



# Macroevolutionary shifts of *WntA* function potentiate butterfly wing-pattern diversity

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**Butterfly wing patterns provide a rich comparative framework to study how morphological complexity develops and evolves. Here we used CRISPR/Cas9 somatic mutagenesis to test a patterning role for *WntA*, a signaling ligand gene previously identified as a hotspot of shape-tuning alleles involved in wing mimicry. We show that *WntA* loss-of-function causes multiple modifications of pattern elements in seven nymphalid butterfly species. In three butterflies with a conserved wing-pattern arrangement, *WntA* is necessary for the induction of stripe-like patterns known as symmetry systems and acquired a novel eyespot activator role specific to *Vanessa* forewings. In two *Heliconius* species, *WntA* specifies the boundaries between melanic fields and the light-color patterns that they contour. In the passionvine butterfly *Agraulis*, *WntA* removal shows opposite effects on adjacent pattern elements, revealing a dual role across the wing field. Finally, *WntA* acquired a divergent role in the patterning of intervein patterns in the monarch, a basal nymphalid butterfly that lacks stripe-like symmetry systems. These results identify *WntA* as an instructive signal for the prepatterning of a biological system of exuberant diversity and illustrate how shifts in the deployment and effects of a single developmental gene underlie morphological change.**

Wnt signaling | pattern formation | evolutionary tinkering | gene co-option | CRISPR mutagenesis

The multitude of patterns found in developing organisms is achieved by a small number of conserved signaling pathways, which raises an important question. How does biodiversity arise from the sharing of constituents across a single tree of life? One explanation for this apparent paradox is that conserved regulatory genes evolve new “tricks” or roles during development (1). Assessing this phenomenon requires comparing the function of candidate genes across a dense phylogenetic sampling of divergent phenotypes. Here, the patterns on butterfly wings provide an ideal test case. The development of scale-covered wings, their structural and pigment complexity, and an elaborate patterning system are key features of the Lepidoptera (moths and butterflies), which form about 10% of all species known to humankind (2). Wing patterns across the group are fantastically diverse and are often shaped by natural and sexual selection (3). Studies in fruit flies, butterflies, and moths have implicated secreted Wnt-signaling ligands as color pattern inducers (4–8). In butterfly wings, two lines of evidence suggest a prominent patterning role for the Wnt ligand gene *WntA* in particular. First, *WntA* was repeatedly mapped as a locus driving pattern-shape adaptations involved in mimicry, and a total of 18 *WntA* causative alleles have been identified across a wide phylogenetic spectrum (9–13). Second, *WntA* expression marks developing wing domains that prefigure the position and shape of pattern elements of various color compositions (10, 14).

The nymphalid groundplan provides a conceptual framework to understand pattern variation in butterflies (3). Under this

framework, patterns are organized into parallel subdivisions of autonomous color pattern complexes known as “symmetry systems,” which are arranged across the dorsal and ventral surfaces of both the fore- and hindwing (14–19) (Fig. 1 A–C). This arrangement is thought to represent a putative archetype of a butterfly wing pattern, and diversity is created by modifying elements within and among these symmetry systems (3). *WntA* is typically expressed in three of the four symmetry systems (14): the small proximal pattern called Basalis (B), the large median pattern called the Central Symmetry System (CSS), and the Marginal Band System (MBS), which features laminar stripes bordering the wing. Here we used CRISPR/Cas9 mutagenesis to impair *WntA* function and assess its patterning roles in Nymphalidae, the largest butterfly family that radiated around 90 Mya (20). We characterize the developmental function of *WntA* in species representative of the nymphalid groundplan and then show that *WntA* has acquired divergent patterning roles in several lineages.

## Results and Discussion

We injected Cas9/sgRNA duplexes into 1–6 h butterfly embryos at a syncytial stage ( $n = 5,794$  eggs). As only a fraction of the dividing nuclei are edited, the resulting mosaicism can bypass the deleterious effects of developmental mutations and yields  $G_0$  escapers that survive until the adult stage for phenotypic analysis (21–23). We performed CRISPR injections in seven nymphalid

## Significance

**Our study assesses the long-held hypothesis that evolution of new gene functions underlies the diversification of animal forms. To do this, we systematically compared the patterning roles of a single gene across seven butterfly species. Under a null hypothesis of gene stasis, each knockout experiment should yield directly comparable phenotypes. We instead observed a varied repertoire of lineage-specific effects in different wing regions, demonstrating that the repeated modification of a key instructive signal was instrumental in the complex evolution of wing color patterns. These comparative data confirm the heuristic potential of CRISPR mutagenesis in nontraditional model organisms and illustrate the principle that biodiversity can emerge from the tinkering of homologous genetic factors.**

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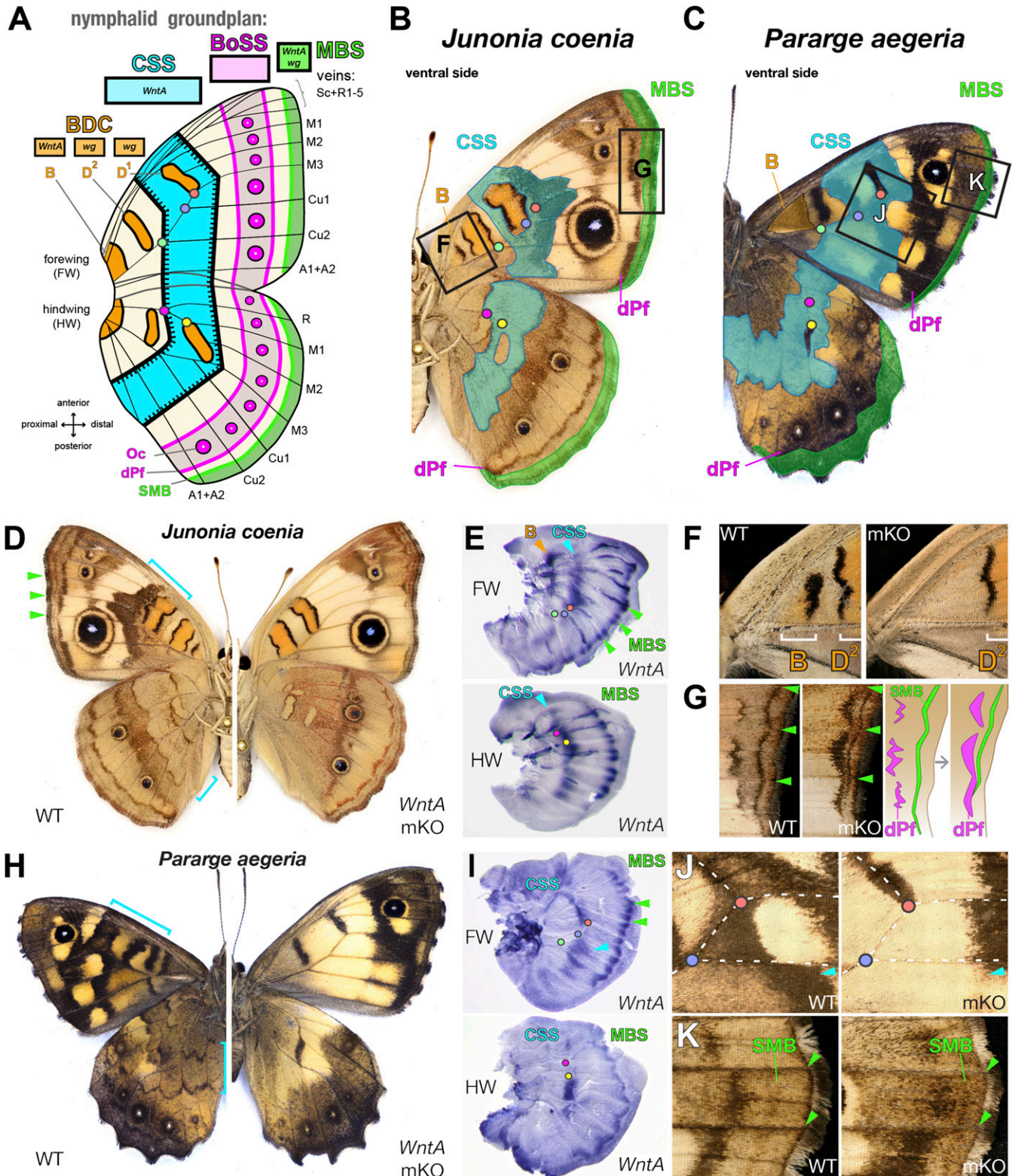
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**Fig. 1.** *WntA* loss-of-function effects in groundplan-like nymphalids. (A–C) The nymphalid groundplan consists of consecutive symmetry systems organized along the antero-posterior axis. Color code indicates groundplan elements in subsequent panels. Orange: Baso-Discal Complex (BDC) patterns; blue: CSS; fuchsia: Bordel Ocelli Symmetry System (BoSS), including dPf; green: MBS, including Sub-Marginal Band (SMB). Dots show wing topological landmarks corresponding to vein crossings. (D–G) *WntA* mKO in *J. coenia* results in the loss of *WntA*<sup>+</sup> patterns. (D) Whole-wing phenotypes. (E) In situ hybridization of *WntA* in WT fifth instar imaginal disks. (F) Blow-up of proximal forewing area showing the loss of B upon *WntA* mKO. (G) Blow-up of proximal forewing area showing the distalization of dPf and SMB elements. (H–K) Replication of the *J. coenia* results in *P. aegeria*. (H) Whole-wing phenotypes. (I) In situ hybridization of *WntA* in WT fifth instar imaginal disks. (J) Loss of the forewing CSS. (K) Distalization of dark-brown dPf and SMB; arrowheads point at corresponding *WntA* expression domains in *I*.

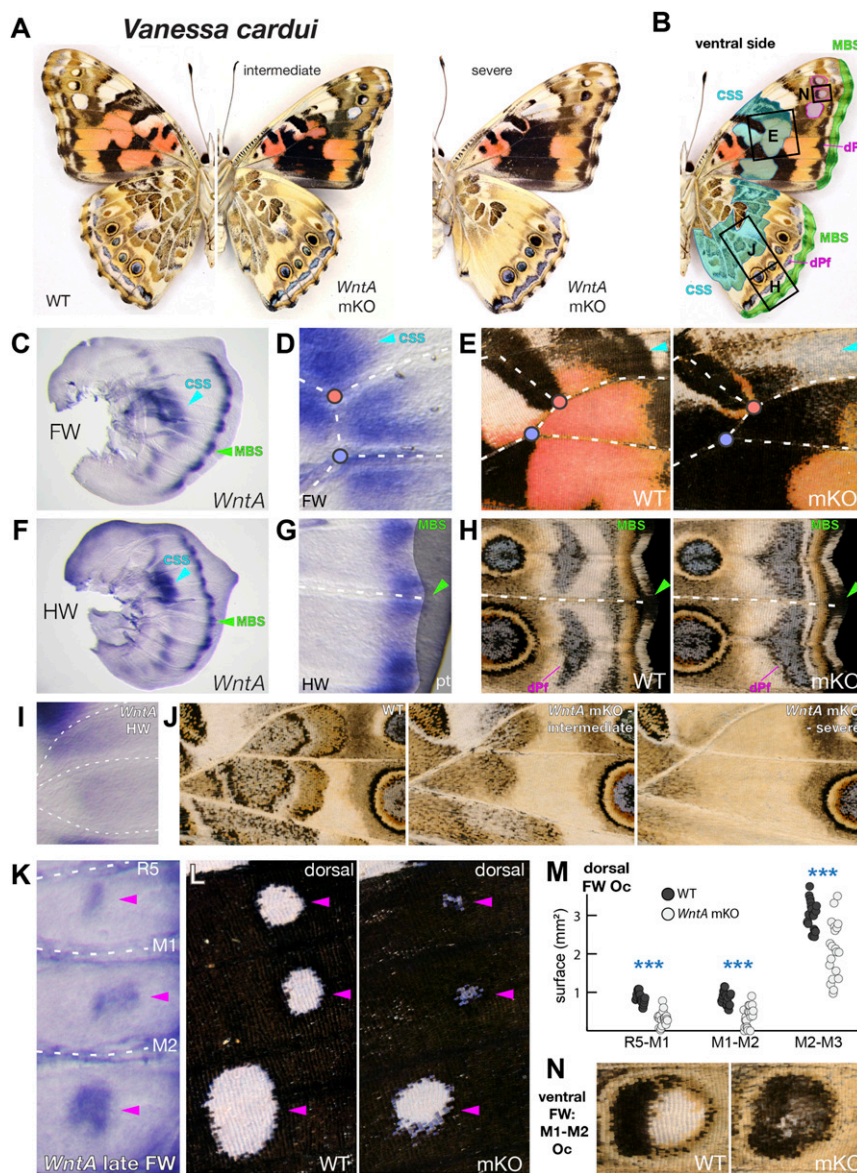


species to induce frameshift mutations in *WntA*-coding exons. About 10% of hatchlings (240 of 2,293 survivors) yielded adult butterflies with mosaic knockout (mKO) pattern defects on their wings (*SI Appendix, Figs. S1–S9 and Tables S1 and S2*).

***WntA* Induces Central Symmetry Systems.** First we used CRISPR to test the effects of *WntA* loss-of-function on the wing patterns of the Common Buckeye *Junonia coenia* (tribe: Junoniini). *WntA* mKOs resulted in a complete loss of the CSS, consistent with *WntA* expression that prefigures its shape and position in the wing imaginal disks (Fig. 1 *D* and *E* and *SI Appendix, Fig. S1*). The *WntA*-positive forewing B element was lost while the *wg*-positive  $D^1$ - $D^2$  elements (8, 24) were unaffected (Fig. 1*F*). The B- $D^1$ - $D^2$  patterns have a similar color composition, indicating that *WntA* and *wg* play interchangeable

roles in their induction. In contrast, the double loss of the distinct B and CSS patterns also illustrates the regional specificity of *WntA*-signaling color outputs across the wing surface. In the marginal section of the wing (Fig. 1*G*), *WntA* mKOs resulted in a contraction of the MBS and in a shift of chevron patterns known as the distal parafoveal elements (dPF) (17, 19). *WntA* may impact these distal elements by participating in complex patterning dynamics in the marginal section of the wing (25).

**Variations on the *WntA* Groundplan Theme.** Next we asked if the instructive roles of *WntA* were phylogenetically conserved, using two other nymphalid butterflies with a groundplan organization, the Specked Wood *Pararge aegeria* (tribe: Satyrini) (15) and the Painted Lady *Vanessa cardui* (tribe: Nymphalini) (18). *WntA*



**Fig. 2.** Conserved and novel aspects of *WntA* function in Painted Lady butterflies. (*A* and *B*) *WntA* mKOs in *V. cardui* result in defects or loss of the CSS, highlighted in cyan in *B*. (*C* and *D*) *WntA* forewing expression is associated with the CSS (magnified in *D*) and the MBS in WT fifth instar wing disks. (*E*) Blow-up of a CSS section showing pattern disruption upon *WntA* mKO. (*F* and *G*) *WntA* hindwing expression in the CSS and the MBS (magnified in *G*; pt, peripheral tissue) in WT fifth instar wing disks. (*H*) *WntA* mKO results in distal shifts of dPF elements. (*I*) Blow-up of *WntA* expression in the hindwing CSS. (*J*) Magnification of intermediate and severe levels of CSS reduction observed upon *WntA* mKO. (*K*) *WntA* expression as observed in the presumptive forewing eyespots in late fifth instar wing disks of *V. cardui*. (*L* and *M*) Reduction of dorsal forewing eyespots following *WntA* mKO. (*N*) Color change in ventral mKO forewing eyespots.







but was subsequently lost due to an episode of high mortality in our stock. Nonetheless, this preliminary result illustrates the potential of CRISPR to induce a variety of loss-of-function alleles, which could be propagated via the germline for tackling future developmental questions where mosaicism is a concern.

**Conclusions.** The Nymphalidae family comprises about 6,000 butterfly species, most of which can be identified by their wing patterns. We used this system as a proxy of morphological evolution and found that a single signal articulates its underlying complexity, as shown by the variety of *WntA* mKO phenotypes obtained across different wing regions and species. Our data highlight three major results. First, *WntA* is associated with multiple pattern elements within the same individual, including within the same wing surface, e.g., both the adjacent Basalis and CSS patterns require *WntA* in *J. coenia* forewings, despite distinct color compositions, whereas CSS stripes often differ between wing surfaces (dorsal vs. ventral, forewing vs. hindwing). Wnt signaling may combine with selector genes that mark distinct wing domains to mediate these regional-specific outputs within a single individual (24, 35). Second, spatial shifts in *WntA* expression cause pattern-shape evolution, exemplified by the multitude of species-specific manifestations of the CSS. *Cis*-regulatory variants of *WntA* (9–12), or alternatively, modulations of the *trans*-regulatory landscape that controls *WntA* expression, may have fashioned these macroevolutionary shifts. Finally, *WntA* evolves new patterning functions. It was co-opted into forewing eyespot formation in the *V. cardui* lineage, evolved a localized pattern-inhibiting role in *A. vanillae*, and was repurposed for the patterning of vein-contouring markings in monarchs. In summary, *WntA* instructs the formation of multiple wing-pattern elements in the nymphalid radiation, demonstrating the importance of prepatterning processes in the unfolding of complex anatomy. The versatility of this signaling factor illustrates how the repeated tinkering of a developmental gene can foster boisterous evolutionary change.

## Experimental Procedures

**Butterflies.** Insect stock origins, rearing conditions, and oviposition host plants are described in *SI Appendix, Table S3*.

**In Situ Hybridizations.** *WntA* cDNA sequences, cloned or amplified with T7 overhang primers, were used as a template to synthesize digoxigenin-labeled RNA probe as described previously (14, 36). Primers for amplification of template DNA are shown in *SI Appendix, Table S4*. In situ hybridization of imaginal discs from fifth instar larvae were performed as described (14).

**Egg Injections.** Butterfly eggs laid on host plant leaves were collected after 1–6 h (*SI Appendix, Tables S2 and S3*). *J. coenia* and *V. cardui* eggs were then washed for 20–100 s in 5% benzalkonium chloride (Sigma-Aldrich), rinsed in water, and dried in a desiccation chamber or by air ventilation for softening the chorion. To soften and separate egg mass in *H. sara*, clumps were treated with a 1:20 dilution of Milton sterilizing fluid (Procter and Gamble) for 4 min, rinsed with water, and dried. Eggs were arranged on a double-sided adhesive tape or glued to a glass slide, usually with the micropyle facing up. CRISPR mixtures containing pre-assembled sgRNAs and recombinant Cas9 protein (PNA Bio) were injected, using pulled quartz or borosilicate needles. The concentration of sgRNAs and Cas9 varied between butterfly species and experiments (*SI Appendix, Table S2*).

**Genotyping.** DNA was extracted from wing muscles or single legs using the Phire animal tissue direct PCR kit (Thermo Fisher Scientific), and amplified using oligonucleotides flanking the sgRNAs target region (*SI Appendix, Table S2*). PCR amplicons were gel-purified, subcloned into the pGEM-T Easy Vector System (Promega), and sequenced on an ABI 3730 sequencer.

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