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Getting the edge: neural precursor selection

Haiwei Pi[1] & Cheng-Ting Chien[2,*]
[1]*Department of Life Science, Chang-Gung University, 259 Wen-Hwa 1st Road, Kweishan, Tao-Yuan, 333, Taiwan;* [2]*Institute of Molecular Biology, Academia Sinica, 128 Academia Road, Nankang, Taipei, 115, Taiwan*

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Abstract

A key issue in development is how to specify single isolated precursor cells to adopt a distinct fate from a group of naive cells. Studies on the development of *Drosophila* external sensory (ES) organs have revealed multiple mechanisms to specify single sensory organ precursors (SOPs) from clusters of cells with equivalent neural potential. Initially single SOPs are selected in part through cell–cell competition from clusters of ectodermal cells that express proneural proteins. To reinforce the singularity, lateral inhibition through the Delta/Notch system and feedback regulations lead to exclusive expression of proneural proteins in SOPs. As transcriptional activators, proneural proteins execute a genetic program in SOP cells for the development of an eventually ES organ. In this article, we will summarize recent advances on how transcriptional regulation, protein degradation, endocytosis and gene silencing by microRNA participate in SOP specification.

Introduction

How cells acquire their distinct fate is the central question in developmental biology. This question is particularly significant in the establishment of nervous systems, as suggested by Anderson that there are probably more different cell types in the nervous system than in all other tissues of the body combined [1]. For the past twenty years, the *Drosophila* external sensory (ES) organ has proven to be an excellent model system to dissect genetic pathways essential for the formation of neural precursors and for the generation of cell diversity by asymmetric cell division (for recent review, please see [2]).

In the development of ES organs, cell fate specification proceeds in three major stages. Initially, the acquirement of neural competence is conferred by two functionally redundant proneural genes *achaete* (*ac*) and *scute* (*sc*) that encode the basic-helix-loop-helix (bHLH) proteins. The proneural proteins form heterodimers with the ubiquitously expressed bHLH protein Daughterless (Da) to function as transcriptional activators to regulate downstream gene expression [3–8]. Expressions of *ac* and *sc* are confined in small clusters of ectodermal cells, the so-called proneural clusters (PCs). While each cell within the PC is endowed with the potential to develop into SOP, only one cell from each PC is conferred with the SOP fate. The neighboring proneural cells (NPCs) are prevented by SOPs from adopting the same fate, a process called lateral inhibition that requires the activity of the ligand Delta in SOPs and the reception of the signal by the Notch receptor in NPCs [9–11]. Thus, when components of the Notch signaling pathway are inactivated, ectopic SOPs and consequently ectopic ES organs form in clusters. Soon after, selected SOPs ensue

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*To whom correspondence should be addressed. Fax: +886-2-27826085; E-mail: ctchien@gate.sinica.edu.tw

asymmetric cell divisions to generate daughter cells of distinct fates. These daughter cells will develop into different components of an ES organ [12]. In this article, we review recent literatures that advance our knowledge on the specification of single SOPs, in the aspect of how distinct cellular activities such as transcription regulation, protein degradation, endocytosis and gene silencing by miRNA are integrated into this developmental process.

Transcriptional regulation

Cascade of transcriptional factors is one of the most utilized strategies to initiate and maintain the fate-specification process. Formation of protein complexes, feedback regulation and autoregulation can be seen as common endeavors of these factors. Proneural proteins are initially expressed in all PC cells, but the expression is gradually confined to one cell, the prospective SOP, with higher protein levels. The then shutout of proneural protein expression in the rest of PC cells renders them to adopt an alternative fate, the epidermal fate in the case of ES organ formation. Two transcriptional factors, Senseless (Sens) and Suppressor-of-Hairless (Su(H)), have been suggested to regulate this binary cell fate decision by either activating or repressing transcription depending on the protein level and associated co-factors.

At the beginning of SOP selection, the zinc-finger protein Sens is specifically expressed in the prospective SOP and a subset of adjacent NPCs. Depending on the protein level, Sens can either activate or repress proneural gene transcription [13]. In NPCs, Sens of low levels directly binds to the DNA motif S-box at the *ac* promoter to represses its transcription. However, it is postulated that the binding site S-box is saturated with high levels of Sens in SOPs. Additional Sens proteins that are not bound at the S-box would interact with the E-box-binding proneural proteins, leading to the synergistic activation of *ac* transcription, a opposite effect by Sens. The mechanism for this switch is still unknown but it has been speculated that the Sens conformation might be changed when bound to proneural proteins [14]. Given that proneural proteins transcriptionally activate Sens, the high- and low-level of Sens protein would lead to more proneural proteins in prospective SOPs and fewer of them in NPCs. Such positive and negative feedbacks in the two neighboring cells would result in more pronounced difference in the levels of proneural proteins and therefore the solidification of SOP fate (see Figure 1).

Su(H) is the primary nuclear effector of the Notch signaling pathway. When the transmembrane Notch receptor is activated in NPCs by the ligand Delta presented by SOPs, the Notch intracellular domain (N^{intra}) is cleaved and migrates into the nucleus where N^{intra} functions as a transcriptional co-activator for the sequence-specific DNA binding protein Su(H) (see Figure 1). Thus, Su(H) acts as a transcriptional activator when associated with N^{intra} in NPCs [15–17]. In SOPs where Notch signaling is inactive and N^{intra} is absent, Su(H) associates with the adaptor protein Hairless that recruits the co-repressor dCtBP and Groucho to repress target genes of the Notch signaling pathway [18–20]. The well characterized targets are genes of the *Enhancer of split Complex* (*E(spl)-C*) that encode transcriptional repressors for *ac* and *sc*. Thus, the effects of repression and activation in SOPs and NPCs, respectively, by Su(H) will eventually converge to the solely presence of proneural proteins in SOPs (see Figure 1).

How do these two binary transcriptional factors generate the binary cell response with precision and robustness? By both theoretical and experimental approaches, it has been shown that binary response can be generated by feedback regulation [21], or by competition of transcriptional factors for a common DNA binding site [22]. In the case of Sens, a bimodal feedback loop is formed between proneural and Sens genes, and in the case of Su(H), the competition between N^{intra} (activator) and Hairless (repressor) for the same DNA-binding protein Su(H) might be able to generate a steep does-response curve in the transcriptional readout based on the competition hypothesis [22]. As a result, an on/off switch of Notch target genes can be established by these competing factors.

Protein degradation

One direct target gene of proneural proteins in SOPs is *phyllopod* (*phyl*), encoding an essential

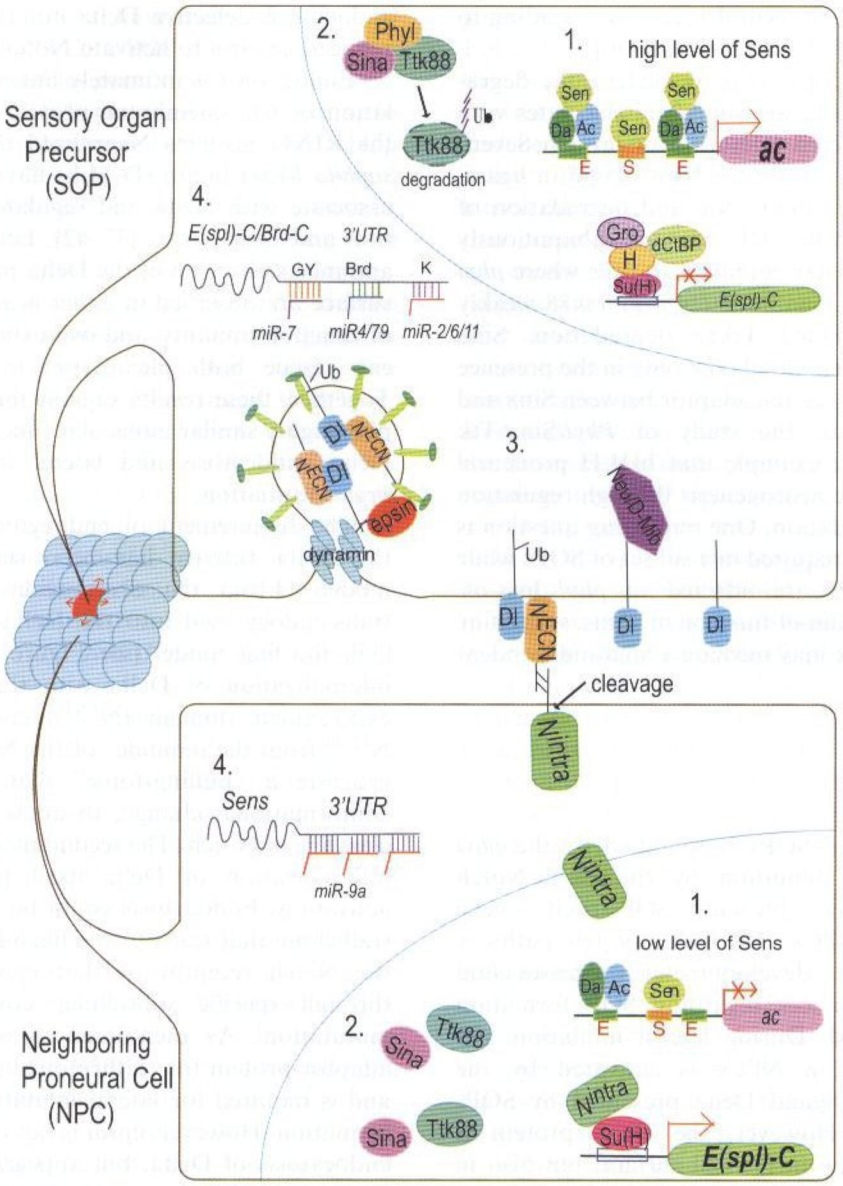

Figure 1. SOP selection and specification is mediated through a network of molecular interactions between and within the cells. The upper cell is an SOP, and the lower cell is a NPC from the same proneural cluster shown on the left. Numbers in the figure indicate the processes of transcriptional regulation (1), protein degradation (2), endocytosis (3) and gene silencing by miRNAs (4). E and S refer to the E box and S box, respectively. Dl is the abbreviation of Delta. Also see text for the other abbreviations and explanation of these regulations.

component in SOP formation [23]. In addition to SOPs, *phyl* is also required for specifying the precursor IIb in the SOP lineage, which divides to generate neurons and sheath cells of ES organs, and the precursors for photoreceptors R1, R6 and R7 in ommatidia. In *phyl* loss-of-function mutants, these neural cells are transformed into cognate non-neural cells [24–26]. On the contrary, the transcriptional repressor Tramtrack (Ttk) is expressed in non-neural cells and misexpression of Ttk inhibits their neural specification [27–30]. Genetic analysis suggests that *phyl* functions upstream of Ttk to inhibit its activity in neural precursors [26]. Mutations in *phyl* results in

expression of Ttk in neural precursors, leading to failure in neural fate specification [30].

One function of *phyl* is to mediate the degradation of the Ttk88 isoform. *Phyl* associates with the highly conserved RING-finger protein Seven-in-absentia (Sina) to form an E3 ubiquitin ligase, and promotes ubiquitination and degradation of Ttk88 [26, 27, 30, 31]. Sina is ubiquitiously expressed during development. In cells where *phyl* is not expressed, Sina interacts with Ttk88 weakly and cannot mediate Ttk88 degradation. Sina strongly associates with Ttk88 only in the presence of *phyl* that serves as the adaptor between Sina and Ttk88 [31]. Thus, the study of *Phyl*/Sina/Ttk provides the first example that bHLH proneural proteins promote neurogenesis through regulation of protein degradation. One remaining question is that Sina is only required in a subset of SOPs while almost all SOPs are affected in *phyl* loss-of-function or Ttk gain-of-function mutants, suggesting that *phyl* and Ttk may mediate a Sina-independent activity.

Endocytosis

While the fate of SOPs is solidified by the *phyl* activity, lateral inhibition by the Delta/Notch signaling pathway prevents SOP-specific gene expression in NPCs. The Delta/Notch pathway regulates diverse developmental processes and only recent advances regarding SOP formation will be discussed. During lateral inhibition, the receptor Notch in NPCs is activated by the transmembrane ligand Delta presented by SOPs (see Figure 1). However, the Delta protein is detected not only on the cell surface, but also in the intracellular endocytic vesicles. Several evidences suggest that endocytosis plays a key role on regulating the signaling activity of Delta. The GTPase protein dynamin, functioning in the pinch-off of clathrin-coated vesicles (CCVs), and the J domain-containing protein auxilin, participating in the uncoating of CCVs, are required for Delta endocytosis and the process of lateral inhibition [32, 33]. Mutant clonal analyses for *dynamin* and *epsin*, the gene encoding the cargo-selective adaptor protein, revealed that endocytosis in signal-sending cells is essential for mediating Notch signaling activity [32, 34]. Furthermore, endocytosis-defective Delta mutant proteins have reduced activity to activate Notch signaling [35].

Endocytosis is intimately linked with ubiquitylation of transmembrane proteins [36]. Recently, the RING proteins Neuralized (Neur) and *Drosophila* Mind bomb (D-Mib) have been found to associate with Delta and regulate its ubiquitination and endocytosis [37–42]. Ectopic ES organs and mislocalization of the Delta protein to the cell surface are observed in either *neur* or *D-mib* loss-of-function mutants, and overexpression of *D-mib* can rescue both phenotypes in *neur* mutants. Together, these results suggest that both proteins play highly similar molecular functions to mediate Delta endocytosis and lateral inhibition in ES organ formation.

The requirement of endocytosis in activating the Delta activity has been suggested in two models. From the observation that Notch is trans-endocytosed into the Delta-expressing cells [35], the first model (see Figure 1) suggests that internalization of Delta with the bound Notch extracellular domain (N^{ECD}) could either clear N^{ECD} from the reminder of the Notch protein, or generate a "pulling-force" that induces Notch conformational change, to allow the subsequent cleavage of Notch. The second model suggests that internalization of Delta itself promotes ligand activation. Endocytosis could be a part of ligand trafficking that leads to the ligand presentation to the Notch receptor or that recycles the ligand through specific subcellular compartments for maturation. As mentioned above, epsin is an adaptor protein for clathrin-mediated endocytosis and is required for lateral inhibition in ES organ formation. However, epsin is not required for bulk endocytosis of Delta, but appears to be essential for targeting Delta to a specific endocytic pathway where the ligand acquires its signaling activity [34, 43].

Gene silencing by miRNAs

The microRNA (miRNA) molecules are 21–23 nucleotides (nts) RNAs that direct gene silencing at a post-transcriptional level. Specific target mRNAs that contain sequences complementary to miRNAs are silenced by RNA cleavage, translation inhibition or both [44]. Over the past three

years, several miRNAs have been identified to target distinct mRNAs in SOP specification.

The miRNA species generated from the *miR-9a* locus down-regulates Sens expression by binding to the 3′ untranslated region (UTR) of Sens [45]. Ectopic Sens expression and ectopic SOPs are found in 14% of the *miR-9a* null mutant flies, consistent with the notion that miRNAs play fine-tuning roles in many developmental processes studied so far.

E(spl)-C and *Bearded complex* (*Brd-C*) are two families of Notch pathway downstream genes in NPCs [15, 17, 46, 47]. Sequence comparison among these two gene families found three conserved motifs of 6–7 nts, named the GY-box, Brd-box and K-box, residing in their 3′-UTRs [48–50]. Mutagenesis study in heterologous transgenes with these 3′-UTRs suggest that all three motifs confer negative post-transcriptional regulation [49, 51]. Subsequently by computational target site prediction, *miR-7* was identified to target several members of *E(spl)-C* and *Brd-C* genes (see Figure 1) [52]. A stretch of sequence in the 5′ region of *miR-7* is found to be complementary to the GY box. Misexpression of *miR-7* in larval imaginal disks reduces the expression of a heterologous transgene in a GY-box dependent manner, and causes ectopic SOPs, a phenotype similar to Notch loss-of-function mutants [51, 52].

In addition, *miR-4*/*miR-79* and *miR-2*/*miR-6*/*miR-11* were also identified to respectively confer Brd-box- and K-box-dependent negative regulation, in a manner similar to the *miR-7* to the GY-box [51]. This study reveals that Notch pathway downstream genes are regulated by at least six different miRNAs. One interesting question arises from these analyses is the usage of multiple miRNAs to target *E(spl)-C* and *Brd-C*. Is it because full strength of down-regulation can only be achieved by six miRNAs working together (quantitative difference), or each miRNA plays specific spatial and temporal roles in development (qualitative difference)? Since all of these analyses are based on miRNA misexpression experiments, loss-of-function analyses of these miRNAs will be needed to further distinguish these two possibilities.

Concluding remark

Although we classify these genes according to their molecular features and cellular activities, these genes cross talk to each other in SOPs and NPCs. For examples, genes of the *E(spl)-C*, regulated by the Notch pathway and miRNAs, negatively control proneural gene expression in NPCs. Also, enriched proneural proteins in SOPs can directly activate *Delta* gene expression to intensify the lateral inhibition process. Thus, these studies show that complex networks of molecular interactions are involved to make single SOPs unique to their neighboring cells, which adopt a completely distinct developmental route. These multiple mechanisms, intertwined by cross regulations, function in amplification once an initial divergence is generated in prospective SOPs and NPCs, thus ensuring the generation of distinct cell fate. Many molecules required for SOP specification are highly conserved throughout evolution and have been found to be involved in vertebrate neurogenesis [53, 54]. In addition to experimental approaches, mathematical models have been built from the ample data obtained from previous genetic and molecular studies [55]. These models would provide many systematic aspects for each component or module in the network, and the necessity of many feedback loops in this process. In the future, the versatility of this system would certainly provide more in-depth information as how cell type diversity is achieved during development.

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