國立臺灣大學醫學院分子醫學研究所

碩士論文

Institute of Molecular Medicine
College of Medicine
National Taiwan University
Master Thesis

極性蛋白 VANG-1 促進 Frizzled 胞吞以拮抗 Wnt 訊息傳遞

The Planar Polarity Protein VANG-1 Antagonizes Wnt Signaling by Promoting Frizzled Endocytosis

何俊緯

Chun-Wei He

指導教授：潘俊良 博士

Advisor: Chun-Liang Pan, M.D., Ph.D.

中華民國 105 年 8 月

August, 2016

doi:10.6342/NTU201602869

doi:10.6342/NTU201602869

國立臺灣大學碩士學位論文
口試委員會審定書

極性蛋白 VANG-1 促進 Frizzled 胞吞以拮抗 Wnt 訊息傳遞
The Planar Polarity Protein VANG-1 Antagonizes Wnt Signaling by Promoting Frizzled Endocytosis

本論文係 何俊緯 君（R03448006）在國立臺灣大學分子醫學研究所完成之碩士學位論文，於民國 105 年 07 月 07 日承下列考試委員審查通過及口試及格，特此證明

口試委員：

_____（簽名）
（指導教授）

_____ _____

_____ _____

_____ _____

_____ _____

系主任、所長 _____（簽名）
（是否須簽章依各院系所規定）

doi:10.6342/NTU201602869

doi:10.6342/NTU201602869

ACKNOWLEDGEMENT

在碩士班兩年中，首先要感謝陳忠寬、陳俊豪和廖健博學長以及指導老師潘俊良老師的建議和指導，讓本篇論文能夠順利進行。謝謝健博學長在做研究時的意外發現形成開起本篇研究的契機，還有在做 co-immunoprecipitation 和 western Blot 的時的指導；也謝謝陳忠寬學長先前的研究讓這篇論文有了一定的基礎，包含了不同基因型的重以及 plasmids，讓我一開始能夠比較快的進入正軌；謝謝俊豪學長在我剛進入實驗室時帶我做實驗還有提供 construct 以及在實驗上給的建議。謝謝指導教授潘俊良老師之前對於 Wnt 調控 AVM、PVM、ALM 和 HSN 發育所做的研究，讓本篇在測試 VANG-1 對於 Wnt pathway 的調控時能夠快點完成。最後感謝我的口試委員吳益群老師、劉雅雯老師和潘俊良老師(指導教授)在進度報告和論文口試時給我的建議和幫助，讓我的研究能夠順利在軌道上並且能夠朝好的方向前進。

一開始在報考分醫所時並不抱有很大的期待會被錄取，因為每年的報考人數很多但是錄取的名額很少。感謝在面試時專任老師們的賞識讓我有這個機會進入分醫所，而當我進來之後看到分醫所的老師們以及學長姐們認真和傑出的表現，更是覺得這一切有多麼的幸運。謝謝 Peter 讓我可以進來 Pan lab 這個大家庭還有在實驗上以他嚴謹細膩的思考邏輯和博學豐富的知識給予的建議以及幫助，甚至在未來人生規畫方面所提供的幫助。也謝謝 Peter 在 Pan lab 給我的一切的訓練，讓我在各方面的能比起兩年前來說都有明顯的進步，例如: 上台報告的能力，製作 PPT 的能力.....等。謝謝在 Pan lab 的每一個人，每天在邊做實驗的同時除了討論實驗之外還有講不完的八卦和垃圾話，讓這看似雷同的每一天都有一點不一樣。我不太會跟陌生人講話，對於一些事情有自己特別的看法，所以在一開始總是很難跟

doi:10.6342/NTU201602869

大家快速的打成一片，謝謝在我剛進來時，健博、均閔用一堆玩笑話和話題讓我可以更快融入氣氛的大家，還有謝謝彥志學長和學妹良憶在實驗上給予的幫助還有和我一起聊遊戲和動畫。最後想要謝謝和我一起在 Pan lab 打拼的同學志達、子庭以及在隔壁實驗室的梅均，謝謝你們在碩一在各種死線前的提醒、一起在期中考前討論考題，還有在各種爆炸前聽我焦慮的碎碎念。

在台北的兩年，從一開始的不適應，一直到現在有點捨不得離開。感謝這兩年來在台北遇到的每一個人，和你們的相遇以及相處絕對是我來唸碩班期間最大的收穫之一，謝謝你們。感謝家人在兩年間給我支持，沒有你們無條件的支持我做的每一個決定，我不能走到今天。

俊緯

2016.8

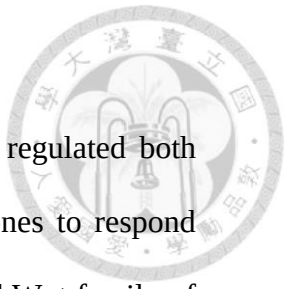
doi:10.6342/NTU201602869

中文摘要

神經對於環境中的導引因子產生反應進而觸發下游訊息傳遞，使神經細胞或軸突生長錐可以移動或延伸到正確的位置。這些導引因子所觸發的訊息傳遞，在時序和空間上須受到嚴密精確的調控，以確保訊息傳遞能夠在正確的時間和位置上活化並適時停止。前人的研究發現，物種間共同性極高的分泌性醣蛋白 Wnt 透過和其受體 Frizzled 的結合，可以調控神經細胞的移動和軸突生長錐的延伸。我們在本篇研究中發現，調控平面細胞極性的關鍵穿膜蛋白 VANG-1(Vangl2/Strabismus)會拮抗並且減弱 Wnt 訊息傳遞，而此作用可發生在線蟲神經母細胞移動或觸覺神經細胞極化並發展出不對稱的突起時。在缺乏 Wnt-Frizzled 訊息時，原本往身體後端移行的 QL 神經母細胞的子代細胞會異常地往身體前端移行，而往前移行的 QR 神經母細胞的子代細胞和 HSN 運動神經元則提早終止其移動，造成其分布位置的異常。*vang-1* 的突變可以改正因缺少 Wnt-Frizzled 訊息時所造成的神經移行或極化的失常，而過度表現 VANG-1 則會引起和 Wnt 或 Frizzled 突變時相近似的缺陷。根據我們所做的遺傳分析結果，我們假設 VANG-1 專一地拮抗 Frizzled 受體。以下所觀察到的兩個現象支持我們的假設 ： 第一，VANG-1 會和 MIG-1 或 MOM-5/Frizzled 形成蛋白質複合體。第二，增加 VANG-1 的表現可促使 Frizzled 進入細胞質；反之，在 *vang-1* 突變的狀態下，Frizzled 滯留在神經細胞膜上的比例有顯著增加。統整所有實驗結果後，我們認為 VANG-1 會促進 Frizzled 受體的胞吞作用，來阻止 Wnt 訊息過度或過久的活化，藉此達到精準控制線蟲中神經母細胞的移動，使其能抵達正確位置。

關鍵字：平面細胞極性、Wnt、Frizzled、胞吞作用、線蟲

doi:10.6342/NTU201602869



ABSTRACT

Signaling of neuronal guidance cues through their receptors is tightly regulated both temporally and spatially, allowing the migrating neurons or growth cones to respond promptly to changing guidance cues in the environments. The conserved Wnt family of secreted glycoproteins control neuronal migration and axon guidance through Frizzled receptors. We report here that VANG-1/Vangl2/Strabismus, a membrane protein important for planar tissue polarity, antagonized Frizzled functions in *C. elegans* neuronal migration. In the absence of Wnt-Frizzled signaling, descendants of the QL neuroblast mismigrated towards the anterior, and those of the QR neuroblast, as well as the HSN motor neurons, stopped prematurely in their anterior migration. Mutations in *vang-1* significantly rescued these phenotypes, and cell-specific overexpression of VANG-1 phenocopied *Wnt* and *Frizzled* mutants for neuronal migration defects. Our genetic analysis suggested that VANG-1 specifically targeted the Frizzled receptors, but not the Wnt ligands or components downstream of the Frizzleds. This conclusion was further substantiated by the following observations: First, VANG-1 formed protein complexes with the Frizzleds MIG-1 and MOM-5. Second, VANG-1 facilitated Frizzled localization to the cytosol, and Frizzleds tended to accumulate on the neuronal membrane in the *vang-1* mutant. We propose that VANG-1 promotes Frizzled endocytosis and prevents prolonged or excessive Wnt signaling to achieve proper regulation of neuroblast migration in *C. elegans*.

Keyword: planar cell polarity, Wnt, Frizzled, endocytosis, *C. elegans*

doi:10.6342/NTU201602869

CONTENTS

doi:10.6342/NTU201602869

doi:10.6342/NTU201602869

doi:10.6342/NTU201602869

Chapter 1 INTRODUCTION

1.1 Neuronal and Growth Cone Migration during Development

Wiring of the nervous system is critical for proper functioning of neural circuits, which control sensory perception, movements, memory and cognition. During early development, neurons migrate from their birthplaces to future locations, and extend neurites to connect with specific target cells. Together with axons branching and synapse formation, neuronal and growth cone guidance set up the fundamental wiring diagram of the nervous system. Neurons and growth cones undergo long-range migration by responding to highly dynamic guidance cues in the environments through which they navigate (Tessier-Lavigne and Goodman 1996). Signaling triggered by these guidance cues needs to be tightly regulated to ensure that neurons and growth cones start and stop migration at the right time, with the right speed, and in the right direction. Defective neuronal migration is a major cause for neuronal heterotopia and lissencephaly, congenital brain disorders characterized by abnormal cortical structures with seizure and profound impairment in sensorimotor and intellectual functions (Marin, Valiente et al. 2010).

Over the past three decades several sets of conserved molecules have been discovered to be essential for neuron and axon guidance (Dickson 2002). In vertebrates, netrin serves as a chemoattractive cue to guide commissural axons of spinal cord neurons to the floor plate of developing embryos by binding to and signaling through its receptor UNC-40/*Deleted in Colorectal Cancer* (DCC) (Hedgecock, Culotti et al. 1990, Kennedy, Serafini et al. 1994, Serafini, Kennedy et al. 1994). In *Drosophila*, netrin is not only important for axon midline-targeting but also plays essential roles in motor

doi:10.6342/NTU201602869

axons navigating towards the muscles (Mitchell and Victor 1996). Work pioneered in *C. elegans* and later confirmed in vertebrates also find that netrin elicits both attractive and repulsive growth cone responses by binding to UNC-40/DCC and UNC-5 receptors, respectively (Chan, Zheng et al. 1996, Norris and Lundquist 2011). Other examples of conserved guidance cues include Slit, Semaphorins and Ephrins (Bagnard, Lohrum et al. 1998, Ozdinler and Erzurumlu 2002, Sakurai, Wong et al. 2002, Miyashita, Yeo et al. 2004).

Wnts are secreted glycoproteins and are known for theirs function in pattern formation during development (Zecca, Basler et al. 1996, Clevers and Nusse 2012). As classical morphogens that specify cell fates, Wnts have also been shown to function as axon guidance cues (Zou 2004). In *Drosophila* embryonic nervous system, repulsive Wnt5 acts through the receptor tyrosine kinase Derailed to control choice of commissural projections at the posterior of the embryonic segments (Yoshikawa, McKinnon et al. 2003). In contrast, in the mouse spinal cord, Wnt4 serves as an attractive cue, by binding to the Frizzled 3 receptor, to guide spinal axons towards the rostral after midline crossing (Lyuksyutova, Lu et al. 2003). Wnts similarly play prominent roles in neuronal and growth cone guidance in *C. elegans*, which will be detailed below.

1.2 Posttranslational Mechanisms That Regulate Signaling Receptors

After guidance cues bind to their receptors, downstream signaling pathways are activated. Signaling from activated receptors needs to be tightly regulated both spatially and temporally. Signaling could be modulated at multiple levels along the transduction cascade, and controlling receptor abundance on the plasma membrane, which represents

doi:10.6342/NTU201602869

the first step in signaling transduction initiated from the receptor-ligand complex, is among the most important mechanisms that tune signaling intensity. Here, I focus on posttranslational mechanisms for regulating receptor level at the plasma membrane.

Regulation of Membrane Targeting of Receptors by Post-Golgi Trafficking

One route to control membrane level of signaling receptors is post-Golgi trafficking, which targets receptors towards the plasma membrane. Midline crossing of commissural axons in *Drosophila* embryos requires that axons are first attracted towards the ventral midline by the ventrally derived attractive cue netrin, and subsequently repelled by the repulsive cue Slit after they cross the midline. Precrossing axons are insensitive to Slit, which is also present at the ventral midline, enabling them to reach the midline under netrin-DCC-dependent attraction. The Slit receptor Robo is produced but prevented from targeting the plasma membrane by interaction with the Golgi protein Commissureless (Comm), which instead targets Robo to the lysosomes for degradation (Keleman, Rajagopalan et al. 2002, Keleman, Ribeiro et al. 2005). Netrin-DCC signaling relieves Robo from Comm-mediated degradation. When commissural axons cross the ventral midline, Robo begins to accumulate at the growth cone membrane such that postcrossing axons are repelled by Slit and prevented from back-crossing.

Regulation of Frizzled Receptor Abundance at the Cell Membrane by Endocytosis

One of the best-studied regulatory mechanisms that control receptor level at the cell membrane is endocytosis (Goldstein and Brown 2009). Internalization of the receptor is an efficient way to downregulate signaling, although in some cases endocytosis does the opposite, facilitating the signaling transduction to continue in the

doi:10.6342/NTU201602869

endosomes (see below). Earlier studies indicate that endocytosis of the Frizzled receptors requires their cytoplasmic adaptors Dishevelleds and β-arrestins, adaptor proteins that tune G protein-coupled receptor (GPCR) signaling (Chen, Kirkbride et al. 2003, Lodowski, Pitcher et al. 2003). In the crypt base columnar (CBC) cells of the intestine, binding of Wnts to Frizzled receptors activates downstream signaling and turns on the transcription of RNF43 E3 ubiquitin ligase. Ubiquitination of Frizzled receptors by RNF43 triggers Frizzled internalization and subsequent degradation, presenting an efficient way to downregulate Wnt signaling. Interestingly, PLR-1, the RNF43 homologue in *C. elegans*, is also shown to downregulate the membrane abundance of Frizzleds through ubiquitination (Moffat, Robinson et al. 2014). These results suggest that ubiquitination-dependent internalization is a conserved mechanism for Frizzled endocytosis.

In addition to classical AP2 endocytosis machinery, β-arrestins and RNF43/PLR-1, recent reports suggest that Dishevelleds and the planar cell polarity (PCP) protein Vangl2 also regulate Frizzled membrane levels (Shafer, Onishi et al. 2011, Onishi, Shafer et al. 2013). In this case, however, endocytosis of Frizzled is thought to activate Wnt signaling. The Dishevelled Dvl1, which acts downstream of Frizzleds, downregulates Wnt signaling by inhibiting Frizzled endocytosis and retaining Frizzleds at the cell membrane by hyperphosphorylation of Frizzled. By contrast, Vangl2, which is predominantly localized at the plasma membrane, antagonizes Dvl1-mediated hyperphosporylation of Frizzleds and promotes Frizzled endocytosis. This PCP-dependent Frizzled endocytosis is suggested to occur at the tips of the growth cone filopodia and plays an important role in steering growth cone turning (Shafer, Onishi et al. 2011). A follow-up study later shows that Dvl2, a component of the mammalian PCP pathway, antagonizes Dvl1 and promotes

doi:10.6342/NTU201602869

endocytosis of Frizzled receptor to facilitate PCP signaling (Onishi, Shafer et al. 2013). This regulation of Frizzled endocytosis is hypothesized to sensitize the growth cone to Wnt gradients along the anterior-posterior axis and facilitate growth cone turning towards the anterior.

1.3 Wnt-Planar Cell Polarity Pathway

Wnts are secreted glycoproteins with lipid modification and are highly conserved from hydra to human (Cadigan and Nusse 1997, Clevers and Nusse 2012). They are well-known morphogens that control morphogenesis and body patterning (Zecca, Basler et al. 1996). Wingless, the *Drosophila* Wnts was first discovered in the landmark screen conducted by Wieschaus and Nusslein-Volhard (Nusslein-Volhard and Wieschaus 1980). The mouse oncogene *int1*, identified by Nusse and Varmus, was found to be Wingless homolog (van Ooyen and Nusse 1984, Nusse 2005). Wnts bind to several classes of receptors, including the seven-transmembrane, G protein-coupled receptor (GPCR)-like protein Frizzleds, the Ror2 receptor tyrosine kinase MuSK/CAM-1, and the Ryk/Derailed/LIN-18 receptor tyrosine kinase (Yoshikawa, McKinnon et al. 2003, Forrester, Kim et al. 2004, Inoue, Oz et al. 2004, Lu, Yamamoto et al. 2004, Janda, Waghray et al. 2012). Binding of Wnts to the Frizzleds activates the cytoplasmic scaffold protein Dishevelleds and signals through either transcription-dependent or transcription-independent pathways. The transcription-dependent pathway, previously known as the canonical pathway, involves β-catenin stabilization, nuclear translocation and activation of target gene transcription by forming a complex with the T Cell Factor (TCF) transcription factor (MacDonald, Tamai et al. 2009). The Wnt pathways that do not involve β-catenin-related transcription include those that control collective polarity

doi:10.6342/NTU201602869

over a plane of tissue, known as the planar cell polarity (PCP), that requires calcium, or that reorganizes the cytoskeleton (Green, Inoue et al. 2008, Angers and Moon 2009). In *Drosophila*, the PCP pathway controls the orientation of wing hairs, sensory bristles or ommatidia of the compound eyes. In vertebrates, an equivalent pathway regulates convergent extension of the embryo gastrulation, involution and closure of the neural tube, as well as arrangement of the inner hair cell of the ear (Maung and Jenny 2011). I will focus on the Wnt-PCP pathway in the following discussion.

The core components of the PCP pathway, including Frizzleds, Dishevelleds, van Gogh/Strabismus, Prickle, Diego and Flamingo, show polarized distribution along the plane of the cell that displays tissue polarity. In the wing of *Drosophila,* van Gogh/Strabismus and Prickle accumulate at the proximal end of the cell which is closer to the body; while Frizzleds, Dishevelleds and Diego accumulate at the distal end and interact with van Gogh and Prickle from the neighboring cell (Vladar, Antic et al. 2009). This polarized distribution is hypothesized to involve mutual exclusion of van Gogh and Frizzleds (Bastock, Strutt et al. 2003). It is thus intriguing how van Gogh excludes Frizzleds from the same cell from the proximal domain, yet interacts with Frizzleds at the distal end of another cell.

In previous *Drosophila* genetic analysis (Adler, Krasnow et al. 1997), they found that wing hairs orient away Frizzled overexpression region and orient towards Vang/Stbm overexpression clone. This result suggested antagonistic relation between Vang/Stbm and Frizzled. In addition, in *Drosophila*, canonical Wnt pathway and PCP pathway use Dfz2 and Fz as a receptor respectively, but both require Dishevelled/DSH to transduce signaling. This suggested that PCP and canonical Wnt pathway could antagonize each other by competing binding of DSH to transduce signaling. Moreover, previous report

doi:10.6342/NTU201602869

showed that DSH is selectively recruited to Fz on the membrane via the DEP domain of DSH, not the PDZ domain, which is known to bind to C-terminus of Frizzled (Wong, Bourdelas et al. 2003), and transduces the PCP signaling (Axelrod, Miller et al. 1998). Besides, a prior study indicates a direct physical interaction between Drosophila van Gogh/Strabismus and the extracellular domain of the Frizzled (Wu and Mlodzik 2008). In their studies, they found that extracellular domain of Frizzled served as ligand of Van Gogh/stbm to transduce PCP signaling.

1.4 Wnt Signaling and Neuronal Development in *C. elegans*

In *C. elegans*, Wnt pathways control many aspects of neuronal development, including neuronal migration, axon guidance, neuronal polarization and synaptogenesis (Eisenmann 2005). The *C. elegans* genome encodes four Frizzleds (*mig-1*, *mom-5*, *lin-17* and *cfz-2*) and five Wnts (*cwn-1*, *cwn-2*, *egl-20*, *mom-2* and *lin-44*). Specificity of Wnt ligands and receptors is well documented in these neuronal developmental processes that highly depend on cellular types, but redundancy between Wnts or Frizzleds is also common. For simplicity below I discuss *C. elegans* Wnt signaling in three neuronal contexts that are directly related to this thesis work.

Wnt Signaling and Migration of the Q Neuroblast Descendants

The Q neuroblasts are born in the posterior of *C. elegans* postembryonically at the right (QR) and the left side (QL), respectively. During the first larval stage (L1), the QR and QL neuroblasts display identical division patterns but migrate in opposite directions. The QR descendants (QR.d) migrate for a long distance to the anterior. In contrast, the QL descendants (QL.d) migrate a short distance to the posterior (Sulston

doi:10.6342/NTU201602869

and Horvitz 1977).

The QL.d migration is controlled by the canonical Wnt pathway that activates β-catenin/BAR-1 and turns on the Hox gene *mab-5* expression (Whangbo and Kenyon 1999, Herman 2001). *mab-5* expression is necessary and sufficient to drive posterior migration of the QL.d (Maloof, Whangbo et al. 1999). EGL-20 is the sole Wnt ligand required to activate *mab-5* and control QL.d migration. MIG-1 is the major receptor for EGL-20, with LIN-17 functions as a minor receptor (Harris, Honigberg et al. 1996).

The QR.d migration is controlled by *cwn-1*/Wnt and *egl-20*/Wnt (Zinovyeva, Yamamoto et al. 2008), and the major receptor is *mom-5*/Fzd (Zinovyeva, Yamamoto et al. 2008). Although MAB-5 overexpression drive QR.d to migrate to the posterior (Maloof, Whangbo et al. 1999), *mab-5* is completely dispensable for QR.d migration, and downstream targets of Wnt signaling in QR.d migration remains elusive. Interestingly, another Hox gene *lin-39* is also essential for the anterior migration of the QR.d (Clark, Chisholm et al. 1993, Wang, Muller-Immergluck et al. 1993, Wang, Zhou et al. 2013), although unpublished data from our lab indicate that *lin-39* expression in the QR.d does not depend on either *egl-20* or *cwn-1* (Chen and Pan, unpublished observations).

Wnt Signaling and HSN Migration

During embryonic development, the hermaphrodite-specific neurons (HSNs) that control egg laying in reproductive adults are born in the posterior of the animal and migrate anteriorly to the position near vulval formation (Sulston and Horvitz 1977, Desai, Garriga et al. 1988, Garriga, Desai et al. 1993). In the *egl-20* and *mig-1* mutants, HSNs show undermigration and this phenotype is enhanced by addition of other *Wnt* or

doi:10.6342/NTU201602869

Frizzled mutation (Forrester, Kim et al. 2004, Pan, Howell et al. 2006, Zinovyeva, Yamamoto et al. 2008). Interestingly, the HSN migration defect of the *mig-1* mutant is more severe than that of the *mig-1;lin-17* double mutant (Pan, Howell et al. 2006). This suggests that *lin-17* could antagonize *mig-1* in HSN migration, although exactly how it happens remains unknown.

Wnt Signaling Controls ALM and PLM Neuronal Polarity

Several studies indicate that polarity of the ALM and PLM mechanosensory neurons is controlled by Wnt signaling (Hilliard and Bargmann 2006, Prasad and Clark 2006, Pan, Baum et al. 2008), although some argue that this is guided outgrowth of the process rather than neuronal polarization (Zheng, Diaz-Cuadros et al. 2015). In mutants that are depleted of extracellular Wnts because of disrupted Wnt secretion, such as the *mig-14*/Wntless, *dpy-23*/AP50 and *vps-35*/retromer, the ALM and PLM neurons have polarity defects (Prasad and Clark 2006, Pan, Baum et al. 2008). *lin-44/Wnt* and *lin-17/Frizzled* regulate PLM has polarity (Hilliard and Bargmann 2006, Prasad and Clark 2006), and the polarity of the ALM neuron is regulated by *cwn-1/Wnt*, *egl-20/Wnt*, *mom-5/Frizzled* and *cam-1/Ror* (Hilliard and Bargmann 2006, Prasad and Clark 2006, Pan, Baum et al. 2008, Chien, Gurling et al. 2015).

Here I report that the *C. elegans* PCP protein VANG-1/van Gogh/Vangl2 inhibits Wnt signaling by facilitating endocytosis of the Frizzled receptors, thereby reducing the level of Frizzleds at the plasma membrane. I provide genetic data suggesting that this regulation is relevant at least in four cellular contexts of neuronal development: migration of the QL.d and QR.d, HSN migration and ALM polarization. VANG-1 overexpression in the neurons phenocopies Wnt or Frizzled mutants. I further

doi:10.6342/NTU201602869

demonstrate physical interaction between VANG-1 and Frizzleds, and this physical interaction does not required the C-terminal domains of Frizzleds or VANG-1. In the *vang-1* mutant, Frizzled localization in the early endosomes is significantly reduced. I discuss how VANG-1 facilitates the internalization of Frizzleds receptors and the implication of this regulation for neuronal development.

doi:10.6342/NTU201602869

Chapter 2 MATERIALS and METHODS

2.1 *C. elegans* Strains and Genetics

Strains were cultured and maintained as described (Brenner 1974). The following are the alleles and transgenes used in this study:

LG I: mig-1(e1787), mom-5(ne12),

LG II: cwn-1(ok546), dsh-2(ok2162), mig-14(ga62), mig-5(rh147)

LG IV: egl-20(n585)

LGX: vang-1(tm1422), vang-1(ok1142), bar-1(ga80), arr-1(ok401)

Transgenes: *zdIs5[Pmec-4::GFP; lin-15(+)]* (*I*), *muIs16[P mab-5::GFP + dpy-20(+)]* (*II*), *rdvIs1[egl-17p::Myri-mCherry::pie-1 3'UTR + egl-17p::mig-10::YFP::unc-54 3'UTR + egl-17p::mCherry-TEV-S::his-24 + rol-6(su1006)]* (*III*), *mgIs42[Ptph-1::GFP + rol-6(su1006)]* (*X*), *zuIs145[Pnmy-2::MOM-5::GFP, unc-119(+)]* (*X*), *twnEx173[Pegl-17::GFP::VANG-1, Pgcy-8::mCherry]*, *twnEx199[Punc-86::GFP(5ng), Pttx-3::GFP]*, *twnEx230[Pmec-7::GFP::VANG-1(5ng), Pdpy-30::NLS::Dsred]*, *twnEx276[Pmec-7::ARR-1::mCherry(5ng), Pgcy-8::mCherry]*, *twnEX288[Pmec-7::GFP::VANG-1(5ng), Pmec-7::::ARR-1::mCherry(5ng), Pgcy-8::gfp]*, *twnEx[Pmec-7::GFP::VANG-1(10ng), Pdpy-30::NLS::Dsred]*, *twnEx[Pmec-7::GFP(5ng), Pdpy-30::NLS::Dsred]*, *twnEx[Pmec-7::GFP(10ng), Pdpy-30::NLS::Dsred]*, *twnEx[Pmec-7::ARR-1::mCherry(10ng), Pgcy-8::gfp]*, *twnEX[Punc-86::GFP(10ng)]*, *twnEx[Punc-86::GFP::VANG-1(10ng)]*

doi:10.6342/NTU201602869

2.2 Plasmid Construction and Molecular Biology

We used standard molecular biology techniques to do cloning and construct plasmids. Constructs used for generating transgenes of the *twnEx* series use the pPD95.77 Fire vector as their backbone. The constructs for expression in the HEK293 cells are in the pCDNA, RK5F or RK5M vector backbone. Information of primers for cloning *arr-1, vang-1, mig-1, dsh-2* and *mom-5* are available upon request. Germ line transformation by microinjection was performed as described (Mello et al., 1991).

2.3 Fluorescence Microscopy of PVM, AVM and HSN and Measurement of Their Positions

We used 1% sodium azide to anesthetize the animals with *zdIs5[Pmec-4::gfp]* to observe PVM and AVM positions and *mgIs42[Ptph-1::gfp]* or *twnEx199[Punc-86::gfp]* to observe HSN positions. These neurons were imaged under the 10X objective of the AxioImager M2 imaging system (Carl Zeiss) and the AxioCam MRm CCD camera (Carl Zeiss). The length of animal body, head to AVM and PVM, vulva to tail and vulva to HSN were further determined by ImageJ.

2.4 Measurement of ALM Polarity

We used 1% sodium azide to anesthetize the animals with *zdIs5[Pmec-4::gfp]* to observe ALM polarity. Neurons were imaged under the 10X objective of the AxioImager M2 imaging system (Carl Zeiss) and AxioCam MRm CCD camera (Carl Zeiss). Bipolar polarity defect is defined as neurons with the normal anterior process and a posterior process longer than 10 times cell diameter. Reversed polarity defect is

doi:10.6342/NTU201602869

defined as ALM neuron without the anterior process but having a long posterior process.

2.5 *mab-5* Expression Measurement

We used 1% sodium azide to anesthetize the animals with *rdvIs1[Pegl-17::myr::mcherry]; muIs16[Pmab-5::gfp]* to observe *mab-5* expression specifically in QL.a and QL.p. Synchronized early L1 larvae (fed 2~3 hours after released from L1 arrest) were observed by the 100X objective of the AxioImager M2 imaging system (Carl Zeiss) and AxioCam MRm CCD camera (Carl Zeiss) with identical imaging parameters. Weak *mab-5* expression is defined as neuron having detecable but dim GFP in the region defined by *rdvIs1*. High *mab-5* expression is defined as neuron with bright GFP signal.

2.6 Confocal Microscopy and Quantification of MOM-5::GFP Distribution

We use the Zeiss LSM 700 Confocal Imaging System to acquire the Z-stack maximum projection images. Pixel-wise GFP fluorescence signal was quantified using the Zeiss Zen imaging software. We used *rdvIs1[Pegl-17::myr::mcherry]* to label the Q cell lineages that highlights the plasma membrane, and *zuIs145[Pnmy-2::MOM-5::GFP]* to observe MOM-5 signal. Early L1 larvae of posthatching 1~2 hours were examined under the 100X objective of the LSM 700 Confocal System (Carl Zeiss) and AxioCam MRm CCD camera (Carl Zeiss). Intensity of MOM-5::GFP signals in QR.a and QR.d cell were measured by the Zeiss Zen imaging software. Intensity of MOM-5::GFP signals in QR.a and QR.d plasma membrane were derived by subtracting cytosolic GFP signals from total GFP signals.

doi:10.6342/NTU201602869

2.7 Co-Immunoprecipitation and Western Blotting

We transfected the constructs into HEK293 cells and lysed them with lysis buffer (50 mM Tris, 150 mM NaCl, 2 mM EDTA pH=8.0, 0.5% Sodium Deoxylcholate, 10 mM phenylmethylsulfonyl fluoride, and 1M Dithiothreitol) with 1% NP-40. For co-immunoprecipitation, we used anti-HA (Invitrogen) or anti-FLAG (Sigma) beads to pull down proteins from cell lysates. Samples for western blot analysis were electrophoresed in 10% SDS-polyacryalamide gel, transferred into PVDF membrane. We used anti-HA, ant-FLAG or anti-Myc antibody to detect protein signal. (I think you need to provide some more details about IP or western procedures)

2.8 RNAi Experiments

We added 1mM IPTG into liquid bacteria with RNAi construct or performed pre-induction for 1.5 hours. Gravid hermaphrodites were bleached in RNAi-NGM plates supplemented with 1mM IPTG. Newly hatched L1 fed on these RNAi plates were allowed to develop to adult, and were then transferred to new RNAi plates for bleaching. Identical procedures were repeated one more time, with gravid hermaphrodites bleached to collected synchronized early L1 larvae. These arrested L1 larvae were released on new RNAi plates and scored at appropriate stages for each phenotype (2-3 hours post-hatching for MOM-5::GFP quantification, and L4 for PVM positions).

doi:10.6342/NTU201602869

Chapter 3 RESULTS

3.1 VANG-1 antagonized Wnt signaling in QL descendants migration in *C. elegans*

In *C. elegans*, the bilaterally symmetric QR and QL neuroblasts were born at similar positions at the right and left side of the worm body and displayed identical patterns of cell divisions, but their descendants migrated towards the anterior and the posterior, respectively (Figure 1). Posterior migration of the QL descendants (noted as QL.d hereafter) was regulated by the Hox gene *mab-5*, whose expression in the QL lineage was controlled by *egl-20*/Wnt signaling (Chalfie, Thomson et al. 1983, Salser and Kenyon 1992). Specifically, while the initial posterior migration of QL was independent of *egl-20* or *mab-5*, subsequent posterior migration of QL.a, QL.p, QL.ap, or QL.pa all required *egl-20* and *mab-5*. A series of genetic studies had identified a β-catenin-dependent Wnt pathway as essential for *mab-5* activation, including genes encoding the Frizzleds (*mig-1* and *lin-17*), the scaffold Dishevelled (*mig-5*), the β-catenin (*bar-1*) and the TCF transcription factor (*pop-1*) (Harris, Honigberg et al. 1996, Eisenmann and Kim 2000, Korswagen, Herman et al. 2000) (Figure 2). Mutations of any of these genes cause a profound decrease or absence of *mab-5* transcription in the QL.d. Loss of *mab-5* expression resulted in abnormal anterior migration of the QL.d, while ectopic expression of *mab-5* in the QR drove the QR.d to migrate posteriorly, similarly to those of the QL.d. Therefore, the status of *mab-5* expression served as a readout for *egl-20/Wn*t activation.

In order to know how Wnt signaling was regulated during QL.d migration, we quantified the position of one of the QL.d, PVM, which was the QL.paa cell and could

doi:10.6342/NTU201602869

be labeled by a specific transgene, *zdIs5[Pmec-4::gfp]*, from L2 to adult. The PVM position was quantified in the L4 animals as a ratio of the anterior-to-PVM length to the anterior-posterior body length of the animal, in which smaller numbers indicate anterior positions and larger numbers indicate posterior positions (Figure 3A). In wild-type animals, PVM was located in the posterior, a result of its posterior migration in early L1. Mutations in the Wnt pathway genes, including *egl-20/Wnt*, *mig-1/Frizzled*, *mig-5/Dishevelled* and *bar-1/β-catenin* all resulted in misplaced PVM in the anterior (Figure 3A). Chien-Po Liao in our laboratory first found that the *tm1422* mutation of *vang-1*, which encodes the four-transmembrane planar cell polarity protein van Gogh/Strabismus/Vangl2 in *C. elegans*, suppressed PVM migration defects in the *mig-1* mutant (data not shown). Chung-Kuan Chen and I independently confirmed this result (Figure 3B and data not shown).

The aforementioned observations raise two possibility. In the first scenario, *vang-1* negatively regulates Wnt signaling. Based on this hypothesis, it is possible to determine the hierarchical order of *vang-1* in the regulation of *egl-20/Wnt* signaling pathway by performing genetic epistasis between *vang-1* and other Wnt pathway mutations. In the second scenario, *vang-1* antagonizes *egl-20/Wnt* signaling through an independent pathway. This model predicts that the *vang-1* mutation should suppressed the PVM phenotypes of the *egl-20*, *mig-1*, *mig-5* and the *bar-1* mutants. To differentiate between these two possibilities, we constructed double mutants between *vang-1* and one of the Wnt pathway mutants and analyzed PVM positions. We found that the *vang-1* mutation failed to suppress PVM migration defects in the *egl-20*, *mig-5* or *bar-1* mutants (Figure 3B). These results suggested that *vang-1* antagonized *egl-20/Wnt* signaling specifically at the level of *mig-1*/Frizzled receptor. In addition, in order to make sure that the rescue

doi:10.6342/NTU201602869

effect of *vang-1*(tm1422) mutation for PVM migration defect in *mig-1* mutant is truly due to loss-of-function *vang-1*, we used another allele *ok1142* of *vang-1* mutation and also used RNAi to knockdown *vang-1* and we found that both of them can rescue PVM migration defect in *mig-1* mutant (Figure 4). This result strongly suggested that loss of function of vang-1 can rescue PVM migration defect in *mig-1* mutant.

That *vang-1* suppressed *mig-1* but not *egl-20*, *mig-5* or *bar-1* suggests that rescue of PVM migration in the *mig-1; vang-1* double mutant requires *egl-20/Wnt* activity, possibly through *lin-17/Frizzled*, which also promotes PVM posterior migration together with *mig-1*. This model predicts that the *mab-5* expression in the QL.d, which was lost in the *mig-1* mutants, could be restored by the *vang-1* mutation. We used the transgenes *rdvIs1[Pegl-17::Myr-mCherry::pie-1 3'UTR + Pegl-17::mig-10::YFP::unc-54 3'UTR + Pegl-17::mCherry-TEV-S::his-24 + rol-6(su1006)*] to label the Q cell descendants and *muIs16[Pmab-5::gfp]* to examine *mab-5* expression level in these cells. We categorized GFP signal in to three groups: strong, weak, undetectable. Strong GFP signal expressed from the *mab-5* promoter could be seen in the wild type but was most lost in the *mig-1* and *egl-20* mutants, as previously reported (Figure 4 and 5)(Whangbo and Kenyon 1999, Pan, Baum et al. 2008). Consistent with our prediction, *mab-5* expression in the QL.d was significantly restored in many *mig-1; vang-1* double mutant animals (Figure 5 and 6). By contrast, *mab-5* expression remained mostly undetectable or weak in the *egl-20; vang-1* double mutant (Figure 5 and 6). These results are consistent with *vang-1* being a negative regulator of the Frizzled.

3.2 *vang-1* Functions Cell-Autonomously in the QL.d

To explore where VANG-1 functions to antagonize MIG-1/Frizzled activity, we

doi:10.6342/NTU201602869

expressed VANG-1 specifically in the Q neuroblast in the *mig-1;vang-1* double mutants, from the *egl-17* promoter (Figure 7) (courtesy of Chung-Kuan Chen). In the *mig-1; vang-1; twnEx173[Pegl-17::gfp::vang-1]* animals, PVMs mismigrated towards the anterior and the positions are similar to those in the *mig-1* single mutant. This result suggests that *vang-1* acts in the Q neuroblast, and likely in the QL.d (Figure 7). Moreover, overexpression of VANG-1 in the Q lineages resulted PVM mismigration in the wild type, and further enhanced PVM migration defects of the *mig-1* mutant (Figure 7). These results indicate that *vang-1* acts cell-autonomously in the Q cell descendants.

3.3 VANG-1 Antagonized Wnt Signaling in QR Descendant Migration in *C. elegans*

Previous studies (Harris, Honigberg et al. 1996, Pan, Baum et al. 2008) suggested that QR descendant migration was also controlled by Wnt signaling. Therefore, we tested whether *vang-1* also antagonized Wnt pathways in migrating QR descendants. The QR neuroblast was born at the right side of the worm and showed identical lineage pattern as that of the QL. QR descendents (noted hereafter as QR.d) migrate to the anterior, and in *Wnt* or *Frizzled* mutants, QR.d terminated their anterior migration prematurely. To understand whether *vang-1* antagonized Wnt signaling during QR.d migration, we quantified the position of the AVM, one of the QR.d (QR.paa) and could also be labeled by *zdIs5[Pmec-4::gfp]* in L4 (Figure 8A). Similar to previous reports, mutations of *egl-20/Wnt, cwn-1/Wnt, mom-5/Frizzled* and *mig-14/Wntless*, which is necessary for Wnt ligands secretion, caused AVM undermigration (Figure 8B). By double mutant analysis, we found that the *vang-1* mutation suppressed AVM migration defect in the *cwn-1*, *egl-20* and *mom-5* mutants, but not that in the *mig-14* mutant

doi:10.6342/NTU201602869

(Figure 7B). This result is consistent with findings in the QL migration that functional Wnts are needed for functional rescue conferred by the *vang-1* mutation. In agreement with the conclusion that *vang-1* antagonized Wnt signaling in AVM (QR.d) migration, overexpression of VANG-1 in the Q neuroblast lineage (*twnEx173*) caused AVM undermigration, phenocopying the *Wnt* and *frizzled* mutants (Figure 8C).

3.4 VANG-1 Antagonized Wnt Signaling in HSN Migration in *C. elegans*

To explore the possibility that VANG-1 broadly antagonizes Wnt signaling in *C. elegans* neuronal migration, we examined the HSN, the motor neuron that controls egg-laying muscles in hermaphrodites and undergoes long-range migration during embryogenesis. The bilaterally symmetric HSNs are born in the tail and migrate anteriorly to reach the positon near the future vulva. Previous reports indicated that HSN migration was controlled by Wnt signaling (Garriga, Desai et al. 1993, Pan, Howell et al. 2006). We used the transgene *mgIs42[Ptph-1::GFP + rol-6(su1006)]* to label the HSNs in L4 or adult animals of wild type and most of the *Wnt* or *Frizzled* mutants. For unknown reason, *mgIs42* was downregulated in the *mig-1 mom-5* mutant, and we used *twnEx199[Punc-86::gfp]* to labeled the HSN in this strain and also in *mig-1 mom-5/hT2; vang-1*. The HSN positons were quantified as a ratio of the vulva-to-HSN length to the vulva-posterior length in young adult animals. Smaller numbers indicate that positions closer to the vulva and hence normal migration. By contrast, larger numbers indicate HSN undermigration (Figure 9A).

In the *cwn-1/Wnt, egl-20/Wnt* and *mig-1/frizzled* mutants, HSN displayed undermigration defects as reported by previous studies. HSN undermigration was more severe in the *cwn-1; egl-20* and *mig-1 mom-5/hT2* double mutants (Figure 9B). We found

doi:10.6342/NTU201602869

that the *vang-1* mutation can suppress migration defects in the *cwn-1*, *egl-20* and *cwn-1; egl-20* double mutants. By contrast, *vang-1* failed to suppress the severe HSN undermigration in the *mig-14* mutant that lost much of the secreted Wnt ligands (Figure 9B). Compared to the *mig-1* single mutant, more HSNs migrated to the normal positon in *mig-1; vang-1* double mutants, suggestive of suppression of the *mig-1* phenotype. However, *vang-1* failed to suppress the defects in the *mig-1 mom-5* mutants (Figure 10A). Together these results indicated that *vang-1* antagonized Wnt signaling in HSN migration, and rescue conferred by *vang-1* mutation requires functional Wnt-Frizzled signaling and would not happen for mutants that lost most Wnt or Frizzled activities. This conclusion was further substantiated by restoring *vang-1* specifically in the HSNs of the *egl-20; vang-1* double mutants, which reversed the suppression and suggested a cell-autonomous role for VANG-1 in modulating Wnt signaling (Figure 10B).

3.5 VANG-1 Antagonized Wnt Signaling in ALM polarity in *C. elegans*

To investigate whether VANG-1 modulates Wnt signaling in contexts other than cell migration, we examined the polarity of another class of touch receptors, the ALM neurons. The bilaterally symmetric ALM neurons extend a long neurite to the anterior and a short posterior process without known functions. Previous studies (Hilliard and Bargmann 2006, Prasad and Clark 2006) had shown that the polarity of ALM was controlled by *Wnt* signaling. In the *cwn-1; egl-20* or the *mig-14* mutants, the ALM neurons often displayed reversed polarity or showed bipolar morphology, with a posterior neurite that was at least 10 cell diameter long (Figure 11A and 11B). The ALM polarity defects in the *mig-14* mutant were significantly worse than those in the *cwn-1; egl-20* double mutant, suggesting that other Wnts also played a role in ALM polarity

doi:10.6342/NTU201602869

control. Chien et al. further showed that *mom-5* and the atypical Wnt receptor gene *cam-1* were both required for normal ALM polarity (Chien, Gurling et al. 2015). We found that the *vang-1* mutation partially suppressed the ALM polarity defects in the *cwn-1; egl-20*, but not in the *mig-14* mutant (Figure 11B). This result was consistent with the model that *vang-1* antagonizes Wnt pathways in ALM polarity control, and that phenotypic suppression by the *vang-1* mutation required active Wnt ligands.

Based on the aforementioned observations, we predicted that VANG-1 overexpression would induce ALM polarity defects. Indeed, when we overexpressed VANG-1 specifically in the touch neurons, we found that ALM posterior processes longer than 5 cell diameter in length became more frequent. This ALM polarity defect was enhanced by further increasing the dosage of VANG-1 overexpression (Figure 12). This result suggested that VANG-1 overexpression phenocopied *Wnt* loss-of-function mutants. Together these results further consolidated the notion that VANG-1 broadly antagonized Wnt signaling in multiple events of neuronal development in *C. elegans.*

3.6 VANG-1 Antagonized Wnt Pathways by Promoting Frizzled Endocytosis

Next, we wanted to know how VANG-1 antagonized Wnt signaling at the level of the Frizzled receptors. Previously Chun-Hao Chen, a former member of our lab, showed that in the *vang-1* mutant, subcellular localization of MIG-1 to the RAB-5(+) early endosomes was decreased in the PLM neuron. By contrast, *vang-1* overexpression increased membrane localization of MIG-1. Based on these results, we hypothesized that VANG-1 antagonized Wnt signaling by promoting Frizzled receptor endocytosis.

A prerequisite of this hypothesis is that Wnt signaling is inhibited by Frizzled endocytosis in cell migration or neuronal polarization. To validate this hypothesis, we

doi:10.6342/NTU201602869

first tested whether ARR-1, which is the β-arrestin2 homologue in *C. elegans* that promoted Frizzled endocytosis, antagonized Wnt signaling in QL.d migration. We found that, similar to the case in the *mig-1; vang-1* double mutant, the *arr-1* mutation partially suppressed PVM migration defects of the *mig-1* mutant (Figure 13A). We further found that PVM migration defects were not further improved in the *mig-1; arr-1 vang-1* triple mutants, compared to those in the *mig-1; vang-1* or *mig-1; arr-1* double mutants (Figure 13A). These data indicated that *arr-1* antagonized Wnt signaling during QL.d migration, and that *arr-1* and *vang-1* acted in a common pathway. Moreover, I found that the *arr-1* mutation largely suppressed the PVM defects caused by VANG-1 overexpression (Figure 13B). This result suggested that *vang-1* acted upstream of *arr-1*/β-arrestin in antagonizing Wnt pathways and possibly also in Frizzled endocytosis.

We further examined whether *arr-1* also antagonizes Wnt signaling in other developmental processes. We found that in QR.d migration, the *arr-1* mutation suppressed AVM migration defects of the *mom-5* mutant (Figure 14A) and those caused by Q-cell specific VANG-1 overexpression (Figure 14B). Moreover, the *arr-1* mutation also suppressed HSN migration defects in the *egl-20* mutant (Figure 15). Suppression of the HSN migration defects is not further improved in the *egl-20; arr-1 vang-1* triple mutants compared to *egl-20; vang-1* or *egl-20; arr-1* (Figure 15), suggesting that *vang-1* and *arr-1* act in a common pathway. In addition to reducing the PVM defects caused by *vang-1* overexpression, the *arr-1* mutation also suppressed the ALM polarity defects caused by *vang-1* overexpression (Figure 16). These results indicate that *arr-1* acts downstream of *vang-1* to antagonize Wnt signaling in multiple aspects of neuronal development in *C. elegans*.

Since *arr-1* acts downstream of *vang-1*, we hypothesized that *vang-1* antagonizes

doi:10.6342/NTU201602869

Wnt signaling by promoting Frizzled endocytosis which required *arr-1*. In order to examine this hypothesis, we used *rdvIs1;zuIs145[Pnmy-2::mom-5::gfp]* to observe MOM-5::GFP distribution between cytosol and membrane labeled by *myr::mCherry* in QR.a and QR.p. We quantified the distribution as a ratio of GFP signal on the membrane to GFP signal in the cytosol, in which smaller numbers indicate more MOM-5 in cytosol and larger numbers indicate more MOM-5 on the membrane. According to the result, we found that there are less MOM-5 in the cytosol in *rdvIs1 ;zuIs145* animals fed with *vang-1* RNAi compared to the animals fed with empty factor (Figure 17).

3.7 VANG-1 forms protein complexes with Frizzleds

Our genetic and cell biological analysis suggests that *vang-1* antagonizes Wnt signaling by promoting Frizzled endocytosis and this process of Frizzled internalization requires *arr-1*. We hypothesize that Frizzled and VANG-1 form protein complexes. To examine this possibility, we tagged VANG-1 or Frizzled with different epitopes, expressed different structures of them in cultured HEK293 cells (Figure 18), and did co-immunoprecipitation. Chun-Hao Chen from our lab had previously shown that MIG-1 formed a complex with VANG-1 (Chen et al., 2015). In addition, I showed that MOM-5 also forms protein complexes with VANG-1 (Figure 19). These experiments indicate that VANG-1 and Frizzleds exist in the same protein complex and likely physically interact with each other.

We next mapped domains of Frizzled and VANG-1 for their physical interaction. We found that VANG-1 that lost most of its C-terminal cytosolic sequence, the VANG-1(1-281), still formed a complex with full-length MIG-1 (Figure 20, Chen et al., 2015) or MOM-5 (Figure 21). Similarly, MIG-1 that lost the short C-terminus tail, the MIG-1

doi:10.6342/NTU201602869

△C, remained in a complex with full length VNAG-1 (unpublished data from Chun-Hao Chen). The C-terminus stretch of MIG-1 contains a putative peptide motif required for interacting with Dishevelleds (Wong, Bourdelas et al. 2003), raising the possibility that VANG-1 and Dishevelleds interact with distinct MIG-1 domains.

Previous studies suggest that the scaffold protein Dishevelled promotes Frizzled endocytosis by recruiting β-arrestin2 (Chen, Kirkbride et al. 2003), and that this Dishevelled-dependent Frizzled endocytosis promotes Wnt signaling. We next investigate whether the Dishevelleds exist in the same complex with VANG-1 and Frizzleds. Our co-immunoprecipitation experiment showed that VANG-1, MIG-1 and DSH-2/Dishevelled existed in the same complex (Figure 22). Interestingly, DSH-2 did not coimmunoprecipitate with the MOM-5/ VANG-1 complex (Figure 23), although *dsh-2* regulates QR.d migration (Pan et al., 2008), a context in which *mom-5* and *vang-1* also play a role. It remains possible that MOM-5 and VANG-1 interact with DSH-1 or MIG-5, two other *C. elegans* Dishevelleds, or that VANG-1 competes for DSH-2 binding to MOM-5. Future studies will address these different possibilities.

doi:10.6342/NTU201602869

Chapter 4 DISCUSSION

Our observations suggest that *vang-1* antagonizes Wnt signaling in multiple cellular contexts of early neuronal development in *C. elegans*. Mechanistically, we provide evidence that VANG-1 downregulates Wnt signaling by facilitating Frizzled endocytosis. These data extend current understanding of VANG-1/Vangl2 functions and add another layer of complexity to interactions between different branches of Wnt signaling.

4.1 Suppression of Wnt Mutant Phenotypes by *vang-1* Mutations

One essential part of our model that VANG-1 downregulates Wnt signaling comes from the observation that phenotypic suppression of Wnt signaling mutants by *vang-1* inactivation depends on remaining Wnt ligands and functional Frizzled receptors. In the case of QL.d or PVM migration, *vang-1* mutant rescues migration defects of the *mig-1* mutant but not those of the *egl-20* mutant. This is explained by remaining LIN-17 Frizzled receptor providing signaling functions in the absence of MIG-1 (Harris, Honigberg et al. 1996, Eisenmann and Kim 2000, Korswagen, Herman et al. 2000). By contrast, EGL-20 is the sole Wnt ligand that drives QL.d migrations. As a result, *vang-1* mutation does not rescue the QL.d defects in the *egl-20* mutant (Chalfie, Thomson et al. 1983, Salser and Kenyon 1992).

A previous report (Pan, Howell et al. 2006) identified MIG-1 and MOM-5 as two major Frizzled receptors required for HSN migration. Consistent with this, more HSN neurons end up in locations near the wild-type positions in the *mig-1; vang-1* double mutants compared to the *mig-1* single mutant, but the *vang-1* mutation does not rescue the HSN defects in the *mig-1 mom-5* double mutant. However, phenotypic suppression of

doi:10.6342/NTU201602869

mig-1 mutants by the *vang-1* mutation regarding the HSN defects was relatively moderate. In one previous report (Pan, Howell et al. 2006), genetic data suggest that LIN-17 may antagonize Wnt signaling. We hypothesize that in the absence of VANG-1, LIN-17 is also upregulated and acts to antagonize Wnt signaling, resulting in a much less impressive rescue of the HSN phenotypes.

4.2 VANG-1 Downregulates Wnt Signaling by Facilitating Frizzleds Internalization

In the report published by Vieira and Schmid in 1996, they found that EGF signaling can be enhanced by promoting EGFR endocytosis. Endocytosis of the receptor is also an important regulation mechanism of signal transduction in Wnt signaling in several developmental contexts, including axon growth cone guidance, planar cell polarization and synaptogenesis (Chen, Kirkbride et al. 2003, Wang and Nathans 2007, Shafer, Onishi et al. 2011, Onishi, Shafer et al. 2013). In different cell types and signaling contexts, endocytosis of surface receptors could either promote or terminate Wnt signaling. Studies in mammalian cells indicate that, after binding of Wnts to the receptors, Wnt signaling is activated, and Dishevelleds, AP-2 and the adaptor protein β-arrestin2 are recruited to the plasma membrane to promote Frizzled endocytosis (Chen, Kirkbride et al. 2003, Yu, Rual et al. 2007). In the work from Chun-Hao Chen, previously from our lab, also it is shown that VANG-1 promotes Wnt signaling by facilitating Frizzled endocytosis and this regulation is important for neurite branching, a later event that occurs after neuronal polarization is established (Chen et al., 2015). These examples highlight the importance of cellular contexts in determining the outcome of signaling after Frizzled receptor internalization. Wnt signal transduction

doi:10.6342/NTU201602869

can additionally be terminated by acidification of the endosomes after being endocytosed in embryonic cells of the imaginal discs in *Drosophila* (Dubois, Lecourtois et al. 2001, Rives, Rochlin et al. 2006).

Although our results suggest that *vang-1* promotes Fizzled endocytosis in a genetic pathway that contains *arr-1*, the mechanism by which VANG-1 promotes Frizzled endocytosis is still unclear. According to the co-immunoprecipitation experiments, we found that MIG-1 forms a complex with VANG-1 and DSH-2. However, DSH-2 is not part of the complex formed by VANG-1 and MOM-5, although previous reports suggest that DSH-2 likely transduces Wnt signaling downstream of MOM-5 in QR.d migration. One provocative hypothesis is that VANG-1 could possibly compete with DSH-2 for binding MOM-5, thus preventing DSH-2 from transducing Wnt signaling. To further examine how Dishevelleds interact with the VANG-1-Frizzled complexes, we plan to test whether MIG-5/Dishevelled forms a complex with VANG-1 and MIG-1, which is the scaffold protein downstream of MIG-1 in QL.d migration. Exclusion of MIG-5 from the VANG-1-MIG-1 complex would further confirm that VANG-1 competes with Dishevelleds for binding Frizzled receptors. Additional biochemical experiments will be necessary to map the interacting domains between VANG-1, Frizzleds and Dishevelleds. We believe that these experiments will greatly advance our understanding how receptor complexes are differentially endocytosed by Dishevelled- or VANG-1-dependent routes, and how these distinct endocytosis routes governs the signaling specificity of Wnts in driving early neuronal development.

doi:10.6342/NTU201602869

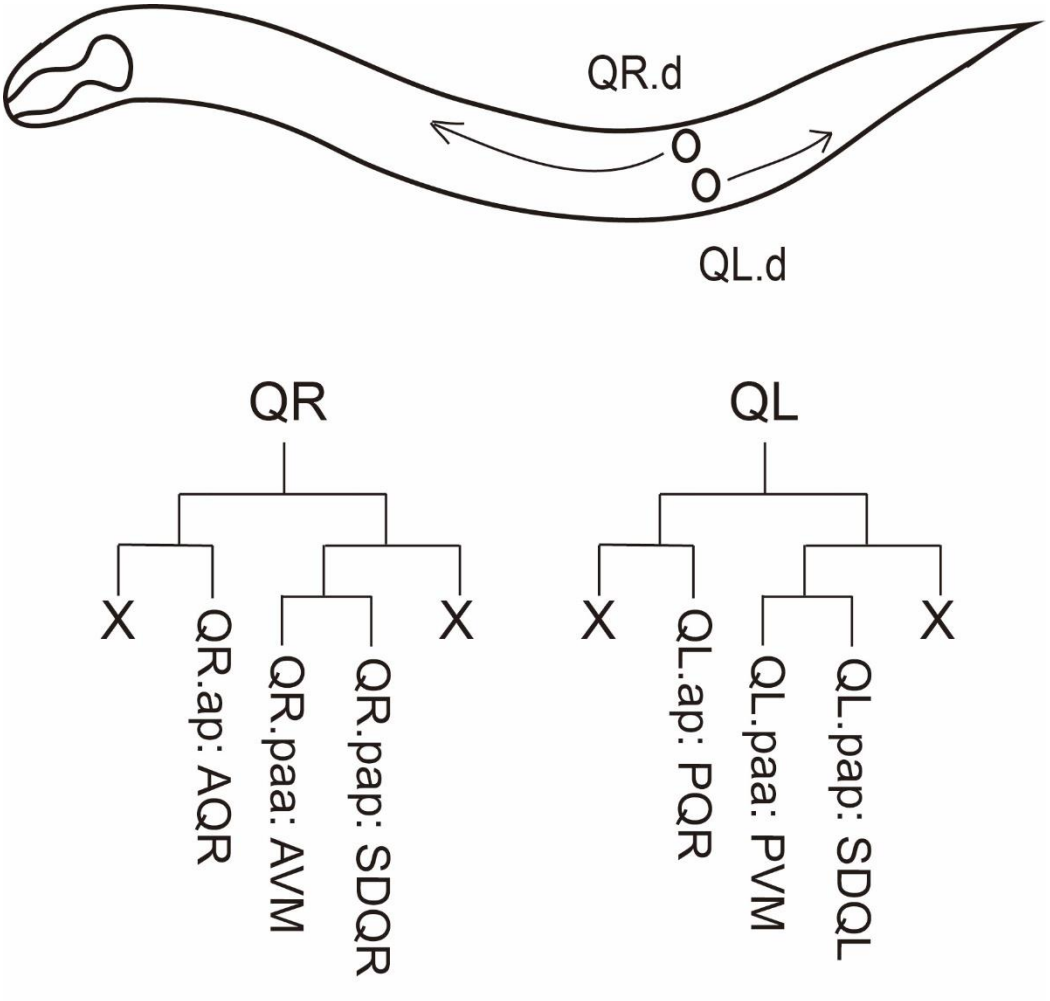
Chapter 5 FIGURES

doi:10.6342/NTU201602869

Figure 1. Development of Q neuroblasts in *C. elegans*

Schematic diagrams of Q neuroblast migration and the Q lineages. (A) QR and QL neuroblasts are born at similar positions at the right and left side of the worm body and their descendants migrate to the anterior and posterior, respectively, during development. (B) The QL and QR display identical patterns of cell division.

doi:10.6342/NTU201602869

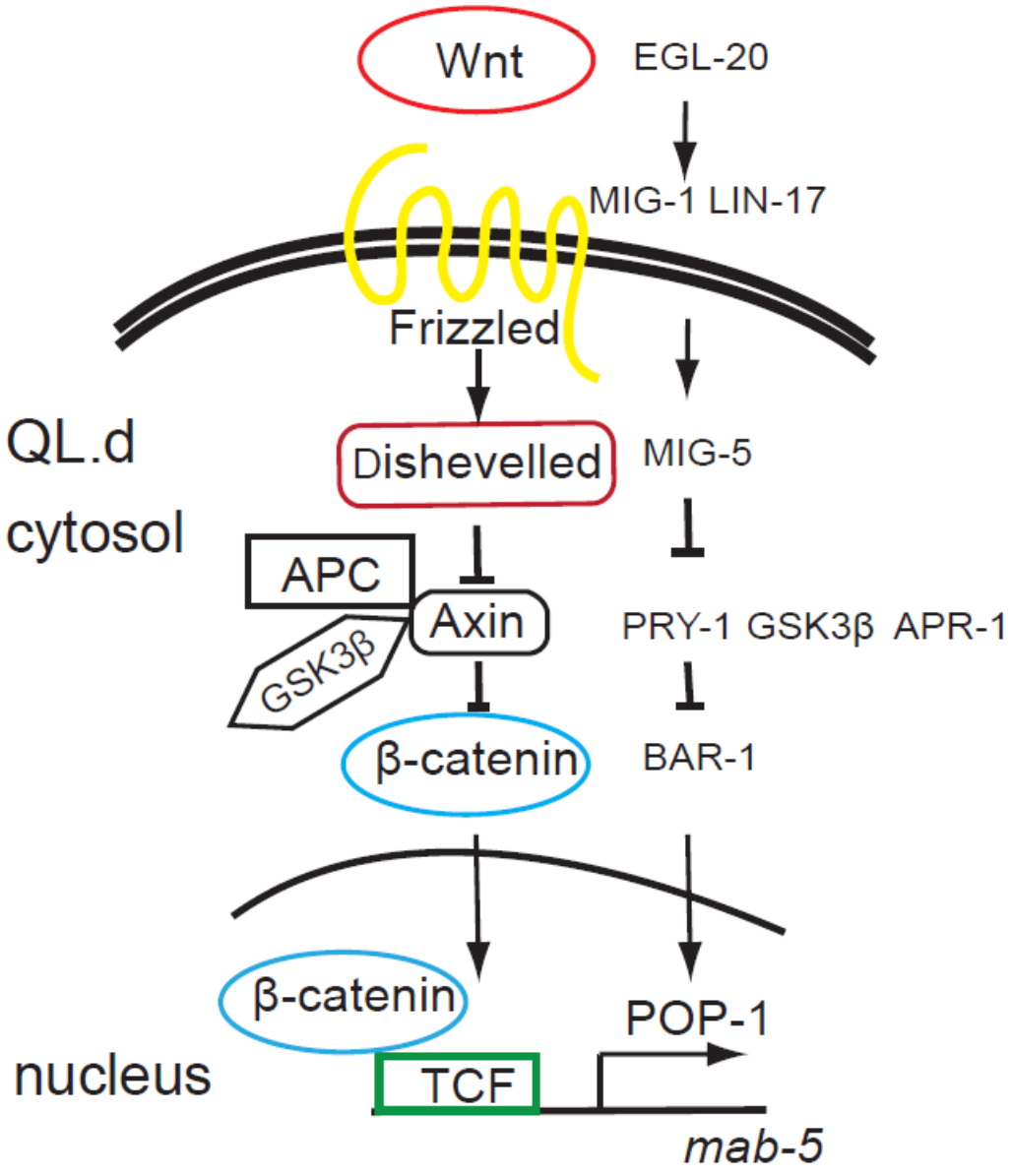


doi:10.6342/NTU201602869

Figure 2. Canonical Wnt signaling

Schematic model of β-catenin-dependent Wnt signaling. *C. elegans* homologs required for QL.d migration are noted on the right.

doi:10.6342/NTU201602869



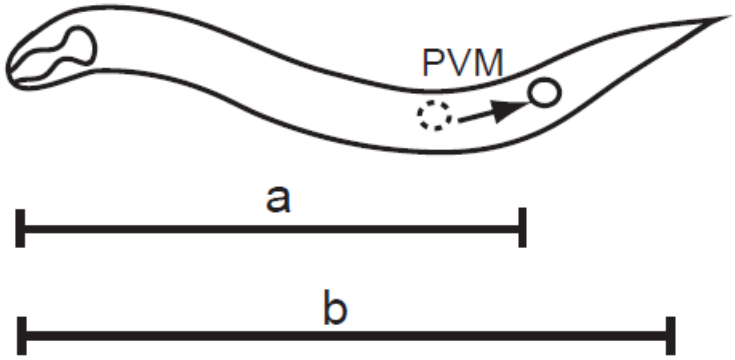
doi:10.6342/NTU201602869

Figure 3. *vang-1* antagonizes Wnt pathwaysignaling at the level of the Frizzled receptor

(A) Schematic diagram of the PVM migration process. The PVM positions are defined as the ratio of the anterior-to-PVM length (a) to the anterior-posterior body length of the animal (b). (B) PVM positions in the wild type and the Wnt pathway mutants. PVM positions are labeled by the transgene *zdIs5[Pmec-4::gfp]*. n.s., not significant. ***, $p < 0.005$, unpaired *t* test. Numbers of neurons scored are indicated behind the genotypes. Error bars represent the S.E.M.

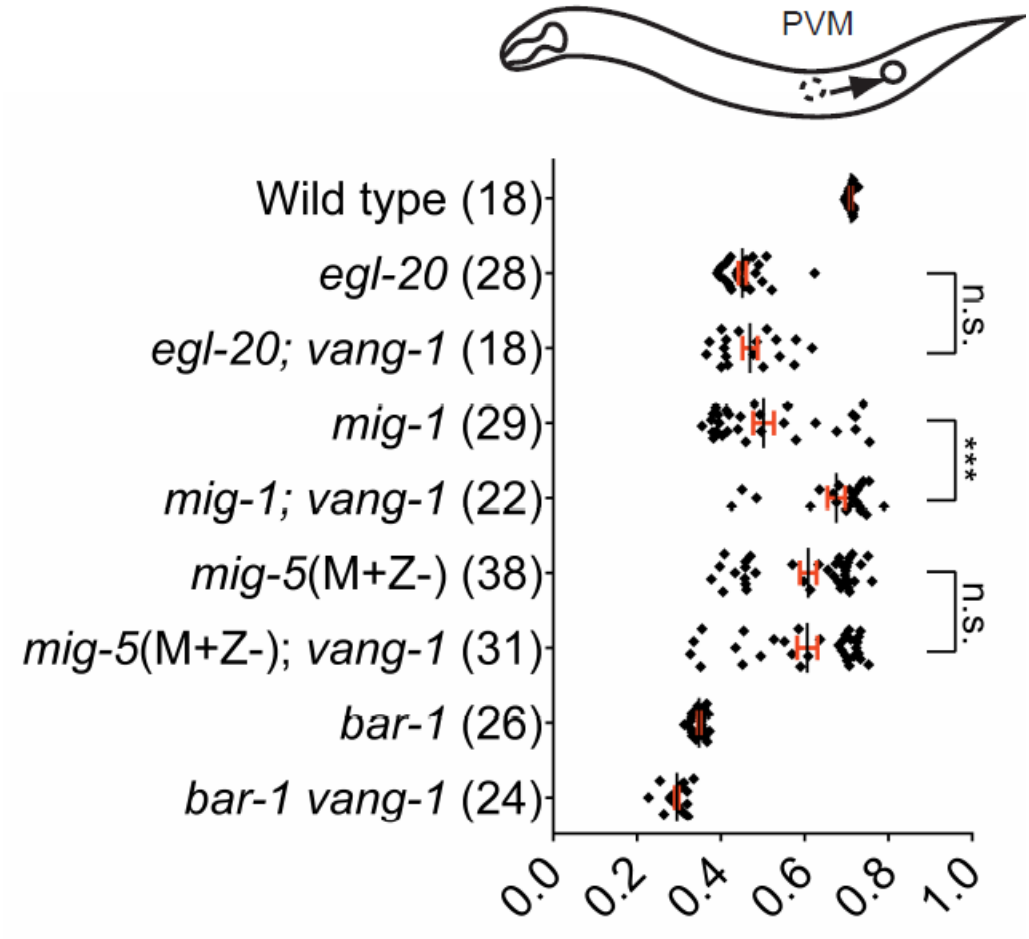
doi:10.6342/NTU201602869

A



PVM position = a/b

B

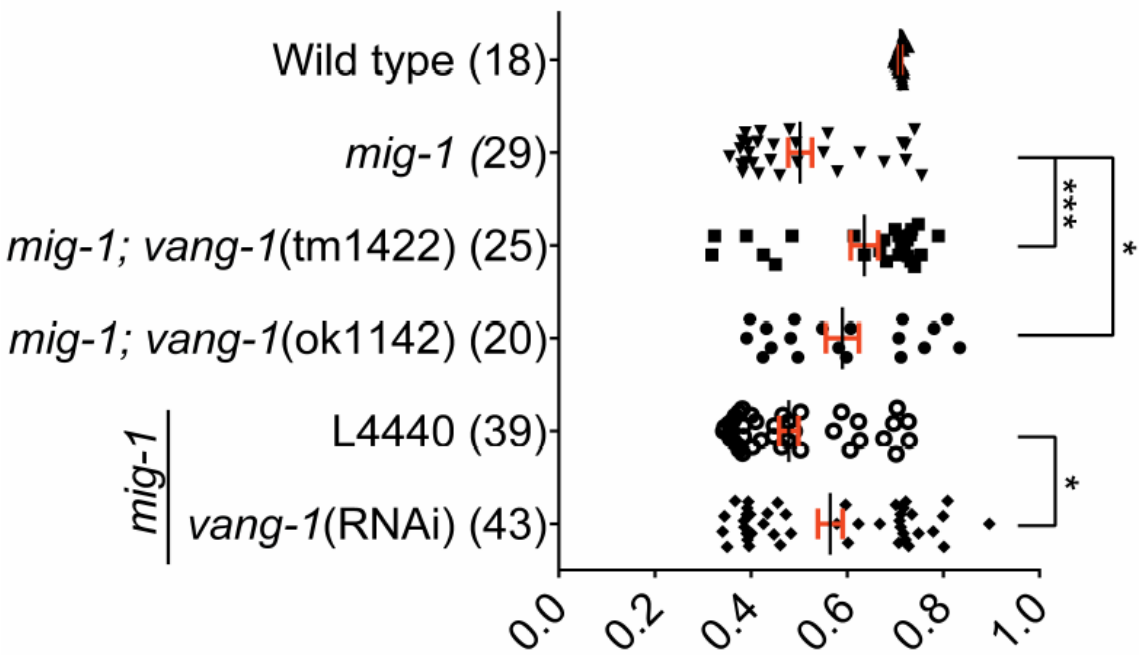


doi:10.6342/NTU201602869

Figure 4. Loss of *vang-1* function recues PVM migration defect in *mig-1* mutant

PVM positions in the wild type and indicated mutants. PVM positions are labeled by the transgene *zdIs5[Pmec-4::gfp]*. *, $p < 0.05$, ***, $p < 0.005$, unpaired *t* test. Numbers of neurons scored are indicated behind the genotypes. Error bars represent the S.E.M.

doi:10.6342/NTU201602869

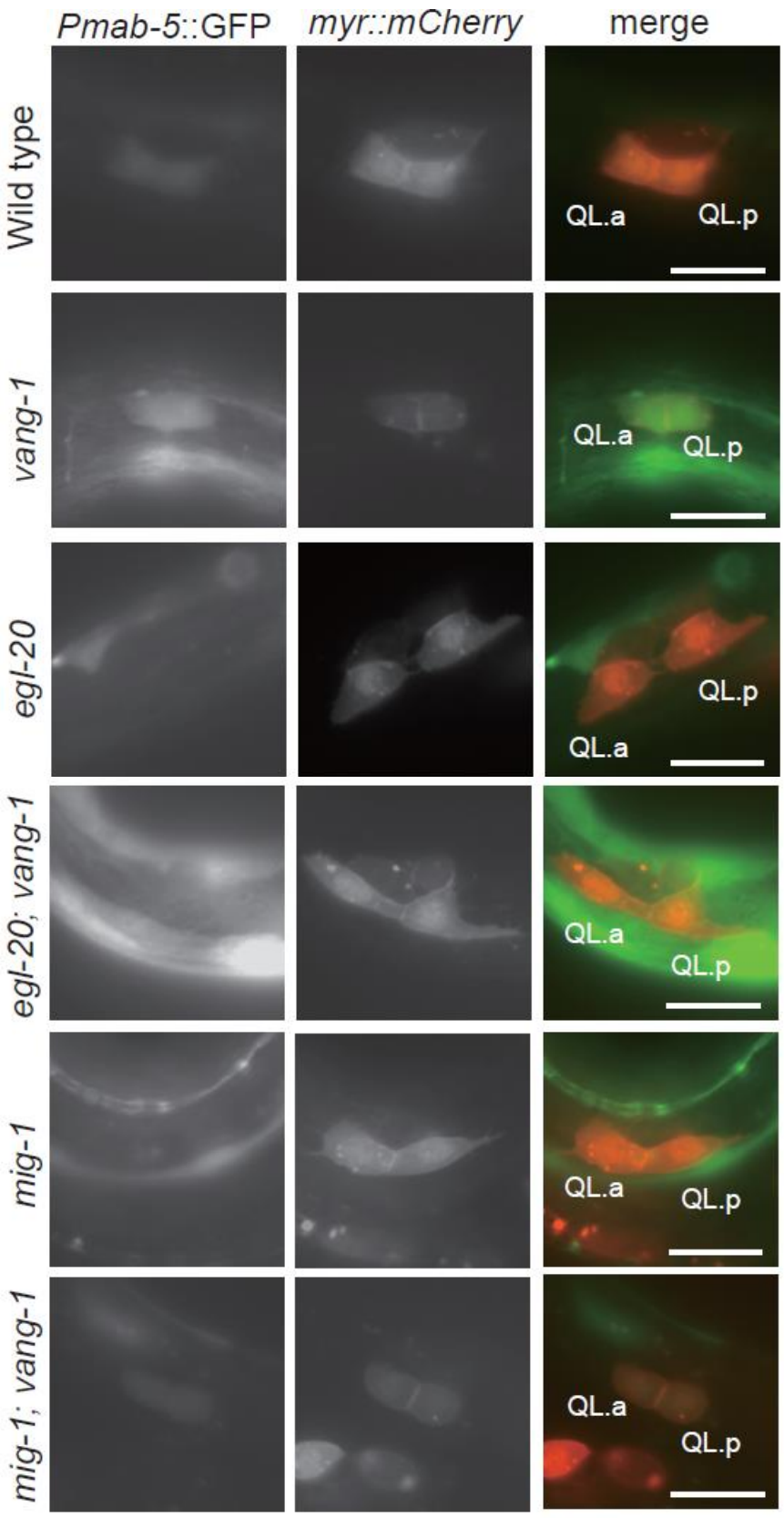


doi:10.6342/NTU201602869

Figure 5. The *vang-1* mutation restores *mab-5* expression in the *mig-1* mutant

Epifluorescence images of QL.a and QL.p in animals expressing *Pmab-5::GFP*. The Q.d cells are labeled by *myr::mCherry* in indicated genotypes. Scale bar = 10μm.

doi:10.6342/NTU201602869

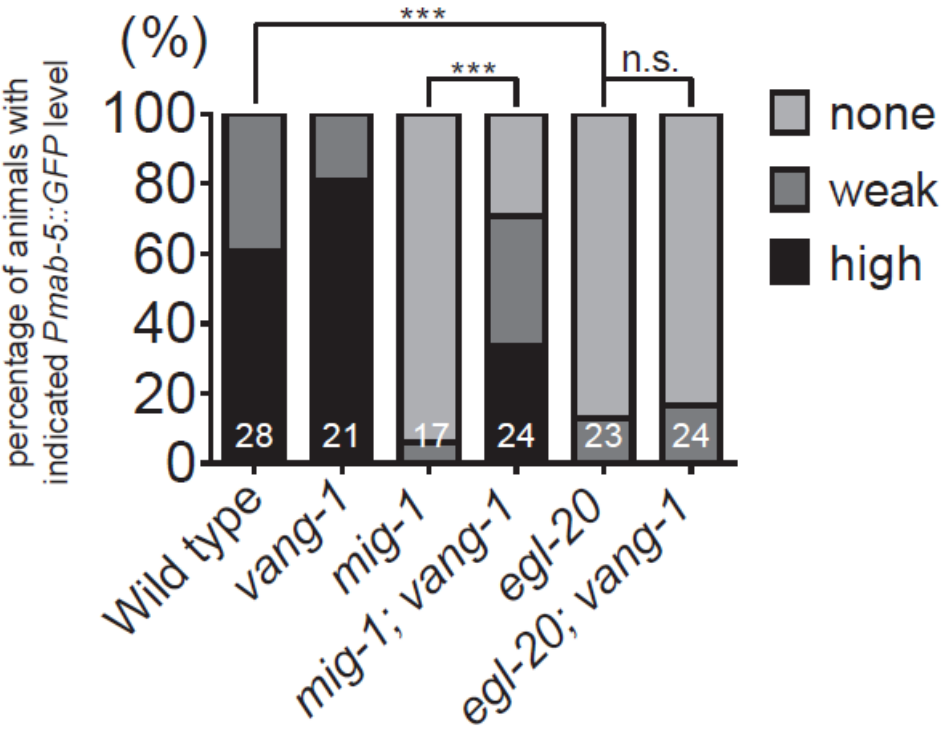


doi:10.6342/NTU201602869

Figure 6. Quantification of *mab-5* expression

Quantification of *mab-5* expression in indicated genotypes. n.s., not significant. ***, $p < 0.005$, Fisher's exact test. Numbers are neurons quantified for *Pmab-5::GFP* intensity.

doi:10.6342/NTU201602869

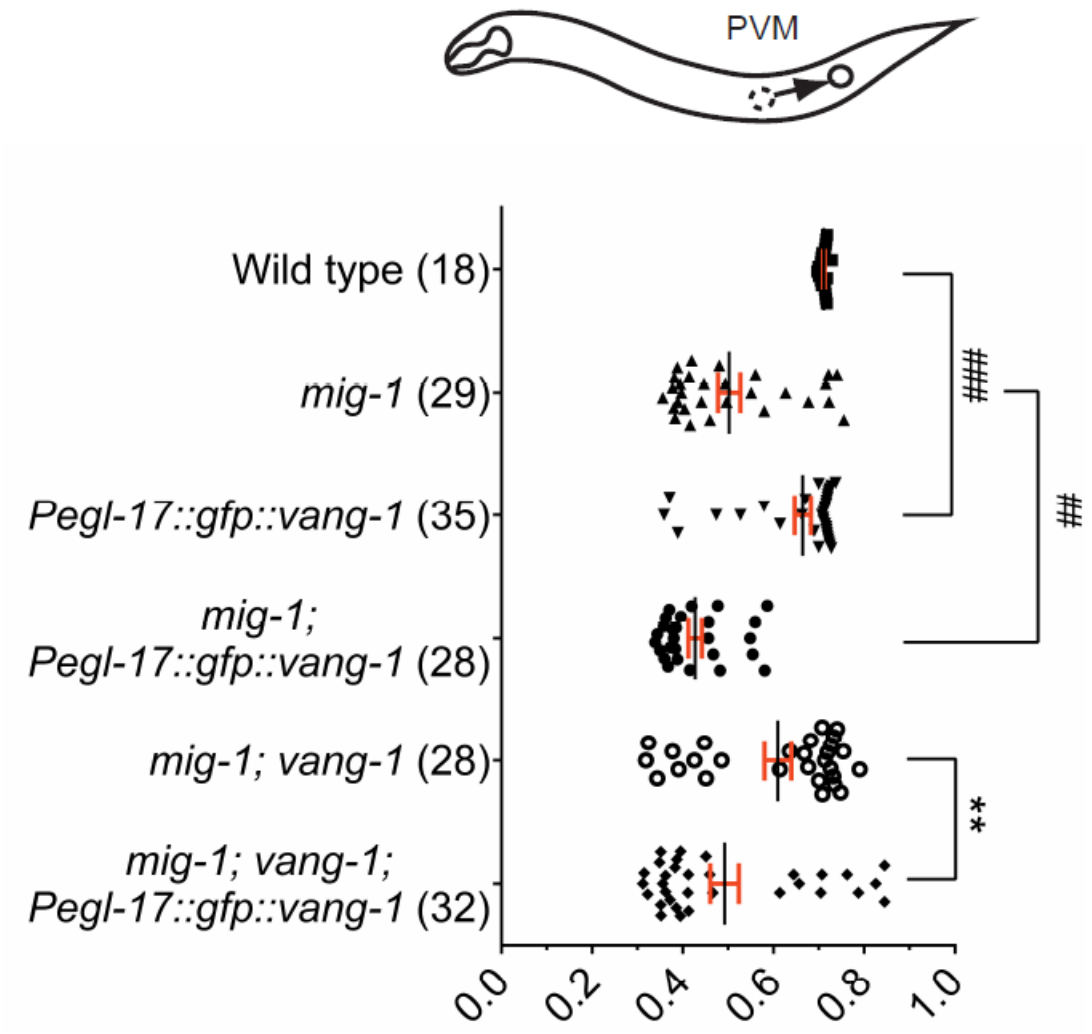


doi:10.6342/NTU201602869

Figure 7. *vang-1* functions autonomously to antagonize Wnt signaling in QL.d migration

Quantification of PVM positions in indicated genotypes. ##, $p < 0.01$; ###, $p < 0.005$, *F* test. **, $p < 0.01$, unpaired *t* test. Numbers of neurons scored are indicated behind the genotypes. Error bars represent the S.E.M.

doi:10.6342/NTU201602869

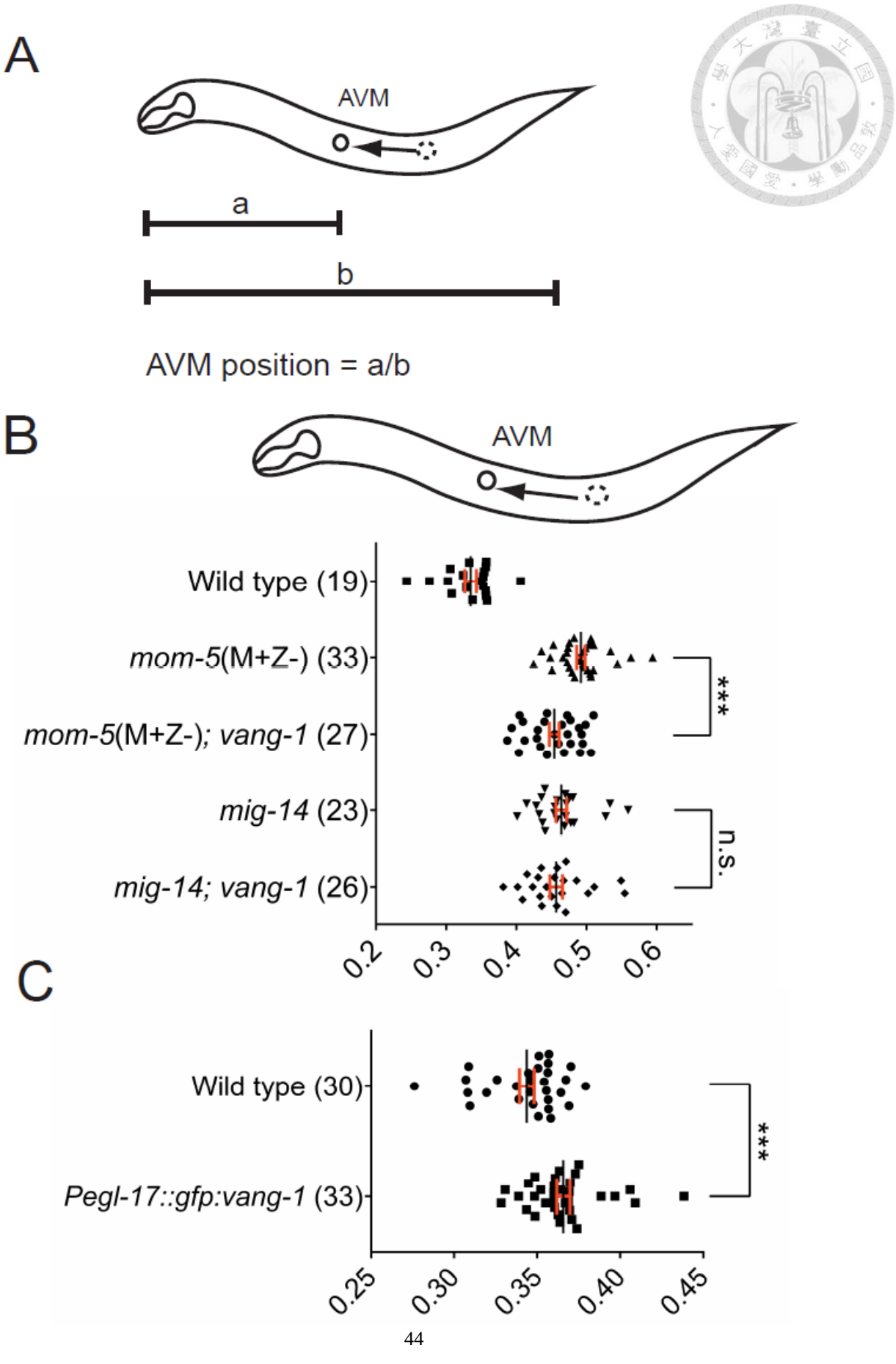


doi:10.6342/NTU201602869

Figure 8. *vang-1* antagonizes Wnt signaling in QR.d migration

(A) Diagram of the AVM migration process. The AVM positions are defined as the ratio of the anterior-to-AVM length (a) to the anterior-posterior body length of the animal (b). (B, C) Quantification of AVM positions in the wild type and Wnt pathway mutants. The AVM positions are labeled by the transgene *zdIs5[Pmec-4::gfp]*. n.s., not significant. ***, $p < 0.005$, unpaired *t* test. Numbers of neurons scored are indicated behind the genotypes. Error bars represent the S.E.M.

doi:10.6342/NTU201602869

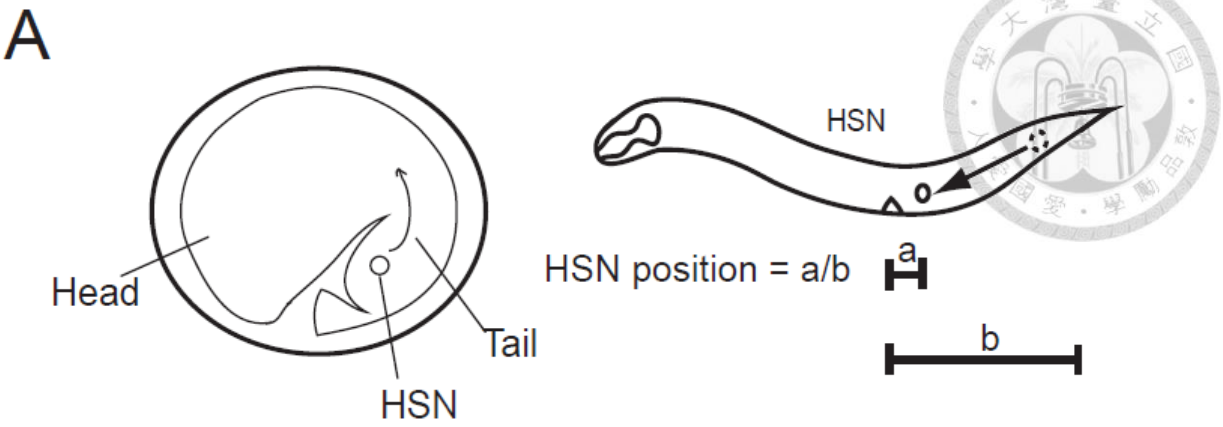


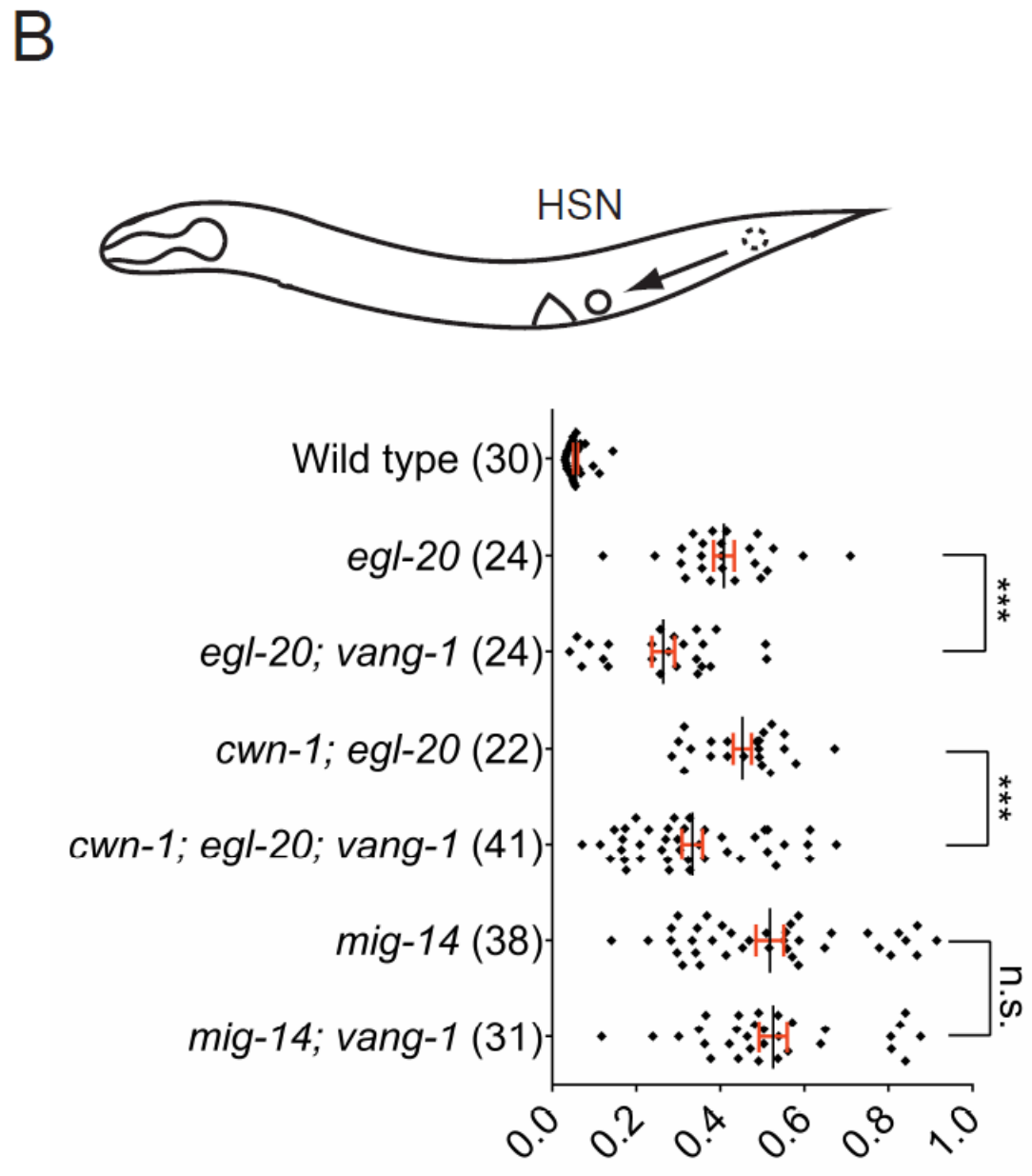
doi:10.6342/NTU201602869

Figure 9. *vang-1* antagonizes Wnt signaling in HSN migration

(A) Diagram of HSN migration during larval development. The HSN positions are defined as the ratio of the vulva-to-HSN length (a) to the vulva-tail body length of the animal (b). (B) Quantificatiion of HSN positions in the wild type and Wnt pathway mutants. The HSN positions are labeled by the transgene *mgIs42[Ptph-1::gfp]*. ***, $p < 0.005$, unpaired *t* test. Numbers of neurons scored are indicated behind the genotypes. Error bars represent the S.E.M.

doi:10.6342/NTU201602869



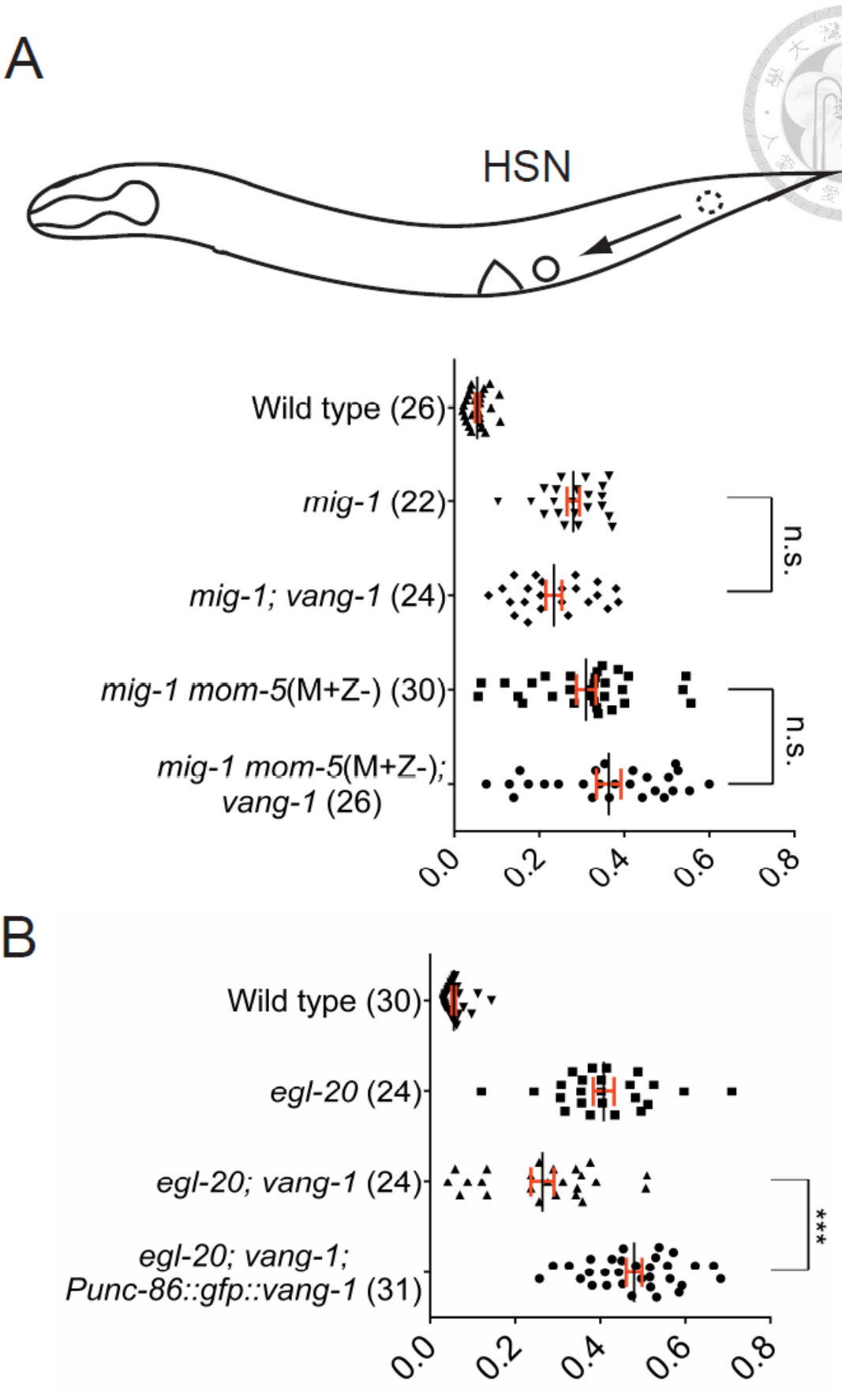


doi:10.6342/NTU201602869

Figure 10. *vang-1* antagonizes Wnt signaling autonomously in HSN migration

(A, B) Quantificatiion of HSN positions in the wild type and Wnt pathway mutants. The HSN positions are labeled by the transgene *mgIs42[Ptph-1::gfp]* in the wild type, *mig-1 and mig-1;vang-1*, and by *twnEx199[Punc-86::gfp::vang-1]* in the *mig-1 mom-5* and *mig-1 mom-5; van.g-1*. n.s., not significant. ***, $p < 0.005$, unpaired *t* test. Numbers of neurons scored are indicated behind the genotypes. Error bars represent the S.E.M

doi:10.6342/NTU201602869

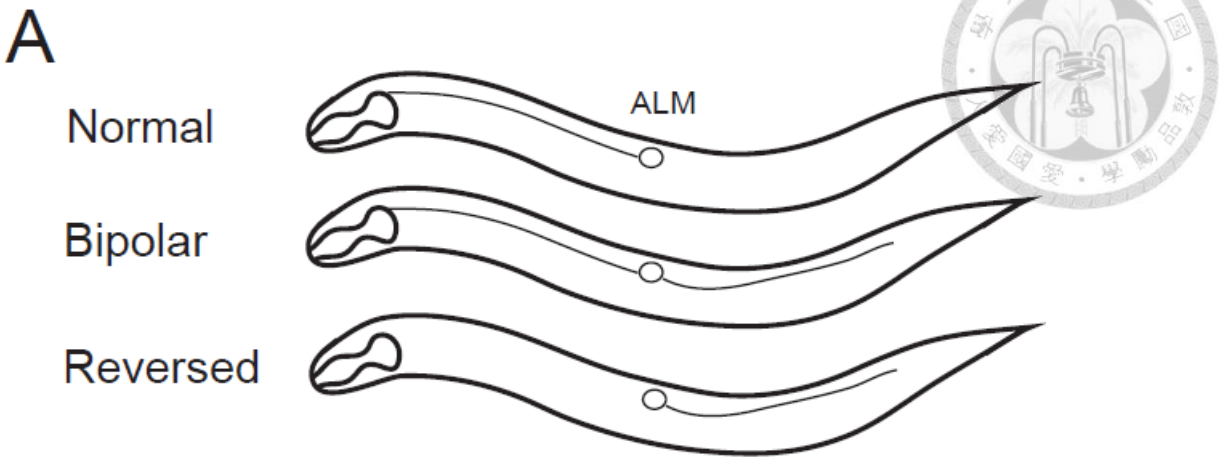


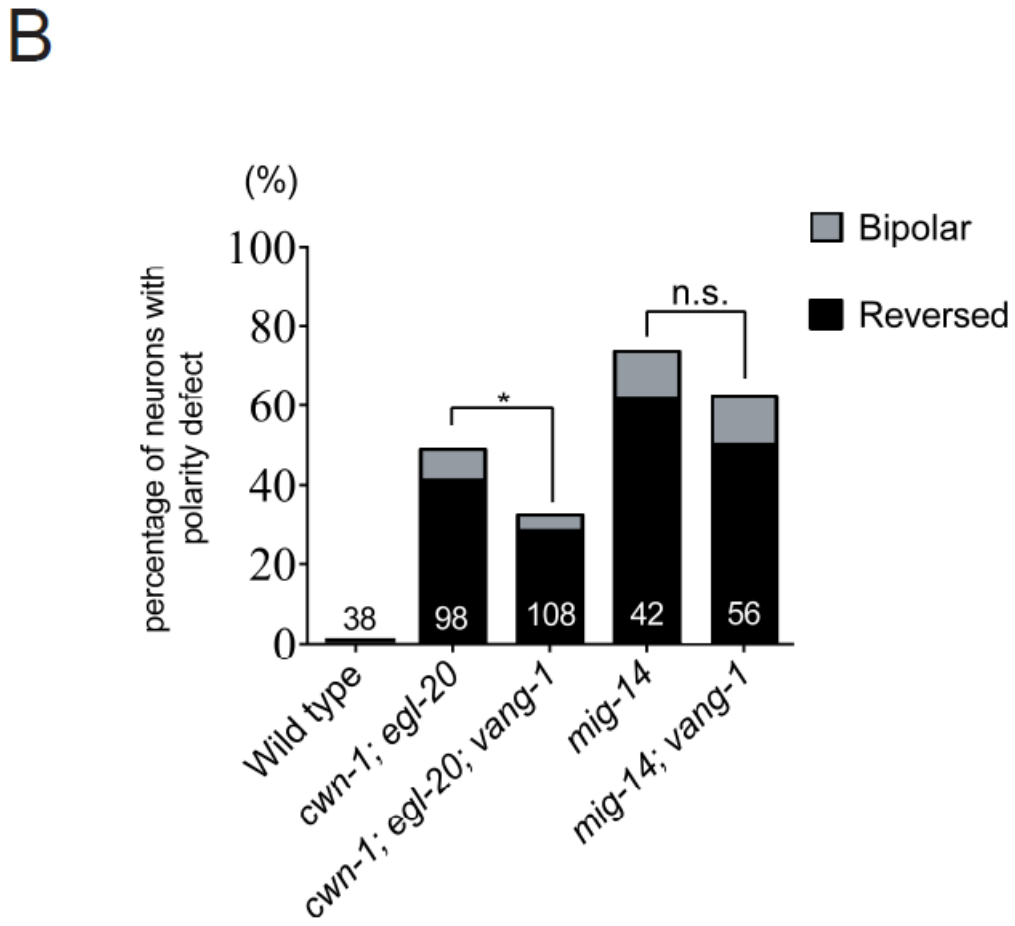
doi:10.6342/NTU201602869

Figure 11. *vang-1* antagonizes Wnt signaling in ALM polarity

(A) Diagram of the ALM polarity phenotypes. (B) Quantification of ALM polarity defects in indicated genotypes. The ALM neurons are marked by the transgene *zdIs5[Pmec-4::GFP]*. n.s., not significance. *, $p < 0.05$; two proportion z test. Numbers are neurons scored.

doi:10.6342/NTU201602869



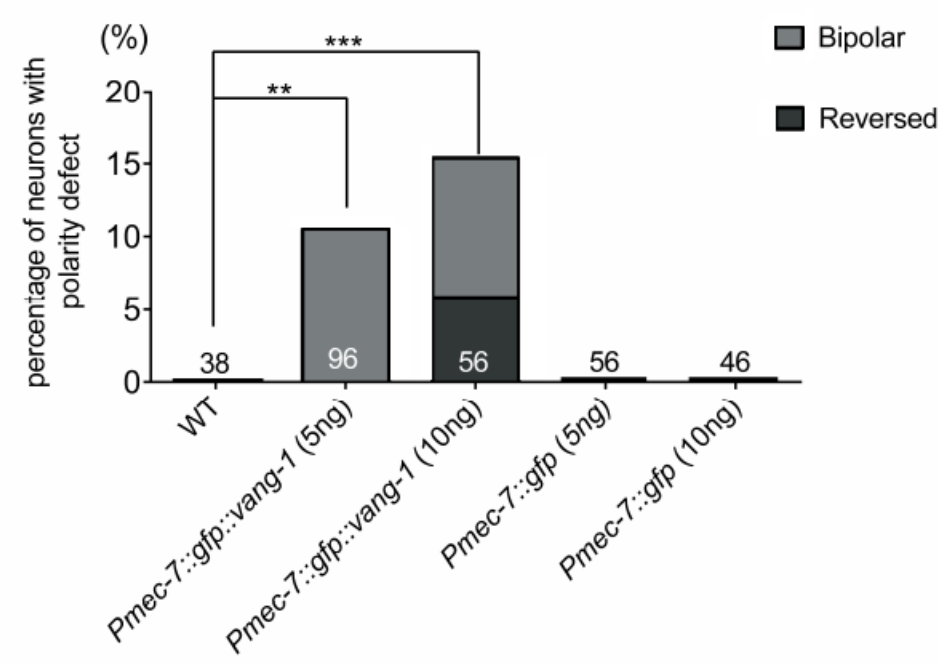


doi:10.6342/NTU201602869

Figure 12. Overexpression of *vang-1* causes polarity defects in the ALM

Quantification of ALM polarity defects in indicated genotypes. **, $p < 0.01$, ***, $p < 0.005$, two proportion z test. Numbers are neurons scored.

doi:10.6342/NTU201602869

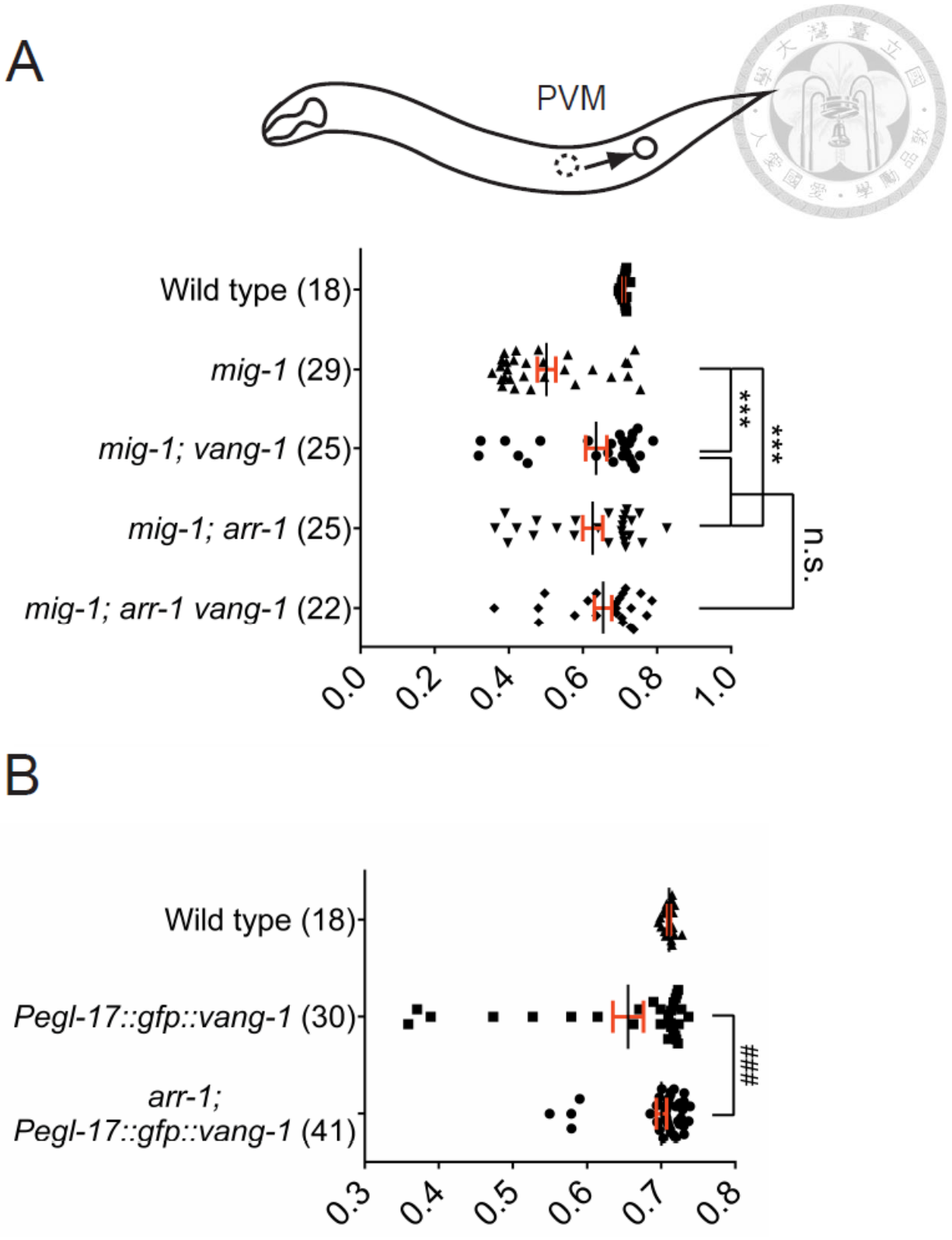


doi:10.6342/NTU201602869

Figure 13. *arr-1* acts downstream of *vang-1* to antagonize Wnt signaling in PVM migration

(A, B) Quantification of PVM positions in indicated genotypes. n.s., not significant. ***, $p < 0.005$; unpaired *t* test; ###, $p < 0.005$, *F* test. Numbers of neurons scored are indicated behind the genotypes. Error bars represent the S.E.M.

doi:10.6342/NTU201602869

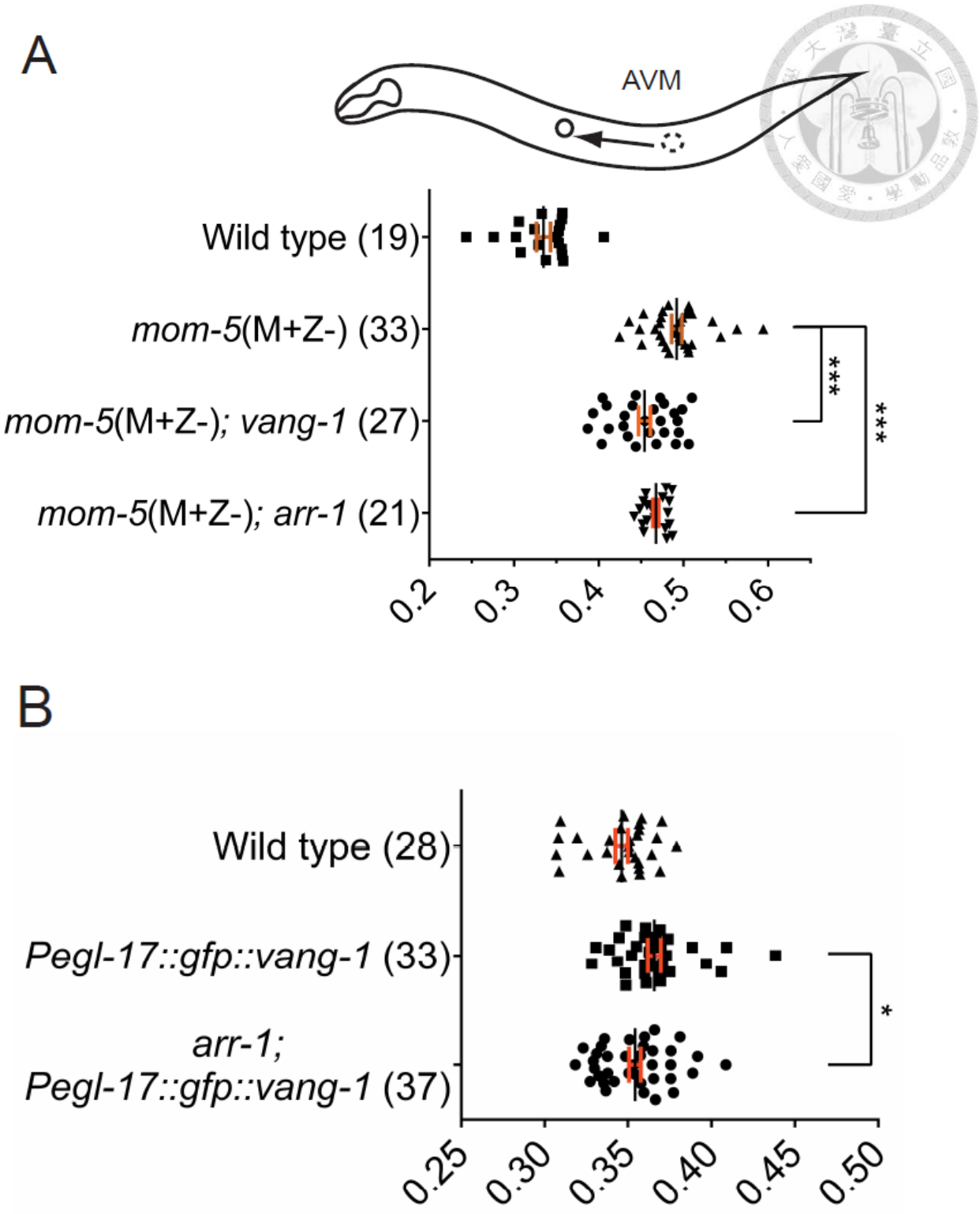


doi:10.6342/NTU201602869

Figure 14. *arr-1* acts downstream of *vang-1* to antagonize Wnt signaling in AVM migration

(A, B) Quantification of PVM positions in indicated genotypes. *, $p < 0.05$; ***, $p < 0.005$, unpaired *t* test. Numbers of neurons scored are indicated behind the genotypes. Error bars represent the S.E.M.

doi:10.6342/NTU201602869

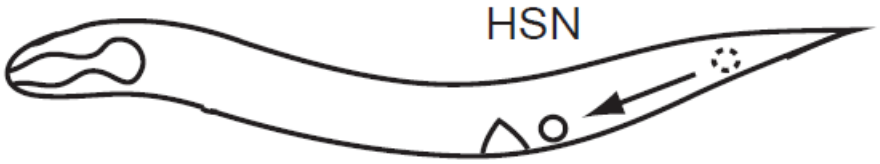


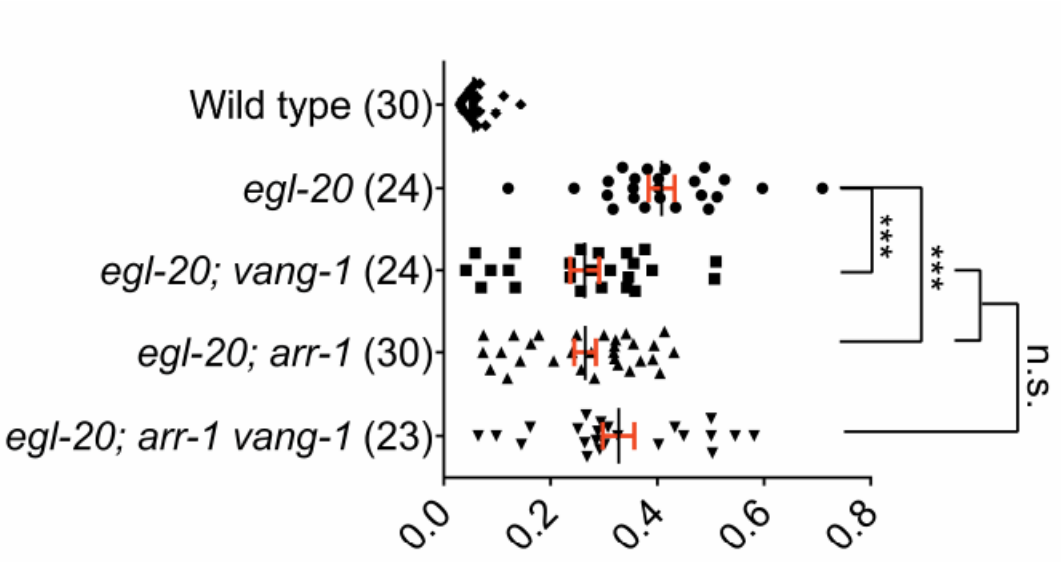
doi:10.6342/NTU201602869

Figure 15. *arr-1* acts in the same pathway with *vang-1* to antagonize Wnt signaling in HSN migration

Quantification of HSN positions, which are labeled by the transgene *mgIs42[Ptph-1::gfp]*. n.s., not significant. ***, $p < 0.005$, unpaired *t* test. Numbers are neurons scored. Error bars represent the S.E.M.

doi:10.6342/NTU201602869



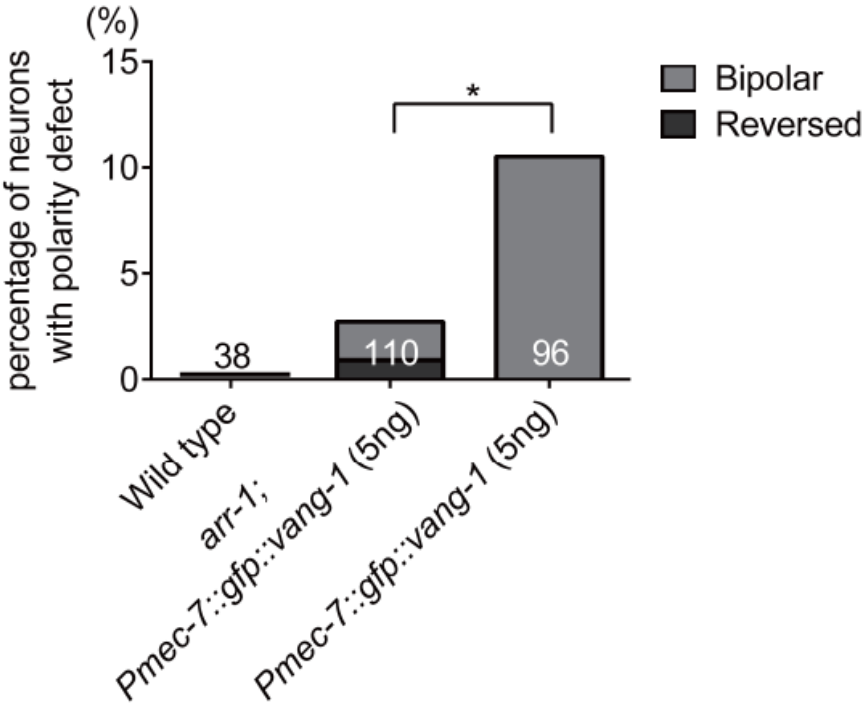


doi:10.6342/NTU201602869

Figure 16. *arr-1* acts downstream of *vang-1* to antagonize Wnt signaling in ALM polarity

Quantification of the ALM polarity defects in indicated genotypes. *, $p < 0.05$, two proportion z test. Numbers are neurons scored.

doi:10.6342/NTU201602869



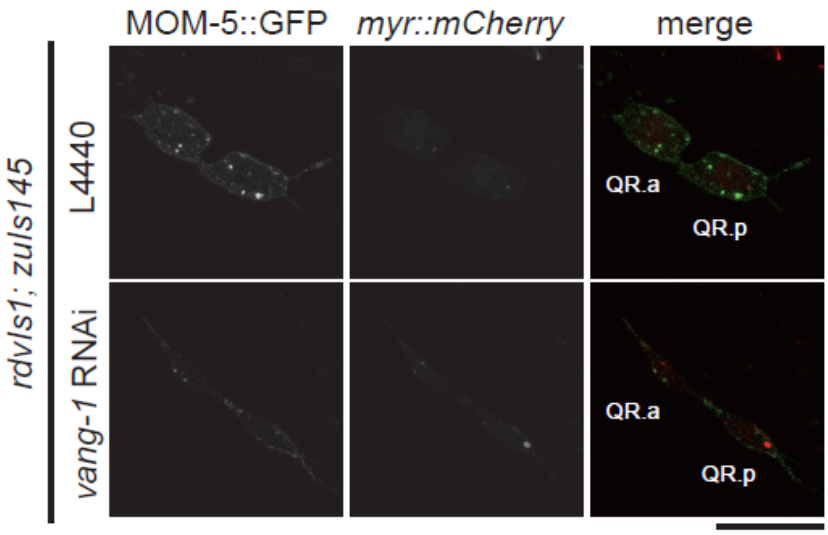
doi:10.6342/NTU201602869

Figure 17. *vang-1* promotes MOM-5 endocytosis.

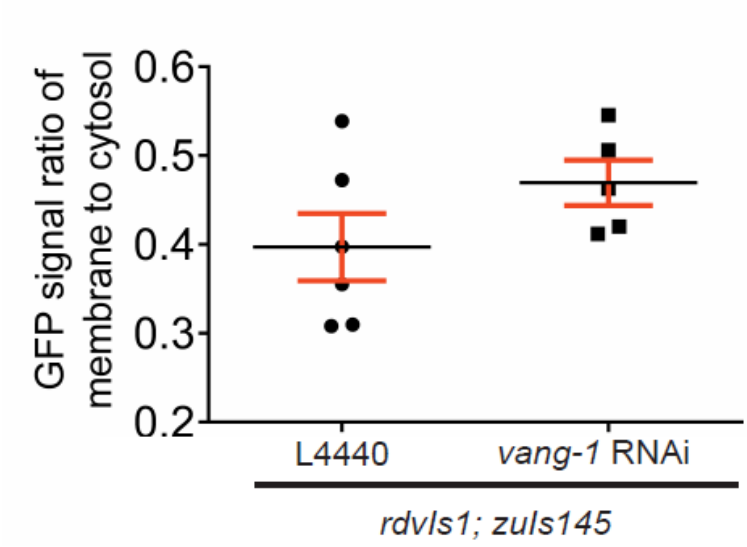
(A) Representative confocal images of animals expressing MOM-5::GFP fed with control or *vang-1* RNAi bacteria. The MOM-5 transgene is *zuIs145*. The Q.d cells are labeled by *rdvIs1*. (B) Quantification of membrane MOM-5::GFP signal as a ratio to MOM-5::GFP signal in the cytosol. Error bars represent the S.E.M.

doi:10.6342/NTU201602869

A



B

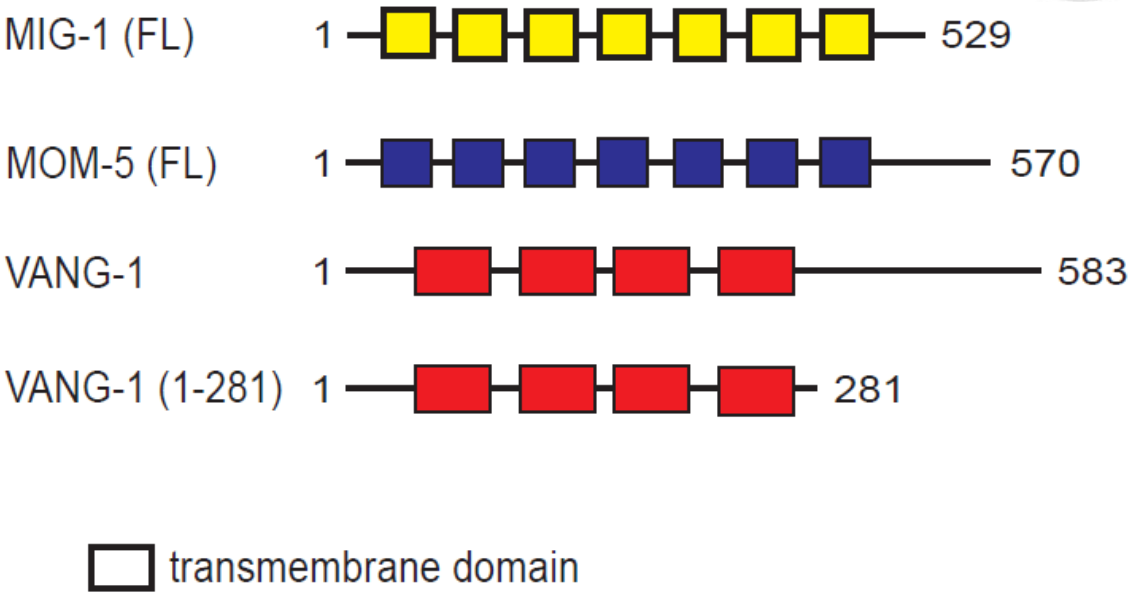


doi:10.6342/NTU201602869

Figure 18. Frizzleds and VANG-1 protein structures

Schematic diagrams of Frizzleds and VANG-1 protein structures. FL, full-length; TM, transmembrane domain.

doi:10.6342/NTU201602869

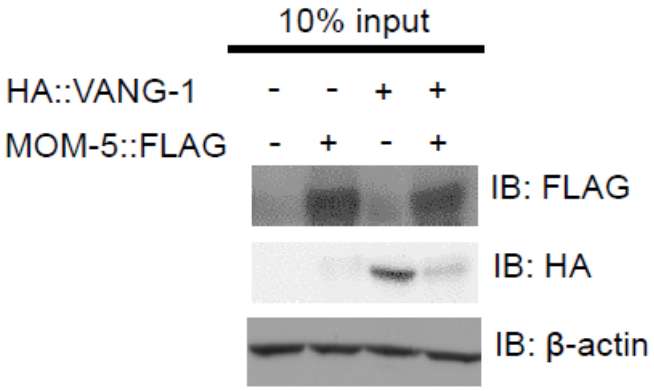


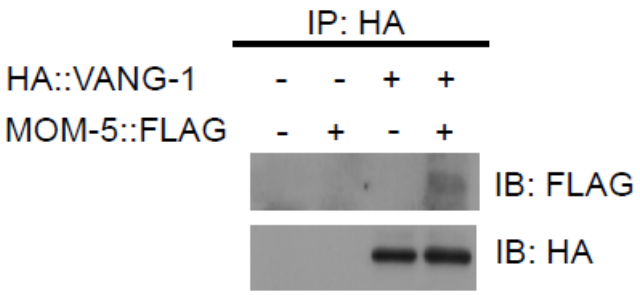
doi:10.6342/NTU201602869

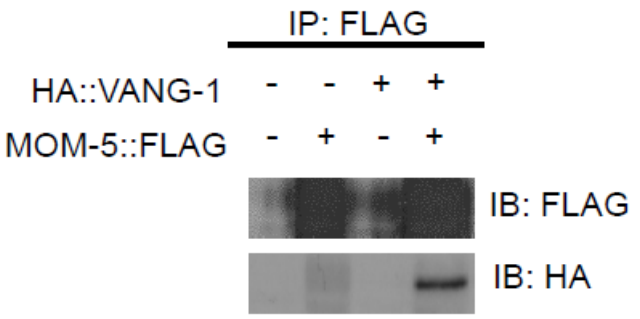
Figure 19. MOM-5 forms a complex with VANG-1

Co-immunoprecipitation of MOM-5::FLAG and HA::VANG-1 expressed in cultured HEK293 cells.

doi:10.6342/NTU201602869





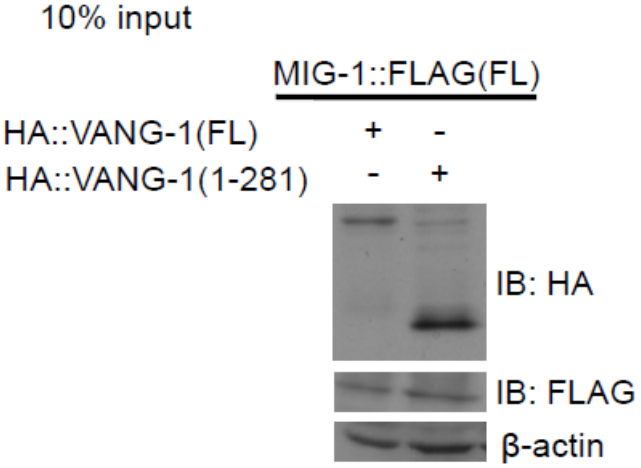


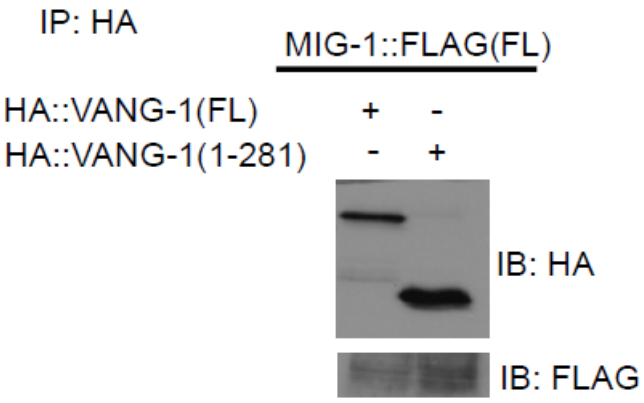
doi:10.6342/NTU201602869

Figure 20. MIG-1 formss complexes with VANG-1 (1-281)

Co-immunoprecipitation of MIG-1::FLAG and HA::VANG-1 (1-281) expressed in the HEK293 cells.

doi:10.6342/NTU201602869



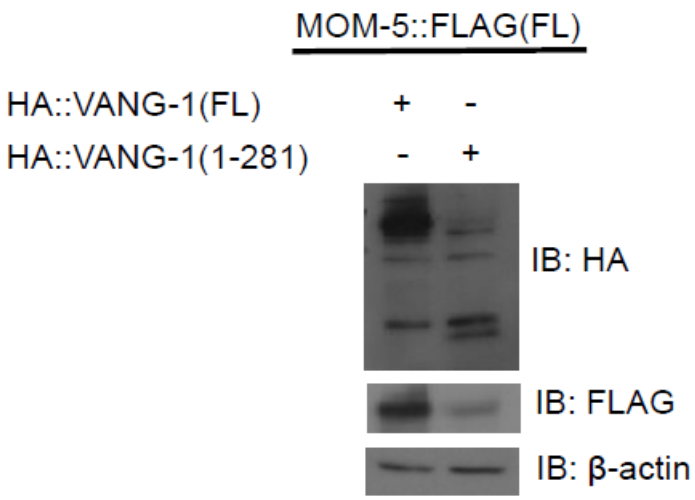


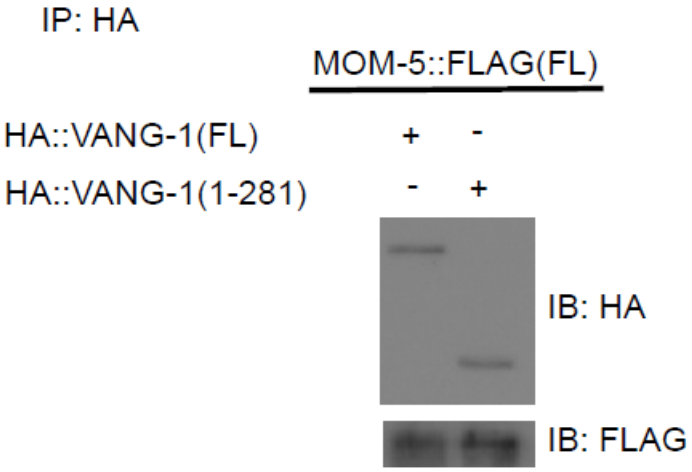
doi:10.6342/NTU201602869

Figure 21. MOM-5 forms complexes with VANG-1 (1-281)

Co-immunoprecipitation of MOM-5::FLAG and HA::VANG-1 (1-281) expressed in the HEK293 cells..

doi:10.6342/NTU201602869



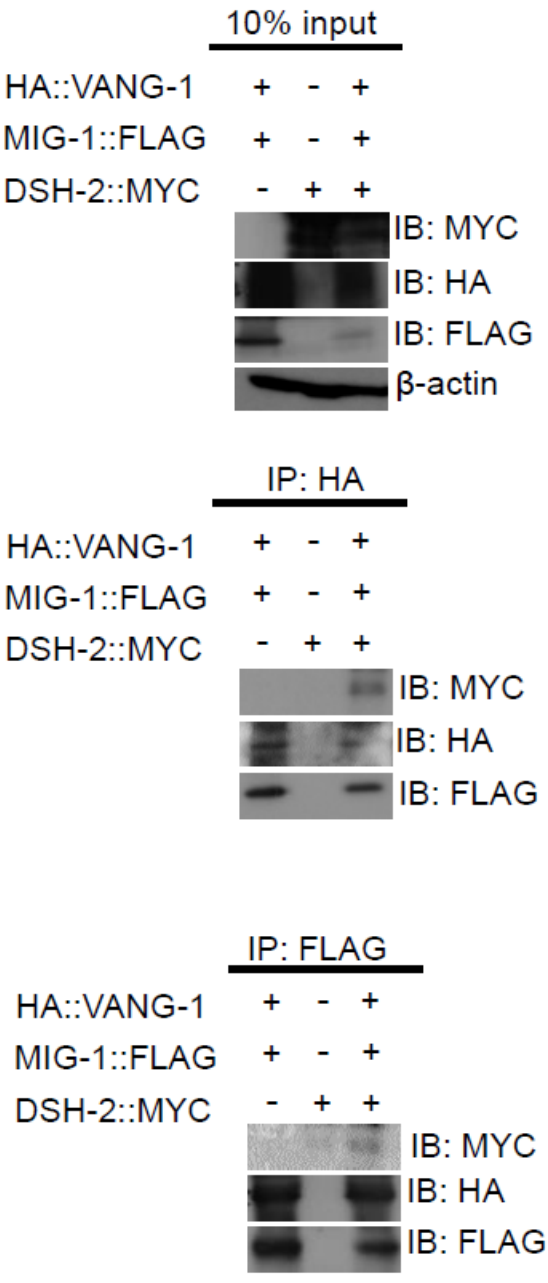


doi:10.6342/NTU201602869

Figure 22. MIG-1 forms a complex with VANG-1 and DSH-2

Co-immunoprecipitation of MIG-1::FLAG, HA::VANG-1 and DSH-2::MYC expressed in the HEK293 cells.

doi:10.6342/NTU201602869

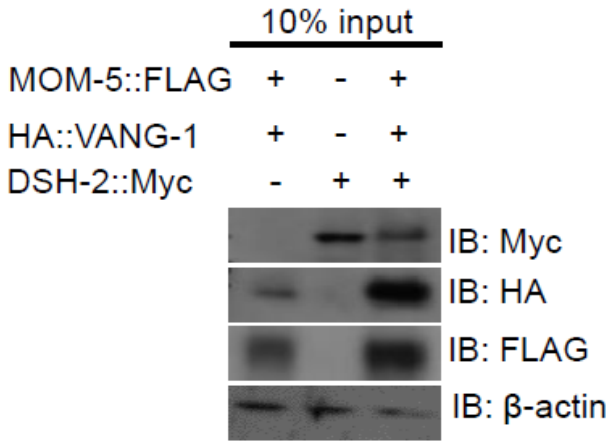


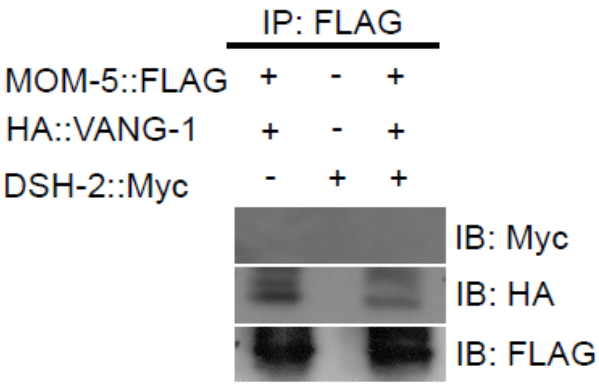
doi:10.6342/NTU201602869

Figure 23. DSH-2 is not part of the MOM-5-VANG-1 complex

Co-immunoprecipitation of MOM-5::FLAG, HA::VANG-1 and DSH-2::MYC expressed in the HEK293 cells.

doi:10.6342/NTU201602869

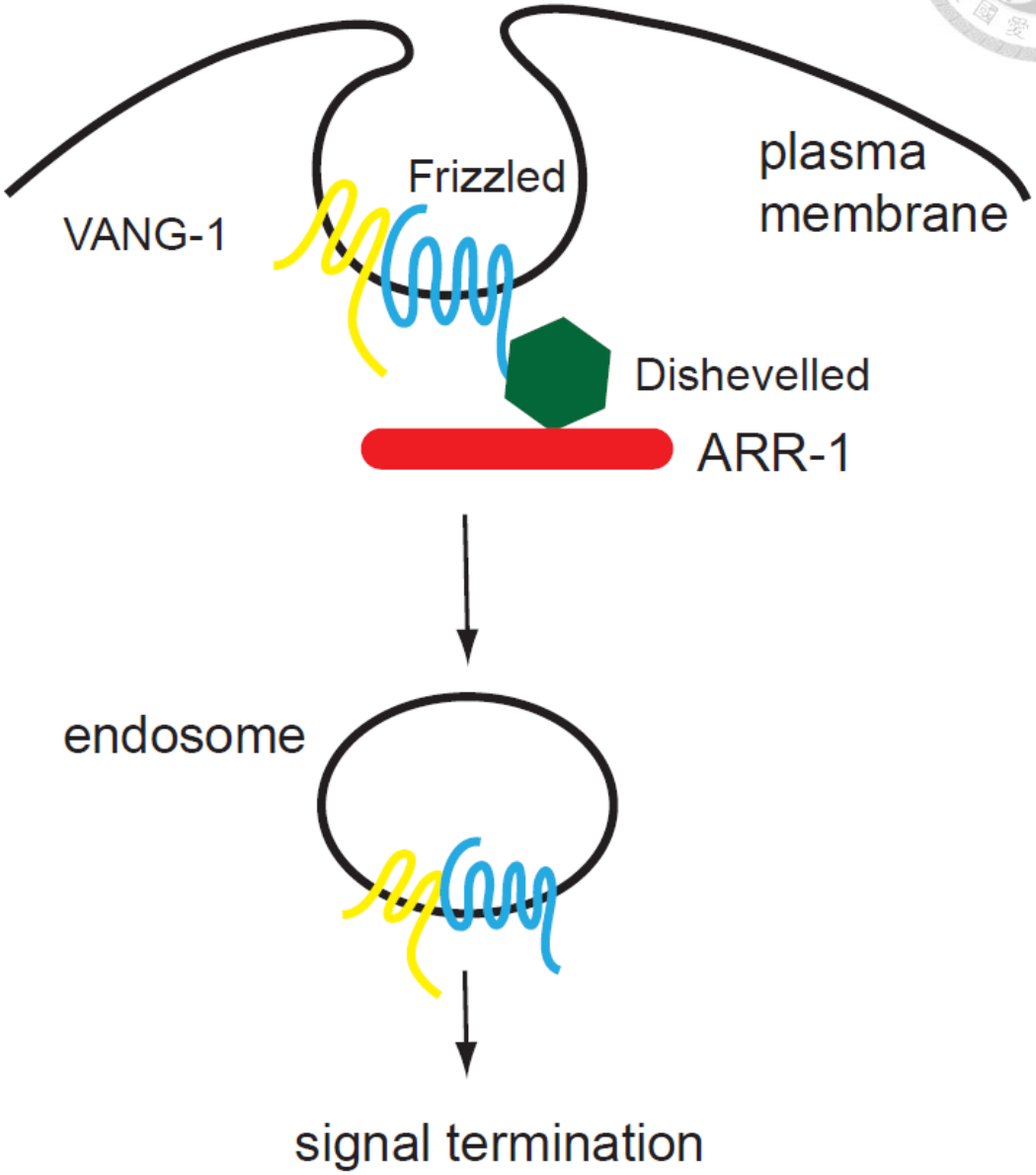




doi:10.6342/NTU201602869

Figure 24. Model of VANG-1 modulation of Wnt signaling.

doi:10.6342/NTU201602869



doi:10.6342/NTU201602869

Chapter 6 REFERENCE

Adler, P. N., R. E. Krasnow and J. Liu (1997). "Tissue polarity points from cells that have higher Frizzled levels towards cells that have lower Frizzled levels." Curr Biol **7**(12): 940-949.

Angers, S. and R. T. Moon (2009). "Proximal events in Wnt signal transduction." Nat Rev Mol Cell Biol **10**(7): 468-477.

Axelrod, J. D., J. R. Miller, J. M. Shulman, R. T. Moon and N. Perrimon (1998). "Differential recruitment of Dishevelled provides signaling specificity in the planar cell polarity and Wingless signaling pathways." Genes Dev **12**(16): 2610-2622.

Bagnard, D., M. Lohrum, D. Uziel, A. W. Puschel and J. Bolz (1998). "Semaphorins act as attractive and repulsive guidance signals during the development of cortical projections." Development **125**(24): 5043-5053.

Bastock, R., H. Strutt and D. Strutt (2003). "Strabismus is asymmetrically localised and binds to Prickle and Dishevelled during Drosophila planar polarity patterning." Development **130**(13): 3007-3014.

Brenner, S. (1974). "The genetics of Caenorhabditis elegans." Genetics **77**(1): 71-94.
Cadigan, K. M. and R. Nusse (1997). "Wnt signaling: a common theme in animal development." Genes Dev **11**(24): 3286-3305.

Chalfie, M., J. N. Thomson and J. E. Sulston (1983). "Induction of neuronal branching in Caenorhabditis elegans." Science **221**(4605): 61-63.

Chan, S. S., H. Zheng, M. W. Su, R. Wilk, M. T. Killeen, E. M. Hedgecock and J. G. Culotti (1996). "UNC-40, a C. elegans homolog of DCC (Deleted in Colorectal Cancer), is required in motile cells responding to UNC-6 netrin cues." Cell **87**(2): 187-195.

Chen, W., K. C. Kirkbride, T. How, C. D. Nelson, J. Mo, J. P. Frederick, X. F. Wang, R. J. Lefkowitz and G. C. Blobe (2003). "Beta-arrestin 2 mediates endocytosis of type III TGF-beta receptor and down-regulation of its signaling." Science **301**(5638): 1394-1397.

Chien, S. C., M. Gurling, C. Kim, T. Craft, W. Forrester and G. Garriga (2015). "Autonomous and nonautonomous regulation of Wnt-mediated neuronal polarity by the C. elegans Ror kinase CAM-1." Dev Biol **404**(1): 55-65.

Clark, S. G., A. D. Chisholm and H. R. Horvitz (1993). "Control of cell fates in the central body region of C. elegans by the homeobox gene lin-39." Cell **74**(1): 43-55.
Clevers, H. and R. Nusse (2012). "Wnt/beta-catenin signaling and disease." Cell **149**(6): 1192-1205.

Desai, C., G. Garriga, S. L. McIntire and H. R. Horvitz (1988). "A genetic pathway for

doi:10.6342/NTU201602869

the development of the Caenorhabditis elegans HSN motor neurons." Nature **336**(6200): 638-646.

Dickson, B. J. (2002). "Molecular mechanisms of axon guidance." Science **298**(5600): 1959-1964.

Dubois, L., M. Lecourtois, C. Alexandre, E. Hirst and J. P. Vincent (2001). "Regulated endocytic routing modulates wingless signaling in Drosophila embryos." Cell **105**(5): 613-624.

Eisenmann, D. M. (2005). "Wnt signaling." WormBook: 1-17.

Eisenmann, D. M. and S. K. Kim (2000). "Protruding vulva mutants identify novel loci and Wnt signaling factors that function during Caenorhabditis elegans vulva development." Genetics **156**(3): 1097-1116.

Forrester, W. C., C. Kim and G. Garriga (2004). "The Caenorhabditis elegans Ror RTK CAM-1 inhibits EGL-20/Wnt signaling in cell migration." Genetics **168**(4): 1951-1962.
Garriga, G., C. Desai and H. R. Horvitz (1993). "Cell interactions control the direction of outgrowth, branching and fasciculation of the HSN axons of Caenorhabditis elegans." Development **117**(3): 1071-1087.

Goldstein, J. L. and M. S. Brown (2009). "The LDL receptor." Arterioscler Thromb Vasc Biol **29**(4): 431-438.

Green, J. L., T. Inoue and P. W. Sternberg (2008). "Opposing Wnt pathways orient cell polarity during organogenesis." Cell **134**(4): 646-656.

Harris, J., L. Honigberg, N. Robinson and C. Kenyon (1996). "Neuronal cell migration in C. elegans: regulation of Hox gene expression and cell position." Development **122**(10): 3117-3131.

Hedgecock, E. M., J. G. Culotti and D. H. Hall (1990). "The unc-5, unc-6, and unc-40 genes guide circumferential migrations of pioneer axons and mesodermal cells on the epidermis in C. elegans." Neuron **4**(1): 61-85.

Herman, M. (2001). "C. elegans POP-1/TCF functions in a canonical Wnt pathway that controls cell migration and in a noncanonical Wnt pathway that controls cell polarity." Development **128**(4): 581-590.

Hilliard, M. A. and C. I. Bargmann (2006). "Wnt signals and frizzled activity orient anterior-posterior axon outgrowth in C. elegans." Dev Cell **10**(3): 379-390.

Inoue, T., H. S. Oz, D. Wiland, S. Gharib, R. Deshpande, R. J. Hill, W. S. Katz and P. W. Sternberg (2004). "C. elegans LIN-18 is a Ryk ortholog and functions in parallel to LIN-17/Frizzled in Wnt signaling." Cell **118**(6): 795-806.

doi:10.6342/NTU201602869

Janda, C. Y., D. Waghray, A. M. Levin, C. Thomas and K. C. Garcia (2012). "Structural basis of Wnt recognition by Frizzled." Science **337**(6090): 59-64.

Keleman, K., S. Rajagopalan, D. Cleppien, D. Teis, K. Paiha, L. A. Huber, G. M. Technau and B. J. Dickson (2002). "Comm sorts robo to control axon guidance at the Drosophila midline." Cell **110**(4): 415-427.

Keleman, K., C. Ribeiro and B. J. Dickson (2005). "Comm function in commissural axon guidance: cell-autonomous sorting of Robo in vivo." Nat Neurosci **8**(2): 156-163.

Kennedy, T. E., T. Serafini, J. R. de la Torre and M. Tessier-Lavigne (1994). "Netrins are diffusible chemotropic factors for commissural axons in the embryonic spinal cord." Cell **78**(3): 425-435.

Korswagen, H. C., M. A. Herman and H. C. Clevers (2000). "Distinct beta-catenins mediate adhesion and signalling functions in C. elegans." Nature **406**(6795): 527-532.

Lodowski, D. T., J. A. Pitcher, W. D. Capel, R. J. Lefkowitz and J. J. Tesmer (2003). "Keeping G proteins at bay: a complex between G protein-coupled receptor kinase 2 and Gbetagamma." Science **300**(5623): 1256-1262.
Lu, W., V. Yamamoto, B. Ortega and D. Baltimore (2004). "Mammalian Ryk is a Wnt coreceptor required for stimulation of neurite outgrowth." Cell **119**(1): 97-108.

Lyuksyutova, A. I., C. C. Lu, N. Milanesio, L. A. King, N. Guo, Y. Wang, J. Nathans, M. Tessier-Lavigne and Y. Zou (2003). "Anterior-posterior guidance of commissural axons by Wnt-frizzled signaling." Science **302**(5652): 1984-1988.

MacDonald, B. T., K. Tamai and X. He (2009). "Wnt/beta-catenin signaling: components, mechanisms, and diseases." Dev Cell **17**(1): 9-26.

Maloof, J. N., J. Whangbo, J. M. Harris, G. D. Jongeward and C. Kenyon (1999). "A Wnt signaling pathway controls hox gene expression and neuroblast migration in C. elegans." Development **126**(1): 37-49.

Marin, O., M. Valiente, X. Ge and L. H. Tsai (2010). "Guiding neuronal cell migrations." Cold Spring Harb Perspect Biol **2**(2): a001834.

Maung, S. M. and A. Jenny (2011). "Planar cell polarity in Drosophila." Organogenesis **7**(3): 165-179.

Mitchell, J. H. and R. G. Victor (1996). "Neural control of the cardiovascular system: insights from muscle sympathetic nerve recordings in humans." Med Sci Sports Exerc **28**(10 Suppl): S60-69.

Miyashita, T., S. Y. Yeo, Y. Hirate, H. Segawa, H. Wada, M. H. Little, T. Yamada, N. Takahashi and H. Okamoto (2004). "PlexinA4 is necessary as a downstream target of

doi:10.6342/NTU201602869

Islet2 to mediate Slit signaling for promotion of sensory axon branching." Development **131**(15): 3705-3715.

Mello, C. C., Kramer, J.M., Stinchcomb, D. and Ambros, V. (1991). Efficient gene transfer in C. elegans: extrachromosomal maintenance and integration of transforming sequences. EMBO J (10): 3959-3970.

Moffat, L. L., R. E. Robinson, A. Bakoulis and S. G. Clark (2014). "The conserved transmembrane RING finger protein PLR-1 downregulates Wnt signaling by reducing Frizzled, Ror and Ryk cell-surface levels in C. elegans." Development **141**(3): 617-628.

Norris, A. D. and E. A. Lundquist (2011). "UNC-6/netrin and its receptors UNC-5 and UNC-40/DCC modulate growth cone protrusion in vivo in C. elegans." Development **138**(20): 4433-4442.

Nusse, R. (2005). "Wnt signaling in disease and in development." Cell Res **15**(1): 28-32.

Nusslein-Volhard, C. and E. Wieschaus (1980). "Mutations affecting segment number and polarity in Drosophila." Nature **287**(5785): 795-801.

Onishi, K., B. Shafer, C. Lo, F. Tissir, A. M. Goffinet and Y. Zou (2013). "Antagonistic functions of Dishevelleds regulate Frizzled3 endocytosis via filopodia tips in Wnt-mediated growth cone guidance." J Neurosci **33**(49): 19071-19085.

Ozdinler, P. H. and R. S. Erzurumlu (2002). "Slit2, a branching-arborization factor for sensory axons in the Mammalian CNS." J Neurosci **22**(11): 4540-4549.

Pan, C. L., P. D. Baum, M. Gu, E. M. Jorgensen, S. G. Clark and G. Garriga (2008). "C. elegans AP-2 and retromer control Wnt signaling by regulating mig-14/Wntless." Dev Cell **14**(1): 132-139.

Pan, C. L., J. E. Howell, S. G. Clark, M. Hilliard, S. Cordes, C. I. Bargmann and G. Garriga (2006). "Multiple Wnts and frizzled receptors regulate anteriorly directed cell and growth cone migrations in Caenorhabditis elegans." Dev Cell **10**(3): 367-377.

Prasad, B. C. and S. G. Clark (2006). "Wnt signaling establishes anteroposterior neuronal polarity and requires retromer in C. elegans." Development **133**(9): 1757-1766.

Rives, A. F., K. M. Rochlin, M. Wehrli, S. L. Schwartz and S. DiNardo (2006). "Endocytic trafficking of Wingless and its receptors, Arrow and DFrizzled-2, in the Drosophila wing." Dev Biol **293**(1): 268-283.

Sakurai, T., E. Wong, U. Drescher, H. Tanaka and D. G. Jay (2002). "Ephrin-A5 restricts topographically specific arborization in the chick retinotectal projection in vivo." Proc Natl Acad Sci U S A **99**(16): 10795-10800.

doi:10.6342/NTU201602869

Salser, S. J. and C. Kenyon (1992). "Activation of a C. elegans Antennapedia homologue in migrating cells controls their direction of migration." Nature **355**(6357): 255-258.

Serafini, T., T. E. Kennedy, M. J. Galko, C. Mirzayan, T. M. Jessell and M. Tessier-Lavigne (1994). "The netrins define a family of axon outgrowth-promoting proteins homologous to C. elegans UNC-6." Cell **78**(3): 409-424.

Shafer, B., K. Onishi, C. Lo, G. Colakoglu and Y. Zou (2011). "Vangl2 promotes Wnt/planar cell polarity-like signaling by antagonizing Dvl1-mediated feedback inhibition in growth cone guidance." Dev Cell **20**(2): 177-191.

Sulston, J. E. and H. R. Horvitz (1977). "Post-embryonic cell lineages of the nematode, Caenorhabditis elegans." Dev Biol **56**(1): 110-156.

Tessier-Lavigne, M. and C. S. Goodman (1996). "The molecular biology of axon guidance." Science **274**(5290): 1123-1133.

van Ooyen, A. and R. Nusse (1984). "Structure and nucleotide sequence of the putative mammary oncogene int-1; proviral insertions leave the protein-encoding domain intact." Cell **39**(1): 233-240.

Vladar, E. K., D. Antic and J. D. Axelrod (2009). "Planar cell polarity signaling: the developing cell's compass." Cold Spring Harb Perspect Biol **1**(3): a002964.

Wang, B. B., M. M. Muller-Immergluck, J. Austin, N. T. Robinson, A. Chisholm and C. Kenyon (1993). "A homeotic gene cluster patterns the anteroposterior body axis of C. elegans." Cell **74**(1): 29-42.

Wang, X., F. Zhou, S. Lv, P. Yi, Z. Zhu, Y. Yang, G. Feng, W. Li and G. Ou (2013). "Transmembrane protein MIG-13 links the Wnt signaling and Hox genes to the cell polarity in neuronal migration." Proc Natl Acad Sci U S A **110**(27): 11175-11180.

Wang, Y. and J. Nathans (2007). "Tissue/planar cell polarity in vertebrates: new insights and new questions." Development **134**(4): 647-658.

Whangbo, J. and C. Kenyon (1999). "A Wnt signaling system that specifies two patterns of cell migration in C. elegans." Mol Cell **4**(5): 851-858.

Wong, H. C., A. Bourdelas, A. Krauss, H. J. Lee, Y. Shao, D. Wu, M. Mlodzik, D. L. Shi and J. Zheng (2003). "Direct binding of the PDZ domain of Dishevelled to a conserved internal sequence in the C-terminal region of Frizzled." Mol Cell **12**(5): 1251-1260.

Wu, J. and M. Mlodzik (2008). "The frizzled extracellular domain is a ligand for Van Gogh/Stbm during nonautonomous planar cell polarity signaling." Dev Cell **15**(3): 462-469.

doi:10.6342/NTU201602869

Yoshikawa, S., R. D. McKinnon, M. Kokel and J. B. Thomas (2003). "Wnt-mediated axon guidance via the Drosophila Derailed receptor." Nature **422**(6932): 583-588.

Yu, A., J. F. Rual, K. Tamai, Y. Harada, M. Vidal, X. He and T. Kirchhausen (2007). "Association of Dishevelled with the clathrin AP-2 adaptor is required for Frizzled endocytosis and planar cell polarity signaling." Dev Cell **12**(1): 129-141.

Zecca, M., K. Basler and G. Struhl (1996). "Direct and long-range action of a wingless morphogen gradient." Cell **87**(5): 833-844.

Zheng, C., M. Diaz-Cuadros and M. Chalfie (2015). "Dishevelled attenuates the repelling activity of Wnt signaling during neurite outgrowth in Caenorhabditis elegans." Proc Natl Acad Sci U S A **112**(43): 13243-13248.

Zinovyeva, A. Y., Y. Yamamoto, H. Sawa and W. C. Forrester (2008). "Complex network of Wnt signaling regulates neuronal migrations during Caenorhabditis elegans development." Genetics **179**(3): 1357-1371.

Zou, Y. (2004). "Wnt signaling in axon guidance." Trends Neurosci **27**(9): 528-532.

doi:10.6342/NTU201602869