Reuse of an insect wing venation gene-regulatory subnetwork in patterning the eyespot rings of butterflies

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Abstract

Novel organismal traits might reuse ancestral gene-regulatory networks (GRNs) in their development, but data supporting this mechanism are still sparse. Here we show the reuse of an ancestral insect venation gene regulatory subnetwork patterning the sharp and distinct rings of color in butterfly eyespots. Using laser microdissection followed by RNA-Seq we first obtained transcriptional profiles of the anterior and posterior compartment of larval wings, and eyespot and adjacent control tissue in pupal wings of *Bicyclus anynana* butterflies. We identified key venation patterning genes such as *Mothers against dpp 6 (Mad6), thickveins, Optix, spalt, optomotor-blind (omb), aristaless, cubitus interruptus,* and *patched* differentially expressed (DE) across compartments, and a sub-set of these genes also DE across eyespot and non-eyespot samples. Fluorescent *in-situ* hybridization (HCR3.0) on the jointly DE genes *Mad6, Optix,* and *spalt*, as well as *dpp* showed clear eyespot-center, eyespot-rings, and compartment-specific expression. Knocking out *dpp* resulted in an individual with venation defects and loss of eyespots, whereas knockouts of *Optix* and *spalt* resulted in the loss of orange scales and black scales, respectively. Furthermore, using CRISPR-Cas9 followed by immunostainings, we showed that Spalt represses *Optix* in the central region of the eyespot, limiting *Optix* expression to a more peripheral ring, which parallels the regulatory interaction found in venation patterning in the anterior compartment of fly larval wings. These network similarities suggest that part of the venation GRN was co-opted to aid in the differentiation of the eyespot rings.

Keywords

Gene-subnetwork co-option, venation patterning, *Optix*, *decapentaplegic*, *spalt*, eyespots, *Bicyclus anynana*

One-sentence summary

We showed the reuse of an ancestral insect wing venation GRN in patterning a novel complex trait in butterflies.

Introduction

While it is possible that novel organismal traits emerge from the gradual construction of dedicated gene-regulatory networks (GRNs), one gene being added at a time, such networks may instead emerge from pre-existing clusters of pre-wired genes that are repurposed during development. So far, a few examples of this mechanism of GRN co-option have been proposed

for the development of novel complex traits. These include the reuse of a GRN that differentiates breathing spiracles in *Drosophila* larvae in the development of the posterior lobe of adult genitalia (Glassford et al., 2015); the reuse of the leg GRN in patterning the wings of insects (Bruce and Patel, 2020; Clark-Hachtel and Tomoyasu, 2020) or the head horns of beetles (Hu et al., 2019; Zattara et al., 2016); the reuse of the wing GRN in the development of a treehopper's helmet (Fisher et al., 2020); or the reuse of an appendage GRN to pattern butterfly eyespot centers (Murugeshan et al., 2022).

Eyespots, unlike the other examples of appendage co-option, where the novel trait protrudes from the body, do not grow out of the wing (Monteiro, 2015). This means that either only a small core network of genes shared between appendages and eyespots was co-opted in the first place, or that the similarities in the development of these traits break apart later in development, after the eyespot centers have differentiated in the late larval stage. One mechanism for this to happen is if another network gets rewired downstream of the shared core network to produce a novel output, i.e., to differentiate rings of color around the central signalling cells rather than tissue outgrowth. Here we explore whether the network of genes involved in early vein patterning in butterflies could have been repurposed to differentiate the color rings in eyespots at later stages of development.

The mechanism of wing vein patterning during larval development in *Bicyclus anynana* butterflies has many similarities to that of *Drosophila*, which is a classic example of positional information in developmental biology (Blair, 2007) (**Figure S1**). In both flies and butterflies, the morphogen Decapentaplegic (Dpp) is expressed in a stripe in the middle of the larval wing, along the Anterior-Posterior (AP) compartment boundary (Banerjee and Monteiro, 2020; Lawrence and Struhl, 1996). In flies, Dpp activates the transcription factors *spalt* and *Optix* (the latter expressed only in the anterior compartment), at different concentration thresholds and distances from the AP boundary (Martín et al., 2017). Subsequently, the cross-regulatory interaction between these two genes determines a sharp boundary that eventually positions longitudinal vein 2, L2 (Martín et al., 2017) (**Figure S1**). In butterflies, the expression of Spalt and Optix proteins is also found straddling a stripe of *dpp* expression, in homologous patterns to those in flies (Banerjee and Monteiro, 2020), suggesting a conserved vein patterning mechanism in both insects at early stages of wing development.

At later stages of wing development, and in butterflies alone, a similar mechanism of positional information has been hypothesized to specify the multiple rings of color in species with eyespots. Here, instead of an AP boundary line source, a central morphogen secreted by the eyespot centers would activate two or more transcription factors at decreasing concentration thresholds and distances from the center (Brunetti et al., 2001; Monteiro et al., 2006; Nijhout, 1978). The similarities between eyespot development and vein patterning, however, rest solely on the expression of Spalt protein in the central black scales of the eyespot (Brunetti et al., 2001), as *Optix, dpp* and other venation candidate genes have never been tested in and around the eyespots during the pupal stage.

Here we tested the hypothesis that a part of the vein patterning GRN, involving the ligand Dpp, the candidate transcription factors Spalt and Optix, as well as other vein-patterning genes, might have been co-opted in the differentiation of the eyespot color rings. We first identified differentially expressed (DE) genes between the anterior and the posterior compartments in mid larval wings, followed by DE genes between pupal eyespot and control tissue in 18-22 hrs pupal wings. We then examined the expression of several candidate Dpp pathway members as well as *spalt* and *Optix,* using enzyme and fluorescent based in-situ hybridization, and tested their function using CRISPR-Cas9. Finally, we targeted both *spalt* and *Optix*, in turn, and followed these disruptions with visualizations of the presence of both proteins in the eyespot

field using immunofluorescence, to test for a potential functional interaction in color ring differentiation similar to that observed in L2 vein differentiation in *Drosophila* (Martín et al., 2017).

Results

Differential gene expression between anterior and posterior compartments of larval wings

To identify and confirm differentially expressed genes between the anterior and the posterior compartments of *B. anynana* mid-larval wings, we used RNA extracted from laser-based microdissections followed by RNA-Seq and DESeq2 analyses (**Figure 1A, Figure S2**). Character-tree analysis showed that the biological replicates from the anterior and the posterior compartment clustered together (**Figure 1B**). Genes that were significantly up-regulated in the anterior compartment included *aristaless, aristaless-like, cubitus-interruptus, patched, Optix,* and *dachshund* (**Figure 1D, Table S1**), whereas posterior compartment up-regulated genes included *engrailed-like, invected-like, hedgehog, hexamine-like, caupolican-like,* and *Toll-like receptor 6* (**Figure 1D, Table S1**). These data are consistent with studies on anterior and posterior compartment-specific genes in insects (Abouheif and Wray, 2002; Banerjee and Monteiro, 2020; Biehs et al., 1998; Bier, 2000; Blair, 2007; Martín et al., 2017).

Differential gene expression between eyespot and control tissue of pupal wings

To identify upregulated genes in eyespots we performed the same laser microdissection experiment in eyespots and adjacent control tissue (**Figure 1, Figure S2**) in 18-22 hrs pupal wings, followed by RNA-Seq and DEseq2 analysis. Character-tree analysis showed that biological replicates from each tissue clustered together (**Figure 1C**). Some of the significantly upregulated genes in the eyespots included Dpp signaling member *Mad6*; Hh signaling members *hedgehog, patched,* and *smoothened*; Wnt signaling members *wnt1, armadillo, pangolin,* and *dishevelled*, and other interesting genes such as *homothorax, dachshund, Ultrabithorax, Sp4-like, twisted gastrulation, grindelwald, Cyclin dependent kinase-14, aristaless-like, spaetzle 3*, and *kayak-like* (**Figure 1E, Table S2**). In the control tissue, highly expressed genes included serine proteases, Wnt repressor *groucho*, *obstructor E-like, peter pan, timeless homolog*, and *henna* (**Figure 1E, Table S2**). Interestingly *dpp, Mad3, Mad4*, and *cubitus interruptus* were not DE across tissues.

dpp and *Mothers against dpp 6 (Mad6)* are expressed in eyespot centers and *dpp* functions in eyespot development

Since we could not identify the presence of *dpp* in our DEseq eyespot data (performed at 18-22 hrs) we conducted hybridization chain reaction (HCR3.0) (Choi et al., 2018), as well as enzyme-based *in-situ* hybridizations at a few earlier and later stages of pupal development. We had inconsistent spatial expression results in 16-24 hrs pupal wings with some showing clear expression of *dpp* in the eyespot centers (**Figure 2F and G, Figure S4 Q and R**), while others not (**Figure S4 A and B**). The 16-24 hrs window overlaps the window of development when protein products of the known Dpp target gene *spalt* are observed in the black scale cells (Monteiro et al., 2006; Brunetti et al., 2001). In wings where *dpp* expression was observed, it localized to only five of the seven eyespot centers, from M1-Cu1 (**Figure 2F and G**). This suggests that the period investigated for our RNA-Seq experiment might be close to the end of Dpp signaling in eyespot centers.

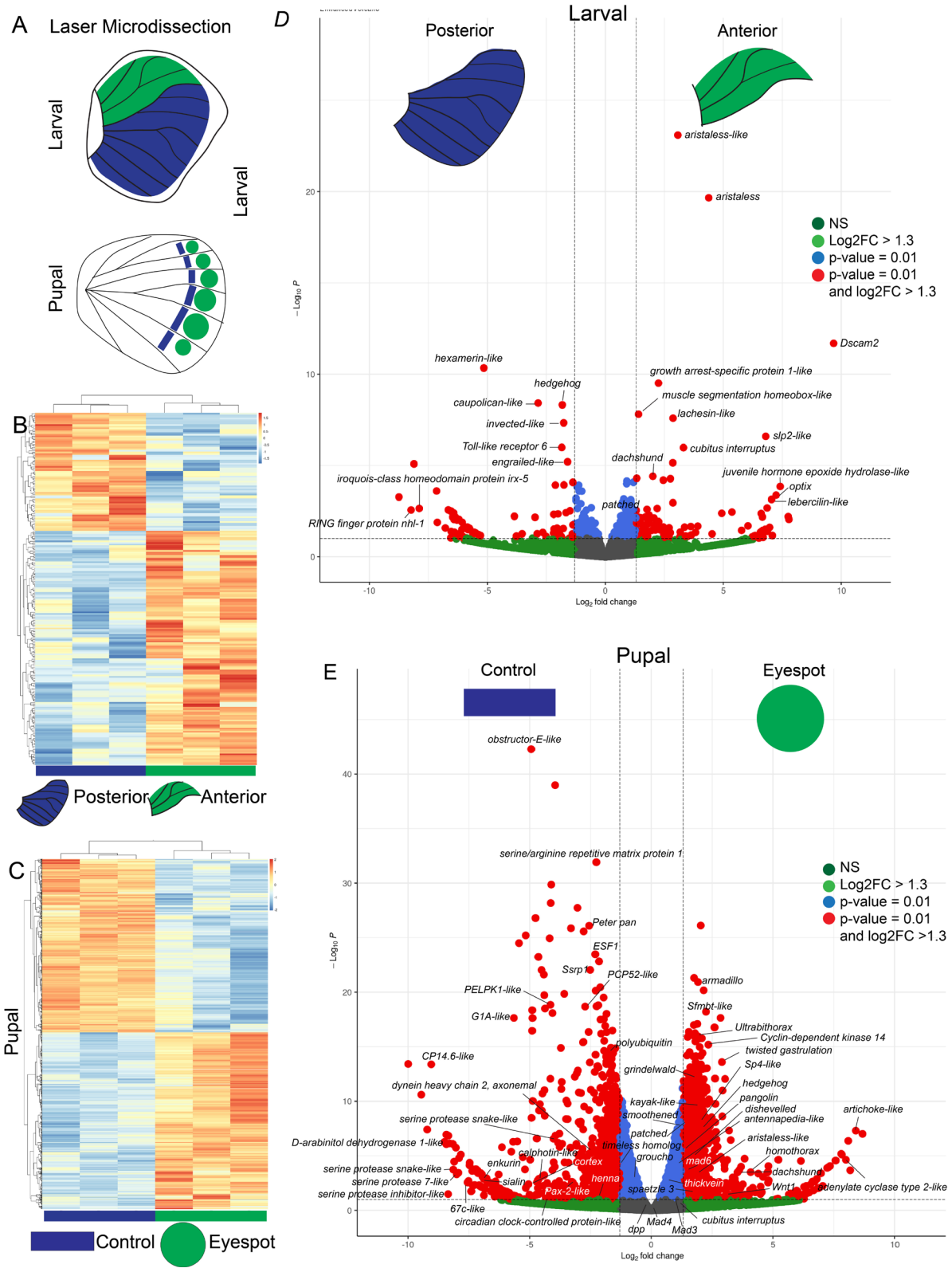

Figure 1. RNA-Seq of the larval anterior and posterior compartment and of pupal eyespot and control tissues dissected with a laser. (**A**) Laser microdissection was performed to isolate the anterior and posterior compartment of larval wing discs, and the eyespot and adjacent control tissue in 18-22 hrs pupal wings. (**B**) Heatmap clustering of the biological replicates of the anterior and the posterior compartments of larval wings and of (**C**) eyespot and adjacent control tissues of pupal wings. (**D**) Volcano plot showing differentially expressed genes

between the anterior and the posterior compartments of larval wings and of (**E**) eyespot and control tissues of pupal wings. Threshold parameters used for the Volcano Plot: Log2FoldChange = 1.3 and p-value = 0.01.

To confirm the deployment of the Dpp signaling pathway in eyespots, we examined the spatial expression of other pathway members, including the receptor *tkv* and the transcription factors *Mad6*, *Mad3* and *Mad4*. During larval wing development, we observed two strong domains of *tkv* away from the strong domains of *dpp* expression (**Figure 2A and B, Figure S3J, N-P**), consistent with data from *Drosophila* where *tkv* is expressed away from the source of Dpp proteins (Zecca et al., 1996). *Mad6* was expressed strongly in a broad domain spanning the AP boundary where its likely transducing the Dpp signaling (**Figure 2C, Figure S3K**), and no expression domains for *Mad3* and *Mad4* were observed (**Figure 2D and E, Figure S3L and M**). In the pupal stages (at 16-24 hrs after pupation) *Mad6* was expressed in the center of the eyespots consistent with the RNA-seq data mentioned above, along with the broad domain spanning the AP boundary conserved from the larval stage (**Figure 2H, Figure S4R**). No specific domain of expression was observed for *tkv* during the pupal stage (**Figure 2K, Figure S4R**).

To test the function of *dpp* in eyespot development we used CRISPR-Cas9. Knocking out *dpp* by injecting a single guide RNA (sgRNA) and Cas9 during the embryonic stages proved to be extremely difficult and resulted in high embryonic mortality. This is likely due to *dpp* being involved in patterning the early embryos (Ashe et al., 2000). Late embryonic knockouts (around 6 hrs) of *dpp* resulted in one individual with the loss of an anterior hindwing eyespot, increases in the size of two other eyespots (one being the large Cu1 eyespot), loss of either M1 or M2 veins, incomplete development of M1/M2 and M3 veins (**Figure S5**), and the appearance of ectopic silver scales (**Figure 2M and N, Figure S5**). These phenotypes are partially like *Ultrabithorax (Ubx)* knockout phenotypes (Matsuoka and Monteiro, 2021), that essentially transform a hindwing into the morphology of a forewing, leading to the ectopic appearance of silver scales, and changes in eyespot number and size.

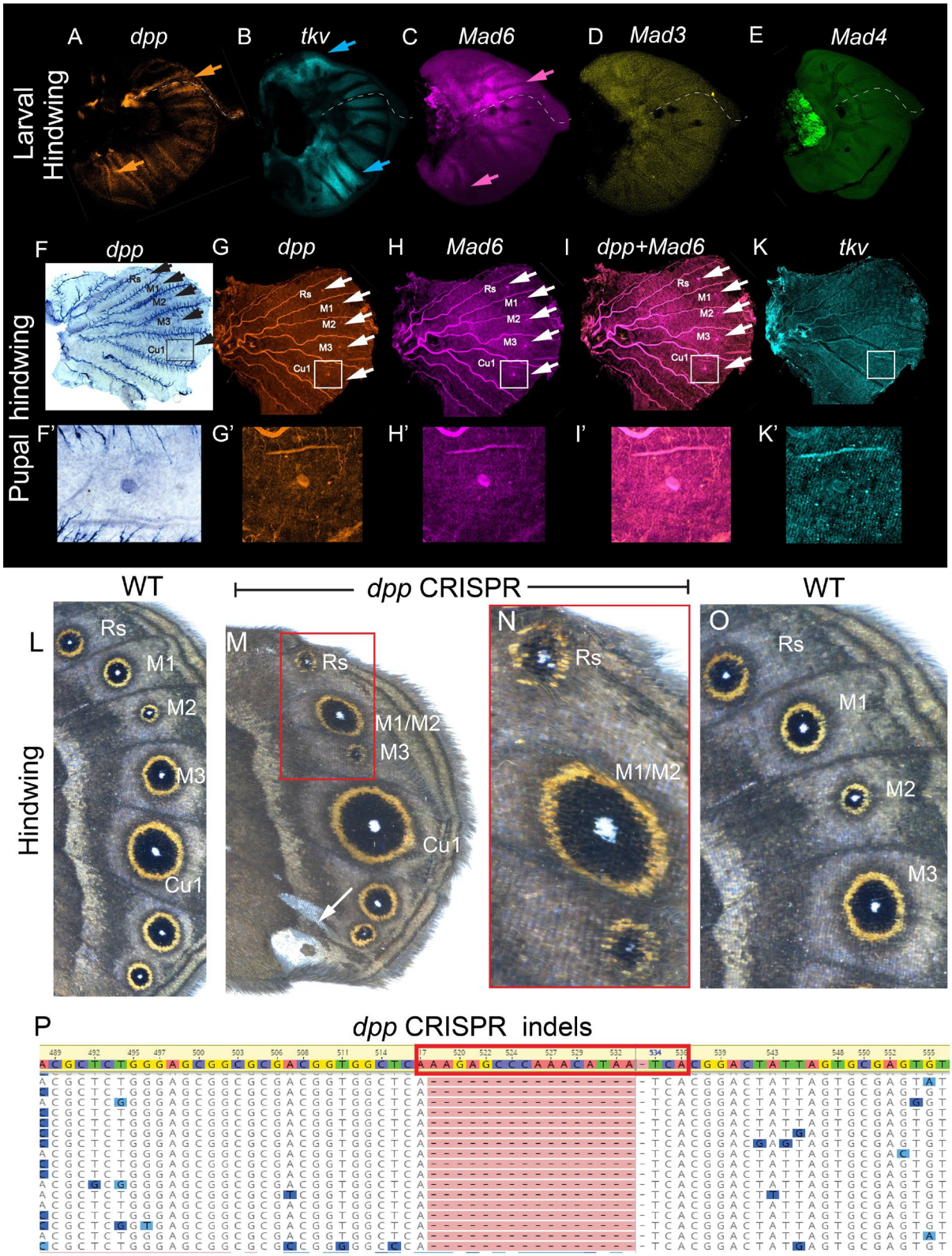

Figure 2: Expression of *decapentaplegic (dpp), thickvein (tkv), Mothers against dpp 6 (Mad6), Mad3,* and *Mad4* in larval and 16-24 hrs pupal hindwings of *B. anynana*. Expression of (**A**) *dpp* in the anterior compartment at the AP boundary (dotted line), (**B**) *tkv* in the anterior and middle posterior wing regions (blue arrows), and (**C**) *Mad6* in a broad domain spanning the AP boundary and in a small lower posterior domain (pink arrow) in the larval hindwings. No expression of (**D**) *Mad3* and (**E**) *Mad4* was observed in larval hindwings. (**F**

and G) Expression of *dpp* in the eyespot center cells, and (**H**) of *Mad6* in the eyespot centers and in a broad domain spanning the AP boundary. (**I**) Co-expression of *dpp* and *Mad6*. (**K**) No specific domain of expression was observed for *tkv*. **(L)** Adult WT. **(M and N)** CRISPR-Cas9 knockout of *dpp* during embryonic development resulted in the loss of either the M1 or M2 eyespot, increases in size of two eyespots M1 (or M2) and Cu1, and ectopic appearance of silver scales (white arrow) in a single individual. (**O**) Adult WT hindwing. (**P**) Deletions (red shaded region) at 3 bps 5' of the PAM sequence of the *dpp* guide RNA site (boxed area) obtained using DNA extracted from the wing shown in panels **M** and **N**.

Optix and *spalt* are expressed in larval wings and in eyespot rings of pupal wings

Next, we examined the expression of *Optix* and *spalt* mRNA using HCR. During larval wing development, *Optix* was expressed strongly in an anterior domain on the hindwing (**Figure 3A**), whereas it was expressed in an additional domain on the forewing, in a posterior region (**Figure S3A and E**). *spalt* was expressed in four distinct domains in both hindwings and forewings (**Figure 3B, Figure S3H**), consistent with a previous study (Banerjee and Monteiro, 2020). In 24 hrs pupal wings, *Optix* was expressed in the upper and proximal margin of the wing, in a region that will produce silver coupling scales (Prakash et al., 2022), in distinct bands along the proximal-distal axis, corresponding to a marginal band and two bands of the central symmetry system, and in scale cells of the eyespot's orange ring (**Figure 3D, Figure S4C, D, K, and L**). *spalt* was expressed in the future black scale cells of the eyespots (**Figure 4E**). Both genes formed a sharp boundary corresponding to the boundary observed between black and orange scale cells in adult individuals (**Figure 3F**). The exact same expression patterns were observed for the protein product of both genes using antibodies (**Figure S6**).

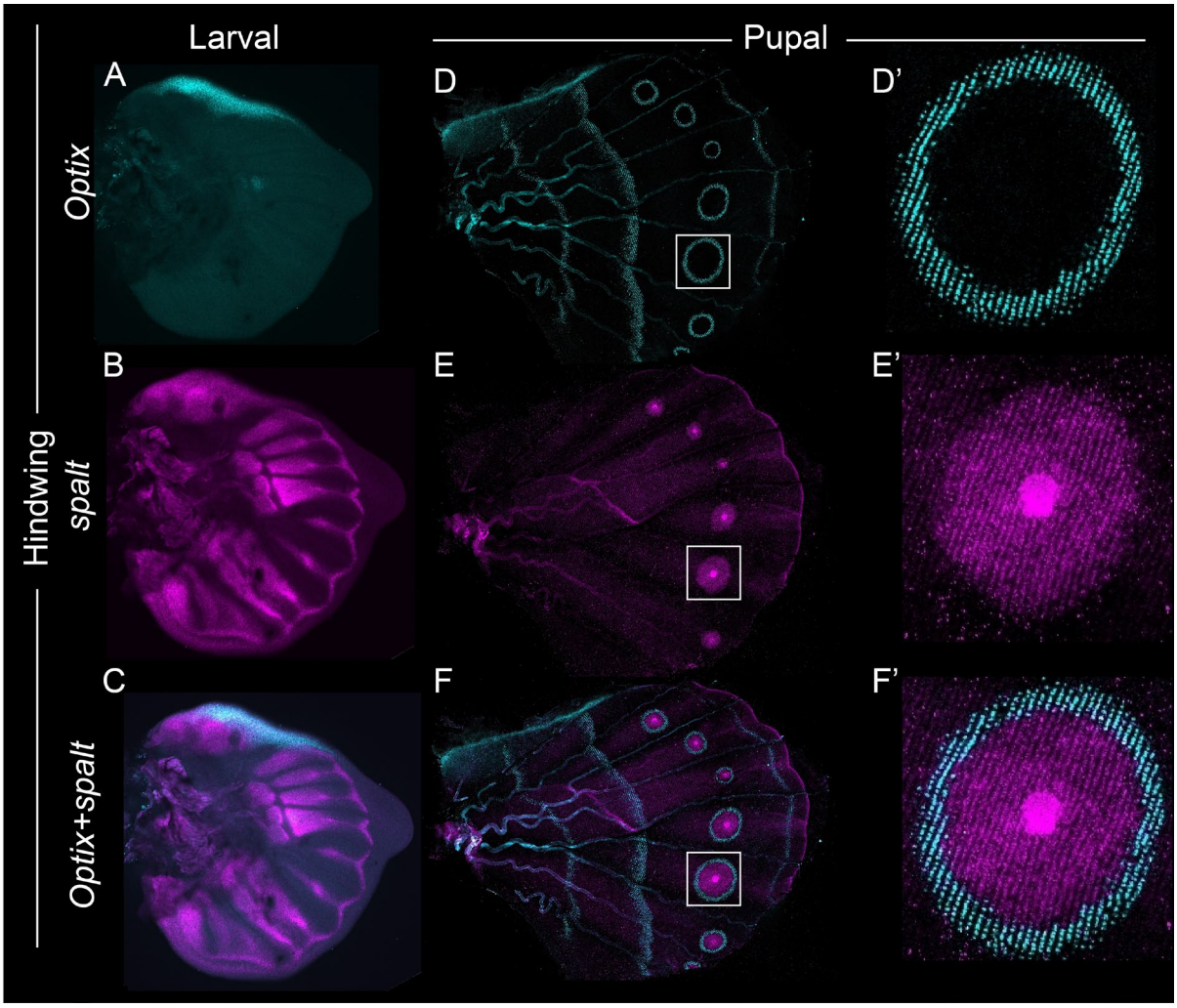

Figure 3: Expression of *Optix* and *spalt* in the larval and pupal wings of *B. anynana*. Expression of (**A**) *Optix* in the upper anterior compartment and (**B**) *spalt* in the four major domain of the larval wing disc. (**C**) Co-expression of *Optix* and *spalt*. Expression of (**D**) *Optix* in the eyespot orange ring cells, in the anterior and proximal margin of the wing, and in three bands transversing the proximal-distal axis, and (**E**) *spalt* in the future black scale cells of the eyespots, discal cell cross-vein, and along the wing margin. (**F**) Co-expression of *Optix* and *spalt*.

***optomotor-blind (omb)* is also expressed in larval wings and in eyespots**

We next investigated the expression of other DE genes, also involved in the vein positional information mechanism in flies, including *al*, *omb*, *ci*, and *ptc* (Bier, 2000; Blair, 2007; De Celis, 2003). Both *al* and *omb* are known targets of Dpp signaling at the AP boundary, with *al* responding to Dpp at lower levels, further away from the boundary (Campbell et al., 1993; Martín et al., 2017). *al* showed upper anterior compartment expression in both larval and pupal wings (**Figure 4B and E**), consistent with a previous study in *B. anynana* (Banerjee and Monteiro, 2020), whereas *omb* was expressed in a large domain spanning the AP boundary in both larval and pupal wings (**Figure 4H and K, Figure S3F**). The small domain of *omb* expression observed in the lower posterior compartment in larval wings is likely activated by the lower posterior band of Dpp signaling that exists in butterfly wings (**Figure 2A, Figure S3J, Figure 4H, Figure S3F**). Faint eyespot center and orange ring expression was also observed for *omb* during the pupal stage (**Figure 4K**). Both *ci* and *ptc* showed anterior

compartment specific expression in larval hindwings and forewings (**Figure 4N and O, Figure S3B and C**), *ptc* being strongly expressed along the AP boundary (**Figure 4O**, **Figure S3C**) where it likely transduces Hh signaling with the help of *ci* (Tanimoto et al., 2000). The anterior expression of *ci* was conserved in the pupal stage (**Figure 4R, Figure S4I and M**). No eyespot-specific expression was observed for *al, ci*, and *ptc* at either the larval or pupal stage (**Figure 4, Figure S4**). In summary, all these fly venation genes show conserved patterns of expression in early butterfly larval wings, indicating potential conservation of function, whereas only *omb* showed a novel expressed in eyespots.

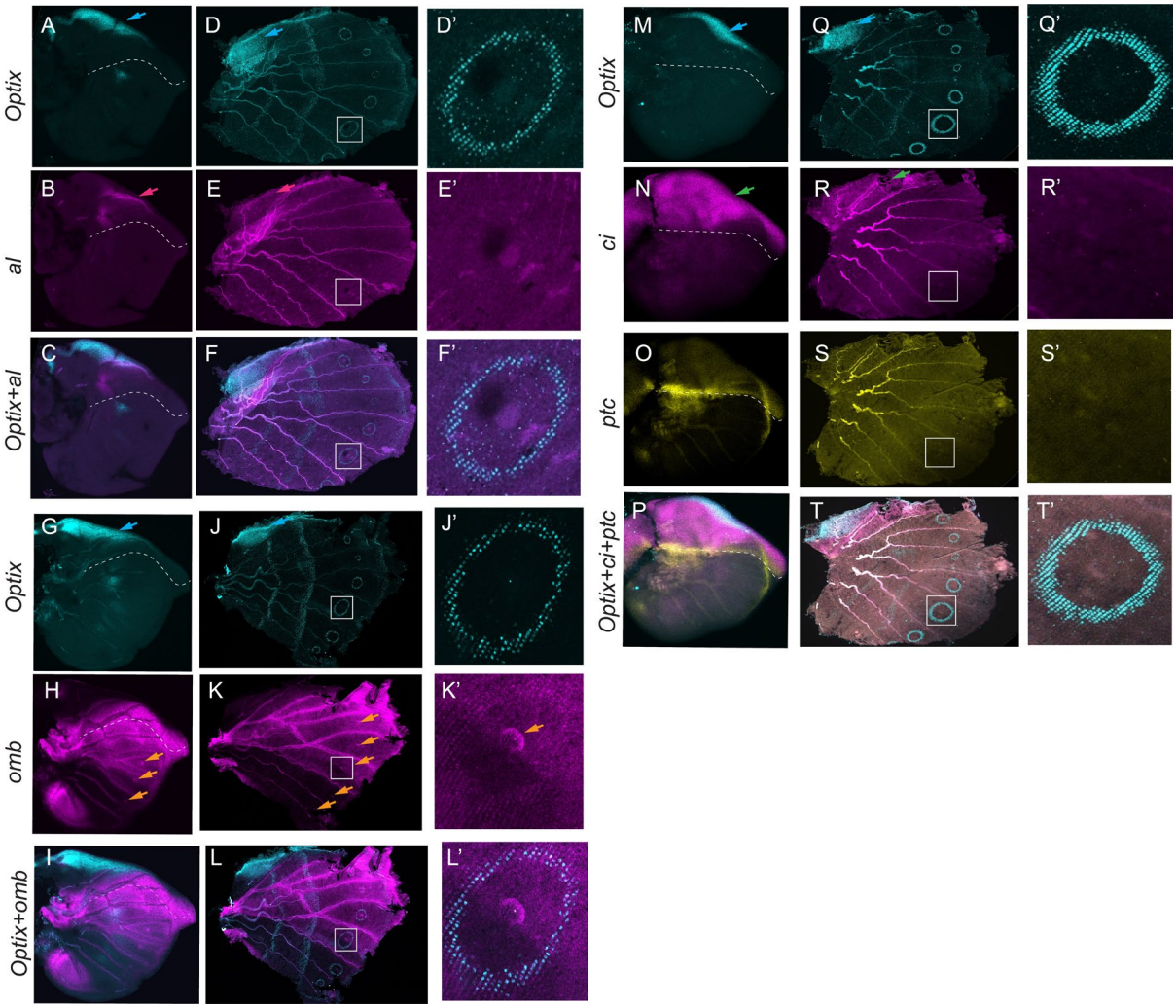

Figure 4: Expression of *Optix, aristaless (al), optomotor-blind (omb), cubitus interruptus (ci),* and *patched (ptc)* in the larval and 18-24 hrs pupal hindwings of *B. anynana*. *Optix* expression (**A, G, and M**) is used as a marker for the other genes. (**B**) *al* is expressed in the anterior compartment (pink arrow) of the larval wing. (**C**) Co-expression of *Optix* and *al*. (**D, J, and Q**) Expression of *Optix* in the eyespot ring and in the proximal-distal bands (blue arrow), and (**E**) *al* in the anterior compartment (pink arrow) in pupal hindwing. (**F**) Co-expression of *al* and *Optix*. (**H**) Expression of *omb* in a broad domain spanning AP boundary and in the lower posterior domain of the larval hindwing. Eyespot center specific expression was also observed during the larval stage (orange arrow). (**I**) Co-expression of *Optix* and *omb* in the larval hindwing. (**K**) In the pupal hindwing *omb* has the conserved AP expression domain along with faint expression in the orange ring and in the eyespot centers (orange arrows). (**N**) Expression

of *ci* in the anterior compartment (green arrow) and of (**O**) *ptc* in the AP boundary and in the anterior compartment of larval hindwings. (**P**) Co-expression of *Optix, ci,* and *ptc* in the larval hindwing. (**R**) Expression of *ci* in the anterior compartment (green arrow) and of (**S**) *ptc* in the pupal hindwing. No specific domain of expression was observed for *ptc*. (**T**) Co-expression of *Optix, ci* and *ptc*. Dashed line in larval wings indicates the AP boundary.

To test if *omb*, *ci,* and *ptc* play a role in eyespot development, we again used CRISPR-Cas9. The single *ci* crispant individual had a defective anterior compartment but no eyespot defects (**Figure S9**), and *omb* and *ptc* didn't showed any observable phenotype (**Table S9 and S10**). This suggests that these genes may play no role in eyespot development.

***Optix* and *spalt* are required for the development of orange and black scales, respectively, and Spalt represses *Optix* expression**

To test the function of *Optix* and *spalt* we injected CRISPR-Cas9 targeting each of the genes separately into early embryos. Individuals that emerged from these injections were mosaics where some cells were disrupted for *Optix* or *spalt* function and others were wildtype (**Figure 5A-F**). Some *Optix* crispants showed transformation of their orange-colored scales to a brown color (**Figure 5 A, B**; **Figure S7**) but did not show changes in the area of black scales, whereas *spalt* crispants developed orange scales in the black scale region of the eyespots (**Figure 5E and F**; **Figure S8**), as previously shown (Murugeshan et al., 2022). We confirmed that knocking out *Optix* and *spalt* led to deletion of nucleotides mostly near the targeted sites (**Figure 5S-U, Figure S7K and L, Figure S8K**). These results show that both *Optix* and *spalt* are required for the development of the orange and black scale cells, respectively. They also suggest that Spalt might be repressing the expression of *Optix* in the central black disc domain, because in *spalt* crispants, these central black scale cells become orange.

To test the regulatory interaction between *spalt* and *Optix* in eyespots, we performed knockouts of each gene in turn, followed by immunostainings targeting the protein products of both genes. *Optix* crispants showed loss of Optix proteins in cells of the orange ring **(Figure 5C, D, and K; Figure S7I),** but did not show changes in the presence of Spalt proteins in those cells (**Figure 5N and Q**). These data indicate that *Optix* is involved in giving the orange ring its color, but this gene is likely not involved in regulating the outer perimeter of the eyespot's black disc nor *spalt* expression in the eyespots. *spalt* crispants showed loss of Spalt protein **(Figure 5O)**, and ectopic levels of Optix protein in the black scale disc region **(Figure 5 L; Figure S8D and E)**. These data indicate that *spalt* represses *Optix* expression in the central black disc region of the eyespot. In the eyespot centers (white pupils), Optix proteins are generally absent, but in some individuals, both Optix and Spalt proteins are found there (**Figure 5L, O, and R**), indicating that in these cells, the regulatory interaction between these genes is different.

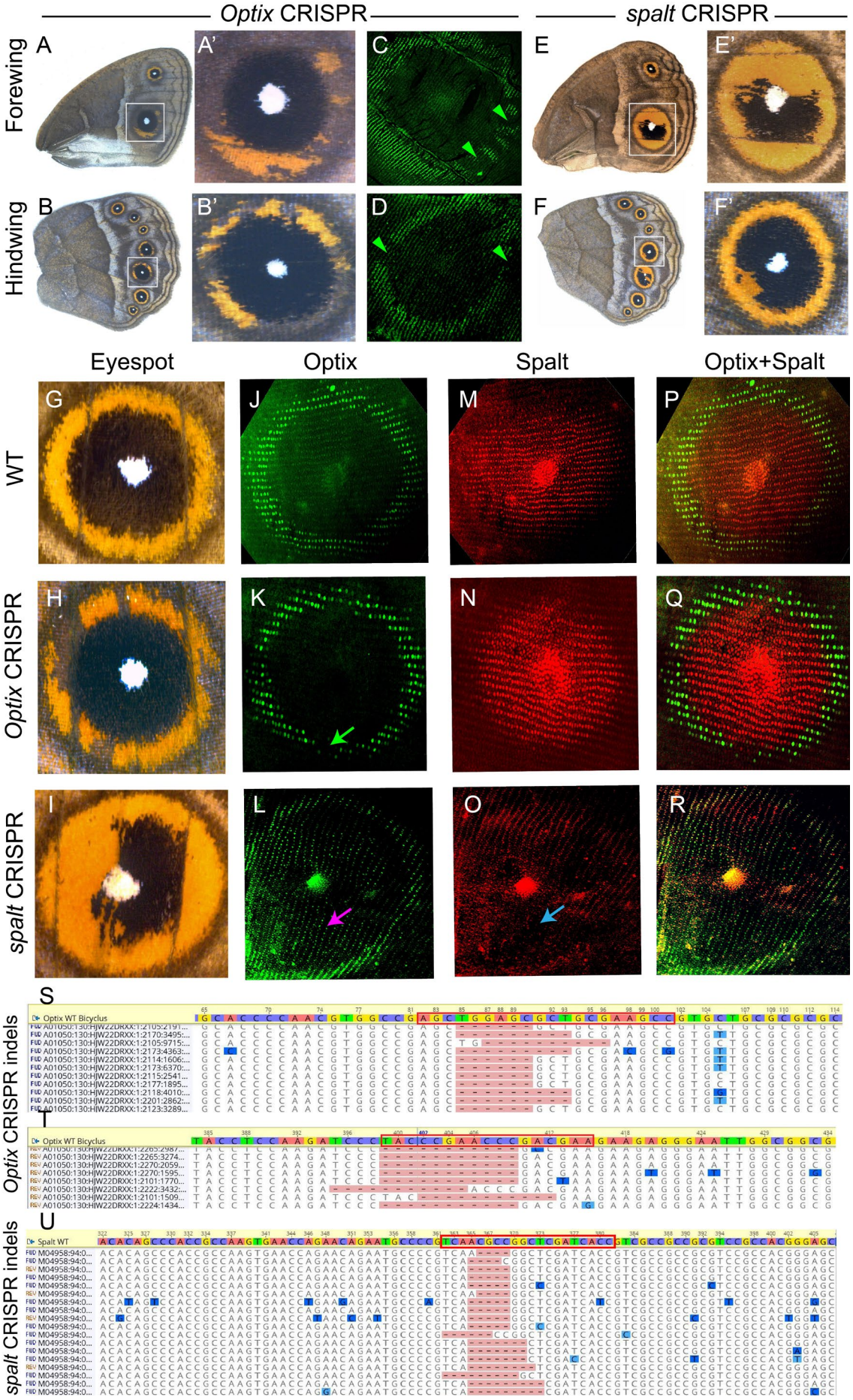
Optix CRISPR
spalt CRISPR
Forewing
Hindwing
A
A'
C
E
E'
B
B'
D
F
F'
Eyespot
Optix
Spalt
Optix+Spalt
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Figure 5. *Optix* and *spalt* are required for the development of orange and black scales, respectively, and Spalt prevents *Optix* from being expressed in the black central region of the eyespot. (**A and B**) CRISPR-Cas9 knockout of *Optix* produced defects in the development of the eyespot's orange ring in both wings (S, T are amplified photos of the eyespots boxed in Q, R). (**C and D**) *Optix* CRISPR results in the loss of Optix protein in cells of the future orange ring area of the eyespots. (**E and F**) CRISPR-Cas9 knockout of *spalt* leads to orange scales developing inside the black scale domain. **(G)** WT eyespot, **(I)** *Optix* crispant, and **(J)** *spalt* crispant. **(J-L)** Eyespots stained with an antibody against Optix in WT, *Optix* crispant, and *spalt* crispant individuals (Note that different individuals are used for adult phenotyping and immunostains). (**L**) *spalt* crispant with Optix protein present in the eyespot central disc. **(M-O)** Eyespots stained with an antibody against Spalt in WT, *Optix* crispant and *spalt* crispant individuals. **(P-R)** Merged channels of Spalt and Optix. **(K)** Green arrow marks the region of missing Optix protein in the *Optix* crispant individual that does not affect Spalt-expressing cells. Pink arrow marks the ectopic presence of Optix, where Spalt proteins are missing (blue arrow). All wings were stained at 16-20 hrs pupal development. CRISPR deletions at the **(S and T)** *Optix* and (**U**) *spalt* target sites in three distinct individuals.

Discussion

Our study showed that several genes previously implicated in venation patterning in *Drosophila* wings are expressed in largely conserved, homologous domains in *B. anynana* butterfly wings, as well as in a novel trait, butterfly eyespots. In both species, at early stages of wing development, *dpp* is expressed along the AP boundary (**Figure 1A, Figure S3J and O**), and the downstream targets *spalt, omb, al,* and *Optix* are expressed in partially overlapping bands, spanning the central Dpp stripe, or some distance away from it (Banerjee and Monteiro, 2020; Blair, 2007; Martín et al., 2017) (**Figure 3C, Figure 4A-C and G-I**). The activation of all these genes is likely carried out by Mad6, a Dpp signal transducer (Matsuda and Affolter, 2017) expressed in a broad domain spanning the AP boundary (**Figure 1C, Figure S3K**). At later stages of development, in the pupal wing, a subset of these genes is also expressed in and around the rings of butterfly eyespots, suggestive of network co-option. In particular, *dpp* is found in the center of the eyespots, and *spalt*, *omb*, and *Optix*, are found in concentric circles of expression at variable distances from the center.

In addition to these suggestive expression patterns, we showed that the genetic circuit involving the repression of *Optix* by Spalt proteins, that sets-up vein L2 in *Drosophila* (Martín et al., 2017), is also conserved in *B. anynana* to set up the sharp boundary between the black and orange color rings in eyespots. In both species, the expression of *Optix* abuts the expression of *spalt* in the anterior compartment of larval wings (**Figure 3A-C**). In *Drosophila* wing discs, down-regulation of *spalt* with RNAi results in the expansion of the Optix domain in the anterior compartment (Martín et al., 2017). However, downregulation of *Optix* with RNAi does not affect the spatial domain of Spalt (Martín et al., 2017). Similarly, in the eyespots, Spalt also represses *Optix's* expression from its own domain (**Figure 5G-R**). We showed that *Optix* is required for orange and *spalt* for black scale differentiation, and disruptions to *Optix* lead to the loss of Optix proteins and orange scales but do not affect the expression domain of Spalt (**Figure 5H, K, N, and O**), whereas disruptions of *spalt* result in the ectopic expression of Optix, and ectopic appearance of orange scales (**Figure 5I, L, O, and R**).

Venation patterning in *Drosophila* also involves other key members from the Dpp and Hh signaling pathways (Blair, 2007) that were DE across anterior and posterior compartments in *B. anynana* larval wings, and further visualized with HCR (**Figure S1, S3, and S4**). Hh, expressed in the posterior compartment in both species (Blair, 2007; Saenko et al., 2011; Tabata et al., 1992) (**Figure S1**), is known to activate the expression of *dpp* at the AP boundary in fly wings by binding to the receptor Patched (Ptc), and transducing the signal via Cubitus interruptus (Ci) (Banerjee and Monteiro, 2020; Bier, 2000; Tanimoto et al., 2000). Correspondingly, both *ptc* and *ci* were expressed strongly in the anterior compartment of *B. anynana* larval wings (**Figure 4N-P**) (Keys et al., 1999; Saenko et al., 2011). Perhaps due to the timing of the stainings, we were unable to localize the transcripts of *ci* and *ptc* in the eyespots during larval and pupal stages with HCR (**Figure 4N, R, O, S; Figure S4I, J, M, and N**). Previous researchers, however, did visualize Ci protein in *B. anynana* eyespot centers (Keys et al. 1999) and *ptc* transcripts in *Junonia coenia* eyespot centers in larval wings. While we were also unable to visualize the Dpp target gene *al* in larval and pupal eyespots (**Figure 4B and E**), *omb* showed faint expression in the center of the eyespot and in the orange ring cells (**Figure 4H and K**). Knocking-out *ci, ptc,* and *omb* using CRISPR-Cas9 didn't result in any eyespot-specific phenotype (**Figure S9**, **Table S9, Table S10**). These results suggest that *al, omb*, and Hh signaling members *ci* and *ptc* may not play a role in eyespot development. Thus, the gene-regulatory network (GRN) patterning the eyespot rings of *B. anynana* likely involves only a subset of the venation GRN with *spalt* and *Optix* being important regulators of the sharp and prominent orange and black rings of color (**Figure 6**).

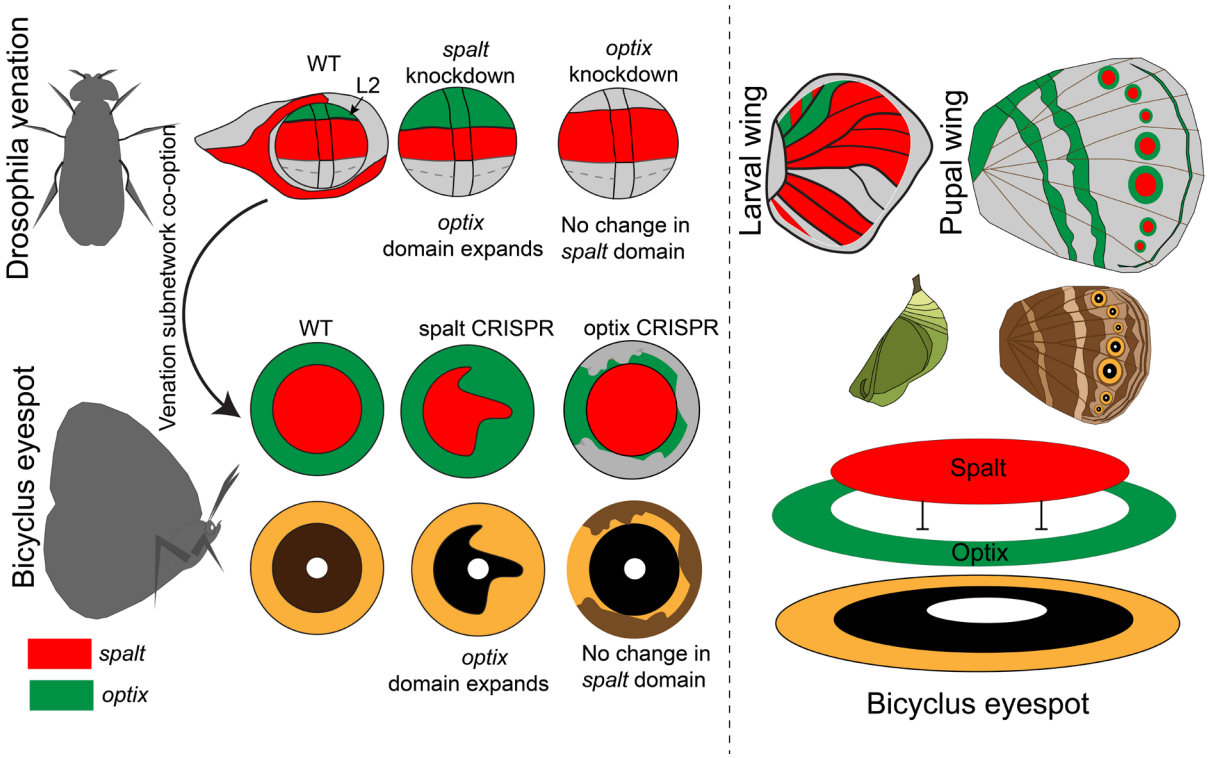

Figure 6. Co-option of a venation subnetwork involving *spalt* and *Optix* in patterning the eyespot rings of *B. anynana*. In *Drosophila*, larval wing venation patterning involves the negative regulation of *Optix* by Spalt in the anterior compartment, at the location of vein L2, where Optix does not cross-regulate the *spalt* expression domain. The same exact regulation is observed in *B. anynana* eyespots where the loss of Spalt proteins results in the ectopic expression of Optix proteins and orange scales, while the loss of Optix only affects the orange

scales without affecting the expression of Spalt proteins or the development of black scales. Spalt thus, represses *Optix* in the black scale region and creates a sharp color boundary in the eyespot rings.

The phenotypes we observed with embryonic *dpp* knockouts suggest that *dpp* has an early role is establishing hindwing identity, in addition to a possible later role in eyespot ring formation. Knockouts of *dpp* during the embryonic stages proved quite challenging due to *dpp's* role in embryonic development (Ashe et al., 2000)). The single crispant individual we obtained had fewer hindwing eyespots but also had other eyespots enlarged, making that hindwing resemble a forewing. The loss of the M1 (or M2) eyespot was likely due to the complete loss of Dpp along one vein (either the M1or M2 vein) (**Figure S5**) (Banerjee and Monteiro, 2020). However, the hindwing acquiring forewing features, such as enlarged M1/M2 and Cu1 eyespots, and the development of silver scales in the posterior compartment (**Figure 2M and N, and Figure S5**), are not consistent with a mere eyespot developmental function for *dpp*. We propose that these features could be due to an interaction between *Ubx* and *dpp,* as a previous *Ubx* crispant in *B. anynana* showed a similar phenotype (Matsuoka and Monteiro, 2021). In *Drosophila, Ubx*, a Hox gene responsible for hindwing identity, controls both the transcription and mobility of Dpp in the haltere (Crickmore and Mann, 2006). We propose that Ubx is playing a similar role in *Bicyclus* hindwings during the larval stage. At this stage, *dpp* expression surrounds the expression of Armadillo (a Wnt transducer) and Distal-less proteins in the eyespot centers (Connahs et al., 2019). The increase in size of M1 and Cu1 eyespots with *dpp* knockouts (**Figure 2M and N**) could be due to the proposed role of Dpp proteins in restricting the expression of these two other genes to the eyespot centers via a reaction-diffusion mechanism (Connahs et al., 2019). If Dpp proteins are removed, the centers might be able to expand, leading to larger eyespots. Also, if Ubx is regulating both *dpp* expression and mobility, *dpp* knockouts might mimic *Ubx* knockouts. Disentangling the early and late roles of *dpp* in eyespot development will only be possible once we are able to knock-out *dpp* in late wing development with somatic CRISPR.

Two roles were also observed for *spalt:* in eyespot center differentiation during the larval stage, and in the differentiation of the black disc during the pupal stage. *spalt* is likely playing a role in a proposed reaction-diffusion mechanisms proposed for focus differentiation during the larval stages (Connahs et al., 2019), because *spalt* knockouts result in loss of eyespots (Murugeshan et al., 2022) or in split eyespot centers (**Figure S8J**). During the pupal stage, however, disruptions of *spalt* lead to transformation of black to orange scales, via the *spalt-Optix* regulatory mechanism identified here.

Several knowledge gaps still remain surrounding eyespot ring differentiation. Support for the vein network GRN co-option proposed in this study would benefit from testing whether *Optix* and *spalt* are responding to Dpp signals produced at the center of the eyespots transduced through the transcription factor Mad6. It is also unclear whether Dpp and Wingless (Wnt1) are jointly required for eyespot ring differentiation. Wnt1 is another proposed morphogen for eyespot development (Monteiro et al., 2006; Özsu et al., 2017) and its function should be newly investigated using knockouts. In the present work we found evidence for the presence of many Wnt signaling members such as *Wnt1, armadillo, pangolin,* and *dishevelled* highly upregulated in the pupal eyespot tissue (**Figure 1E**). In addition, the role of *engrailed* (*en*) in eyespot ring differentiation, a gene co-expressed in the same orange cells as *Optix*, is still unclear (Brunetti et al., 2001) (**Figure S10**). Multiple paralogs are expressed in eyespots (Banerjee et al., 2020), and disruptions of all paralogs simultaneously may need to be performed to overcome functional redundancy.

Methods

Rearing *Bicyclus anynana*

Bicyclus butterflies were raised in the lab at 27°C, 60% humidity and 12-12 hrs day-night cycle. Larvae were fed young corn leaves and adults were fed mashed bananas.

CRISPR-Cas9

Optix, *spalt, ci, knirps, ptc, dpp,* and *omb* CRISPR-Cas9 experiments were carried out based on a protocol previously described (Banerjee and Monteiro, 2018). Guides were designed to target the coding sequence of the genes (see the supplementary file for sequences and the regions targeted). A solution containing Cas9 protein (IDT, Cat No. 1081058) and guide RNA, each at a concentration of 300ng/ul, diluted in Cas9 buffer, and a small amount of food dye was injected into 1509 embryos for *Optix*, 2474 embryos for *spalt,* 2012 embryos for dpp, 283 embryos for *ci,* 306 embryos for *ptc*, 1259 embryos for *knirps,* and 734 embryos for *omb* (**Table S4-S10**). A few of the *spalt* and *optix* injected individuals were dissected at 24 hrs after pupation to perform immunostainings, while the rest were allowed to grow till adulthood. After eclosing, the adults were frozen at -20° C and imaged under a Leica DMS1000 microscope.

Dpp synthetic guide based CRISPR-Cas9 experiment were carried out at embryonic developmental stages. The experiment was conducted in the same way as mentioned above except a synthetic guide from Integrated DNA Technologies (IDT) was used. Briefly a 20 bp region was selected using the IDT custom guide tool (AAAGAGCCCAAACATAATCA). For the preparation of the synthetic guide-Cas9 mixture, the *dpp* guide (crRNA) was mixed with equimolar amount of tracrRNA (IDT), heated at 95°C for 5 mins and cooled to room temperature. Afterward Cas9 enzyme was added and left for 20 mins at room temperature. Non-toxic food dye was added to the mix and injected into the embryos. After eclosion, the adults were frozen and imaged under a Leica DMS1000 microscope.

For identification of the indels, DNA from the mosaic CRISPants wings were isolated using Omega Tissue DNA extraction kit (Cat No: D3396-01). Afterwards PCR was performed to amplify the region of interest. PCR products were purified and sent to the next generation sequencing facility at Genewiz. After sequencing, the reads were aligned using Geneious 10.0 to the reference WT gene sequences.

Immunostainings

Pupation time was recorded using an Olympus tough tg-6 camera, and pupal wings were dissected at different timepoints after pupation under a Zeiss Stemi 305 microscope in 1x PBS at room temperature based on a protocol previously described (Banerjee and Monteiro, 2020b). Wings were fixed using 4% formaldehyde in fix buffer (see **Table S11** for details), followed by four washes in 1x PBS, five mins each. After the washes, the wings were incubated in block buffer (see **Table S11** for details) at 4ºC overnight. The next day primary antibodies against Optix (1:3000, rat, a gift from Robert D. Reed) and Spalt (1:20000, guinea pig GP66.1), were added in wash buffer and incubated at 4ºC for 24 hrs. The next day anti-rat AF488 (Invitrogen, #A-11006) and anti-guinea pig AF555 (Invitrogen, # A-21435) secondary antibodies at the concentration of 1:500 in wash buffer were added followed by four washes in wash buffer, 20 mins each. Wings were then mounted on an inhouse mounting media (see **Table S11** for details) and imaged under an Olympus fv3000 confocal microscope.

Enzyme based *In-situ* hybridization

For the visualization of *dpp* expression in pupal wings of *Bicyclus*, pupation time was recorded, and the wings were dissected from 18-24 hrs old pupae in 1x PBS under a Zeiss stemi 305 microscope based on a previously described protocol (Banerjee and Monteiro, 2020b) and transferred to 1xPBST with 4% formaldehyde. After fixation for 30 min the wings were washed three times in 1x PBST for 5 mins each. The wings were then treated with proteinase K and glycine and washed again three times using 1x PBST. After, the wings were gradually transferred into pre-hybridization buffer (see **Table S12** for composition) and heated at 65ºC for 1 hr. Hybridization buffer with a *dpp* probe (see **Table S12** for composition) was added to the wings and they were incubated for 16 hrs at 65ºC. Wings were then washed five times for 30 mins each in pre-hybridization buffer. After washing, wings were moved to room temperature and gradually transferred to 1x PBST and washed in 1x PBST. Afterwards, the wings were incubated in block buffer (see **Table S12** for composition) for 1 hr, followed by addition of anti-digoxygenin (Sigma-Aldrich, Cat No. 11093274910) diluted 1/3000 times in block buffer. After 1 hr of incubation the wings were washed five time, five mins each in block buffer. Finally, wings were transferred to alkaline-phosphatase buffer (see supp table S4 for composition) supplemented with NBT-BCIP (Promega, Cat No. S3771) and incubated in the dark till the development of color. The wings were imaged under a Leica DMS1000 microscope.

Hybridization chain reaction (HCR3.0)

HCR3.0 was carried out based on the protocol described in Choi et al., 2018 with the modifications for butterfly wing tissue described below. Larval and pupal wings were dissected in 1x PBS followed by fixation in 4% formaldehyde in 1x PBST. Afterwards wings were washed in 1x PBST permeabilized in a detergent solution (Bruce et al., 2021) and washed with 5x SSCT. Wings were incubated for 1 hrs in 30% probe hybridization buffer followed by overnight incubation in 30% probe hybridization buffer supplemented with HCR probes (see supp section for sequences) at 37°C. After incubation wings were washed five times with 30% probe wash buffer at 37°C. Afterwards wings were brought back to room temperature and washed twice with 5x SSCT followed by incubation in amplification buffer and overnight incubation in amplification buffer supplemented with fluorescent hairpin probes (Molecular instruments) in dark for 16-20 hrs. The next day wings were washed four times with 5x SSCT mounted in an inhouse mounting media and imaged under an Olympus fv3000 microscope.

Laser micro-dissection

Laser microdissections of the anterior and posterior compartments of larval wings and eyespot and adjacent control tissue from pupal wings were carried out based on the protocol described in (Banerjee et al., 2022). Briefly, wings were dissected in 1x PBS and mounted in PEN membrane slides (Cat No.: LCM0522). Wings were washed with cold 50%, 75% and 100% ethanol followed by staining using Histogene staining solution (Cat. No.: KIT0415) for 20-30 seconds. Wings were washed twice with cold 100% ethanol and cold acetone. The slides were either kept at -80°C or immediately dissected using Zeiss PALM microbeam-Laser microdissection microscope using the parameters described in (Banerjee et al., 2022). 12 hindwings in the larval stage and 4 hindwings in the pupal stage were used for each biological replicate, respectively. A total of three biological replicates were used for downstream analysis.

RNA-isolation from the laser dissected samples.

Microsections of the larval and pupal wings were picked up from the membrane slide using a pair of fine forceps (Dumont, Cat. No. 11254-20) and transferred to 200 µl tubes with 50 µl distilled water. Afterwards, 100 µl of LCM lysis buffer (Banerjee et al., 2022) was added and the tubes were incubated at 55°C in a water bath for 6 mins with vortexing in between. The

samples were then homogenized with 0.1mm zirconium beads (BioSpec, Cat. No. 11079101z) in a homogenizer (Next Advance Bullet Blender) for 3 mins followed by RNA isolation using a Qiagen RNA isolation kit (Cat. No. 74136). RNA samples were lyophilized in Genewiz RNA stabilization tubes and sent for sequencing (Next Generation Sequencing, Azenta US, Inc).

RNA-seq analysis

Raw reads were analyzed using fastqc and cleaned using bbduk (Bushnell, 2014)with the parameters ktrim= r, k = 23, mink = 11, hdist = 1, tpe, tbo. Cleaned reads were aligned using *B. anynana* ncbi reference genome assembly (txid: 110368) using Hisat2 (Kim et al., 2019) and mapped to reference *B. anynana* gtf file obtained from ncbi (txid: 110368) using stringtie (Pertea et al., 2015). A gene count matrix table was created from the stringtie files using pydr.py (Pertea et al., 2015) package using python2.7 followed by analysis using DESeq2 in R (Love et al., 2014). Volcano plots were created using EnhancedVolcano package in R.

Data availability

The sequencing data generated in the paper has been submitted to NCBI under the bio-project PRJNA948390.

Authors contribution

TDB and AM designed the experiments, analysed the results, and wrote the manuscript. TDB performed the experiments. Both the authors read and agreed to the final version of the manuscript.

Funding

TDB was supported by a Yale-NUS scholarship. This work was supported by the National Research Foundation, Singapore, Investigatorship award NRF-NRFI05-2019-0006 to AM.

Acknowledgements

We would like to thank Robert Reed for the anti-Optix antibody. Suriya Narayanan Murugesan and Shen Tian for helpful discussion on the manuscript. We would like to thank DBS-CBIS confocal facility and Ms. Tong Yan for access to the Olympus fv3000 confocal microscope. The authors declare no conflict of interest.

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Supplementary materials

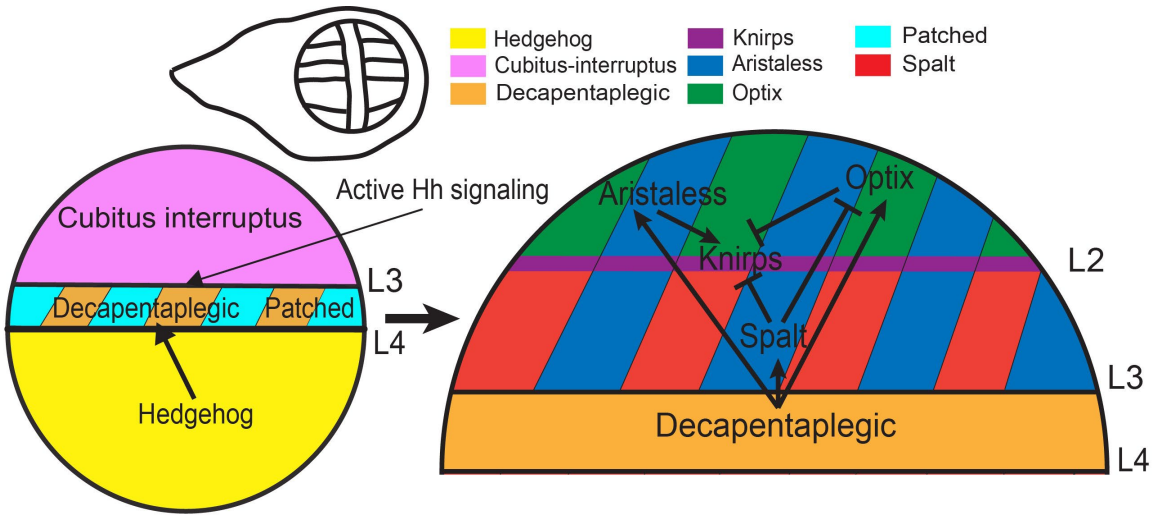

Figure S1: Venation patterning in the anterior compartment of *Drosophila melanogaster* (redrawn from Banerjee and Monteiro, 2020). Hedgehog (Hh), a short-range morphogen expressed in the posterior compartment of the larval wing disc in the presence of its receptor Patched and Cubitus interruptus activates Dpp signaling in a thin stripe of tissue in the anterior compartment. Dpp is capable of diffusing to the most anterior part of the larval wing disc where it activates key genes such as *aristaless* and *Optix* at low, and *spalt* at high concentration thresholds. Spalt protein represses the expression of *Optix* in the posterior part of the anterior compartment. Aristaless is responsible for the activation of another transcription factor *knirps* while both Spalt and Optix repress the activation of *knirps*. As a result, *knirps* is only activated in a thin stripe which forms the L2 vein of the *Drosophila* wing.

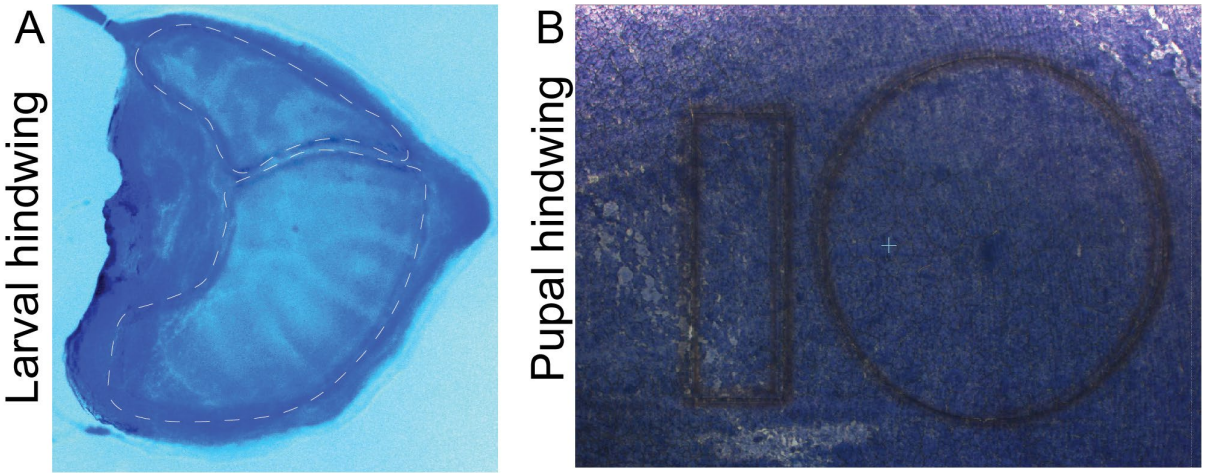

Figure S2: Areas dissected using laser microdissection in (A) Larval wing and (B) pupal wing of *B. anynana*.

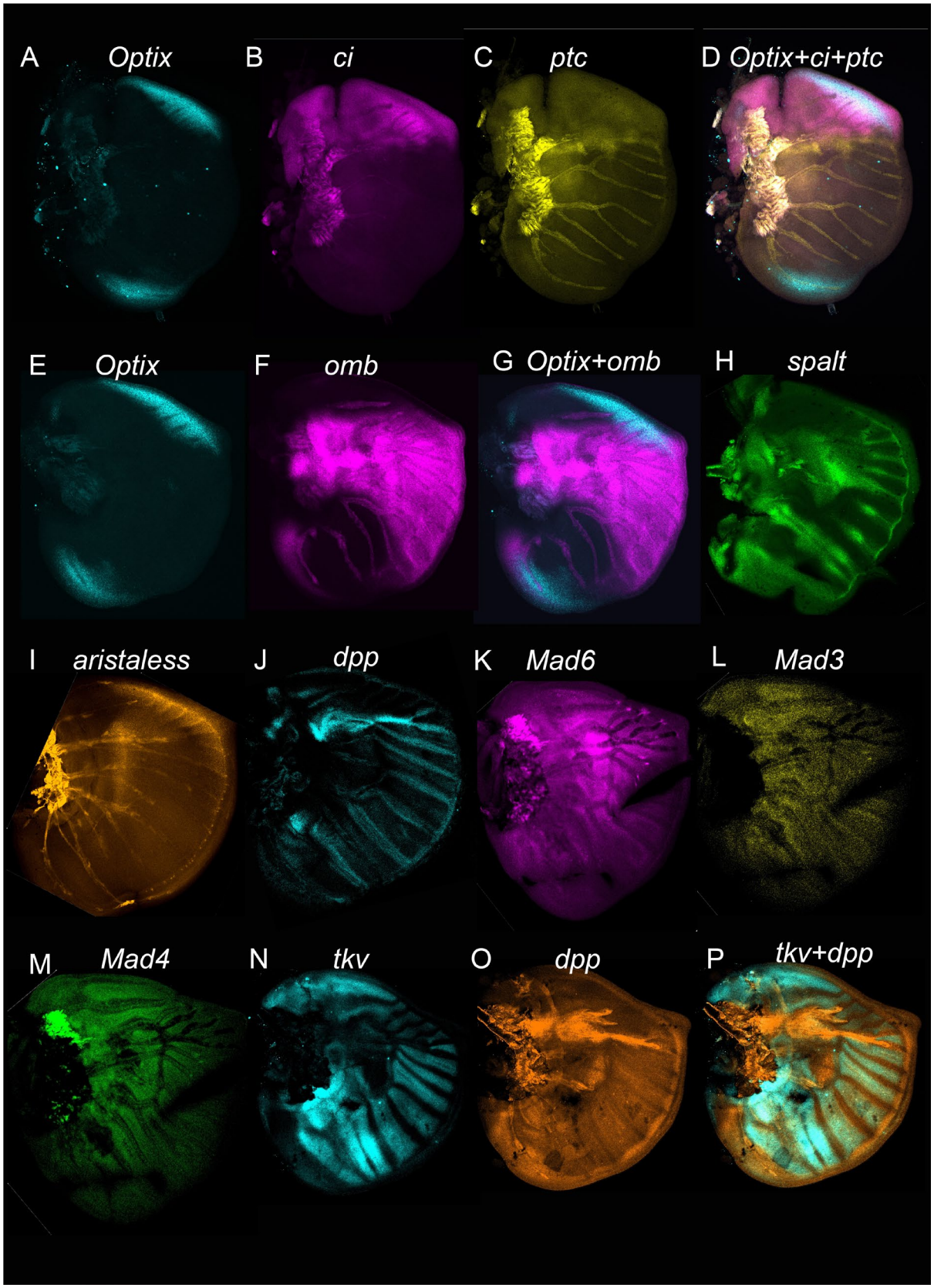

Figure S3: Expression of *Optix, ci, ptc, omb, spalt, aristaless, dpp, tkv, Mad6, Mad3,* and *Mad4* in the larval forewing of *B. anynana*. Expression of (**A**) *Optix* in the upper anterior and the lower posterior compartment, (**B**) *ci* in the anterior compartment, (**C**) *ptc* in the AP stripe and the anterior compartment, (**D**) Co-expression of *Optix*, *ci,* and *ptc*. Expression of (**E**) *Optix*

in upper anterior and lower posterior domain and (**F**) *omb* in a broad domain spanning the AP boundary and the lower posterior compartment. (**G**) Co-expression of *Optix* and *omb*. Expression of (**H**) *spalt* in four main domains, (**I**) *al* in the anterior compartment, (**J**) *dpp* in the AP boundary stripe and in the lower posterior compartment, (**K**) *thickveins* in the upper anterior and in a domain spanning two sectors in the posterior compartment, (**L**) *Mad6* in the domain spanning the AP boundary. No expression of (**M**) *Mad3* and (**N**) *Mad4* was observed.

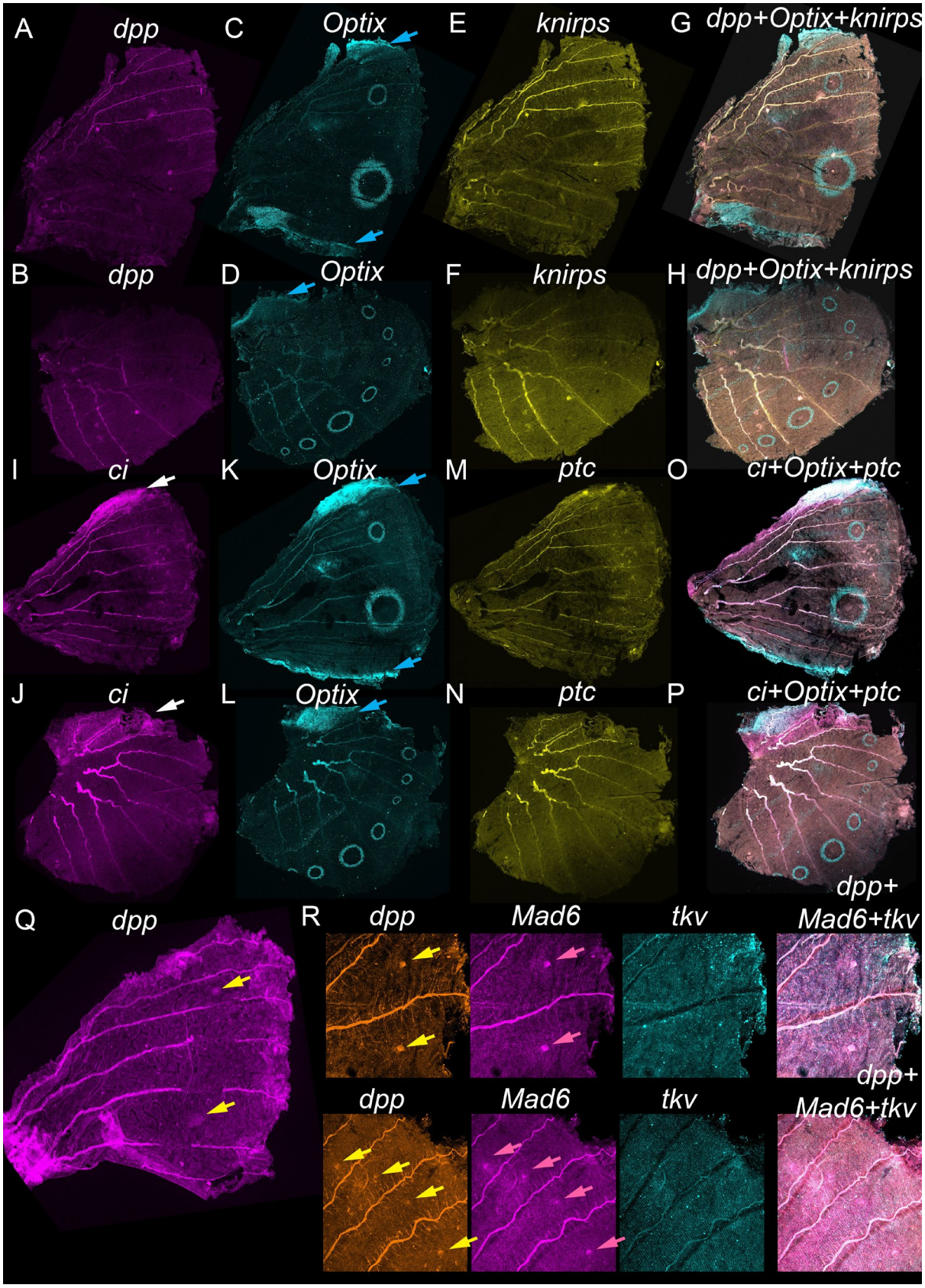

Figure S4. Expression of *dpp, knirps, Optix, ci, ptc*, *Mad6*, and *tkv* in 16-24 hrs pupal forewings and hindwings of *B. anynana*. Expression of *dpp* in **(A)** forewings and (**B**) hindwings showing no eyespot specific expression. Expression of *Optix* in (**C and K**) forewings showing expression in the upper anterior and posterior compartment and in the

eyespot orange ring, and in (**D and L**) hindwings, showing upper anterior compartment specific expression, along three bands crossing the proximal-distal axis of the wing, and in the eyespot orange ring. No specific expression domain of *knirps* was observed in the (**E**) forewing, and (**F**) hindwing. Co-expression of *dpp, Optix*, and *knirps* in the (**G**) forewing and (**H**) hindwing. Expression of *ci* in the (**I**) forewing and (**J**) hindwing showing expression in the upper anterior compartment. No specific expression domain was observed for *ptc* (**M**) in forewings and (**N**) hindwings. Co-expression of *ci, Optix*, and *ptc* in the (**O**) forewing and (**P**) hindwing. (**Q**) Expression of *dpp* in a pupal forewing showing eyespot specific expression (yellow arrow). (**R**) Expression of *dpp, Mad6*, and *tkv* in pupal hindwing. *dpp* and *Mad6* shows expression in the eyespot center. While *tkv* doesn't show any specific expression domain.

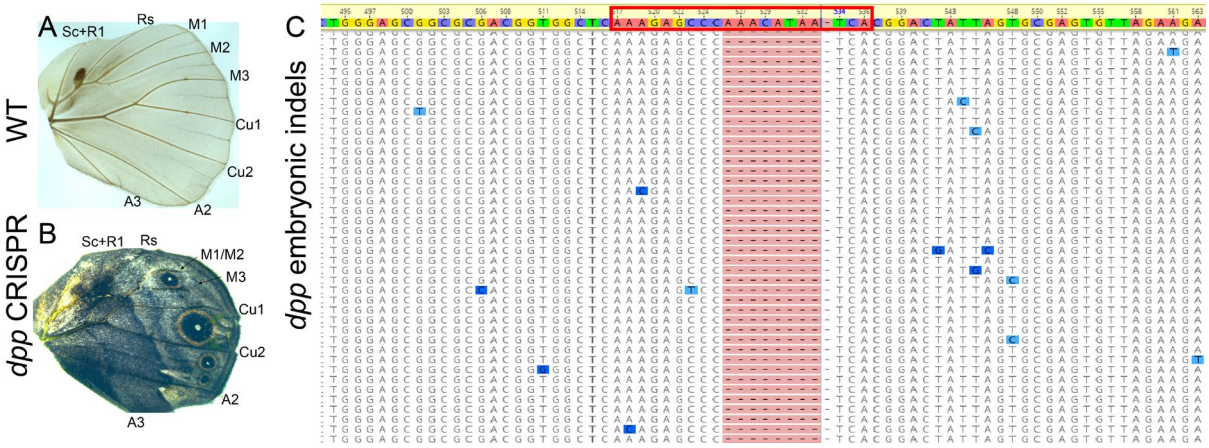

Figure S5: Effect of *dpp* CRISPR on venation and eyespot development. (**A**) WT adult hindwing venation. (**B**) Effect of disruptions to *dpp* on hindwing venation. We observed loss of M1 or M2 veins and incomplete formation of the M1/M2 and M3 veins. Loss of an eyespot, enlargement of eyespots, and ectopic silver scale development was observed in the hindwing of the *dpp* crispant. (**C**) Indels at the site of *dpp* CRISPR from the wing in panel **B**.

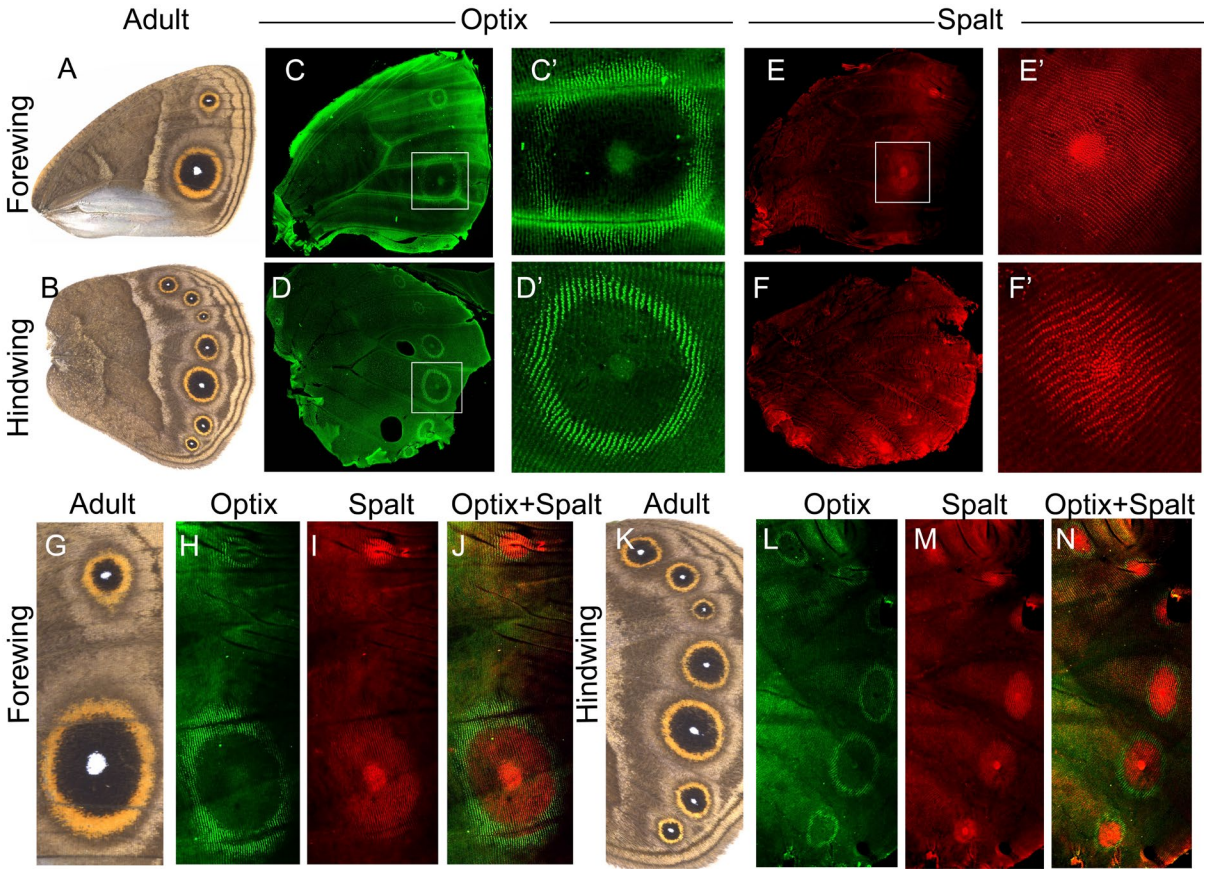

Figure S6. *B. anynana* adult wings and the localization of Optix and Spalt proteins in 16-44 hrs old pupal wings. (A) WT forewing, **(B)** WT hindwing. **(C)** The presence of Optix proteins in the forewing and **(D)** in the hindwing. **(E and F)** Presence of Optix in the orange ring of Cu1 eyespots (boxed in C and D). (**G**) Presence of Spalt protein in the forewing and **(H)** in the hindwing. **(I and J)** Presence of Spalt protein in the black disc of Cu1 eyespots. (**G**) Adult forewing. (**H**) Optix localization in the forewing. (**I**) Spalt localization in the forewing. (**J**) Merged channels of Optix and Spalt. (**K**) Adult hindwing. (**L**) Optix localization in the hindwing. (**M**) Spalt localization in the hindwing. (**N**) Merged channels of Optix and Spalt.

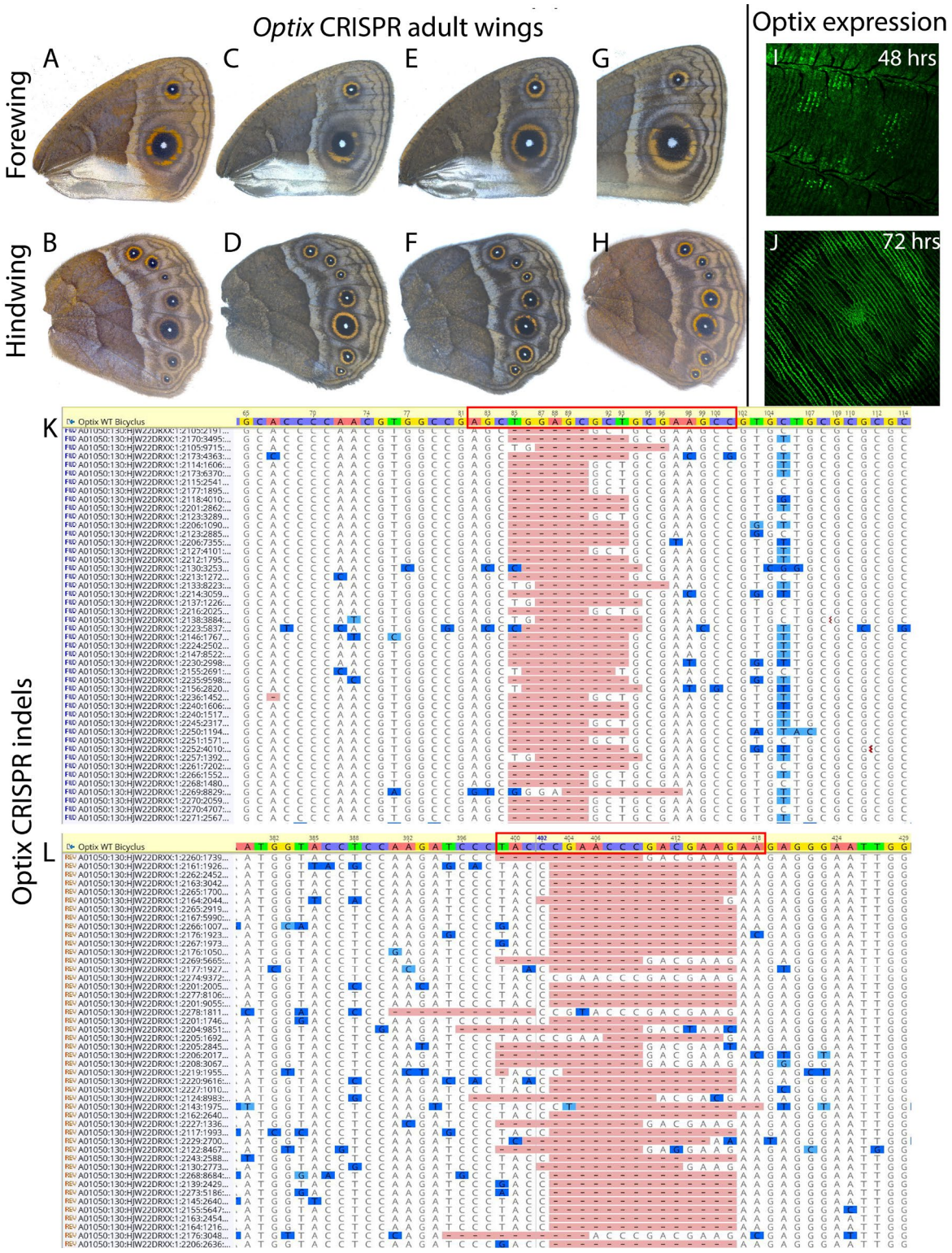

Figure S7. Function of *Optix* in *Bicyclus anynana* butterflies and localization of Optix protein in pupal wings. (A-H) *Optix* CRISPR adult wings. *Optix* CRISPR results in the conversion of orange scales into brown scales in the eyespots. **(I and J)** Antibody staining of Optix proteins in an *Optix* CRISPR individual at 48 hrs and a WT 72 hrs old pupal wing. **(K and L)** Deletions at the two sites targeted for *Optix* CRISPR.

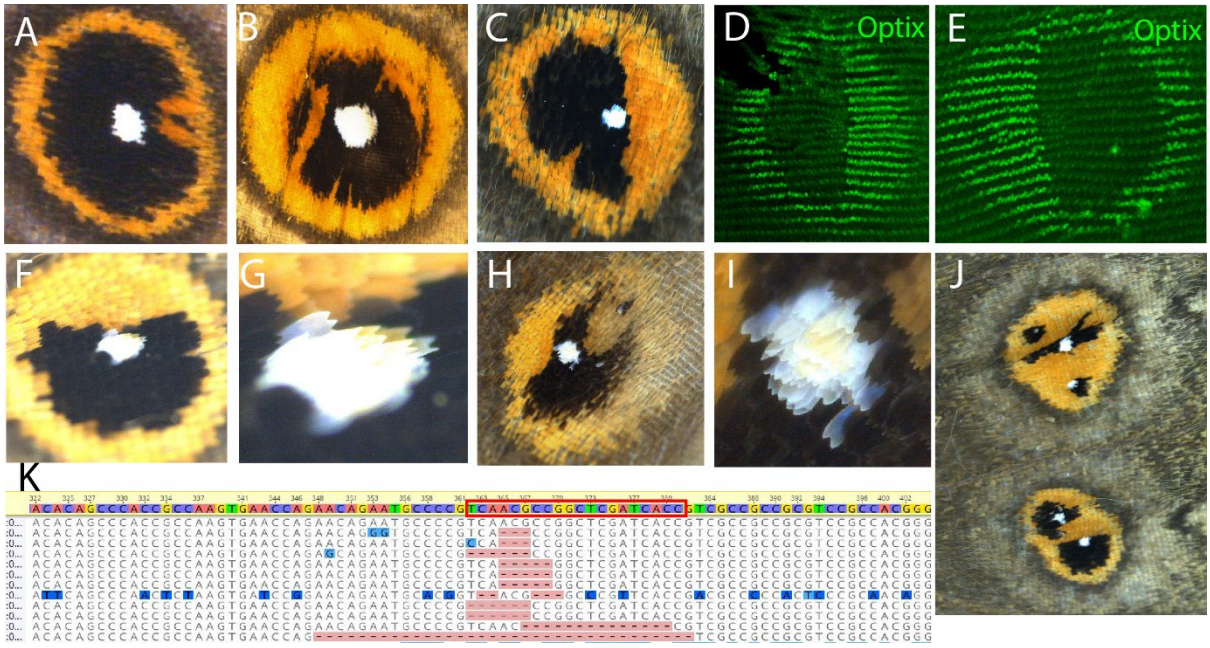

Figure S8: Effect of *spalt* CRISPR on the eyespots. (**A-C**) Loss of Spalt results in the development of orange scales in the black scale region due to the presence of (**D and E**) Optix in that region. (**F-I**) Loss of *spalt* results in the development of yellow scales in the white center scale region of the eyespot. (**J**) Loss of *spalt* results in the split of eyespot foci with distinct domains of the white, black, and orange scales. (**K**) Deletions at the site of *spalt* CRISPR (red box).

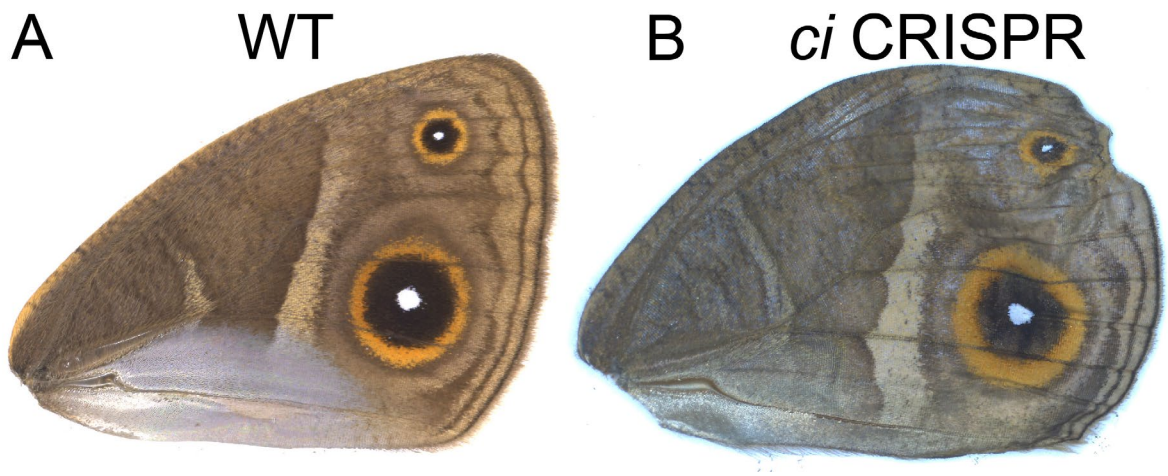

Figure S9. Effect of *ci* CRISPR in the adult forewing. (A) Adult WT forewing. (B) *ci* CRISPR forewing showing defect in the anterior compartment. No defect was observed in the eyespots.

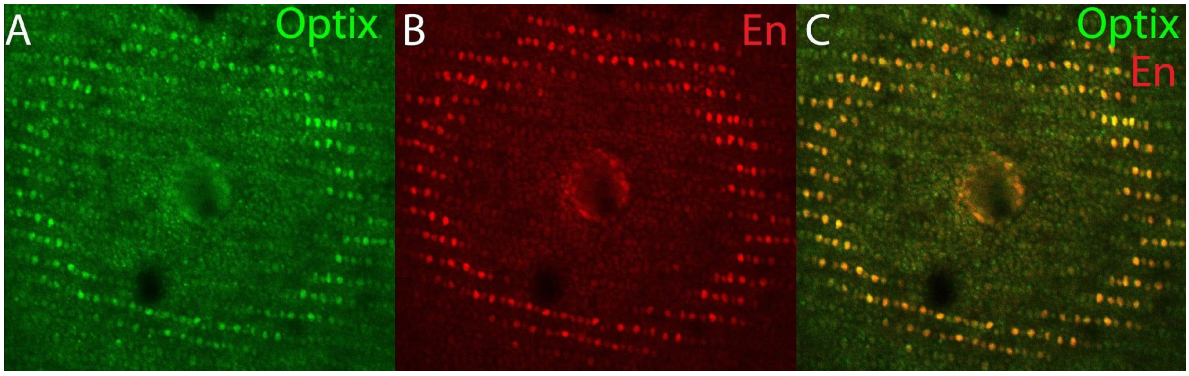

Figure S10. Co-localization of Optix and Engrailed (En) in the orange ring of the eyespots. Both (**A**) Optix and (**B**) En proteins are present in the cells that will form the orange scales of the eyespot. (**C**) Co-expression of Optix and En.

Table S1: Top differentially expressed genes in the anterior and posterior compartment of larval *B. anynana* wings.

Gene_ID	Description	log2FoldChange	pvalue	padj
LOC112054675	homeobox protein aristaless-like	3.073633	7.99E-24	8.79E-20
LOC112056678	homeobox protein aristaless	4.381925	2.21E-20	1.22E-16
LOC112043786	Down syndrome cell adhesion molecule-like protein Dscam2	9.676908	2.08E-12	7.62E-09
LOC112045125	hexamerin-like	-5.15048	4.66E-11	1.28E-07
LOC112057967	growth arrest-specific protein 1-like	2.251076	3.16E-10	6.96E-07
LOC112045741	homeobox protein caupolican-like	-2.84906	3.91E-09	7.17E-06
LOC112042874	tiggy-winkle hedgehog protein	-1.82419	4.87E-09	7.65E-06
LOC112047854	muscle segmentation homeobox-like	1.406152	1.58E-08	2.17E-05
LOC112046560	transcriptional activator cubitus interruptus isoform X1	2.869324	2.62E-08	3.20E-05
LOC112053006	homeobox protein invected-like isoform X2	-1.76609	4.74E-08	5.21E-05
LOC112048837	fork head domain transcription factor slp2-like	6.798241	2.60E-07	0.00026
LOC112057951	toll-like receptor 6	-1.84605	1.04E-06	0.000903
LOC112042983	lachesin-like	3.311257	1.07E-06	0.000903
LOC112053007	segmentation polarity homeobox protein engrailed-like	-1.60007	6.43E-06	0.005049
LOC112048760	neuroligin-2-like	2.85999	7.27E-06	0.005329
LOC112058231	uncharacterized protein LOC112058231	-8.11071	8.41E-06	0.005777
LOC112044740	dachshund homolog 2 isoform X2	2.011952	4.03E-05	0.026096

LOC128199477	#N/A	2.75725	5.41E-05	0.031285
LOC112053108	uncharacterized protein LOC112053108	1.331493	5.12E-05	0.031285
LOC112058471	carbonic anhydrase-related protein 10 isoform X1	2.456293	6.50E-05	0.035719
LOC112048640	T-box transcription factor TBX3 isoform X7	0.909847	7.43E-05	0.038906
LOC112054669	heat shock protein 67B1-like	1.189768	8.54E-05	0.041189
LOC112055169	ras-related protein Rab-32 isoform X5	-1.37732	8.61E-05	0.041189
LOC128198260	#N/A	0.903109	0.000101	0.046358
LOC112047794	rhomboid-related protein 3 isoform X1	-1.12963	0.000121	0.049466
LOC112047931	zinc finger protein ush isoform X1	-2.11728	0.00012	0.049466
LOC112045732	homeobox protein araucan-like isoform X1	-1.7553	0.000118	0.049466
LOC112051985	juvenile hormone epoxide hydrolase-like	7.410346	0.000143	0.055979
LOC112058319	muscle-specific protein 20-like	-1.19289	0.000153	0.058117
LOC112043273	uncharacterized protein LOC112043273	-7.15354	0.000255	0.089987
LOC112045364	uncharacterized protein LOC112045364	-0.99816	0.000262	0.089987
LOC112048596	putative inorganic phosphate cotransporter isoform X1	-1.10908	0.000261	0.089987
LOC112044525	homeobox protein SIX6-like	7.236613	0.000433	0.144285
LOC128199516	#N/A	-8.74784	0.000549	0.177527
LOC112058262	muscle, skeletal receptor tyrosine-protein kinase-like isoform X1	-1.24171	0.000646	0.203115
LOC112047337	toll-like receptor 7	0.78013	0.000702	0.214365
LOC112058222	lebercilin-like protein	7.045822	0.000741	0.220344
LOC112043001	uncharacterized protein LOC112043001	1.07768	0.000776	0.224482
LOC112048768	POU domain, class 6, transcription factor 2 isoform X1	-1.24136	0.000854	0.236967
LOC112054415	uncharacterized protein LOC112054415	0.83795	0.000862	0.236967
LOC112048478	protein mab-21	1.079178	0.000969	0.259859
LOC112050186	zinc finger protein 177-like	0.7199	0.001095	0.286586
LOC112051041	endocuticle structural glycoprotein ABD-5-like	2.864416	0.001126	0.287911
LOC112052161	protein patched isoform X1	0.902902	0.001162	0.290375

LOC112050109	probable chitinase 10	-1.04185	0.00126	0.308028
LOC112053786	uncharacterized protein LOC112053786	1.102876	0.001338	0.319755
LOC112055429	alaserpin-like isoform X1	0.69989	0.001534	0.358876
LOC112048344	cholinesterase 2-like	0.728368	0.00161	0.368844
LOC112043999	large neutral amino acids transporter small subunit 1	-0.5963	0.002085	0.467831

Table S2: Top differentially expressed genes in the eyespot and control tissue of *B. anynana* pupal wings.

Gene_ID	Description	log2FoldChange	pvalue	padj
LOC112055331	protein obstructor-E-like	-4.938008557	5.16E-43	5.45E-39
LOC128199092	#N/A	-3.961160116	1.04E-39	5.48E-36
LOC112046720	serine/arginine repetitive matrix protein 1 isoform X1	-2.265975363	1.09E-32	3.83E-29
LOC128199053	#N/A	-4.121680054	1.38E-30	3.64E-27
LOC128199054	#N/A	-4.144273478	6.78E-29	1.43E-25
LOC112053683	G-patch domain and KOW motifs-containing protein, partial	-3.038345973	1.78E-28	3.14E-25
LOC128199095	#N/A	-4.765663963	1.63E-27	2.46E-24
LOC112050679	ras GTPase-activating protein-binding protein 2	2.030696914	7.08E-27	9.10E-24
LOC112053205	protein Peter pan	-2.555856714	7.76E-27	9.10E-24
LOC112043100	uncharacterized protein LOC112043100	-3.306811444	1.37E-26	1.45E-23
LOC112051683	uncharacterized protein LOC112051683	-2.781028756	2.57E-26	2.46E-23
LOC112054299	chromatin complexes subunit BAP18 isoform X2	-5.162649496	6.39E-26	5.62E-23
LOC128199083	#N/A	-4.188832582	1.17E-25	9.48E-23
LOC128199074	#N/A	-5.439863652	3.29E-25	2.48E-22
LOC112043561	ESF1 homolog	-2.309304506	3.19E-24	2.24E-21
LOC128199091	#N/A	-4.649886991	5.92E-24	3.91E-21
LOC112047762	MKI67 FHA domain-interacting nucleolar phosphoprotein-like	-2.158151544	1.45E-23	8.98E-21
LOC112048198	FACT complex subunit Ssrp1	-2.512173077	8.91E-23	5.22E-20
LOC128199080	#N/A	-4.523848077	9.43E-23	5.24E-20
LOC128199096	#N/A	-4.42987424	2.48E-22	1.31E-19
LOC112053191	elongin-B isoform X2	1.755529117	4.52E-22	2.27E-19
LOC112048594	armadillo segment polarity protein isoform X2	1.916341092	1.11E-21	5.33E-19
LOC112056205	U3 small nucleolar ribonucleoprotein protein MPP10	-2.110440974	3.38E-21	1.55E-18
LOC112045285	ethanolaminephosphotransferase 1-like	2.150614042	6.41E-21	2.82E-18
LOC112054861	RNA-binding protein 28	-2.286205593	7.02E-21	2.97E-18
LOC128199067	#N/A	-3.583703971	1.44E-20	5.83E-18
LOC128199079	#N/A	-4.411317293	1.91E-20	7.46E-18

LOC112046874	zinc finger protein on ecdysone puffs-like isoform X1	-1.971125298	2.98E-20	1.12E-17
LOC112050585	protein PELPK1-like	-4.155464865	1.45E-19	5.28E-17
LOC112049406	pre-mRNA-splicing factor Slu7	-2.192332327	1.52E-19	5.35E-17
LOC112056909	probable ATP-dependent RNA helicase Dbp45A isoform X2	-2.252771749	1.92E-19	6.55E-17
LOC112045966	pupal cuticle protein PCP52-like	-2.72140226	2.08E-19	6.87E-17
LOC128199087	#N/A	-4.381284748	3.21E-19	1.03E-16
LOC128199101	#N/A	-4.898938121	4.58E-19	1.42E-16
LOC112044446	polycomb protein Sfmbt-like	2.247138379	6.07E-19	1.83E-16
LOC128199088	#N/A	-4.071406839	8.35E-19	2.45E-16
LOC112046711	nucleolar protein 8	-1.850564311	9.17E-19	2.62E-16
LOC112055320	tyrosine-protein phosphatase non-receptor type 9	2.834548157	2.27E-18	6.31E-16
LOC112044989	pupal cuticle protein G1A-like	-5.652936924	2.36E-18	6.38E-16
LOC112044964	uncharacterized protein LOC112044964	-4.887939958	2.42E-18	6.39E-16
LOC112058154	calcium homeostasis endoplasmic reticulum protein isoform X1	-1.958560549	2.84E-18	7.30E-16
LOC112052258	protein SDA1 homolog	-2.10131782	3.14E-18	7.88E-16
LOC112045120	uncharacterized protein LOC112045120	1.889710604	7.80E-18	1.91E-15
LOC112051814	protein TIS11	1.745206078	9.87E-18	2.37E-15
LOC112048699	DNA-(apurinic or apyrimidinic site) lyase-like	-1.905162827	1.20E-17	2.81E-15
LOC112048503	ubiquitin-conjugating enzyme E2-24 kDa isoform X1	2.592535368	1.69E-17	3.88E-15
LOC112046604	surfeit locus protein 6 homolog	-1.606657809	3.10E-17	6.97E-15
LOC128199052	#N/A	-4.901297572	3.52E-17	7.74E-15
LOC112046338	failed axon connections	1.536392399	4.11E-17	8.84E-15
LOC112047699	uncharacterized protein LOC112047699	-1.849746015	4.41E-17	9.13E-15
LOC112049355	pre-mRNA-splicing factor CWC22 homolog	-2.576363811	4.40E-17	9.13E-15
LOC112047981	uncharacterized protein C14orf119 homolog	1.793680976	5.61E-17	1.14E-14
LOC112052912	pre-rRNA processing protein FTSJ3	-2.073395186	6.26E-17	1.25E-14
LOC112055040	peptidyl-prolyl cis-trans isomerase D	1.708616076	8.66E-17	1.69E-14
LOC112057782	homeotic protein ultrabithorax	1.919667063	1.10E-16	2.12E-14
LOC112046508	uncharacterized protein LOC112046508	1.48893366	1.21E-16	2.27E-14
LOC112050572	sister chromatid cohesion protein PDS5 homolog B-like	2.07540653	1.66E-16	3.07E-14
LOC112045283	partitioning defective 6 homolog beta	1.992281153	1.71E-16	3.11E-14
LOC112053604	arginine/serine-rich protein PNISR	-1.94743651	2.81E-16	5.03E-14

Table S3: Primer table

Sl. No.	Name	Sequence
1.	dpp_insitu_F	GTTCTTCAACGTAAGCGGCG
2.	dpp_insitu_R	CCACAGCCTACCACCATCAT
3.	dpp_sequencing_F	GCCTGTTCTTCAACGTAAGC

4.	dpp_sequencing_R	CTCCGTGTACAGCATGAGC
5.	dpp_sequencing300_F	ACCGGCAGACAGAGACTG
6.	dpp_sequencing300_R	CCACTCCTCCTCCTCGTC
5.	Optix_sequencing_F	AGACGCTGGAGGAGAGC
6.	Optix_Sequencing_R	CGCTCGGTCTCTTTGC
7.	spalt_sequencing_F	GCATCGACAAGATGCTGAAA
8.	spalt_Sequencing_R	TTCATTTAGGGACGGTGGAG
9.	dpp_CRISPR_1	GAAATTAATACGACTCACTATAGGAAAGAGCCCAAACATAATCA CGTTTTAGAGCTAGAAATAGC
10.	dpp_CRISPR_2	GAAATTAATACGACTCACTATAGGTAAAAACTATCAGGACATGAGTTTTAGAGCTAGAAATAGC
11.	dpp_CRISPR_3	GAAATTAATACGACTCACTATAGGCGGACGTGGGCTGGAGCGACGTTTTAGAGCTAGAAATAGC
12.	dpp_CRISPR_4	GAAATTAATACGACTCACTATAGGGGCTCGTTGGAACGAGAGGTGTTTTAGAGCTAGAAATAGC
13.	dpp_CRISPR_5	GAAATTAATACGACTCACTATAGGTAATAGTCCGTGATTATGTTGTTTTAGAGCTAGAAATAGC
14.	dpp_CRISPR_6	GAAATTAATACGACTCACTATAGGGATATAGATGAGAGTTGCGTGTTTTAGAGCTAGAAATAGC
15.	dpp_CRISPR_7	GAAATTAATACGACTCACTATAGGCAGCAGCTGCTTCTCGGCGGGTTTTAGAGCTAGAAATAGC
16.	Optix_CRISPR_1	GAAATTAATACGACTCACTATAGGGGCTTCGCAGCGCTCCAGCTGTTTTAGAGCTAGAAATAGC
17.	Optix_CRISPR_2	GAAATTAATACGACTCACTATAGGTTCTTCGTCGGGTTCGGGTAGTTTTAGAGCTAGAAATAGC
18.	spalt_CRISPR_1	GAAATTAATACGACTCACTATAGGTGATCGAGCCGGCGTTGAGTTTTAGAGCTAGAAATAGC
19.	omb_CRISPR_1	GAAATTAATACGACTCACTATAGGTTAATGATATAGCCCGAGAGGTTTTAGAGCTAGAAATAGC
20.	omb_CRISPR_2	GAAATTAATACGACTCACTATAGGGGCTCGGACTCGTCGTGCTCGTTTTAGAGCTAGAAATAG
21.	ci_CRISPR_1	GAAATTAATACGACTCACTATAGGCCCCACCGGATAACCTTAGAGTTTTAGAGCTAGAAATAGC
22.	ci_CRISPR_2	GAAATTAATACGACTCACTATAGGAACAACAAACCTGTGTATGCGTTTTAGAGCTAGAAATAGC
23.	ci_CRISPR_3	GAAATTAATACGACTCACTATAGGATAACTTGTTCCGAAAGAGGGTTTTAGAGCTAGAAATAGC
24.	knirps_CIRSPR_1	GAAATTAATACGACTCACTATAGGAGGCGTATCGGGTTTTGGACGTTTTAGAGCTAGAAATAGC
25.	knirps_CIRSPR_2	GAAATTAATACGACTCACTATAGGAGCTTTTTCGTCTAGTTTAGGTTTTAGAGCTAGAAATAGC
26.	patched_CRISPR_1	GAAATTAATACGACTCACTATAGGGAGCTGTCCCACGGATGTTAGTTTTAGAGCTAGAAATAGC
27.	patched_CRISPR_2	GAAATTAATACGACTCACTATAGGATAGTGCCGTTCCTAGCTCTGTTTTAGAGCTAGAAATAGC

28.	CRISPR_reverse	AAAAGCACCGACTCGGTGCCACTTTTT CAAGTTGATAACGGACTAGCCTTATTT TAACTTGCTATTTCTAGCTCTAAAAC

Table S4. Optix CRISPR-Cas9 injection table

Sl. No.	Concentration	Date	Eggs Injected	Hatchlings	% Hatchlings
1.	300 ng/µl	6th June 2020	326	65	19.9
2.	300 ng/µl	9th July 2020	923	126	13.6
3.	300 ng/µl	27th Aug 2020	389	88	22.6

Table S5. spalt CRISPR-Cas9 injection table

Sl. No.	Concentration	Date	Eggs Injected	Hatchlings	% Hatchlings
1.	300 ng/µl	23rd June 2020	276	39	14.1
2.	300 ng/µl	28th Aug 2020	593	74	12.5
3.	300 ng/µl	8th Sep 2020	879	106	12.1
4.	300 ng/µl	24th Sep 2020	726	65	9.0

Table S6. dpp CRISPR-Cas9 injection table

Sl. No.	Concentration	Date	Eggs Injected	Hatchlings	% Hatchlings
1.	300 ng/µl	23rd Jan 2020	683	89	13.0
2.	300 ng/µl	3rd March 2020	923	117	12.7
3.	300 ng/µl	11th Feb 2021	282	51	18.1
4.	300 ng/µl	6th July 2021	124	15	12.1
5.	300 ng/µl	23rd July 2021	472	29	6.14
6.	300 ng/µl	27th July 2021	326	43	13.2
7.	50ng/µl	6th Sept 2022	472	65	13.7
8.	100 ng/µl	27th Sept 2022	326	57	17.5
9.	100ng/µl	9th Feb 2022	186	24	12.9

Table S7. ci CRISPR-Cas9 injection table

Sl. No.	Concentration	Date	Eggs Injected	Hatchlings	% Hatchlings
1.	300 ng/µl	8th July 2021	283	35	12.0

Table S8. knirps CRISPR-Cas9 injection table

Sl. No.	Concentration	Date	Eggs Injected	Hatchlings	% Hatchlings
1.	300 ng/µl	15th Feb 2020	725	128	17.6
2.	300 ng/µl	12th Feb 2020	534	97	18.2

Table S9. omb CRISPR-Cas9 injection table

Sl. No.	Concentration	Date	Eggs Injected	Hatchlings	% Hatchlings
1.	300 ng/µl	9th Feb 2020	734	85	11.6

Table S10. patched CRISPR-Cas9 injection table

Sl. No.	Concentration	Date	Eggs Injected	Hatchlings	% Hatchlings
1.	300 ng/µl	23rd Nov 2020	306	78	25.5

Table S11. Immunofluorescence Buffers

Buffers	Chemicals	Amount
Fix buffer (30 ml)	0.1M PIPES pH 6.9 (500 mM)	6 ml
	1 mM EGTA pH 6.9 (500mM)	60 µl
	1% Triton x-100 (20 %)	1.5 ml
	2 mM $MgSO_4$ (1M)	60 µl
	37% Formaldehyde	55 µl per 500 µl of buffer
	dH_2O	22.4 ml
Block buffer (40 ml)	50 mM Tris pH 6.8 (1 M)	2 ml

	150 mM NaCl (5 M)	1.2 ml
	0.5% IGEPAL (NP40) (20%)	1 ml
	5 mg/ml BSA	0.2 gr
	H2O	35.8 ml
Wash buffer (200 ml)	50mM Tris pH 6.8 (1 M)	10 ml
	150 mM NaCl (5 M)	6 ml
	0.5% IGEPAL (20 %)	5 ml
	1 mg/ml BSA	0.2 gr
	dH_2O	179 ml
Mounting media	Tris-HCl (pH 9.2)	20 mM
	N-propyl gallate	0.5%
	Glycerol	60%

Table S12. *In-situ* hybridization Buffers

Buffers	Chemicals	Amount
10X PBS (500 ml) * Sterilize by autoclaving.	K_2HPO_4	5.34 g
	KH_2PO_4	2.64 g
	NaCl	40.9 g
	DEPC treated H_2O	To 500 ml
1X PBST (50 ml)	1X PBS	50 ml
	Tween® 20	50 µl
20X SSC (1000 ml) *Adjust the pH to 7.0 with 1M HCl and sterilize by autoclaving.	NaCl	175.3 g
	Trisodium citrate	88.2 g
	DEPC treated H_2O	Till 1000 ml
Pre-hybridization buffer (40 ml)	Formamide	20 ml
	20X SSC	10 ml
	DEPC treated water	10 ml
	TWEEN20	40 µl
Hybridization buffer (40 ml)	Formamide	20 ml
	20X SSC	10 ml
	DEPC treated water	10 ml

	TWEEN20	40 µl
	Spaltmon sperm	40 µl
	Glycine (100mg/ml)	40 µl
Block buffer (50 ml)	1X PBS	50 ml
	TWEEN20	50 µl
	BSA	0.1 gm
Alkaline phosphatase buffer (20 ml)	Tris-HCl (pH 8.0)	2 ml
	NaCl (5M)	400 µl
	$MgCl_2$ (200mM)	250 µl
	DEPC treated water	Till 20 ml
	TWEEN20	20 µl

Sequence of *decapentaplegic* used for enzyme based *in-situ* hybridization.

GTTCTTCAACGTAAGCGGCGTACCGGCCGACGAGGTGGCGCGCGGCGCCGACCTCTCGTT
CCAACGAGCCGTCGGCACCACCGGCAGACAGAGACTGTTGTTGTACGACGTGGTGCGCC
CTGGCCGCCGCGGCCACTCCGAGCCGATCCTGCGGCTGCTGGACTCCGTTCCGCTCCGGC
CCGGGGAGGGAATCGTCAACGCCGACGCTCTGGGAGCGGCGCGACGGTGGCTCAAAGA
GCCCAAACATAATCACGGACTATTAGTGCGAGTGTTAGAAGAAGACGCCGCGAGTGCGA
GCAGGGACGCGAAGTTCCCGCACGTGCGCGTGCGCAGACGCGTCACGGACGAGGAGGA
GGAGTGGCGGACGGCGCAGCCGCTGCTCATGCTGTACACGGAGGACGAGCGCGCGCGCG
CGTCGCGGGAGACGAGCGAGCGGCTGACGCGCAGCAAGCGCGCGGCGCAGCGGCGGGG
GCACCGCGCGCACCACCGCCGCAAGGAGGCGCGCGAGATCTGCCAGCGCCGCCCGCTGT
TCGTCGACTTCGCGGACGTGGGCTGGAGCGACTGGATCGTGGCCCCGCACGGCTACGAC
GCGTACTACTGCCAGGGCGACTGCCCCTTCCCGCTGCCGGACCACCTCAACGGCACGAAC
CACGCGATAGTGCAGACTCTGGTCAACTCAGTGAACCCCGCGACGGTGCCCAAAGCGTG
CTGCGTGCCGACGCAACTCTCATCTATATCTATGTTATATATGGACGAAGTGAACAATGT
GGTGCTTAAAAACTATCAGGACATGATGGTGGTAGGCTGTGG

Region of *Optix* used for CRISPR-Cas9 (Highlighted in red)

ATGCGCGGCTCCTGGGACGAGTCCACGACGGCGGCGCTGCACGCGCGCATCCTGGAGGC
GCACCGCGGGTCCGCCGCGCCCGACCGCGCCGAGCCCGCGTGCGAGCCTCCGCCGCTGA
CGCTGGGCGCGCTGGAGCTGGCGGCGCCCACGCCGCTGCTGCCGCTGCCCACGCTGAGC
TTCAGCGCCGCGCAGGTGGCCACCGTGTGCGAGACGCTGGAGGAGAGCGGCGACGTGGA
GCGCCTGGCGCGCTTCTTGTGGTCGCTGCCCGTGGCGCACCCCAACGTGGCCGAGCTGGA
GCGCTGCGAAGCCGTGCTGCGCGCGCGCCGTCGTCGCCTTCCACGCCGGCCGCCACCG
CGAGCTGTACGCCATCCTCGAGCGCCACCGCTTCCAGCGCTCCAGCCACGCCAAGCTGCA
AGCGCTGTGGCTGGAGGCGCACTACCAGGAGGCTGAGCGCCTGCGCGGCCGTCCGCTGG
GCCCCGTCGACAAGTACCGCGTGCGGAAGAAGTTCCCGCTCCCGAGGACGATCTGGGAC
GGCGAGCAGAAGACGCACTGTTTCAAGGAGCGGACGCGATCTCTACTCCGAGAATGGTA
CCTCCAAGATCCCTACCCGAACCCGACGAAGAAGAGGGAATTGGCGGCGGCGACGGGTC

TGACGCCGACGCAAGTCGGCAACTGGTTCAAAAACCGACGGCAAAGAGACCGAGCGGC
CGCCGCCAAGAACCGCTCCGCCGTGCTGGGCAGAGGATAA

Region of *spalt* targeted by CRISPR-Cas9 (location of guide RNA highlighted in red)

GCATCGACAAGATGCTGAAAATAATAATAGTCTCGAAGACGGCGAGGCCGAAATACCTG
AAGCCGACATGCCCCCCGTGGGTCTGCCGTTCCCTTTGGCAGGACACGTTACTCTTGAGG
CTCTACAAAATACGAGAGTAGCGGTCGCCCAATTCGCTGCAACAGCGATGGCAAATAAT
GCGAATAACGAAGCTGCTATACAAGAATTACAAGTGTTACACAACACTCTATACACTTTA
CAGTCACAACAAGTATTTCAACTTCAGTTAATACGTCAGCTTCAGAATCAGTTATCTCTA
ACTCGACGGAAAGAAGACGATCCACACAGCCCACCGCCAAGTGAACCAGAACAGAATG
CCCCGTCAACGCCGGCTCGATCACCGTCGCCGCCGCGTCCGCCACGGGAGCCGTCGCCTG
TTATACCCTCTCCTCCTACTAGCCAAAGTTTGCCGTCGACTCACACACATCACACACCCA
AAACTGAACAGATATCTATCCCTAAGATTCCAACTTCCTCACCATCTTTAATGACCCACC
CACTTTATAGTTCAATTTCTTCGTCATTAGCATCTTCCATCATAACAAACAATGATCCTCC
ACCGTCCCTAAATGAA

Region of *dpp* used for CRISPR-Cas9 (Highlighted in red)

ATGCGTGGGGCGTGCGCGTGCGCGGTGGTGTGCGCGTTGGTGGCGCTGTGCGCGGCGCG
GCTGGACGAGTCCGCGCGCGCCGCCGCCGAGAAGCAGCTGCTGGCACTGCTGGGCCTGC
CGCGCCGGCCGCCGCCGCGCGCCCGCCCGCCGCCCGTGCCGCGCGCGCTGCGCGTGCTGT
ACGACTCGCGCGCGCTGCCCGCCGCCGCCGCCAACACGGCGCGCTCCTTCCACCACACGC
CCACGCCGCTCGACGAGCGCTTCCCCGGCGACCACCGCTTCCGCCTGTTCTTCAACGTAA
GCGGCGTACCGGCCGACGAGGTGGCGCGCGGCGCCGACCTCTCGTTCCAACGAGCCGTC
GGCACCACCGGCAGACAGAGACTGTTGTTGTACGACGTGGTGCGCCCTGGCCGCCGCGG
CCACTCCGAGCCGATCCTGCGGCTGCTGGACTCCGTTCCGCTCCGGCCCGGGGAGGGAAT
CGTCAACGCCGACGCTCTGGGAGCGGCGCGACGGTGGCTCAAAGAGCCCAAACATAATC
ACGGACTATTAGTGCGAGTGTTAGAAGAAGACGCCGCGAGTGCGAGCAGGGACGCGAA
GTTCCCGCACGTGCGCGTGCGCAGACGCGTCACGGACGAGGAGGAGGAGTGGCGGACG
GCGCAGCCGCTGCTCATGCTGTACACGGAGGACGAGCGCGCGCGCGCGTCGCGGGAGAC
GAGCGAGCGGCTGACGCGCAGCAAGCGCGCGGCGCAGCGGCGGGGGCACCGCGCGCAC
CACCGCCGCAAGGAGGCGCGCGAGATCTGCCAGCGCCGCCCGCTGTTCGTCGACTTCGC
GGACGTGGGCTGGAGCGACTGGATCGTGGCCCCGCACGGCTACGACGCGTACTACTGCC
AGGGCGACTGCCCCTTCCCGCTGCCGGACCACCTCAACGGCACGAACCACGCGATAGTG
CAGACTCTGGTCAACTCAGTGAACCCCGCGACGGTGCCCAAAGCGTGCTGCGTGCCGAC
GCAACTCTCATCTATATCTATGTTATATATGGACGAAGTGAACAATGTGGTGCTTAAAAA
CTATCAGGACATGATGGTGGTAGGCTGTGGCTGCCGATGA

Region of *omb* used for CRISPR-Cas9 (Highlighted in red)

ATGCATCATCTCGAGAATTTCTCAATAAGCCGTGGTGCCTGTGGGGCCGACGCGGGCGCC
GCCCCGCGGCGAGTCGGTGGCGCCTCTATCGATTATTCGGGCCTCTCGGTCGCGCGTAGC
GCCGCGTCCCGCTCGCGCCGCGCGGGTGGCGCCCGCCGCGTCATGCGCGACCACCGCGC
GACTGGCGCGACCTCGCGCGAGCCGCTCTCGGGCTATATCATTAATCTATTACTCCGGGA
AGGTGGGCTCGTACGTAAGGAGCTCATCTATAGCCAAAGTCAACGGATGTTGTCAAAGT
TGCTGTGGCCAAACCCAGTGCCTAGTCCGCCCAGAGACCCCCACTCAGATCATTTGACGA
TCCTGAACTCGATGCACAAGTACCAGCCACGGTTCCACCTGGTGCGAGCCAACGACATCC
TCAAGCTGCCCTACTCCACCTTCCGCACCTACGTATTCAAGGAGACCGAGTTCATCGCCG
TCACCGCCTACCAGAACGAGAAGATAACGCAGCTGAAAATCGACAACAACCCCTTCGCG
AAAGGCTTCCGAGACACGGGGGCGGGGAAGCGGGAGAAGAATCTGTCCGTGTACCGCA
GGCAGGCGCTGCTGACGGCGCGGTCGGACGCCCGCGAGGACGACGACGAACGTCCACTA
GACGTTGGCGGACCCTCCAGCCCGCCGCCGCCCGCGACGCAACACACGAGCAGCTCGTG
GTTCAGTTCGAGTGGAGGCGGCGCGGACTCCGGTCCAGAGGAAGCCGGCTCGGACTCGT

CGTGCTCCGGCCCCGCGCGCGCTCCCTCGCCCCCCGCCGGGCCCTCGCGGCCTTCTCCCG
CCCCTGACGTGTCTCTCGGCCCGCCGGTGCAGCCCCCCCTCCTGCCCTACCTGTACCCGCC
TTCGCTGTACCCGCCACCGTTCTTCCCGCCACACCAAATGCCGCCAGGTCTGTTGTTCAAC
CTGCATCCCCTCCTGCAGCAGTACTCGTTGCCCCCTCCCCTCGCGCCGCCGACCCCCACGT
CCGCGCCTTCTCTGAGCAAGACGCACAGGTTCGCGCCGTACGCGCTGCCCGGGTTAGGGT
CTGCGTTCGAGCAGGTCGCGCCCAGAGCGAGGAGCCTCAGTTCGTCGCCGGCGCGGCCG
CGGGTGGGGTCGCCTCCCACGAGAGCGGCGTCAGCGGACCCCCCGCCGGACGCGCCGAC
GTCGACCACCTCCGCCGCGACCCCTCCCGCGTCCGACCTCAAGAGCATCGAGCGTATGGT
CAACGGCTTGGACGTGGAGACGCAGGACTGA

Region of *ci* used for CRISPR-Cas9 (Highlighted in red)

ATGGGCTTAGAATATTTAAGTGCAGCGAGGAGTCTACATCCAGAACTTCATGCAGGCAG
TACGTTAGCGAGTCAAGAGTTCCAACTCAGTTTAGAAGGATCAAGAATAGCCTCTCAGA
ACCGTCTAAGGTTATCCGGTGGGGCGATCAGTGCGTCAGCTAACAGGAAGAGGGCGGTT
TCCTGGAGCCCCTACTCGGCGGAGTCCCTGGACTTGGCTGCTGTGATACGCGCGTCCCCG
GCCAGCCTCGCTGTGAGGGCCCCTTCTGCGGCCTCTACTGGCAGCTATGGACATCTCAGT
GCAGGAGCAATATCCCCAGCGCTCGGCAGCGGCAGAGGCGGCACACCAGGCCGCGCTCG
CGCATCTGGTGGCCGGCATACACAGGTTTGTTGTTGTGTGCTGACGACGAGCGGCAACAA
CGTCATCCCTGGCATCGTGCTGCTCGACCCCGCGCAGCAGCAGGCGGCAGCGCATGAGC
ATCTGATGGCTGGCATACACAGATCTCACATATCGTCACCGACTCAGCTGCTGATCGCGC
CGGTGGACGTGCGGCCCGGCCTGGGGCTGGACGGCACGCCCCCTCACATGCAGCAACCG
CATCAGCAACCTGAAATCACCAGTGTTATGGAGGCTGATAGTGCATCAACGGCTCTCAAC
CAGCGCAAGTCCCCACAAGTGCTGATATCACACCGAGAGAACATGAACAGCAACAAGCC
ATTGTCGGCGGCGGCAGAGAGCACGGTCCACGACGGGCTGGACTCTAAGGATGAGCCTG
GAGACTTCATTGAGACTAACTGCCACTGGGTGGACTGTAAGCTGGAGTTCCCAACACAG
GACGATCTAGTAAAGCACATCAACACAGACCACATCCACGCCAGCAAGAAAGCCTTCGT
CTGCCGCTGGGTCAGCTGCTCCAGGGACGAGAAGCCTTTCAAAGCGCAGTACATGCTTGT
GGTGCATATGAGACGCCACACTGGAGAAAAGCCTCATAAGTGTACATTCGAGGGATGCT
GCAAAGCTTACTCCCGGCTCGAGAACCTGAAGACCCACCTGCGGAGTCACACCGGGGAG
AAGCCCTACACGTGCGAATACCCCGGCTGTGCGAAGGCTTTCTCCAACGCCAGCGATCG
GGCGAAGCATCAGAACAGAACTCATAGTAATGAGAAACCGTACGTATGTAAGGCTCCAG
GCTGCACGAAGCGGTACACAGACCCGTCCTCCCTCCGCAAGCATGTGAAGACTGTACAT
GGTGCTGAGTTCTACGCCAGCAAGAAACATAAAGGGTGCAGTCGAGGAGACGATTCAGC
CGAGTCTGGGGGTGGTGGCGCGGGATCTTCGCCACGGTCGGAGGAGGGTGGAGTTCCTA
TGGGGGTGAGGGGTCACACGTCCTCGGCCTCTGTCAAGAGTGAGAGCCCAGCTTCACCCT
TACCTCATGGTCTACATACACCGGCTCATCAGTTATCAGCACAATGTGGTGGTGAACTGG
ACTTCGGTGTGTCTGGTTTGGGCGGGTTTAGTGATGAAAACGGCGCTCCCTACTTCAGAC
TAGATGGCGAGGTGGAACAGGAAGTGGTAGGGGAAGTGGGACAACTGCCCCTCATGCTG
CGCGCTATGGTAGCGATAGGGGAGCCGCGCGCCCATCATCACGCGCCGCGCTTCGGACA
CAAGATGGGAGTGGGGAGACTGATGCCGCCCATACACGGGGCAGATGTTGGTGGAGGTG
GTATTCAAGGGCGGACAGAAATAGGAGGTACAAACGTAGCGGTAGAACTGAAGACCGG
ACTGCCCAACACGAGGCGAGACTCTGGAATCTCTTCTGGAAGCAGTTTGTATAGTGCTAG
ATCGTCGGATATCTCGCGCAAGAGCAGCCAGGCGTCAGTGGTGTCGGGCGCGGTGGTCA
CCACCACAGGCGTGGCCGGGCAGCAGAGGCTAGTCGCGCAGCATGCGGCCATTTATGAC
CAACTGTCACCTGACAGCAGTCGGAGATCTAGTCAAGTTTCCTGCGTAGGCTACGCCCCG
CCGCCGTCGTCAGCATTGGCTGCAGTTCAAGCTGTAAGGACCTCACAAGGCAATCAGGCT
GTACTGCTTCGAGGAGTCACGTGTTCAGAAGTGAGAGCAGAAGAGTTAGCCCTAGAACT
GGATCCTAACGTGCAAGTGAAGGAGGAAGCCAGGAGATTGTCAGAGCAATCCAACCTCA
GTGACCAGGCTCAGGGATATCAGCCATATCCATGCACCAATGACGATGTGGGTGATGCT
CCCTTCCCATTCAAGACAGAAGATGATCATGTGATATCGTATAAAGAATCTCGATCAAAT

TCCACAAACACTGTGGTTATCACCACAGCACAAGTTCATCACCCAAATCAAGAAGTCAAT
CTTGAACAGGTCGCGGAAGGAGAAATGGTGGAGAACAAGCTGGTGATACCAGACGAAA
TGATGCAATATCTTAACCAATCAATATTGGGAACCGACTCGGTAACACCAGCAAAAACG
GACTCCAGCAATCCTGACCCAGAAGGAGCAAACAAAGAAAAAGATACAACAACCAAAG
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GTGATATTCAAGTTGATTTAAGGTCATTAGATGTTAGTATGTCTGGTAACAGCGGCTCTC
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AAGTTACACCAGAAACATGTGAACAAGAATTCTCAAAACCCCCGACAAGTAATGTTGTT
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CATGACACAAACTATCATGAGTCCCAGTTTAGCACATAACATGTTAAGTCCTCAGAGTCT
ACCGCATAGTTCAATGAGCCCACAGAGCATCAGAAGCCCTCAGCATATGCCTCAAAGCA
TCATGAGCCCACCAAGCGTATACAATGTCATGAGTCCGCAAAGTGTCATGAGCGTCATGT
CTCCACAGCACAATGCAATGAGCCCACAAAGTATGCAGAGTTTATTGAGCCCTCAGATG
CCAAATCAAATGATAATGAGTCCCCGACACAACAACATTGGAAGTCCTCATTCCCAGAA
CATCGCAAGTCCCATGATGAACATGGCAAGTCCAATGACGCAGAACATTGCTAGTCCGA
TGAGTCATGGCATGCCAAGTCCAATGCATCCTGGCCTTCAAAGCCCAATAACAAGTCCTA
TGGTACAAAACATGTCCAATATGACGATGCAACCAGTGCCAAATCAAAATGCGAATATA
ATGATGAGTAACATGAATCCTCCTCAACAAATGACGGCAGCACCACCCTATAATAACCG
TCCCAACTGTCCTCCAAAACTTCCAAACAAAAACTTTAGTAACGTTCCTAATCAATACCA
AAACCAAAACTACGGCCCAGCGCCACCTTATCCCATACAAAACCAAGTCAATGTAAACA
TGAGAAACCAAAACATTCAACAGTACCAGATGATGCAACACTATAATCCTAATCAACCA
CAGGTGACAATGATGCAAAACAACCATCAAAACATGGTTTATAATAATCAAATGACAAA
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ACGATCTTCAGTAATGAGCGTCGATAACAGTGGAAACATGAGTCGTGGTGCTATGAACA
GTTACTGTGAACAACAGAATGTATGTCCTCCCATGCAAAACGTACAATATAACCAGAAC
ATGCAATATCCTCAACCGCCTCCATACAATTCTGTAGCGAACGCTGCTAACGTAATGGGT
CCTCCACCACCAAAGAATAACCATCAGTACAACCAGGCAATGATGAACAATAACCAATA
CTACAATCATCAGAGATCTTACAACCAATGGGACTATCCTGGCAACCAATTCAACAAGC
ACAACATGCAAAAATCTGGTCAGAATTCAGTCAATATGTCTACAGGTAGTCAAAAACCT
ATAAACGGTGCGAGAATGTCAATGAATTGCAATCAGATGGTCAAAAATGCTGCGGAGCA
ACAAGCGGACTGCAGTATGAATAGTTTGAGAAGCCAAAACAACCAAACTGATGTTCAAG
TTTGGGATATCTCTCAGTCCCAAATAGAGGCTACGAATGGAAGAAAGAAAAACCAAAAT
ACCATGCGTCAGGAGACATATCAAAGGACCTTGGAATACGTAGAGAACTGTGAGAATTG
GAAGAGTTCTGAAATGGTATCCAGTAGCACACATCCTCTACAGGGCGGTGACAATATGG
TTGTGAACGATCTCCAAACTTCTCTATCTTCGTTTTATGAAGAAAATCAATATCTTCAGAT
GATACAATAG

Region of *knirps* used for CRISPR-Cas9 (Highlighted in red)

ATGGCTGATGGAAGTGGGAAGAGACGGGGTCATAGAAACACAGGTGCATTCAAAACAC
GAGACCGGTCCAAAACCCGATACGCCTGTAAAACTACGACCGATCATTCATATATTTGGG
TGGCAATAAATTTCCCTAAGAATGGATCCATCAGTGAAGTCACACCCATTAAACTAATGG
CAGATTTATTCGATCGCGAGGAGATTTACGATGTCGGCATCTGTTTACCGACTTCGCCCA
AGGTGGACAGCTTTGTGCAGATACAACGGCCTGCAATCGCCAGACATACCTCATTGTATG
TAATAATCTGTCAGCTCGTGCTAGTGTTAAAACACAGCTTTTATAGAAGCGGCAGCGGGC
GCCGGCTCTATCGGTCATTCTTCGGGCGCTCCTACAACAACCTGAACTCCATCACCGAGT
GCAAGAACAACGGGGAGTGCGTCATCAACAAAAAGAACCGCACGGCGTGCAAAGCGTG
TCGACTGCGCAAATGCCTCATGGTGGGCATGTCCAAATCCGGCTCCAGATACGGAAGAC

GATCCAACTGGTTCAAGATACACTGCCTTCTGCAAGAACAGCAACAAGCCGCCCAGGCG
CACTCTCCCCCTAGAGTACCGCAGTCGCCGCACTTAGCGCCGCCTTTTCCACCTCACCTCT
TCCCTGGACTGGCGCGACCGAGAACCAAAGAGGAACTCGCTCTGTTAGGCCTCGACGAT
TACAAAGCACCCTGCTCAGGATCCCCGGACTCTCAACGAAGCGGCTCATCCCCTAAACTA
GACGAAAAAGCTCGCATCACTCACCGACCGCCTGACCGCCCTCTGACGCCACCCAGAGA
CTCCTTCCTCCCTTTACCTTTAGCCTTGCCGCACTTTCCACACTCACCGTTTCTCCATCCGC
AGCACTTCAACCCGTTCCCGCCGAATCACCACCTCCTCTTCCCGCCAGGGTTCCACCCGA
TATACTCCAGACATCTATTAGACCACGCCGCACTCAGACAAGTGGCTGAAAATAACAAC
GACGTGAGAATCGACGACAATAACACGGAATCATCGAAGCGCTTCTTTTTGGATGAGAT
TCTTAAGCAGCAACGATCTGCGCAGCCTACGCCGCAAGAAGATGTGATATCTGAAGCAG
AATTTGTACCCACTCCGCCGGCGGAAAGGAGAACGTCGGAGTCACCTCTGCAAGAGAAT
CCAATGGATCTATCCGTCAAATCTGACGGAAGATCGAGTTCGGCGCGACGGCGGTCCGA
CGACAGCGAGGTGATCGCCCCGGACAACGATGACCCAGAATCCGGGAGTGACCGAGCGT
CCGCCAGTGAAGAAGAAGATTTATCGTACTCCCAAATAAAGAGGATCAAACTCCACCCT
TTAGATCTGACAACCAAAGTCTGA

Region of *ptc* used for CRISPR-Cas9 (Highlighted in red)

ATGTACGACATCGAGTGGCGGCTCAAGGACCTCTGCTACAGCCCCAGCATCCCGGACTTC
GAGGGCTACCACCACATCGAGTCCATCATAGACAACGTCATCCCGTGCGCAATCATCAC
GCCGCTCGACTGCTTCTGGGAGGGGTCCAAGTTGCTTGGTCCTGAATATCCTATATTTGT
ACCTCATCTAAAAAACAAACTACAATGGACTCATTTGAACCCGCTCGAAGTATTAGAGG
AAGTGAAGAAGCTAAAGTTCCAGTTCCCTCTGAGCACAATGGAGGCGTACATGAAGAGG
GCCGGCATCACGTCCGCTTACATGAAGAAGCCGTGCTTAGACCCCACCGACCCTCATTGT
CCAGACACGGCGCCGAACAAAAAATCAGGACATATTCCAGATGTAGCGGCAGAGCTGTC
CCACGGATGTTACGGTTTCGCGGCTGCGTACATGCACTGGCCGGAGCAGTTGATAGTGGG
CGGAGCGACGAGGAACTCCACATCAGCTCTGAGGAGTGCGCGCGCCCTGCAGACCGTCG
TCCAGTTGATGGGCGAGCGAGAAATGTACGAATACTGGGCCGACCACTACAAAGTGCAC
CAAATTGGTTGGAATCAAGAAAAAGCCGCCGCCGTACTTGATGCTTGGCAGAGAAAGTT
TGCAGCTGAAGTAAAAAAGATGACTACCTCAAGTTCAGTGTCAGCAGCGTACAGCTTCT
ACCCGTTTTCGACCTCAACTTTGAATGACATACTCGGAAAATTCTCGGAAGTCTCACTAA
AGAACATTATTTTGGGATACATGTTTATGTTAATTTATGTTGCTGTAACGTTAATACAATG
GCGAGATCCAATTCGTTCTCAAGCTGGAGTGGGTATAGCCGGAGTATTGCTTCTGTCGAT
CACAGTAGCCGCTGGCTTAGGCTTTTGTGCATTATTAGGCATACCATTCAATGCATCGAG
TACACAAATAGTGCCGTTCCTAGCTCTCGGACTAGGTGTTCAAGATATGTTCCTTCTCACT
CACACATACGTTGAACAAGCGGGAGATGTGCCGAGAGAAGAGAGAACCGGACTGGTAC
TGAAAAAGAGCGGACTGAGCGTTCTACTGGCCTCTCTGTGCAATGTTATGGCGTTCTTAG
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CGTAGATCAGCGGCGAGAGCCGATCTATTGTGTTGTTTGATGCCAGAAAGTCCGCTTCCC
AAAAAGAAAATTCCAGAGCGAGCTAAATCAAGGAGTGGAAAGACTGATAAGAACAATA
GGTTAGACACGACTCGGCAACCATTAGATCCAGATGTGACGGGCGAGCAACCCAAAGCC
TGTTGCCTCAGCGTGTCGCTCACCAAGTGGGTCAAGAACCAATACGCGCCCTTCATAATG
CGCCCCGCTGTTAAGGTAACATCAATGCTAGCGTTGATAGTAGTGATTTTGGCGAGTGTT
TGGGGAGCCACGAAAGTCAAAGATGGATTGGATTTAACAGACATAGTACCAGAACATAC
TGATGAACGCGAATTTCTTACGCGCCAAGAGAAATACTTCGGCTTCTACAATATGTATGC
TGTGACGCAGGGCGACTTTGAATATCCCACGAATCAGAAATTGTTATACGAATATCACGA
TCAATTTGTTCGAATACCGAATATTATTAAAAACGACAACGGTGGACTCACAAAGTTTTG
GTTGGGTCTATTTCGCGACTGGTTACTGGATTTACAGGATGCATTCGACAAAGAAGTAGC
CAGTGGCTGTATCACTCAAGAATACTGGTGTAAGAATGCAACTGATGAAGGAATATTAG

CTTATAAGCTTATGGTACAGACGGGTCATGTAGACAATCCGATTGACAAATCACTCATTA
ATTCTGGTCACAGATTAGTCGATAAAGATGGGATTATAAATCCTAAAGCGTTTTACAACT
ATCTATCAGCATGGGCAACGAATGACGCATTAGCGTATGGAGCATCTCAAGGAAATCTG
AAACCACAACCCCAAAGATGGATTCATTCTCCGGAAGACGTCCATTTAGAAATAAAGAA
GTCATCACCGTTAATCTACACTCAATTACCGTTTTATTTATCGGGGCTGAGTGATACTGAC
AGTATAAAGACATTGATAAGGTCAGTTCGAGAGCTTTGTTTGAAGTACGAGGCGAAAGG
ATTACCGAACTTTCCTTCTGGAATACCGTTCTTGTTTTGGGAACAGTATCTGTATTTGAGG
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TGCTCTTGAATGCATGGGCGGCGGTGTTAGTGACTCTATCATTAGCCACTTTGGTGCTGC
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TGGTACTGGCTGTTGGACGAGGCGTACATTTTACTGTCCATTTATGTTTGGGTTTCGTTAC
TTCAATAGGTTGCAAGCGTCGCCGAGCGTCGCTAGCTCTAGAGGCAGTGCTCGCGCCGGT
GGTGCACGGAGCGCTGGCCGCTGCGCTGGCAGCCTCCATGCTAGCTGCCAGCGAGTTCG
GCTTCGTGGCACGACTGTTCCTCAGACTGCTACTGGCACTCGTGGTGCTTGGATTAGTTG
ACGGATTGCTGTTCTTCCCTATCATACTGTCGATATTGGGACCGGCTGCTGAGGTGCAAC
CTTTGGAGCATCCCGAACGATTGTCAACACCTTCACCGAAAAGTTCGCCCGTCCATCCTC
GGAAATCAAGTTCTGGTTCAAACGGAGGCGACAAATCCAGTCGAACCAGCAAAACGGCT
CCACGACCTTCCGGACCATCATTGACAACTATAACAGAAGAGCCATCCAGTTGGCACAG
CTCCAACCACTCTGTTCAGTCGTCAATGCAGTCCATAGTGGTTCAACCGGAGGTGGTGGT
AGAGACGACTACGTATAACGGAAGCGATTCTACTTCTGGCAGATCAACGCCGACTTCGA
AATCGACGCACACTGGAGCTGTCACTACAACTAAGGTAACCGCAACAGCGAATATAAAA
GTTGAGGTAGTAACGCCAAGCGACAGAAAATCACGACGCTCATATCATTACTACGACCG
TCGAAGGGATCGAGATGATGACAGAGATAGAGAAAGAGATCGTGATCGGGATCGAGAT
AGAGACAGAGATCGTGAAAGAGATCGTGATAGAGACAGAGAGAGATCTAGAGAACGGG
ACCGAAGGGACAGGTATAGGGAAGAGAGAGATCATCGCGCTTCGCCGAGAGAAAACGG
TCGTGATTCTGGGCATGAAAGTGATTCTTCCCGACACTGA

Sequence of the genes and the oligos used for HCR3.0

>decapentaplegic_B1_XM_052883655.1

GTTCTTCAACGTAAGCGGCGTACCGGCCGACGAGGTGGCGCGCGGCGCCGACCTCTCGTT
CCAACGAGCCGTCGGCACCACCGGCAGACAGAGACTGTTGTTGTACGACGTGGTGCGCC
CTGGCCGCCGCGGCCACTCCGAGCCGATCCTGCGGCTGCTGGACTCCGTTCCGCTCCGGC
CCGGGGAGGGAATCGTCAACGCCGACGCTCTGGGAGCGGCGCGACGGTGGCTCAAAGA
GCCCAAACATAATCACGGACTATTAGTGCGAGTGTTAGAAGAAGACGCCGCGAGTGCGA
GCAGGGACGCGAAGTTCCCGCACGTGCGCGTGCGCAGACGCGTCACGGACGAGGAGGA
GGAGTGGCGGACGGCGCAGCCGCTGCTCATGCTGTACACGGAGGACGAGCGCGCGCGCG
CGTCGCGGGAGACGAGCGAGCGGCTGACGCGCAGCAAGCGCGCGGCGCAGCGGCGGGG
GCACCGCGCGCACCACCGCCGCAAGGAGGCGCGCGAGATCTGCCAGCGCCGCCCGCTGT
TCGTCGACTTCGCGGACGTGGGCTGGAGCGACTGGATCGTGGCCCCGCACGGCTACGAC
GCGTACTACTGCCAGGGCGACTGCCCCTTCCCGCTGCCGGACCACCTCAACGGCACGAAC
CACGCGATAGTGCAGACTCTGGTCAACTCAGTGAACCCCGCGACGGTGCCCAAAGCGTG
CTGCGTGCCGACGCAACTCTCATCTATATCTATGTTATATATGGACGAAGTGAACAATGT
GGTGCTTAAAAACTATCAGGACATGATGGTGGTAGGCTGTGG

Dpp1_HCR_P1B1: gAggAgggCAgCAAACggAAACCTCGTCGGCCGGTACGCCGCTTA

Dpp1_HCR_P2B1: TGGAACGAGAGGTCGGCGCCGCGCGTAgAAgAgTCTTCCTTTACg

Dpp2_HCR_P1B1: gAggAgggCAgCAAACggAACAGTCTCTGTCTGCCGGTGGTGCCG

Dpp2_HCR_P2B1: GCCAGGGCGCACCACGTCGTACAACTAgAAgAgTCTTCCTTTACg

Dpp3_HCR_P1B1: gAggAgggCAgCAAACggAACAGCCGCAGGATCGGCTCGGAGTGG

Dpp3_HCR_P2B1: CCCGGGCCGGAGCGGAACGGAGTCCTAgAAgAgTCTTCCTTTACg

Dpp4_HCR_P1B1: gAggAgggCAgCAAACggAAGCCGCTCCCAGAGCGTCGGCGTTGA

Dpp4_HCR_P2B1: TGTTTGGGCTCTTTGAGCCACCGTCTAgAAgAgTCTTCCTTTACg

Dpp5_HCR_P1B1: gAggAgggCAgCAAACggAACGCCACTCCTCCTCCTCGTCCGTGA

Dpp5_HCR_P2B1: TACAGCATGAGCAGCGGCTGCGCCGTAgAAgAgTCTTCCTTTACg

Dpp6_HCR_P1B1: gAggAgggCAgCAAACggAATCTCCCGCGACGCGCGCGCGCGCTC

Dpp6_HCR_P2B1: GCTTGCTGCGCGTCAGCCGCTCGCTTAgAAgAgTCTTCCTTTACg

Dpp7_HCR_P1B1: gAggAgggCAgCAAACggAAGCCCACGTCCGCGAAGTCGACGAAC

Dpp7_HCR_P2B1: GTGCGGGGCCACGATCCAGTCGCTCTAgAAgAgTCTTCCTTTACg

Dpp8_HCR_P1B1: gAggAgggCAgCAAACggAAGGGGCAGTCGCCCTGGCAGTAGTAC

Dpp8_HCR_P2B1: GCCGTTGAGGTGGTCCGGCAGCGGGTAgAAgAgTCTTCCTTTACg

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