordinate spatially restricted generation of a signal in one type of cell, with the response to that signal in another.

Future studies will show whether this principle holds true in mammalian systems. Both Hox genes and the role of Wnt4 in NMJ development are conserved between Drosophila and mammals (McGinnis and Krumlauf, 1992; Strochlic et al., 2012). Furthermore, Wnt4 expression in mammalian muscles is temporally regulated in a manner similar to that in Drosophila embryos (Strochlic et al., 2012), suggesting that the same mechanism may indeed coordinate muscle innervation in mammals.
Material and methods:

Fly stocks and genetics:
The following fly stocks were used: Oregon R, w^{118}, Ubx^{1}/TM6B, Tb, Sb, Dfd-lacZ and UAS-Ubx (kindly provided by L. S. Shashidara, IISER, Pune, India), Ubx^{5.28}/TM6B, Tb, Sb, Dfd-lacZ, abdA^{MX1}/TM3, Sb, Kr-Gal4, UAS-GFP (Sánchez-Herrero et al., 1985), dHb9-Gal4/TM3, Sb, ftz-lacZ (Broihier et al., 2002), 24B-Gal4, UAS-mCherry/TM6B (kindly provided by S. Merabet, IGFL, Lyon, France), Mef2-Gal4, UAS-CD4::td-tom.FP/TM6B (kindly provided by O. Vef, University of Mainz, Germany), Act5C-Gal4/CyO, Wnt4EMS23, bw/CyO, hh-lacZ, arm^{4}/FM7, grh-lacZ, arm^{8}/FM7c, Dfd-GMR-nvYFP, UAS-mCherry RNAi, UAS-Ubx RNAi, UAS-arm RNAi, UAS-sgg.B (UAS-GSK3), UAS-dTCFΔN (UAS-dTCF.DN), UAS-CD4::tdGFP and UAS-CD8::GFP (Bloomington Stock Center, Indiana, USA). arm^{8}/X or arm^{8}/X; Ubx^{1}/+ animals were identified by the anti-Sex lethal signal.

The UAS-Ubx RNAi insertion on the second chromosome (attP40) was generated using the shUbx RNAi (HMS01403) construct in pValium20 (Ni et al., 2011) (kindly provided by the TRiP consortium, Harvard, USA). All experiments were performed at 25°C except for the RNAi and dominant-negative experiments, which were incubated at 29°C.

Immunohistochemistry

For antibody staining, embryos were dechorionated, fixed and immunostained following previously published protocols (Becker et al., 2016), except that embryos were fixed for 22 minutes. Staging of embryos was done according to Campos-Ortega and Hartenstein (Campos-Ortega and Hartenstein, 1997). Early stage 17 was staged according to Pereanu et al. (Pereanu et al., 2007).

The following primary antibodies were used: chicken anti-Beta-Gal (1:1000) (Abcam #ab9361), rabbit anti-Beta-Gal (1:1000) (Cappel #55976), mouse anti-GFP (1:250) (Roche #11814460001), rabbit anti-GFP (1:500) (Torrey Pines Biolabs #TP401), mouse anti-Fas2 1D4 (1:10), mouse anti-Cut 2B10 (1:20) (DSHB), mouse anti-Sxl M18 (1:10), mouse anti-Ubx FP3.38 (1:20) (all from Developmental Studies Hybridoma Bank), rat anti-Myosin (1:500) (Abcam #ab51098), rabbit anti-dHb9 (1:2000) (kindly provided by J. B. Skeath, Washington University in St. Louis, USA) and guinea pig anti-Ubx (1:200) (kindly provided by I. Lohmann, University of Heidelberg, Germany).

As fluorescent secondary antibodies we used anti-guinea pig Dylight 405, anti-chicken Alexa Fluor 647 (both Jackson Immuno Research), anti-mouse Alexa Fluor 488, anti-rabbit Alexa Fluor 488, anti-mouse Alexa Fluor 568, anti-rabbit Alexa Fluor 568, anti-rat
Alexa Fluor 633 (all from Thermo Fisher Scientific) in a 1:500 dilution. All secondary antibodies were used according to the manufacturer's protocols.

**in situ hybridisation**

For *in situ* hybridisation, the *Dsulf1* probe was generated via PCR as reported previously (Weiszmann et al., 2009). The *Wnt4* probe was made from EST clone RE26454 (Stapleton et al., 2002) upon digestion with BsgI (New England Biolabs). Primer sequences are available upon request. Both probes were labelled using the DIG-RNA Labelling Kit (Roche). The hybridisation on embryos was carried out as described before (Tautz and Pfeifle, 1989). Probes were detected using anti-DIG AP (Roche).

**Image acquisition**

The non-fluorescent stainings were documented on a Zeiss Axioplan. Fluorescent confocal images were acquired on a Leica TCS SP5 microscope. Laser intensities were kept constant between experiments and controls. Image processing was done using ImageJ, Adobe Photoshop CS4 and Adobe Illustrator CS4.

**Statistical analysis**

Statistical analysis of categorical data was performed using $\chi^2$ tests for pairwise comparisons to controls. Correct contacts were tested against the combination of wrong contacts. No blinding was done.
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Competing interests

The authors declare no competing or financial interests

Author Contributions

Conceived and designed the experiments: ARO, CH, GMT. Performed the experiments: CH. Analysed the data: CH, ARO, GMT. Wrote the paper: CH, ARO, GMT.

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References

Ai, X., Do, A. T., Lozynska, O., Kusche-Gullberg, M., Lindahl, U. and Emerson, C. P. (2003). QSulf1 remodels the 6-O sulfation states of cell surface heparan sulfate proteoglycans to promote Wnt signaling. *J. Cell Biol.* 162, 341–351.

Baek, M. and Mann, R. S. (2009). Lineage and birth date specify motor neuron targeting and dendritic architecture in adult Drosophila. *J. Neurosci.* 29, 6904–6916.

Baek, M., Enriquez, J. and Mann, R. S. (2013). Dual role for Hox genes and Hox cofactors in conferring leg motoneuron survival and identity in Drosophila. *Development* 140, 2027–2038.

Baëza, M., Viala, S., Heim, M., Dard, A., Hudry, B., Duffraisse, M., Rogulja-Ortmann, A., Brun, C. and Merabet, S. (2015). Inhibitory activities of short linear motifs underlie Hox interactome specificity in vivo. *Elife* 4, 1–28.

Bate, M. (1993). The mesoderm and its derivatives. In *The Development of Drosophila melanogaster* (ed. Bate, M. and Martinez-Arias, A.), pp. 1013–1090. New York: Cold Spring Harbor Laboratory Press.

Becker, H., Renner, S., Technau, G. M. and Berger, C. (2016). Cell-Autonomous and Non-cell-autonomous Function of Hox Genes Specify Segmental Neuroblast Identity in the Gnathal Region of the Embryonic CNS in Drosophila. *PLOS Genet.* 12, e1005961.

Bell, E., Wingate, R. J. T. and Lumsden, A. (1999). Homeotic Transformation of Rhombomere Identity After Localized Hoxb1 Misexpression. *Science* 284, 2168–2171.

Bondos, S. E., Tan, X.-X. and Matthews, K. S. (2006). Physical and genetic interactions link hox function with diverse transcription factors and cell signaling proteins. *Mol. Cell. Proteomics* 5, 824–834.

Bossing, T., Udolph, G., Doe, C. Q. and Technau, G. M. (1996). The embryonic central nervous system lineages of Drosophila melanogaster. I. Neuroblast lineages derived from the ventral half of the neuroectoderm. *Dev. Biol.* 179, 41–64.
Broihier, H. T., Skeath, J. B. and Louis, S. (2002). Drosophila Homeodomain Protein dHb9 Directs Neuronal Fate via Crossrepressive and Cell-Nonautonomous Mechanisms. Neuron 35, 39–50.

Campos-Ortega, J. A. and Hartenstein, V. (1997). The embryonic development of Drosophila melanogaster. 2nd ed. Berlin, Heidelberg, New York: Springer.

Catela, C., Shin, M. M., Lee, D. H., Liu, J.-P. and Dasen, J. S. (2016). Hox Proteins Coordinate Motor Neuron Differentiation and Connectivity Programs through Ret/Gfra Genes. Cell Rep. 14, 1901–1915.

Charron, F., Stein, E., Jeong, J., McMahon, A. P. and Tessier-Lavigne, M. (2003). The Morphogen Sonic Hedgehog Is an Axonal Chemoattractant that Collaborates with Netrin-1 in Midline Axon Guidance. Cell 113, 11–23.

Chen, J., Park, C. S. and Tang, S.-J. (2006). Activity-dependent synaptic Wnt release regulates hippocampal long term potentiation. J. Biol. Chem. 281, 11910–11916.

Choi, J. C., Park, D. and Griffith, L. C. (2004). Electrophysiological and Morphological Characterization of Identified Motor Neurons in the Drosophila Third Instar Larva Central Nervous System. J. Neurophysiol. 91, 2353-2365.

Dani, N., Nahm, M., Lee, S. and Broadie, K. (2012). A Targeted Glycan-Related Gene Screen Reveals Heparan Sulfate Proteoglycan Sulfation Regulates WNT and BMP Trans-Synaptic Signaling. PLoS Genet. 8, e1003031.

Dasen, J. S., Tice, B. C., Brenner-Morton, S. and Jessell, T. M. (2005). A Hox regulatory network establishes motor neuron pool identity and target-muscle connectivity. Cell 123, 477–491.

Deshpande, N., Dittrich, R., Technau, G. M. and Urban, J. (2001). Successive specification of Drosophila neuroblasts NB 6-4 and NB 7-3 depends on interaction of the segment polarity genes wingless, gooseberry and naked cuticle. Development 128, 3253–3261.

Dhoot, G. K., Gustafsson, M. K., Ai, X., Sun, W., Standiford, D. M. and Emerson, C. P. (2001). Regulation of Wnt signaling and embryo patterning by an extracellular sulfatase. Science 293, 1663–1666.
Dickson, B. J. (2002). Molecular mechanisms of axon guidance. *Science* **298**, 1959–1964.

Dixit, R., VijayRaghavan, K. and Bate, M. (2008). Hox genes and the regulation of movement in Drosophila. *Dev. Neurobiol.* **68**, 309–316.

Fellgett, S. W., Maguire, R. J. and Pownall, M. E. (2015). Sulf1 has ligand-dependent effects on canonical and non-canonical Wnt signalling. *J. Cell Sci.* **128**, 1408–1421.

Friedrich, J., Sorge, S., Bujupi, F., Eichenlaub, M. P., Schulz, N. G., Wittbrodt, J. and Lohmann, I. (2016). Hox Function Is Required for the Development and Maintenance of the Drosophila Feeding Motor Unit. *Cell Rep.* **14**, 850–860.

Gorczyca, M. G., Phillis, R. W. and Budnik, V. (1994). The role of tinman, a mesodermal cell fate gene, in axon pathfinding during the development of the transverse nerve in Drosophila. *Development* **120**, 2143–2152.

Graba, Y., Gieseler, K., Aragnol, D., Laurenti, P., Mariol, M. C., Berenger, H., Sagnier, T. and Pradel, J. (1995). DWnt-4, a novel Drosophila Wnt gene acts downstream of homeotic complex genes in the visceral mesoderm. *Development* **121**, 209–218.

Hirth, F., Hartmann, B. and Reichert, H. (1998). Homeotic gene action in embryonic brain development of Drosophila. *Development* **125**, 1579–1589.

Hoang, B. and Chiba, A. (2001). Single-Cell Analysis of Drosophila Larval Neuromuscular Synapses. *Dev. Biol.* **229**, 55-70.

Hooper, J. E. (1986). Homeotic gene function in the muscles of Drosophila larvae. *EMBO J.* **5**, 2321–2329.

Hsiao, H.-C., Gonzalez, K. L., Catanese, D. J., Jordy, K. E., Matthews, K. S. and Bondos, S. E. (2014). The Intrinsically Disordered Regions of the Drosophila melanogaster Hox Protein Ultrabithorax Select Interacting Proteins Based on Partner Topology. *PLoS One* **9**, e108217.

Inaki, M., Yoshikawa, S., Thomas, J. B., Aburatani, H. and Nose, A. (2007). Wnt4 is a local repulsive cue that determines synaptic target specificity. *Curr. Biol.* **17**, 1574–1579.
Inaki, M., Shinza-Kameda, M., Ismat, A., Frasch, M. and Nose, A. (2010). Drosophila Tey represses transcription of the repulsive cue Toll and generates neuromuscular target specificity. Development 137, 2139–2146.

Jones, W. M., Chao, A. T., Zavortink, M., Saint, R. and Bejsovec, A. (2010). Cytokinesis proteins Tum and Pav have a nuclear role in Wnt regulation. J. Cell Sci. 123, 2179–2189.

Kerr, K. S., Fuentes-Medel, Y., Brewer, C., Barria, R., Ashley, J., Abruzzi, K. C., Sheehan, A., Tasdemir-Yilmaz, O. E., Freeman, M. R. and Budnik, V. (2014). Glial wingless/Wnt regulates glutamate receptor clustering and synaptic physiology at the Drosophila neuromuscular junction. J. Neurosci. 34, 2910–2920.

Klassen, M. P. and Shen, K. (2007). Wnt signaling positions neuromuscular connectivity by inhibiting synapse formation in C. elegans. Cell 130, 704–716.

Kleinschmit, A., Koyama, T., Dejima, K., Hayashi, Y., Kamimura, K. and Nakato, H. (2010). Drosophila heparan sulfate 6-0 endosulfatase regulates Wingless morphogen gradient formation. Dev. Biol. 345, 204–214.

Kurusu, M., Cording, A., Taniguchi, M., Menon, K., Suzuki, E. and Zinn, K. (2008). A Screen of Cell-Surface Molecules Identifies Leucine-Rich Repeat Proteins as Key Mediators of Synaptic Target Selection. Neuron 59, 972–985.

Lacombe, J., Hanley, O., Jung, H., Philippidou, P., Surmeli, G., Grinstein, J. and Dasen, J. S. (2013). Genetic and Functional Modularity of Hox Activities in the Specification of Limb-Innervating Motor Neurons. PLoS Genet. 9, e1003184.

Landgraf, M., Bossing, T., Technau, G. M. and Bate, M. (1997). The origin, location, and projections of the embryonic abdominal motorneurons of Drosophila. J. Neurosci. 17, 9642–9655.

Loureiro, J. and Peifer, M. (1998). Roles of Armadillo, a Drosophila catenin, during central nervous system development. Curr. Biol. 8, 622–633.

Lyuksyutova, A. I., Lu, C.-C., Milanesio, N., King, L. A., Guo, N., Wang, Y., Nathans, J., Tessier-Lavigne, M. and Zou, Y. (2003). Anterior-Posterior Guidance of Commissural Axons by Wnt-Frizzled Signaling. Science 302, 1984–1988.
Maro, G. S., Klassen, M. P. and Shen, K. (2009). A beta-catenin-dependent Wnt pathway mediates anteroposterior axon guidance in C. elegans motor neurons. *PLoS One* 4, e4690.

Marqués, G. (2005). Morphogens and synaptogenesis in Drosophila. *J. Neurobiol.* 64, 417–434.

Matthes, D. J., Sink, H., Kolodkin, a L. and Goodman, C. S. (1995). Semaphorin II can function as a selective inhibitor of specific synaptic arborizations. *Cell* 81, 631–639.

Mauss, A., Tripodi, M., Evers, J. F. and Landgraf, M. (2009). Midline Signalling Systems Direct the Formation of a Neural Map by Dendritic Targeting in the Drosophila Motor System. *PLOS Biol.* 7(9): e1000200.

McGinnis, W. and Krumlauf, R. (1992). Homeobox genes and axial patterning. *Cell* 68, 283–302.

Michelson, A. M. (1994). Muscle pattern diversification in Drosophila is determined by the autonomous function of homeotic genes in the embryonic mesoderm. *Development* 768, 755–768.

Miguel-Aliaga, I. and Thor, S. (2004). Segment-specific prevention of pioneer neuron apoptosis by cell-autonomous, postmitotic Hox gene activity. *Development* 131, 6093–6105.

Mitchell, K. J., Doyle L., J. L., Serafini, T., Kennedy, T. E., Tessier-Lavigne, M., Goodman, C. S. and Dickson, B. J. (1996). Genetic analysis of Netrin genes in Drosophila: Netrins guide CNS commissural axons and peripheral motor axons. *Neuron* 17, 203–215.

Ni, J.-Q., Zhou, R., Czech, B., Liu, L.-P., Holderbaum, L., Yang-Zhou, D., Shim, H.-S., Tao, R., Handler, D., Karpowicz, P., et al. (2011). A genome-scale shRNA resource for transgenic RNAi in Drosophila. *Nat Meth* 8, 405–407.

Nose, A. (2012). Generation of neuromuscular specificity in Drosophila: novel mechanisms revealed by new technologies. *Front. Mol. Neurosci.* 5, 1–11.
Packard, M., Koo, E. S., Gorczyca, M., Sharpe, J., Cumberledge, S. and Budnik, V. (2002). The Drosophila Wnt, wingless, provides an essential signal for pre- and postsynaptic differentiation. Cell 111, 319–330.

Parker, L., Ellis, J. E., Nguyen, M. Q. and Arora, K. (2006). The divergent TGF-beta ligand Dawdle utilizes an activin pathway to influence axon guidance in Drosophila. Development 133, 4981–4991.

Peifer, M. and Wieschaus, E. (1990). The segment polarity gene armadillo encodes a functionally modular protein that is the Drosophila homolog of human plakoglobin. Cell 63, 1167–1176.

Pereanu, W., Spindler, S., Im, E., Buu, N. and Hartenstein, V. (2007). The emergence of patterned movement during late embryogenesis of Drosophila. Dev. Neurobiol. 67, 1669–1685.

Philippidou, P. and Dasen, J. S. (2013). Hox genes: choreographers in neural development, architects of circuit organization. Neuron 80, 12–34.

Philippidou, P., Walsh, C. M., Aubin, J., Jeannotte, L. and Dasen, J. S. (2012). Sustained Hox5 gene activity is required for respiratory motor neuron development. Nat. Neurosci. 15, 1636–1644.

Prokop, A. (1999). Integrating bits and pieces: synapse structure and formation in Drosophila embryos. Cell Tissue Res. 297, 169–186.

Prokop, A. and Technau, G. M. (1994). Early tagma-specific commitment of Drosophila CNS progenitor NB1-1. Development 120, 2567–2578.

Reynaud, E., Lahaye, L. L., Boulanger, A., Petrova, I. M., Marquilly, C., Flandre, A., Martinez, T., Privat, M., Noordermeer, J. N., Fradkin, L. G., et al. (2015). Guidance of Drosophila Mushroom Body Axons Depends upon DRL-Wnt Receptor Cleavage in the Brain Dorsomedial Lineage Precursors. Cell Rep. 11, 1293–1304.

Rogulja-Ortmann, A., Renner, S. and Technau, G. M. (2008). Antagonistic roles for Ultrabithorax and Antennapedia in regulating segment-specific apoptosis of differentiated motoneurons in the Drosophila embryonic central nervous system. Development 135, 3435–3445.
Sánchez-Herrero, E., Vernós, I., Marco, R. and Morata, G. (1985). Genetic organization of Drosophila bithorax complex. *Nature* **313**, 108–113.

Santiago, C. and Bashaw, G. J. (2014). Transcription factors and effectors that regulate neuronal morphology. *Development* **141**, 4667–4680.

Schnorrer, F. and Dickson, B. J. (2004). Axon Guidance: Morphogens Show the Way. *Curr. Biol.* **14**, R19–R21.

Serpe, M. and O’Connor, M. B. (2006). The metalloprotease tolloid-related and its TGF-beta-like substrate Dawdle regulate Drosophila motoneuron axon guidance. *Development* **133**, 4969–4979.

Stapleton, M., Liao, G., Brokstein, P., Hong, L., Carninci, P., Shiraki, T., Hayashizaki, Y., Champe, M., Pacleb, J., Wan, K., et al. (2002). The Drosophila Gene Collection: Identification of Putative Full-Length cDNAs for 70% of D. melanogaster Genes. *Genome Res.* **12**, 1294–1300.

Strochlic, L., Falk, J., Goillot, E., Sigoillot, S., Bourgeois, F., Delers, P., Rouvière, J., Swain, A., Castellani, V., Schaeffer, L., et al. (2012). Wnt4 participates in the formation of vertebrate neuromuscular junction. *PLoS One* **7**, e29976.

Studer, M., Lumsden, A., Ariza-McNaughton, L., Bradley, A. and Krumlauf, R. (1996). Altered segmental identity and abnormal migration of motor neurons in mice lacking Hoxb-1. *Nature* **384**, 630–634.

Tan, Y., Yu, D., Busto, G. U., Wilson, C. and Davis, R. L. (2013). Wnt Signaling Is Required for Long-Term Memory Formation. *Cell Rep.* **4**, 1082–1089.

Tautz, D. and Pfeifle, C. A. (1989). Non-radioactive in situ hybridization method for the localization of specific RNAs in Drosophila embryos reveals translational control of the segmentation gene hunchback. *Chromosoma* **98**, 81–85.

Technau, G. M., Rogulja-Ortmann, A., Berger, C., Birkholz, O. and Rickert, C. (2014). Composition of a Neuromere and Its Segmental Diversification under the Control of Hox Genes in the Embryonic CNS of Drosophila. *J. Neurogenet.* **28**, 171–180.
Tran, K. D. and Doe, C. Q. (2008). Pdm and Castor close successive temporal identity windows in the NB3-1 lineage. Development 135, 3491–3499.

Tran, T. H., Shi, X., Zaia, J. and Ai, X. (2012). Heparan sulfate 6-O-endosulfatases (Sulfs) coordinate the Wnt signaling pathways to regulate myoblast fusion during skeletal muscle regeneration. J. Biol. Chem. 287, 32651–32664.

Weiszmann, R., Hammonds, A. S. and Celniker, S. E. (2009). Determination of gene expression patterns using high-throughput RNA in situ hybridization to whole-mount Drosophila embryos. Nat. Protoc. 4, 605–618.

Yoshikawa, S., McKinnon, R. D., Kokel, M. and Thomas, J. B. (2003). Wnt-mediated axon guidance via the Drosophila Derailed receptor. Nature 422, 583–588.

Zarin, A. A., Asadzadeh, J. and Labrador, J. P. (2014). Transcriptional regulation of guidance at the midline and in motor circuits. Cell. Mol. Life Sci. 71, 419–432.

Zheng, C., Diaz-Cuadros, M. and Chalfie, M. (2015). Dishevelled attenuates the repelling activity of Wnt signaling during neurite outgrowth in Caenorhabditis elegans. Proc. Natl. Acad. Sci. 112, 13243–13248.
Fig. 1. Morphological characterisation of ventro-lateral muscles and their innervation pattern in the late embryo. Shown are thoracic (T1-T3) and abdominal (A1-A7) segments with focus on the innervation of the ventro-lateral muscle group. (A) External view of a wildtype whole-mount embryo (w¹¹¹⁸). Stained are motoneuronal tracts using anti-Fas2 (green), muscles using anti-Myosin (magenta), and anti-Ubx (blue). Ubx is expressed at high levels within the CNS. The white rectangle marks the area depicted in the scheme in (C). (B-B") Filet preparation of an early stage 17 embryo. The innervation pattern is visualized using membrane-bound UAS-CD4::tdGFP expressed under dHb9-Gal4 control. Arrowheads mark the innervations of VL1 in T3 and A1. Noteworthy are the segment-specific differences with regard to the ISNb contact with LBD in T3 and A1 (arrows in B and B’). The curved arrow indicates innervation of...
muscle VI1 by the DC MN in A1. (C) A scheme of the internal view on the muscle field. Relevant muscles are coloured in magenta. Segment-specifically modified muscles in T3 and A1 are coloured in purple. Those muscles, whose morphology and identity was difficult to determine are encircled with a dashed line. The Transverse Nerve (TN) is missing in thoracic segments and A1. Anterior is to the left in all panels, dorsal is up. Abbreviations: TN transverse nerve, ISN intersegmental nerve, SN segmental nerve, LBD lateral bipolar dendritic neuron. Scale bar represents 40 µm in (A) and 20µm in (B).
Fig. 2. Ubx expression in VL muscles and RP neurons. (A) Shown is a filet preparation of a late stage 14 embryo with the indicated genotype and stained with anti-Ubx (blue), anti-GFP (yellow), anti-Fas2 (green) and anti-Myosin (magenta). Ubx expression can be observed in VL1-4 of A1 to A7. (B) Magnified view of area marked with white square in (A). Muscles VL1-4 are encircled with a white stippled line. In A1 expression levels are lower than in A2 (B’). Growth cones of the ISN and SN have entered the peripheral muscle field (B” and B’’). (C) Filet preparation of an early stage 17 embryo with the indicated genotype stained with anti-Ubx (blue) and anti-GFP (green). Ubx is expressed within RP MNs (encircled) until late developmental stages. Highest expression levels are observed in A1, whereas T2 and T3 show weak Ubx expression (see insets). White dashed line marks the midline. Anterior is up in all images. Scale bar represents 20 µm.
Fig. 3. *Ubx* contributes to the correct innervation pattern of the ventro-lateral muscle group. Shown are filet preparations of early stage 17 embryos stained with anti-Fas2 (green) and anti-Myosin (magenta). The genotypes are indicated above each panel. (A–A') In wildtype embryos VL1-MNs form a T-shaped ending on VL1 in segments A2 to A7 (indicated by white arrowheads). (B–B') Homozygous *Ubx* mutants show defective innervation of VL1 (white arrows) in A2 to A7. In (A) and (B), VL1 muscles are encircled.
with a stippled line. In all insets, the dotted line marks the ventral VL1 border. Anterior is to the left, dorsal is up in all images. Scale bar represents 20 µm. (**A**-**B**) Schematic depictions of the observed defects. ISNb is shown in dark green and the ventro-lateral muscles in magenta. (**C**) Quantification of the ISNb defects in the different genetic backgrounds. *Ubx* mutants show significant defects compared to WT with regard to VL1 innervation. Data are presented as categories. For statistical analysis, correct contacts were compared to the combination of wrong contacts (misrouting, no contact with VL1, reduced contact with VL1) and significance of the data sets was tested using the $\chi^2$-test. ***p<0.005. n denotes the number of evaluated hemisegments: WT: n=125, *Ubx$^1$/Ubx$^1$: n= 97, *Ubx$^1$/Ubx$^{6.28}$: n = 95.
Fig. 4. *Ubx* is necessary and sufficient for the expression of *Wnt4* and *Dsulf1*. Shown are filet preparations of late stage 14 embryos after *in situ* hybridisation against *Wnt4* and *Dsulf1*. Arrowheads indicate the signal of the respective factor in the ventro-lateral muscle field. To the right, higher magnifications of areas marked with the black rectangle are shown. (A-A') The expression of *Wnt4* can be observed as a stripe in VL2 and VA1 (arrowheads) in balanced embryos (controls) within segments A2-A7. In A1 this expression is only weak or not detectable. (B-B') In *Ubx*^1^ mutants, *Wnt4* expression in muscles is almost completely missing, whereas the expression in the CNS is not detectably affected. (C-C') In control, i.e. balanced, embryos the expression of *Dsulf1* can be detected in ventro-lateral muscles, including VL2 (arrowheads). Furthermore, it can
be detected in more dorsal regions, which are probably the lateral-transverse muscles. (D-D’) In Ubx^1 mutants the expression is reduced in a graded fashion, whereby only the expression levels in A6 and A7 appear equal to those in control embryos. In A1 and A2 expression is completely abolished and in A3-A5 it is clearly reduced. (E-E’) Thoracic segments lack expression of Wnt4 in VL muscles under wildtype conditions. (F-F’) Mesodermal expression of Ubx in 24B > Ubx embryos results in ectopic expression of Wnt4 in thoracic segments (arrowheads). (G-G’) Thoracic segments show very low levels of Dsul1 expression under wildtype conditions. (H-H’) Mesodermal expression of Ubx in 24B > Ubx embryos results in ectopic expression in thoracic segments (arrowheads). The stippled line marks the ventral midline. Anterior is up in all images.
Fig. 5. Manipulating downstream components of canonical Wnt signalling in dHb9-positive MNs causes VL1 innervation defects. Shown are filet preparations of early stage 17 embryos stained with anti-Fas2 (green) and anti-Myosin (magenta). Genotypes are indicated above each panel. (A-A') In control embryos (dHb9 > mCherry RNAi) VL1-
MNs make a T-shaped ending on VL1 (white arrowheads, see insets). (B-B') In arm⁸ mutants innervation of VL1 is strongly reduced (white arrows) and that of VL2 thicker (curved arrow). (C-C') Knock-down of arm in dHb9-positive MNs produces similar defects in VL1 innervation (white arrows). In (A), (B), and (C), VL1 muscles are encircled with a stippled line. In all insets, the dotted line marks the ventral VL1 border. The scale bar represents 20 µm. Anterior is to the left and dorsal is up in all images. (A’’-C’’) Scheme of the observed defects. The ISNb is coloured in dark green and the ventro-lateral muscles in magenta. (D) Quantification of the ISNb defects in the different genetic backgrounds. Mutants for arm⁸ and overexpression of UAS-arm RNAi, UAS-GSK3 or UAS-dTCF.DN show significant defects in VL1 innervation compared to control embryos (dHb9-Gal4 > UAS-CD4::tdGFP or dHb9 > mCherry RNAi). For statistical analysis, correct contacts were compared to the combination of wrong contacts (misrouting, no contact with VL1, reduced contact with VL1) and significance of the data sets was tested using χ²-test **p<0.05, ***p<0.005. n denotes the number of evaluated hemisegments: dHb9-Gal4 > UAS-CD4::tdGFP: n= 81 (control), arm⁸: n=143, dHb9-Gal4 > UAS-dGSK3: n= 94, dHb9-Gal4 > UAS-dTCF.DN: n=105, dHb9-Gal4 > UAS-mCherry RNAi: n= 78, dHb9-Gal4 > UAS-arm RNAi: n=142.
Fig. 6. *Ubx* genetically interacts with the Wnt4 pathway to control proper innervation of VL1. Shown are filet preparations of embryos in early stage 17 stained with anti-Fas2 (green) and anti-Myosin (magenta). The genotypes are given above each panel. (A-A’) In heterozygous *arm^{+}/X* controls VL1 innervation shows a wildtype pattern.
In heterozygous \textit{Ubx}^{1+/+} controls innervation of VL1 is also unchanged (white arrowheads, see also inset). \textbf{(B-B')} In heterozygous embryos innervation of VL1 is defective in A2-A7 (white arrows, see also inset). In \textbf{(A)}, \textbf{(B)}, and \textbf{(C)}, VL1 muscles are encircled with a stippled line. In all insets, the dotted line marks the ventral VL1 border. Anterior is to the left, dorsal is up in all images. The scale bar represents 20 µm. \textbf{(A'’-C’’)} Schematic depictions of the observed defects. ISNb is shown in dark green and the ventro-lateral muscles in magenta. \textbf{(D)} Quantifications of the ISNb defects in the evaluated genetic backgrounds. Genetic interactions between \textit{Ubx} and \textit{Wnt4}, the \textit{arm}^{4} null allele and \textit{arm}^{8} as a specific mutant of the canonical Wg-signalling, were tested. All double-heterozygous mutants show significant defects compared to their single-heterozygous controls with respect to VL1 innervation. Correct contacts were tested versus the combination of wrong contacts (misrouting, no contact with VL1, reduced contact with VL1) and significance of the data sets were tested using $\chi^2$-test. *** $p<0.005$. \textit{n} denotes the number of evaluated hemisegments: \textit{Ubx}^{1+/+}: \textit{n}=123, \textit{Wnt4}^{EMS23+/+}: \textit{n}=156, \textit{Wnt4}^{EMS23+/+;Ubx}^{1+/+}: \textit{n}=103, \textit{arm}^{4/X}: \textit{n}=94, \textit{arm}^{4/X;Ubx}^{1+/+}: \textit{n}=107, \textit{arm}^{8/X}: \textit{n}=60, \textit{arm}^{8/X;Ubx}^{1+/+}: \textit{n}=70.
Fig. 7. RNAi depletion indicates cell autonomous requirements for Ubx and arm in VL1-MNs to control correct innervation of VL1. Shown are quantifications of the VL1 innervation rate in early stage 17 embryos after knock-down of the indicated factors in MNs using dHb9-Gal4. Knock-down of Ubx leads to defects in VL1 innervation compared to a control RNAi construct (UAS-mCherry RNAi). The effect of the Ubx single knock-down can be increased by a combined knock-down of Ubx and arm. Parallel expression of a UAS-Ubx construct with UAS-Ubx RNAi leads to an almost complete rescue, indicating the specificity of the Ubx RNAi construct. Correct contacts were tested versus the combination of wrong contacts (misrouting, no contact with VL1, reduced contact with VL1) and significance of the data sets were tested using $\chi^2$-test. *** p<0.005, n.s. not significant. n denotes the number of evaluated hemisegments: dHb9-Gal4 > UAS-mCherry RNAi: n=78, dHb9-Gal4 > UAS-Ubx RNAi: n= 107, dHb9-Gal4 > UAS-Ubx RNAi: n= 83, dHb9-Gal4 > UAS-Ubx RNAi; UAS-arm RNAi: n= 95, dHb9-Gal4 > UAS-Ubx; UAS-Ubx RNAi: n= 120.
**Fig. 8.** *Ubx* is needed from A2 to A7 in both VL1-MNs and VL1 muscles to ensure a correct innervation pattern. Shown are filet preparations of early stage 17 embryos stained with anti-Fas2 (green) and anti-Myosin (magenta). The genotypes are given above each panel. (A-A’) Restoring expression of Ubx in all muscles using 24B-Gal4
cannot rescue VL1 innervation defects in *Ubx* mutants (white arrows). (B-B') Neuronal *Ubx* expression using *dHb9-Gal4* cannot rescue VL1 innervation defects (white arrows mark defective contacts on VL1, white arrowhead marks correct contact, see inset). (C-C') Restoring *Ubx* expression ubiquitously using *Act5C-Gal4* rescues VL1 innervation defects (white arrow marks defective contact on VL1, white arrowheads mark correct contacts, see insets). (A’’-C’’) Schematic depictions of the observed defects. ISNb is shown in dark green and the ventro-lateral muscles in magenta. In (A), (B), and (C), VL1 muscles are encircled with a stippled line. In all insets, the dotted line marks the ventral VL1 border. Anterior is to the left, dorsal is up in all images. The scale bar represents 20 µm. (D) Quantifications of the ISNb defects in the different genetic backgrounds. Driving *Ubx* expression in an *Ubx*1 mutant background using either muscle-specific drivers (*24B-Gal4, Mef2-Gal4*) or drivers for early (*sca-Gal4*) or late (*dHb9-Gal4*) neuronal expression cannot rescue VL1 defects. Only ubiquitous expression of *Ubx* in the *Ubx*1 mutant background can rescue contacts with VL1 up to almost 40%. Correct contacts were tested versus the combination of wrong contacts (misrouting, no contact with VL1, reduced contact with VL1) and significance of the data sets were tested using χ²-test. ***p<0.005, n.s. not significant. n denotes the number of evaluated hemisegments: WT: n= 125, *Ubx*1: n= 97, *24B-Gal4 > UAS-Ubx, Ubx*1: n=75, *Mef2-Gal4 > UAS-Ubx, Ubx*1: n=53, *dHb9-Gal4 > UAS-Ubx, Ubx*1: n=72, *sca-Gal4 > UAS-Ubx, Ubx*1: n=117, *Act5C-Gal4 > UAS-Ubx, Ubx*1: n=168.
Fig. 9. A combined role of Ubx in muscles and neurons during muscle innervation.

During late stage 14 Ubx regulates the expression of Dsulf1 and Wnt4 in muscle VL2. These factors are secreted by VL2 to signal to the arriving growth cones. In VL1-MNs, Wnt4 is sensed by its receptors Fz-2 and Drl-2, the first one probably triggering the canonical Wnt4 signalling pathway in these neurons. Arm translocates to the nucleus, where it either interacts with dTCF and Ubx to control potential target genes or acts with dTCF in parallel to Ubx to regulate the status of VL1-MNs. This process triggers the repulsion by the Wnt4 signal and leads to extension of VL1-MN axons towards VL1.
**Fig. S1. Ubx mutants show no detectable morphological defects in T3 and A1.** (A-A’’) Shown are filet preparations of early stage 17 embryos stained with anti-GFP (yellow), anti-Fas2 (green) and anti-Myosin (magenta). The genotype is indicated above the panel. With membrane-tagged GFP the morphology of VL1-MN projections could be followed in an Ubx\(^1\) mutant background (white arrowheads). No clear differences in T3 and A1 can be detected compared to controls (Fig. 1B-B’’). Anterior is to the left, dorsal is up. Scale bar represents 20 μm. (B) Schematic depictions of the observed defects. VL1-MN projections are shown in yellow and the ventro-lateral muscles in magenta.
Fig. S2. VL1 innervation defects in abdA and Wnt4 mutants. (A-B’) Shown are filet preparations of early stage 17 embryos stained with anti-Fas2 (green) and anti-Myosin (magenta) respectively. Genotypes are indicated above each panel. (A-A’) In abdA^{MX1} mutants innervation of VL1 is only slightly affected (white arrowheads indicate normal VL1 innervation). The ISNb sometimes curves dorsally towards the LBD, as in T3 or A1 (curved arrow). (B-B’) In Wnt4^{EMS23} mutants, innervation of VL1 is reduced (white arrows) and contacts on VL2 are expanded (curved arrows). In (A) and (B) VL1 muscles are encircled with a stippled line. In all insets, the dotted line marks the ventral VL1 border. Anterior is to the left, dorsal is up in all images. Scale bar represents 20 μm. (A”, B”) Schematic depictions of the observed defects. ISNb is shown in dark green and the ventro-lateral muscles in magenta. (C) Quantification of the ISNb defects in the different genetic backgrounds (see also Table S1). Mutants for abdA^{MX1} show no significant defects
whereas Wnt4EMS23 mutants show defective VL1 innervation. Data are presented as categories. n denotes the number of evaluated hemisegments: abdA^{MX1}: n = 127, Wnt4EMS23: n = 86.

**Fig. S3. RP5 is present in Ubx^1 mutants.** (A-B') Shown are ventral nerve cords of late stage 16 embryos. The overviews show separations which comprise the RP MNs. RP MNs can be identified based on their stereotypic dorsal position in the CNS, and the expression of dHb9. RP5 can be distinguished by the co-expression of Cut (encircled with a dashed line, see also magnifications of single focal planes to the right). In Ubx^1 mutants, all RP MNs, including RP5 can be identified. The stippled white line marks the midline. Anterior is up. Scale bar represents 20μm.
Fig. S4. Comparison of Ubx expression levels in controls and Act5C > Ubx, Ubx<sup>1</sup> embryos. (A-B') Shown are filet preparations of early stage 17 embryos stained with anti-Ubx (blue) and anti-Myosin (magenta). (A-A') Controls (w<sup>1118</sup>) show strong Ubx expression in neurons and weak expression in muscles. (B-B') In the ubiquitous Ubx<sup>1</sup> mutant rescue conditions (Act5C > Ubx, Ubx<sup>1</sup>) expression levels are weaker, except in a few cells in the epidermis. White stippled line marks the midline. Anterior is to the left and dorsal is up. Scale bar represents 20 μm.
Table S1. Distribution of phenotypes (%) for all genetic backgrounds analysed.

| genotype                        | correct contact on VL1 (%) | reduced contact on VL1 (%) | no contact on VL1 (%) | misrouting | n  |
|---------------------------------|-----------------------------|----------------------------|-----------------------|------------|----|
| Oregon R                        | 88.8                        | 8.0                        | 1.6                   | 1.6        | 125|
| Wt<sup>1118</sup>               | 88.1                        | 6.0                        | 1.2                   | 4.8        | 84 |
| Ubx<sup>1</sup>                 | 10.3                        | 25.8                       | 53.6                  | 10.3       | 97 |
| Ubx<sup>1</sup>/Ubx<sup>6-22</sup> | 29.5                        | 33.7                       | 24.2                  | 12.6       | 95 |
| Wnt<sup>4-323</sup>             | 40.7                        | 25.6                       | 12.8                  | 20.9       | 86 |
| arm<sup>9</sup>                 | 34.3                        | 49.0                       | 13.3                  | 3.5        | 143|
| abdA<sup>666</sup>              | 83.5                        | 0.8                        | 2.4                   | 13.4       | 127|
| Ubx<sup>1</sup>/+                | 69.9                        | 22.0                       | 4.9                   | 3.3        | 123|
| Wnt<sup>4-323</sup>/+           | 71.2                        | 12.8                       | 6.4                   | 9.6        | 156|
| arm<sup>9</sup>/X               | 74.5                        | 20.2                       | 4.3                   | 1.1        | 94 |
| Wnt<sup>4-323</sup>/+; Ubx<sup>1</sup>/+ | 63.3               | 28.3                       | 5.0                   | 3.3        | 60 |
| arm<sup>9</sup>/X; Ubx<sup>1</sup>/+ | 38.3                        | 35.5                       | 21.5                  | 4.7        | 107|
| arm<sup>9</sup>/X; Ubx<sup>1</sup>/+ | 21.4                        | 54.3                       | 15.7                  | 8.6        | 70 |
| UAS-mCherry RNAi<sub>III</sub> x dHb9-Gal4 | 73.1                        | 23.1                       | 1.3                   | 2.6        | 78 |
| UAS-Ubx RNAi<sub>III</sub> x dHb9-Gal4 | 28.0                        | 52.3                       | 8.4                   | 11.2       | 107|
| UAS-Ubx RNAi<sub>III</sub> x dHb9-Gal4 | 27.7                        | 51.8                       | 18.1                  | 2.4        | 83 |
| UAS-arm RNAi<sub>III</sub> x dHb9-Gal4 | 10.5                        | 41.1                       | 42.1                  | 6.3        | 95 |
| UAS-arm RNAi<sub>III</sub> x dHb9-Gal4 | 29.6                        | 49.3                       | 13.4                  | 7.7        | 142|
| UAS-Ubx; UAS-Ubx RNAi<sub>III</sub> x dHb9-Gal4 | 64.2                        | 23.3                       | 1.7                   | 10.8       | 120|
| UAS-CD4::tdGFP x dHb9-Gal4       | 81.5                        | 13.6                       | 0.0                   | 4.9        | 81 |
| UAS-GSK3 x dHb9-Gal4             | 20.2                        | 55.3                       | 17.0                  | 7.4        | 94 |
| UAS-dTCF.DN x dHb9-Gal4          | 59.0                        | 26.7                       | 10.5                  | 3.8        | 105|
| UAS-Ubx<sup>1</sup>; UAS-Ubx<sup>1</sup>; Ubx<sup>1</sup>/+ | 12.0                        | 4.0                        | 30.7                  | 53.3       | 75 |
| UAS-Ubx<sup>1</sup>; MeF2-Gal4, UAS-CD4::td-FP, Ubx<sup>1</sup>/Ubx<sup>1</sup> | 1.9                        | 3.8                        | 58.5                  | 35.8       | 53 |
| UAS-Ubx<sup>1</sup>; dHb9-Gal4, Ubx<sup>1</sup>/Ubx<sup>1</sup> | 6.9                        | 19.4                       | 70.8                  | 2.8        | 72 |
| UAS-Ubx/sc-Gal4; Ubx<sup>1</sup>/Ubx<sup>1</sup> | 8.5                        | 9.4                        | 65.0                  | 17.1       | 117|
| UAS-Ubx/Act5C-Gal4; Ubx<sup>1</sup>/Ubx<sup>1</sup> | 38.7                        | 22.6                       | 23.2                  | 15.5       | 168|