Temporal flexibility of gene regulatory network underlies a novel wing pattern in flies

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Organisms have evolved endless morphological, physiological, and behavioral novel traits during the course of evolution. Novel traits were proposed to evolve mainly by orchestration of preexisting genes. Over the past two decades, biologists have shown that cooption of gene regulatory networks (GRNs) indeed underlies numerous evolutionary novelties. However, very little is known about the actual GRN properties that allow such redeployment. Here we have investigated the generation and evolution of the complex wing pigmentation pattern of the fly Samoaea leonensis. We show that the transcription factor Engrailed is recruited independently from the other players of the anterior-posterior specification network to generate a new wing pattern. We argue that partial cooption is made possible because 1) the anterior-posterior specification GRN is flexible over time in the developing wing and 2) this flexibility results from the fact that every single gene of the GRN possesses its own functional time window. We propose that the temporal flexibility of a GRN is a general prerequisite for its possible cooption during the course of evolution.

cooption | novelty | gene network | drosophilids | pigmentation

Organisms have evolved endless morphological, physiological, and behavioral novel traits during the course of evolution (1, 2). Novel traits are defined here as characters that are qualitatively novel features of an organism (3). Theory predicts that such innovations unlock ecological opportunity and allow organisms to invade new ecological niches (4, 5). Birds have evolved feathers, which allowed them to fly (6), plants evolved flowers and subsequent reproductive success (7), turtles evolved shells (8), and beetles have grown horns to fight over females (9). The way that most of these and other adaptations first evolved, however, is still largely unknown.

Even if such innovations can originate through the emergence of de novo genes (10–13), cooption of preexisting genes or GRNs has been emerging as a widespread evolutionarily relevant mechanism. Just to cite a few examples, gene cooption underpins butterfly wing pattern variation (14), wasp venom diversification (15), convergent expression of caffeine in plants (16), and the emergence of numerous morphological traits (reviewed in refs. 17–20).

The Drosophila model system has contributed significantly to our understanding of how the cooption of key developmental (or toolkit) genes underlies the emergence of evolutionary novelties. For example, the diffusible morphogen Wingless was coopted to generate discrete black dots on Drosophila guttata wings (21), and the homeodomain protein Distal-less was coopted to make the male wing black spot in Drosophila biarmipes (22). Other examples include not only the redeployment of a single gene, but the reuse of a network of a larval breathing structure to evolve an adult genital lobe (23) and the redeployment of the EGFR and Dpp pathways to evolve respiratory appendages of the Drosophila eggshell (24). The cooption of toolkit genes or GRNs leads to the idea that such redeployment is possible through changes in cis-regulation (19, 25). Indeed, the spatiotemporal expression of toolkit genes is often controlled by modular and independent enhancers (26–28). This regulatory logic is thought to facilitate the gain or loss of enhancers over evolutionary time (25).

Nevertheless, it does not explain how the new expression of the coopted toolkit genes does not interfere with the development of the tissue. Some authors have suggested that the reuse of toolkit genes might only happen during late development after completion of the early function of the redeployed genes (21, 22, 29). However, little is known about the properties of a GRN that allow the cooption of one or several of its components/genes without impairing the development of the tissue.

In this study, we use the complex wing pigmentation pattern of the fly species Samoaea leonensis as a model to address how the temporal flexibility of GRN underlies the cooption of toolkit genes to make a complex color pattern. We argue that the traditional view of a GRN overlooks the temporal nature of development. We show that 1) the anterior-posterior specification GRN is flexible over time in the developing Drosophila wing and 2) this flexibility results from the fact that every single gene of the GRN possesses its own functional time window. We hypothesize that this flexibility allows the transcription factor Engrailed to be individually coopted to generate a novel wing color pattern during evolution. We propose that the temporal flexibility of a
GRN is a general prerequisite for gene cooption during the course of evolution.

Materials and Methods

Animal Collection and Rearing. *Samoai a attenuata*, *Samoai a ocellaris*, and *Samoai a hirta* were collected by net sweeping in American Samoa. The *Samoai a* species were grown on Wheeler–Clayton food in the laboratory at room temperature. Paper was added along the side of the vial and wetted with an antifungal agent. It helped to maintain a moist environment and to facilitate pupation.

Fly Stocks. *Chommyza amoena*, *Drosophila deflacta*, *Drosophila funebris*, *D. guttifera*, *Drosophila quadrilineata*, *S. leonensis*, and *Zaprionus ghesquièrei* were obtained from *Drosophila* Species Stock Center (http://flybase.org/). Additional *S. leonensis* individuals were obtained from Masayoshi Watada (Ehime University, Matsuyama, Japan), *Drosophila grimmshawi*, *Drosophila hawaiiensis*, and *Drosophila silvestris* were obtained from Kenneth Kaneshiro (University of Hawai‘i, Mānoa, Hawai‘i).

The following transgenic lines were used: UAS-c (30), UAS-cd (TRI JF01272), UAS-DI (BDSC no. 5612), UAS-Dp (TRI HM01390), UAS-Dl (31), UAS-Dp (BDSC no. 1486), UAS-Dp (TRI JF02455), UAS-en (32), UAS-ey (TRI VDRC GD35695), UAS-hh (33), UAS-hh (TRI 25794), UAS-M (TRI JF01356), UAS-p (BDSC no. 5817), UAS-p (TRI JF0322), UAS-v (TRI JF02126), UAS-wg (BDSC no. 5919), UAS-wg (TRI HM00794), hh-lacZ (34), nabb (DSHB, 35), and tub-gal80 (BDSC no. 7018).

Data Collection and Phylogenetic Analysis. Phylogenetic markers were identified in several complete genomes by BLASTN using *Drosophila melanogaster* sequences as a probe. *D. grimmshawi*, *S. melanogaster*, *Drosophila mojavensis*, *Drosophila pseudoobscura*, *Drosophila virilis*, and *Drosophila willistoni* genomes were retrieved from FlyBase (http://flybase.org/). Alternatively, markers were amplified by PCR using degenerate primers (SI Appendix, Table S1) in species for which genomic data were not available. The sequences reported in this paper have been deposited in public databases (SI Appendix, Table S2).

Nucleotide sequences for individual markers were aligned with MUSCLE (36) and manually adjusted, and selected blocks obtained for each marker were concatenated and used for phylogenetic reconstruction. Maximum-likelihood (ML) searches were performed using PhyML v3.1 under the GTR+Γ model (37). One hundred bootstrap replicates were conducted for support estimation. Bayesian phylogenetic analyses were performed using MrBayes 3.2 under the GTR+Γ+I model (38). We ran four independent chains for at least 100,000 generations and discarded the first 25,000 generations as burn-in. The nucleotide sequence alignment and tree files are downloadable from the Dryad Digital Repository at http://dx.doi.org/10.5061/dryad.pri4xqkhs.

In Situ Hybridization and Immunostaining. In situ hybridization was carried out as previously described (39), using digoxigenin (DIG)-labeled RNA probes at a hybridization temperature of 65 °C. Stained tissues were mounted in 80% glycerol and then photographed using an Olympus SZX16 stereo microscope equipped with an Olympus DP71 digital camera.

Results and Discussion

The *Samoai a* Clade: A Model to Study Evolution of Pigmentation. *Samoai a* is a small genus of seven described species endemic to the Samoan Islands in the central South Pacific (45, 46). This genus is undoubtedly embedded within the family Drosophilidae, although its exact phylogenetic position remains debated (47). Moreover, the relationships remain unresolved within the genus *Samoai a*.

We employed a phylogenomic approach relying on a set of phylogenetic markers we developed in this study. We assembled a data set of 12 nuclear genes from 12 species of drosophilids, including four *Samoai a* species. Phylogenetic analyses support the *Samoai a* clade as sister to *D. quadrilineata* (Fig. L4), confirming the topology previously found by Yassin and colleagues (48). Our phylogeny also provides insights into the internal structure of the *Samoai a* clade and the evolution of wing coloration. The black-wing species *S. hirta* and *S. attenuata* display a more primitive wing pattern, whereas the spotted-wing species *S. ocellaris* and *S. leonensis* display a more derived pattern (Fig. LB). This finding suggests that spotted wings derive from the addition of white spots to uniformly black wings. Furthermore, ancestral reconstruction infers that wings were likely translucent in the last common ancestor of Drosophilidae (49). We hypothesize that an initial evolutionary change consisted in producing black pigment uniformly through the wing of the last common ancestor of the *Samoai a* species. Thus, the *Samoai a* clade represents a unique case study for the step-wise evolution of wing pigmentation. Our study primary aims at understanding how the spotted wing pattern is generated in the species *S. leonensis*.

Cooption of Engrailed For Making a Complex Wing Pigmentation Pattern. The *S. leonensis* wing pigmentation consists of a complex white and black spot pattern (Fig. LB). White color results from the absence of melanin and the presence of a translucent wing membrane. We first focused on the generation of the black spots of the wing. In insects, black color very often results from the cuticular accumulation of melanin. The protein Yellow is required for the biosynthesis of melanin on fly wings (50). Similarly, we found that the spatial expression pattern of yellow prefigures the adult melanin wing pattern (SI Appendix, Fig. S1A). Then, we tested for putative candidate genes that could be involved in making the white spots. In pupal wings, the *S. leonensis* engrailed (en) transcript and protein are expressed where the adult white spots will appear (Fig. IB and SI Appendix, Fig. S1B). The white spots are located both on the anterior and posterior sides of the wing, meaning that the expression of *En* is not restricted to the posterior compartment in the *S. leonensis* pupal wing. This finding is remarkable since *en* has been so far described as specifying posterior identity of embryonic segments (51) and wing discs (52) in *Drosophila* early development.

To test whether *en* has a totally different developmental function in *S. leonensis*, we investigated the dynamics of *en* expression (both at the transcript and protein level) during wing development in this species. In wing discs, the expression of *En* is homogeneous and restricted to the posterior compartment (Fig. 2), similarly to what is known in *D. melanogaster* (52). In early pupal wings (stage P5ii), the expression fades away in some regions, which leads to a nonuniform expression of the transcripts (*SI Appendix, Fig. S1 B and C*) and the protein *En* (Fig. 2) in the posterior compartment. Expression in spots shows up in the posterior region first (stage P6), and then in the anterior part of the developing pupal wing (stage P7; *SI Appendix, Fig. S1 D–G*). Taken together, our data suggest that the early function of *En* as a posterior identity gene is conserved between *D. melanogaster*.
and S. leonensis. As for the novel function of En as a putative repressor of Yellow (53), it takes place during late pupal stages (stages P6/7). In other words, the cooption of the identity gene en to make pigmentation is possible during late pupal wing development without affecting the overall morphology of the wing.

We hypothesize that there is a critical time point beyond which en is no longer required in maintaining the posterior identity of a developing wing. We functionally tested this hypothesis in D. melanogaster because of the genetic tools available in this model.

**Late Redeployment of Engrailed Does Not Affect Wing Morphology in D. melanogaster.** First, we used RNAi to deplete En function in the wing disc and the pupal wing strictly. The inducible expression system GAL4/GAL80 allowed us to control the time of the RNAi hairpin expression (Fig. 3A). Silencing en 1 h after pupariation formation (APF) led to a drastic effect on the overall morphology of the adult wing (Fig. 3B). The wing tended to be symmetrical on either side of the first row of posterior cells, and the posterior cross-vein was absent. This phenotype is very similar to the one obtained when using the wing driver MS1096-Gal4 (54), validating the use of the nabN1555-Gal4 driver in our study. When en knockdown was triggered between 18 and 21 h APF, this led to milder effects in the posterior margin (Fig. 3B). This time-course experiment identified 21 h APF as a critical time point that corresponds to the end of posterior identity function of En in D. melanogaster. Following the same approach, we overexpressed en throughout the whole larval wing disc or pupal wing. Overexpressing en until 32 h APF led to defaults in the adult wings, such as modified cross-veins and weak pigmentation (Fig. 3B). After 32 h APF, the overexpression of en did not affect wing morphology. Our data show that there is a critical time point during D. melanogaster wing development after which tinkering with en expression, by both loss or gain of function, does not affect wing morphology. We hypothesize that the developmental and evolutionary cooption of en for a new function in wing pigmentation is more likely to occur after this critical time because it does not impair its old function. In D. melanogaster, the gene en does not act independently but rather as a component of the Hedgehog circuit. We therefore investigated the role of the other genes of the Hedgehog circuit in S. leonensis.

**Engrailed Is Partly Recruited Independently of the Hedgehog Circuit.** Hedgehog (Hh) is a major secreted morphogen involved in development (55). In Drosophila wing imaginal discs, hh is expressed exclusively in cells of the posterior compartment. The definition of this cellular boundary is initiated by the asymmetric expression of en in posterior cells (32), which induces, cell-autonomously, hh expression (reviewed in ref. 56). Hh produced in the posterior compartment diffuses into the anterior compartment (57, 58). On the contrary, En represses the expression of Cubitus interruptus (Ci) (59), an essential downstream component of Hh signaling (60, 61). The first steps in the reception and transduction of the Hh signal are mediated by its receptor Patched (62) and the seven-transmembrane-domain protein Smoothened (63, 64). Hh controls the expression of the secreted signaling molecule Decapentaplegic (Dpp) in a thin stripe of anterior cells along the anterior–posterior boundary (65). Dpp acts as a symmetrical long-range morphogen to organize the growth and patterning of surrounding tissue (66).

We investigated the expression of the main components of the Hedgehog circuit in the S. leonensis pupal wing. The expression of hh is restricted to the posterior compartment (Fig. 4), and does not follow the anterior en expression in spots. Moreover, in the posterior compartment, the down-regulation of en expression in discrete sites does not induce a similar effect on hh expression (Fig. 4). The gene ci is strictly expressed in the anterior compartment (Fig. 4). Its expression is not uniform and correlates with black regions where en is not expressed (Fig. 4). This observation suggests that En might still repress Ci in the anterior part of the wing, showing therefore that the Hedgehog circuit might be partly active. The gene ptc is strongly expressed along the anterior–posterior (AP) compartment border, and shows also a weak expression through the whole pupal wing (Fig. 4). The expression of ptc in S. leonensis pupal wing is reminiscent of ptc expression in D. melanogaster at similar developmental stages (Fig. 4, Inset). Thus, ptc is not involved in making the novel S. leonensis wing color pattern through the cooption of en. Finally, the gene dpp is expressed in the pupal veins like in

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**D. melanogaster** (Fig. 4). The expression of *dpp* is totally independent from *en* expression in the pupal wing, suggesting that *dpp* is not reused along with *en* to generate the novel color pattern in *S. leonensis*. Taken together, these results suggest that *en* is recruited independently of the Hedgehog circuit during *S. leonensis* pupal wing development to make the wing white spots. Contrary to the Hedgehog circuit in the wing disc, with its components interacting with each other, the same circuit might be more flexible during *S. leonensis* pupal development. We tested this hypothesis by comparing the function of the Hedgehog circuit components between wing disc and pupal development in *D. melanogaster*. We performed time-course silencing/overexpression of the Hedgehog circuit components and investigated the underlying molecular changes.

**The Hedgehog Circuit Is Flexible over Time in *D. melanogaster* Wing.** Before 59 h APF, depletion of *dpp* function generates incomplete vein development in the distal part of the wing (t = 1 and 54 h APF; Fig. 5), whereas proper wings are obtained after this critical time point. Early overexpression of *dpp* causes an

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**Fig. 3.** Time course of inducible misexpression of *en* in *D. melanogaster*. (A) Detailed methodology. *nab-Gal4-Gal80ts>* UAS-transgene flies were kept at 18 °C to develop until the third-instar larva stage. Wandering larvae were then collected and placed back to 18 °C (control) or transferred to 30 °C to switch the expression of the transgene on. (B) Depletion of *en* before 21 h APF and overexpression of *en* before 32 h APF led to wing defects (Left). The phenotypes range from severe A–P polarity defects to weak vein malformation (Right).

**Fig. 4.** In situ hybridization of key genes of the Hedgehog circuit in late pupal wings. Large pictures show expression patterns in *S. leonensis*, and Insets depict pattern for homologs in *D. melanogaster*. The gene *hh* shows a similar expression pattern in both species, whereas *en* is expressed in spots at this stage in *S. leonensis*. This result suggests that *hh* is no longer activated by En in *S. leonensis* late pupal wing. On the contrary, *ci* seems to be still repressed by En in the anterior compartment. The localization of some of the future anterior white spots is indicated by the white dotted line.
overall change of the wing morphology and the differentiation of most wing tissue as vein \((t = 1 \text{ h and } 43 \text{ h APF}; \text{Fig. 5})\). Later overexpression leads to minor modifications of the veins \((t = 72 \text{ h}; \text{Fig. 5})\), whereas no effects are detected from 74 h APF.

In the case of hh depletion, we only obtained a phenotype showing an absence of the anterior cross-vein when the RNAi was induced 1 h APF \((t = 1 \text{ h APF}; \text{Fig. 5})\). Up to 39 h APF, overexpression of hh causes incomplete vein formation in the anterior wing compartment \((t = 19 \text{ h and } 35 \text{ h APF}; \text{Fig. 5})\). Interestingly, the gene ci behaves similarly to hh. Prior to 39 h APF, overexpression of ci leads to minor defects in vein formation \((t = 1 \text{ h and } 38 \text{ h APF}; \text{Fig. 5})\), whereas ci depletion only shows vein defects when induced very early \((t = 1 \text{ h APF}; \text{Fig. 5})\). These findings indicate that hh and ci lose their developmental function early during pupal wing development, whereas these two genes are key developmental players in the larval wing disc (reviewed in refs. 67 and 68).

Depletion of ptc transcripts causes severe vein defects \((t = 1 \text{ h APF}; \text{Fig. 5})\) or mild ones \((t = 32 \text{ h APF}; \text{Fig. 5})\) until a loss of phenotype from 33 h APF. Early overexpression of ptc results in an abnormal wing shape, reduction in size, and absence of veins \((t = 1 \text{ h APF}; \text{Fig. 5})\). Later overexpression leads to minor defaults in the anterior cross-vein \((t = 24 \text{ h APF}; \text{Fig. 5})\) and no visible defects from 27 h APF.

As for the gene en, we identified a critical time point during pupal development for key components of the hedgehog signaling, where their function is no longer needed to regulate vein and wing morphology. Whereas ci and hh lose their developmental function early during pupal wing development, ptc and dpp maintain a role in vein patterning until a later stage. Except for ci and hh, whose functions seem to remain synchronized in time, the different players of the Hh circuit have their own function and dynamics during pupal development.

In order to confirm the flexibility of the Hedgehog circuit over time, we also investigated the interaction of the different partners at the molecular level. First, we assessed the expression of En, Hh, Ptc, Ci, and Dpp proteins in third-instar wing discs. As previously described, the depletion of En protein affects the expression of the other partners in the wing disc (SI Appendix, Fig. S3, Top Right). The Ci and Ptc proteins are no longer repressed by En, resulting in their expression domains expanding to the posterior compartment (32). The Dpp protein becomes expressed at the border between \(en^+\) and \(en^-\) cells (69, 70). Our study shows the repression of Ci by En is not active anymore in the pupal wing. Indeed, Ci expression is restrained in the anterior compartment of the late pupal wing in absence of En protein (SI Appendix, Fig. S3, Top Right). Interestingly, we found that Ci
is no longer repressed by En in the *S. leonensis* pupal wing (Fig. 4).

We also studied the expression of these different proteins in a context of overexpression of En. As expected, the ectopic expression of En protein affects the expression of the other players in the wing disc (*SI Appendix, Fig. S3, Bottom Right*). Hh is now activated and expressed in both the anterior and posterior compartments (70); Dpp is expressed in newly + cells in the anterior compartment (70), and Ci is partly repressed in the anterior compartment (71). However, our study brings insights into the interaction between those proteins in the developing pupal wing. Whereas the protein En is still overexpressed throughout the whole wing, Hh remains expressed strictly in the posterior compartment (*SI Appendix, Fig. S3, Bottom Right*). Similarly, Ci remains only expressed in the anterior compartment (*SI Appendix, Fig. S3, Bottom Right*). Dpp is mainly expressed at the A–P boundary, with discrete expression sites along the anterior margin. Again, Ci and Hh do not respond to En in the late pupal wing, as they do in the wing disc.

Taken together, these results show that the Hedgehog circuit is flexible over time and emphasize the possibility that *en* is coopted partly independently from its wing disc network in *S. leonensis* to its novel expression pattern prefiguring the wing white spots. Examples of partial GRN recruitment are scarce, but such molecular events have been proposed to underlie the development of abdominal appendages in sepsid flies (72, 73), the formation of eyespots in butterflies (74, 75), and the origin of gin-trap in beetle pupae (76).

So far, and based on their expression pattern, we only identified *y, en, and ci* as putative genes involved in wing pigmentation in *S. leonensis*. Are there additional genes involved in making the wing spot pattern in *S. leonensis*?

**Several Developmental Genes Are Redeployed in the *S. leonensis* Wing.** We tested for additional candidate genes that might be reused to make the wing color pattern in *S. leonensis*. In *Drosophila*, the protein Mothers against dpp (Mad) is required for both transduction of Dpp signals (77) and Wg signaling (78) during the formation of the wing anterior–posterior and proximo-distal (PD) axes, respectively. In *S. leonensis*, the phosphorylated form pMad is expressed in spots that prefigure some of the distal black spots on the adult wing (Fig. 6), whereas pMad is restricted to the wing margin in *D. melanogaster* (Fig. 6, Inset). This result suggests that pMad might also be involved in the color wing pattern in *Samoaia*.

We have also identified relevant expression changes for the homeodomain protein Distal-less (Dll). In *Drosophila*, Dll plays a role in patterning the wing through the differentiation of the wing margin (79). The protein Dll is similarly expressed in the wing margin and along longitudinal veins in *D. melanogaster* (Fig. 6) and *S. leonensis* (Fig. 6, Inset). However, the *S. leonensis* Dll has extra expression domains in spots that correspond to the black patches on the adult wing (Fig. 6, white arrowheads). Interestingly, two previous studies have demonstrated that Dll is recruited to make the wing black spot in the *D. biarmipes* adult male (22) and dark pigmentation in *Bicyclus anynana* butterflies (80, 81). These observations raise the question of the evolutionary origin of the link between the melanin pathway protein Yellow and Dll. Has Dll been independently recruited several times during the course of evolution? We searched for developmental genes whose expression pattern prefigures wing black ornamentation outside the subgenus *Sophophora*. We uncovered a strict correlation between melanin and Dll expression patterns in the Hawaiian drosophilids, as well as in *Chymomyza amoena* (*SI Appendix, Fig. S4*), suggesting that Dll has been recruited independently several times to make black pigmentation during evolution.

The *nubbin* (*nub*) gene encodes a POU-domain protein required for proximal–distal patterning in the *Drosophila* wing (82). Here it is also interesting to note that indirect evidence suggests that nub might regulate the expression of *en* (83). Both *S. leonensis* and *D. melanogaster* Nub proteins are expressed in the wing margin, as well as along the veins of the pupal wing (Fig. 6). This vein expression is compatible with the putative role of Nub in wing vein formation in *D. melanogaster* (84, 85). The *S. leonensis* Nub protein is additionally expressed at the distal tip of the longitudinal vein L2 and in a few spots in the posterior compartment (Fig. 6, white arrowheads). Interestingly, the Nub expression domains specific to *S. leonensis* coincide with specific expression domains of the receptor Notch (N), and wingless to some extent, in the same species (Fig. 6, black arrowheads). It has been previously shown that Nub represses N-dependent target genes in the *D. melanogaster* wing disc (86). Our data suggest that the interaction between Nub and Notch activity is still functional in the *S. leonensis* pupal wing.

**Fig. 6.** Expression of genes putatively involved in the wing pigmentation in *S. leonensis*. Large pictures show expression patterns in *S. leonensis*, and Insets depict pattern for homologs in *D. melanogaster*. The localization of some of the future anterior white spots is indicated by the white dotted line.
However, the ligand Delta (Dll) is the component of the Notch signaling pathway that shows a striking correlation between its expression and the location of white spots on the S. leonensis wing (Fig. 6). This expression totally differs from the Dl expression in D. melanogaster that is mainly restricted to the veins (Fig. 6, Inset), suggesting that Dl has been coopted along with en to make the white wing spots in S. leonensis. What do we know about a possible interaction between en and Dl in development? Very little is known except that Drosophila hindgut patterning requires the repression of the gene Dl by En (87, 88). Our study clearly unravels a case where Dl and En are coexpressed in the same populations of cells, suggesting that En does not repress the expression of Dl in the Samoain pupal wing. This same coexpression is also seen in the center of eyespot patterns in multiple species of butterfly (89). Further investigation would be required to elucidate the nature of the direct/indirect interaction between En and the Notch signaling pathway in the present context.

By performing similar time course experiments, we identified specific critical time points for these additional investigated genes in D. melanogaster (SI Appendix, Fig. S5). Remarkably, we showed that the depletion of wg and Dll transcripts rapidly stops having a phenotypic effect during pupal wing development (SI Appendix, Fig. S5, Top). The early functional “relaxation” of wg and Dll during pupal development might have facilitated their cooption in the making of wing pigmentation in D. guttata (21), and in D. biarmipes (22), the Hawaiian flies (SI Appendix, Fig. S4), the genus Chrynomona (SI Appendix, Fig. S4), respectively. It would be interesting to test whether this characteristic of early “relaxation” is a general theme in other cases of morphological parallel evolution (90–93).

Conclusion

Key developmental genes are organized in networks or circuits. Several studies have shown that the heterotopic redeployment of such GRNs underlies the emergence of evolutionary novelties. Remarkably, the expression of a coopted gene does not impair the overall development of the novel location (tissue). We propose that 1) temporal flexibility is a property of GRN that is fundamental to allow cooption during the emergence of novel traits and 2) this flexibility results from the fact that every single gene of the GRN possesses its own functional time window. Moreover, differences in the time of functional relaxation of toolkit genes might explain why some genes are more easily coopted than others (SI Appendix, Fig. S6). In other words, relaxation time as an intrinsic property of a given GRN could explain why the same genes have been independently coopted during the course of evolution.

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