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Dscam2訊號機制對限制視覺神經樹突場尺寸之研究

Signaling Mechanisms of the Down Syndrome Cell  
Adhesion Receptor Dscam2 in Restricting Dendritic Field  
Expansion of Visual Neurons

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## Dedication and acknowledgement



I dedicate my thesis to my mother- Mrs. Esther Oyewale (of blessed memory) who passed on to glory on February 1, 2022. Her commitment to my career is beyond mere description. Through her resolute determination to ensure I attain the peak of my chosen career path, I have come this far. Her labor and love speak volumes and resonate in my heart till eternity.

I attribute all successes in my life to the gracious help I received from God Almighty. I also deeply appreciate the invaluable contributions and support from many people around me. I recognize the advisory role of my advisor- Dr. Chi-Hon Lee for the privilege of undertaking my PhD studies in his lab. Through his tutelage and constructive critique of my progress, I was able to develop abilities for critical thinking and explore several aspects of molecular and cellular neuroscience. In addition to this was the support I received from the members of Lee's lab. To mention a few: Dr. Tzu-Yang Lin, for his generous intellectual contributions to my project, generation of TrioGEF point mutations and also for sharing the Dscam2 $\Delta$ ICD line; Yenny Zhang, who is now an alumnus of the lab, for sharing her data on Src RNAi experiments and genetic interaction of Dscam2 and Src; Dr. Pei-Ju Chen for writing the python code for the dendritic field size quantification.

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Thank you all.

## 摘要

過去研究認為 Down syndrome cell adhesion molecule 2 (Dscam2) 通過介導神經突之間的同型交互作用，來指引神經迴路的組裝。然而，此訊號傳遞機制尚不明確。在本篇論文中，我們使用果蠅視覺神經作為模型，證明 Dscam2 對於髓質投射神經元 Tm20 的樹突場尺寸擴展與正確的平面投射方向不可或缺。這些 Dscam2 的細胞自主功能具異構特性：Dscam2 10B 異構體可限制樹突場尺寸，而 10A 和 10B 皆會調控平面投射的方向。除了 Dscam2 細胞膜外域的調控機制，Dscam2 細胞質域的訊號傳遞對於 Tm20 形成正確樹突型態也是必要的。接著我們描繪 Dscam2 在細胞內的訊號途徑，並發現非受體酪胺酸激酶 Src2，和 Dscam2 有遺傳交互作用，參與指示應有的樹突型態。通過篩選候選基因，我們認為 Rac1 是 Src 在 Dscam2 路徑的下游的潛在標的，可被核苷酸轉換因子 Trio 調控，推測它會調節樹突發育過程中的肌動蛋白骨架。除此之外，我們發現與先前認知不同，PI3K 並非 Dscam2 下游限制樹突發展的因子。我們的研究為更深入了解 Dscam2 訊號路徑建立基礎。

關鍵字: Cell adhesion molecule, 樹突發育, 神經迴路的組裝, 訊號傳遞機制, 果蠅視覺神經

## Abstract

It has been proposed that the Down syndrome cell adhesion molecule 2 (Dscam2) mediates homotypic interactions between neurites to guide neural circuit assembly. However, its signaling mechanism is unknown. In this thesis, using the *Drosophila* visual neurons as a model, we demonstrate that Dscam2 is required in the medulla projection neurons, Tm20, for proper expansion of dendritic field sizes and correct planar projection. These cell-autonomous functions of Dscam2 are isoform-specific: Dscam2 10B isoform restricts dendritic field sizes while both 10A and 10B isoforms regulate the direction of dendritic planar projection. Aside the mechanism utilized by the ectodomains, Dscam2's cytoplasmic domain is essential to elicit signal transduction for proper dendritic patterning of Tm20 neurons. We then, delineated Dscam2's intracellular signaling pathway and discovered the involvement of the non-receptor tyrosine kinase, Src42 which genetically interacts with Dscam2 to instruct appropriate dendritic arbor of Tm20 neurons. By screening candidates, we identified Rac1 as the potential target of Src in Dscam2 pathway, which itself is regulated by its nucleotide exchanger Trio, presumably, to modulate actin cytoskeleton during dendrite development. In addition, we determined that unlike previous suggestion, PI3K, does not act downstream of Dscam2 to restrict dendritic elaboration. Our findings pave the path to elucidating the signaling pathway of Dscam2.

Keywords: Cell adhesion molecule, dendrite development, neural circuit assembly, signaling mechanism, *Drosophila* visual neurons

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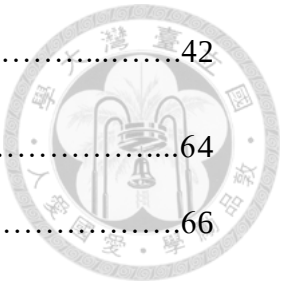
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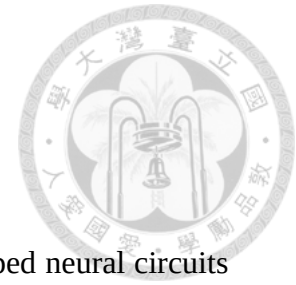
## CHAPTER ONE

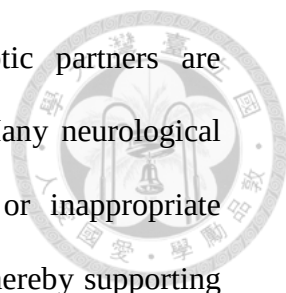
### INTRODUCTION

#### 1.1 CNS development and neural circuit assembly

The central nervous system (CNS) consists of complex and stereotyped neural circuits that develop through series of morphological and functional changes under the control of diverse classes of molecules (Pan & Monje, 2020; Peng et al., 2009). These molecules include but not limited to transcription factors, cell surface proteins, kinases and actin regulatory proteins that work together to guide neural circuit assembly (Shree et al., 2022; Xie et al., 2022). This molecular collaboration instructs the precise coordination of the development of the brain, whose functions ultimately impacts animal behaviors (Richter & Gjorgjieva, 2017). There is a shared similarity in the construction and composition of the brains among different species; the adult human brain consists of above 80 billion neurons while the *Drosophila* brain has about 200,000 neurons. This abundant pool of neuronal cells establishes complicated neural circuits by forming numerous connections to ensure propagation of electrotonic impulses that results in organismal behaviors (Phelps et al., 2021; Raji & Potter, 2021).

Over the decades, committed efforts have been directed towards deciphering the events unfolding during neural circuit (NC) formation and the surrounding factors that coordinate these events, precisely, on elucidating how these events affect the units of NC- axons and dendrites (Chao et al., 2009; Lu et al., 2009; Weiner et al., 2013). Both the axons and dendrites develop in a pattern that is spatiotemporally controlled by cascade of molecular agents (Furusawa & Emoto, 2020; Schelski & Bradke, 2017). During the neural circuit assembly, dendrites of neurons form stereotypic morphological patterns that depict their uniqueness in form and function (Jan & Jan, 2010; Marrs et al., 2001). Proper spacing of dendrites of same cell types and their correct routing to form

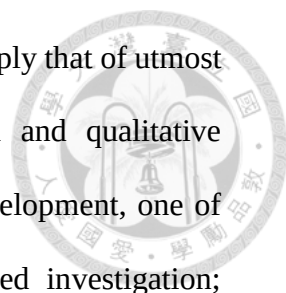




appropriate receptive field sizes with axons of their presynaptic partners are prerequisites for normal physiological behavior of an organism. Many neurological challenges have been linked directly to improper development or inappropriate connection between pre and post synaptic units of a neural circuit thereby supporting the necessity for proper connectivity and functionality of the NC units as a fulcrum to biological systems operations (Forrest et al., 2018; Kulkarni & Firestein, 2012). Development of axons have been deeply investigated with abundant evidences to support the notion that axonal growth, targeting and connection to their definitive post synaptic partners is a requirement for normal neuronal function. On the other hand, due to the complexity in size and shape of dendrites, despite numerous studies, much less is understood about the precise molecular events leading to their normal morphogenesis (Lin et al., 2020). Therefore, it is vital to dissect the mechanisms underlying the proper formation of dendritic arbors.

### **1.2 Dendrite development: a critical component of neural circuit assembly**

Dendrites form a vital component of the neural circuit and their development impacts neuronal functions. Dendritic morphology is unique in shape and size to specific neuronal cell type, and the form of dendritic patterning adapted to by neurons largely determine their spatial allocation and number of inputs they could process during synaptic transmission (Arikkath, 2012; Dong et al., 2015; Gao, 2007; Puram & Bonni, 2013). Studies have shown that these type-specific arrangements of dendrites are vital prerequisites of synaptic connectivity and neural computation (London & Häusser, 2005). The uniqueness of dendrites from cell to cell makes results of the assessment of their morphologies features differ among studies; some dendrites could be assessed in two dimensions (2D) while others require three-dimensional (3D) assessment to gain

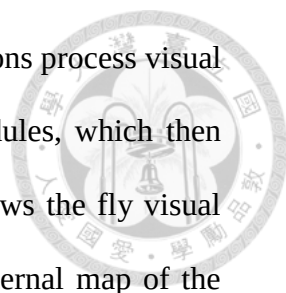


full understanding of their morphologies (Ting et al., 2014). These imply that of utmost importance is a need for standard techniques for computational and qualitative delineation of dendritic properties. To fully understand dendrite development, one of these aspects of dendrite morphology will be required for detailed investigation; dendrite initiation point, branching and termination frequency, layer specification and arbor formation. Specification of dendritogenesis site is under the tight control of intra and extra cellular programs. Dendrites restrict their outgrowth point to a specific site of the neuron by rearrangement and remodeling of their cytoskeleton during morphogenesis (Jan & Jan, 2001; Scott & Luo, 2001). Signaling pathways regulating this aspect of dendrite growth have been well-investigated and documented, showing that transcription factors and cell surface receptors have taken the leading edge. Small GTPases have also been shown to be intersectional in these pathways by controlling membrane trafficking during neuronal development both spatially and temporally (Kamiyama et al., 2015; Spiering & Hodgson, 2011).

Dendrites branch out to expand their receptive fields, and termination allows them to restrict the size of their dendritic fields and prevent overlapping with neighboring cells of the same type. This indicates that during morphogenesis, dendrites decide whether to keep branching or to stop; this decision determines their size and the inputs they can receive and integrate during synaptic transmission. Expanding their dendritic arbors involves a preference for continued branching, which delays termination. Conversely, terminating involves ceasing branching. These attributes of dendrites are regulated by cell-type specific or graded expression of guidance molecules (Ting et al., 2014).

### 1.3 *Drosophila* visual system: A model for dissecting neural circuit assembly

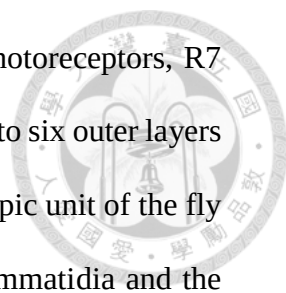
The fruit fly, *Drosophila melanogaster* has proven to be one of the invaluable model organisms for studying various biological processes, including the intricate and highly evolved visual system. More so, vertebrates and fly visual systems share common stereotyped features in complexity and organization including structural and functional patterning (Clandinin & Zipursky, 2002; Jolly et al., 2016; Sato et al., 2013). The fruit fly optic lobe houses visual neurons that are well-distributed among four neuropils including, lamina, medulla, lobula and lobula plate (Figure 1A). Each neuropil in the optic lobe is well-organized into modules to confer a precise visual retinotopic map in the fly's eye to ensure visual acuity. This modular organization is well represented in the synaptic orderliness and relationship seen in the optic lobe with functional impact in the wiring mechanism of the visual system. Following that organizational pattern, *Drosophila*'s compound eye is grouped into repeated units called ommatidia. Each ommatidium contains eight photoreceptors (R1-R8). These photoreceptor neurons (otherwise known as R cells) express different opsins including the broad-spectrum opsin (Rh1) expressed by R1-R6 photoreceptors, UV-sensitive opsins (Rh3/4) expressed by R7, and the R8 photoreceptors express blue/green (Rh5/6) opsins. R7 and R8 play active roles in the chromatic functions, whereas R1-R6 function in the motion detection aspect of the visual process by connecting to the lamina neurons, L1-L6 (Apitz & Salecker, 2014; Ngo et al., 2017; Shinomiya et al., 2019). The lamina, the neuropil just below the retina is also organized into synaptic units called cartridges which contain information been relayed by the retinal ommatidia. Specifically, lamina neurons L1-5 receive motion information from R1-R6 and further relay it to the medulla by their axonal projections into the layers of the medulla neuropil. On the other hand, chromatic information is directly conveyed to the medulla neurons by R7 and R8 photoreceptors



whose axons terminate at the M3 and M6 respectively. Medulla neurons process visual information through diverse neural populations organized into modules, which then relay the information to higher brain centers. This arrangement allows the fly visual system to form precise synaptic units, creating a comprehensive internal map of the external visual field captured by its compound eye (Plazaola-Sasieta et al., 2017). Interestingly, the visual systems of flies and vertebrates share several common features. These similarities include: 1) layer and columnar organization, 2) a large variety of neuron subtypes derived from a few major neuronal cell types within the neuropils, 3) multiple synaptic input sites between pre- and postsynaptic neurons, 4) neuron arrangements that map specific patterns of convergence and divergence, and 5) segregation between synaptic regions and regions occupied by soma (Sanes & Zipursky, 2010; Takemura et al., 2008).

#### **1.4.0 Medulla: The largest neuropil of *Drosophila* optic lobe**

The medulla forms the largest neuropil in the fly's optic lobe; it is organized into layers and columns reminiscent to the retina and mammalian cortex (Morante & Desplan, 2008). The medulla, being the largest of the neuropils contains different cell types with unique morphological characteristics (Li et al., 2013). Many studies have therefore characterized these features and used them as model tools for studying dendrite development (Takemura et al., 2008). The medulla of the optic lobe houses different cell types ranging from the axonal extensions and bulbs of photoreceptors (R7 and R8), axons and terminals of lamina neurons (L1-L5), medulla intrinsic neurons (Mi), projection neurons (Tm and TmY) and distal medulla neurons (Li et al.) (Suzuki et al., 2013). The fly medulla neuropil is divided into layers and columns reminiscent to the retina and mammalian cortex (Holguera & Desplan, 2018); it contains 10 layers (M1-



M10) and 750 columns. The distribution of axon terminals of the photoreceptors, R7 and R8, and the arbors of lamina neurons, L1-L5 divide the medulla into six outer layers (Takemura et al., 2008). The columns of medulla form single retinotopic unit of the fly visual system as a continuous visual unit pathway from the retina ommatidia and the lamina cartridges. Each column contains several cell-types in a well-organized fashion such that there's almost no or minimal overlapping with neighbors (Courgeon & Desplan, 2019). These columnar arrangements enable medulla neurons to maintain proper intra and inter connections and consequently adopt unique receptive field sizes. This stereotype feature is agreed to be tightly under the control of homophilic recognition molecules that afford neurons with the ability to elicit mutual repulsion between same cell-type and adhesion between different cell-types (Hakeda-Suzuki & Suzuki, 2014).

Through several steps and processes, the medulla develops into structured layer neuropil. Cells from the retina and lamina neuropils as well as the medulla neurons pattern the structural description of the medulla neuropils. Broadly, the medulla is divided into the proximal and distal segments, and having ten layers in all named as M1-M10. The M1-M6 layers form the distal segment while the remaining layers form the proximal part. The layers are characterized with the terminals of neurons or the routing of dendritic arbors within them. The M1-M5 layers have the terminals of the L1-L5 lamina neurons axons. Other intrinsic, tangential and distal medulla neurons define the other layers of the medulla. The M3 layer is characterized with the R8 terminal bulb while the M6 layer is defined by the axon terminal of the R7 photoreceptors. Most medulla neurons could be differentiated from one another by the layers their dendritic arbor occupy and the direction they project (Sanes & Zipursky, 2010; Takemura et al., 2008).

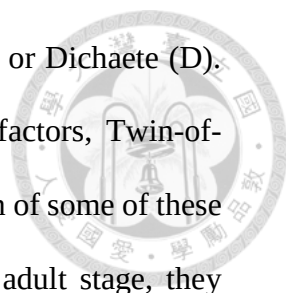
#### 1.4.1 Medulla neurons

The medulla has two broad classes of neurons; medulla projection neurons and interneurons occupy majority of the neuropil. Each group contains different cell-types that arborize at various layers of the medulla to form their appropriate receptive field sizes. The medulla interneurons (Mis) connect within and between layers in the neuropil while the transmedulla (Tms) projection neurons expressly extend their axons through the outer medulla into the lobula complex of the proximal centers of the optic lobe. The other group of neurons are the widefield distal medulla neurons, like Dm8, Dm9; they elaborate their dendritic arbors over several medulla columns (Takemura et al., 2017). Studies have shown that medulla neurons differ extensively in shape and sizes, resulting in unique morphological and functional characteristics among the cell-types. However, these neurons cluster to form repetitive unit of the medulla columns in sync with the inputs from the distal part of the optic lobe to ensure wiring acuity.

The functional properties of the medulla neurons vary as well; studies have revealed that some of the cells are sensitive to different wavelength of the light spectrum. These spectral properties are believed to guide and control different aspect of the fly behaviors. The wide field distal neuron, Dm8, specifically has been shown to exhibit spectral opponency thereby becoming the first neuron in the class of invertebrates to manifest such chromatic property (Li et al., 2021). This and many more make the medulla the most functionally mysterious region of the optic lobe (Gao, 2007).

#### 1.4.2 Tm20 neurons: a model for investigating dendrite development

Tm20 neurons are one of the medulla projection neurons with unique morphological features that afford them to be a suitable model for studying dendrite development. These neurons in addition with Tm5 neurons are derived from late stage medulla



neuroblasts that express the transcription factors, Sloppy-paired (Slp) or Dichaete (D). At early stage of morphogenesis, they express other transcription factors, Twin-of-eyeless (TOY) and Apterous (Apt). They later switch-off the expression of some of these transcription factors as morphogenesis progresses, such that at the adult stage, they neither express Slp nor D. These unique transcriptional code gives specific identity to Tm20 neurons and help to differentiate them from other medulla neurons (Konstantinides et al., 2022). Morphologically, Tm20 neurons are medulla projection neurons; they have their cell bodies outside the medulla and extend their neurites into the distal and proximal segment of the medulla neuropil. A single Tm20 neuron projects its axon through the medulla neuropils lying anterior to the R7 and R8 photoreceptors axons in its home column before terminating at the lobula of the higher center in the optic lobe. Uniquely, their dendritic architecture makes them different from other medulla projection neurons; They have two main dendritic branches shooting out at the M3 and M8 layers of the medulla. The main dendritic branch at the M3 layer of the medulla routes up the photoreceptors in the home column, branching along the way to form Tm20's main dendritic arbor which lies within the M1-M3 layers of the medulla (Ting et al., 2014). Tm20 neurons are therefore monocolumnar cells, restricting their dendritic field sizes to a single medullar column with minimal overlapping with their neighbors of the same cell-type. Functionally, Tm20 neuron, being itself a cholinergic neuron, receives ON- and OFF-inhibitory inputs from diverse cells from other neuropils as well as from other medulla neurons. Precisely, they make active synapses with R8 photoreceptors, L2, L3 lamina neurons, intrinsic medulla neurons (Mi1), transmedulla neurons (TmY) and also make connections with other Tm20 neurons. In addition, the planar projection of Tm20 dendrites is predominantly from the anterior to the posterior. These unique morphological features of Tm20 neurons make them easily identifiable

and differentiated from other closely related medulla projection neurons such as the Tm2 and Tm9 neurons (Ting et al., 2014).



### **1.5 Wiring mechanisms of the neural circuits**

Underlying the development of the brain is a well-controlled assembly of the neural circuits. This process involves the navigation of axons and the routing of dendrites in a complex but highly coordinated manner. Each axon finds its post synaptic target dendrite by navigating through the neural environment through the guidance of its growth cone at the leading edge; a process tightly under the controls of axon guidance molecules such as Ephrins, Nephrens, Slits, Semaphorins and their receptors. Some of these guidance molecules act to either restricts the growing axons from unintended target or promote the location of the right target by guidance cues during axonal growth (Vartak et al., 2023). For example, in the mammalian olfactory system, different classes of secreted semaphorins bind to specific receptors like Neuropilins and members of plexin family during the assembly of their neural circuit to establish and maintain connectivity (Pasterkamp et al., 1999). In *Drosophila*, semaphorins have been involved in axon branching, growth and targeting through their activation of specific signaling pathways during nervous system development (Nguyen et al., 2022; Zwarts et al., 2016).

On the other hand, the routing of dendrites is also under the control of the cell intrinsic programs and their extrinsic factors (Puram & Bonni, 2013). Dendrite morphogenesis involves series of actions by either secreted molecules such as insulin or membrane-bound molecules like cell adhesion molecules that elicit the activation specific signaling pathways to regulate actin cytoskeleton organization and rearrangement. The interplay between extracellular factors and the cell intrinsic factors affords neurons a controlled wiring of their postsynaptic components with the proper presynaptic partners. This

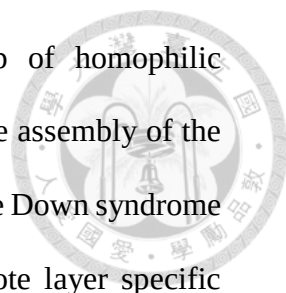
phenomenon has been well investigated but still far from complete comprehension due to the complexity of dendritic morphology (Lin et al., 2020).

Thus, the assembly of the neural circuits features mainly two important components; the growth, pathfinding and targeting of the axons and the sprouting, branching and routing of dendrites through the release of guidance cues and the programmed control from the cell genetic makeup. The resultant effect of this biological process is the accurate construction of synaptic components and the appropriate functioning of the body system. Disruption of the neural circuits is implicated in many neurodevelopmental and neuropsychiatric disorders (Forrest et al., 2018; Kulkarni & Firestein, 2012)

### **1.6 Homophilic recognition molecules: role in neural circuit assembly**

Proper cell-cell communication lies at the central mechanism for neural circuit assembly. These communications involve interplay between cell external cues and internal responses that elicit specific signal transduction to instruct appropriate cell morphology and physiology. Many studies have shown that the intrinsic cellular program and extrinsic factors act in sync to direct dendritic patterning during dendrite morphogenesis; some of these factors function in different aspect of dendrite development including size of dendritic arbors, planar direction and specificity in post synaptic targeting (Lin et al., 2020; Sanes & Zipursky, 2020). Among the extrinsic factors requisite for proper dendritic patterning is the homophilic immunoglobulin superfamily proteins. Members of this family such as cadherins and Dscams are important cell-surface receptors regulating the proper development of dendrites.

Homophilic recognition enables neurons to avoid connection with same cell-type but with different cell-types (i.e. self and non-self-recognition) thereby establishing precise connection and definite arbor formation (Corty et al., 2009; Han et al., 2012). The



immunoglobulin superfamily (IgSf) proteins form a large group of homophilic recognition molecules that mediate repulsion or adhesion to guide the assembly of the neural circuit in both vertebrates and invertebrates. In this group are the Down syndrome adhesion molecules, sidekicks and protocadherins. Sidekicks promote layer specific arborization of dendrites and synapse formation in distinct population of retinal neurons. Protocadherins isoforms are expressed in Purkinje cells and amacrine neurons of the mouse retina to afford a mechanism for self-avoidance of same cells and tiling of different cells via stochastic expression of the splice variants (Yamagata & Sanes, 2008). Studies have supported the idea that alternative splicing of cell recognition molecules is a required mechanism for proper neuronal wiring of the nervous system.

### **1.7 Down syndrome cell adhesion molecule 2 (Dscam2)**

Dscam2 is a cell surface protein belonging to the immunoglobulin superfamily of homophilic recognition proteins that function in cell-cell communication to mediate several cellular processes involved in neural circuit assembly. Functionally, Dscam2 has been suggested to mediate homotypic adhesion or repulsion between neurites through its homophilic recognition properties (Odierna et al., 2020). The *Drosophila melanogaster* genome consists four Dscam genes (Dscam, and Dscam2–4) that encode type I transmembrane proteins comprising of ten immunoglobulin (Ig) and six fibronectin type III repeats in their extracellular domain with a single pass transmembrane domain. The cytoplasmic tails of these proteins differ in length. The genomic organization of individual member of fly Dscam family largely differs likewise; Dscam1 has a function in forming self-avoidance between neurons during the development of the CNS. Through alternative splicing, it generates 38,016 different proteins from four cassettes of alternative exons. Dscam isoforms bind homophilically,

and in vivo studies indicate that these interactions promote repulsion. However, this expression of the different isoforms is stochastic making it rather almost impossible to study the functions of each of the isoform. Dscam2–4 do not show extensive isoform diversity, and in this way these family members are more similar to mammalian DSCAMs (Schmucker & Chen, 2009; Tadros et al., 2016).

Down syndrome cell adhesion molecule (Dscam2) was originally identified as an open reading frame in a region of human chromosome 21 susceptible to numerical chromosomal aberration known as Down syndrome. *Drosophila* Dscam2, an orthologue of human DSCAM, contains only two alternative immunoglobulin 7 domains that share about 50% sequence identity and are referred to as Dscam2 10A and Dscam2 10B (Millard et al., 2007). Either of the isoforms possesses homophilic binding properties to instruct cell recognition during development. Among the Dscam family, Dscam2 has been shown to mediate homotypic and heterotypic interactions between neurites to control dendritic shape and arbor in a context-dependent manner both in CNS and PNS of fruit fly (Hattori et al., 2007). Dscam2 is capable of mediating adhesion or repulsion in different cell-types. In contrast to Dscam1 which only functions as self-avoidance molecule, Dscam2 functions in both self and non-self-avoidance (Millard et al., 2007). These self and non-self-recognition is the underlying mechanisms in neural circuit assembly. Through this avoidance mechanisms, Dscam2 serves as a tiling receptor to guide the proper assembly of the neural circuit. From the large pool of studies on Dscam2, its cellular functions have been well investigated, however, its molecular mechanism is still unknown.

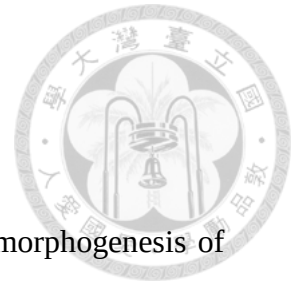
## 1.8 Aims

The specific aims of this study include:

1. To investigate the cellular functions of Dscam2 during dendrite morphogenesis of Tm20 neurons.
2. To examine the cytoplasmic domain requirement of Dscam2 in Tm20 dendritic field size restriction.
3. To determine Dscam2 intracellular signaling pathway in restricting expansion of Tm20 receptive field sizes.
4. To assess the connectivity and functional consequences of Dscam2 mutation in Tm20 neurons.

## 1.9 Significance of this study

It has been proposed that Dscam2 functions as a tiling receptor, mediating either adhesion or repulsion between neurites to guide neural circuit assembly. Although previous studies have demonstrated these diverse roles of Dscam2 in CNS development, its molecular mechanism remains unclear. In this study, we investigated the signaling mechanisms of Dscam2 and identified its potential interacting partners. Through a series of genetic manipulations and morphological analyses, we determined the signaling pathway of Dscam2 in restricting the dendritic field sizes of *Drosophila* visual neurons. This study is pivotal and paves the way for future advancements in understanding how homophilic recognition molecules regulate the assembly of neural circuits during CNS development.



## CHAPTER TWO



### 2.0 MATERIALS AND METHODS

#### 2.1 *Drosophila* stocks and genotypes

Flies were raised on standard cornmeal/molasses medium at 12h/12h light-dark cycle at 25°C (except otherwise stated for specific experiments). The full genotypes of samples shown in figures are provided in Table. In this study, the following fly lines were used: *R35H02-Gal4* (Slp1-Gal4; BDSC\_49923), *OrtC1-Gal4*, *hsFLP1*, *hsFLP122*, *UAS-Dscam2<sup>RNAi</sup> TriP.HMC03411* (BDSC\_51839), *UAS-Dscam2<sup>RNAi</sup> P{GD111}v1003* (VDRC\_1003), *FRT2A* (BDSC\_1997), *FRT82*, *Dscam2<sup>1</sup>* (Millard et al., 2007), *Dscam2 10A* (Lah et al., 2014), *Dscam2 10B* (Lah et al., 2014), *FRT43D*, *mb1<sup>E27</sup>* (BDSC7318), *Dscam2ΔICD* (this study), *R33H10-LDBD* (this study), *R16H03-LexAp65AD* (BDSC\_52510), *R39D12-LexAp65AD* (BDSC\_52681, Pfeiffer et al., 2010), *UAS-CD4-spGFP1-10*, *Rh3/4-syb-spGFP1-10*, *LexAop-syb-spGFP1-10*, *PI3K<sup>df</sup>*, *FRT82* (Luo et al., 2020), *FRT40*, *Rho1* (BDSC\_41771), *UAS-Rho1* (BDSC\_7334), *Rac1<sup>J11</sup>*, *FRT2A* (BDSC\_6674), *UAS-Rac1* (BDSC\_6293), *Cdc42<sup>4</sup>*, *FRT40* (BDSC\_9106), *Trio<sup>1</sup>* (BDSC\_9129), *TrioGEF1QA* (this study), *TrioGEF2QA* (this study), *UAS-Trio(wt)*, *UAS-Trio<sup>muGEF1</sup>*, *UAS-Trio<sup>muGEF2</sup>* (gifts from Ed Giniger) (Shivalkar & Giniger, 2012).

#### 2.2 MARCM-based mosaic analysis

To label single neurons we used the *hsFLP*-based mosaic analyses in combination with cell-specific drivers at either second or third instar larvae. To activate Flipase expression, larvae bearing these transgenes were transferred to a PCR tube and subjected to a 60~90s heat shock in a 37°C water bath. To generate the Tm20 MARCM clones, *hs122-Flp* and *ortC1-Gal4* were used with the appropriate FRT chromosomes. To generate single

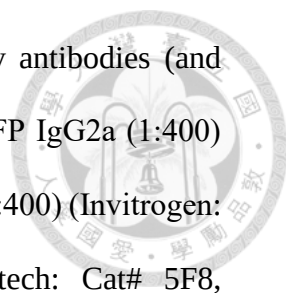
Tm20 clones, late third-instar larvae were transferred to a PCR tube and subjected to heat shock in a 37°C water bath. A 1-2min heat shock was administrated to hs122-Flp-bearing flies and a 15-min heat shocks was used for those bearing hs-Flp1. Under these conditions, over 95% of the MARCM clones consisted of Tm20 neurons. In some conditions, heat shock was carried out at the second instar larvae due to the timing of protein synthesis.

### 2.3 Generation of TrioGEF point mutations

Using seamless GRISPR/Cas9 gene editing by homology-dependent repair (HDR) provided by WellGenetics Inc., we introduced point mutations Q1417A and Q2078A into the GEF1 domain and GEF2 domain of *trio\_RA* (*trio*/CG18214) respectively. To generate TrioGEF1QA, a PBacDsRed cassette containing Piggy Bac 3' terminal repeats, 3xP3-DsRed and Piggy Bac 5' terminal repeats was used to introduce Q1417 to A, CAG to GCG of *trio\_RA*. And to generate TrioGEF2QA, a PBacDsRed cassette containing Piggy Bac 3' terminal repeats, 3xP3-DsRed and Piggy Bac 5' terminal repeats was used to introduce Q2078 to A, CAG to GCG of *trio\_RA*. Each transgenic fly was sequence validated by Sanger sequencing.

### 2.4 Immunostaining

Immunohistochemistry was performed on adult flies of 1-5 days old. Whole-mount fly were decapitated in fixative containing 4% paraformaldehyde (PFA) in 0.1M PB. Prefixed brains were gently removed and post-fixed in fixative for 30mins. Afterwards, fixed brains were washed in PBT (phosphate buffered saline solution (PBS) in 0.5% TritonX-100) for 5 mins three times. They were later blocked in 1% normal goat serum (NGS) in PBT for 30mins before subsequently incubated in primary antibodies (3-5



days) and secondary antibodies (overnight). The following primary antibodies (and concentrations) were used: mAb24B10 (1:100) (DSHB); mouse  $\alpha$ GFP IgG2a (1:400) (Invitrogen: Cat# A-11120, RRID:AB\_221568); rabbit mAb  $\alpha$ GFP (1:400) (Invitrogen: Cat# A-11122, RRID:AB\_221569); rat  $\alpha$ RFP (1:400) (Chromotech: Cat# 5F8, RRID:AB\_2336064),  $\alpha$ hCD4 (1:400) (abcam: Cat# ab133616); guinea pig Toy and guinea pig  $\alpha$ Dichaete (1:100) (a generous gift from Claude Desplan). The secondary antibodies from Invitrogen include: Alexa Flour 488 goat anti-mouse (Cat# A-21131, RRID: AB\_2535771), goat anti-rabbit Alexa Flour 568 (Cat# A-11036, RRID:AB\_10563566), and Alexa Flour 647 goat anti-mouse (Cat# A21240, RRID: AB\_2535809), Alexa Flour 647 goat anti-rabbit (Cat# A21245, RRID:AB\_2535813), Alexa Flour 647 goat anti-guinea (Cat# A21450, RRID: AB\_2535867) were used at a dilution of 1:400.

## 2.5 Image acquisition

Confocal images were acquired using Zeiss LSM880 laser-scanning microscope. Using a 40x oil objective with NA-1.2 (except for ExM were 20x water lens was used), Z-stack images captured by ZEN black software had a resolution at 512 x 512 x N voxels (the number of Z-sections, N, typically ~150), 16 bits (voxel size = 0.11 $\mu$ m x 0.11 $\mu$ m x 0.2  $\mu$ m), and two- or three-color channels. Images acquired were later deconvolved on ZEN blue (ZEISS) or Huygens Professional (Scientific Volume Imaging, Hiversum, Netherlands) softwares and viewed on Imaris 9.6.0.

## 2.6 GFP reconstitution across synaptic partners (GRASP)

Synaptic activity-dependent GRASP (synGRASP) for detecting active connections between L2/R7 and Tm20 neurons was performed using a synaptobrevin-fused spGFP1-10 expressed in all L2 lamina neurons or R7 photoreceptors and the UASmCD4::spGFP11 driven by the OrtC1-Gal4 was tethered to the membranes of single Tm20 clones. The native fluorescence signal of reconstituted GFP was used. In the case of weak signal, the anti-GFP was used to enhance reconstituted GFP signal with minimal background noise generated but could be differentiated from real GRASP signals. Synaptic GRASP for detecting active synapses based on the reconstitution of syb-spGFP1-10 and spGFP11 was performed in 0-12d old flies, and native fluorescence signal of GFP was used (Macpherson et al., 2015). Syb::GFPsp1-10 was preferentially detected by rabbit polyclonal anti-spGFP1-10 antibody. For the 12hr/12hr light-dark cycle, flies used were at 25°C after they were first heat-shocked at third instar wandering larvae while in dark-dark cycle, they were rather raised and dissected under absolute dark condition.

## 2.7 Dendritic field sizes quantification

As previously described in Ting et. al., (2014) and (Ting et al., 2017) with slight modifications, landmark-based registration methods, implemented in MIPAV (Medical Image Processing, Analysis, and Visualization), were used to register the combined 3D images and skeletonized dendritic trees to the reference columns. To register a single Tm20 neuron, we assigned 27 reference points on M1, M3 and M6 medulla layers of 9 photoreceptor axons surrounding the Tm20 neurons in the order of dorsal, anterior-dorsal, anterior, anterior-equatorial, equatorial, posterior-equatorial, posterior,

posterior-dorsal and central. Coordinates of these 27 reference points were then used to register each neuron. The dendritic arbor of the registered Tm20 neuron would then be cropped in three dimensions to restrict visualization of affine-transformed neuron to the receptive field size of the observed Tm20 neuron. Through python programming, registered Tm20 neurons were affine-transformed and then visualized by Napari for subsequent dendritic field sizes quantification and analysis.

## **2.8 Statistics**

Statistical analysis of dendritic field sizes and synaptic GRASP puncta value of different genotypes were done on GraphPad prism v10. Unpaired student test was used to compare two groups, one-way analysis of variance (ANOVA) was used to check statistical significances among multiple groups. Except otherwise indicated, all data are presented as mean value  $\pm$  S.E.M.

## CHAPTER THREE



### 3.0 RESULTS

#### 3.1.0 Patterning of Tm20 dendrites requires Dscam2

To determine potential genes regulating dendrite morphology during development, we screened a number of transmembrane proteins previously shown to function in dendrite development. Previous studies have shown that CSPs are key players in dendrite morphogenesis. We knocked down each of the candidate genes in developing Tm20 neurons and carefully characterized their dendritic morphology. To effectively knockdown each CSP in developing Tm20 neurons, we used sloppy-paired GAL4 (Slp1-Gal4) to drive both UAS-RNAi and UAS-dicer2. Sloppy-paired transcription factor drives the cell lineage from which Tm20 neurons are derived and therefore serve as a good developmental driver for them (Xie et al., 2022). Sparse labeling of Tm20 neurons expressing UAS-RNAi was achieved through a 17D01-Flp recombinase transgene, inducing excision of the FRT site-flanked transmembrane reporter mCD8GFP driven by the OrtC1a-LexA::VP16/LexAop system (Figure 1B). To achieve sparse labelling, flipase expression was driven stochastically by 17D01 promoter in some Tm20 neurons. Only cells with successful excision of the stop cassette by flipase will drive the expression of GFP while cells with intact stop cassette will not be labelled. Hence, the labelling of Knocked-down cells will be sparsely distributed across the neuropil.

We observed that knocking down the cell surface receptor, Dscam2, extensively perturbed the normal dendritic patterning of Tm20 neurons with unique characteristic phenotypes. Two independent RNAi lines (UAS-Dscam2 RNAi<sup>TRiP.HMC03411</sup> and UAS-Dscam2 RNAi<sup>GD111</sup>) targeting different sequence regions of *Dscam2* genome showed

aberrant dendritic phenotypes compared to their control (Figures 1C-F'). This suggests that *Dscam2* is required for proper dendritic patterning during the development of Tm20 neurons. We then asked whether these observed effects were cell-autonomous for *Dscam2*. To critically investigate this presumed cellular function of *Dscam2*, there is need to observe the phenotypes at single cell resolution. We therefore, proceeded to generate single mutant clones using the mosaic analysis technique.

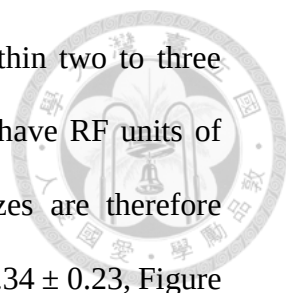
### 3.1.1 *Dscam2* restricts dendritic field sizes of Tm20 neurons

RNAi-mediated knockdown phenotypes justified that *Dscam2* is required in Tm20 neurons for proper development of their dendrites. To have a comprehensive knowledge of the cellular functions of *Dscam2* in Tm20 dendrite development, we generated single mutant clones using the technique of mosaic analysis with repressible cell marker (MARCM) which allows the determination of the effects of specific gene at single cell resolution (Lee & Luo, 1999). Following this method, we assessed the phenotypes of *Dscam2*<sup>1</sup> mutant Tm20 compared to their controls. *Dscam2*<sup>1</sup> is a null mutant but viable (Millard et al., 2007). We first observed that *Dscam2* null mutant Tm20 neurons have three major characteristic aberrant dendritic phenotypes: they showed abnormally enlarged dendritic arbors occupying 4-8 medulla columns (100%, n = 20, Figure 3B-B'') in contrast to control that occupies 1-2 medulla columns (Figure 3A-A''), thereby transforming Tm20 neuron to a multicolumnar cell (Figures 3A' and B'). Secondly, in *Dscam2* mutants, although their receptive field maintained correct layer arborization, they were instead transformed into cells with multiple main dendritic branches (MMDB: 2(0%), 3(43.75%), 4(35.50%), 5(6.50%) (Figures 3B and C), which completely differ from controls that extended a single main dendritic branch at the third medulla layer (M3) and rout a single medulla column (100%; Figure 3A). Ultimately, *Dscam2* mutants

had error in their dendritic planar projection direction (DPD) because unlike controls in which DPD projects from the anterior to the posterior, they project anteriorly (40%). These results justified that *Dscam2* cell-autonomously regulates the dendritic patterning of Tm20 neurons.

In our previous study, we developed tools to adequately quantify dendritic field sizes using the dendritic field units of the medulla columns (Ting et al., 2014). Using this analysis tool enabled us to generate morphometric attributes of dendrites of Tm neurons including Tm20. However, due to the overly enlarged receptive field sizes and the complexity of the dendritic trees observed in *Dscam2* mutants, our previous quantification tools and metrics fail to apply efficiently. Therefore, to overcome these challenges we modified our quantification method to characterize these features of the mutants. In this new morphometric analysis, we represented the medulla columns as a hexagonal array similar to the rhombic array in the previous design; each hexagon represents one medulla column which depicts a retinotopic unit of the fly visual system (Figure 2C) (Malin & Desplan, 2021; Sato et al., 2013). The receptive field (RF) of a neuron has its home column photoreceptor surrounded by eight neighboring photoreceptors. Each hexagon is subdivided into twelve segments to enable a detailed assessment of the extent to which the hexagon is occupied by dendrites during quantification. The dendritic field size (DFS) of a neuron is then calculated as the number of hexagonal segments invaded by its dendritic arbor.

To derive the DFS of a neuron, we first registered the cell by assigning it to 27 reference columns at M1, M3 and M6 layers of the medulla representing the terminal points of L1, R8 and R7 respectively (Figure 2A). The registered cell is then subjected to affine transformation before quantification is done (Figures 2B-E). Using this morphometric



analysis, the control Tm20 neurons restrict their dendritic arbor within two to three medulla columns ( $2.99 \pm 0.97$  DFS,  $n=20$ ) while *Dscam2* mutants have RF units of approximately ( $6.39 \pm 0.33$  DFS,  $n=20$ ) (Figure 3C); the RF sizes are therefore significantly enlarged in *Dscam2* mutant Tm20 neurons (difference;  $3.34 \pm 0.23$ , Figure 3C). Knowing that the number of inputs a neuron can receive and subsequently integrate during synaptic transmission highly depends on its receptive field size (Apitz & Salecker, 2014; Grewe et al., 2006), we presumed that increased RF in *Dscam2* mutants could therefore imply that they developed capacity for more inputs from their presynaptic partners, such as L3 or L2 lamina neurons, and may thereby disrupt the normal retinotopic mapping of the visual system as a connectivity consequence. The connectivity implication would be presented in the Figure 5. Taken together, we propose that *Dscam2* homophilic recognition effects restrict the elaboration of the dendritic arbors Tm20 neurons during development in a cell autonomous fashion.

### **3.1.2 *Dscam2* mutation does not alter the normal cell fate of Tm20 neurons**

To ascertain the cell fate of *Dscam2* mutants, we examined the expression of cell-type specific transcription factors for Tm20 morphogenesis. Cell-type specific transcriptional code is unique to individual medulla neurons during development and their expressions pattern the development of *Drosophila* neuroblasts temporally. Developmental patterning of medulla neurons has been well-investigated; each medulla neuron expresses one or sets of transcription factors (Tfs) at different stages of development by switching-on or off these Tfs until they achieve stably differentiated cells (Li et al., 2013).

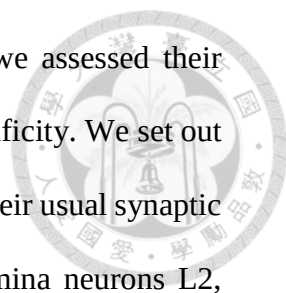
Following this temporal pattern, Tm20 neurons at early stage of development express the transcription factors- Sloppy-paired (Slp), Twin-of-eyeless (TOY), Dichaete (D) and

Apterous (Ap). At the adult stage however, they neither express *Slp* nor *Dichaete* anymore, making them  $D^{-ve}$  but  $Toy^{+ve}$  cells (Konstantinides et al., 2022). We therefore examined the cell fate of both the controls and *Dscam2* mutant clones as judged by the expression of their positive marker (*Toy*) and their negative marker (*D*). We observed that even though *Dscam2* loss-of-function perturbs the normal dendritic patterning of Tm20 neurons, they still maintained their normal cell fate as confirmed by their cell-type specific transcription factor expression; *Dscam2* mutants expressed *Toy* just as their controls (Figures 4A and B) but did not express *Dichaete* just as is the case with their control (Figure 4C and D). Besides, the promoter- *OrtC1* which was used to drive Gal4/UAS-mCD8GFP in Tm20 expresses at late developmental stage and mainly in adult Tm20 neurons.

After verifying that *Dscam2* mutation did not alter transcriptional program of Tm20 neurons, we went on to examine the connectivity implications that could result from developing aberrantly large receptive field sizes.

### **3.2.0 *Dscam2* regulates wiring mechanisms of Tm20 neurons**

To decipher the role of *Dscam2* in the wiring mechanism of Tm20 neurons, we examined the synaptic properties of *Dscam2* mutants compared to their controls. Observing that *Dscam2* mutants displayed aberrantly enlarged dendritic field sizes, we presumed that this could potentially result in increased receptive capacity compared to their controls. Based on electron microscopy (EM) reconstruction, Tm20 neuron receives both on and off-inhibitory inputs from neurons within the optic lobe neuropils including L3, L2, Mi4, C3, R8, Dm9, Tm1 and sometimes from neighboring Tm20s (Chklovskii et al., 2010; Nern et al., 2015; Takemura et al., 2017; Takemura et al., 2015). The number of inputs from these synaptic partners varies and is contingent on the location of the Tm20 neurons within the medulla neuropil.



To determine the connectivity consequences of *Dscam2* mutants, we assessed their wiring in terms of synaptic inputs precision and synaptic partner specificity. We set out to test whether *Dscam2* mutants make aberrant synapses with either their usual synaptic partner or non-synaptic partner. For the former, we focused on lamina neurons L2, which are one of the major inputs to Tm20 neurons, and for the latter, we examined R7 photoreceptors, knowing that they do not typically have synaptic inputs to Tm20 neurons (Takemura et al., 2017). To test this hypothesis, we utilized activity-dependent synaptic GFP reconstitution across synaptic partners (synGRASP) to monitor active inputs to Tm20 neurons from candidate neurons (Feinberg et al., 2008; Macpherson et al., 2015).

We anchored UAS-CD4::splitGFP11 to the membrane of Tm20 neurons while simultaneously, all L2 or R7 cells expressed synaptobrevin-tagged splitGFP1-10 to restrict expression to synaptic vesicles (Figure 5B). These split GFPs reconstitute upon active synaptic transmission. Using this method, we first examined whole pattern expression of GRASP in either L2-Tm20 or R7-Tm20 controls. We observed GFP signals only in L2-Tm20 but not in R7-Tm20 confirming the synaptic partner specificity for Tm20 neurons (Figure 5C – D’).

### **3.2.1 *Dscam2* regulates synaptic partner input precision**

To check the behavior in single Tm20 mutants, we exposed each group to a 12-hour light/dark (LD) cycle from eclosion (day 0) till 12 days posteclosion, and monitored their synaptic activities as reflected by the reconstituted GRASP signals. In *Dscam2* mutant Tm20 neurons, aberrant connections were observed with their ectopic branches with L2 neurons at their invaded column contrary to their controls that make connections only at their cognate column (Figure 6A and B). Ultimately, at 12 days old, there was a

significant decrease in the synapses of *Dscam2* mutants compared to their control suggesting that the loss-of-function of *Dscam2* affected the receptive field capacity of Tm20 neurons (In control, 3-20 GRASP puncta  $n = 21$ , in *Dscam2*<sup>1</sup>, 0 -10 puncta,  $n = 24$ , Figure 6E). In addition, the kinetics of synaptic inputs from L2 to Tm20 rises with increasing age while in *Dscam2* mutants, it first rises till 3days after eclosion but then decline afterwards (Figure 6G).

### 3.2.2 *Dscam2* controls synaptic partner specificity

To investigate the potential loss of synaptic partner specificity, we examined the likelihood of aberrant synapses between *Dscam2* mutant Tm20 and R7 photoreceptors. In control, single Tm20 neuron did not exhibit any GRASP signal, indicating the absence of inputs from R7 (Figure 6C). In contrast, *Dscam2* mutants were observed forming aberrant synapses with R7 along the shafts of their axons in the presence of light as stimuli (0-12 GRASP puncta,  $n = 21$ , Figure 6D, F). The GRASP kinetics of *Dscam2* mutants is however lower in R7-Tm20 compared to L2-Tm20 (Figure 6H). Interestingly, *Dscam2* mutants L2/R7 - Tm20 synapses exhibited random wiring patterns with surrounding columns where their ectopic main dendritic branches invaded contrary to their controls; these were mostly observed at the posterior equatorial region, central and equatorial columns. Fewer contacts were observed in the remaining columns, with no contact at the posterior and the anterior equatorial column (Figure 6B' and D'). Under total dark condition, insignificant GRASP signals were detected (except in R7-Tm20 at 12d, Figure 7B) suggesting that the observed activity-dependent synapses in majorly from light stimulation (Figure 7C, D).

Worthy of note is that the number of GRASP puncta observed in this connectivity experiment is slightly lower than the EM reconstruction finding, potentially owing to

resolution discrepancies in confocal microscopy images. Nevertheless, taken together, the loss-of-function of Dscam2 in Tm20 neurons resulted in diminished wiring precision in both synaptic inputs and partner specificity. This underscores the importance of proper connectivity for circuits functionality, which is influenced in part by cell adhesion molecules (Plazaola-Sasieta et al., 2017).

### 3.3 Extracellular molecular diversity is required for Dscam2 functions

Dscam2 is a cell surface protein with ten immunoglobulin (Ig) domain, six fibronectin type III repeats, a single transmembrane domain and a cytoplasmic tail at its C-terminal similar to other Dscam1 (Wojtowicz et al., 2004). Through alternative splicing of the exon 10 belonging to immunoglobulin 7 (Ig7) of its extracellular domain, Dscam2 generates two exclusive homophilic isoforms, 10A and 10B (Figure 8A) (Lah et al., 2014; Tadros et al., 2016). Studies have shown that most neurons express either of the isoforms while some neurons express both isoforms. This alternative splicing is regulated by a well-known RNA binding protein- muscle blind (Mbl). During the development of *Drosophila* visual neurons, the splicing factor, Mbl, promotes the selection for exon 10B while repressing 10A expression (Lah et al., 2014). Compared to the control, mutants carrying only exon 10A showed aberrant dendritic phenotypes in the similitude of Dscam2 null and not with single isoform mutants for 10B (Figures 8B-E), which suggests that in Tm20 neurons, Dscam2 10B essentially regulates dendritic field sizes (Figure 8G). However, both isoforms (10A and 10B) determine dendritic planar projection direction (Figure 8D and 8E). To confirm the results of this isoform specificity, we examined the phenotypes of *mbl* loss-of-function. *mbl* mutant Tm20 neurons completely phenocopied *Dscam2* null mutant for dendritic phenotypes (Figure 8C, C', 8F and F'). Based on the expressions of Dscam2 and Mbl from the

developmental transcriptome of Tm20 neurons, we suspect an isoform switch from 10A to 10B at about 40 hr APF (after pupal formation). Knowing that Dscam2 mediates homophilic binding between similar cells, we propose that repulsion between neighboring Tm20 neurons is mediated by 10B isoform recognition while heterotypic adhesion between Tm20 neurons and other medullar neurons such as laminar neurons regulate the direction of planar projection. Combined, these results support the notion that molecular diversity of Dscam2 extracellular domain is an active mechanism for its actions.

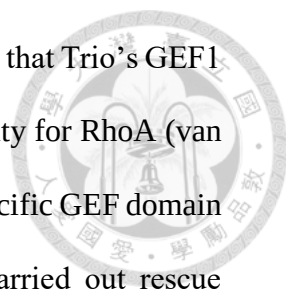
### 3.4 Cytoplasmic domain is required

To delineate the signaling mechanism of Dscam2 aside extracellular domain isoform variations, we investigated its cytoplasmic domain requirement in Tm20 dendrite development. The cytoplasmic domain of Dscam2 span its exons 18-22 with two alternatively spliced exons- 18 and 19 that generate long and short forms of the cytoplasmic domain isoforms. These isoforms are categorically grouped into two; the short forms (GIKLM) and the long forms (P and Q). The function of Dscam2 cytoplasmic domain is unknown (Figure 11A). And how Dscam2 mediates repulsion and/or adhesion is totally unknown. We therefore, sought to determine whether different cytoplasmic domain isoforms or their specific regions could contribute to the function of Dscam2. We propose that Dscam2 transduce signals through its intracellular domain components to restrict dendritic field sizes and pattern dendritic planar direction of Tm20 neurons during morphogenesis. To test this hypothesis, we CRISPR knock-in an RFP marker to replace exons 18-22 and used recombinase mediated cassette exchange (RMCE) to successfully remove the cytoplasmic domain's coding region (Figure 9A). This mutant Tm20 carrying intracellular domain deletion was tagged *Dscam2AICD*.

Single *Dscam2 $\Delta$ ICD* mutants showed dendritic phenotypes indifferent from *Dscam2* null (100%, n=14, Figures 9C, C'). These results show that Dscam2 cytoplasmic domain is required for dendritic field size control. Taken together, these findings support the notion that Dscam2 restricts the elaboration of dendritic arbors through its cytoplasmic domain function potentially involving tyrosine phosphorylation to trigger intracellular signal transduction. We therefore, turned to dissect potential intracellular interacting partners for Dscam2.

### 3.5 Trio activates Rac1 activity to modulate dendrite morphology

To identify potential intracellular interacting partners of Dscam2, we used candidate approach to knock down potential signaling molecules implicated in dendrite development. The distinctive dendritic phenotypes of Dscam2 mutants suggest a need for actin cytoskeleton rearrangement and remodeling during Tm20 dendrite morphogenesis. Consequently, we examined molecules that might regulate actin cytoskeleton dynamics during dendrite development. Through this approach, we identified Trio, a guanine nucleotide exchange factor (GEF) as a potential interacting partner of Dscam2 for Tm20 dendrite morphogenesis control. Single mutant Tm20 devoid of Trio completely phenocopied *Dscam2* null for the major dendritic phenotypes ( $5.78 \pm 0.35$  DFS, 2-5 MMBD, n = 16 Figure 10C, C' and J). *Drosophila* Trio is a multidomain nucleotide exchanger with a high resemblance in protein structure compared with its mammalian and nematode orthologues; it comprises amino terminus ~230 amino acids, eight spectrin repeats, GEF1, an SH3-domain and GEF2 (Malartre et al., 2010; Schmidt & Debant, 2014; Tao et al., 2019). Trio specifically functions via its GEF domains to activate small GTPases by exchanging their GDP for GTP (Cherfils & Zeghouf, 2013). Therefore, we presumed that Trio restricts expansion of dendritic



arbors through either of its GEF domains. Previous studies have shown that Trio's GEF1 is specific for Rac1 activation while its GEF2 has more binding affinity for RhoA (van Rijssel et al., 2012; van Rijssel & van Buul, 2012). To ascertain the specific GEF domain essential for Trio's function in Tm20 dendrite development, we carried out rescue experiments with wild type Trio and with either of the Trio's GEF domains by overexpression in a *Trio*<sup>1</sup> mutant background. Surprisingly, all the Trio derivatives rescued *Trio*<sup>1</sup> mutants to a large degree: rescuing with wild-type Trio restored the dendritic field sizes and reduced development of ectopic main branches of *Trio* mutants almost entirely (Figure 10D), while rescuing mutant Trio with defective Rac1GEF (GEF1) domain also restored the receptive field sizes significantly but retained the aberrant axonal phenotypes (Figure 10E). Lastly, rescuing with mutant Trio contain defective Rho1GEF (GEF2) domain did restore the DFS of *Trio*<sup>1</sup> mutant better and completely rescued its abnormal axonal pretermination to that of the control (Figure 10F). These overexpression results do not differentiate the function of Rac1 and Rho1 GEFs for proper Tm20 dendrite development. We therefore, generated individual point mutations on either of the GEF domains by exchanging the glutamine residues- 1417 (on GEF1) and 2078 (on GEF2) for alanine thereby impairing their catalytic activities as shown in previous studies (Newsome et al., 2000) (Figure 10A). Strikingly, significant dendritic abnormalities in similar fashion to *Dscam2*'s was only observed in *TrioGEF1QA* mutant but not in *TrioGEF2QA* (Figure 10F – G' and J); suggesting that Trio potentially modulates actin cytoskeletal dynamic of Tm20 dendrites through its Rac1-specific GEF (GEF1) domain.

### 3.6 Trio instructively regulates dendritic arbor restriction in Tm20 neurons

We further noted that overexpressing wild-type Trio (UAS-trio) results in significant reduction of the dendritic field sizes of Tm20 neurons (DFS:  $2.48 \pm 0.79$ , Figure 11B and B'). In addition, overexpressing either of the trio's GEF mutants (i.e. UAS-muGEF1 and UAS-muGEF2) also resulting in significant decrease in dendritic field sizes (DFS:  $2.54 \pm 0.79$ , and  $2.56 \pm 0.77$  resp., Figure 11C – D'). There was also error in dendritic planar projection directions in the GEF mutants' overexpression except in the wild-type Trio. However, none of the Trio's derivatives show any multiple main dendritic branches nor any axonal phenotypes (Figures 11B - D).

### 3.7 Rac1 activation is required for proper dendritic development of Tm20 neurons

Having observed the critical involvement of Trio in the hypothetical signaling pathway of Dscam2, we further attempted to decipher specific member of the small GTPases that is likely involved Tm20 dendrite development. Previous studies have shown the vital roles of small GTPases in actin cytoskeletal reorganization and remodeling during development (Hall & Nobes, 2000; Spiering & Hodgson, 2011). They are known to serve as molecular switches modulating several cellular functions including mitosis, proliferation, migration and apoptosis (Gonzalez-Billault et al., 2012). Importantly, the functions of Rho family of GTPases consisting of RhoA, Cdc42 and Rac1 during vertebrate and invertebrate neurite morphogenesis have been extensively investigated (Edlund et al., 2002). They are known to exist in two different forms- GDP-bound and GTP-bound, which equally confer to them their inactivation and activation respectively (Bernards & Settleman, 2004; Cherfils & Zeghouf, 2013; Yang et al., 2012). These two different forms cycle between the cell membrane and cytoplasm through the binding effects of other proteins that regulate their activities. Specifically, Cdc42 participates in

specifying dendrite initiation site (Kamiyama et al., 2015), while RhoA and Rac1 function in axon guidance by directing the contraction and protrusion of growth cones (Spiering & Hodgson, 2011). They perform these functions by propagating signal transduction from cell surface receptors to other intracellular effectors that modulate actin cytoskeleton (Kotelevets & Chastre, 2020). Many studies have examined the actions of small GTPases in axonal growth and development, but much is still unknown of their functions in dendrite development especially their involvement in dendritic arbor patterning.

As a first step to dissecting the potential roles in the development of Tm20 neurons, we examined their active and inactive locked forms (i.e. constitutively active (CA) and dominant negative (NA) respectively). We did not observe any significant effects in dendritic field sizes expressing either dominant negative or constitutive active forms of Rho1 and Rac1 in Tm20. However, *Rac1* mutant Tm20 neurons had significantly perturbed dendritic patterning; *Rac1* mutant Tm20 developed aberrantly enlarged dendritic field sizes slightly lower than but not significantly different from that of *Dscam2* null and with fewer ectopic main dendritic branches ( $4.81 \pm 0.34$  DFS (62.3%), 2 MMDB (29.5%),  $n = 17$ , Figure 12D, D' and E). In addition, *Rac1* mutant Tm20 neurons still perturbed the dendritic planar projection directions of Tm20 neurons (18%, Figure S2E).

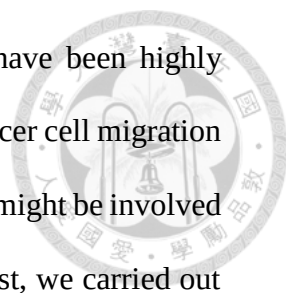
Rac1 has been implicated in several cell biological processes including cell proliferation, cell survival, cell-to-cell contacts, cell motility and invasiveness (Kotelevets & Chastre, 2020). Rac1 in its on state (i.e. GTP-bound) decreases the incidence of depolymerization of actin filaments, while stimulating the production of new actin filaments and promoting the uncapping and extension of already synthesized filaments (Newsome et

al., 2000). Rac1 acts downstream Sema3A-Nrp1/PlxnA4 signaling pathway to control cortical neuron basal dendrite arborization (Danelon et al., 2020), activation of Rac1 regulates the development of dendritic spines in rat primary cortical culture (Mi et al., 2017). However, due to the weak phenotypes observed in Rac1 mutants, it is still unclear whether and how other members of the small GTPases might be involved in Tm20 dendritic morphology. We therefore proceeded to examine mutants of Rho1 and Cdc42. We however, did not record nor observe any significant aberration in dendritic phenotypes in the similitude of Dscam2's (Figures 12B – 12C').

Interestingly, we found that overexpressing wild-type Rac significantly rescued *Trio* mutants' dendritic phenotypes but not the axonal phenotype. We noted that Trio with intact GEF1 domain completely rescued axonal phenotype (Figure S2D). Our submission is that Rac1 may likely regulate axonal development exclusively and that their activation by Trio confers on them more effective involvement in axonal growth and termination in addition to other factors. We therefore, deemed them unlikely to be actively occupying a significantly leading role in Tm20 dendritic patterning. In conclusion, Rac1 is the downstream effector for Trio in Dscam2 signaling mechanism for dendritic patterning.

### **3.8 Src genetically interacts with Dscam2 to pattern Tm20 dendritic morphology**

In an attempt to determine the potential molecule downstream Rac1 activation in the proposed Dscam2 pathway, we further identified the non-receptor tyrosine kinase, Src42, whose knock-down resulted in aberrant dendritic expansion phenotypes similar to Dscam2's (Figures 13B – C', F). Src kinases are well-known oncogenes involved in different intracellular signaling pathways. They are capable of regulating various aspect of cell physiology either by autophosphorylation or by phosphorylating their



downstream effectors (Anguita & Villalobo, 2018). Their actions have been highly implicated in different cancer cells where they are found to control cancer cell migration and infiltration (Guarino, 2010). We attempted to understand how Src might be involved in Dscam2 signaling mechanism to pattern dendritic morphology. First, we carried out genetic interaction between Src and Dscam2. Our results using weak RNAi lines to generate double knockdown of *Dscam2* and *Src42* showed that both genes genetically interact to restrict dendritic field sizes and pattern the dendrite projection direction of Tm20 neurons (Figures 13D and D'). Whether a physical association exist between these two proteins is yet to be proven. Besides, Rac1 has been shown to interact with Src kinases to propagate signal transduction (Chang et al., 2011; Servitja et al., 2003). Src is able to phosphorylate Rac1 guanine nucleotide exchanger, Trio, thereby modulating their activities in controlling actin cytoskeletal dynamics in different cell physiology (Bandekar et al., 2022). Therefore, it is also possible that Rac1 regulates Tm20 dendrite development by interacting with Src. Altogether, our findings suggest that Src42 kinase activity is essentially required in Tm20 dendrite patterning.

A recent study claimed that Dscam2 signaling involves PI3 Kinase-dependent endosomal pathway in suppressing synaptic strength at the neuromuscular junction of the fruit fly (Odierna et al., 2020). We then presumed that PI3K might also be a potential interacting partner of Dscam2. To confirm this assumption, we examined *Pi3k<sup>df</sup>* mutants Tm20s and assessed their dendritic morphologies. We indeed found that Pi3K is likely involved in Tm20 dendritic patterning by regulating their planar projection directions. However, their effects on Tm20 dendritic field sizes appeared opposite to that of Dscam2's; they rather contribute to promoting the expansion of receptive field sizes instead of restricting them (Figure 13E and E'). This suggests that they are likely not in the same linear pathway with Dscam2. One previous study had shown that PI3 kinase

functions to promote elaboration of dendritic arbors in wide-field amacrine medulla neurons, Dm8 (Luo et al., 2020). Therefore, we regard it unlikely that they are downstream to Dscam2 in dendritic field size control. Nonetheless, we propose that Dscam2 interacts with Src kinase to elicit signal transduction that is required to pattern the development of dendritic arbors.

## CHAPTER FOUR



### 4.0 DISCUSSION

#### **4.0 Signaling mechanisms of Dscam2**

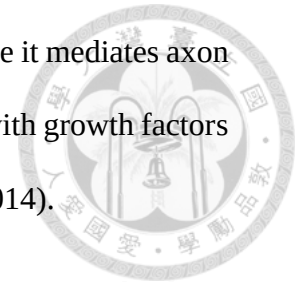
In this thesis, we unveil a potential signaling mechanism of the cell adhesion molecule, Dscam2 in guiding the assembly of the neural circuit during CNS development. Having found that Dscam2 restricts expansion of dendritic field sizes not by transcriptional regulation, we delved into the potential signaling mechanism through which it mediates its functions. We showed that Dscam2 restricts expansion of dendritic arbors via homotypic repulsive interaction between neighboring Tm20 neurons that transmits signal essentially through its cytoplasmic domain. We further speculated and found that this signal transduction is propagated by intracellular signaling molecules that act in similar pathway with Dscam2 to regulate dendritic patterning during morphogenesis.

#### **4.1 Trio instructively regulates dendritic field sizes**

Our data suggests that Trio, a guanine nucleotide exchange factor (GEF), plays a pivotal role in regulating the development of Tm20 dendrites. GEFs are known for their ability to catalyze the exchange of GDP for GTP on small GTPases, thereby activating them and initiating downstream signaling pathways (Cherfils & Zeghouf, 2013). In the context of dendritic development, the activity of small GTPases like Rac1 and Rho1 is crucial for cytoskeletal dynamics, which in turn influence dendrite morphology (Gonzalez-Billault et al., 2012).

The observation that overexpression of Trio's nucleotide exchange function affects Tm20 dendrite morphogenesis underscores the importance of precise regulation of GTPase activity during this process (Figure 11). Previous studies have demonstrated the

involvement of Trio in different aspect of neuronal development where it mediates axon outgrowth and guidance and also synapse physiology by interacting with growth factors or guidance cues like cell adhesion molecules (Schmidt & Debant, 2014).



The finding that either of Trio's GEF domains may be involved in restricting dendritic field sizes suggests a complex interplay between different signaling pathways mediated by distinct GTPases. This was similar to the rescue scenario by both the two GEFs of Trio observed in the work of Kashef and colleagues (Kashef et al., 2009) where Trio serve as downstream to x-cadherin-11 in mediating protrusive motility of cranial neural crest cells in *Xenopus*. Besides, these rescue abilities could suggest that Trio functions in dendrite development by utilizing many functional domains with a possibility of partial functional redundancy with its GEF domains. That necessitated the generation of single point mutations on each GEF domain to disrupt their specific catalytic function and decipher their contribution in Tm20 dendrite development.

The subsequent investigation into the specific roles of Trio's GEF domains through point mutations provides valuable insights (Figure 10). The discovery that the GEF1 domain is indispensable for Trio's function in restricting dendritic arbor elaboration highlights the selective involvement of Rac1 activation in this process. Rac1 is known to promote actin polymerization and branching, which are essential for dendritic growth and complexity, and previous studies have shown that Trio regulates dendrite outgrowth essentially through Rac1-mediated activation (Briançon-Marjollet et al., 2008; Neubrand et al., 2010). In our study, we showed that Trio rather restricts elaboration of Tm20 dendritic arbors via Rac1-specific GEF domain (Figure 10H). This discrepancy may suggest that different cells utilize similar molecule in divergent fashion to pattern their morphology during development. Nonetheless, the association between Trio's

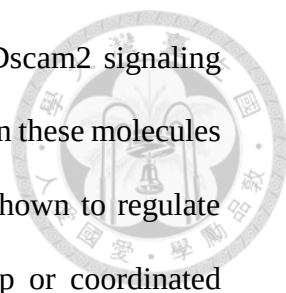
GEF1 domain and Rac1 activation aligns with previous studies in neurite development in *Drosophila*, where Trio has been implicated in regulating Rac1-mediated cytoskeletal dynamics during neuronal development (Awasaki et al., 2000). This finding not only adds to our understanding of the molecular mechanisms underlying dendritic morphogenesis but also suggests potential therapeutic targets for conditions characterized by aberrant dendrite development, such as neurodevelopmental disorders.

Overall, these finding sheds light on the intricate signaling networks involved in dendrite morphogenesis and highlights the importance of Trio and its GEF1 domain-mediated Rac1 activation in this process. Further research into the downstream effectors and regulatory mechanisms of Trio signaling may uncover novel insights into neural development and potential avenues for therapeutic intervention.

#### **4.2 Rac1 activation is indispensable**

The results indicate a potential interaction between Dscam2 and Src, which in turn activates Rac1, a small GTPase critical for actin cytoskeleton dynamics during Tm20 dendrite morphogenesis. This suggests a complex signaling network underlying dendrite development, where Dscam2 may serve as a key regulator orchestrating cytoskeletal rearrangements through the activation of Rac1 via Src.

Small GTPases, such as Rac1, Rho1, and Cdc42, are known to regulate various aspects of actin cytoskeleton dynamics by acting as molecular switches. In this context, the observed lack of active involvement of Rho1 and Cdc42 in field size restriction during Tm20 dendrite development suggests a selective role for Rac1 in this process. However, it's important to acknowledge that Rho1 and Cdc42 may still play roles in other aspects of Tm20 development, highlighting the complexity and redundancy within signaling pathways regulating dendrite morphogenesis.



The proposed interaction between Src and Rac1 in the context of Dscam2 signaling aligns with previous studies indicating the regulatory interplay between these molecules in cellular physiology, particularly in cell migration. Src has been shown to regulate Rac1 activity, and vice versa, suggesting a potential feedback loop or coordinated regulation between these signaling molecules (Ouyang et al., 2008). However, in this study, we are yet to decipher the hierarchy nor elucidate a linear signaling between Rac1 and Src in Dscam2 signaling pathway for dendritic patterning. The possibilities remain that either of them could be an activator or effector.

Overall, these findings provide valuable insights into the molecular mechanisms governing dendrite morphogenesis, with implications for understanding neurodevelopmental processes and potential avenues for therapeutic intervention. Further investigation into the precise molecular interactions and downstream signaling pathways regulated by Dscam2, Src, and Rac1 will be essential for elucidating the full scope of their roles in dendrite development and neuronal function.

#### **4.3 Intracellular signaling requires activation by tyrosine kinases**

The non-receptor tyrosine kinase Src42 regulates the dendritic morphology of Tm20 neurons as suggested by its RNAi-mediated knockdown (Figure 10C). Src kinases perform diverse cellular roles; they function by autophosphorylation or by phosphorylating other proteins. Several cancer studies have identified the unique roles of Src kinases in cellular differentiation, migration and invasion (Anguita & Villalobo, 2018). In Tm20 dendrite morphogenesis, Src42 genetically interacts with Dscam2 to pattern Tm20 dendrite development (Figure 10). Contrary to the suggestion that Dscam2 mechanism is PI3K-dependent (Odierna et al., 2020), we determined that Pi3K does not involve in the pathway utilized by Dscam2 to restrict dendritic field sizes. However,

Pi3k could be involved in the dendritic planar projection directions of Tm20 neurons, and rather promotes elaboration of their receptive field sizes (Figures 10E and 10E'). Previous studies indeed showed that PI3K promotes elaboration of dendritic field sizes in the wide-field amacrine distal medulla neurons, Dm8 (Luo et al., 2020). Therefore, we deemed it unlikely that Dscam2 restricts dendritic field expansion through Pi3 kinase but rather through Src kinases.

#### **4.4 Molecular diversity of Dscam2 is required for its function**

Many previous studies have shown that the actions of Dscam2 in regulating dendrite morphogenesis is usually in most scenario, isoform specific. We therefore tested whether the roles of Dscam2 in Tm20 is isoform specific or isoform dominant. Both isoforms are expressed in Tm20 neurons. Single isoform mutants' expression reveals that Dscam2 10B is essentially required in Tm20 for dendritic field size restrictions. Both Dscam2 10A and Dscam2 10B are required for planar projection direction of Tm20 dendrites (Figure 5). This isoform specificity is confirmed by the RNA binding protein, Muscle blind which is the splicing factor. From the single cell transcriptomic analysis of Tm20 neurons, we noted that Dscam2 and Mbl are expressed throughout their developmental time points with less expression in Dscam2 compared with Mbl. But, at 40 hr after pupal formation (40h APF), Mbl expression decreases while Dscam2 expression increase. We suspect a switch in isoform at this developmental time point. The splicing factor, Mbl is known to repress the expression of 10A isoform and promote the expression of 10B isoform. We therefore, conclude that both extracellular domain isoforms of Dscam2 play certain roles Tm20 development at certain developmental stage of morphogenesis but there is an essential requirement for isoform 10B for appropriate dendritic arbor as reflected in the rescue experiments (Figures 7 and 8).

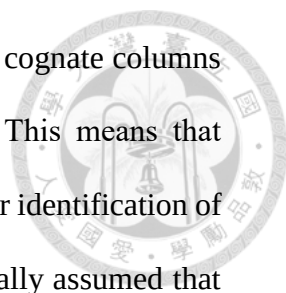
#### 4.5 Dscam2 restricts elaboration of dendritic arbors

Previous studies proposed that Dscam2 mediates homophilic interactions between neurons by adhesion or repulsion in a context-dependent manner. It could therefore be assumed that each Tm20 neuron in the medulla neuropil is able to restrict its dendritic field size by three-dimensional homotypic repulsion of neighboring cells during development. However, our preliminary data on the non-cell autonomous effects of Dscam2 show that this is likely one of the mechanisms but not exclusive.

It could be thought that Dscam2 functions to pattern dendritic architecture by regulating Tm20 cell transcription during development. However, we found that Dscam2 does its actions in Tm20 neurons without altering their cell fate as judged by the expression of the Tf Toy in Dscam2 mutants and lack of the expression of Dichaete which is normally absent in adult Tm20 neurons (Figure 4). In addition, the OrtC1 driver used in this study is a key driver of Tm20 neurons. — By these findings, we have clearly examined the cellular functions of Dscam2 in developing Tm20 neurons and found that Dscam2 is cell-autonomously required in Tm20 projection neurons for the development of their proper dendritic field sizes, columnar restrictions of their main dendritic branches and their correct planar projection directions. However, whether and how these functions are exclusively through homotypic repulsion is still yet to be well-elucidated.

#### 4.6 Dscam2 regulates synaptic partner specificity and selectivity

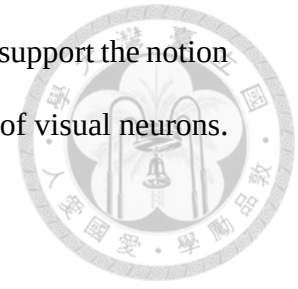
Wiring specificity is required for effective nervous system functions (Li et al., 2018). Therefore, we investigated the likely connectivity consequences that could result from aberrantly large receptive field sizes which is a consequence of Dscam2 mutation. Through the activity-dependent synGRASP method, we clearly showed that Dscam2 regulates the connectivity of Tm20 neurons with their synaptic partners. The aberrant



synapses *Dscam2* mutant Tm20s make with L2 neurons outside their cognate columns suggest a random wiring due to loss of specificity (Figure 7B'). This means that *Dscam2*'s homophilic recognition properties is also required for proper identification of synaptic partners during the assembly of the neural circuits. We initially assumed that since *Dscam2* mutants developed enlarged dendritic field sizes (Figure 3C), it would also mean that they developed unusual capability for more inputs from their presynaptic partners. However, our findings disproved that assumption because even though *Dscam2* mutants have aberrantly enlarged receptive field sizes, at their full adulthood (which should be the stage for highest synapses), the number of inputs they ultimately received is lower compared to their controls (Figures 7E). In addition, we discovered that *Dscam2* mutant Tm20 loss their synaptic partner specificity by making aberrant connections with R7 photoreceptor which on the normal situation is not a presynaptic partner (Figure 7D). In addition, the fact that loss of *Dscam2*'s functions resulted in connection with non-synaptic partner, R7, also suggest that *Dscam2* could be required not just for specificity in partner choice but also for selectivity of appropriate synaptic partners. It is an ongoing debate that whether recognition molecules mediate specificity or selectivity of synaptic partners. Evidences for both phenomena exist while it remains unclear which could be predominant (Ashley et al., 2019; Sanes & Zipursky, 2020; Xu et al., 2019)

We infer from these observations that *Dscam2* regulates the wiring properties of Tm20 neurons, however, how *Dscam2* does these functions is still not totally understood. We therefore, propose that *Dscam2* controls the synaptic functions of Tm20 neurons by modulating the conformational changes of the synaptic proteins around the synaptic sites. In the future, we will critically examine the ultrastructure of *Dscam2* mutants in

comparison with the wild types. Nevertheless, these findings strongly support the notion that Dscam2 controls synaptic input precision and partner specificity of visual neurons.



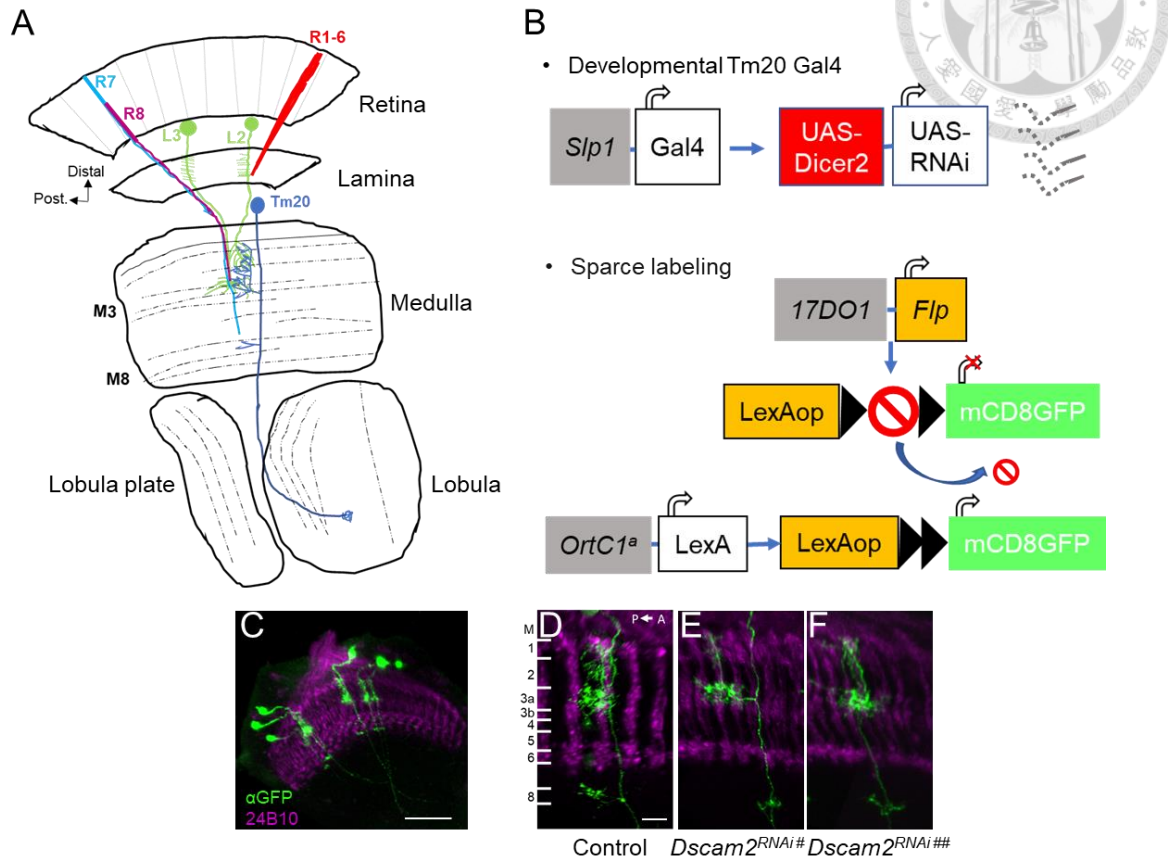
#### **4.7 Model of Dscam2 signaling mechanisms**

This study proposes a signaling mechanism by which Dscam2 restricts the expansion of dendritic field sizes using the medulla projection visual neuron Tm20 as a model. The preference for extracellular isoforms during development initiates the first stage of this mechanism. Specifically, the Dscam2 10B isoform is essential for homotypic interactions while the cytoplasmic domain is necessary to initiate intracellular signaling. Activation of Trio is likely required to propagate signals intracellularly through Rac1 and Src activations that would lead to the regulation of actin cytoskeletal dynamics during dendrite morphogenesis (Figure 14).

#### **4.8 Concluding remarks**

Moving forward, it is important to identify the potential actin modulatory proteins that are regulated by Dscam2 signaling. Our preliminary findings point towards the involvement of actin nucleators – cappuccino and spire whose knockdown perturbed the dendritic patterning of Tm20 neurons and also showed some potential genetic interactions with Dscam2 and with its interacting partner. However, further evidences of association and involvement would be essential to substantiate this.

## Figures



**Figure 1. Dscam2 is required for proper dendrite patterning of Tm20 neurons.**

(A) Schematic diagram of *Drosophila* visual system comprising the retina (divided into ommatidia) and its optic lobe neuropils; lamina (arranged as cartridges), medulla (organized into layers and columns), lobula plate (Lop) and Lobula. R7 and R8 (light blue and magenta, resp.) projects from the retina through the lamina to reside in the medulla. R7 axons terminate at the M6 layer while the R8 axonal terminal is at the M3 layer of the medulla. The lamina neurons, L2 and L3 (neon green) extend the axons into the medulla and elaborate their dendritic arbors at the M2 and M3 layers of the medulla respectively. Tm20 neuron (Marrs et al.) has its cell body outside the medulla but projects its axons through the medulla neuropil to terminate at the Lobula.

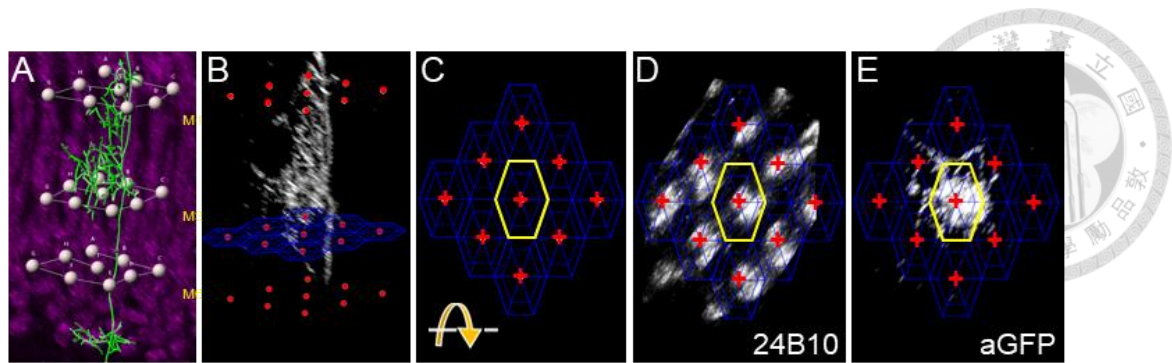
(B) Schematic of the design for RNAi-mediated screening of potential regulators of Tm20 neuron; two binary systems- UAS/Gal4 and LexA/LexAop to enable knockdown of specific genes during the development of Tm20 neurons and the visualization of their adult cells, respectively, for dendritic morphological assessment. The Sloppy-paired 1(Slp1) enhancer was used to drive RNAi expression in developing Tm20 neurons. Slp is a transcription factor that is expressed at the early stage of Tm20 neuron. Sparse labeling of adult Tm20 neurons was achieved through stochastic expression of flipase driven by the 17D01 promoter which enables the flip out of the stop cassette in order that OrtC1-driven LexAVP16 could drive GFP expression in adult cells.

(C) Sparsely labelled Tm20 neurons. A single Tm20 has its cell body outside the medulla and projects its axon through the medulla neuropil to terminate at the lobula of the optic lobe. Anti-GFP to label Tm20 neurons (green cells) while the photoreceptors (magenta) were visualized by 24B10 anti-body. Scale bar, 30µm.

(D) UAS-Dicer2 control Tm20 neurons with characteristic dendritic features; dendritic arbor within M1-M3 medulla layers.

(E) and (F) Dscam2 RNAi-mediated knock down in Tm20 neurons altered their normal dendritic patterning suggesting that they are required for Tm20 dendritic morphogenesis.

# TRiP.HMC03411, ## GD111. Scale bar, 5µm.



**Figure 2. Registration and affine transformation for quantification of dendritic field sizes.**

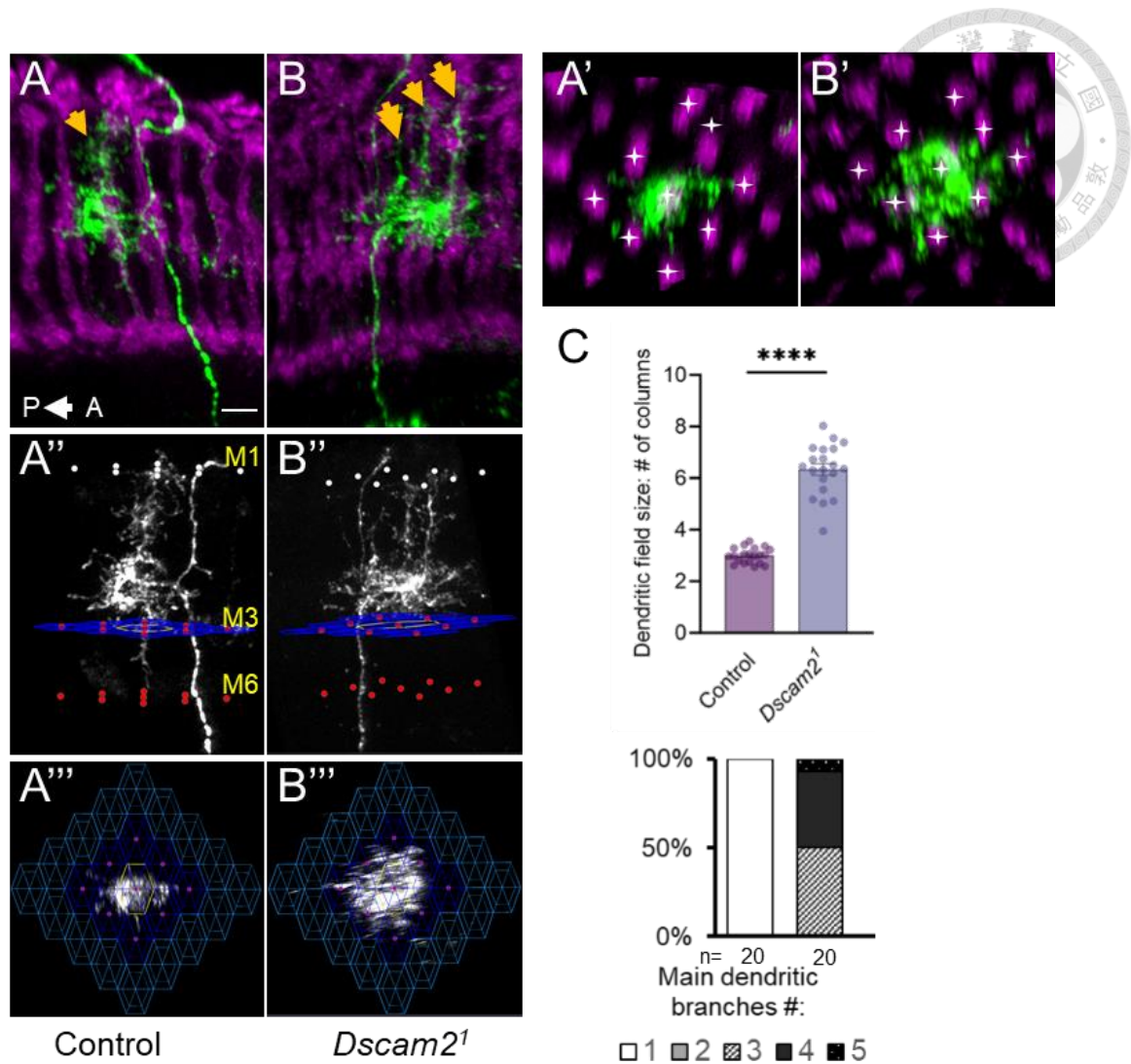
(A) Registered single Tm20 neuron assigned to 27 reference columns (white spheres) after neuron tracing (Marrs et al.). The photoreceptors (magenta) serve as the reference columns with nine reference assigned reference points at each medulla layer M1, M3 and M6 respectively.

(B) Tm20 neuron after affine transformation.

(c) Horizontal view of the hexagonal array of reference columns.

(D) photoreceptors used as reference columns in the hexagonal array stained with 24B10 antibody.

(E) dendritic arbor of Tm20 neuron restricts its dendritic field size to its cognate column with minimal lateral extensions to neighboring columns.

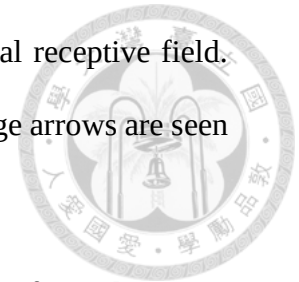


**Figure 3. The cell adhesive receptor *Dscam2* cell-autonomously restricts dendritic field sizes of Tm20 neurons.**

(A) Dorsal-ventral view of single Tm20 neuron generated by mosaic method shows the normal dendritic morphology, with their dendritic arbor surrounding approximately single photoreceptor which is surrounded by eight other photoreceptors seen in proximal-distal view (A'). White plus indicates the reference columns at the M3 medulla layer.

(B) Single *Dscam2* mutant Tm20 neuron shows aberrant dendritic phenotypes compared to its control; enlarged dendritic field size (DFS) as seen in (B' and B''') in which

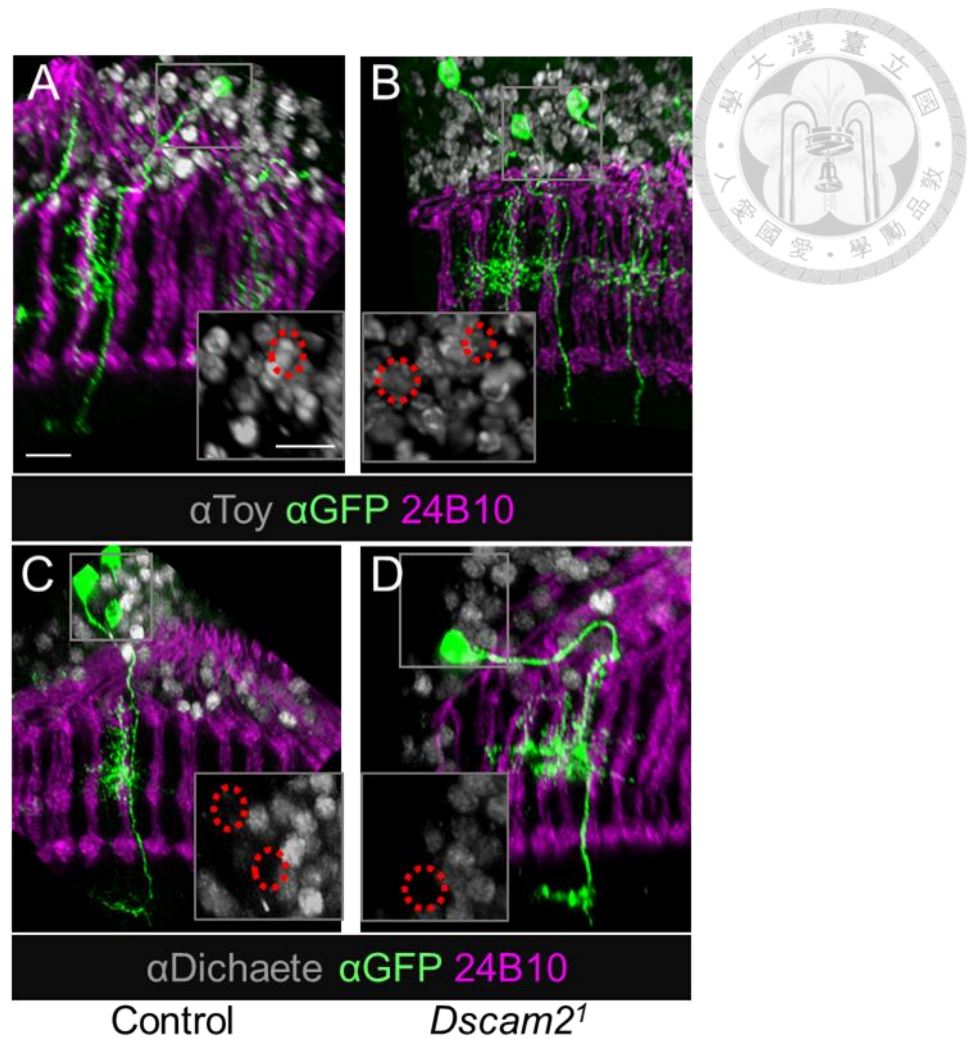
dendritic arbor extends beyond the nine photoreceptors of the normal receptive field. Multiple main dendritic branches (MMDB) indicated by double orange arrows are seen to develop in *Dscam2* mutants



(A'') Dorsal-ventral view and (A''') proximal-distal view of affine-transformed neurons of (A) after registration; twenty-seven photoreceptors from M1, M3 and M6 medulla layers serve as reference columns. Each medulla column is represented by a hexagon. Tm20 neurons project from anterior to posterior (white arrow), they restrict their dendritic arbor to average of three medulla columns having their main dendritic field within M1-M3 medulla layers.

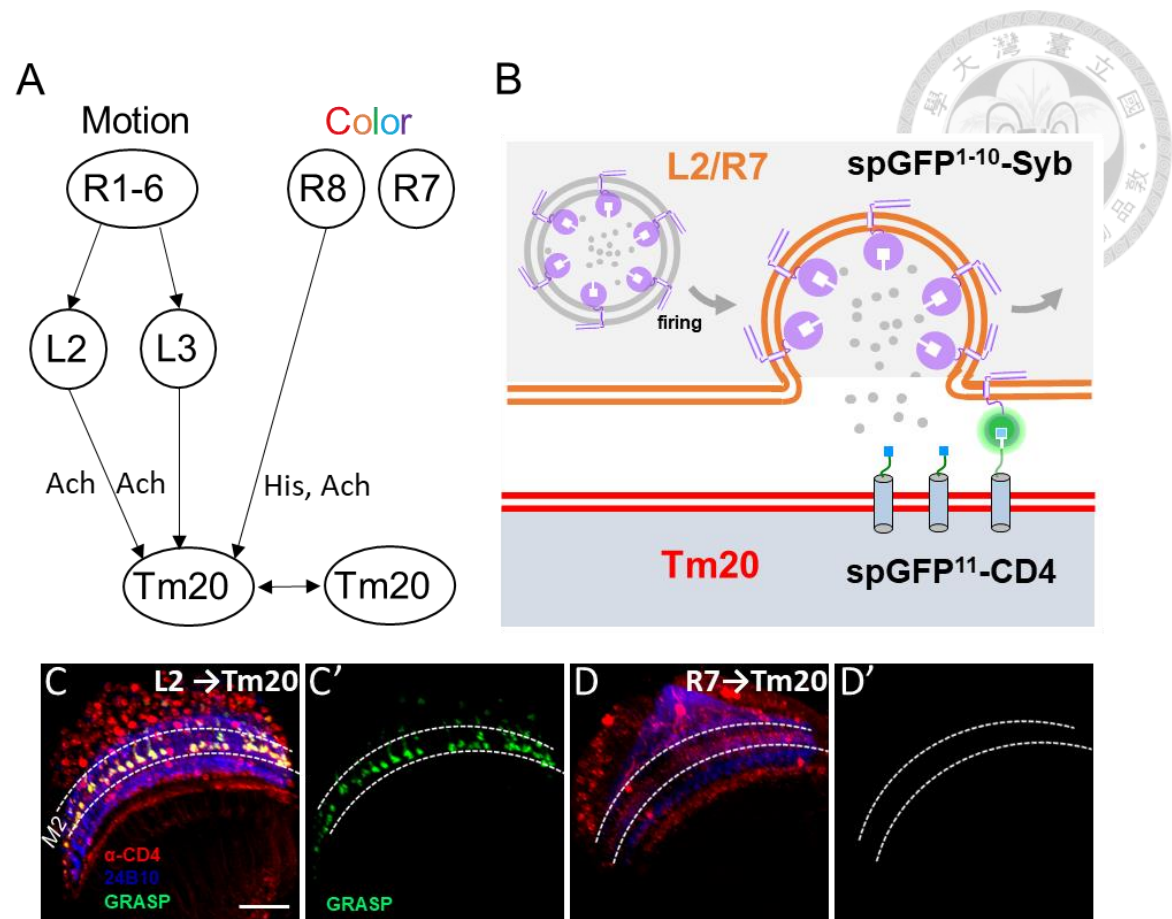
(B'') Dorsal-ventral view and (B''') proximal-distal view of affine transformed neurons of (B) showing the disruption of proper dendritic morphology of Tm20 neurons in *Dscam2* mutants. Scale bar, 5 $\mu$ m (A-B')

(C) Quantification of dendritic phenotypes in *Dscam2* mutant and its control; *Dscam2* mutant has significant increase in dendritic field size and generates multiple ectopic main dendritic branches compared to its control. \*\*\*\*P<0.0001.



**Figure 4. *Dscam2* mutant Tm20 maintains normal cell fate.**

(A-D) Expression of transcription factors Toy (twin-of-eyeless; A, B) and Dichaete (C, D) was examined by immunohistochemistry (grey). (A, C) Control and (B, D) *Dscam2* mutant Tm20 clones were generated and labeled by the mCD8GFP marker (Marrs et al.) using the MARCM technique. Cell bodies position are indicated by red circles to show presence of Toy and absence of Dichaete in controls and *Dscam2* mutants. Photoreceptor axons, visualized by 24B10 antibody staining, were used as landmarks. As in the control, *Dscam2* mutant Tm20 expressed Toy but not Dichaete. Inset: high magnification views of the main images. Scale bar: 10µm and 5µm for the main image and insets respectively.



**Figure 5. Connectivity of Tm20 neurons.**

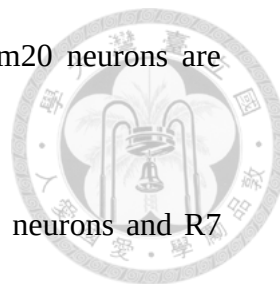
(A) Synaptic partners of Tm20 neurons; it receives inputs from and release outputs to several optic lobe neurons including lamina neurons, photoreceptor, medulla projection neurons, distal medulla neurons and medulla intrinsic neurons.

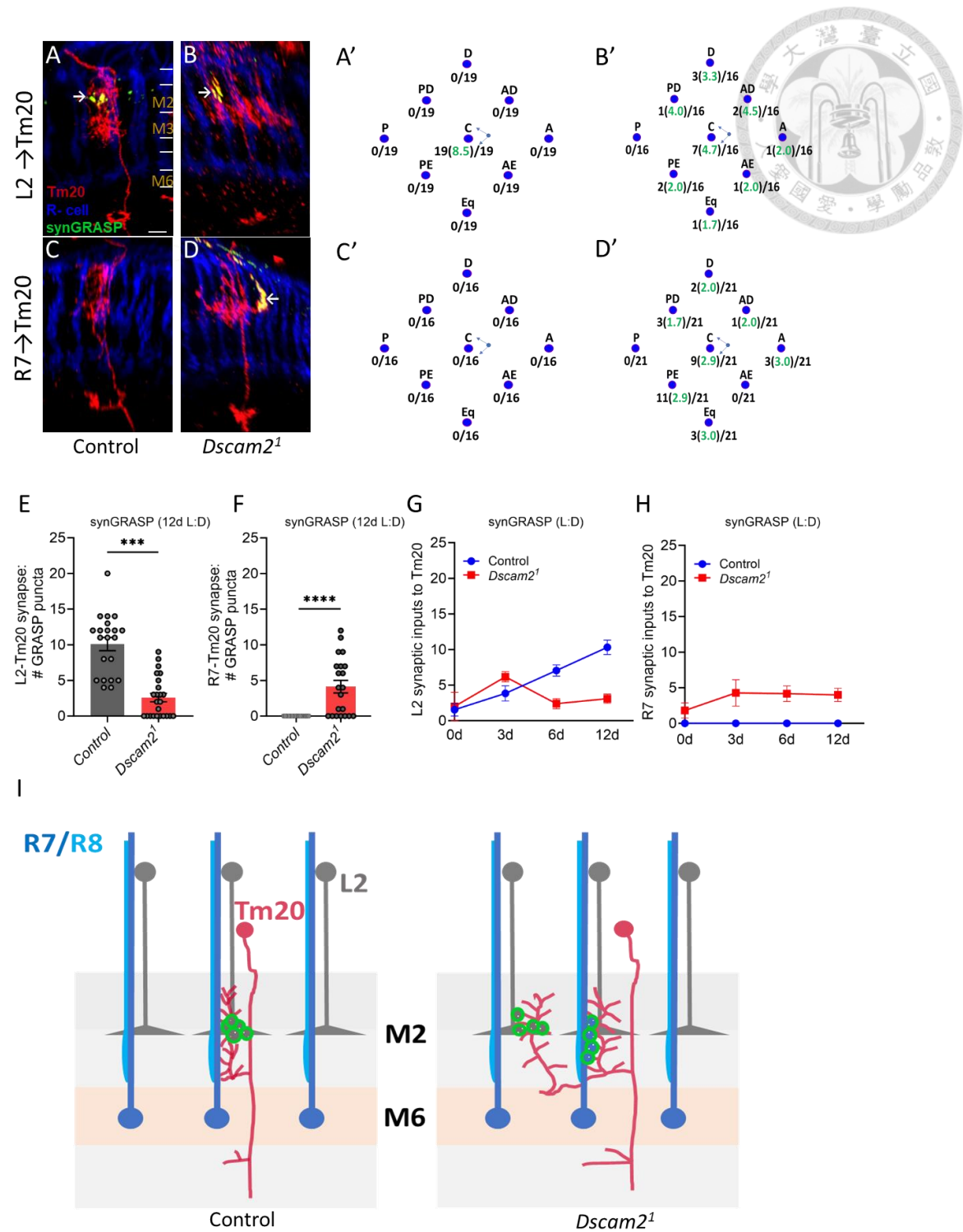
(B) schematic of synaptic activity-dependent GFP reconstitution across synaptic partners (synGRASP). Synaptobrevin-tagged splitGFP1-10 fused to the presynaptic vesicles release neurotransmitters upon light stimulation to reconstitute with GFP11 tethered to the receptor channel on the membrane of Tm20 dendrites.

(C and C') Whole pattern activity-dependent GRASP (Marrs et al.) between lamina neurons, L2 and Tm20 neurons (red-labelled cells) to serve as positive control. Photoreceptors (blue) serve as landmark. Synaptic connections between L2 and Tm20

are within the M2 medulla layer (marked by white dash lines). Tm20 neurons are labelled with anti-CD4.

(D and D') No synaptic connections are seen with wild-type Tm20 neurons and R7 photoreceptors. Scale bar, 30  $\mu\text{m}$ .

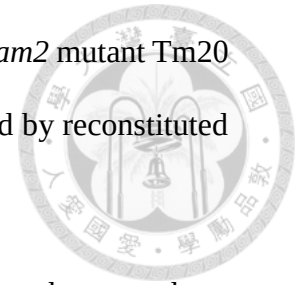




**Figure 6. *Dscam2* mutants make aberrant connections.**

(A) Single cell activity-dependent GFP reconstitution across synaptic partners (GRASP) between Tm20 (red) and L2 lamina neuron within its cognate column. GRASP (Marrs et al.) indicated by white arrow head. Land mark photoreceptors (blue).

(B) Aberrant synapses made by the ectopic main branch of single *Dscam2* mutant Tm20 neuron with L2 at the invaded column. Functional inputs are reflected by reconstituted GFP (Marrs et al.) indicated by white arrow head.



(C) Control for R7-Tm20 GRASP reveals no active synaptic connections between these two cells at any medulla layers.

(D) Ectopic main dendritic branch of single *Dscam2* mutant Tm20 neuron makes aberrant synapses (yellow) with the shaft of R7 photoreceptor at wrong column.

(A', B') columnar inputs from L2 to Tm20 neurons in the control and *Dscam2* mutant resp. at 12days old flies. Medulla columns as represented by the photoreceptors (blue), number of cells (blue), average of columnar active synaptic inputs from L2 to Tm20 neurons (green fonts).

(C', D') Columnar synaptic inputs representation between R7 and Tm20. R7 is not presynaptic partner to Tm20 neurons, therefore, they show no synaptic connections. However, *Dscam2* mutants make several misconnections with R7 by their ectopic dendrites.

(E) Quantification of synaptic GRASP (synGRASP) between L2 and Tm20 neurons at 12-day-old flies under a 12hr light/dark cycle (L:D). \*\*\*P<0.0001.

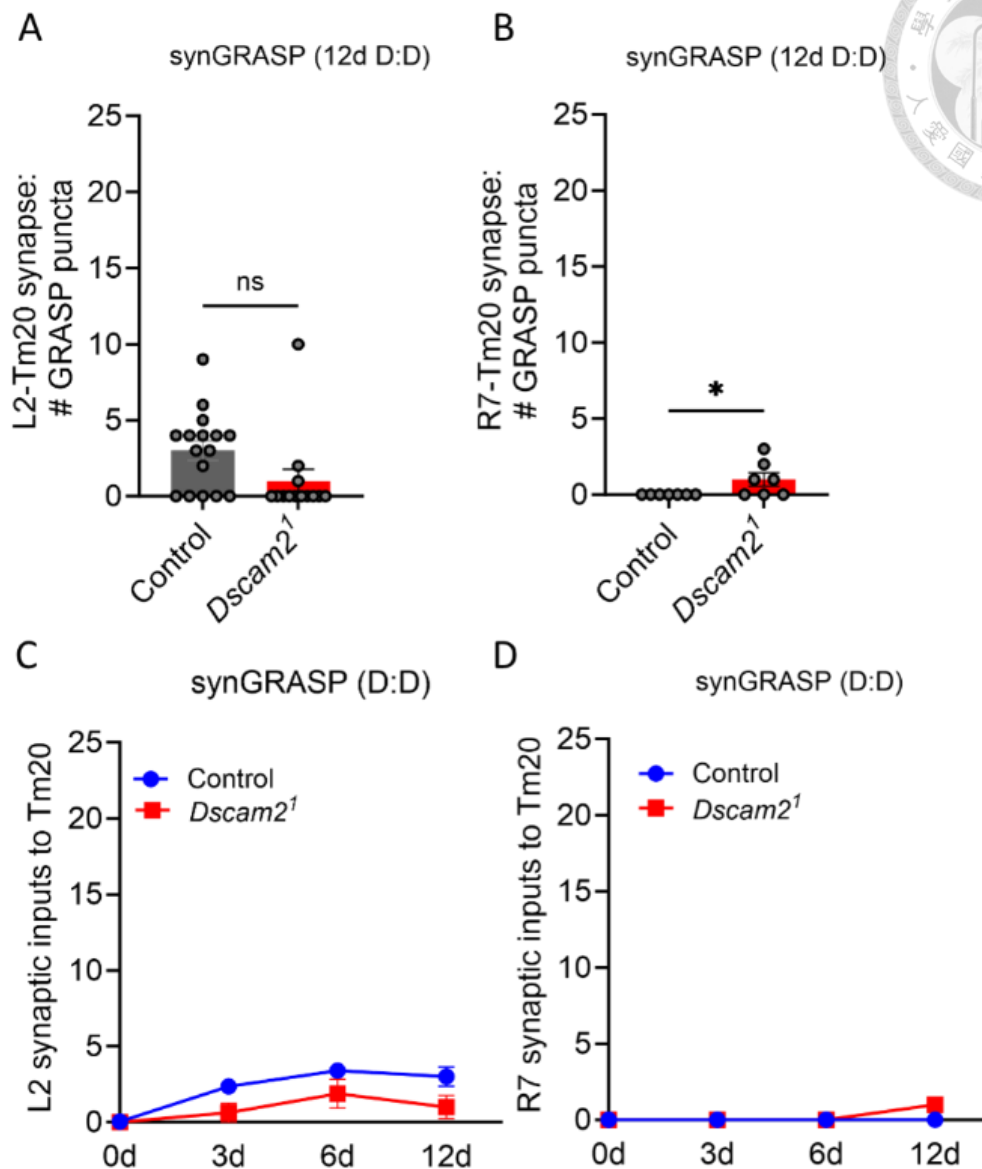
(F) Quantification of synGRASP between R7 and Tm20 neurons at 12-day-old flies under a 12hr light/dark cycle (L:D). \*\*\*P<0.0001.

(G) Kinetics of synGRASP in 0d to 12day old flies; Control shows a gradual increase in active inputs into Tm20 neurons from their presynaptic partner L2. *Dscam2* mutants show increase in synaptic inputs until 3-days after eclosion followed by gradual

decrease till 6-days before stability is achieved in old adult flies. Error bars represent S.E.M.

(H) Synaptic GRASP kinetics of aberrant connections form R7 to Tm20. GRASP signals build until 3-days old and decline afterwards to stabilize through advanced stage of adulthood. Error bars indicate S.E.M. Scale bar, 5 $\mu$ m.

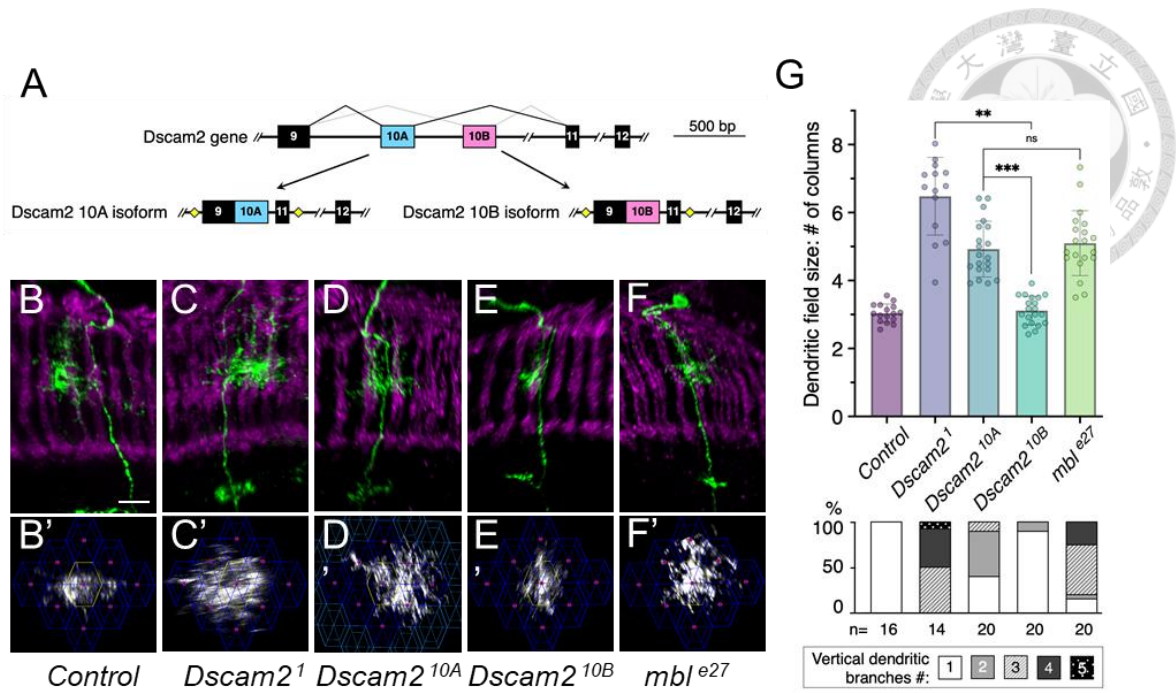
(I) Schematic of connectivity consequences of *Dscam2* mutation. In the control, Tm20 makes synaptic connections with L2 neurons, and at their cognate column (L2-Tm20 GRASP is represented with green circle with gray fill). In *Dscam2* mutants however, aberrant synaptic connections are generated with the axonal shaft of R7 photoreceptors (R7-Tm20 GRASP indicated by green circle with deep blue fill), and ectopic main dendritic branch that invades neighboring column also make aberrant connections with L2.



**Figure 7. Synaptic activities in dark condition.**

(A-B) Aberrant synapses of *Dscam2* mutant Tm20 compared to its control in 24 dark-dark condition from L2 (A) and R7 (B) at 12-day post eclosion period. \* $P < 0.05$ , ns; not significant.

(C-D) Kinetics of synapses under absolute dark condition in L2-Tm20 (C) and in R7-Tm20 (D). x-axis indicates days after eclosion and y-axis represent number of GRASP puncta. Error bars indicate S.E.M.



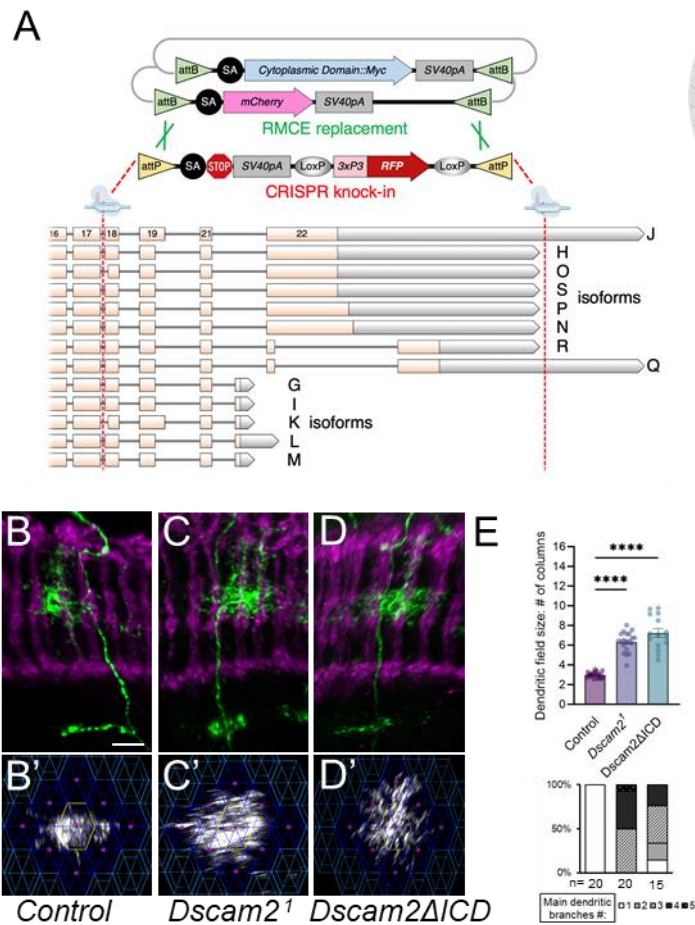
**Figure 8. Extracellular domain isoform specificity.**

(A) Schematic design for generating single isoform mutants of Dscam2. Alternatively-spliced exon 10 generates two isoforms of – Dscam2 10A and Dscam2 10B.

(B-F) Single mutant Tm20 neurons generated by the mosaic method; control (A), *Dscam2* mutant with aberrant dendritic morphology (B), single Dscam2 isoform mutant containing alternatively spliced exon 10A only (C), single Dscam2 isoform mutant having only the exon 10B (D), mutant of the splicing factor, Mbl (E). Scale bar, 5µm.

(B'-F') Affine transformed images of A-E in proximal-distal view.

(G) DFS quantification; *Dscam2* 10B only has no significant difference compared to control, *Dscam2* 10A has significantly increased DFS compared to control but indifferent from *Dscam2* null and *Mbl* mutants whose DFS significantly increased compared to control. \*\*\*P<0.001, \*\*P<0.001, ns; not significant.



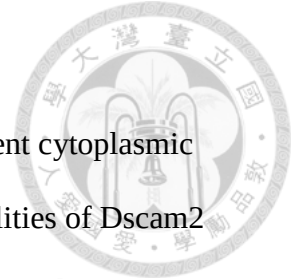
**Figure 9. Cytoplasmic domain requirement.**

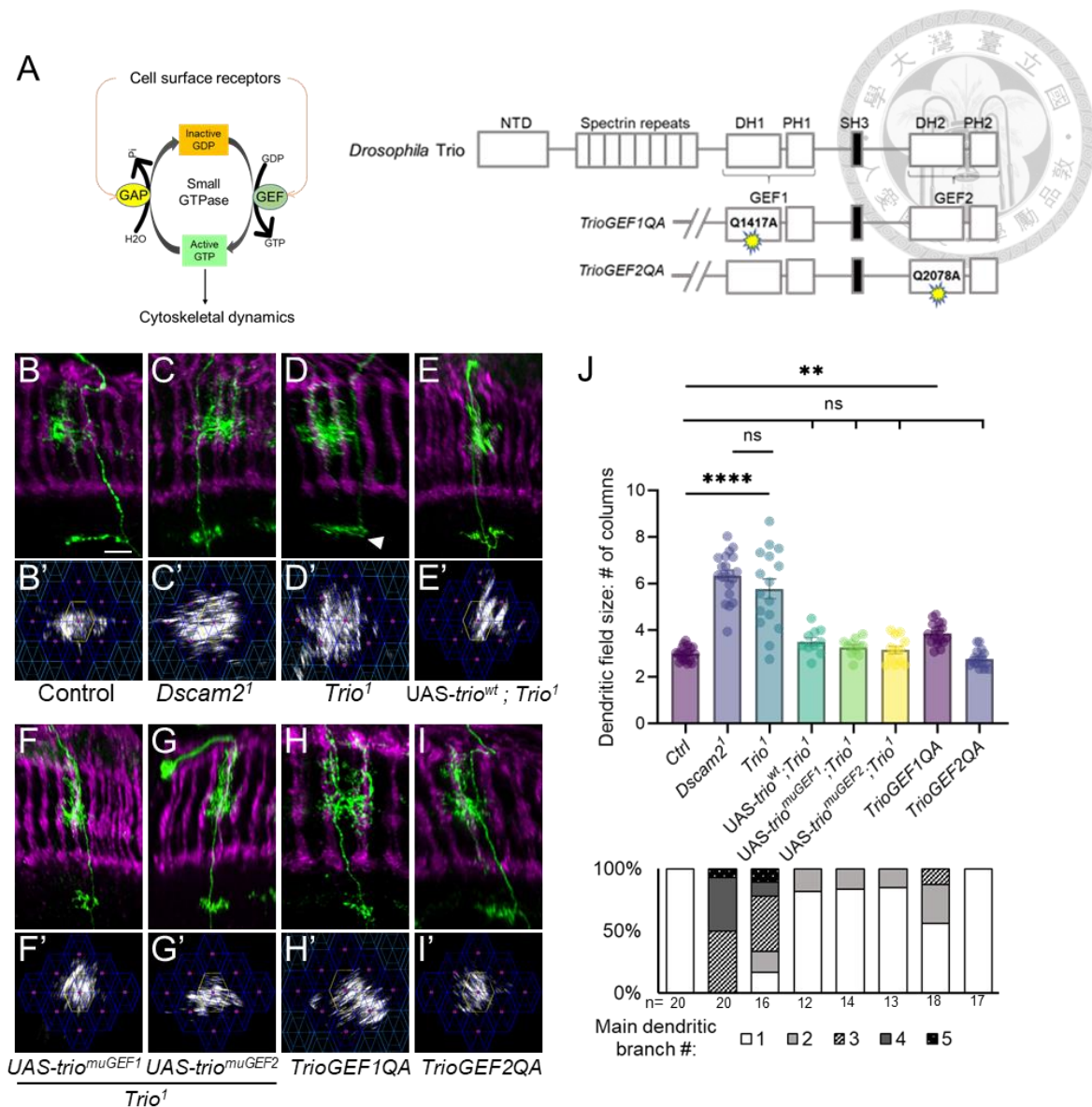
(A) RMCE CRISPR knock-in design for investigating cytoplasmic domain isoform requirement. Cytoplasmic domain span exons 18-22 with variations at exons 18 and 19 to generate short or long forms of transcripts. *Dscam2* intracellular domain deletion is generated by this design to assess the involvement of the intracellular domain regions.

(B-G) Single Tm20 mutants of control (A), *Dscam2* null mutant (C), *Dscam2* intracellular deletion phenocopying *Dscam2* null for all dendritic abnormalities (D), and rescue with Q form (E), rescue with P form (F) and rescue with P form with tyrosine replacement with phenylalanine (G), in proximal-distal view. Scale bar, 5 μm.

(B'-G') dorsal-ventral view of B-G after affine transformation.

(H) Dendritic field sizes quantification of mutants; rescue with different cytoplasmic domain isoforms confer varied degree of rescue to dendritic abnormalities of Dscam2 null. Best rescue was observed with the P-form. Multiple main dendritic phenotypes penetrance for all mutants (panel below H).





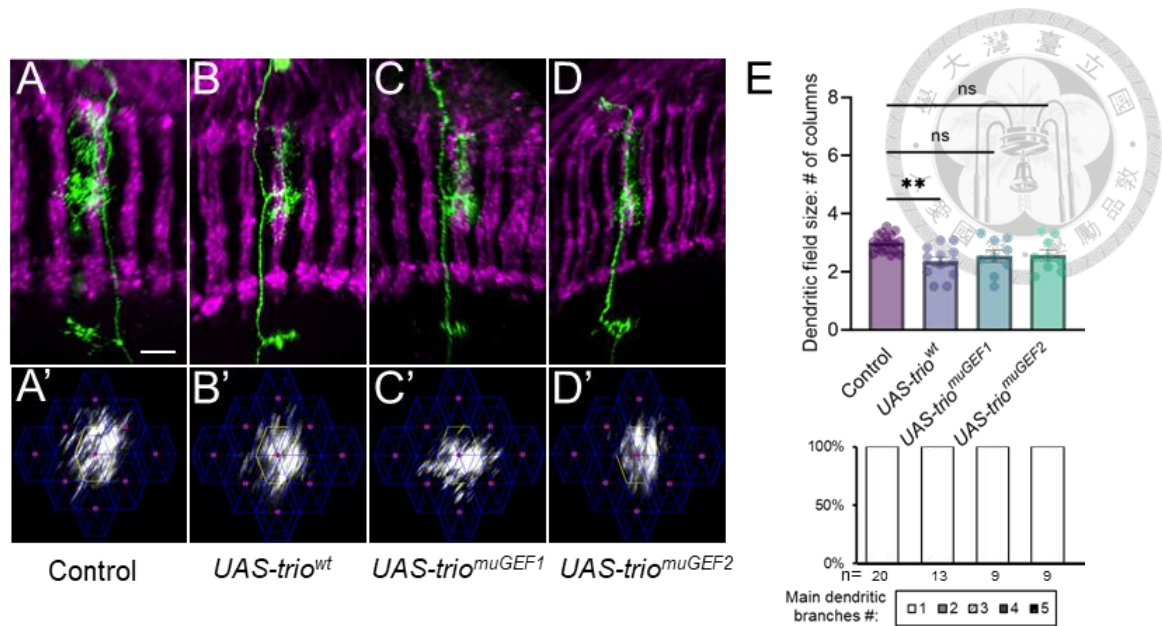
**Figure 10. Trio restricts dendritic arbor through Rac1-specific domain.**

(A) Schematic showing the regulation of small GTPases (left) and the design for generating TrioGEF point mutations (right). Two separate point mutations (yellow star) on Trio GEF domains were introduced to interfere with its nucleotide exchange functions. Point mutations on GEF1 and GEF2 domains of Trio were generated by amino acid exchange to impair the nucleotide exchange function of Trio for Rac1 and Rho1 respectively (details in methods).

(B-I) single Tm20 clones in; control (B), *Dscam2* mutant with aberrant dendritic phenotypes (C), *Trio* mutants phenocopying *Dscam2* null for all dendritic phenotypes, in addition premature axonal termination at the M8 layer (D), overexpression of wild-type *Trio* rescued *Trio* mutant dendritic abnormalities (E), *Trio*<sup>1</sup> rescue with overexpression of *Trio* with defective Rac1-GEF domain (F), rescue with intact Rac1-GEF domain (G), *TrioGEF1QA* mutant, carrying a point mutation that disrupts TrioGEF1 (Rac1-specific GEF) domain phenocopied *Dscam2* mutants' aberrant dendritic morphologies (H), *TrioGEF2QA* mutant, carrying a point mutation that impairs TrioGEF2 (Rho1-specific GEF) domain indifferent from control Tm20 dendritic morphologies (I). White arrow head indicates premature axon termination at M8 layer of the medulla. Scale bar, 5μm.

(A'-H') proximal-distal view of A-H after affine transformation.

(I) Quantification of DFS; overexpression of *Trio* derivatives significantly rescued *Trio* mutant dendritic field sizes. *Trio* and *TrioGEF1QA* mutants DFS significantly increased compare to control. \*\*\*\*P < 0.0001, \*\*P < 0.01, ns; not significant.

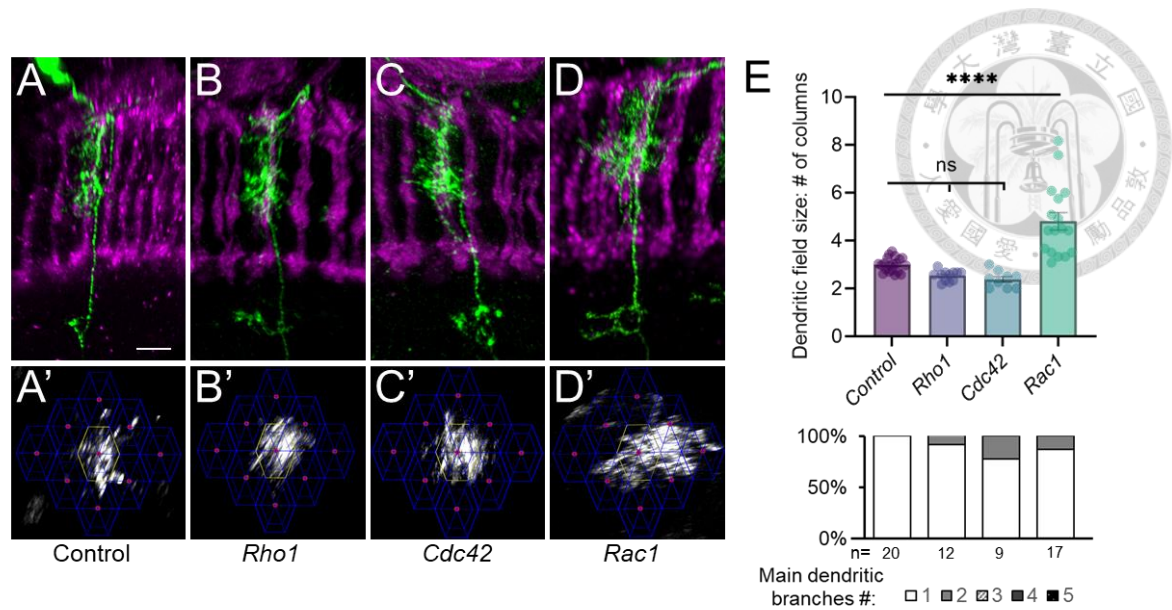


**Figure 11. Overexpression of Trio shrinks dendritic field sizes.**

(A-F) Dendritic field sizes reduced in overexpression of mutant Trio. Single Tm20 of control (A), overexpression with Trio in wild-type Tm20 significantly shrinks receptive field sizes (B), Trio overexpression with defective Rac1 GEF domain (C), Trio overexpression with intact Rac1 GEF domain (D). Scale bar, 5 $\mu$ m.

(A'-D') affine transformed images of A-D in proximal-distal view.

(E) DFS quantification shows decrease in receptive field sizes, significant in *UAS-Trio<sub>wt</sub>* but neither in GEF1 or GEF2 mutants even though there were decreases in the field sizes. \*\* $P < 0.01$ , ns; not significant. No ectopic main dendritic branch in all mutants.

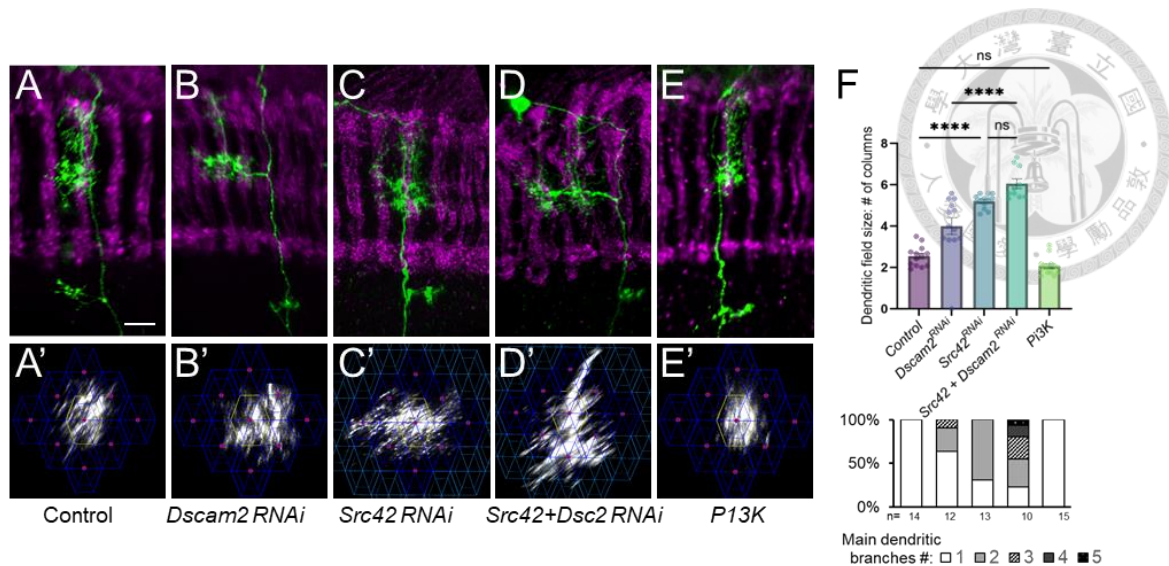


**Figure 12. Dscam2 restricts dendritic field sizes through Rac1 signaling.**

(A-D) Rac1 small GTPase functions Dscam2 signaling pathway in restricting dendritic field sizes. Single Tm20 neurons in wild-type control (A), expression of mutant alleles of Rho1 in Tm20 (B) is indifferent from control dendritic phenotypes, mutant alleles of Cdc42 had no aberrant dendritic phenotypes (C), *Rac1* mutants in (D) showed abnormal dendritic morphologies that were similar is phenotypes to Dscam2's.

(A'-E') Affine-transformed images of A-E used in quantification of dendritic field sizes.

(E) Quantification of DFS shows significant increase with *Rac1* mutant, but not in other mutants, compared to control. \*\*\*\* $P < 0.0001$ , ns; not significant. Scale bar, 5 $\mu$ m.



**Figure 13. Src interacts genetically with Dscam2 to pattern Tm20 dendrite morphology.**

(A) Control with normal dendritic morphology.

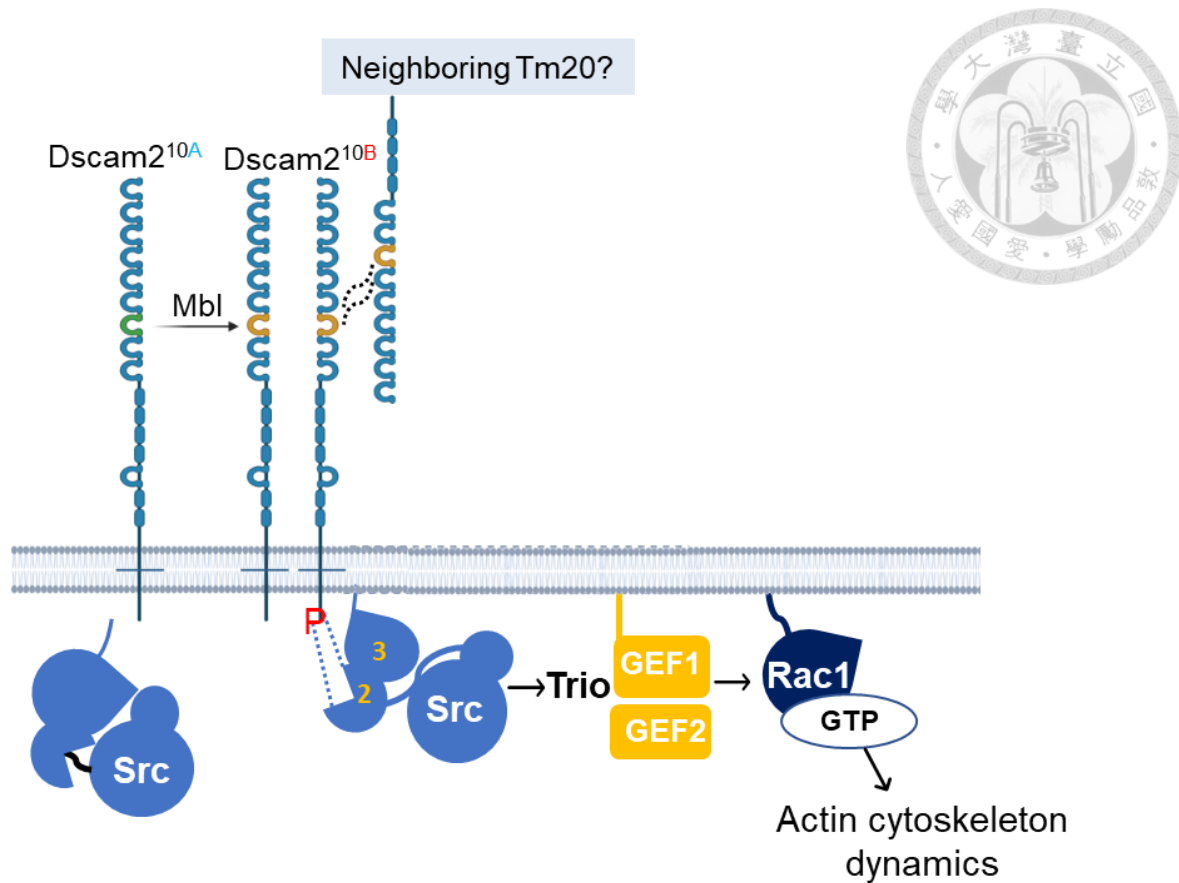
(B-C) *Dscam2* RNAi and *Src4a* RNAi respectively have aberrant dendritic phenotypes indifferent from each other.

(D) Double knockdown of weak RNAi lines of *Dscam2* and *Src42* showed enhanced aberrant dendritic phenotypes suggesting that these two genes genetically interact to instruct appropriate dendritic arbors of Tm20 neurons; hence, could potentially be in the same pathway. Scale bar, 5µm.

(E) *Pi3K* mutants showed no enlarged dendritic field sizes.

(A'-E') Affine-transformed representation of A-D.

(F) Quantification of DFS and ectopic main dendritic branches. \*\*\*\*P<0.0001, ns; not significant.



**Figure 14. Model of Dscam2 signaling pathway.**

Schematic depicting a hypothetical mechanism of the cell adhesive receptor Dscam2 in restricting dendritic field sizes (DFS) and regulating dendritic planar projection direction (DPD). Homotypic interaction between neighboring Tm20 neurons is mediated by the extracellular expression of Dscam2 10B isoform followed by signal transmission through the cytoplasmic domain to activate potential intracellular signaling partners which include non-receptor tyrosine kinase Src, small GTPase Rac1 and its nucleotide exchanger Trio, for rearrangement and reorganization of actin cytoskeleton during dendrite morphogenesis.

Table



Table: Genotype

| Figure                  | Genotype                                                                                                                                                                       |
|-------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 1C-D'                   | <i>UAS-Dcr2; Ort<sup>C1a</sup>-LexA::VP16, R17D01-FLP/+; slp<sup>R35H02</sup>-GAL4, pLot-FSF-CD8GFP/+</i>                                                                      |
| 1E, 1E'                 | <i>UAS-Dcr2; Ort<sup>C1a</sup>-LexA::VP16, R17D01-FLP/+; slp<sup>R35H02</sup>-GAL4, pLot-FSF-CD8GFP/UAS-Dscam2<sup>RNAi TriP.HMC03411</sup></i>                                |
| 1F, F'                  | <i>UAS-Dcr2; Ort<sup>C1a</sup>-LexA::VP16, R17D01-FLP/+; slp<sup>R35H02</sup>-GAL4, pLot-FSF-CD8GFP/ UAS-Dscam2<sup>RNAi P{GD111}v1003</sup></i>                               |
| 2A-E                    | <i>HsFLP<sup>122</sup>; Ort<sup>C1a</sup>-GAL4&gt;mCD8GFP/+; Tub-GAL80, FRT2A/FRT2A</i>                                                                                        |
| 3A-3A''', 4A, 4C        | <i>HsFLP<sup>122</sup>; Ort<sup>C1a</sup>-GAL4&gt;mCD8GFP/+; Tub-GAL80, FRT2A/FRT2A</i>                                                                                        |
| 3B-B''', 4B, 4D, 8B, 9B | <i>HsFLP<sup>122</sup>; Ort<sup>C1a</sup>-GAL4&gt;mCD8GFP/+; Tub-GAL80, FRT2A/Dscam2<sup>1</sup>, FRT2A</i>                                                                    |
| 6C, C'                  | <i>Yw; LexAop.syb.spGFP1-10, Ort<sup>C1a</sup>-GAL4&gt; UAS-CD4.spGFP11 / R16H03-LexA; +/- FRT2A, R39D12-LexA</i>                                                              |
| 6D, 6D'                 | <i>Yw; LexAop.syb.spGFP1-10, Ort<sup>C1a</sup>-GAL4&gt; UAS-CD4.spGFP11 / Rh3/4.Syb.GFP1-10, UAS-CD4.spGFP11; +/- FRT2A</i>                                                    |
| 7A, 7A'                 | <i>HsFLP<sup>122</sup>; LexAop.syb.spGFP1-10, Ort<sup>C1a</sup>-GAL4&gt; UAS-CD4.spGFP11 / R16H03-LexA; Tub-GAL80, FRT2A/ FRT2A, R39D12-LexA</i>                               |
| 7B, 7B'                 | <i>HsFLP<sup>122</sup>; LexAop.syb.spGFP1-10, Ort<sup>C1a</sup>-GAL4&gt; UAS-CD4.spGFP11 / R16H03-LexA; Tub-GAL80, FRT2A/ Dscam2<sup>1</sup>, FRT2A, R39D12-LexA</i>           |
| 7C, 7C'                 | <i>HsFLP<sup>122</sup>; LexAop.syb.spGFP1-10, Ort<sup>C1a</sup>-GAL4&gt; UAS-CD4.spGFP11 / Rh3/4.Syb.GFP1-10, UAS-CD4.spGFP11; Tub-GAL80, FRT2A/ FRT2A</i>                     |
| 7D, 7D'                 | <i>HsFLP<sup>122</sup>; LexAop.syb.spGFP1-10, Ort<sup>C1a</sup>-GAL4&gt; UAS-CD4.spGFP11 / Rh3/4.Syb.GFP1-10, UAS-CD4.spGFP11; Tub-GAL80, FRT2A/ Dscam2<sup>1</sup>, FRT2A</i> |
| 8D, 8D'                 | <i>HsFLP<sup>122</sup>; Ort<sup>C1a</sup>-GAL4, UAS-mCD8GFP /+; Tub-GAL80, FRT2A/Dscam2<sup>10A</sup>, FRT2A</i>                                                               |
| 8D, 8D'                 | <i>HsFLP<sup>122</sup>; Ort<sup>C1a</sup>-GAL4, UAS-mCD8GFP /+; Tub-GAL80, FRT2A/ Dscam2<sup>10B</sup>, FRT2A</i>                                                              |
| 8E, 8E'                 | <i>Mbl<sup>E27</sup>; hs122; FRT42, mbl<sup>E27</sup>/FRT42, Tub-GAL80; Ort<sup>C1a</sup>-GAL4, UASmCD8GFP/UASmCD8GFP</i>                                                      |
| 9A, 9A'                 | <i>HsFLP<sup>122</sup>; Ort<sup>C1a</sup>-GAL4&gt;mCD8GFP/+; Tub-GAL80, FRT2A/Dscam2ΔICD, FRT2A</i>                                                                            |
|                         |                                                                                                                                                                                |

|           |                                                                                                                                                                                                                                            |
|-----------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 10D, 10D' | <i>HsFLP<sup>122</sup></i> ; <i>Ort<sup>C1a</sup>-GAL4</i> , <i>UAS-mCD8GFP</i> ; <i>Tub-GAL80</i> , <i>FRT2A/Trio<sup>1</sup></i> , <i>FRT2A</i>                                                                                          |
| 10E, 10E' | <i>HsFLP<sup>122</sup></i> ; <i>Actin-GAL4</i> , <i>UAS-LexADB</i> , <i>Ort<sup>C1a</sup>-LexA::VP16</i> , <i>LexAop-rCD2GFP/UAS-trio<sup>(wt)</sup></i> ; <i>FRT2A</i> , <i>Tub-GAL80/Trio<sup>1</sup></i> , <i>FRT2A</i>                 |
| 10F, 10F' | <i>HsFLP<sup>122</sup></i> ; <i>Actin-GAL4</i> , <i>UAS-LexADB</i> , <i>Ort<sup>C1a</sup>-LexA::VP16</i> , <i>LexAop-rCD2GFP/UAS-trio<sup>(GEF1mu)</sup></i> ; <i>Tub-GAL80</i> , <i>FRT2A/Trio<sup>1</sup></i> , <i>FRT2A</i>             |
| 10G, 10G' | <i>HsFLP<sup>122</sup></i> / <i>UAS-trio<sup>(GEF2mu)</sup></i> ; <i>Actin-GAL4</i> , <i>UAS-LexADB</i> , <i>Ort<sup>C1a</sup>-LexA::VP16</i> , <i>LexAop-rCD2GFP/Tub-GAL80</i> , <i>FRT2A/Trio<sup>1</sup></i> , <i>FRT2A</i>             |
| 10H, 10H' | <i>HsFLP<sup>122</sup></i> ; <i>Ort<sup>C1a</sup>-GAL4</i> , <i>UAS-mCD8GFP/+</i> ; <i>Tub-GAL80</i> , <i>FRT2A/TrioGEF1QA</i> , <i>FRT2A</i>                                                                                              |
| 10I, 10I' | <i>HsFLP<sup>122</sup></i> ; <i>Ort<sup>C1a</sup>-GAL4</i> , <i>UAS-mCD8GFP/+</i> ; <i>Tub-GAL80</i> , <i>FRT2A/TrioGEF2QA</i> , <i>FRT2A</i>                                                                                              |
| 11B, 11B' | <i>UAS-Dicer2</i> ; <i>Ort<sup>C1a</sup>-LexA::VP16</i> , <i>R17D01-FLP/ UAS-trio<sup>GEF1mu</sup></i> ; <i>slp<sup>R35H02</sup>-GAL4</i> , <i>pLot-FSF-CD8GFP/+</i>                                                                       |
| 11C, 11C' | <i>UAS-Dicer2</i> ; <i>Ort<sup>C1a</sup>-LexA::VP16</i> , <i>R17D01-FLP/ UAS-trio<sup>GEF1mu</sup></i> ; <i>slp<sup>R35H02</sup>-GAL4</i> , <i>pLot-FSF-CD8GFP/+</i>                                                                       |
| 11D, 11D' | <i>UAS-trio<sup>GEF2mu</sup></i> ; <i>Ort<sup>C1a</sup>-LexA::VP16</i> , <i>R17D01-FLP/+</i> ; <i>slp<sup>R35H02</sup>-GAL4</i> , <i>pLot-FSF-CD8GFP/+</i>                                                                                 |
| 12B, 12B' | <i>HsFLP<sup>122</sup></i> ; <i>Tub-GAL80</i> , <i>FRT40/Rho1</i> , <i>FRT40</i> ; <i>Ort<sup>C1a</sup>-GAL4</i> , <i>UAS-mCD8GFP</i> /+                                                                                                   |
| 12C, 12C' | <i>HsFLP<sup>122</sup></i> ; <i>Tub-GAL80</i> , <i>FRT40/Cdc42</i> , <i>FRT40</i> ; <i>Ort<sup>C1a</sup>-GAL4</i> , <i>UAS-mCD8GFP</i> /+                                                                                                  |
| 12D, 12D' | <i>HsFLP<sup>122</sup></i> ; <i>Ort<sup>C1a</sup>-GAL4</i> , <i>UAS-mCD8GFP</i> /+; <i>Tub-GAL80</i> , <i>FRT82/Rac1<sup>J11</sup></i> , <i>FRT2A</i>                                                                                      |
| 13C, C'   | <i>UAS-Dcr2</i> ; <i>Ort<sup>C1a</sup>-LexA::VP16</i> , <i>R17D01-FLP/ UAS-Src42<sup>RNAi</sup></i> <i>P{KK108017}VIE-260B</i> ; <i>slp<sup>R35H02</sup>-GAL4</i> , <i>pLot-FSF-CD8GFP/+</i>                                               |
| 13D, D'   | <i>UAS-Dcr2</i> ; <i>Ort<sup>C1a</sup>-LexA::VP16</i> , <i>R17D01-FLP/ UAS-Src42<sup>RNAi</sup></i> <i>P{KK108017}VIE-260B</i> ; <i>slp<sup>R35H02</sup>-GAL4</i> , <i>pLot-FSF-CD8GFP/ UAS-Dscam2<sup>RNAi</sup></i> <i>P{GD111}v1003</i> |
| 13E, E'   | <i>HsFLP<sup>122</sup></i> ; <i>Ort<sup>C1a</sup>-GAL4</i> , <i>UAS-mCD8GFP</i> /+; <i>Tub-GAL80</i> , <i>FRT82/Pi3K<sup>df</sup></i> , <i>FRT82</i>                                                                                       |
|           |                                                                                                                                                                                                                                            |

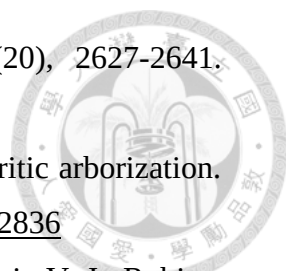


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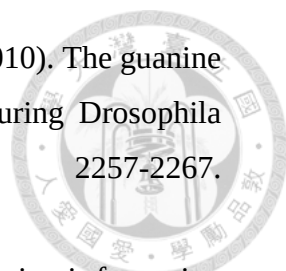
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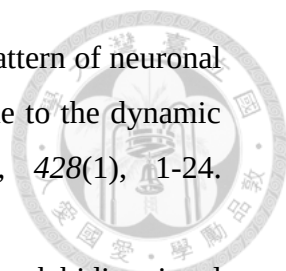
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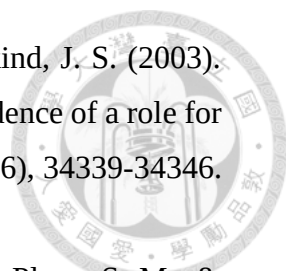
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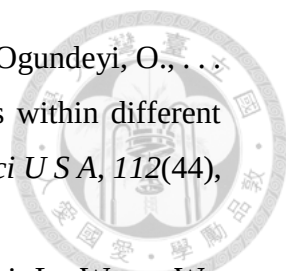
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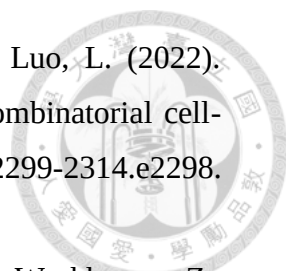
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