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SHORT COMMUNICATION

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# Evidence for Notch-mediated lateral inhibition in organizing butterfly wing scales

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**Abstract** Here I present gene expression data that implicate a Notch-mediated lateral inhibition process in the spatial organization of butterfly wing scales. During early pupal development the receptor molecule Notch is expressed in a grid-like pattern in the wing epithelium, resulting in parallel rows of uniformly spaced cells with low Notch expression. Previous work has shown that these low-Notch cells express a homolog of the *achaete-scute* proneural transcription factors and develop into scales. All of these observations are consistent with the *Drosophila* model of Notch-mediated bristle determination and support the hypothesis that bristles and scales share an underlying patterning mechanism.

**Keywords** *Heliconius* · Bristle · Achaete-scute · Evolution · Development

## Introduction

The majority of moth and butterfly wings are covered with thousands of flat, overlapping scale cells (Fig. 1A). These scales may serve multiple functions including thermoregulation, pheromone dispersal, and color pattern formation (Scoble 1992). Mayer (1896) noted the developmental similarity between lepidopteran scales and insect sensory bristles and concluded that the two structures are homologous, a hypothesis supported by subsequent observations (Overton 1966; Wigglesworth 1972; Galant et al. 1998). There is molecular evidence for scales being evolutionarily derived from bristles in that a homolog of the *achaete-scute* (*ac-sc*) transcription factors is expressed in scale-forming cells (SFCs) of the nymphalid butterfly *Junonia* (*Precis*) *coenia* (Galant et al. 1998). *ac-sc* expression promotes the determination of neural precursor cells, including bristle precursors, in *Drosophila melanogaster* (Calleja et al. 2002), and the expression of an *ac-sc* homolog in SFCs suggests that bristles and scales share an underlying specification mechanism (Galant et al. 1998). In this report I explore the possibility that scales and bristles may be spatially organized through a shared mechanism.

In most butterfly species, wing scales occur in parallel rows (Nijhout 1991). Perhaps not coincidentally, notal bristles of *D. melanogaster* and many other muscomorph flies are also spaced in parallel rows (Simpson et al. 1999). Bristle spacing in *D. melanogaster* is organized through lateral inhibition activity of the Notch signaling pathway (Artavanis-Tsakonas et al. 1999; Simpson et al. 1999). In this process, cells expressing Delta, the membrane-bound Notch ligand, activate the Notch receptor in neighboring cells thereby triggering a positive feedback increase of Notch expression in those neighboring cells. The resulting pattern consists of spaced, isolated Delta-expressing cells surrounded by Notch-expressing cells. Because Notch activation results in repression of *ac-sc*, only the central Delta-expressing cells express *ac-sc* and are specified as neural precursors. On a larger scale, this process contributes to generating a pattern of bristles spaced in rows on the fly's notum. Significantly, detailed mathematical models have demonstrated that a Notch-like lateral inhibition process is sufficient to explain the morphological differentiation of butterfly SFCs during early pupal development (Honda et al. 2000).

Because of (1) the common ancestry of wing scales and sensory bristles, (2) the similar requirements of scales and bristles to be patterned on an epidermal monolayer, (3) the expression of the Notch target *ac-sc* in SFCs, and (4) the applicability of lateral inhibition models to patterns of SFC differentiation, it was apparent that Notch signaling was a strong candidate for playing a role in scale organization. To assess this I examined the expression of Notch in butterfly wings during early SFC determination.

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## Materials and methods

### Immunohistochemistry

Antibody stains were performed on forewing tissue of 16-h pupal *Heliconius erato petiverana* (Nymphalidae) using a previous protocol (Brunetti et al. 2001). Antibodies for the double stain were a mouse monoclonal antibody C17.9C6 that recognizes the Notch intracellular domain (Fehon et al. 1990) and a rabbit polyclonal antibody that recognizes the transcription factor Distal-less (Panganiban et al. 1995). Secondary staining was done using Cy3-conjugated goat anti-mouse and Cy2-conjugated goat anti-rabbit antibodies (Jackson Laboratories). Slide-mounted samples were visualized on a confocal microscope.

In larval moth and butterfly wing discs, the C17.9C6 Notch antibody localizes in patterns consistent with Notch's known roles in vein and margin patterning (unpublished observation). Furthermore, high magnification images from larval wing disc stains show C17.9C6 localization on the apical cell membranes, consistent with Notch's identity as a membrane-bound receptor (unpublished observation). Other preliminary data show that the C17.9C6 Notch antibody stains developing grasshopper legs in patterns similar to those described from *Drosophila* (B. Blachuta, personal communication). Together, these observations suggest that the C17.9C6 Notch antibody effectively recognizes the Notch molecule across multiple insect orders.

The use of a Distal-less antibody as a nuclear marker in this study was possible because the Distal-less protein is expressed over a portion of the *H. erato* pupal wing epidermis. Preliminary data suggest that this expression may be associated with the adult color pattern, but further work is required to establish this with certainty.

### Scanning electron microscopy

For the scanning electron micrograph a melanic portion of an adult *H. erato* forewing was dissected from a deceased butterfly. Scales were carefully brushed off some of the wing membrane to reveal the underlying socket arrangement. The sample was mounted dorsal side-up on an aluminum stub with adhesive tape and then sputter coated with 30 nm gold. The sample was visualized on a scanning electron microscope at ×170 magnification and the image was captured on Polaroid film.

## Results and discussion

### Notch expression is consistent with a lateral inhibition model

Sixteen hours after pupation, when SFCs are known to be determined in nymphalid butterflies (Galant et al. 1998), Notch is upregulated in a grid-like pattern in the butterfly wing epidermis (Fig. 1B). This results in parallel rows of uniformly spaced cells with downregulated Notch expression. Consistent with molecular and theoretical models of lateral inhibition (Simpson et al. 1999; Honda et al. 2000), low-Notch cells never neighbor each other and are always encircled by 7–10 high-Notch cells. The low-Notch cells also have larger nuclei and diameters than the high-Notch cells (inferred from the nuclear stain, Fig. 1B), unambiguously identifying these cells as the nascent polyploid SFCs (Nijhout 1991; Galant et al. 1998). While the grid-like pattern is consistent across the wing epidermis, there is variation in the specific number of cells surrounding each SFC, indicating a certain level of stochasticity in the SFC patterning process. It would be predicted that a membrane-bound Notch ligand such as Delta is expressed in the low-Notch cells; however this could not be determined because a Delta marker has not yet been developed for butterflies.

The observed Notch expression pattern is suggestive of a process that may produce a repetitive and highly ordered arrangement of specified cells from a morphologically undifferentiated epithelium. In a pure lateral inhibition model the degree to which SFCs are organized in parallel rows appears to be primarily dependent upon two variables: (1) the size of the nascent SFCs during the lateral inhibition process, and (2) the ratio between the number of SFCs and their surrounding undifferentiated cells (Honda et al. 2000). Although Honda et al. (2000) did not thoroughly explore the relationship between parameter space and the emergent parallel organization of SFCs, their simulation with the lowest values for the two above parameters produced a SFC pattern similar to that observed in Fig. 1B. It is worth noting that differentiation of SFCs appears to occur simultaneously across the wing, ruling out the possibility that developmental asymmetry between wing regions is a mechanism for row formation.

In summary, the association between Notch expression and previous theoretical, developmental, and gene expression studies strongly implicates a Notch-mediated lateral inhibition process in the spatial organization of SFCs in butterfly wings (Fig. 1C).

### Scale and bristle organization: a developmental basis for parallel evolution?

In most moths, wing scales are not organized in any apparent pattern beyond being evenly spaced (Nijhout 1991). Most parameter combinations applied to the Honda et al. (2000) lateral inhibition model produce a semi-random arrangement of SFCs reminiscent of ancestral moth scale patterns. Pending Notch expression data from moths, it is reasonable to speculate that in moths an ancestral Notch pattern generator produces an evenly spaced, but otherwise unorganized, arrangement of SFCs. Subsequently, selection may have acted on "parameters" of SFC development to tune the lateral inhibition process to produce parallel rows as in butterflies.

There are several unrelated butterfly lineages in which scales do not form parallel rows (Nijhout 1991), suggesting that the scale patterning system may be evolutionarily labile. Furthermore, there is cryptic variation in early SFC development between lepidopteran families. In the nymphalids *J. coenia* and *H. erato*, SFCs occur in distinct parallel rows when they are first differentiating (Fig. 1B; Nijhout 1991; Galant et al. 1998). In contrast, initial SFC differentiation in the pierid *Pieris rapae* appears to occur in a spatially random pattern, with SFCs only later becoming arranged into rows through an as-of-yet unknown process (Yoshida and Aoki 1989; Honda et al. 2000). In the sphingid moth *Manduca sexta,* one of the

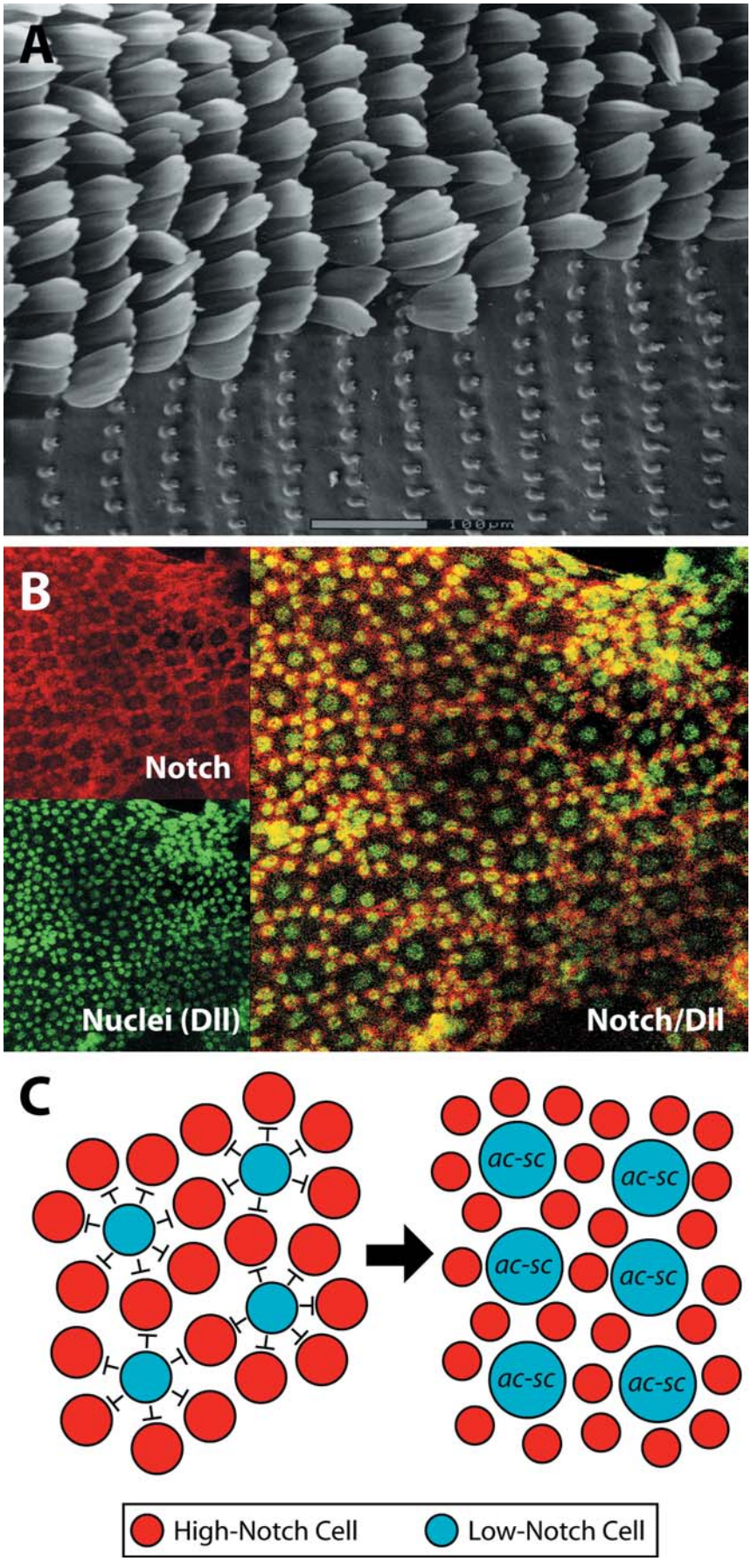


**Fig. 1A–C** Notch expression supports a lateral inhibition model of butterfly wing scale determination. **A** Scales in most butterflies are arranged in parallel rows, as seen in this electron micrograph of a *Heliconius erato* forewing. Scales towards the bottom of the micrograph have been brushed off to reveal the parallel arrangement of sockets. Both scales and sockets are derived from a mother scale-forming cell (SFC) during pupal development. **B** Sixteen hours after pupation, during early SFC differentiation, Notch is upregulated in a grid across the wing epithelium of *H. erato*. The cells with low Notch expression are nascent SFCs. Nuclei are stained by an antibody to the transcription factor Distal-less. **C** The grid-like Notch expression pattern is consistent with the lateral inhibition model of neural precursor determination from *Drosophila melanogaster,* and with the previously described expression of *ac-sc* in SFCs 24 h after pupation in the butterfly *Junonia coenia*. In this hypothetical model, low-Notch cells inhibit expression of *ac-sc* genes in neighboring cells while uninhibited *ac-sc* expression in low-Notch cells results in an SFC fate

minority of moths with parallel scales, SFCs become arranged into rows through cell migration (Nardi and Magee-Adams 1986). Inferring the evolutionary history of lepidopteran scale patterning awaits a more thorough phylogenetic treatment, but ultimately may provide an excellent context for studying the evolution of a patterning system.

On a broader scale, the evolutionary transition(s) from random to parallel scales on the lepidopteran wing is remarkably similar to the transition from random to parallel bristles on the dipteran notum (Simpson et al. 1999). Another similarity between bristle and scale evolution is the derivation of row spacing. Aligned SFCs in *P. rapae* and *H. erato* are spaced by only one or two cells, which is likely the ancestral condition. In contrast, aligned SFCs in *J. coenia* are spaced by two to four cells (Nijhout 1991; Galant et al. 1998; Koch et al. 2003). This presumably derived state in *J. coenia* is similar to the spacing of fly bristle rows by four to five cells. How rows may become spaced in the context of Notch-mediated lateral inhibition is unknown.

The similar sequence of innovations in the evolution of bristle and scale pattern development is striking, and is suggestive of parallel evolution. This begs the question: can the mechanistic qualities of a conserved signaling pathway promote the evolutionary re-emergence of stereotyped, but non-homologous, patterns?

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