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 interveinous 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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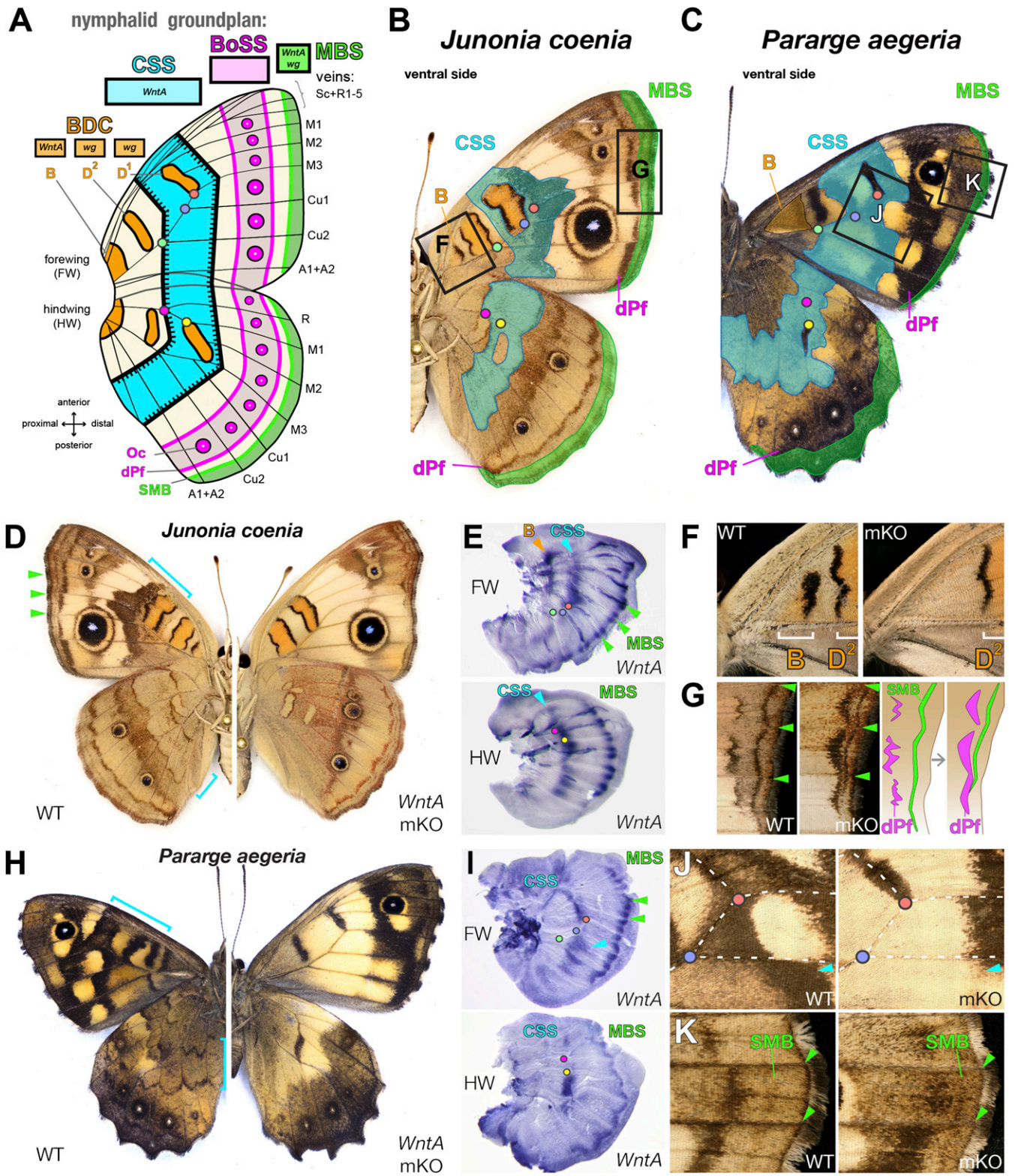

Fig. 1. *WntA* loss-of-function effects in groundplan-like nymphalids. (*A*–*C*) The nymphalid groundplan consists of consecutive symmetry systems organized along the antero-posterior axis. Color code indicates groundplan elements in subsequent panels. Orange: Baso-Discal Complex (BDC) patterns; blue: 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*$^{+}$ 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 parafocal 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*

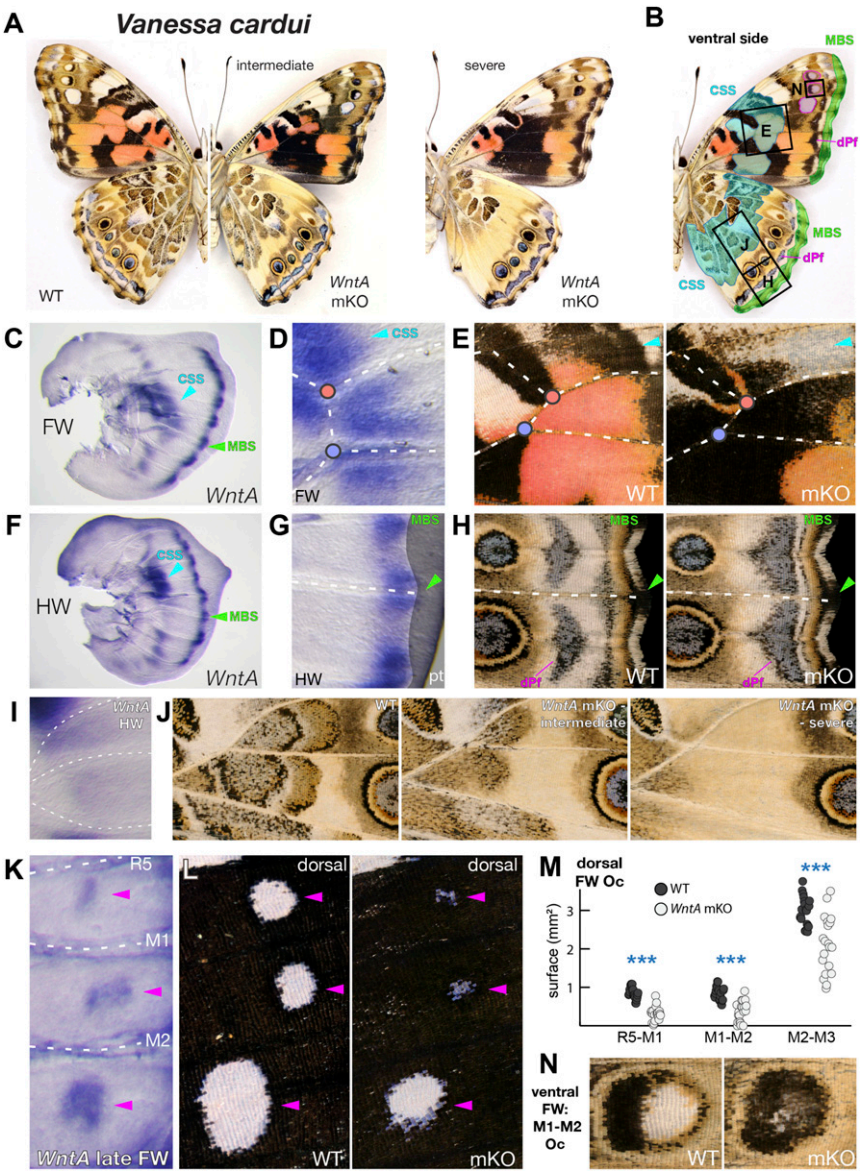

Fig. 2. Conserved and novel aspects of *WntA* function in Painted Lady butterflies. (*A* and *B*) *WntA* mKOs in *V. cardui* result in defects or loss of the CSS, highlighted in cyan in *B*. (*C* and *D*) *WntA* forewing expression is associated with the CSS (magnified in *D*) and the MBS in WT fifth 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.

mKOs yielded consistent effects by eliminating the CSS and distalizing the parafocal elements in these two species (Figs. 1 *H–K* and 2 *A–H* and *SI Appendix*, Fig. S10). Of note, in the *V. cardui* hindwing, the complex wave-like patterns of the CSS were lost upon severe *WntA* mKO and reduced in more intermediate forms (Fig. 2 *I* and *J*). These two species also highlighted other aspects of *WntA* phenotypic effects. In *P. aegeria* hindwings, the mKO-mediated disruption of the marginal system resulted in an apparent expansion of the eyespot outer rings (*SI Appendix*, Fig. S10*D*). *V. cardui WntA* mKOs resulted in the reduction of each dorsal forewing eyespot (P values $< 10^{-4}$; Fig. 2 *K–M*) and generated color composition defects in the ventral forewing eyespots (Fig. 2*N* and *SI Appendix*, Fig. S3). Only *V. cardui* forewings are known to express *WntA* in their eyespots (14). We thus infer that *WntA* was co-opted in the eyespot gene regulatory network of the *V. cardui* lineage to elaborate upon the patterning of this complex feature (26). Overall, comparisons in three species show that multifaceted modulations of *WntA* function have shaped variations on the basic nymphalid groundplan theme.

***WntA* Induces Pattern Boundaries in *Heliconius*.** We next focused on species that departed more markedly from the nymphalid

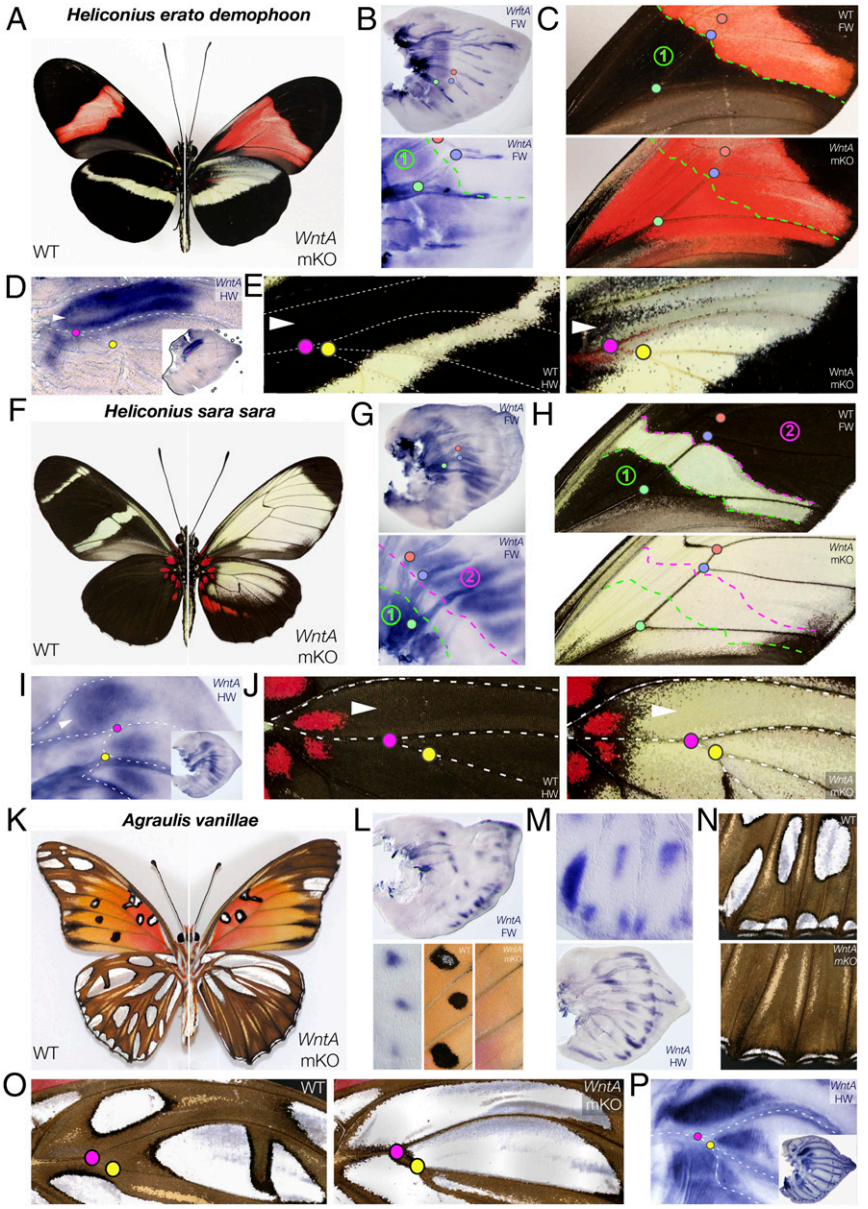

Fig. 3. Variegated *WntA* loss-of-function phenotypes in passionvine butterflies. (*A–E*) Effects of *WntA* mKO in *H. e. demophoon*. (*A*) Whole wings. (*B*) Detection of WT proximal *WntA* expression by larval forewing in situ hybridization (zone 1). (*C*) Loss of proximal pattern boundary in *WntA*-positive zone 1. (*D*) Antero-proximal expression of *WntA* in WT late larval hindwings. (*E*) Loss of antero-proximal pattern boundary in mKO hindwings. (*F–J*) Effects of *WntA* mKO in *H. sara*. (*F*) Whole wings. (*G*) Detection of proximal (zone 1) and median (zone 2) *WntA* in larval forewings. (*H*) Loss of proximal (green line) and median (fuchsia line) pattern boundaries resulting in loss of melanic identity in zones 1 and 2. (*I*) Antero-proximal expression of *WntA* in larval hindwings. (*J*) Widespread antero-proximal color identity shift in mKO hindwings. (*K–P*) Effects of *WntA* mKO in *A. vanillae*. (*K*) Whole wings. (*L*) Silver-spot–related expression of *WntA* in larval forewings and loss in mKO forewings. (*Bottom*) M3-A1 spot triad. (*M* and *N*) Silver spot-related expression of *WntA* in larval hindwings (*M*) and loss/reduction in mKO hindwings (*N*). (*Top*) M3-A1 spot complex. (*O*) Silver-spot pattern expansion in proximal mKO hindwings. (*P*) Secondary expression of *WntA* in the proximal region of late larval hindwings. Colored dots: wing topological landmarks (vein crossings).

groundplan configuration, starting with the hyperdiverse *Heliconius* clade (tribe: Heliconiini). We performed CRISPR mKOs in Central American morphs of two species, *Heliconius erato demophoon* and *Heliconius sara sara*. *WntA* removal resulted in an expansion of light-color patterns in both cases (Fig. 3 *A* and *F*). In *H. erato demophoon*, *WntA* expression marked melanic patches that contour forewing red and hindwing yellow stripes (Fig. 3 *B* and *D*). Predictably, its loss-of-function resulted in the loss of the corresponding boundaries with black contours being replaced by expansions of red or yellow (Figs. 3 *C* and *E*). *H. sara* forewing disks showed a proximal and central *WntA* expression domains that each correspond to melanic fields that frame the signature yellow stripes of this butterfly (Fig. 3*G*). Both melanic intervals were lost following *WntA* mKOs (Fig. 3*H*), yielding an almost uniformly yellow forewing surface. Hindwings showed a similar effect with *WntA* deficiency resulting in melanic-to-yellow switches in the anteroproximal half of the wing (Fig. 3*J*). Interestingly, this treatment also revealed a cryptic stripe of red patches. A similar phenotype is observed in subspecies of *H. sara*, as well as in its sister species *Heliconius leucadia* (*SI Appendix*, Fig. S11), suggesting that modulations of Wnt signaling could underlie these cases of natural variation. Overall, these data support previous predictions that groundplan elements such as the CSS can be homologized to what form the apparent contours of *Heliconius* patterns (27–29). *WntA* is best thought as a prepatterning factor that determines boundaries between color fields, a view that is compatible with the replacement effects of mKOs, where *WntA*-deficient cells acquire the color fate of the adjacent territory. This property may explain why *cis*-regulatory tinkering of *WntA* expression seems to underlie the repeated modification of color pattern shapes across this explosive radiation (9–12), as it allows the coordinated modulation of color fate on either side of a moving boundary.

Antagonistic Roles of *WntA* in Adjacent Patterns. Compared with *Heliconius*, the closely related Gulf Fritillary butterfly (*Agraulis vanillae*) has modified the nymphalid groundplan differently to produce its distinctive wing pattern (27). Rather than continuous stripes, *A. vanillae* shows dispersed silver spots of identical color composition, each consisting of a core of highly reflective "mirror" scales (30) and an outline of black scales. A subset of silver spots express *WntA* or *wg* (14), and accordingly, all of the $WntA^+$ patterns contracted or disappeared in *WntA* mKOs (Fig. 3 *K–N* and *SI Appendix*, Fig. S6). Among the wg^+ elements (forewing D^1 and D^2), only D^1 coexpressed *WntA* and was specifically reduced in *WntA* mKOs (*SI Appendix*, Fig. S12), suggesting that silver spots respond to overall Wnt dosage. *WntA* mKOs also resulted in a drastic expansion of *WntA*-free ($WntA^-$) patterns (Fig. 3*O*). Importantly, butterflies treated with exogenous heparin, a ligand-binding molecule with Wnt gain-of-function effects (9, 14, 31, 32), showed the opposite outcome: expanded $WntA^+$ and reduced $WntA^-$ patterns (14). These reverse effects of CRISPR loss-of-function vs. heparin gain-of-function suggest that *WntA* activates and represses two distinct sets of patterns, and the repressed domain in fact shows a secondary wave of *WntA* expression in late larval instar wing disks (Fig. 3*P*). This observation leads us to propose that the dual effect of *WntA* may be due to a biphasic deployment, with a first wave of *WntA* pattern-activating expression followed by an inhibitory event in the Wnt-repressed territory. Testing this working model will require the identification and expression profiling of *WntA*-signaling targets in *A. vanillae*.

Repurposing of *WntA* in a Reduced Groundplan. Finally, we used the lack of visible CSS in monarchs (*Danaus plexippus*; tribe: Danaini) as an example of extreme divergence from the nymphalid groundplan. *WntA* lacked a CSS median stripe expression as expected and was instead detected around the presumptive veins, indicative of a potential role in the induction of vein-dependent patterns (33). *WntA* mKO adults showed drastic expansions of the white interveinous patterns (Fig. 4), which are usually visible as thin outlines of the veins in WT ventral wings. In addition, white dot elements that ornate the marginal region expanded and fused following *WntA* mKO. Other *WntA* mKO monarchs showed a small dorsal patch of ectopic interveinous scales in the crossvein region, demonstrating maximal *WntA* expression in hindwings (*SI Appendix*, Fig. S13). Consistent with a Wnt loss-of-function, this mild phenotype was reproduced by injection of dextran sulfate, a drug treatment that emulates Wnt signal inhibition in other butterflies (14, 32) (*SI Appendix*, Fig. S14). Overall, expression and functional data suggest that *WntA* was again repurposed, in this case as a repressor of interveinous white scales in the monarch lineage.

Lessons from Somatic CRISPR Phenotypes. Somatic mutagenesis yielded loss-of-function data in the G_0 adults of seven butterfly species, an achievement that would have been unrealistic in the pre-CRISPR era. Experimental replication using various single-guide RNA (sgRNA) targets ruled out a contribution of off-target lesions, and genotyping experiments revealed a predominance of frameshift, presumably null *WntA* alleles (*SI Appendix*, Figs. S8 and S9). Variations in clone size, allelic dosage, and the possible occurrence of hypomorphic mutations could underlie complex cases of mosaicism, explaining the range of observed effects (Fig. 2*J* and *SI Appendix*, Figs. S1–S7). Inferring the allelic composition of wing mutant clones from their genotyping is complicated by the movement of insect wing epithelial cells following adult emergence (34), as well as by the presence of cell contaminants that are unlikely to underlie the pattern phenotype (e.g., tracheal cells, neurons, hemocytes). We attempted the generation of germline mutations in *V. cardui* to bypass the experimental limitations of somatic heterogeneity. Following the injection of a single sgRNA targeting the *WntA* stop codon, we obtained an adult female bearing a modification of the forewing CSS (*SI Appendix*, Fig. S15). Six G_1 offsprings displayed the same phenotype and were all heterozygous for a 16-bp indel mutation, resulting in a C-terminal Cys-Asn-Stop → Gly-Ser-Arg-Stop editing of the predicted WntA protein. This allele was passed to a second generation

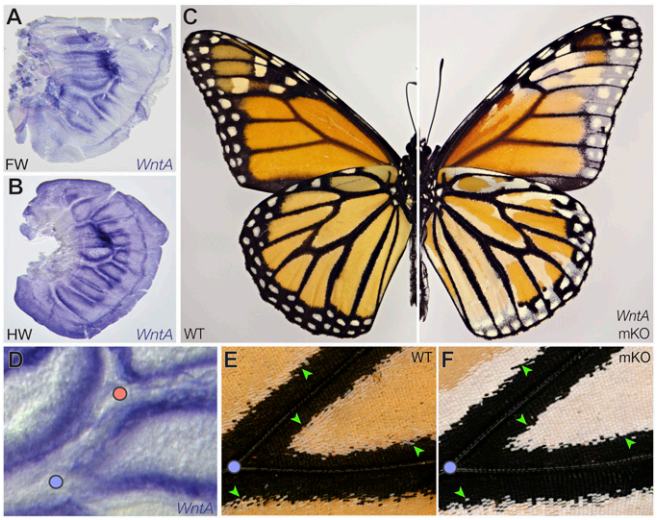

Fig. 4. *WntA* loss-of-function in monarch butterflies induces interveinous white scales. (*A* and *B*) In situ detection of *WntA* in *Danaus plexippus* last larval instar forewing (*A*) and hindwing disks (*B*). (*C*) Whole-wing phenotypes following *WntA* mKO. All effects consist of expansion of white scale patterns. (*D*) Close-up view of larval hindwing *WntA* interveinous expression in the periphery of tracheal vein precursors. (*E* and *F*) Expansion of interveinous white scale fate in the hindwing region corresponding to *D*. Colored dots: wing topological landmarks (vein crossings).

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 preassembled 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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