# The *ivory* lncRNA regulates seasonal color patterns in buckeye butterflies

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**This PDF file includes:**

Main Text
Figures 1 to 5
Supplemental Figure S1-S6

**Abstract**

Long non-coding RNAs (lncRNAs) are transcribed elements increasingly recognized for their roles in regulating gene expression. Thus far, however, we have little understanding of how lncRNAs contribute to evolution and adaptation. Here we show that a conserved lncRNA, *ivory*, is an important color patterning gene in the buckeye butterfly *Junonia coenia*. *ivory* overlaps with *cortex*, a locus linked to multiple cases of crypsis and mimicry in Lepidoptera. Along with the Livraghi et. al companion paper, we argue that *ivory*, not *cortex*, is the color pattern gene of interest at this locus. In *J. coenia* a cluster of *cis*-regulatory elements (CREs) in the first intron of *ivory* are genetically associated with natural variation in seasonal color pattern plasticity, and targeted deletions of these CREs phenocopy seasonal phenotypes. Deletions of different *ivory* CREs produce other distinct phenotypes as well, including loss of melanic eyespot rings, and positive and negative changes in overall wing pigmentation. We show that the color pattern transcription factors Spineless, Bric-a-brac, and Ftz-f1 bind to the *ivory* promoter during wing pattern development, suggesting that they directly regulate *ivory*. This case study demonstrates how *cis*-regulation of a single non-coding RNA can exert diverse and nuanced effects on the evolution and development of color patterns, including modulating seasonally plastic color patterns.

**Significance**

The genomic locus hosting the *cortex* gene has been linked to numerous cases of color pattern adaptation in moths and butterflies, including crypsis, mimicry, and seasonal polyphenism. Here we show in buckeye butterflies that the actual color pattern gene at the *cortex* locus is an evolutionarily conserved long non-coding RNA (lncRNA), dubbed *ivory*, that overlaps with *cortex*. Compared with other wing pattern genes, *ivory* stands out because of the highly nuanced, quantitative changes in pigmentation that can be achieved by manipulating adjacent *cis*-regulatory sequences. This study highlights how lncRNAs can be important factors underlying morphological evolution, and emphasizes the importance of considering non-coding transcripts in comparative genomics.

## Main Text

### Introduction

Seasonal cycles are deeply ingrained in the genomes of many animals. Evolutionary adaptation to annual fluctuations in conditions such as daylength, temperature, and precipitation is often realized in the form of phenotypic plasticity, where trait expression is determined in response to external cues. While the physiological and endocrine mechanisms underlying plasticity have been well characterized in many systems, there are still relatively few case studies that highlight the genetic basis of plasticity in natural populations. Buckeye butterflies, *Junonia coenia*, have been a useful model in this regard. Across much of their range, these butterflies are polyphenic, and show distinct seasonal color patterns – in the summer they are a light tan color, while in the autumn they are dark red and absorb and retain heat more efficiently (1). This polyphenism is mediated by seasonal changes in daylength and temperature, and natural genetic variation in this response has been mapped to three loci: *herfst*, *trehalase*, and *cortex* (2). Of these, the *cortex* locus is of particular interest because it has also been implicated in adaptive melanism in moths (3), leaf mimicry in *Kallima* (4), and color pattern mimicry in *Heliconius* (5, 6). It remains a question of fundamental interest how a single locus like this can control such a wide variety of adaptive variation between species, populations, and even seasons. Such multifunctionality is presumably driven by *cis*-regulatory innovation, but we still have a poor understanding of how adaptive changes, including plasticity, are distributed across *cis*-regulatory regions of individual genes. Genetic association mapping coupled with chromatin accessibility comparisons in *J. coenia* have linked several *cis*-regulatory elements (CREs) at the *cortex* locus to plasticity, which now motivates us to functionally characterize how adaptive color pattern variation is encoded at this locus (2).

While there has been significant interest in the *cortex* locus for the reasons described above, functional studies have, in fact, proven vexing. First, expression work looking at *cortex* mRNA and protein localization in developing butterfly wings has largely failed to show any obvious associations with color patterns (5, 6), or have proven unrepeatable in our hands. Second, mosaic deletion phenotypes occur at such low frequencies they could possibly be explained by spurious long deletions affecting adjacent genes (7). And last, and most importantly, Cas9-induced germ line deletions in *cortex* show no phenotype in F1 individuals (8). Remarkably, as we show below, and is also shown in a companion paper by Livraghi et al. (8), this all seems to be explained by the fact that *cortex* is not the color pattern gene we once thought it was – rather, the actual gene of interest is a long non-coding RNA (lncRNA), dubbed *ivory*, that partially overlaps with the *cortex* gene on the antisense strand. This is interesting because few lncRNAs have thus far been implicated in adaptation, even though lncRNAs are of major emerging interest in developmental biology due to their roles as chromatin and spatially restricted transcription regulators (9, 10).

The goals of this study, then, were threefold. First, we sought to provide functional validation of the *ivory* lncRNA as a color patterning gene in the buckeye butterfly *J. coenia*, with a focus on seasonally

plastic wing pattern phenotypes (4, 6, 10). Second, we aimed to characterize some of the *cis*-regulatory architecture of the *ivory* locus using both comparative and functional approaches. Last, using a deletion line, we aimed to assess the effects of *ivory* on gene expression during wing development. We show that the *ivory* lncRNA is a fundamental regulator of wing pigmentation with a deeply conserved *cis*-regulatory region. Deletions at different regulatory regions of the *ivory* locus not only phenocopy seasonal color pattern phenotypes but can have broader positive and negative quantitative effects on pigmentation. This work, accompanied by Livraghi et al.'s comparative study (8), calls for a major revision of our models of butterfly wing pattern development, and more generally demands that researchers working on similar biological questions must be vigilant for non-coding RNA molecules that may not be properly annotated in current genome assemblies.

## Results

### The *ivory* lncRNA promoter is conserved in butterflies and is bound by color pattern factors

As described above, efforts to characterize the function of the *cortex* gene in wing pattern development have proven inconsistent. We thus sought to identify previously unrecognized features of the locus through a more careful annotation of transcribed elements and chromatin marks within the *cortex* topologically associated domain (TAD) (Fig. 1A). We generated long-read PacBio transcript sequences from a collection of tissues that included wings at different stages of development (Table S1). These data identified a lncRNA that partially overlaps with the 5' end of the *cortex* gene (2). The 3' region of this predicted lncRNA also overlaps with the previously described 78kb *ivory* mutation – a spontaneous deletion identified in *Heliconius melpomene* that produced largely unpigmented butterflies (11) – thus, this novel lncRNA is dubbed *ivory* after the original *Heliconius* mutation.

We next annotated the *ivory* region with RNA Polymerase II (Pol II) ChIP-seq data to identify potential transcription start sites (promoters) within the TAD (Fig. 1A). We identified a major Pol II binding site during pupal development, which occurred at the 5' end of the first exon of *ivory*. To identify CREs of potential interest, we generated and/or compiled pupal wing ChIP-seq data from several transcription factors implicated in pigment regulation (Table S2). We looked at previous ChIP-seq data for the wing pattern transcription factors Spineless (12), which produces similar wing depigmentation mutant phenotypes as *ivory* (13), and generated new ChIP-seq data for Fushi tarazu-F1 (Ftz-F1) and Bric-a-brac (Fig. 1B), for which mutants have globally reduced and enhanced pigmentation, respectively (Fig. S1). All three transcription factors showed strong binding signals at the presumptive *ivory* promoter, as well at a handful of other apparent presumptive CREs within the TAD, as inferred from ATAC-seq data (Table S3) (Fig. 1). Spineless, in particular, showed a strong cluster of binding sites in the first intron of *ivory*, in an interval previously identified as showing nucleotide polymorphism associations with seasonal polyphenism in *J. coenia* (2). A broad comparison with ATAC-seq-annotated genomes of multiple nymphalid butterflies showed that the first exon of *ivory* is conserved across the family, along with many conserved CREs, as inferred from the chromatin accessibility profiles (Fig. 1B, Table S4). Together, these

data demonstrate activity of the conserved *ivory* lncRNA promoter and transcript during pupal wing development, and that *ivory* is directly bound by broad color pattern transcription factors Spineless, Ftz-F1, and Bric-a-brac, including Spineless binding at CREs associated with seasonal polyphenism.

### *ivory* is expressed in color patterns

The above data, in the context of previous mapping studies from many different butterfly and moth species, identify *ivory* lncRNA as a strong candidate gene for wing pattern adaptation. This lncRNA was overlooked in previous mRNA-seq studies, however, and therefore escaped annotation. Our Iso-Seq data allowed us to identify a complete *ivory* transcript, for which our Pol II data imply transcription is occurring in developing pupal wings. To characterize spatial expression of *ivory* we performed hybridization chain reaction *in situ* hybridizations in early pupal wings. We observed high expression levels of *ivory* lncRNAs in specific color pattern elements, especially the outer boundaries of the discal bands (Fig. 2A) the eyespot central and outer rings (Fig. 2B-D). This strong association with melanic color pattern elements was consistent with chromogenic ivory *in situs* in other butterflies, as shown in Livraghi et al. (8), and suggests a positive role for *ivory* in regulating these patterns.

### *ivory* plays roles in wing pigmentation and eyespot development

To assess the role of *ivory* in color pattern development we generated two mutant deletion lines using CRISPR/Cas9. We targeted the promoter (P187) and first exon of ivory with three single guide RNAs (sgRNAs) (Fig. 3A, TableS5, S6). After inter-crossing mKO parents, progeny with strong ventral wing pigmentation phenotypes were selected and crossed. We generated two lines: one an 18bp deletion ($ivory^{\Delta 18bp}$) of the core promoter that does not affect the first exon of ivory (Fig. 3A), and one with a 17kb deletion ($ivory^{\Delta 17kb}$) that spans the promoter and first exon, but that does not affect any *cortex* coding regions (Fig. 3B). The $ivory^{\Delta 18bp}$ lines presented an overall faded phenotype, but still appeared to have most pigment types represented (Fig. 3C).

The $ivory^{\Delta 17kb}$ line, which was confirmed through whole genome sequencing, produced nearly pigmentless butterflies (Fig. 3C). This phenotype resemlbed the original *H. melpomene ivory* spontaneous mutants (11), where almost all melanin appeared to be lost, and only weak traces of orange presumptive ommochrome pigmentation remained. Heterozygous $ivory^{\Delta 17kb}$ mutants showed an intermediate phenotype of partially faded pigmentation. $ivory^{\Delta 17kb}$ did not prove to be embryonic lethal, although homozygous butterflies were flightless and not as active as heterozygotes or wildtypes. We were able to maintain this line for several generations by occasional backcrossing to the wildtype line and genotyping.

Transcription of *ivory* in melanic eyespot rings during early pupal development shows that this gene has a specific regulatory program to activate expression in these pattern elements. We were thus intrigued to note that while the *ivory*$^{\Delta 18bp}$ line showed the faded pigmentation phenotype typical of various *ivory* knockouts, it nonetheless retained fully pigmented melaic center rings in the major eyespots (Fig. 3C). These central eyespot elements either appeared to be fully pigmented, or completely absent as in *ivory*$^{\Delta 17kb}$ homozygotes (Fig. 3C). These results do not identify a specific eyespot regulatory element, but they do suggest that the pigment fading effect can be decoupled from *ivory*'s role in determining internal melanic eyespot rings.

Importantly, none of the mutants we observed appeared to show any change in the size or location of identifiable color pattern elements themselves, leading us to infer that the main function of *ivory* lies in regulating pigmentation, and not pattern formation *per se*.

### *cis*-Regulatory deletions replicate alternate seasonal phenotypes

*J. coenia* presents different ventral hindwing pigmentation depending on seasonal conditions (Fig. 4A). As described above, a region of open chromatin and Spineless binding in the first intro of *ivory* has been genetically linked to natural variation in this polyphonic response (Fig. 1A, Fig. 4B) (2). To determine how this and other *ivory cis*-regulatory regions may be involved in color pattern regulation, we used CRISPR/Cas9 to generate "shotgun deletion" somatic mosaic knockouts (mKO) at five presumptive *ivory* regulatory regions: the promoter, one upstream element (E149), and three CREs in the polyphenism-associated interval (E205, E209, and E230) (Fig. 4A, Table S5). As previously described, this CRISPR/Cas9 approach targets a CRE with multiple sgRNAs to induce a spectrum of mutations of differing lengths, thus generating $G^0$ somatic mosaics that represent a diversity of mutant alleles that allow us to survey the breadth of effects that regulatory mutations can produce (13).

This exercise produced three distinct phenotypes (Fig. 4C,D). The first, and most compelling, class of phenotypes is the replication of seasonally plastic color patterns that originally led to the mapping of this locus in *J. coenia*. Specifically, deletions at all elements was capable of generating mosaic clones with partially hypomorphic reduction of dark red pigmentation on the ventral hindwing in a fashion that phenocopies summer phenotypes of *J. coenia* (Fig. 4C, D). In these mutants the "background" beige and brown coloration was minimally affected, while red interspersed through an otherwise beige background field was reduced. Many of these mutants show reduced or undetectable effects on the dark red umbral band as well (e.g., Fig. 4C), in a manner highly reminiscent of natural seasonal variation (Fig. 4A). Thus, mutant butterflies that would otherwise show an autumn phenotype have mutant clones that resemble the summer phenotype. This contrasts with *ivory* exonic knockouts (e.g. *ivory*$^{\Delta 17kb}$), or strong CRE hypomorphs, as described below, which show a uniform loss of almost all pigmentation across the ventral hindwing, including the umbral band (Fig. 4D).

Second, deletions at all elements were all capable of producing strongly hypomorphic mutant clones that resembled the *ivory*$^{\Delta 17kb}$ line described above, and as evidenced in other species (6, 8) – an overall fading or complete loss of most pigmentation on both dorsal and ventral wing surfaces (Fig. 4D). We infer that these elements function as enhancers, or positive regulators, of *ivory* transcription, and that the fading phenotypes are likely a result of decrease in *ivory* transcription or dosage. The final class of phenotypes, seen in a subset of E209 and E230 mutants, were pigmentation hypermorphs that showed darkening of both dorsal and ventral pattern elements (Fig. 4D). We infer that these CREs possess some silencer-like functionality, and that their deletion likely results in increased *ivory* dosage.

Together, these results demonstrate that *ivory* can be regulated by non-coding sequences in a refined fashion, likely through dosage-like effects on global pigment intensity, but also showing evidence of potentially uneven or pattern-specific strength-of-effect (e.g. umbral band and eyespot centers). Importantly, the replication of nuanced seasonal phenotypes serves to validate previous genetic mapping work linking this gene to color pattern plasticity (2).

### *ivory* regulates a large and dynamic cadre of transcripts during wing development

The diverse effects of *ivory* on wing coloration, including regulation of multiple pigment types, implies that this lncRNA directly or indirectly regulates multiple biological processes. To assess the transcriptional impact of *ivory* loss of function, we characterized the wing transcriptome at three stages of development in the *ivory*$^{\Delta 17kb}$ line: (1) last (5th) instar wing imaginal discs, when pattern formation begins; (2) pupal wings two days after pupation when patterns are finalized, but before scales mature, and when our *in situ* hybridizations in Fig. 2 were performed; and (3) late pupal wings, 6-7 days after pupation, when scales are nearly matured and pigments are being synthesized. We compared wild type and mutant transcriptomes and identified the most differentially expressed genes at each timepoint (Tables S8-S11). There was an overall large effect of the *ivory* knockout on gene expression during wing development with thousands of significantly differentially expressed transcripts (5$^{th}$: 518; Day 2: 3629; Day 6: 469) at FDR of $p^{adj}$ =0.01 (Fig. 5).

We looked specifically at differentially expressed genes with known or suspected roles in wing pigmentation and/or patterning (Table S8) to ask if the *ivory* knockout phenotype is consistent with functions of these genes. In this respect, there were a number of pigmentation genes that were downregulated in knockouts, including the ommochrome gene *kynurenine formamidase*, and melanin genes including *laccase2*, *dopa decarboxylase*, *black*, and *yellow* gene family members *yellow-d2* and *yellow-c* (14). *kynurenine formamidase* was of particular interest because of its high normalized read counts in late pupal wild type wings (> 2k), implying very high expression, perhaps consistent with the whole-wing effects of the ivory loss of function phenotype. Interestingly, there were also a few pigment-related genes upregulated in mutants, including the melanin-related genes *dopamine N-acetyltransfrease*

(*Dat*), *mamo*, *tan*, and multiple *prophenoloxidase*- and *yellow*-family genes. *trehalase* and several homologs of *tret 1-2*, a predicted trehalose transporter, were also highly upregulated in early pupal wings, which is notable because *trehalase* knockouts present a summer-morph phenotype similar to *ivory* CRE knockouts (6). A >10 fold change increase in *trehalase* and *tret 1-2* expression in early pupal wings of *ivory*$^{\Delta 17kb}$ thus provides extra support for a functional connection between these loci in the context of wing pigmentation, yet the inverse correlation between their expression suggests a more nuanced relationship than previously surmised.

In terms of known color pattern genes that were differentially expressed, the best supported candidates included *spineless*, *engrailed*, *washout/domeless*, *herfst/crooked legs*, *bar-H1*, and *ecdysone receptor* (2, 13, 15-18). While it is perhaps premature to speculate about the functional connection between *ivory* and most of these genes, *spineless* stands out for three reasons: [1] knocking it out produces global, wing-wide effects on pigmentation just as *ivory* does; [2] it binds to the *ivory* promoter (Fig. 1B), suggesting reciprocal regulation; and [3] it has surprisingly high expression levels in mutant wings during early pupal development, with normalized read counts exceeding 10k in *ivory*$^{\Delta 17kb}$ wings (Tables S3, S6). *ecdysone receptor* is also an attractive candidate because titers of the steroid hormone ecdysone determine seasonal wing pattern phenotypes (19), thus providing a possible mechanism for *ivory*'s role in color pattern polyphenism.

A final notable pattern that emerged from the RNA-seq analysis was the strong upregulation of multiple *Osiris*-family genes in in *ivory*$^{\Delta 17kb}$ wings during early pupal development, in terms of fold change, significance, overall read count magnitude, and overall number of *Osiris* genes (Table S10). This gene family is thought to encode transmembrane transporters, although *Osiris* genes have not been well characterized outside of tracheal function in *Drosophila (20)*, and they have not previously been linked to butterfly wing pigmentation. This gene family may provide candidates for future functional assessment.

## Discussion

In this study we show that the lncRNA *ivory* regulates *J. coenia* color patterns, and that perturbation of *ivory* CREs can produce nuanced color pattern variations, including replicating seasonal phenotypes (2, 21). In terms of color pattern evolution, these results are of broad interest because they prompt a rewrite of our understanding of the *cortex* locus, which has received significant attention for its association with wing pattern adaptation across numerous species of moths and butterflies. Our findings, particularly in the context of recent *cortex* deletion lines lacking color pattern phenotypes (8), show that *ivory*, not *cortex*, is the color pattern gene of interest. Therefore, important molecular case studies of industrial melanism (3), Müllerian mimicry (5), and crypsis (4) should perhaps be revisited. Our work provides a cautionary example for anyone working to identify genes underlying variation that we need to look more carefully for evidence of causative non-coding transcripts.

More broadly, *ivory* represents a potentially novel case study of deeply conserved non-coding RNA underlying repeated instances of adaptation. Non-coding RNAs are now known to be abundant in genomes, and are being increasingly recognized as important regulators of gene expression (10). lncRNAs like *ivory* are thought to act as *trans*-regulators of gene expression through both pre-transcriptional and post-transcriptional effects that may include various protein, RNA, and DNA interactions. While lncRNAs have been linked to various human health conditions, including cancer and neurodevelopmental disorders (9, 22), little is known about how they may contribute to adaptation or plasticity. One of the few case studies that directly implicates any kind of non-coding RNA in trait adaptation concerns the role of small RNAs at the *YUP* locus in monkeyflowers, which affect carotenoid flower pigmentation (23). It is interesting that the *YUP* case study involves pigmentation, as does our *ivory* study. The similarities end there, however, as the *YUP* locus encodes multiple small RNAs that are evolutionarily labile, and individual RNAs can be specific to individual species. The *ivory* gene appears to behave in an evolutionary mode more consistent with other wing pattern adaptation loci like *optix* (a transcription factor) or *WntA* (a ligand), where a deeply conserved gene with a core developmental role diversifies phenotypes via *cis*-regulatory innovation (7). *ivory* presents an unparalleled opportunity to learn more about the contributions of non-coding RNAs to both evolutionary adaptation and phenotypic plasticity.

Our study was prompted by *J. coenia* DNA sequence associations that link a cluster of intronic CREs to variation in seasonal color pattern plasticity (2). Targeted deletions in this regulatory region caused a switch from autumnal color pattern to a summer color pattern, and thus appear to confirm a role for these CREs in modulating seasonal color patterns. What is interesting beyond simply being a validation of previous association work, however, is that these phenotypes also demonstrate that CREs of *ivory* can exercise nuanced, quantitative control over the color identity of large fields of cells. Clearly, natural butterfly color pattern diversity consists of much more than binary cell fate decisions across a fixed palette of colors - there is also natural quantitative variation in hue, intensity, and semi-stochastic expression of local color schemes across specific wing fields. Thus far, however, we know very little about the developmental genetic basis of variation in these kinds of quantitative color qualities (24). *ivory* stands out for playing this kind of role, and we wonder whether its identity as a lncRNA may be tied to this nuanced functionality in color regulation.

lncRNAs are widely known for their gene regulatory functions, but they can also play other functional roles within cells as well in protein interactions, organelle regulation, etc. (10). We were thus curious to determine to what extent differential expression of *ivory* affected gene expression. Does it regulate many genes, or only a handful of key pigmentation genes? Or perhaps it has no effect on gene regulation, and plays a direct role in pigment synthesis, precursor transport, or other aspects of scale cell biology. Our developmental RNA-seq data from wings of $ivory^{\Delta 17kb}$ individuals, with nearly pigmentless *ivory* loss-of-function phenotypes, show clearly that *ivory* has strong effects on transcript abundance during wing development, and that many genes show evidence of both upregulation and downregulation

in mutant wings (Fig. 4). The transcripts include a number of candidate pigmentation and wing patterning genes that are suggestive for explaining the pigment-related phenotypes (Table S8). Downregulation of various melanin and ommochromes genes (e.g. *dopa decarboxylase*, *black*, *kynurenine formamidase*) in *ivory* mutants makes immediate sense because of the pigment loss phenotypes, but also compelling is the upregulation of other pigmentation genes, such as *mamo*, a melanin repressor (25). Of course, however, we do not know which genes or transcripts are direct targets of *ivory* activity versus indirect downstream effectors. Livraghi et al. (8) speculate that a primary function of *ivory* function lies in regulating melanin synthesis genes, which we think is likely to be true. But our RNA-seq data also make it clear that *ivory* has a strong and broad influence over expression of many genes, even in larval imaginal discs, which may help explain its apparent effects on other non-melanic pigment types as well. Future work on this lncRNA would benefit from characterizing its molecular mode of action and identifying its direct targets during wing development. Through more functional work we will be able to understand how lncRNAs like *ivory* facilitate adaptive variation and plasticity in rapidly evolving gene regulatory networks.

## Materials and Methods

### Butterfly husbandry

Our primary *J. coenia* stock originated from Durham, North Carolina, and was reared in the lab at 27°C, 16 : 8 hrs light : dark on an artificial wheat germ diet supplemented with dried *Plantago lanceolata*, as previously described (26). We used a genetic assimilation “red line” of this stock with constitutive autumn red ventral hindwing coloration (2) to enhance detection of CRISPR/Cas9 mutation effects on red pigment patterns. Genetic mutant lines were visually determined by a fading in the ventral wing (Fig. 4C).

### Iso-Seq long read transcriptome annotation

For whole organism long-read RNA annotations we sampled an array of tissues at different developmental stages (Table S1). Tissues were dissected into Trizol and stored at -80 °C. RNA was extracted using RNeasy Mini Kit (Qiagen) with on column DNase treatment and quantified with a Qubit RNA BR Assay kit (Molecular Probes). RNA samples were sent to Maryland Genomics (University of Maryland School of Medicine) for quality control (RIN = 9.9), full length cDNA synthesis and Iso-Seq library pre. Libraries were sequenced on a PacBio Sequel II 8M SMRT Cells on a 30 hour movie run. The sequencing run provided 1.1 TB of data that is concatenated using the PacBio SMRT IsoSeq pipeline (https://www.pacb.com/support/ software-downloads/) to generate a circular consensus sequence file (.ccs) along with high quality (hq) and low quality (lq) full length transcript files. A SMRT PacBio wrapper (PBMM2) based on pairwise alignment (MINIMAP2) script was applied using the .css file against the jcgenv2.fa genome (Lepbase). The generated alignment file (.bam) was used for downstream transcriptome re-annotation using the BRAKER3 pipeline (17). Briefly, BRAKER 3 incorporates transcript selector algorithm TSEBRA with AUGUSTUS predictions through BRAKER1 and BRAKER2 pipelines. This allowed us to combine long read sequences (this manuscript) with previous short read data (12) to

produce an updated version 3 *J. coenia* transcriptome annotation (Jcv2_OGSv3.gff). Briefly, mRNAseq libraries from (2) were compiled for gene annotations using BRAKER I, and protein predictions from BRAKER II were compiled by lepidopteran and dipteran NCBI predicted protein sequences. Assessment of completeness was performed with BUSCO v5.4.7 with complete BUSCO score of 97.9 % (Table S1). The v3 *J. coenia* gene predictions are available on the Dryad repository: _____

**Chromatin immunoprecipitation and sequencing**

Pupal wing tissue for chromatin immunoprecipitation was collected and processed as previously described (12, 27). Further information on antibodies, pipelines and dataset sources used for this study are found in Table S3. ChIP-seq and input libraries were prepared using an NEBNext DNA Ultra II kit without size selection. Libraries were sequenced using the Cornell Biotechnology Resource Center Genomics Facility on an Illumina NextSeq 500/550 at 20 million bp reads for treatment (ChIP) and 30 million for control (Input).

**ATAC-seq, ChIP-seq, and Hi-C dataset analysis**

Chromatin profiled TAD boundaries and comparative ATAC-seq alignments within the *cortex* locus (Fig. 1) were accessed and adapted from Mazo-Vargas et al. (7). Comparative noncoding genome alignments for Scaffold 15 were performed using CACTUS/HALPER on *J. coenia* wing tissue ATAC-seq peakcalls (MACS2) (7). Peak calls representing sites of open chromatin are given a score from (0.001 – 1.000) the lowest score representing regions of least conservation and a score of 1.0 representing conservation in (8/12) species used in the alignment. Pupal wing ATAC-seq datasets of representative nymphalid clade butterfly species were gathered from previous studies: *J. coenia, V. cardui*, *H. himera*, and *D. plexippus* (Table S4). Peak calls for regions of high ATAC-peak conservation were imported into Geneious Prime (v2023.1.1) and manually annotated for position and chromatin accessibility (i.e. ATAC-seq signal) Table S5. ATAC-seq peaks below (RPKM normalized count values < 10) were characterized as not accessible and marked with a gray line in Fig 1.

ChIP-Seq alignment data for the transcription factor Spineless was taken from van der Burg et al, (12). We generated new Bric-a-brac, Ftz-F1, and Pol II ChIP-seq data as described above. ChIP libraries were aligned to the lepbase.org *J. coenia* genome version 2 (jcv2). Alignment, duplicate removal and peak calling was done as previously described (27).

**Hybridization chain reaction *in situ* hybridization**

The first exon of *ivory* was used to design hybridization chain reaction probe pairs with sequences unique to the *ivory* transcript. Wings were removed from 2-day old pupae and *in situs* were performed following the protocol of Chatterjee et al. (28). B1 hairpin amplifiers were tagged with AlexaFlour 647 fluorophores, and wings were imaged on a Leica Stellaris 5 confocal microscope.

### CRISPR/Cas9 targeted mutagenesis and genotyping

To generate *ivory* loss-of-function lines we designed two sgRNAs targeting the 5' promoter where Pol II binds (Table S10). We carried out sgRNA-Cas9 embryo injections in *J. coenia* as previously described (29), and selected $G^0$ adults with large clonal mosaics for sib mating. From the resulting $G^1$s we purified two lines: one with a nearly complete loss of pigmentation, and one with highly faded pigmentation. Whole genome Illumina sequencing of three individuals from each line confirmed that the former had an 17kb deletion (*ivory*$^{\Delta 17kb}$) and the latter an 18bp promoter deletion (*ivory*$^{\Delta 18bp}$) (Fig. 3A, B).

To test the function of *ivory* CREs in red line *J. coenia* we generated somatic mKO mutants using sgRNA pairs targeting selected ATAC-seq peaks, as previously described (7). This approach generates mosaic G0 individuals, where bilateral asymmetry of mKO clones provides a useful assay for ruling out natural variation or other non-CRISPR effects that would otherwise produce symmetrical phenotypes (14). Cas9 targeting was confirmed by PCR and sequencing of selected mutants for each locus (Table S6, S7).

### *ivory*$^{\Delta 17kb}$ mRNA sequencing and expression analysis

We dissected wings from late 5$^{th}$ instar larvae, day 2 pupae, and day 6 pupae of red line and *ivory*$^{\Delta 17kb}$ *J. coenia.* Each of the three experimental replicates for each stage represented all forewings and hindwings pooled from three individuals. PolyA-enriched transcripts were isolated and bidirectionally sequenced as previously described (TableS3). *ivory*$^{\Delta 17kb}$ mutants were genotyped with TaqPhire Pol Direct-Tissue PCR (Invitrogen), DNA was extracted from a third to fourth instar caterpillar spine and added to a reduced 20 µl lysis reaction mix, 1µl of this lysis reaction was used as template for PCR. RNA-Seq datasets (Novogene) were aligned using HiSAT2 on jcgenv2.fa genome followed by Featurecounts using the IsoSeq informed v3 annotations (Table S1).

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## Figures and Tables

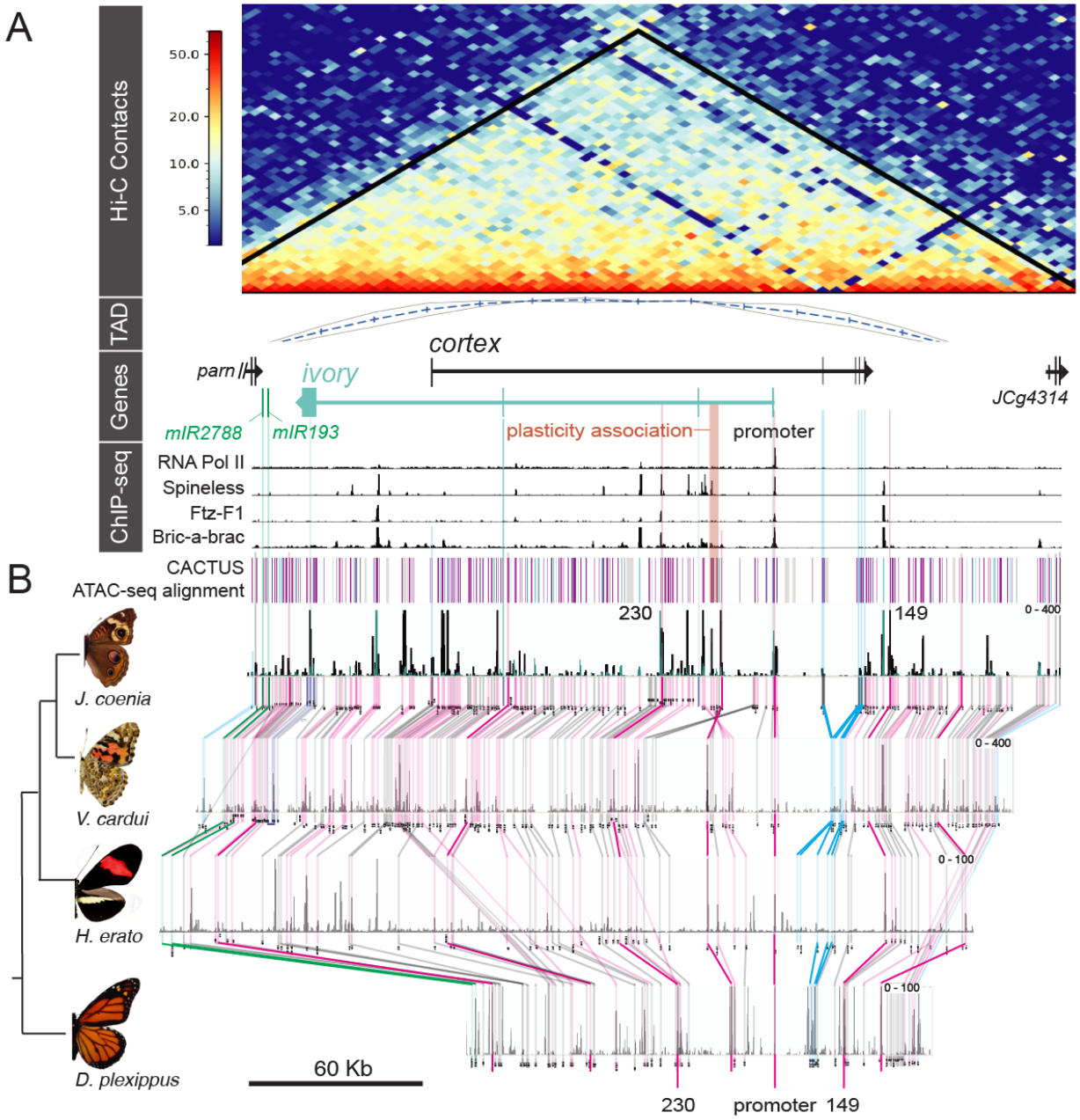


**Figure 1.** The *ivory* / *cortex* locus is deeply conserved in butterflies. (A) Hi-C chromatin conformation capture reveals a topologically associating domain (TAD) that centers on the overlapping *ivory* and *cortex* genes. The heat map displays chromatin interaction strength. ChIP-Seq annotates binding of Pol II and four transcription factors during pupal wing development. The orange column highlights a region of nucleotide variant associations linked with natural variation in seasonal polyphenism of *J. coenia* wing coloration (2). (B) Wing ATAC-seq data from four nymphalids highlights deep conservation of CREs across species. Orthology of specific sequences is indicated by color coded lines (green: micro RNAs; magenta to gray: ATAC-seq peaks; turquoise: *ivory* exons; light blue: *cortex* exons).

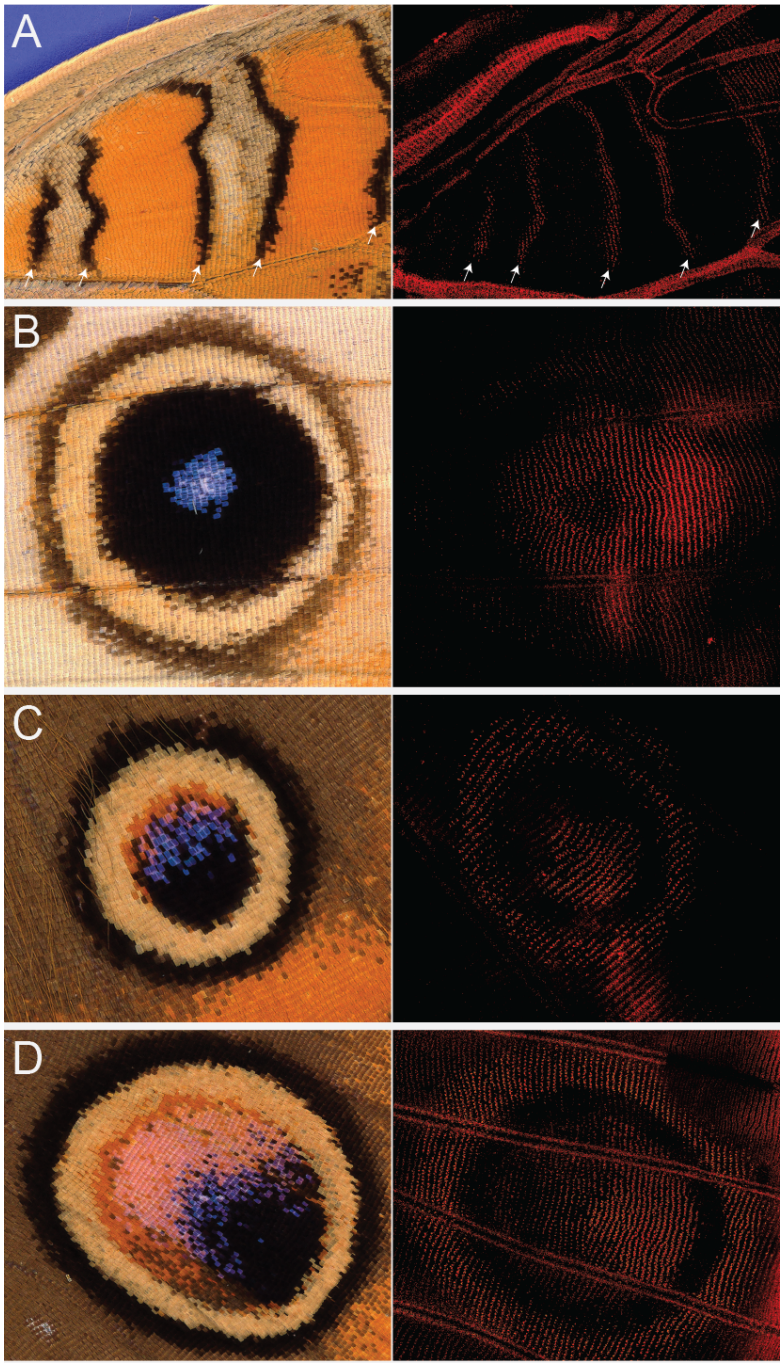


**Figure 2.** *ivory* is expressed in color pattern elements. Pictured are HCR *in situ* hybridizations of early pupal wings revealing *ivory* transcripts in (A) black borders of ventral discal bands (white arrows), (B) center ring of ventral forewing eyespot, and center and rings of dorsal hindwing (C) anterior and (D) posterior eyespots.

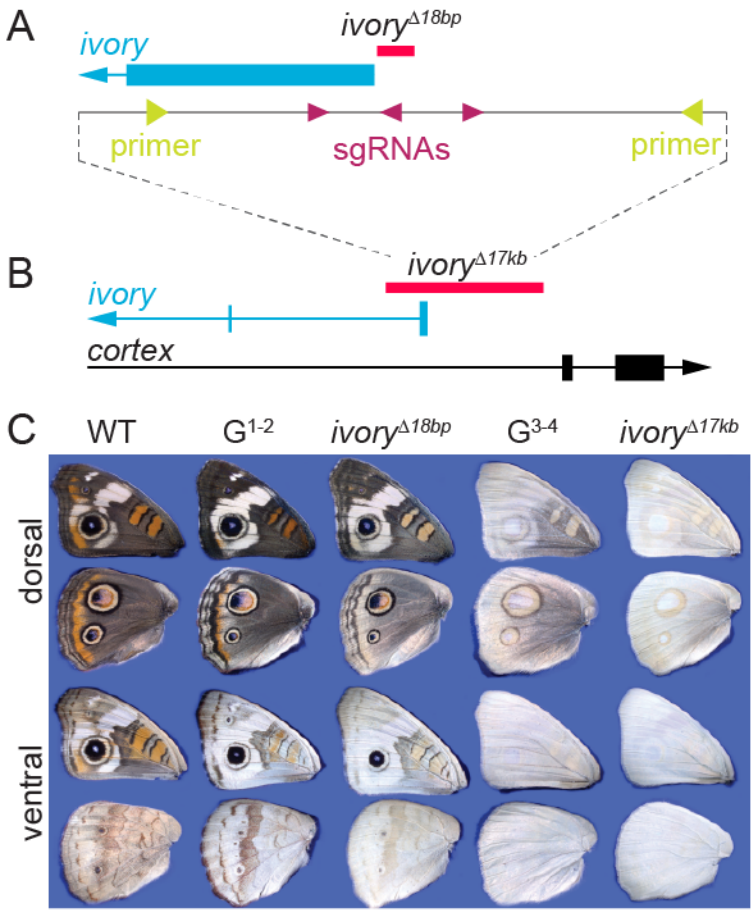


**Figure 3.** *ivory* promoter deletion lines show reduction and loss of pigmentation. (A) Adjacent to the *ivory* 5' exon, in the promoter, is the with *ivory*$^{\Delta p18bp}$ deletion (red). Location of PCR genotyping primers (green) and sgRNAs (magenta) is shown. (B) Expanded view of the *ivory* 5' region showing the location of the *ivory*$^{\Delta 17kb}$ deletion. (C) Phenotypes from promoter deletion line purification process. Pictured are representative intermediate individuals (likely heterozygotes), along with pure *ivory*$^{\Delta p18bp}$ and *ivory*$^{\Delta 17kb}$ individuals. Notable is the quantitative variation in overall pigmentation, but consistent presentation of the melanic center rings of the large eyespots.

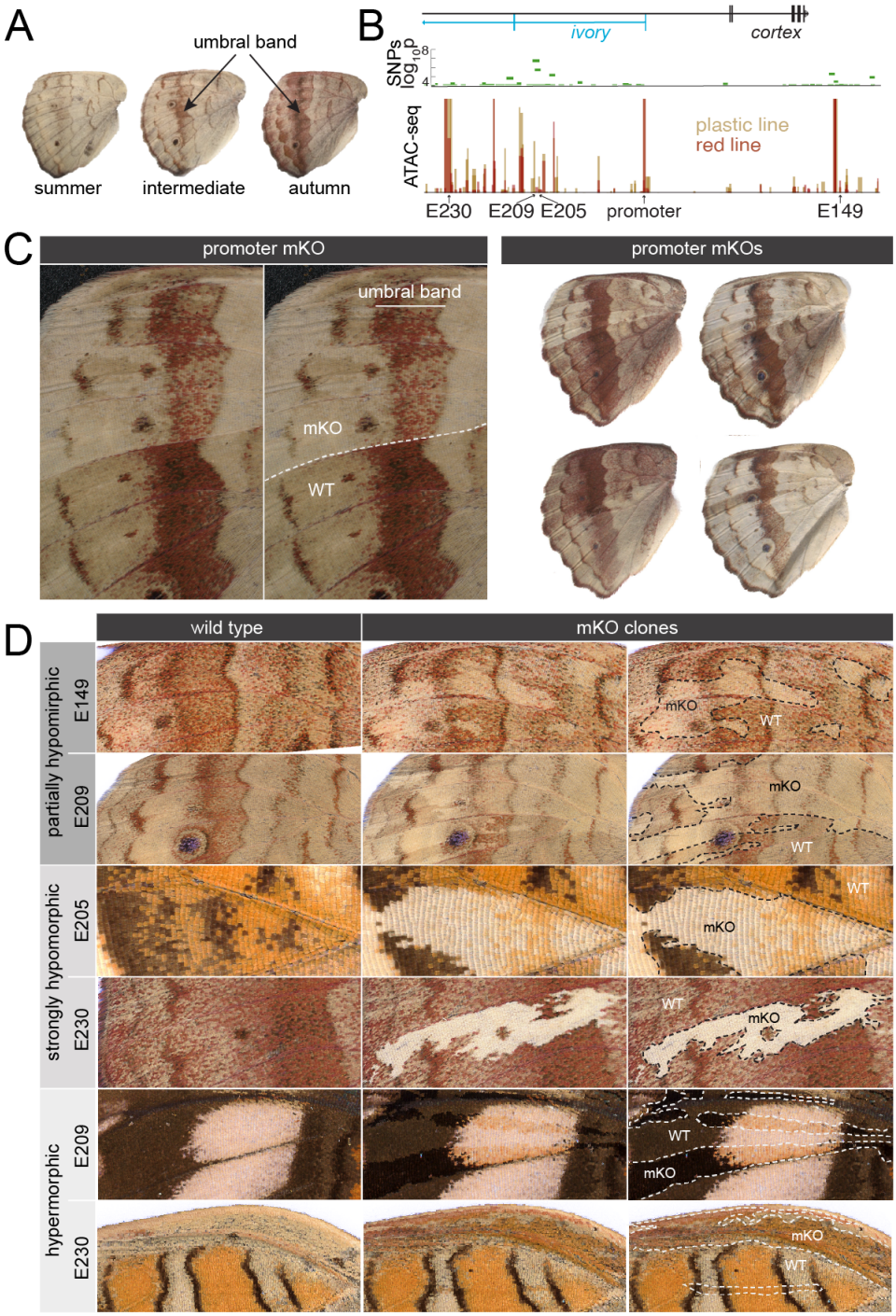


**Figure 4.** CRISPR mKOs of *cis*-regulatory regions result in several distinct phenotypes. (A) Examples of natural seasonal ventral hindwing phenotypes, including an intermediate form. (B) Locations of single nucleotide polymorphisms (SNPs) linked to variation in seasonal plasticity, along with ATAC-seq peaks from previously published "plastic" and "red" selection lines (2). Targets of CRISPR-Cas9 mKOs are annotated relative to SNPs and ATAC-seq. (C) Examples of promoter mKOs showing partially hypomorphic phenotypes resembling seasonal variation. (D) Examples of the different classes of mKO phenotypes from deletions in the targeted CRE regions.

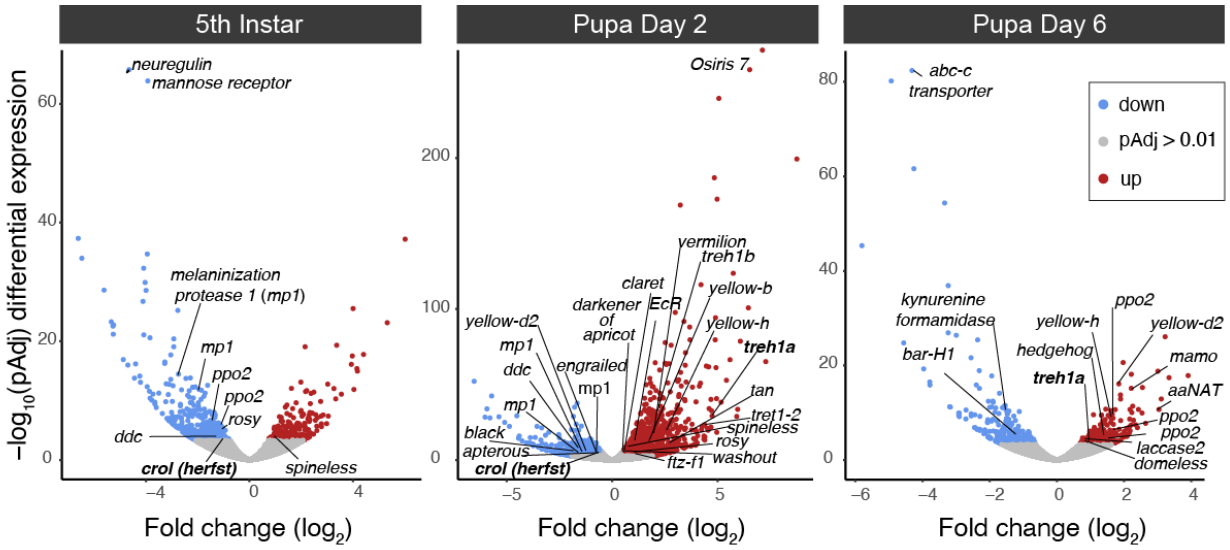


**Figure 5.** *ivory* loss-of-function results and major changes in gene expression during wing development. Volcano plots illustrate differences in transcript abundance in wild type versus pigmentless *ivory*$^{\Delta 17kb}$ wings at three stages of development. Blue dots mark genes downregulated in mutants and red dots mark genes upregulated in mutants. Genes potentially implicated in pigmentation or wing pattern development are highlighted. *ddc*: *dopa-decarboxylase*; *EcR*: *ecdysone receptor*; *ppo*: *prophenoloxidase*.

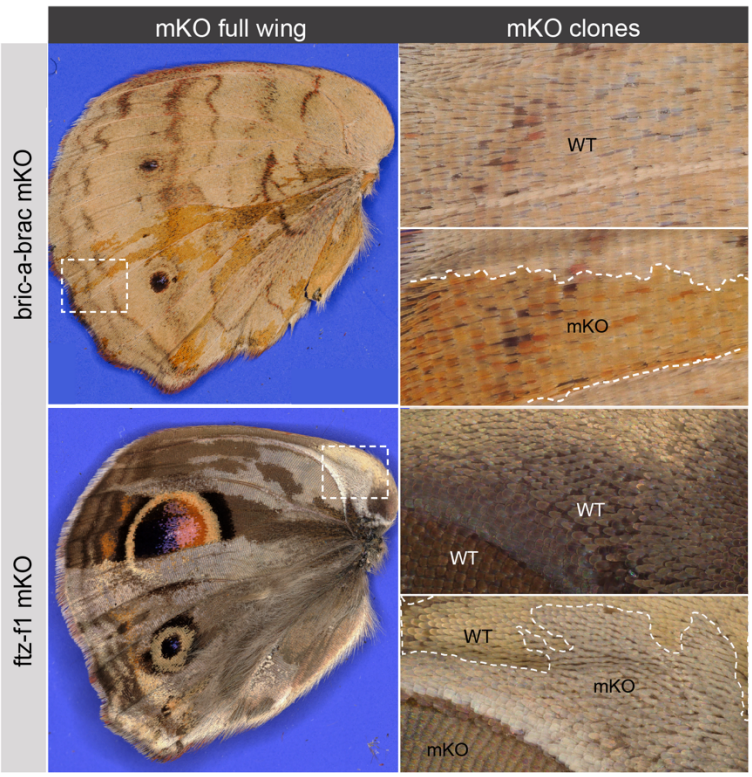


**Figure S1. *ivory* locus transcription factor genes are involved in ommochrome and melanin pigment in butterfly wings.** mKO of the bric-a-bac transcription factor shows hyperpigmentation phenotypes in ventral hindwings. *Ftz-f1* is required for morphological and cellular scale pigment development. mKO clones on select hindwing surfaces show both minor and major changes in scale morphology and reductions of pigmentation.

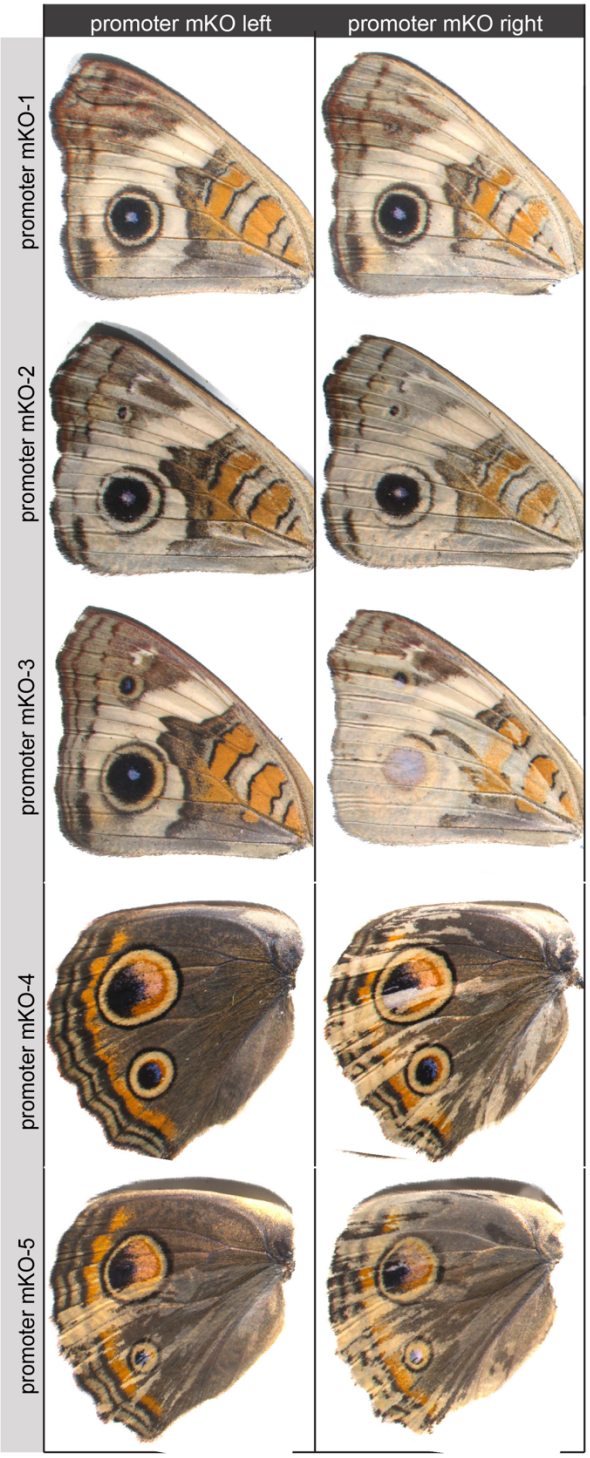


**Figure S2.** mKOs from sgRNAs targeting the *ivory* promoter (187).

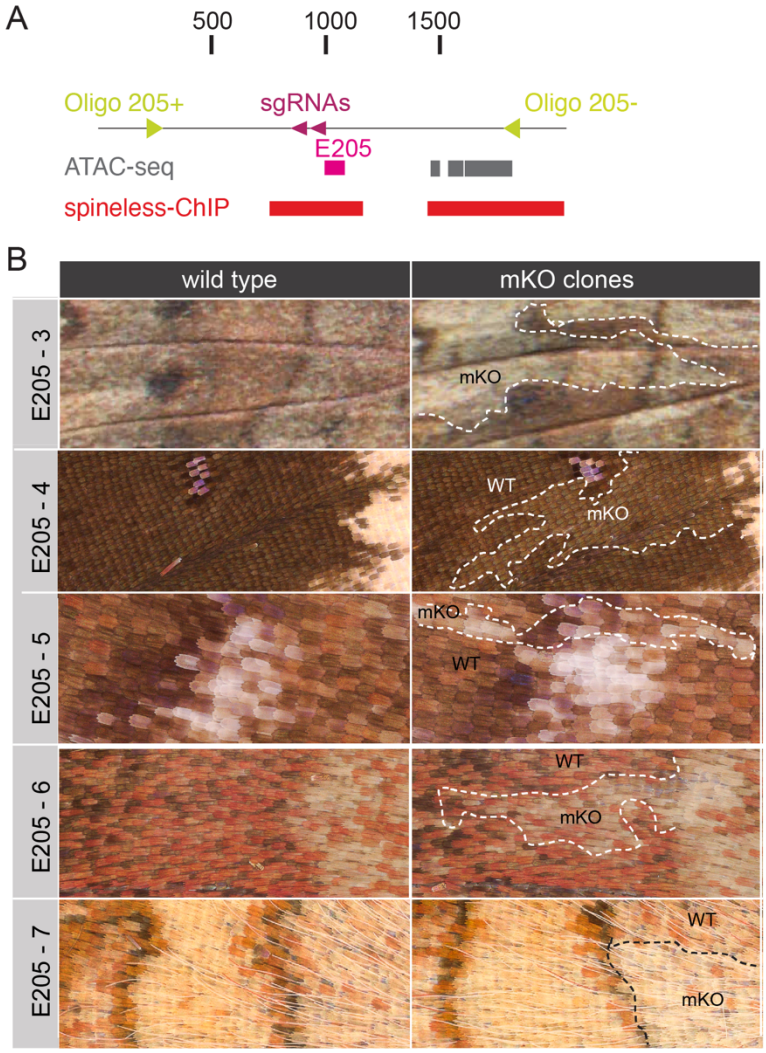


**Figure S3.** mKOs phenotypes from CRISPR targeting of CRE 205. (A) A map of sgRNAs, including Spineless ChIP-seq peaks. (B) mKO individuals showing pigmentation phenotypes.

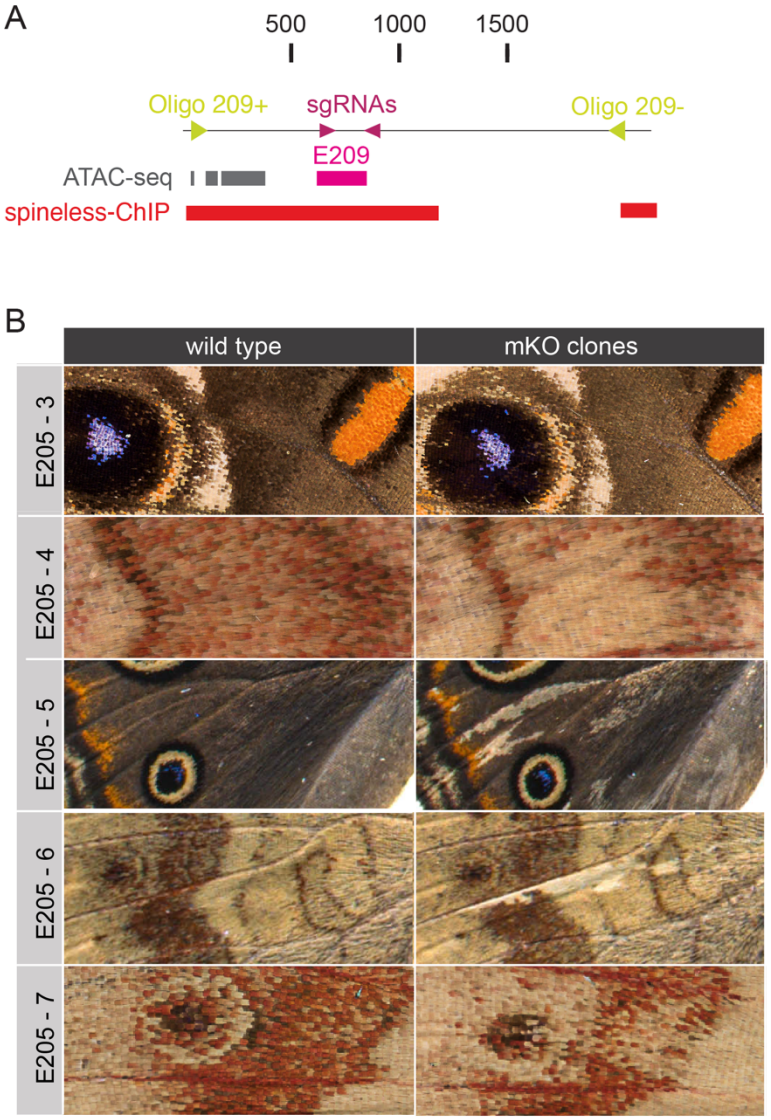


**Figure S4.** mKOs phenotypes from CRISPR targeting of CRE 209. (A) A map of sgRNAs, including spineless ChIP-seq peaks. (B) mKO individuals showing pigmentation phenotypes.

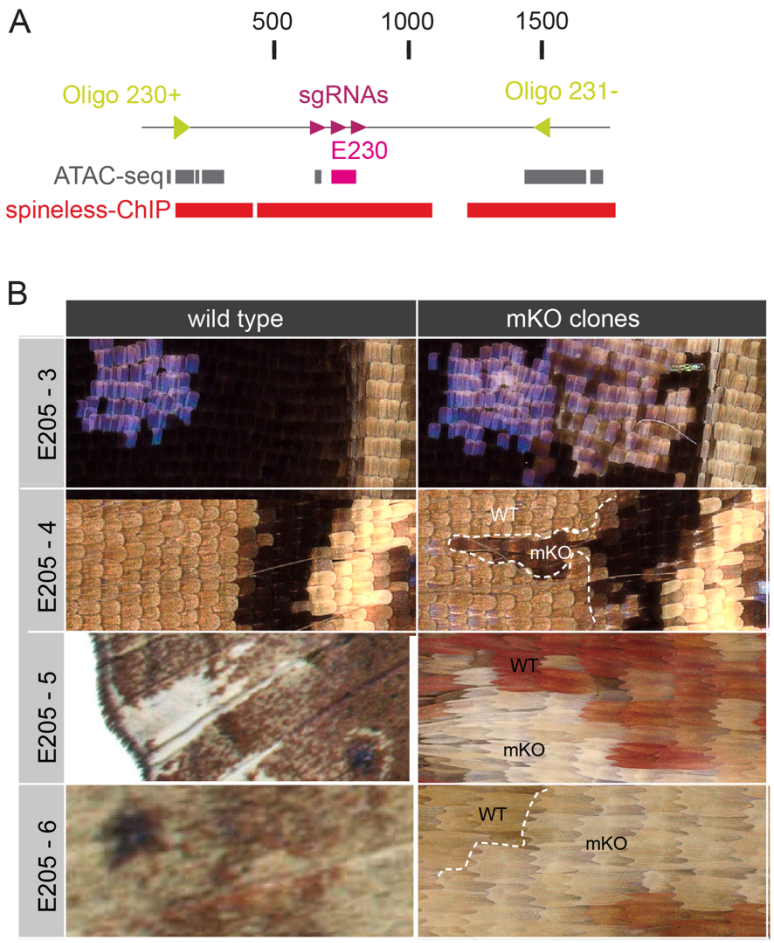


**Figure S5.** mKOs phenotypes from CRISPR targeting of CRE 230. (A) A map of sgRNAs, including spineless ChIP-seq peaks. (B) mKO individuals showing pigmentation phenotypes.

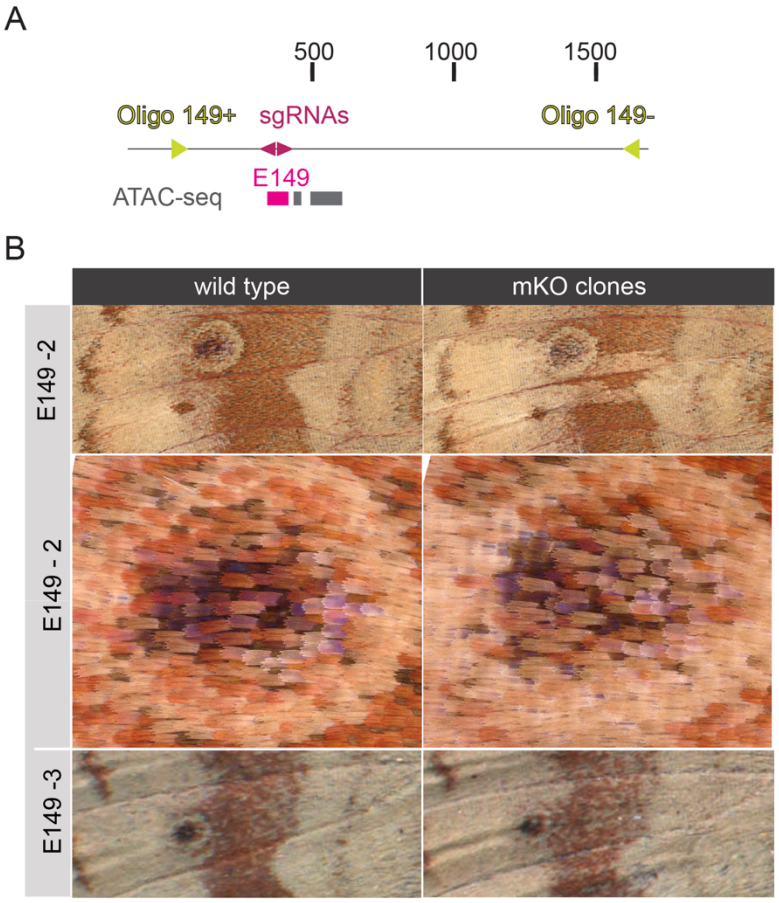


**Figure S6.** mKOs phenotypes from CRISPR targeting of CRE 149. (A) A map of sgRNAs, including spineless ChIP-seq peaks. (B) mKO individuals showing pigmentation phenotypes.