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Recruitment of a *hedgehog* Regulatory Circuit in Butterfly Eyespot Evolution

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The origin of new morphological characters is a long-standing problem in evolutionary biology. Novelties arise through changes in development, but the nature of these changes is largely unknown. In butterflies, eyespots have evolved as new pattern elements that develop from special organizers called foci. Formation of these foci is associated with novel expression patterns of the Hedgehog signaling protein, its receptor Patched, the transcription factor Cubitus interruptus, and the *engrailed* target gene that break the conserved compartmental restrictions on this regulatory circuit in insect wings. Redeployment of preexisting regulatory circuits may be a general mechanism underlying the evolution of novelties.

To understand the origin of evolutionary novelties, the developmental and genetic mechanisms that produce new structures and patterns must be elucidated (*1*). One fundamental issue to be addressed is the extent to which novelty arises from either the redeployment of preexisting developmental programs or the separate recruitment of selected individual genes into new programs. The eyespots on butterfly wings are a recently derived evolutionary novelty that arose in a subset of the Lepidoptera and play an important role in predator avoidance (*2–4*). The production of the eyespot pattern is controlled by a developmental organizer called the focus, which induces the surrounding cells to synthesize specific pigments (*5–7*). The evolution of the developmental mechanisms that establish the focus was therefore key to the origin of butterfly eyespots. Here, we identify changes in development and gene expression associated with the evolution of eyespots.

Our screen for genes involved in eyespot evolution was based on the hypothesis that one or more of the signaling molecules that pattern the insect wing blade might also be involved in eyespot development. In *Drosophila*, two orthogonal systems of short-range [Hedgehog (Hh) and Serrate/Delta] and long-range [Decapentaplegic (Dpp) and Wingless (Wg)] signaling proteins organize wing imaginal disc growth, patterning, and gene expression (*8*). If any of these signals were involved in eyespot development, then modulation of their expression patterns near the position of eyespot foci should be observed. To test this hypothesis, we cloned the *Precis coenia* orthologs of the *dpp*, *wg*, and *hh* genes (*9*) and examined their expression patterns by in situ hybridization in fifth (last) larval instar wing imaginal discs, when establishment of the foci occurs (*5*). Only *hh* expression is modulated near developing foci.

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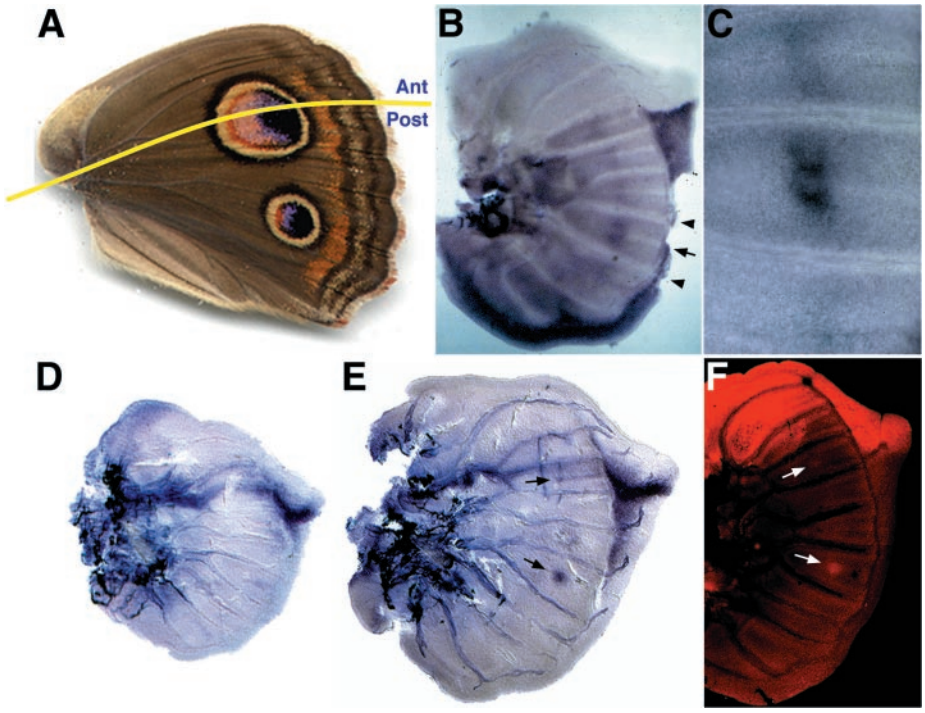

Fig. 1. Hh signal transduction is associated with the establishment of eyespot foci. (**A**) Dorsal surface of the hindwing. The A/P border is indicated (Ant/Post). (**B** and **C**) In situ hybridization with a probe for *hh* RNA. (B) In the mid-fifth instar, *hh* transcripts are detected throughout the posterior compartment. However, *hh* transcription is decreased in the midline (arrow) between the veins (arrowheads) and increased in regions flanking the positions of each potential focus. The heavily stained distal region (damaged in this specimen) is fated for cell death. (C) In the late fifth instar, *hh* transcription is sharply increased in cells flanking those destined to form an active focus (center). No increase is seen in positions that will not form active foci (top and bottom). (**D** and **E**) In situ hybridization with a probe for *ptc* RNA. (D) In the mid-fifth instar, *ptc* transcripts are detected in cells just anterior to the A/P boundary. (E) In the late fifth instar, *ptc* transcripts are also detected in the posterior compartment in eyespot foci (arrows). (**F**) Immunofluorescent detection of Ci protein reveals expression throughout the anterior compartment as well as in the posterior compartment in both eyespot foci (arrows). The anterior part of the wing is toward the top and distal is to the right in all panels.

In mid-fifth instar imaginal discs, *hh* is expressed in all cells of the posterior compartment (Fig. 1B), as in *Drosophila* (*10*), but *hh* transcript levels are increased in a striking pattern in cells just outside of the subdivision midlines at specific positions along the proximodistal axis of the wing. These domains of increased *hh* transcription flank cells that have the potential to form foci. Later in the fifth instar, higher levels of *hh* transcripts accumulate specifically in cells that flank the developing foci (Fig. 1C).

To test whether this increased level of *hh* transcription around developing foci is indicative of active Hh signaling, we examined the expression patterns of genes known to be modulated in response to the Hh ligand, namely, the Hh receptor encoded by *patched* (*ptc*) and the signal transducer encoded by *cubitus interruptus* (*ci*) (*11*, *12*). In the presence of high levels of Hh, Ptc function is inhibited, resulting in the accumulation of the activator form of Ci. Because *ptc* is a direct target of Ci (*13*), cells that receive and transduce the Hh signal have increased levels of *ptc* transcription.

In early and mid-fifth instar *P. coenia* wing discs, high levels of *ptc* transcripts are present along the anteroposterior (A/P) boundary of the wing (Fig. 1D), and *ci* transcripts are limited to the anterior compartment of the disc (*14*). These patterns are homologous to those observed in *Drosophila* wing discs (*15*–*17*). However, later in development, activation of *ptc* (Fig. 1E) and *ci* transcription (*14*), accompanied by accumulation of Ci protein (Fig. 1F) (*18*), occurs in the posterior compartment of the butterfly wing disc in cells that are flanked by the domains of highest *hh* transcription and are destined to become eyespot foci. These results indicate that the Hh signal is received and transduced by cells that will differentiate as foci.

The expression of *ptc* and *ci* in eyespot foci within the posterior compartment of the butterfly wing breaks the A/P compartmental restrictions on gene expression known from *Drosophila*. In *Drosophila*, Engrailed (En) acts in the posterior compartment to restrict Ptc and Ci to the anterior compartment through direct repression of *ci* transcription (*15*, *17*), which in turn prevents *ptc* expression (*19*, *20*). *ptc* and *ci* expression in the posterior compartment of *P. coenia* wing discs indicates that during the evolution of eyespots, either the repression of *ci* by En or the expression pattern of the *en* gene has diverged from that observed in *Drosophila* wings.

We examined whether the expression of *en*, or of its paralog *invected* (*inv*), was modulated during focal establishment. In early fifth instar wing discs, these genes are expressed evenly throughout the posterior compartment (*14*). However, later in development, En and Inv are expressed at much higher levels in foci (Fig. 2A) (*21*) and are completely coincident with the expression of Ci (Fig. 2B). In pupal wing discs, when focal establishment is complete, expression of En/Inv is maintained in the focus and expands to cells around it (Fig. 2C). These results suggest that during the course of eyespot

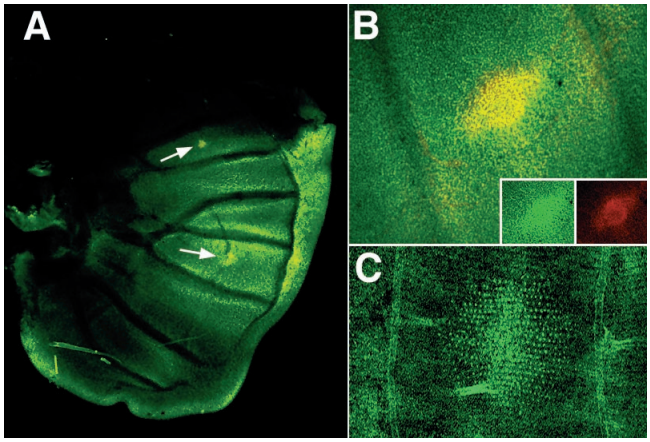

Fig. 2. En/Inv is expressed at high levels in eyespot foci. (**A**) Detection of En/Inv with the monoclonal antibody 4F11 reveals expression throughout the posterior compartment with higher levels present in eyespot foci (arrows). *en/inv* transcripts are expressed in identical patterns (*14*). (**B**) Double immunofluorescent detection of Ci (red) and En/Inv (green) shows that focal expression of these proteins is completely coincident. (**C**) After pupation, En/Inv protein is present at high levels in the eyespot focus and in surrounding scale-building cells (aligned in rows).

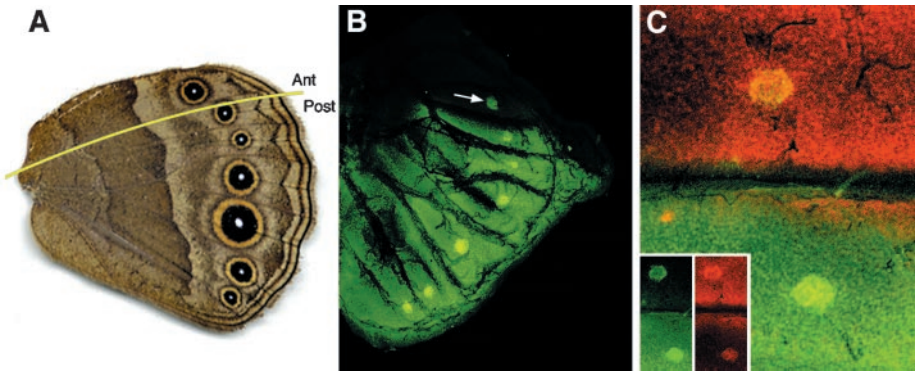

Fig. 3. Hh signal transduction is associated with the establishment of all eyespot foci, regardless of their anteroposterior location. (**A**) Eyespots occur in both the anterior and posterior compartments (border indicated) on the ventral surface of the *B. anynana* hindwing. (**B**) In the late fifth instar wing imaginal disc, En/Inv is expressed at high levels in all eyespot foci. En/Inv in the anteriormost focus (arrow) is well within the anterior compartment of the wing and does not contact cells in the posterior compartment. (**C**) High-magnification view of the region surrounding the A/P boundary reveals that En/Inv (green) and Ci (red) expression are coincident in the eyespot foci.

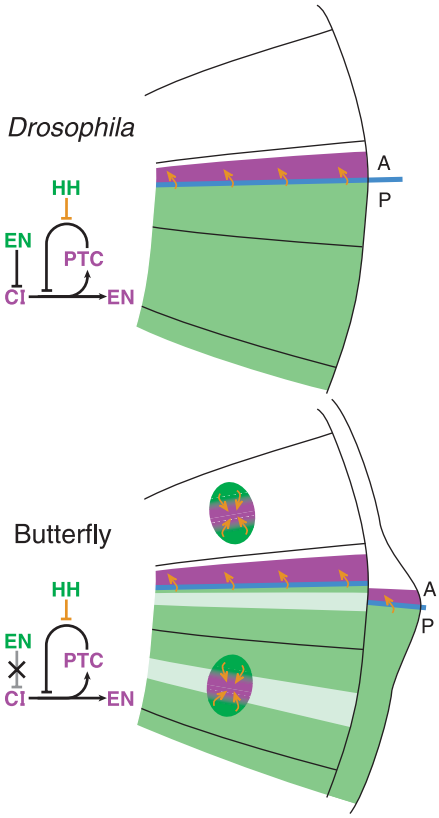

Fig. 4. Model for the recruitment of the Hh signaling pathway in the evolution of butterfly eyespot foci. Hh induction of *en/inv* expression in cells just anterior to the A/P boundary is a conserved feature of wing development. This activation is dependent on binding of the Hh ligand to the membrane receptor Ptc and the conversion of the transcription factor Ci to its activator form. In eyespot-bearing butterflies, the same Hh-regulated pathway is used to establish eyespot foci. *hh* expression is modified in butterflies through repression along the midline of each wing subdivision and increased transcription in cells flanking the eyespot foci. In butterflies, repression of *ci* in the posterior compartment is relaxed for these cells to express *ptc* and transduce the Hh signal.

evolution, there was a relaxation of the strict En-mediated repression of *ci* that occurs in the posterior compartment of *Drosophila*. Furthermore, the coincidence of the expression domains of *en/inv* and *ci*, rather than of *en/inv* and *hh*, indicates that during focal establishment *en* and *inv* are targets, rather than inducers, of Hh signaling.

In *P. coenia*, as in most species of butterflies, eyespots are found only in the posterior compartment of the wing, the normal domain of *hh* and *en/inv* expression. However, a few butterfly families contain species with eyespots in the anterior compartment. If *hh* signaling plays a role in focal establishment and all butterfly eyespot foci develop by the same mechanism, as is suggested by correlations in their variance (*22*), then expression of Hh signaling components and *en/inv* should be associated with foci located in the anterior as well as the posterior compartment. To test whether Hh signaling accompanies focal establishment regardless of its location in the wing, we examined hindwing imaginal discs of *Bicyclus anynana*, a species that forms eyespots on both sides of the A/P compartment border (Fig. 3A). Both En/Inv and Ci are coexpressed in all *B. anynana* eyespot foci, including the one in the anterior compartment (Fig. 3, B and C). Thus, the expression of the Hh signaling pathway and *en/inv* is associated with the development of all eyespot foci and has become independent of A/P compartmental restrictions.

The novel expression patterns of *hh*, *ptc*, *ci*, and *en/inv* could result from independent or dependent changes in their regulation during eyespot evolution. Experimental evidence from *Drosophila* and comparative analysis of butterflies leads us to infer that some changes in the expression of *hh* pathway components were primary whereas others were secondary consequences. For instance, beginning in the late third instar of the developing *Drosophila* wing disc, the ability to express *en/inv* in response to Hh signaling is a general property of Ci-expressing cells. This competence is present throughout the anterior compartment (*23*) but is only used just anterior to the A/P boundary to pattern intervein tissue (*24*, *25*). Expression of *en/inv* just to the anterior of the A/P boundary in *P. coenia* and *B. anynana* indicates that this regulatory circuit is conserved in butterflies (note overlap of En/Inv and Ci in Fig. 3C). The similarity between the induction of *en/inv* by Hh at the A/P boundary and in eyespot foci in late wing development suggests that during eyespot evolution, the Hh-dependent regulatory circuit that establishes foci was recruited from the circuit that acts along the A/P boundary of the wing (Fig. 4).

For this Hh regulatory circuit to operate in focal development, two primary spatial regulatory changes must have evolved. First, mechanisms must have evolved that modulate levels of *hh* expression along the proximodistal axis of the wing field. Second, because reception of the Hh signal depends on expression of the Ptc receptor, which in turn depends on Ci function, the restriction of *ci* from the posterior compartment must have been relaxed. *ptc* and *en/inv* expression would then evolve as secondary consequences of these regulatory changes. This recruitment of an entire regulatory circuit through changes in the regulation of a subset of components increases the facility with which new developmental functions can evolve and may be a general theme in the evolution of novelties within extant structures.

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Turning Brain into Blood: A Hematopoietic Fate Adopted by Adult Neural Stem Cells in Vivo

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Stem cells are found in various organs where they participate in tissue homeostasis by replacing differentiated cells lost to physiological turnover or injury. An investigation was performed to determine whether stem cells are restricted to produce specific cell types, namely, those from the tissue in which they reside. After transplantation into irradiated hosts, genetically labeled neural stem cells were found to produce a variety of blood cell types including myeloid and lymphoid cells as well as early hematopoietic cells. Thus, neural stem cells appear to have a wider differentiation potential than previously thought.

Stem cells have been identified in adult tissues that undergo extensive cell replacement due to physiological turnover or injury such as the hematopoietic, intestinal, and epidermal systems (*1*). These cells have been found in the central nervous system (CNS) (*2*), a tissue thought to be capable of extremely limited self-repair. CNS stem cells can generate the three major cell types found in the adult brain: namely, astrocytes, oligodendrocytes, and neurons (*3*). This is consistent with the view that the developmental potential of stem cells is restricted to the differentiated elements of