POU domain motif3 (Pdm3) induces wingless (wg) transcription and is essential for development of larval neuromuscular junctions in Drosophila

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Wnt is a conserved family of secreted proteins that play diverse roles in tissue growth and differentiation. Identification of transcription factors that regulate wnt expression is pivotal for understanding tissue-specific signaling pathways regulated by Wnt. We identified pdm3m7, a new allele of the pdm3 gene encoding a POU family transcription factor, in a lethality-based genetic screen for modifiers of Wingless (Wg) signaling in Drosophila. Interestingly, pdm3m7 larvae showed slow locomotion, implying neuromuscular defects. Analysis of larval neuromuscular junctions (NMJs) revealed decreased bouton number with enlarged bouton in pdm3 mutants. pdm3 NMJs also had fewer branches at axon terminals than wild-type NMJs. Consistent with pdm3m7 being a candidate wg modifier, NMJ phenotypes in pdm3 mutants were similar to those of wg mutants, implying a functional link between these two genes. Indeed, lethality caused by Pdm3 overexpression in motor neurons was completely rescued by knockdown of wg, indicating that Pdm3 acts upstream to Wg. Furthermore, transient expression of Pdm3 induced ectopic expression of wg-LacZ reporter and Wg effector proteins in wing discs. We propose that Pdm3 expressed in presynaptic NMJ neurons regulates wg transcription for growth and development of both presynaptic neurons and postsynaptic muscles.

Transcription factors play essential roles by inducing genes during the formation of body plans, organ development, tissue specificity, and generation of diverse cell types. Numerous transcription factors are grouped based on similarity in their sequences and domain structures. Pituitary-specific positive transcription factor 1, Octamer transcription factor-1, Uncoordinated-86 domain (POU) transcription factors belong to a subfamily of homeodomain transcription factors, and are highly conserved in all metazoans. POU domain consists of two DNA binding domains, POU homeodomain and POU specific domain, and these two domains are linked by a flexible linker. Based on sequence homology of the POU domain and the linker, POU proteins are grouped into six classes. POU proteins are often expressed in spatiotemporally restricted patterns during development, implying that they may be specialized for differentiation of specific cells or tissues by activating required signal transduction pathways.

The class VI Drosophila POU domain motif3 (Pdm3) protein is reported to function in olfactory receptor neurons (ORNs) by regulating olfactory receptor gene expression and axon targeting, and in ring (R) neurons by regulating the development of ellipsoid body (EB) and axon targeting to EB in the central brain. Pdm3 is also important for the axon targeting of a type of tracheal dendrite (td) neurons. In particular, td neurons that normally form synapse in the nerve cord change their target to the central brain by ectopic expression of Pdm3. Besides the neuronal functions of Pdm3, Pdm3 also acts as a repressor of abdominal pigmentation in D. melanogaster, and plays a role in female-limited color dimorphism in abdomen of D. montium. Despite these studies, it is still unknown how Pdm3 performs these neuronal and non-neuronal functions.

pdm300828 and pdm31 homozygotes exhibit defects in axon targeting, odor perception, and locomotion. pdm300828 allele has insertion of a piggyback element in an intron near the 3′ end of the pdm3 gene, and pdm31 has...
that the lethal region in a mapping line (BDRC #4347) that contains multiple morphological markers. The meiotic mapping revealed complementation analysis were performed. Meiotic mapping was carried out by crossing the suppressor with m7 Ci

induced lethality, and 28 suppressors were maintained for further analysis (Fig. 1A). All suppressors showed Sco/CyO D/TM6 and survivors were balanced with females. These 30A-Gal4 on the late-pupal lethality induced by Sona driven by 30A-Gal4 (Fig. 1A). 89 rare survivors were obtained among mutants show reduction in bouton number but increase in bouton size20–24. Components in Wg signaling such as Arrow (Arr) that positively regulates Wg signaling as a coreceptor of Wg also shows its mutant phenotype similar to wg, but Shaggy (Sgg/GSK3β) that negatively regulates Wg signaling as a kinase shows opposite phenotype to wg22,25. Thus, dynamic regulation of Wg signaling is essential for the development of NMJ.

Secreted Wg also signals to the presynaptic motor neuron to regulate Futsch, one of the microtubule-associated proteins (MAPs)26. Futsch is a homolog of mammalian MAP1B, and both Futsch and MAP1B are phosphorylated at a conserved site by Sgg/GSK3β27. The phosphorylated MAP1B does not bind microtubules, which results in reduced stability of microtubules3,28. Therefore, localization of Futsch at NMJ faithfully reflects the stability of microtubules that is dynamically regulated by Wg signaling. Loss of futsch phenotype is similar to the loss of wg phenotype in NMJ26.

We report here that pdm3 is identified as a suppressor of Sona-induced lethality. Based on the involvement of Sona in Wg signaling and the neuronal role of Pdm3, we specifically studied the roles of Pdm3 in NMJ. Similar to loss of wg, loss of pdm3 in NMJ caused decrease in number but increase in size of boutons. Lethality induced by overexpressed Pdm3 was completely rescued by the knockdown of Wg in motor neurons but not vice versa. This indicated that Pdm3 functions upstream to Wg, and prompted us to test whether Pdm3 can induce Wg transcription. Indeed, transient expression of Pdm3 in wing discs induced Wg transcription and Wg effector proteins. Based on these data, we propose that one of the main functions of Pdm3 in NMJ is to induce wg transcription.

**Results**

**pdm3 and sono have a positive genetic interaction.** As a first step toward understanding the function of sono, we carried out a lethality-based genetic screening using ethyl methanesulfonate (EMS) as a mutagen based on the late-pupal lethality induced by Sona driven by 30A-Gal4 (Fig. 1A). 89 rare survivors were obtained among 18,000 progeny from the cross between EMS-treated 30A-Gal4 males and untreated UAS-sono females. These survivors were balanced with Sco/CyO and D/TM6 for the establishment of suppressor lines whose mutations are in the second and third chromosomes, respectively. Established lines were retested for the suppression of Sona-induced lethality, and 28 suppressors were maintained for further analysis (Fig. 1A). All suppressors showed lethality, and a few suppressors produced rare homozygous adults.

To map the position of the lethal site in suppressor m7, meiotic and deficiency mappings as well as complementation analysis were performed. Meiotic mapping was carried out by crossing the m7 suppressor with a mapping line (BDRC #4347) that contains multiple morphological markers. The meiotic mapping revealed that the lethal region in m7 is located in between the cinnabar (cn) and curved (c) (Fig. 1B). Subsequent deficiency mapping identified two different regions that are responsible for lethality, one near cn and the other near c. Complementation analysis then showed that m7 has two independent mutations in pdm3 and arrow (arr) genes on the right arm of the second chromosome (Fig. 1B). Pdm3 is a class VI POU domain transcription factor, and Arr is a co-receptor of Wg ligand and essential for transduction of canonical Wg signaling8. The m7 suppressor was crossed with Canton-S (CS) and their progeny was checked by complementation test with pdm300828 and arr mutants in order to find flies with a single mutation, pdm300828 and arr00828.

Genomic sequencing revealed that pdm3500828 has a defective hobo element in the first exon of the pdm3 gene that is upstream of the initiation codon (Fig. 1C) while arr500828 has a point mutation in the arr gene (in preparation). None of the other suppressors had the hobo element in the pdm3 gene, indicating that insertion of the hobo element is unique to the m7 suppressor, and occurred subsequent to the point mutation in the arr gene. We found that the level of Pdm3 is extremely low in pdm300828 wing discs, establishing that insertion of the hobo element negatively affects the expression of Pdm3 (Fig. S1). Transheterozygotes of the two available deficiencies, Df(2R) BSC267 and Df(2R)BSC267 Df(2R) Exel6058, were missing only the pdm3 gene in the entire genome, so Df(2R)BSC267 Df(2R) Exel6058 flies were used as a deletion mutant of pdm3 in this study (Fig. 1C).

We found that not only pdm300828 but also pdm300828, pdm3 and pdm3 RNAi driven by 30A-Gal4 completely suppressed the Sona-induced pupal lethality (n > 200 each). Thus, pdm300828 is an authentic sono suppressor, and pdm3 shows a positive genetic interaction with sono.

**Boutons of pdm3 NMJs are decreased in number but increased in size, similar to wg NMJs.** Further analysis of sono suppressors revealed that sono itself and most suppressors are linked to Wg signaling2,23, which raised an interesting possibility that Pdm3 is also involved in Wg signaling. We noticed that pdm3 mutant
larvae are slow in locomotion (Movie 1), implying a potential role of pdm3 in NMJ. To address the relationship between pdm3 and wg in NMJ, we stained pdm31 and wtgs/wgCX4 NMJs of the late 3rd instar larvae for a presynaptic marker Horseradish peroxidase (HRP) and a postsynaptic marker Dlg to detect Type Ib boutons at muscles 6 and 7 in the 2nd abdominal (A2) and the 3rd abdominal (A3) segments20.

We found that number of boutons in pdm31 NMJ was reduced by 40% and 16% compared to the wild-type counterparts in A2 and A3 segments, respectively (Fig. 2A,B,F). Bouton numbers in pdm3f00828 NMJ were reduced by 27% in A2 segment and those in pdm3 m7 NMJ were only mildly reduced (Fig. S2; data not shown), so we focused our analysis on pdm31 NMJ that shows the most pronounced phenotype. Consistent with the previous report, number of boutons in wtgs/wgCX4 NMJ was reduced by 12% and 11% in A2 and A3 segments, respectively (Fig. 2C–E,H).

We then carried out quantitative analysis on size of boutons in pdm3 and wg NMJs. To measure the size of boutons, serial images of boutons were taken, the images were combined, and then area of the most distal bouton in the combined image was measured (Fig. 2G). Size of pdm31 distal boutons was increased by 30% at A2 but was not increased at A3 segments compared to wild-type (Fig. 2G). Size of wtg+/wtgCX4 boutons was increased by 32% and 11% in A2 and A3 segments, respectively, compared to the heterozygous controls, +/wtgCX4 and +/wtg (Fig. 2I). This is in line with a previous report that wtg+ boutons are noticeably larger20.

Figure 1. A lethality-based genetic screen for sona suppressors and characterization of the m7 suppressor. (A) Scheme of a genetic screen for identifying suppressors that survive against late pupal lethality induced by Sona overexpression. EMS was used as a mutagen, and obtained suppressors from the screen were crossed with second and third chromosome balancers before further testing. (B) The m7 suppressor was mapped by meiotic mapping, deficiency mapping, and complementation test. Multiple morphological markers are present in the second chromosome of a mapping line BDRC 4347, and the two markers, cinnabar (cn) as an eye color mutation and curved (c) as a wing shape mutation were identified as sites closely located to the two independent lethal sites of m7 suppressor. Two lethal sites were separated by recombination with CS and subsequent complementation test with pdm3g0025x and arr identified that m7 has two lethal mutations in pdm3 and arr genes. (C) Two deficiency lines used for mapping are shown with deleted regions in red. Transheterozygotes obtained by crossing the two deficiency lines do not have the pdm3 gene. pdm3m7 has a defective hobo element inserted in an exon that represents the 5′ untranslated region. The blue boxes indicate remaining parts of the inserted hobo element. A red arrow marks the initiation codon and a red asterisk marks the termination codon. The scale bar is for the hobo element only.
Some pdm3¹ boutons were not clearly separated from neighboring boutons (Fig. 2B"), which is also reported in wg boutons20. We defined the axon branch in which more than 50% of boutons are unseparated as ‘fused’ branch while those in which less than 50% of boutons are fused as ‘normal’ branch. We found that 30.5% of axon branches in A2 segments of pdm3¹ NMJs are fused (36 out of 118). Only 3% of wild-type axon branches was fused based on this definition (8 out of 282).

One unique phenotype of pdm3¹ boutons was an abnormally high level of Dlg in 42.9% of NMJs examined (33 out of 77, Fig. S3). Neither wild-type NMJs (0 out of 88) nor wg NMJs (0 out of 92) had high level of Dlg. This suggests that SSR is not properly developed in pdm3¹ NMJs. We also checked the localized pattern of a glutamate

Figure 2. Boutons in pdm3¹ and wg⁶/wgCX⁴ mutant NMJs are decreased in number but increased in size. CS and pdm3¹ were cultured at 25°C whereas wg mutants were cultured at 18°C in all figures. Type lb boutons of NMJs at muscles 6/7 were stained for HRP (green) and Dlg11. Boxed regions in (A–E) are magnified in (A‘–E‘). (A–E) Boutons of NMJs in the A2 segment of control (A) and pdm3¹ (B). Boutons of NMJs in the A2 segment of heterozygous controls (C,D) and wg⁶/wgCX⁴ (E). Shorter and thicker branches and loosely organized boutons of pdm3¹ and wg⁶/wgCX⁴ are marked with arrowheads (B‘,E‘). (F–I) Number (F) and size (G) of terminal boutons of NMJs in A2 and A3 of wild-type and pdm3¹. Number (H) and size (I) of terminal boutons of NMJs in A2 and A3 of controls and wg⁶/wgCX⁴, n = 51 for A2 and 37 for A3 of CS, 37 for A2 and 40 for A3 of pdm3¹, n = 21 for A2 and 19 for A3 of +/wgCX⁴, n = 30 for A2 and 25 for A3 of +/wg⁶, n = 44 for A2 and 48 for A3 of wg⁶/wgCX⁴, n = 52 for A2 and 49 for A3 of +/wgCX⁴, and n = 50 for A2 and 43 for A3 of +/wg⁶ and wg⁶/wgCX⁴. *Represents p < 0.05; ** represents p < 0.01; *** represents p < 0.0001. Data are presented as mean ± SEM. Scale bars: 10 μm.
receptor GluRIIA in pdm3 and wg boutons. It has been shown that the GluRIIA pattern in wild-type bouton is cluster-like but that in wg boutons is diffused without any clusters28. Consistent with this report, cluster-like pattern of GluRIIA was found in CS, -/+ wg CX4, and -/+ wg1 control boutons but wg+/- wg CX4 boutons showed diffused pattern (Fig. S4C–E). Unlike wg NMJs, the pattern of GluRIIA in (Pdm3+1) NMJs was not noticeably different from control NMJs (Fig. S4A,B). Taken together, the loss of pdm3 or wg phenotype decreased number but increased size of boutons, but pdm3 and wg NMJs were dissimilar in the level of Dlg and the pattern of GluRIIA.

**Number of axon branches in pdm3** NMJ is reduced. Wg signaling is required for the formation of new branches from an existing axon, and these new branches can be visualized by Futsch20,22. We found that number of axon branches in pdm3 NMJs was decreased by 25% at the A2 segment and was unchanged at the A3 segment (Fig. 3A”.B”,F). wg+/- wg CX4 NMJs showed 40% and 33% reduction in number of axon branches at the A2 and A3 segments, respectively (Fig. 3C”–E”,H). Therefore, Pdm3 is important in A2 and Wg is important for both A2 and A3 for the formation of axon branches.

Stable microtubule-bound Futsch appears as a filamentous bundle that pass through the center of NMJ axon20,22,26. Interestingly, the distal bouton at the end of each axon branch visualized by Futsch shows four distinct shapes: a bundled shape and three types of unbundled shapes such as looped, splayed, and diffused/punctate28. Splayed or diffused/punctate axon terminals indicate that microtubules are unstable due to transition to new axonal growth, while looped axon terminals indicate paused growth cones20,28. Proportion of distal boutons with unbundled shape is increased by mutations that affect NMJ expansion such as wg and futsch20,22. Magnified images of wild-type NMJs showed that less than 10% and 20% of the axon terminals at A2 and A3 are unbundled, respectively (Fig. 3A”.A”–G). In contrast, number of unbundled terminals was increased 7.3 and 3.4 times in A2 and A3 segments of pdm3 NMJs compared to wild-type, respectively (Fig. 3B”–B”; red arrow and arrowheads). All unbundled axon terminals in pdm3 NMJs were either splayed or diffused/punctate, and looped axon terminals were not detected. Number of splayed or diffused/punctate terminals in wg NMJs was also increased about two times in A2 and A3 segments compared to wild-type (Fig. 3I). Thus, proportion of splayed or diffused/punctate terminals in A2 and A3 segments was significantly increased in both pdm3 and wg NMJs.

Increase in number of splayed or diffused/punctate terminals in pdm3 NMJs suggests that microtubules in pdm3 NMJs are unstable. To directly address this point, we visualized axon terminals with α-Tubulin and Futsch staining were much weaker in axon branches of pdm3 NMJ compared to wild-type (Fig. S5A,B). In case of wg NMJs, signal from α-Tubulin staining was substantially reduced in entire axon compared to wild-type (Fig. 5E–F). In summary, microtubules become unstable, which may lead to reduced number of axon branches in both pdm3 and wg NMJs.

**Pdm3 expression in motor neuron is important for NMJ growth.** Wg secreted from motor neuron and glia is important for growth and differentiation of presynaptic terminals20,24. To figure out which cell type among motor neuron and glia expresses Pdm3, we expressed pdm3 RNAi in motor neurons by the OK6-Gal4 driver and in glia by the repo-Gal4 driver (Fig. 4A,B). These two Gal4 lines have been used to show cell specification of a given protein in numerous reports23,24–26. Knockdown of pdm3 by OK6-Gal4 caused 10% reduction in bouton number, suggesting that pdm3 is required in neurons for NMJ growth (Fig. 4A). Knockdown of pdm3 by repo-Gal4 did not change bouton number, suggesting that Pdm3 expression in glia is not required for NMJ growth (Fig. 4B).

We then asked whether expression of Pdm3 by OK6-Gal4 rescues pdm3 NMJ phenotype. To this end, we generated UAS-pdm3 pdm3/Cyo-GFP and OK6-Gal4 pdm3/Cyo-GFP flies and checked the phenotype of their progeny, UAS-pdm3 pdm3/OK6-Gal4 pdm3. Unexpectedly, pdm3 homozygotes were cold sensitive and could not grow at the temperature lower than 22 °C, but Pdm3 overexpression by OK6-Gal4 induced lethality at the temperature higher than 24 °C. Due to this temperature restraint, UAS-pdm3 pdm3/OK6-Gal4 pdm3 larvae were obtained only at 23 °C at a very low frequency. NMJs of these larvae showed increase in bouton number, and decrease in bouton size, and normalized level of Dlg compared to pdm3 NMJs (Fig. S6). Taken together, overexpressed Pdm3 in motor neurons rescued the loss of pdm3 phenotype in NMJs.

Our results so far have shown that bouton number and size of pdm3 NMJs are more severely affected in A2 than A3, so we examined the expression pattern of Pdm3 in ventral ganglion where cell bodies of motor neurons are present in order to examine the level of Pdm3 along the anterior-posterior (AP) axis. We found that Pdm3 is expressed more in the anterior part than posterior part of ventral ganglion, which is consistent with severer pdm3 phenotype in A2 than A3 (Fig. S7). Further analysis with more refined markers will help understand the effect of this AP gradient of Pdm3 on NMJ growth.

**Pdm3 acts upstream to Wg in neurons.** Similarity between pdm3 and wg NMJs prompted us to examine the genetic relationship between pdm3 and wg by co-expression of the two among pdm3, GFP-wg, pdm3 RNAi, and wg RNAi (Fig. 4C). As controls, bouton number of NMJs in these UAS lines was counted, which turned out to be similar to that of CS (Fig. S8). When we overexpressed pdm3 or wg by OK6-Gal4, pdm3 caused larval lethality, and wg increased bouton numbers in both A2 and A3 segments (Fig. 4C). When we knocked down pdm3 or wg by the same Gal4, the bouton number of the A2 segment was reduced by 10%, but that of the A3 segment was not changed in both cases. When GFP-wg and pdm3 RNAi were co-expressed, increase in bouton number by GFP-Wg was not affected by pdm3 RNAi (Fig. 4C). When pdm3 and wg RNAi were co-expressed, however, lethal phenotype by overexpressed pdm3 was completely rescued by knockdown of wg. Therefore, wg is epistatically downstream to pdm3. This result raised an interesting possibility that Pdm3 may regulate wg transcription.

**pdm3 adults exhibit defects in locomotion, planar cell polarity and wing posture.** Pdm3 has both neuronal and non-neuronal roles in fly development (see Introduction). 100% of pdm3OK6, pdm3OK6, and
Figure 3. Decreased number of axon terminals and unstable microtubules in pdm3¹ and wgts/wgCX4 NMJs. The boutons of NMJs in muscles 6/7 at the A2 were stained for HRP (green) and Futsch (white). The boxed regions in (A–E) are magnified in (A″–E″). The white arrowhead and arrows indicate bundled Futsch-positive terminals, and red arrowheads and arrows indicate unbundled Futsch-positive terminals. Number of Futsch-positive terminals represents the number of branches in NMJs. (A,B) NMJs of the control and pdm3¹ stained for HRP (A,B) and Futsch (A″,B″). (C–E) NMJs of the +/wgCX4 (C), +/wgts (D) and wgts/wgCX4 (E) stained for HRP. (F,G) Number of Futsch terminals (F) and percentage of unbundled axon terminals (G) in the type Ib boutons of control and pdm3¹. (H,I) Number of Futsch-positive terminals (H) and percentage of unbundled axon terminals (I) in the type Ib boutons of +/wgCX4, +/wgts, and wgts/wgCX4. n = 51 for A2 and 37 for A3 of CS, 37 for A2 and 40 for A3 of pdm3¹; n = 21 for A2 and 19 for A3 of +/wgCX4; n = 30 for A2 and 25 for A3 of +/wgts, 44 for A2 and 48 for A3 of wgts/wgCX4. *Represents p < 0.05; ** represents p < 0.01; *** represents p < 0.001. Data are presented as mean ± SEM. Scale bars: 10 µm.
pdm³ adults (n = ~50 each) had other defects such as wing drooping (Fig. 5A,B), planar cell polarity (PCP) phenotype in a posterior region near the L3 vein (Fig. 5C,D), and incomplete adhesion between dorsal and ventral blades of wings (Fig. S9, Table 1 in Supplementary Information). These phenotypes of pdm³ mutants suggest that Pdm³ plays previously unidentified roles in wing development. Therefore, we decided to use wing discs to study the relationship between pdm³ and Wg.

Pdm³ was highly expressed in both proximal and distal hinge regions in and near where patched (ptc) is expressed (Fig. S10A). This Pdm³ pattern is genuine because Pdm³ was not detected in the ptc region of ptc > pdm³ discs (Fig. S10B). Expression of Pdm³ in the hinge region may be responsible for the wing drooping phenotype of pdm³ mutants although there were no visible defects in adult wing hinges (Fig. 5C,D). No change in the level of Wg was observed in the ptc > pdm³ wing discs, suggesting that loss of pdm³ does not affect Wg transcription in the DV midline of the wing pouch region (Fig. S11).

**Transient expression of Pdm³ induces Wg transcription.** To understand the role of Pdm³ in relation with Wg, we carried out gain of function analyses using multiple Gal4 lines. Overexpression of Pdm³ induced embryonic to pupal lethality with all Gal4 lines used in this study (Table 2 in supplementary information). In case of en-Gal4 driver that caused embryonic lethality, some rare larval escapers were shorter than controls and had abnormal denticle patterns in the ventral epidermis (Fig. 5E,F). Pdm³ overexpression by other tissue-specific Gal4 lines also reduced size of affected tissues. For instance, Pdm³ expression by GMR-Gal4 generated small eyes (Fig. 5G,H), and that by nub-Gal4 caused mostly pupal lethality and loss of wings in rare adults (Table 2 in supplementary information). Consistent with this phenotype of nub > pdm³ wings, size of all nub > pdm³ wing discs examined was smaller than control wing discs (n = 14 each, Fig. 6A,B).

One interesting finding was increase in the level of Wg at the DV midline of nub > pdm³ wing discs (Fig. 6A,B). To examine this phenomenon further, we transiently expressed pdm³ with Gal80° system using ptc-Gal4 for 6, 12, 24, 36 and 48 hours at 30°C in order to avoid lethality by Pdm³ overexpression, and checked the level of Wg-LacZ as a marker for wg transcription. We found that wg-LacZ was ectopically expressed in the ptc region after 36 or 48 hours but not before 36 hours of transient Pdm³ expression (Fig. 6C,D). The downstream effector proteins of Wg signaling, Distal-less (Dil) and Sensless (Sens), were also induced at the ptc region (Fig. 6E,F). Thus, pdm³ directly or indirectly activates transcription of Wg.

**Discussion**

We report here that Pdm³ regulates growth and development of NMJs. pdm³ mutants showed increase in bouton size and decrease in bouton number, which are similar to the phenotype of wg mutants. Lethality induced by the overexpression of Pdm³ was rescued by knockdown of Wg in NMJ, indicating that Pdm³ functions upstream to

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**Figure 4.** Pdm³ is required in motor neurons and acts upstream to Wg. (A,B) The number of boutons in OK6-Gal4/+ as a control (A) or in repo-Gal4/+ as a control (B) is set at 100% and those in OK6 > pdm³i were divided by the control bouton number and multiplied by 100 for bar graphs. n = 27 for control, 32 for OK6 > wgi, and 18 for OK6 > pdm³i. n = 23 for control, 21 for repo > wgi, and 34 for repo > pdm³i. (C) The number of boutons in the A2 and A3 segments when GFP-wg, wgi, pdm³i, singly expressed or coexpressed by OK6-Gal4. n = 27 for A2 and 34 for A3 of CS, 18 for A2 and 17 for A3 of GFP-wg, 32 for A2 and 30 for A3 of wgi, 18 for A2 and 20 for A3 of pdm³i, 22 for A2 and A3 of GFP-wgpdm³i, 22 for A2 and 20 for A3 of GFP-wgpdm³i.

*Represents p < 0.01; ** represents p < 0.001. Data are presented as mean ± SEM.
Wg. Furthermore, overexpression of Pdm3 induced \(wg\) transcription in wing discs. We propose here that a major function of Pdm3 in motor neurons is to induce \(wg\) transcription, and secreted Wg from motor neurons regulates growth, development, and maturation of both pre- and post-synaptic regions of NMJ.

The mammalian homolog of Pdm3 is Brain-5 (Brn-5)/POU class 6 homeobox 1 (POU6F1) mainly expressed in brain and spinal cord. Brn-5 is heavily expressed in embryonic brain but also expressed in adult brain and multiple adult organs such as kidney, lung, testis, and anterior pituitary \(^35\). In developing brain, Brn-5 is expressed in postmitotic neurons after neuronal progenitor cells exit cell cycle in the early process of terminal neuronal differentiation \(^36\). Therefore, both Pdm3 and Brn-5 function in differentiation of neurons. Interestingly, ectopic expression of Brn-5 inhibits DNA synthesis \(^37\), which is similar to cell cycle arrest phenotype by Wg overexpression \(^38\). Given the homology between Pdm3 and Brn-5 as well as functional similarities, Brn-5 may also induce \(wnt\) transcription.

Most of Pdm3 functions identified so far are related to the maturation of neurons such as olfactory neurons, R neurons and td neurons as well as their postsynaptic partners \(^7\)\(^-\)\(^9\). Ectopic expression of Pdm3 induced lethality without exception, indicating that expression of Pdm3 in fly tissues is generally repressed in vivo in order to express Wg under the strict spatiotemporal control. An important question is whether Pdm3 directly transcribe \(wg\). We found that \(wg\) transcription is induced only after 36 hours of transient overexpression of Pdm3. It is possible that the level of Pdm3 needs to be over a threshold to induce \(wg\) transcription. Alternatively, Pdm3 may need to turn on other components to indirectly induce \(wg\) transcription. DNA sequence of Brn-5 binding site has been reported \(^39\)\(^-\)\(^41\), so analysis on \(wg\) and \(wnt\) regulatory regions will help understand the mechanism of \(wnt\) induction by Pdm3 and Brn-5.

We consistently found more significant NMJ phenotypes in A2 than A3 in both \(pdm3\) and \(wg\) mutants. Therefore, \(pdm3\) and \(wg\) may play more prominent roles in the A2 than the A3 segment. In fact, the level of Pdm3 was higher in the anterior region than the posterior region of ventral ganglion, which suggests that more Wg may be present in the NMJs of anterior abdominal segments. Consistent with this idea, the number of type Ib boutons in the A2 segment was 1.8 times more than A3 segment. One difference between \(pdm3\) and \(wg\) mutants is the lack of certain phenotypes in the A3 segment of \(pdm3\) NMJs: the size of boutons and the number of axon terminals in A3 were not affected in \(pdm3\) mutant. It is possible that Pdm3 turns on both common and segment-specific...
genes besides wg, and A3 segment-specific components may alleviate the loss of wg phenotype in the A3 segment. Similarly, other proteins induced by Pdm3 may also play important roles in NMJ growth, differentiation and maintenance. In fact, multiple signaling pathways including Glass-bottom-boat (Gbb) pathway also play roles in NMJ development\(^42,43\). Gbb is secreted from muscles and induces development of both pre- and post-synaptic structures, similar to Wg signaling.

We identified a defective hobo element in the \(pdm3\)\(^m7\) allele. The hobo element belongs to Ac family found in maize and has short inverted terminal repeats\(^44\). Laboratory and wild strains of \(D.\) melanogaster have average 28 and 22 copies of hobo elements in the genome that are either full-length or defective, respectively\(^45,46\). Because other suppressors identified in the genetic screen using Sona overexpression did not have hobo element in the \(pdm3\) gene, the transposition of the hobo element to the \(pdm3\) gene may have occurred subsequent to the generation of a point mutation in the arr gene by EMS. Since both arr and pdm3 are positively involved in Wg signaling, this hobo insertion may have helped the original arr\(^m7\) mutation to further decrease the activity of Wg signaling under the condition of Sona overexpression.

Besides the neuronal roles of Pdm3, all \(pdm3\) mutants show minor but consistent defects in planar cell polarity in a restricted region of the wing as well as adhesion between the dorsal and ventral wing blades. Other phenotypes such as wing drooping and premature death were also observed in all \(pdm3\) mutants, but these may be due to malformation of synaptic structures. Pdm3 also plays a role in female-limited color dimorphism in abdomen of \(D.\) montium\(^11\). The authors found in sexually dimorphic females that the first intron of the \(pdm3\) gene has four tandem sets with predicted binding sites for the HOX gene Abdominal-B (Abd-B) and the sex determination gene doublesex (dsx). Interestingly, it has been shown that Wg expression is repressed by the combinatory work of Abd-B and Dsx proteins\(^47\). Taken together, it is possible that transcription of wg and pdm3 is co-repressed by Abd-B and Dsx. Such co-repression of wg and pdm3 transcription may be also required for synaptic growth and differentiation in neurons. Further studies on Pdm3 will help understand how this understudied transcription factor is involved in the final differentiation of various cell types.

**Materials and Methods**

**Fly strains.** Ok6-Gal4, BG57-Gal4 and repo-Gal4\(^48\) were obtained from S.-B. Lee’s lab, and UAS-pdm3\(^7,8\), UAS-GFP:wg\(^49\), pdm3\(^m0028\) and pdm3\(^17,3\) were obtained from the labs that produced them. UAS-sona\(^12\) is produced in our lab. UAS-wg RNAi (#48898-3) was obtained from Fly Stocks of National Institute of Genetics. All other lines such as UAS-GFP (#1533), UAS-GFP:CD8 (#5130), UAS-pdm3 RNAi (#26749), Df(2R)BSC267, Df(2R)Exl6058, were obtained from Bloomington Drosophila Stock Center.

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Figure 6. Pdm3 induces wg transcription. (A,B) nub > pdm3 wing discs (B) have smaller wing pouch than control nub-Gal4/+ (A). (C,D) Induction of Wg-LacZ (arrows) after 36 hours of pdm3 expression in ptc > pdm3 Gal80\(^{ts}\) discs (D) compared to the control discs (C). (C,D) are black and white images of (C,D). (E,F) Induction of Sens (F) and Dll (F’) marked with arrows, compared to control (E’F’).
Immunohistochemistry. Immunohistochemical staining of larval wing discs was performed as described\(^\text{13}\), and that of NMJs was performed as described with slight modifications\(^\text{60,61}\). To obtain larvae for NMJ analysis, flies were cultured at 25 °C except wg mutants at 18 °C. To control the population size of larvae in each vial, eggs were harvested on grape plates and incubated at 25 °C for 24 hours. Then, 30 larvae were transferred to a food vial, and were cultured until the wandering larval stage for dissection. In case of wg larvae, 30 larvae were cultured at 18 °C until dissection. For NMJ staining, larvae were dissected with HL3.1 solution on sylgard plates and fixed for 20 minutes in 5% formaldehyde/HL3.1 solution\(^\text{35}\) or Bouin’s solution\(^\text{36}\). Fixed samples were rinsed 3 to 4 times with PBS or HL3.1 and then were blocked in 5% BSA in PBS before antibody treatment. The following antibodies were used: anti-Pdm3 (rat, 1:100)\(^\text{37}\), anti-β-Gal (chicken, 1:100; Abcam ab134435), anti-Dlg (rabbit, 1:500)\(^\text{35}\), anti-HRP-Cy3 (1:100; Jackson ImmunoResearch), anti-Wg (mouse, 1:1,000; DSHB 4D4), anti-Futsch (mouse, 50:1; DSHB 22C10), anti-α-Tubulin (mouse, 1:200; Sigma MAB1864), anti-Glutamate receptor IIA (mouse, 1:10; DSHB concentrated 88B4D2 (MH2B)). For GlurHIA staining, the samples were fixed by Bouin’s solution.

Image capture and quantitative analysis of boutons. We stained the late 3rd instar larvae for a presynaptic marker HRP and a postsynaptic marker Dlg to detect NMJs. Type Ib boutons have more extensive SSR compared to other bouton types (I, II, and III), so are easily detected by the high level of Dlg\(^\text{19}\). Therefore, type Ib boutons are defined as round-shaped structures in NMJ branches that are stained with HRP and have high level of Dlg, and only ones that were qualified to this definition were counted as boutons. To obtain images containing boutons, type Ib boutons visualized with HRP and Dlg were taken with 1 μm interval for 7–10 Z stacks at 400X magnification by a confocal laser microscope of Carl Zeiss (NFEC-2010-09-141569) with Zen 2009 program. To manually count number of boutons, all Z stack images were then merged and type Ib boutons at muscles 6 and 7 in A2 or A3 segments in a given image were counted. The number of images used for counting for each genotype was 17–52. To measure size of boutons, images of terminal boutons were captured at 2,000X magnifications and then area of boutons in merged images was measured by Zen 2009 program.

Cuticle preparation of larvae. The cuticle preparation was performed as described with slight modifications\(^\text{34}\). Flies were put into a chamber with a grape juice-containing agar plate that has yeast paste at the center. After 4 hours of egg-laying, plates were incubated for 20 hours at 25 °C. Larvae were transferred to distilled water on cover glass, and washed again with distilled water. Water was then removed and the 1:1 mixture of lactic acid and Hoyer's mount solution was applied. After waiting for about 1 minute, the sample on a cover glass was placed on the slide glass, and then incubated for overnight at 65 °C.

Statistical analysis. Statistical analysis was performed using ANOVA to compare different genotypes to a wild-type control within experimental groups. Data are presented as mean ± SEM. To determine statistical significance, t-test and one-way ANOVA of Microsoft Excel 2019 were used.

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Author contributions
Y. Kim and K.-O. Cho designed experiments, analyzed data and wrote the paper. Y. Kim conducted experiments.

Competing interests
The authors declare no competing interests.

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