Review

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Review

Notch Signaling in Insect Development: A Simple Pathway with Diverse Functions

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Abstract: Notch signaling is a highly conserved pathway which occurs between adjacent cells to distinguish them. The Notch signaling cascade operates in a simple mechanism, yet Notch plays remarkably diverse roles in development to regulate cell fate determination, organ growth and tissue patterning. Originally isolated and characterized in the model insect *Drosophila melanogaster*, recent studies in various insect species have revealed that Notch signaling is broadly involved in the construction of insect tissues. This review focuses on providing a molecular mechanistic and developmental picture regarding the roles of Notch pathway in insects. The functions of Notch in the formation and patterning of insect embryo, wing, leg, ovary and several specific structures as well as in stress responses are summarized. These results are discussed in developmental context with an eye toward understanding the functionality of Notch signaling pathway in different insect species.

Keywords: Notch; insect; development; *Drosophila*

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1. Introduction of the Notch signaling pathway

A small number of signaling pathways are iteratively used to regulate cell fate determination, organ growth and tissue patterning during insect development. One of the most important pathways is mediated by Notch, which functions to distinguish adjacent cells in numerous developmental processes [1]. The Notch signaling cascade operates in a remarkably linear fashion without signal amplification steps which are normally found in other pathways [2]. The core components of Notch signaling pathway include the ligands of Delta and Serrate (known as Jagged in vertebrates), the Notch receptor and the transcription factor of the CBF1/Su(H)/LAG1 (CSL) family [3]. Both Notch and Delta/Serrate (Dl/Ser) proteins contain large extracellular domain composed of epidermal growth factor-like repeats which function to mediate their direct contact. Notch recognizes Dl/Ser presented at the surface of neighboring cells, and binding with the ligands triggers proteolytic cleavage of the Notch protein [4]. As a result, the intracellular domain of Notch (NICD) is released and translocated into the nucleus, whereas NICD forms a transcription activation complex with CSL to drive the transcription of downstream target genes [5]. In the absence of signal input, NICD could not be produced and CSL recruits a variety of co-repressor factors to suppress the expression of Notch targets. Thus, Notch senses the information sent by neighboring cells, transduces the signal and adjusts the cell state accordingly (Figure 1).

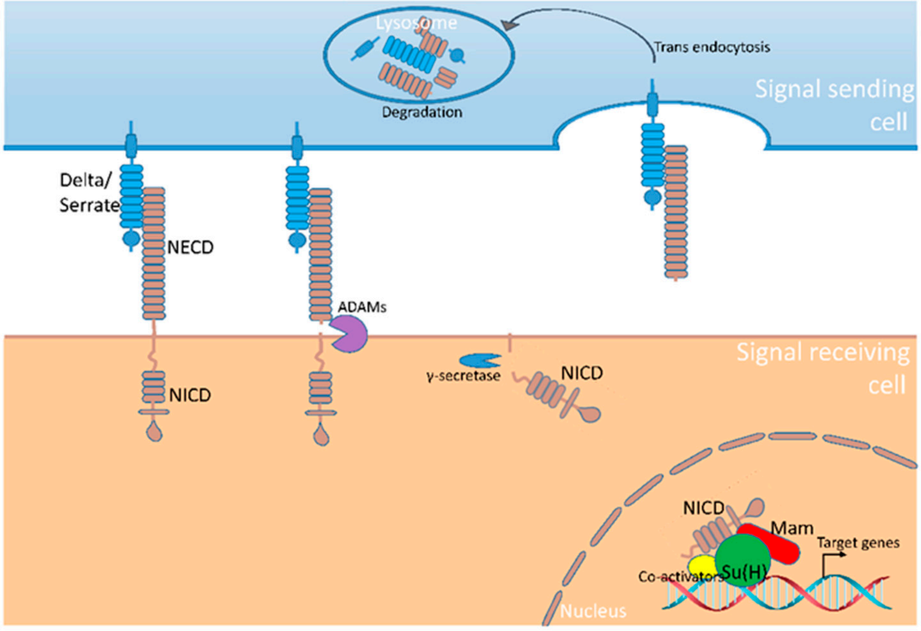

Figure 1. A simplified model of the Notch signaling transduction cascade in the fruit fly. The signal sending cell is in blue and the signal receiving cell is in earthy yellow. Upon binding with the Delta/Serrate ligands, the Notch receptor sequentially processed by metalloproteases of the ADAM/TACE family and the γ-secretase, resulting the release of NICD from the membrane. In the nucleus, NICD assembles with the CSL family protein Su(H) and the co-activator Mam to form a complex that regulates target gene expression.

Notch signaling is highly conserved across the animal kingdom, and a functional Notch pathway with true homologs of receptors and ligands could be identified in most metazoan species with available genomic information [6–8]. Notch signaling plays crucial roles in various developmental events such as cell fate determination, cell cycle progression, cell death and stem cell homeostasis [1–5]. Therefore, it is not surprising that mutations of genes encoding Notch signaling components are involved in various human diseases, including birth defects and malignant tumors [9–12]. Many aspects of the Notch signaling pathway, such as the signal transduction machinery, the regulatory network, as well as the involvement in human diseases have been elaborately reviewed in previous articles [13–20]. This review will focus on the roles of Notch signaling in different stages and tissues during insect development, coving recent findings in various insect species.

2. A brief history of Notch signaling studies in *Drosophila*

Over 100 years ago, Thomas H. Morgan chose *Drosophila melanogaster*, the fruit fly, as a model animal to study the fundamental law of heredity. The famous white eyed mutant fly led to a series of significant discoveries, and ultimately the establishment of the modern theory of the gene [21,22]. Along with the eye color mutants, various mutants affecting body color and wing morphology were isolated and characterized by Morgan and his students [23]. In 1911, Morgan reported the generation of "beaded wings" mutants by radium ray irradiation, and recorded that the marginal vein and wing blade in these mutants were eliminated to various degrees [24]. In 1914, John S. Dexter isolated one mutant strain from the beaded stocks which he named as "Perfect Notched", and demonstrated that the wing notches in the tips is caused by a dominant sex-linked factor [25]. However, the "Perfect Notched" stock was lost and Dexter was not able to further examine which factor causes the notched wing phenotype. Later on, Morgan and his students identified additional *Notch* mutants and Calvin B. Bridges mapped the approximate position of *Notch* in the X chromosome [23]. In 1919, Otto L. Mohr recovered a novel *Notch* allele, and showed for the first time that deficiency of a small region of the X chromosome likely caused the *Notch* loss-of-function mutation [26]. In the next seven decades, the number of *Notch* alleles and related phenotypes continued to grow, while the

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biochemical nature of Notch protein remained a mystery [27]. Molecular cloning of the *Notch* locus marked a new stage of Notch signaling research, and the finding that *Notch* gene encoded a transmembrane receptor inspired the subsequent studies about cell interaction and signal transduction [28–31]. Thereafter, detailed molecular genetic analyses have defined the core components and the canonical signal transduction cascade of the Notch signaling pathway.

Mutations of Notch pathway components are found to affect a broad spectrum of developmental events such as cell differentiation, tissue pattern formation and stem cell self-renew [32–34]. Now there is a common understanding that Notch signaling is crucial for the development of most, if not all, organs in the fly [35–38]. Three major modes of Notch actions have been summarized, including lateral inhibition, boundary induction and lineage decision [32]. A historical description of the "lateral inhibition" concept appears in study of cuticle patterning in the bloodsucking bug *Rhodnius prolixus* [39]. In the dorsal abdomen of *R. prolixus*, bristles arise from little mounds of smooth cuticle termed plaques, and each plaque appears to exert an inhibitory influence to prevent the development of new plaques within a certain radius [39]. Lateral inhibition takes place within a cluster of cells with initially similar fate and potential, whereas Notch signaling amplifies small differences within them. As a result, one cell with low/no Notch activity acquires a specific fate and prevents the surrounding cells from adopting the same developmental route [32]. When occurring between two populations of cells, Notch signaling establishes a boundary to segregate the two cell groups, which is generally utilized to subdivide developmental fields during tissue formation. Through asymmetrical distribution of signaling regulators which leads to differential signaling activity, Notch controls binary cell fate specification between daughter cells in various cell lineages [32]. It has become evident that Notch functions at different stages and in many tissues during fly development, sometimes with distinct modes of action even within one tissue. Benefited from the abundant genetic resources and exquisite genetic tools, systematic *in vivo* screens have identified large sets of genes that are involved in Notch signaling in different developmental contexts [40–45]. Investigation of the molecular mechanisms between Notch and these genes will help to elucidate how Notch signaling fulfills such sophisticated roles.

The diversified developmental functions of Notch signal have been explored in depth in the model insect *D. melanogaster*. Genes encoding the receptor and ligands of Notch pathway are found in major groups of insects (Figure 2). Recent advances in genomic resources and methodologies have allowed investigations of Notch function among a broad range of insect species, which will be discussed in the following chapters.

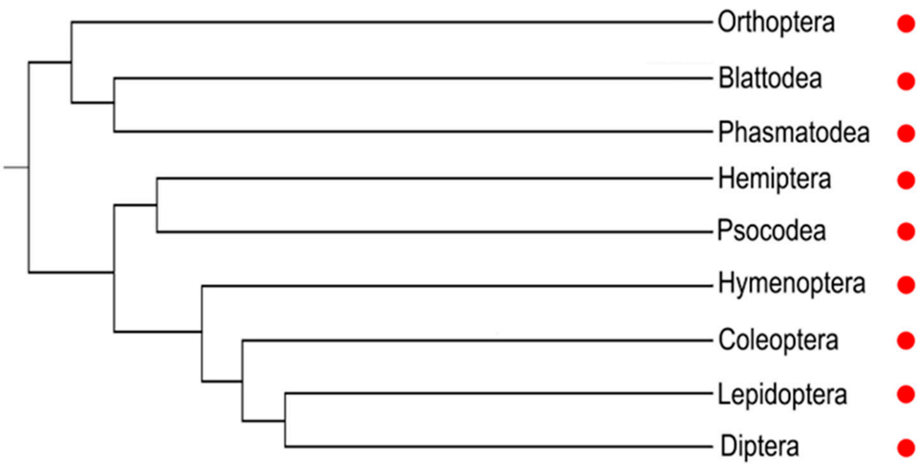

Figure 2. Notch signaling is highly conserved among insects. The cladogram shows that homologs of Notch, Delta and Serrate are found in major groups of insects (red dots).

3. Notch signaling in insect embryo development

Donald F. Poulson is generally regarded as the pioneer who established the link between Notch and insect embryo development. In the 1930s, Poulson described a unique "neurogenic" phenotype in *Notch* mutant *D. melanogaster* embryos, which failed to develop mesodermal and endodermal tissue while produced hypertrophic nervous system [46,47]. Genetic screens looking for similar neurogenic phenotypes led to the identification of *Dl*, *mastermind* (*mam*), and *Enhancer of Split* (*E(spl)*), which were later proven to encode core Notch signaling components [48,49]. Further studies revealed that Notch signaling acts during multiple stages to control embryonic nervous system development, including selection of neural progenitor cells (neuroblasts; NBs), control of NBs daughter cells proliferation, neuronal cell fate specification, glia development and axon pathfinding [50]. NBs are selected from a uniform layer of cells called neuroectoderm by the lateral inhibition process [51]. Expression of proneural genes defines stereotypically spaced "proneural clusters" in the neuroectodermal cell sheet, each proneural cluster consists of 6-8 cells with similar potential to develop into NBs [52]. Inside the proneural clusters, the cellular interactions mediated by Notch signal results in the selection of one cell with the lowest Notch activity to become NB. Activation of Notch signaling in cells surrounding NB results in the expression of the *E(spl)* gene complex encoded transcription factors which directly repress the proneural genes and further prevent these cells from becoming NBs [52]. Thus, in mutants of *Notch* and many other Notch pathway genes, excessive NBs are formed due to the lack of lateral inhibition inside the proneural cluster [53].

NBs were recognized as distinct cell population with specific characteristics of cell size, cell shape, and nuclear position by histological methods in the embryos of several insect species more than 130 years ago [54]. Similar number and arrangement of NBs were found in the embryos of insects with different developmental modes and life histories, including *D. melanogaster*, the bloodsucking bug *R. prolixus*, cockroaches (*Blatta germanica* and *Periplaneta americana*), locusts (*Locusta migratoria*, *Melanoplus femurrubrum*, *Schistocerca americana* and *Schistocerca gregaria*), potato beetle (*Doryphora decemlineata*), red flour beetle (*Tribolium castaneum*), stick insect (*Carausius morosus*), silverfish (*Ctenolepisma longicaudata*), tobacco hornworm (*Manduca sexta*) and yellow mealworm (*Tenebrio molitor*) [54–66]. These observations indicate that selection of NBs during insect embryonic development might be mediated by conserved mechanisms [67–69]. The landmark laser ablation experiments performed in locust (*S. americana*) embryo demonstrated that lateral inhibition by the enlarging NB ensures development of only one NB in each proneural cluster [59,66]. In cricket (*Gryllus bimaculatus*) and cockroach (*P. americana*) embryos, RNAi knock-down of *Notch* and/or *Dl* caused classic neurogenic phenotype in early stages and cell apoptosis in later stages [70–72]. Knock-down of *Notch* and *E(spl)* by RNAi in red flour beetle embryo led to similar neurogenic phenotype with increased number of NBs [73]. Computational analyses suggest that the *E(spl)* gene family is quite conserved among insects [74–80]. It is possible that Notch signaling and the *E(spl)* gene family are generally utilized to determine NB fate in insect embryos [81].

Other than the common role in neurogenesis, Notch signaling also regulates different developmental events in different insects. It appears that Notch signaling is critical for embryo segmentation in several insect species, but is dispensable for this process in other insects [82]. Segmentation is a fundamental process that divides the developing body into separate units which can undergo independent developmental programs [83]. Two distinct modes of embryo segmentation have been reported in insects [84]. In long germ insects such as *D. melanogaster*, all segments are specified almost simultaneously within the blastoderm before gastrulation takes place. On the other hand, only segments of the head region are specified in the blastoderm in short and intermediate germ insects, whereas the remaining segments arise sequentially from a posterior segment addition zone using a clock and wave front mechanism similar as that in vertebrates [84]. Originally identified from a genetic screen in *D. melanogaster*, many segmentation factors are found to function in a conserved fashion among insects with different germ band types [82–85]. In vertebrates and several sequential segmenting arthropods such as brine shrimp, water flea, centipede and spider, Notch signaling plays critical roles in segmentation [82]. It is proposed that Notch signaling represents an ancestral mechanisms controlling segmentation in arthropods and vertebrates [86]. However, whether and how Notch signaling is involved in embryo segmentation in insects is still under debating.

It is well accepted that Notch signal is not required for embryo segmentation in *Drosophila*. The segment morphology and expression pattern of segmentation factors were not affected in *Notch* mutant fly embryos, despite that remarkable neurogenesis defects would emerge in later stage [87,88]. In another long germ insect, the honeybee (*Apis mellifera*), Notch signaling was not involved in segmentation as well [89]. In the short germ hemipteran milkweed bug *Oncopeltus fasciatus*, *Dl* was not expressed in the right time and right space to regulate embryo segmentation [90,91]. Importantly, RNAi knock-down of *Dl* in *O. fasciatus* embryo did not affect the expression pattern of other segmentation factors [92]. In the short germ red flour beetle, no evidence could support a role of Notch signaling in segmentation [93]. The *fringe* (*fng*) gene encodes a conserved modifier of the Notch receptor [94,95], and is required for segmentation in mice and chicken [96,97]. Expression of *fng* is not detected until after segment boundaries are established in the embryo of the short germ locust *S. gregaria*, ruling out a role in embryo segmentation [98]. The germ band morphology is highly dynamic in the silkworm *Bombyx mori*, but the available molecular data suggest that the majority of segments are not patterned prior to the onset of gastrulation, which fits with the short germ type [84]. In *B. mori* embryos, *Notch* RNAi caused patterning defects without affecting the formation of segments [99], but *Dl* RNAi led to loss of posterior segments and disruption of segment boundaries [100]. The cockroach *P. americana* belongs to short germ type, and *Notch* RNAi resulted in segment morphology defects along with changes of segmentation factor expression pattern [72,86]. In the intermediate germ cricket *G. bimaculatus*, Notch signaling was maternally required for morphogenesis of segments and formation of posterior segments in the embryo [70]. However, a subsequent study argued that zygotic Notch signaling was not required for early segment patterning or morphological formation of segment boundaries, and the authors pointed out that apoptosis and neurogenesis defects in early stages might cause secondary defects in segment morphologies [71]. In conclusion, remarkable evolutionary flexibility exists among the insects in terms of dividing segments in the embryo, and there is no corresponding relationship between the germ band type and the involvement of Notch signaling in embryo segmentation.

4. Notch signaling in insect wing development and patterning

Insects are the only group of invertebrates that have wings, which are considered as a key evolutionary innovation that allowed insects to become the most diverse and abundant animal group [101]. The evolutionary origin of insect wings is an important yet highly debated mystery, but the key steps and signaling pathways underlying wing development are quite conserved among winged insects [101–103]. Our understanding of how Notch signal regulates insect wing development largely come from studies in *D. melanogaster*. In the wings of fruit fly, the developmental events regulated by Notch signal include, but are not limited to wing margin formation, wing growth, vein patterning and sensory organ specification [104,105].

The *Notch* gene is named after the phenotype of "one or more incisions at the end of wings", and nicked wing margin is arguably the most common and conspicuous defect observed in survivable *Notch* mutant flies [22]. But it took more than a century to figure out how Notch signal regulates various aspects of fly wing development at the cellular and molecular level [23,104]. As a typical holometabolous insect, the fruit fly undergoes complete metamorphosis which means that the larvae have no resemblance to the adult and the transformation to adult occurs during the pupal stage. The precursors of adult wing are maintained during larval stages as distinct clusters of undifferentiated cells called wing imaginal disc (also known as wing disc) [105]. Despite the significant difference in cell number, cell size, cell identity and tissue morphology between wing disc and adult wing, most of the patterning events take place in the wing discs during the larval stage [104]. In the developing wing disc, cells utilize Notch signaling to establish the boundary between the dorsal and ventral (D/V) compartment. Cells in the two compartments develop into two apposed epithelial sheets in the adult wing blade, while the wing margin is formed by the D/V boundary cells [105]. Notch is activated along both sides of the D/V boundary by two different ligands, Dl activates Notch in dorsal boundary cells and Ser activates Notch in ventral boundary cells [106–108]. Glycosylation in the extracellular domain by fng sensitizes Notch towards binding with Dl while inhibits binding with Ser, thus differentiating its affinity and responses from the two ligands [109–116]. The expression of both *Ser* and *fng* is under the control of a dorsal-specific transcription factor apterous (ap), and feedback loops among these genes further strengthen the D/V boundary [110,117–120]. In the D/V boundary cells, Notch signal promotes their proliferation and survival through activating the expression of target genes such as *vestigial* (*vg*), *wingless* (*wg*) and *cut* [106,107,121–131]. In addition to the cell autonomous functions, vg and wg also regulate the growth of cells away from the D/V boundary [106,121,128,129]. Mutations impairing Notch signal activity disrupt the segregation of D/V compartment as well as the overall growth in the wing [105].

The fly wing blade consists two main cell types—vein and intervein, and the veins serve as structural supports for the wing blade and as vessels for trachea, nerves and hemolymph [104]. Notch signaling promotes intervein fate and inhibits vein fate so that the boundary between the two types of cells could be established [132–135]. Mutations dampening Notch signal activity during the larval and pupal stages result in uniformly thickened veins with deltas at the tips, while abnormal activation of Notch signaling can remove adult veins [118,132–134,136]. The wing disc contains small number of sensory organ precursor (SOP) cells which will form sensory bristles in the notum and along the anterior edge of the wing margin in adult flies [105]. SOPs are selected through a lateral inhibition process mediated by Notch signaling, after which they undergo stereotyped asymmetric divisions to form the mechanosensory organ and the fates of daughter cells are also regulated by Notch signaling [37,122,137–139]. Disruption of Notch activity at different developmental stages could disrupt bristle pattern, bristle number as well as cell fate specification [1,139]. The phenotypes in wing margin, veins and sensory bristles have been used as easy-to-spot and reliable readout for genetic screens that aim to identify Notch signaling modulators [140–142].

Notch signaling is required for wing development in several Dipteran insects. In two fly species closely related with *D. melanogaster*, *Drosophila hydei* and *Drosophila virilism*, mutant alleles of *Notch* and other genes in the pathway led to similar wing margin, vein and bristle defects [143–150]. Mutation stocks displaying nicked wing margins have been isolated in the housefly *Musca domestica*, which were later mapped as *Notch* and *cut* mutants [151–153]. With the completion of *M. domestica* genome sequencing and success of Cas9-mediated genome editing, further molecular genetics analysis will provide insights about the roles of Notch signaling in house fly development [154,155]. Many mutations affecting wing development have been isolated and characterized in the Australian sheep blowfly, *Lucilia cuprina* [156–158]. The *Scalloped wings* (*Scl*) loss-of-function mutants displayed wing notching, vein thickening and bristle abnormalities, and the *Scl* gene has been molecularly identified as the homolog of *Notch* [159,160].

Lepidopteran insects such as butterflies and moths normally have two pair of wings (forewings and hindwings) covered by microscopic dust-like scales. Although the wing structure and morphology are very different from the flies, a series of works have demonstrated the importance of Notch signaling during *B. mori* wing development. The *flügellos* (*fl*) mutant of the silkworm produce wingless pupae and moths because wing discs cannot respond to ecdysone during metamorphosis. The *fl* mutant wing discs develop normally until the fourth larval instar and developmental defects emerge in the fifth larval instar and pupae [161–164]. Molecular mapping and cloning have shown that the gene at the *fl* locus is actually the homolog of *fng* [165]. Whole-mount *in situ* hybridization confirmed that *fng* is expressed in the dorsal layer, while *BmWnt1*, the homolog of *wg*, is expressed along the D/V boundary in wing discs [165,166]. In *fl*/*fng* mutant wing discs, the expression of *BmWnt1* is diminished [165]. The microRNA *mir-2* was found to target *fng*, and over-expression of *mir-2* and somatic mutagenesis of *fng* using the CRISPR/Cas9 system resulted in similar defects of wing morphology and inhibition of *BmWnt1* expression [167]. The selector gene *ap* was found to be expressed in the dorsal layer of wing disc and *vg* mutants exhibit small wing phenotype similar as seen in the fly [168]. These findings indicate that *B. mori* likely employs the same regulatory cascade which includes the compartment selector (ap), Notch signaling and downstream targets (vg and wg) to control D/V boundary formation and wing growth.

Butterflies are well known for their colorful wings with pattern elements of many types and colors. In the best studied model species *Precis coenia*, the buckeye butterfly, the expression of *ap* was restricted in dorsal cells while *wg* was expressed in cells along the future wing margin in fifth instar wing discs [169]. Such expression pattern is comparable to that of fruit fly and silkworm [165,169]. Recent studies have shown that the up-regulation of *Notch* likely represents an early developmental event in the formation of eyespots and other wing color patterns in multiple butterfly species [170–177]. The expression pattern of Notch protein in the pupal wing of *Heliconius erato* indicates that Notch mediated lateral inhibition might play a role in organizing butterfly wing scales [178]. However, functional studies are required to demonstrate the exact roles of Notch signaling during butterfly wing development.

The highly specialized forewings called elytra is considered as an important trait driving the successful radiation of Coleoptera (beetles), one of the most successful animal groups on the planet [179]. Compared with the hindwings, elytra in beetles consist thick, hardened, and pigmented cuticles and many morphologically distinct features. Nevertheless, the expression pattern of many key regulatory genes in the elytra and hindwings of *T. castaneum* was surprisingly similar [180]. The expression of Notch target genes *wg* and *cut* were found in D/V boundary cells in both elytra and hindwing discs [180]. Comparative developmental analysis found that the *abrupt* gene encodes a novel regulator of Notch signaling and plays an essential role for proper wing vein patterning in both the red flour beetle and fruit fly [181]. The beetle E(spl) proteins suppressed the formation of bristles and veins when ectopically expressed in fly wing disc [73]. The red flour beetle has emerged as an important model insect with elaborate genetic toolkit and imaging techniques [182,183]. In the foreseeable future, studies in *T. castaneum* will bring up new insights about the roles of Notch signaling in different types of insect wings.

A recent study revealed that in the brown planthopper, *Nilaparvata lugens*, the *Notch* locus contains a complex alternatively spliced gene encoding a series of protein isoforms with different expression patterns [184]. When dsRNAs targeting different transcripts were injected into the planthopper nymphs, a certain degree of functional differences among Notch variants was observed. Several planthopper Notch variants were involved in bristle, vein and wing blade development [184]. Expression of Notch pathway genes was detected in the wing discs of various insect species, but their functions in wing development need further exploration [185,186].

5. Notch signaling in insect leg development

Notch pathway plays diverse and fundamental roles in *D. melanogaster* leg patterning and growth [187]. The fruit fly legs are composed of ten segments, and each segment is separated by a flexible joint. Fusion of leg segments and reduction of leg growth have been noticed as common defects in *Notch*, *Dl* and *Ser* mutants [188–190]. On the other hand, ectopic activation of Notch within the leg were sufficient to induce formation of extra segment borders (joints) and local cell growth [191–193]. The separation between leg segments is determined in the larval stage, at which time *Notch*, *Ser*, *Dl*, and *fringe* are expressed in a segmentally repeated pattern in the imaginal leg disc [191–194]. Interestingly, Notch is asymmetrically activated at the distal side of the *Ser* and *Dl*-expressing domain, forming nine rings along the Proximal-Distal (P-D) axis in the leg disc [195]. Such polarized activation of Notch is critical for proper leg development, continuously activation of Notch across segments led to shortened legs with segmentation defects [191–193]. Numerous factors and pathways were found to interact with Notch signaling to establish the boundaries between leg segments, but the underlying mechanisms are still not fully elucidated [196–209]. Proper activation of Notch pathway is also instructive for the formation of the joints and for the correct growth of the leg [210]. The fly leg joints can be subdivided into two classes, the proximal “true joints” and the distal “tarsal joints”. The morphology of true joints varies from one to another, while the tarsal joints are identical and are composed of a “socket” in the proximal part and an interlocking “ball” in the distal part [195]. Both types of joints are shaped by Notch signaling, while distinct target genes are separately activated in true joints and tarsal joints [187,195,211]. During tarsal joints morphogenesis, Notch signaling is required for the fate specification of socket cells and ball cells, as well as for the extensive cell shape changes and cell movements which are essential for forming the joint structures [212,213]. How Notch controls leg growth are mostly unknown, while some reports indicate that the interaction with Hippo pathway is involved [192,197].

The roles of Notch in leg segmentation, joint formation and leg growth are quite conserved among different insect species. Fusion of the tarsal elements in the leg was found in *D. hydei* Notch mutants [146]. In the cricket *G. bimaculatus*, RNAi knock-down of *Notch* led to reduction of leg length and loss of joints [70]. In the red flour beetle, *Notch* RNAi eliminated most joints from the leg but the leg length was not affected, while *Ser* RNAi eliminated nearly all the joints and reduced the overall length of the leg [214,215]. The *nubbin* (*nub*) gene was expressed in a series of concentric rings in fly leg disc, and mutation of *nub* resulted in shortened legs [216,217]. Notch signaling directly regulates the expression of *nub* in fly leg disc [192,203]. Expression of *nub* homologs were found in the developing legs in several insect species, including the cockroach *P. americana*, the milkweed bug *O. fasciatus* and the primitively wingless firebrat *Thermobia domestica* [218]. RNAi knock-down of *nub* in *O. fasciatus* embryos led to shortened thoracic legs and growth of ectopic appendages on abdominal segments [219]. In the house cricket *Acheta domesticus* and the cockroach *P. americana*, *nub* was required for the growth of specific leg segments and joint formation [220]. The finding that the levels of *nub* expression were reduced after *Notch* RNAi indicates that Notch signaling might also regulate *nub* expression in *P. americana* [220]. Several signaling pathways, including Notch were upregulated during regenerative patterning and growth in ladybird beetle (*Harmonia axyridis*) legs [221]. Notch mediated segmentation of the appendages has been proposed as a defining character and molecular phylotypic trait of arthropods, which could be further tested in other insect species [222].

6. Notch signaling in insect reproduction

Notch signaling is essential for ovary development in *D. melanogaster*, particularly during egg chamber formation and the assembly and maintenance of the ovarian germline stem cell (GSC) niche [33,223,224].

Oogenesis in fruit fly begins in the germarium located at the anterior tip of the ovariole, where GSCs divide asymmetrically to give rise to cystoblasts. Each cystoblast undergoes four rounds of incomplete cell division to produce a germline cyst containing 16 interconnected cells. The germline cyst is then encapsulated by somatically derived follicle cells, and the collection of germline and follicle cells at this point is known as an “egg chamber.” In the egg chamber, only one cell within a germline cyst will develop into the oocyte, while the remaining 15 cells become nurse cells to provide RNAs and proteins for the oocyte. An egg chamber progresses through numerous developmental stages while moving toward the posterior of the ovary before finally becoming a mature egg and entering the oviduct [224]. Using temperature sensitive mutant alleles, mutation of *Notch* and *Dl* were found to significantly inhibit the number of eggs laid by female flies, and defects in both follicle cell development and the establishment of anterior-posterior polarity in the oocyte were observed [225]. Subsequent studies demonstrated that Notch activity is required in the follicle cells for their transition from mitosis to endocycling, which is regulated by Dl expressed in the germline cells [226–238]. The egg chamber possesses intrinsic anterior-posterior (AP) polarity, with the nurse cells being anterior and the oocyte posterior. This stereotypical position plays a key role in generating AP polarity in both the oocyte and the surrounding follicle cells [239]. Interestingly, such AP polarity is established through a relay mechanism which propagates asymmetry from older to younger cysts. During early stage of oogenesis, a germline cyst signals through the Dl-Notch pathway to induce the formation of the anterior polar cells when it buds from the germarium. The anterior polar cells in turn express the JAK/STAT signaling ligand Unpaired and signal toward the adjacent anterior stalk/polar cell precursors to induce the stalk cell fate. The stalk induces the positioning of the oocyte at the posterior pole in the adjacent younger cyst by adhesive interactions. As the oocyte is being positioned, a new round of Dl-Notch signaling takes place in that cyst to induce the anterior polar cells [240,241]. Thus, each cyst polarizes the next cyst through a series of posterior to anterior induction events [242,243].

The concept of stem cell niche was proposed around half a century ago to describe the tissue microenvironment capable of supporting the self-renew and maturation of haemopoietic stem cells, which was later extended to other tissues that contain stem cells. However, the ability of the niche to support stemness was firstly demonstrated by studies of fly ovarian GSC niche [244]. At the anterior tip of the germarium, the terminal filament cells and the cap cells form the niche which supports two to three ovarian GSCs. The formation of both terminal filament cells and cap cells rely on proper Notch signaling [244–253]. Importantly, the strength of Notch signaling controls the size of the ovarian GSC niche. Hyperactivation of Notch signaling leads to the formation of more cap cells and bigger niches, which support more GSCs. Whereas dampened Notch signaling results in decreased cap cell number and niche size, and consequently less GSCs [246]. In mature adult flies, Notch signaling also transmits the effects of diet and age on GSC niche activity and GSC maintenance [254–258]. .

Two major types of ovarioles, panoistic ovarioles and meroistic ovarioles, are found in insects. In panoistic ovarioles, all progenies of germline stem cell become functional oocytes and no nurse cells are formed. Meroistic ovarioles contain nurse cells and can be further classified into polytrophic ovarioles and telotrophic ovarioles by morphological characters. In polytrophic ovarioles, nurse cells are found in the egg chamber and transport mRNA and proteins to oocyte through ring canals. While in teleotrophic ovarioles, nurse cells are located in the germarium and are connected to oocytes in early stages by nutritive cords in a distance [259]. Although it is generally believed that the panoistic ovaries represent the ancestral type from which the meroistic types had derived, there is no exact correspondence between ovariole type and phylogenetic position [260]. In the panoistic ovariole of the cockroach *Blattella germanica*, inhibition of Notch signaling activity caused defects in stalk formation, follicle cell proliferation and follicle cell differentiation [261–264]. In the telotrophic ovariole of *T. castaneum*, Notch signaling is required for stalk formation, follicle cell proliferation and AP axis establishment [265,266]. Interestingly, the role of Notch signaling in follicle cells differ significantly in distinct insect species. In *D. melanogaster*, Notch promotes the switch from mitosis to the endocycle in follicle cells, whereas in *B. germanica* and *T. castaneum* Notch signaling is required for maintaining the mitotic cycle of follicle cells. The relationship between the role of Notch in follicle cells and ovariole structure remains an open question. Notch was found to regulate vitellogenesis in the fat body of *L. migratoria*, which is crucial for oocyte maturation and ovarian growth [267]. Vitellogenesis is generally required for insect oogenesis and egg production, whether Notch signaling is involved in this basic physiological event in other insects is worth for further examinations [268]. In the honeybee *A. mellifera*, oogenesis in the germarium of worker bees were repressed by Notch signaling [269,270]. In the honeybee queens, Notch pathway genes were dynamically expressed in the ovariole at different oviposition phases, but their functions were not examined [271,272]. Highly abundant Notch-like proteins were detected during the initial stages of ovary development in *Bactrocera dorsalis*, indicating a potential role in oriental fruit fly oogenesis [273]. Taken together, Notch signaling is likely generally required for oogenesis in insect species, but we have just scratched the surfaces on how Notch executes specific roles in distinct cell types, developmental stages and insect species during ovary development and reproduction.

7. Notch signaling in insect stress responses

Insects encounter numerous stresses in the real world, including but not limited to temperature fluctuation, nutrition deficiency, pathogen infection and toxin exposure. Recent studies have discovered that Notch signaling is involved in stress responses under several conditions.

Nutrition is one of the most important environmental variables impacting insect life cycles, and insects adjust their behavior, physiological activities and metabolic programs in respond to changes of food quality and quantity [274]. The insulin pathway functions as nutritional sensor to coordinate the metabolic requirement and other biological events [268]. In female insects, reproduction requires a massive input of nutrition resources to produce eggs with a large amount of nutrient reserves [275]. Insulin signal pathway relays the effect of diet on reproduction via regulation of Notch signaling in *D. melanogaster* ovary [254–256,258,276]. In somatic tissues such as gut, muscle and neuronal system, insulin signaling affects Notch activity by various means [277–279]. A recent study found that dietary cholesterol affects the level and duration of Notch signaling through modulating Dl and Notch stability and trafficking, which further influences cell differentiation in fly adult midgut and alters the metabolic program [280]. The expression of Notch pathway genes was upregulated when high quality diet was provided for honeybee *A. mellifera*, but the underlying mechanisms and the functional importance is still unknown [281]. In the larval guts of Asian honey bee, *Apis cerana*, the Notch pathway appeared to be targeted by miRNAs and piRNAs at different developmental stages [282,283]. The lifespan of fruit fly adults was extended by supplementation of pterostilbene, fucoxanthin and a traditional Chinse herb *Cistanche tubulosa*, and the expression of Notch pathway genes was increased upon ingestion of these substances [284–286]. These studies highlight the role of Notch signaling during response to nutrition status in insects.

Many viruses are dangerous pathogens for human beings and could be transmitted by vector insects such as mosquitoes [287]. After infection by the alphavirus Sindbis, expression of Notch pathway genes was up-regulated in S2 cells, leading to the presumption that Notch signaling may be involved in establishment of virus persistence in insect cells [288]. Induction of Notch pathway genes was also observed upon Dengue virus infection in *Aedes albopictus* cells [289]. Infection of Dengue virus and Chikungunya virus resulted in up-regulation of Notch pathway genes and midgut cell division in the vector mosquito *Aedes aegypti* [290–292]. Knocking-down the expression of *Dl* by RNAi inhibited midgut cell division induced by infection, but significantly increased the susceptibility of refractory *Aedes aegypti* strain to Dengue virus [293]. The human malaria parasite *Plasmodium berghei* also induced midgut cell division and Notch pathway activation in the vector mosquito *Anopheles albimanus* [294,295]. In the midguts of *Anopheles gambiae*, *Plasmodium falciparum* infection induced chromatin changes in regulatory elements of Notch pathway genes, but the significance of these observations needs to be further explored [296]. When wild silk moth *Antheraea yamamai* was infected by the pathogenic nucleopolyhedrovirus, expression of several Notch pathway genes was up-regulated in the larval midgut [297]. Inhibition of Notch signaling was associated with midgut development defects in locust *L. migratoria* and the yellow fever mosquito *Aedes aegypti* [298,299]. In fruit fly midgut, Notch signaling promotes asymmetric division in the intestine stem cells to control tissue homeostasis and responses to various stimulations [300–304]. Whether virus and pathogen infection would trigger Notch related responses in intestine stem cells could be further tested in other insects.

Notch plays a central role in hematopoietic cell fate determination and maintenance in larvae lymph gland, a vital organ of the immune defense system in *D. melanogaster* [35,305–308]. Fungal infection and wasp parasitization led to downregulation of Notch signaling and triggered specific immune responses in fruit fly [308,309]. Stimulation by Gram-negative bacteria led to up-regulation of Notch pathway genes in honey bee workers [310]. Several lncRNAs were found to control immune priming in *T. castaneum* likely by regulating expression of Notch pathway genes [311]. These studies indicate that Notch signaling might be involved in certain immune responses upon pathogen infection in insects.

Using the developing wing as a model system, the anthrax toxins and cholera toxins were found to inhibit endocytic trafficking of Notch signaling components and impair Notch activity in *D. melanogaster* [312,313]. In a Zika virus infection model, the virus non-structural protein NS4A was shown to restrict fly eye growth through regulation of JAK/STAT signaling and inhibit wing growth via affecting Notch activity [314]. The heavy metal mercury led to neurogenesis defects in fly embryo and marginal nicks in the wing strongly through inhibiting Notch cleavage and NICD production [315]. Treatment with methylmercury, an organic form of mercury easily absorbed by the intestinal trac and an environmental pollutant, led to increased levels of Notch signaling in fly cells and embryos [316–319]. The response of Notch target genes to mercury showed variations in different cell types, upon treatment of organic or inorganic forms, and was sometimes independent of the Notch receptor [316–319]. Pesticide exposure also affects Notch signaling in several insects. Feeding fly larvae with sublethal level of chlorfenapyr resulted in wing and leg developmental defects and disruption of Notch signal activity [320]. Exposure to four different adverse environmental factors, including low dose of gamma-irradiation, formaldehyde, toluene and dioxin, commonly affected Notch signaling in adult flies [321]. Harmine, a natural β-carboline, inhibits *Drosophila* development by negatively affecting the developmental signaling network including Notch signal pathway [322]. Fipronil exposure decreases the expression and activity of Notch in the brain and Malpighian tubules of the stingless bee *Partamona helleri* [323,324]. Sublethal level of fluralaner impaired larval development of the common cutworm *Spodoptera litura* and led to wing notches in the surviving adults, but whether Notch signaling was affected is not examined [325]. The Notch pathway was responsive to ultraviolet irradiation and metamorphosis oxidative stress in *B. mori* [326]. Together, these results suggest that Notch signaling may be involved in response to hazardous factors in insects.

Insects would go through functional hypoxia when oxygen supply is insufficient for metabolism demand, and respond to hypoxia via multiple strategies [327]. Hypoxia tolerance is regulated by Notch signaling in *D. melanogaster*, flies with impaired Notch activity had a significant reduction in hypoxia tolerance, but those with hyperactivated Notch signaling had a dramatic opposite effect [328–332]. It would be exciting to examine whether such strong effects of Notch signaling in hypoxia tolerance is also present in other insects, especially those experience environmental hypoxia at certain stages in their life history.

8. Notch signaling in less studied but more interesting tissues

Insects have evolved to acquire many fascinating novel structures while keeping a steady basic body plan, and the mechanisms underlying the evolution of morphological novelties remain enigmatic [333]. From a developmental biology point of view, as one of the fundamental regulator units of tissue development and growth, the involvement of Notch signaling in construction of such morphological novelties is actually not surprising at all.

The bull-headed dung beetle *Onthophagus taurus* and many other scarab beetle species possess rigid projections of the exoskeleton referred to as horns, which is a highly diversified and evolutionarily novel morphological structure evolved from wing serial homologs [334]. Notch pathway genes were expressed in the developing dung beetle horn [335,336]. Importantly, Notch signaling was identified as a regulator of the dramatic diversity of horn sizes and shapes across dung beetle males [337]. In the Asian rhinoceros beetle *Trypoxylus dichotomus*, *Notch* RNAi disturbed horn primordial furrow depth which led to defects in the horn shape in the pupae [338].

The dorsomedial and the abdominal support structurea are two types of body wall projections commonly observed in the pupae of scarab beetles. These two structures were identified as wing serial homologues in *Onthophagus taurus*, and *Ser* RNAi disrupted the formation of both structures [339]. Gin-trap is as structure found exclusively on pupae of the closely related beetle families Tenebrionidae and Colydiidae, which is believed to be evolved after the radiation of holometabolous insects. Gin-traps function as defensive organs to grasp the appendages of predators in the pupae. In the beetle *T. castaneum*, RNAi knock-down of Notch pathway components disrupted the formation of gin-traps [340].

In dung beetles, the fore tibia has become a specialized digging tool which may have allowed them to access the compacted soil as habitat. The dung beetle fore tibia is a flattened and enlarged segment characterized by four to five prominent tibial teeth which could enhance the digging performance. RNAi of *Ser* and downstream genes of Notch pathway resulted in fusion of all leg segments accompanied by a reduction of tibial teeth in *Onthophagus taurus* [341]. These findings indicate that Notch signaling is repeatedly recruited to regulate the development of evolutionarily novel morphological structures in beetles.

As the major olfactory sensory organ, antennae are crucial for insects to locate food resources, mating partners and oviposition sites, as well as to evade predators and toxic substances. The insect antennae are highly diversified with astonishing variations in shapes, structures and sizes [342]. In *D. melanogaster*, the fate of antenna cells is determined by several selector genes, while Notch signaling regulates cell proliferation, tissue growth and formation of boundary between antenna segments [192,203,343–345]. In the beetle *T. castaneum*, *Ser* RNAi resulted in strong reduction in antenna length and complete absence of joints, while *Notch* RNAi led to the absence of most antennal joints without significant reduction of antenna growth [346]. However, *Notch* RNAi, but not *Ser* RNAi decreased the density of sensory bristles on the antenna [346]. The expression pattern of *Notch* and *Dl* in the developing antenna in the cricket *G. bimaculatus* was suggestive for a role in segmentation [70].The *nub* gene is a down-stream target of Notch signaling during leg development in *D. melanogaster* and *P. americana* [220]. In the milkweed bug *O. fasciatus*, *nub* RNAi resulted in deficient sensory bristles formation without significant affecting segmentation and growth [219]. Depletion of *nub* by RNAi in *Acheta domesticus* and *P. americana* led to fusion of segments in antenna [220]. The Expression of Notch target gene *E(spl)mβ* was detected in specific segments in *B. mori* larval antennal primordium, which develops as the feather like antenna in adults [347]. Upon *Notch* RNAi, the *B. mori* antenna segments were drastically fused and the overall length along the PD axis was extensively reduced, and milder defects such as fusion of neighboring lateral branches were also observed [348]. These findings support a general requirement of Notch signaling in insects antenna growth and segmentation.

Insects show a remarkable diversity of mouthpart morphology, and the genetic regulatory network for mouthpart development is not completely understood. RNAi knock-down of *Dl* in the silkworm and honeybee caused mild alterations in embryonic labrum shape [89,100]. Knockdown of two components of the Notch pathway, *Ser* and *mib1*, led to loss of the labrum in *T. castaneum* larvae likely due to defects in cell proliferation [215]. RNAi of *Notch* and *Dl* also disrupted sensory organ development in the *T. castaneum* labrum [349]. A role of Notch signaling during mouthpart construction in the fruit fly and other insects is expected but has not been reported yet.

The diverse color patterns of insects often function as camouflage to protect them from predators [350]. In the Asian swallowtail butterfly, *Papilio xuthus*, young larvae from the first to the fourth instar show black and brown patterns to mimic bird droppings but change their color pattern to mimic host plants in the final instar. Such color pattern switch in *Papilio xuthus* caterpillar was initiated at the early fourth instar by the juvenile hormone, and both *Dl* and *E(spl)mβ* were found to be specifically expressed in the epidermis of certain color marking region during the pattern switch period [351,352]. Functional analysis demonstrated that Notch signaling defines the edge and pigmentation area in the final color patterns [353]. RNAi knock-down of *Notch* and *Dl* led to expansion of pigmentation area and disruption of border lines in the fifth instar larvae. RNAi knock-down of *Dl* in *Papilio machaon*, a species closely related to *Papilio xuthus*, led to similar, albeit more subtle phenotypic changes of larvae color pattern. Interestingly, in the silkworm *L* mutant, which shows pairs of black brown twin spots on each larva body segment, knockdown of *Notch* but not *Dl*, *Ser* or *fringe* caused pigmentation loss in the twin spots [353]. These findings demonstrated the importance of Notch signaling in the adaptive evolution of camouflage formation in caterpillars, which encourages further examination of the roles of Notch signaling during color pattern formation in other insect species.

9. Conclusions

It has been more than one century since the first *Notch* mutant was isolated in the fruit fly, which has been used as a leading model system to investigate the developmental functions of Notch signaling. Comprehensive studies in this little inconspicuous insect has led to impressive progresses in Notch signaling mechanisms. The crucial components, signal transduction cascade and the major modes of action of Notch pathway are likely highly conserved among insect species, and the involvement of Notch signaling during the development of various insect tissues have been validated.

Nevertheless, in distinct insect species there are too many fascinating developmental events which are absent in the fruit fly [58,84,342,350,354,355]. Recent advances in genome editing and other functional genetics technologies have made it possible to identify novel genetic factors and evaluate the contribution of Notch signaling pathway underlying diverse developmental phenomena in 'non-model' insect species (Figure 3). Future studies will undoubtedly help us to better understand the extensive roles of Notch in the formation and evolution of insect tissues, and may also reveal novel regulators, functions and signaling mechanisms. On the other hand, these knowledges could be readily converted to design genetic control methods such as RNA pesticide [356], genetic sexing [357] and gene drive system [358] to protect crops and humans from insect pests.

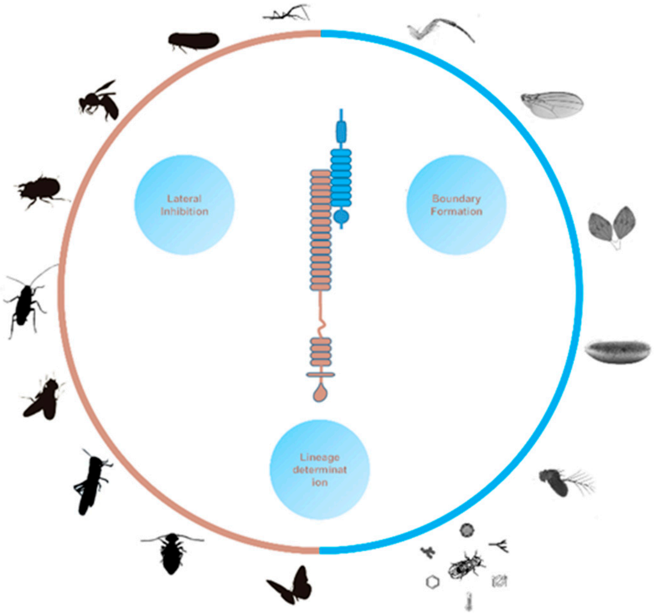

Figure 3. Roles of Notch signaling in insect development. The Notch signaling pathway primarily functions through three major modes, including lateral inhibition, boundary formation, and lineage determination. Widely present in major insect taxa, Notch signaling plays a crucial role in the development of embryos, wings, legs, ovaries and antennae, as well as in the insect's response to stress.

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