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RESEARCH ARTICLE

Ultrabithorax modifies a regulatory network of genes essential for butterfly eyespot development in a wing sector-specific manner

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ABSTRACT

Nymphalid butterfly species often have a different number of eyespots in forewings and hindwings, but how the hindwing identity gene *Ultrabithorax* (*Ubx*) drives this asymmetry is not fully understood. We examined a three-gene regulatory network for eyespot development in the hindwings of *Bicyclus anynana* butterflies and compared it with the same network previously described for forewings. We also examined how *Ubx* interacts with each of these three eyespot-essential genes. We found similar genetic interactions between the three genes in fore- and hindwings, but we discovered three regulatory differences: *Antennapedia* (*Antp*) merely enhances *spalt* (*sal*) expression in the eyespot foci in hindwings, but is not essential for *sal* activation, as in forewings; *Ubx* upregulates *Antp* in all hindwing eyespot foci but represses *Antp* outside these wing regions; and *Ubx* regulates *sal* in a wing sector-specific manner, i.e. it activates *sal* expression only in the sectors that have hindwing-specific eyespots. We propose a model for how the regulatory connections between these four genes evolved to produce wing- and sector-specific variation in eyespot number.

KEY WORDS: Butterfly, CRISPR, Eyespot, Hox, Novel trait

INTRODUCTION

Hox genes are general transcription factors that are often used to promote or repress the development of traits. When Hox genes are manipulated, traits might become reduced or disappear, or become enlarged. For example, the Hox gene *Ultrabithorax* (*Ubx*) is responsible for repressing the growth of the dorsal appendages of the third thoracic segment of *Drosophila*, and shaping them into the small haltere balancing organs (Lewis, 1978; Crickmore and Mann, 2006; de Navas et al., 2006). The removal of *Ubx* from halteres makes these appendages develop into large flight wings (Lewis, 1978). Conversely, the large hindlegs of crickets (Mahfooz et al., 2007) also owe their appearance to *Ubx*, which functions as a growth-promoting gene in this species; hindlegs become smaller when *Ubx* is downregulated. So, Hox genes can function as either promotors or repressors of traits, depending on the trait and species in question.

Recently, however, we described a system whereby Hox gene manipulations affect the same type of trait in different ways, depending on the location of that trait within the body. In *Bicyclus anynana* butterflies, CRISPR-Cas9 experiments targeting *Ubx* led to an expected homeotic transformation, i.e. hindwing patterns were modified into those of the forewing (Matsuoka and Monteiro, 2021). This homeotic transformation led both to the enlargement of some eyespots as well as the disappearance of other eyespots on the hindwing. Eyespots that were unique to hindwings, i.e. without a corresponding serial homolog in forewings, required *Ubx* to differentiate, and did not differentiate when *Ubx* was disrupted. Eyespots with a forewing serial homolog, by contrast, were repressed in size by *Ubx*. These hindwing eyespots, which are naturally smaller than their counterparts on the forewing, enlarged to forewing size when *Ubx* was disrupted. This indicated that *Ubx* is both a size repressor as well as an essential gene for eyespot development, and these two functions vary with the location of the eyespot on the hindwing.

Similar experiments targeting the Hox gene *Antennapedia* (*Antp*) also led to different effects on eyespots, depending on whether the eyespots were on the forewings or hindwings. Antp protein is present in the center of all eyespots (Fig. 1); however, *Antp* disruptions have been shown to lead to the disappearance of all forewing eyespots, but only to the disappearance of the white centers and to a size reduction of hindwing eyespots (Matsuoka and Monteiro, 2021). This indicated that *Antp* is essential for eyespot development on forewings, but merely required for the differentiation of the white central scales and for enlarging eyespots on hindwings.

In contrast to the effects of disruptions of these two Hox genes, CRISPR-Cas9 targeted disruptions of two other eyespot-associated genes, *Distal-less* (*Dll*) and *spalt* (*sal*), in different nymphalid species have shown each gene to be essential for the development of all eyespots, on both forewings and hindwings, regardless of the wing sector in which the eyespot was found (Zhang and Reed, 2016; Connahs et al., 2019; Murugesan et al., 2022). This suggests that some genes have a global effect on eyespot development, whereas the Hox genes have a more limited, wing- or wing sector-specific role.

Recently, we studied how three of these eyespot-essential genes, *Antp*, *Dll* and *sal*, interacted with each other on the forewings of *Bicyclus anynana* butterflies (Murugesan et al., 2022). We discovered that *Dll* is upstream of both *sal* and *Antp*, and is required for upregulation of these genes in eyespot centers during the larval stages. We also discovered that *sal* and *Antp* upregulate each other. The output of this regulatory network, however, must be modified on the hindwing by *Ubx*, as *Antp* no longer functions as an essential gene for eyespots to develop on this wing. In addition, *Ubx* is required for the development of eyespots that are unique to hindwings, but it is unclear whether this gene interacts with or activates any of the other three genes in those wing sectors.

Given that *Ubx* is essential for the development of some hindwing eyespots (Matsuoka and Monteiro, 2021), we hypothesized that *Ubx* might interact with at least one of the other

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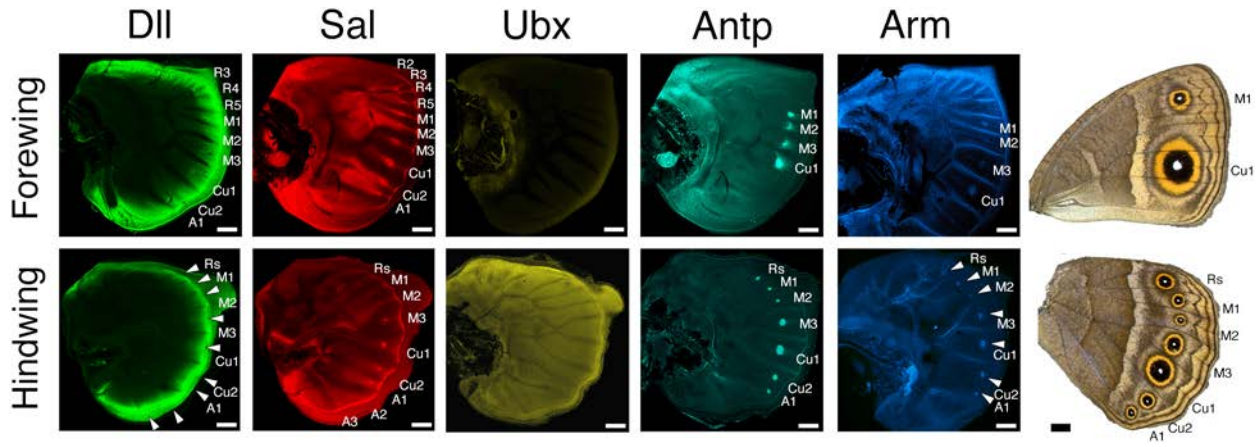

Fig. 1. Differential expression pattern of eyespot-associated genes between fore and hindwing. The Dll protein pattern is similar across both wings. Dll protein is present in the wing margin, along a finger pattern from the wing margin into nine wing sectors, and in the foci mapping to future eyespot centers. The Sal protein pattern is also similar in both wings. Sal shows a wing sector-specific pattern of expression, with two outer domains, in the most anterior and most posterior wing sectors, and two more central domains, one spanning wing sectors R2 to M3, and the other spanning Cu2 to A2 wing sectors (see Banerjee and Monteiro, 2020). Fingers and foci patterns are similar to those of Dll. Ubx protein is ubiquitously present across the hindwing but was not detected in forewings. Ubx expression in the eyespot foci is stochastic (Fig. S5). In late larval wings, Ubx is sometimes downregulated in the foci. Antp protein pattern is different between fore- and hindwings. Antp is present only in four eyespot foci in the forewing, but in seven in the hindwing. Arm shows a similar pattern in eyespot foci as Antp, but Arm is also expressed in the fingers leading to the foci. Scale bars: 100 µm (larval wings); 5 mm (adult wings).

three (forewing) essential genes, *Antp*, *Dll* or *sal*, in hindwing eyespots, functioning either upstream or downstream of these genes. We also hypothesized that *Ubx* might have a distinct interaction with these genes in sectors in which *Ubx* displays a merely repressive effect on eyespot size. To test these hypotheses, we first described patterns of gene expression for all four genes in hindwings and compared them with forewings. Then, we generated mosaic crispants with the CRISPR-Cas9 system, targeting *Ubx*, *Antp*, *Dll* and *sal* in turn. We followed the effects of these perturbations on the protein levels of the targeted gene, as well as the other three proteins on the hindwing, using double immunostaining. We focused our investigation on the larval stages of hindwing development when eyespot centers, also called the foci, are being differentiated. We examined interactions between the four genes specifically in the foci, as well as in other parts of the wing.

RESULTS

Differences in expression pattern of Dll, Sal, Antp and Ubx proteins between fore- and hindwings

To test whether there were differences in the expression pattern of Dll, Sal and Antp proteins between fore- and hindwings, we examined larval wings using immunohistochemistry. Because we are primarily interested in the function of these genes during eyespot center specification, we only examined gene expression patterns during larval wing development. The adult forewing has only two eyespots in M1 and Cu1 wing sectors, whereas the hindwing has seven eyespots (Fig. 1). We found that the expression pattern of Dll and Sal proteins was similar in both fore- and hindwings, even though wing shape and final eyespot number are different (Fig. 1, Fig. S1). In early larval wings, both Dll and Sal proteins showed a similar finger pattern of expression in both fore- and hindwings, with an enlarged area at the center of nine potential eyespots on each wing (Fig. 1, Fig. S1). In contrast, the expression pattern of Antp protein was different between fore- and hindwings. Antp protein was expressed in seven eyespot foci in hindwings, never in the finger pattern, but was only expressed in four foci, from M1 to Cu1 eyespots, in forewings (Fig. 1). Later in larval development, Antp expression was retained in M1 and Cu1 foci (Figs S2-S4), but lost from the middle two wing sectors.

To explore further differences between the eyespot gene regulatory network (GRN) between fore- and hindwings, we examined the expression pattern of Armadillo (Arm) protein, a mediator of Wnt signaling that is involved in eyespot development (Özsu and Monteiro, 2017; Banerjee and Monteiro, 2020; Connahs et al., 2019). Whereas Arm was expressed in all seven eyespot foci in hindwings, in forewings Arm had a dynamic expression pattern, initially expressed in four foci and later only in two (Fig. 1, Fig. S4). Furthermore, Arm expression was localized within the nucleus of cells in eyespot foci, whereas it was localized at the cell membrane outside of eyespot foci (Fig. S4D′). These staining patterns suggest that Dll and Sal proteins are not sufficient, on their own, to activate future eyespot centers on forewings; the presence of Antp and Arm proteins, throughout the middle and late stages of larval wing development, might be necessary for the differentiation of an eyespot focus.

Ubx showed a typical protein expression pattern restricted to the hindwing (Fig. 1), as previously shown (Weatherbee et al., 1999, Tong et al., 2014). Ubx protein was ubiquitously present across the hindwing at the early larval stage (Fig. S5). As the wing developed, Ubx protein disappeared from the future eyespot centers in some wings, whereas in other wings, Ubx was retained in these centers (Fig. S5). These variations, which seem somewhat stochastic and not dependent on development time, affected all hindwing eyespots in the same way. It is unclear what this variation in Ubx expression has on adult phenotypes as ventral eyespots are always constant in number.

To examine the regulatory interactions between *Ubx*, *Dll*, *sal* and *Antp* in eyespot development in hindwings, we used embryonic injections of Cas9 mRNA and short guide RNAs targeting one gene at a time. We primally focused on the establishment of eyespot foci; therefore, we examined gene interactions during the larval stage, when these genes are fully expressed in the foci, particularly at the time point when trachea have spread throughout the larval wing.

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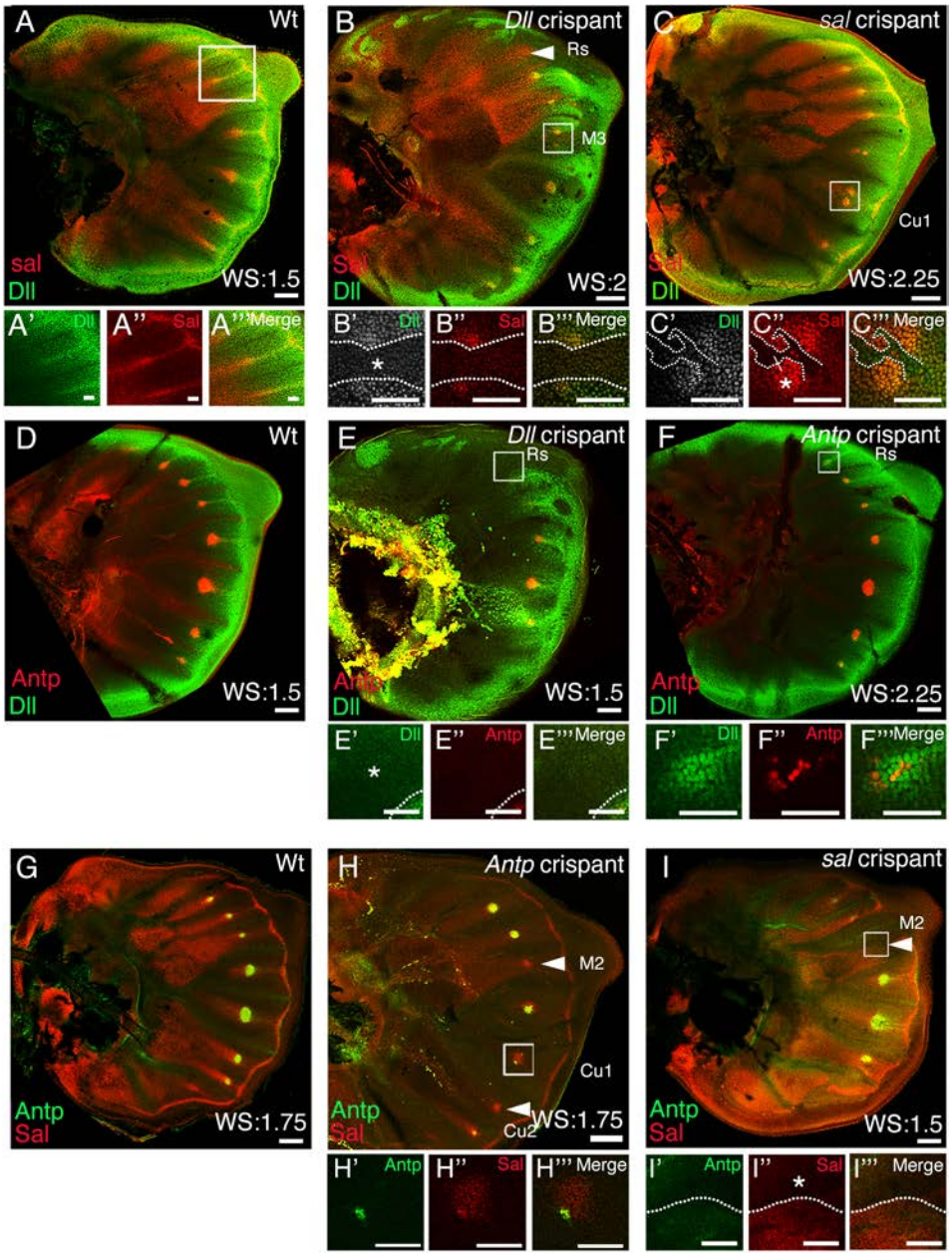

Fig. 2. Regulatory interactions between *Dll*, *sal* and *Antp* on the hindwing. (A-C‴) Expression pattern of Dll and Sal proteins in a Wt hindwing (A-A‴), a *Dll* crispant hindwing (B-B‴) and a *sal* crispant hindwing (C-C‴). (D-F‴) Expression pattern of Dll and Antp in a Wt hindwing (D), a *Dll* crispant hindwing (E-E‴) and an *Antp* crispant hindwing (F-F‴). (G) Expression pattern of Antp and Sal in a Wt hindwing (G), an *Antp* crispant hindwing (H-H‴) and a *sal* crispant hindwing (I-I‴). Boxed areas are shown at higher magnification below, as indicated. Arrowheads point to missing eyespot foci. Dotted lines delineate regions where the protein from the targeted gene is missing, marked with asterisks. All the wings are larval wings at the indicated wing stage (WS) (Reed et al., 2007). Scale bars: 100 µm (whole wing images); 50 µm (high-magnification images).

Genetic interaction between *Dll* and *sal*

We first examined the relationship between *Dll* and *sal* in hindwings (Fig. 2A-A‴). In *Dll* crispants with broad mosaics showing absence of Dll protein, Sal protein was broadly overexpressed in distal wing areas with no clear delineation of expression in the midline fingers and eyespot centers (Fig. 2B-B‴, Fig. S6). This indicates that *Dll* is repressing *sal* in the distal part of the wing. In other *Dll* crispants, *Dll* crispant mosaics overlapping the eyespot foci and fingers disrupted Sal protein expression and resulted in no fingers and in a split eyespot focus (Fig. 2B-B‴). In addition, Sal protein was not detected in the *Dll* crispant mosaics in the chevron patterns along the wing margin (Fig. S6), indicating that *Dll* is required for proper *sal* expression in chevrons along the wing margin, midline fingers and eyespot centers.

We then examined whether *sal* also regulates *Dll*. *sal* crispants with broad *sal* crispant mosaics showed normal Dll protein expression along the midline finger and wing margin (Fig. S7C). However, both types of eyespot centers were split in two, when smaller *sal* crispant mosaics transected those cells (Fig. 2C, Fig. S7A,B). These results suggest that *Dll* is upstream of *sal*, as also observed in forewings (Murugesan et al., 2022), but *sal* is also regulating the focal expression of *Dll* in hindwings. Taken together, *Dll* appears to be an activator of *sal* in the eyespot centers, midline fingers, and chevrons along the wing margin, even though Sal protein starts to be visibly expressed in eyespot foci earlier than Dll protein (Fig. S1). *sal* also regulates *Dll* in the foci. The regulatory relationship between these genes is distinct in the general distal wing region, where *Dll* is repressing *sal*.

Genetic interaction between *Dll* and *Antp*

Next, we examined the relationship between *Dll* and *Antp* (Fig. 2D). In *Dll* crispants, Antp protein expression was lost in *Dll* crispant mosaics (Fig. 2E-E‴, Fig. S8), whereas in *Antp* crispants, Dll protein expression was not affected in either type of eyespot foci of *Antp* crispant mosaics (Fig. 2F-F‴, Fig. S9). These results suggest that *Dll* upregulates *Antp* in the hindwing eyespots as also observed in forewings (Murugesan et al., 2022). From the above results, we

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identified *Dll* as an upstream regulator of both *Antp* and *sal* genes in hindwing eyespots, as also observed in forewing eyespots.

Genetic interaction between *Antp* and *sal*

Next, we examined the relationship between downstream genes *Antp* and *sal* (Fig. 2G). In *Antp* crispants, Sal protein levels were mostly unaffected (Fig. S10A,C), but in some wings they were slightly dampened (but not lost) in *Antp* crispant mosaics (Fig. 2H-H‴, Fig. S10B), whereas in *sal* crispants, Antp protein levels were lost in either type of eyespot foci of *sal* crispant mosaics (Fig. 2I-I‴, Fig. S11). These results indicate that *sal* is essential for activation of *Antp*, but *Antp* merely upregulates *sal*, and is not essential for *sal* activation. The regulatory interaction between these two genes is different from the interaction observed in forewings, where *Antp* is an essential gene for *sal* activation (Murugesan et al., 2022). Taken together, the regulatory interactions involving two of the three genes are different between forewings and hindwings.

Genetic interaction between *Ubx* and *Dll*

To examine whether *Ubx* was involved in mediating these differences in regulatory interactions, we extended our investigation to *Ubx*. We first tested the specificity of the UbdA antibody, which is known to recognize both Ubx and Abd-A proteins (Kelsh et al., 1994). In *Ubx* crispants, we found patches of hindwing cells that clearly lost all fluorescence (Fig. 3C, Figs S13, S15, S16), indicating that the UbdA antibody detects Ubx proteins, and that Abd-A is not likely to be co-expressed with Ubx in the hindwing.

We then examined the relationship between *Ubx* and *Dll* (Fig. 3A). In *Dll* crispants, Ubx protein expression was reduced in *Dll* crispant mosaics overlapping the wing margin (Fig. 3B, Fig. S12). In *Ubx* crispants, the levels of Dll protein were not affected in either type of eyespot foci, those with forewing homologs and those present only on hindwings (Fig. 3C). Dll was also not affected in *Ubx* crispant mosaics along the wing margin (Fig. S13). However, it is unclear whether loss of *Ubx* activity affects the *Dll* pattern in the eyespot foci as Ubx expression was stochastic at the stage when we examined the relationship between the two genes. These results suggest that *Dll* positively regulates *Ubx* along the wing margin, but *Ubx* does not regulate *Dll* expression.

Genetic interaction between *Ubx* and *sal*

Next, we examined the relationship between *Ubx* and *sal* (Fig. 3D). In *sal* crispant cells, Ubx protein levels were not affected (Fig. 3E, Fig. S14). However, in *Ubx* crispants, Sal protein levels were reduced, but not lost, in cells in the center of the wing (Fig. S15). In the eyespot foci, the interaction of *Ubx* with *sal* was sector specific and different between the sectors containing eyespots on both wings and sectors with hindwing-specific eyespots. Sal protein expression in the Rs and M2 eyespot foci (an eyespot present only on hindwings) was lost in *Ubx* crispant mosaics (Fig. 3F, Fig. S15D), whereas Sal protein expression in the Cu1 eyespot (an eyespot with a forewing serial homolog) was not affected in *Ubx* crispant mosaics (Fig. 3F). These results suggest that *Ubx* is required to activate *sal* in the wing sectors with hindwing-specific eyespots, but not in the wing sectors with eyespots on both wings.

Genetic interaction between *Ubx* and *Antp*

We next examined the relationship between *Ubx* and *Antp* (Fig. 3G). We found that Ubx protein levels were not affected in *Antp* crispant mosaics (Fig. 3H, Fig. S16). However, *Ubx* regulates *Antp* in a distinct way in different wing regions. In the distal margin, Antp protein was upregulated in mosaics of *Ubx* crispant mosaics, with the level of ectopic Antp expression being comparable to the level observed in eyespot foci (Fig. 3I, Fig. S17). In the foci, Antp protein expression was lost in both forewing serial homologs and hindwing-specific eyespot foci (Fig. 3I). These results suggest that *Ubx* is required for *Antp* activation in the eyespot foci, but it represses *Antp* along the wing margin.

In summary, we found that in hindwings *Ubx* is required for *sal* activation in the hindwing-specific eyespot foci, and required for *Antp* expression in both kinds of eyespot foci. The main differences between the eyespot GRN in forewings and hindwings are that *Antp* is no longer essential to activate *sal* in hindwing foci, *Ubx* upregulates *Antp* in all hindwing eyespots, and *Ubx* upregulates *sal* in a sub-set of eyespots (those that are hindwing specific). Outside the foci, *Dll* upregulates *Ubx* expression along the wing margin, Ubx upregulates *sal* in more central wing regions, and represses *Antp* along the wing margin and other areas of the wing.

DISCUSSION

Differences in the differentiation of eyespot foci between fore- and hindwings

B. anynana gradually differentiates two eyespot foci in larval forewings and seven foci in hindwings to produce a corresponding number of eyespots on the adult wings (Fig. 1). The genetic mechanism that limits the development of eyespots in the M1 and Cu1 wing sectors on the forewing is not understood. In this study, we found that eyespot focal differentiation involves (1) Dll and Sal initially showing a similar protein expression pattern in both fore- and hindwings across most wing sectors, (2) Antp and Arm proteins only becoming expressed in four of those sectors in forewings (M1, M2, M3 and Cu1), but in seven sectors in hindwings, and (3) the initially competent four middle forewing sectors becoming further reduced to two sectors (M1 and Cu1), with the disappearance of Antp and Arm proteins (as well as Sal, Dll, Engrailed and Notch proteins; Monteiro et al., 2013) from those sectors. A previous study identified a candidate locus, *Spotty*, that performs this last role – of repressing eyespot development from the middle M2 and M3 sectors in forewings – but its molecular identity is still unclear (Monteiro et al., 2003).

The gene expression interactions examined here give us an insight about the genetic mechanisms underlying eyespot focus establishment across different wings and wing sectors. There are, at least, three different genetic mechanisms taking place in three sets of wing sectors; the M1 and Cu1 wing sectors, the M2 and M3 wing sectors, and the rest of the wing sectors where eyespots are present in hindwings but absent in forewings (sectors Rs, Cu2 and A1). The M1 and Cu1 sectors, and the M2 and M3 sectors, share the same mechanism for eyespot development until the stage when Antp and Arm proteins disappear from the M2 and M3 sectors in forewings (Fig. S4). The remainder forewing sectors express Dll and Sal proteins, as in other sectors, but not Antp, suggesting that Dll and Sal, which are required for eyespot development (Murugesan et al., 2022), are not sufficient for inducing eyespot development. These results suggest that Antp is indispensable for eyespot development on forewings.

Temporal expression dynamics of eyespot genes revealed differences in eyespot foci establishment between fore- and hindwings. Expression of eyespot genes appeared at the same time in forewings, whereas they appeared gradually in hindwings, and first in M3 and Cu1 sectors. It is possible that the time lag in hindwings is correlated with the relative differences in final size of hindwing eyespots, where M3 and Cu1 eyespots are among the largest.

We found a stochastic pattern of Ubx expression in foci whereby expression is sometimes downregulated in foci (Fig. S5). Oscillations in gene expression have been observed in various model systems,

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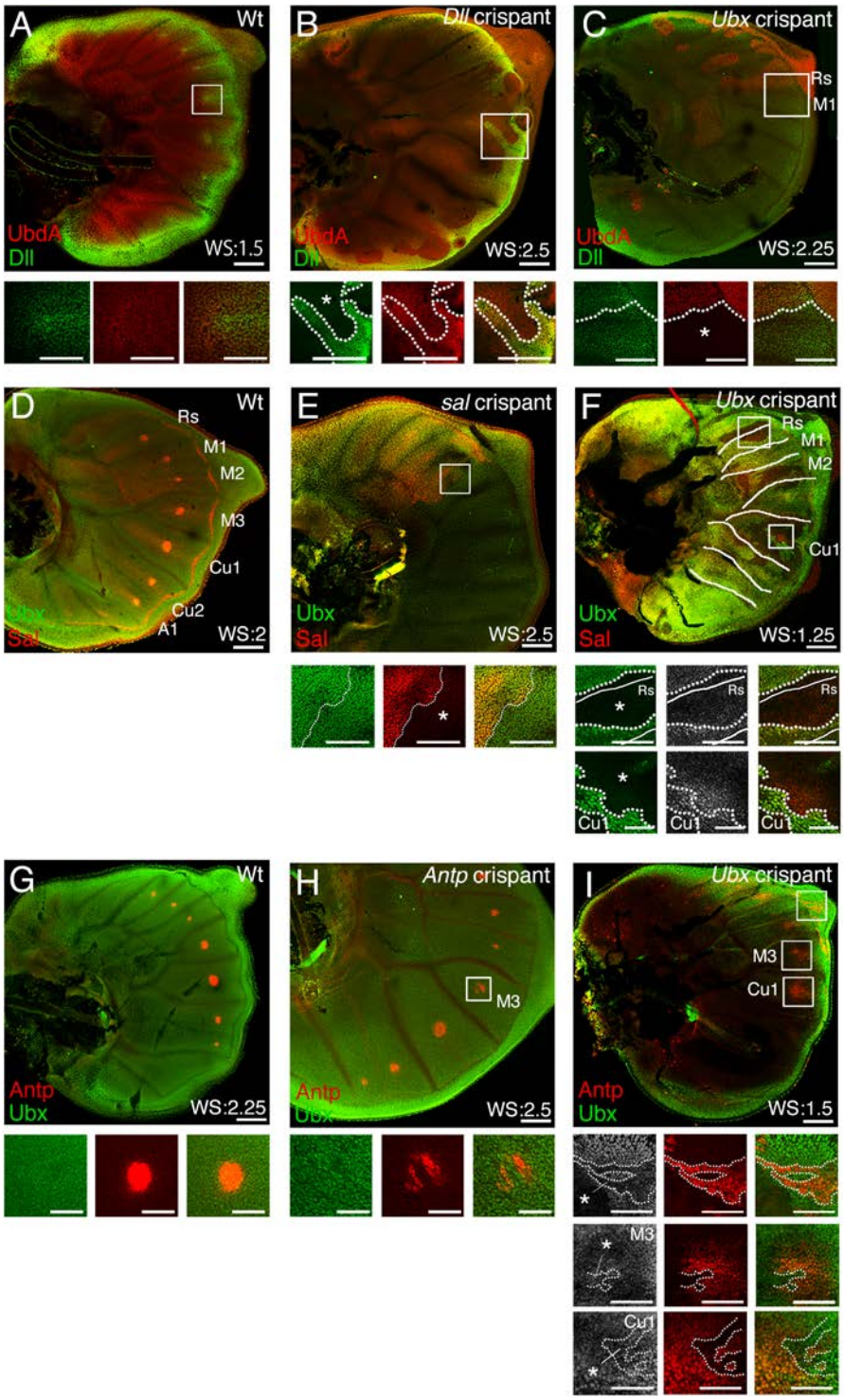

Fig. 3. Regulatory interactions of *Ubx* with *Dll*, *sal* and *Antp* on the hindwing. (A-C) Expression pattern of Dll and Ubx protein (detected using a UbdA antibody) in a Wt hindwing (A), a *Dll* crispant hindwing (B) and a *Ubx* crispant hindwing (C). (D-F) Expression pattern of Ubx and Sal in a Wt hindwing (D), a *sal* crispant hindwing (E) and a *Ubx* crispant hindwing (F). (G-I) Expression pattern of Antp and Ubx in a Wt hindwing (G), an *Antp* crispant hindwing (H) and a *Ubx* crispant hindwing (I). Boxed areas are shown at higher magnification below, as indicated. Dotted lines delineate regions where the protein from the targeted gene is missing, marked with asterisks. Solid lines in F represent veins. All the wings are larval wings at the indicated wing stage (WS) (Reed et al., 2007). Scale bars: 100 µm (whole wing images); 50 µm (high-magnification images).

including in somitogenesis of vertebrates (Aulehla et al., 2003; Riedel-Kruse et al., 2007), and several signaling molecules (Wnt and Notch signaling) known to show oscillations are active during eyespot development (Reed and Serfas, 2004; Monteiro et al., 2006). A future study could try to address these stochastic patterns further.

Differences in the gene regulatory interaction of eyespot essential genes between fore- and hindwings

The differences in the dynamic patterns of focal differentiation between forewings and hindwings stem from the exclusive presence of Ubx proteins in hindwings, as disruptions of this gene transform hindwing patterns into forewing patterns (Matsuoka and Monteiro, 2021). These homeotic transformations include the removal of hindwing-specific eyespots, and the enlargement of two eyespots that have forewing homologs, indicating that Ubx interacts with these two types of eyespots in different ways. In this study, we examined how *Ubx* interacts with three other genes with an essential function in eyespot development in forewings (*Antp*) or both wings (*Dll* and *sal*), and how these interactions differ between forewings and hindwings and between hindwing sectors. We found six main

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results that are summarized in Fig. 4A-C: (1) *Dll* is required for both *Antp* and *sal* activation in hindwing eyespot foci, as also observed in forewings; (2) *sal* is required for *Antp* activation, as in forewings; (3) *sal* can impact *Dll* expression in the foci; (4) *Antp* upregulates *sal* in forewings eyespot foci, but is no longer required for *sal* activation in hindwings; (5) *Ubx* is required to activate *Antp* expression in all eyespot foci on hindwings; and (6) *Ubx* is required for *sal* activation only in hindwing-specific eyespots. We will discuss these six results in turn.

Results (1) and (2) indicate that the eyespot GRN is largely similar between forewings and hindwings, that *Dll* is a top regulator, impacting both the expression of *sal* and *Antp*, and that *sal*, together with *Dll*, is essential for *Antp* activation.

Result (3) is novel as we observed that *sal* can also impact *Dll* expression in the foci (Fig. 2C), and split the foci into two, when specific *sal* crispant mosaics transect the foci (Fig. S18; Murugensan et al., 2022). However, we speculate that this regulatory interaction might also be present in forewings. In the previous study, the *sal* crispant mosaics examined covered the whole eyespot region, and Dll expression in the fingers was not visibly affected, but it might be possible that the level of Dll is affected (Murugesan et al., 2022). We propose that *sal* regulates *Dll* expression in the foci through the reaction-diffusion mechanism proposed for focus differentiation (Connahs et al., 2019). When this mechanism is disrupted, via loss of *sal*, the foci split apart. Further modeling of the reaction-diffusion mechanism, including *sal*, should be performed in future.

Result (4) shows that whereas *sal* is still essential for *Antp* expression in hindwings, *Antp* merely upregulates *sal* expression in the foci (Fig. 2H,I), rather than being essential for *sal* expression, as in forewings (Murugesan et al., 2022). We speculate that this regulatory difference between wings is, in part, due to redundant functions of *Antp* and *Ubx*, with *Ubx* also being involved in activating *sal* in some wing sectors, as discussed below.

Result (5) – *Ubx* being required to activate *Antp* expression in all hindwing eyespot foci – is novel. This positive regulation is contrary to what is normally observed for Hox genes, i.e. more-posterior Hox genes (such as *Ubx*) are often found to negatively regulate the more-anterior Hox genes (such as *Antp*), at least in embryonic stages of insect development (Gummalla et al., 2014). Although this negative cross-regulation is still observed overall on the hindwing (discussed below), it is not present in the focal cells. This indicates that the regulation of *Antp* by *Ubx* in the foci is unique and different from how these genes interact elsewhere on the wing.

In result (6), we found that *Ubx* regulates *sal* expression in a hindwing-sector specific manner. *Ubx* is essential for *sal* expression in the hindwing-specific eyespots, but does not visibly affect *sal* expression in the hindwing eyespots that have forewing homologs (Fig. 3F). This result can help explain why *Ubx* disruptions led to loss of hindwing-specific eyespots alone, as *sal* is an essential gene for eyespot development, making *Ubx* also an essential gene for eyespot development in these wing sectors (Matsuoka and Monteiro, 2021). How eyespots are negatively regulated by *Ubx* in the M1 and Cu1 hindwing sectors, however, is unclear. This negative regulation was shown in a previous study in which M1 and Cu1 eyespots increased in size after *Ubx* function was removed in crispant mosaics (Matsuoka and Monteiro, 2021). We found that Sal expression in M1 and Cu1 eyespots was maintained in *Ubx* crispants, but *sal* might be upregulated after the loss of *Ubx* activity. In this scenario, *Ubx* might negatively regulate eyespot development by negatively regulating *sal* expression in these wing sectors. As we cannot distinguish whether the level of Sal protein is affected or not, a future study may address this possibility further.

Antp is no longer an essential gene in eyespot development in hindwings because disruptions of this gene merely result in loss of the white centers and in smaller eyespots (Matsuoka and Monteiro, 2021). One possible scenario is that this could be due to *Ubx* filling a partially redundant function with *Antp* in eyespot regulation in the

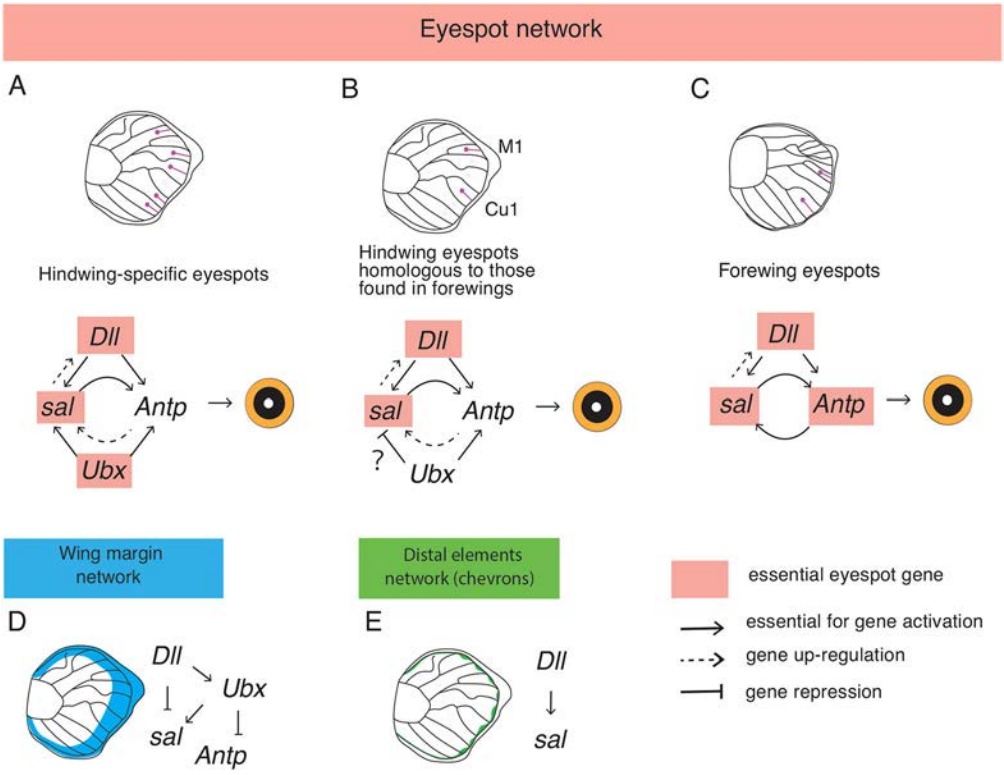

Fig. 4. Schematic of genetic interactions among *Dll*, *sal*, *Antp* and *Ubx* in the hindwing. (A) In the focal region of hindwing-specific eyespots, *Ubx* is necessary for eyespot development, and *Ubx* activates *sal* expression. (B) In the focal region of hindwing eyespots that have forewing homologs, *Ubx* negatively regulate eyespot size, possibly via *sal* repression. (C) Genetic interaction in the eyespots on the forewing (from Murugesan et al., 2022). (D) Genetic interaction in the wing margin region. (E) Genetic interaction in the distal elements (chevrons) found in the wing margin.

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hindwing-specific eyespots, perhaps via the joint regulation of *sal*. This could happen due to the undifferentiated binding of both Hox genes to the same regulatory DNA sequences (Slattery et al., 2011). In forewings, *Antp* would be essential to activate *sal*, whereas in hindwings, *Ubx* could partially take over the role of *Antp*, making *Antp* no longer essential for eyespot foci differentiation.

Gene-regulatory interactions outside of the eyespot foci

In this study, we also examined regulatory interactions between the four genes outside the eyespot regions in hindwings of *B. anynana* (Fig. 4D,E). These data gave us extra insights into the GRN involved in general wing development and patterning, as this GRN is not well studied in insects other than *Drosophila*.

Here, we show that *Dll* functions as both an activator as well as a repressor of *sal* outside of the foci (Fig. 2B, Fig. 4D,E, Fig. S6). *Dll* functions as an activator of *sal* along the midline fingers and marginal chevrons (Fig. 4A,E). Previous computer simulations demonstrated that the finger and focal pattern of Dll is likely produced via a reaction diffusion mechanism that starts with Dll being uniformly expressed in the margin of the wing (Connahs et al., 2019). Our results suggest that eyespot foci, finger patterns, and margin chevrons might be the result of the same GRN, as also suggested recently in independent modeling work (Nijhout, 2017). In the broader wing margin, however, outside the defined Sal stripe along the chevrons, *Dll* functions as a repressor of *sal* (Fig. 2B, Fig. 4D, Fig. S6). To our knowledge, the interaction between *Dll* and *sal* on the wing of insects has not been examined even in *Drosophila*. *Drosophila Dll* mutants show subtle changes in bristle formation along the wing margin that scarcely affect wing development (Campbell and Tomlinson, 1998). By contrast, in a previous study, we found that loss of *Dll* affects wing shape, in addition to the loss of wing scales and eyespots in *B. anynana* (Connahs et al., 2019). Our data suggests that *Dll* defines the distal limit of *sal* expression in butterfly wings, because *Dll* disruptions led to ectopic *sal* expression in the distal wing region, and resulted in deformed wings. These results suggest that the mechanism for wing margin development might be different between butterflies and flies.

Counteracting the repressive effects of *Dll* effects on *sal* in the wing margin, *Ubx* positively regulates *sal* outside of the eyespot foci (Fig. 3F). A similar regulatory interaction is observed in *Tribolium*, where *Ubx* positively regulates *sal* in the flight wing (Tomoyasu et al., 2005). In the haltere of *Drosophila*, however, *Ubx* negatively regulates *sal* (Galant et al., 2002), so we speculate that this repressive function of *Ubx* is a derived feature of flies.

Interestingly, *Dll* also upregulates *Ubx* in the distal wing margin, but *Ubx* does not regulate *Dll* (Fig. 3B,C, Fig. 4D). This finding is surprising as *Ubx* is usually considered to be a modifier of the wing GRN, rather than being incorporated into the wing GRN and being itself modified by it. However, Ubx protein was not completely absent in *Dll* crispant mosaics (Fig. 3B), so *Dll* is not necessary for the activation of *Ubx* on the hindwing, but *Dll* increases *Ubx* expression along the margin. It is possible that this regulatory interaction between *Dll* and *Ubx* might have aided in the origin of eyespots initially restricted to hindwings, as discussed below.

We found that *Ubx* represses *Antp* in wing regions outside of the eyespot foci (Fig. 3I, Fig. S17). The same regulatory interaction is observed in *Drosophila* (Tsubota et al., 2008; Domsch et al., 2019). The upregulation of *Antp* in *Ubx* crispants gives us an insight into the genetic mechanism behind the typical homeotic transformation in *Ubx* crispants, including that previously described for *B. anynana*, whereby hindwings were transformed into forewings (Matsuoka and Monteiro, 2021). It has been believed that the forewing of most insects is in a Hox-free state, and that *Ubx* gives hindwings their unique identity. However, in this study we found that Antp proteins were elevated in *Ubx* crispant mosaics (Fig. 3I, Fig. S17), uncovering a repressive role of *Ubx* on *Antp* and the likely expression of *Antp* (albeit at low levels) in forewings as well (Fig. 4D). These results indicate that *Antp* is likely necessary for *B. anynana* forewing differentiation, as has been recently shown for *Bombyx*, *Drosophila* and *Tribolium* (Paul et al., 2021; Fang et al., 2022). In a previous study, we also showed that Sal proteins were lost in *Antp* crispant mosaics outside of the eyespot region in forewings (Murugesan et al., 2022), suggesting that Antp proteins are present in the wing at low levels, and are required for regulating *sal*. In addition, the shape of the forewing was slightly deformed in *Antp* crispants (Matsuoka and Monteiro, 2021). These results indicate that *Antp* plays a role in forewing differentiation in *B. anynana*, and that butterfly forewings do not represent a Hox-free state.

Overall, we uncovered a partial gene regulatory network for wing development in *B. anynana* butterflies, and showed that, although the expression of wing patterning genes is highly conserved, their genetic relationship is slightly different from that of *Drosophila* and beetles.

Possible genetic mechanism underlying eyespot origins and eyespot number evolution

Ancestral state reconstructions on a large phylogeny of ∼400 genera suggested that eyespots first originated in four to five wing sectors on the ventral side of the hindwings of an ancestral lineage of nymphalid butterflies, before appearing on forewings and on dorsal sides of both wings (Oliver et al., 2014; Schachat et al., 2015). Recently, we proposed a possible function of *Antp* and *Ubx* as required genes for eyespot evolution (Matsuoka and Monteiro, 2021). Here, we provide further insight into the genetic mechanism that may have allowed the origin of eyespots on hindwings first, followed by eyespot origins on forewings.

Prior to the partial appendage GRN co-option event, proposed to have led to eyespot origins (Murugesan et al., 2022), nymphalid butterflies had no eyespots (Fig. 5A). We propose that after co-option of the appendage GRN, the genes *Dll* and *sal* gained a novel expression domain in the eyespot foci in fore- and hindwings, which, together with essential *Ubx* input restricted to hindwings, allowed eyespots to emerge in hindwings only (Fig. 5B). These eyespots might have originally lacked a white center. The origin of forewing eyespots in satyrid butterflies might be connected to *Antp* having acquired a novel expression domain in eyespot foci, initially dependent on *Ubx*, which subsequently allowed *Antp* to take on the eyespot-activating function of *Ubx* in forewings (Fig. 5C). In addition, the expression of *Antp* in eyespots might have aided the origin of the white centers (Fig. 5C) as *Antp* crispants lose these white centers in hindwing eyespots (Matsuoka and Monteiro, 2021). Eyespot number and size were further modified in each lineage and species probably through the introduction of mutations in genes such as *Spotty* (Fig. 5D). The wild-type (Wt) version of the *Spotty* gene represses eyespot development in the same two central wing sectors (M2 and M3) on both forewings and hindwings (Monteiro et al., 2007). *Ubx*, however, might partially repress *Spotty*, leading to the development of eyespots in those wing sectors in hindwings. *Ubx* also acquired a novel function to negatively regulate the size of the hindwing eyespots that have forewing counterparts (M1 and Cu1). This could be through a negative regulation of *sal* expression, but more evidence is needed here.

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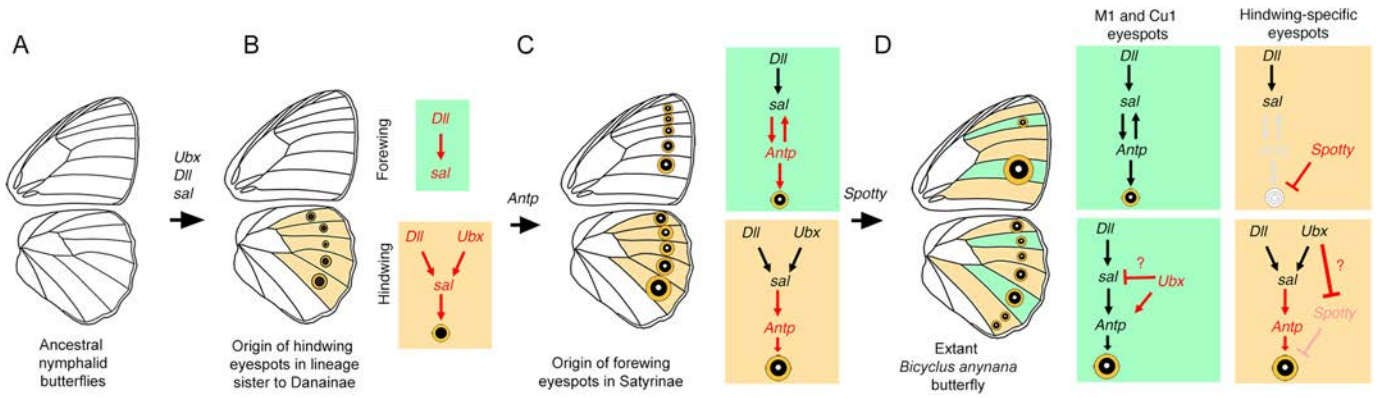

Fig. 5. Proposed evolution of genetic interactions underlying the origin of eyespots. (A) Common ancestral nymphalid butterflies did not have eyespots on their wings. (B) The first eyespots likely appeared on the hindwing through the co-option of an appendage GRN. This might have led to the novel expression of *Dll* and *sal*, in the eyespot foci of hindwings alone, as Ubx is also essential for *sal* expression in most of the hindwing eyespots. (C) Once *Antp* was co-opted to the eyespot GRN, under *Ubx* regulation, the first eyespots would have originated on the forewing, using *Antp* as the essential gene for *sal* activation in forewings. Eyespots might have also gained a distinct center at this stage. (D) The number and size of eyespot were likely modified in each lineage using sector-specific genes. The Wt version of the *Spotty* locus led to the loss of eyespots in the M2 and M3 wing sectors on the forewing.

In conclusion, in this study we have begun to address why a different number of eyespots develop on fore- and hindwings of *B. anynana* butterflies by directly examining the regulatory interactions of a few eyespot essential genes, and their interaction with a hindwing-specific selector gene, *Ubx*. We uncover part of a mechanism for wing sector-specific regulation of eyespot development directed by *Ubx*, and propose a more detailed molecular mechanism to explain the hindwing-specific origin of eyespots. Future work, such as an analysis of the total RNA species present in each wing sector, as well as functional experiments across species, may further advance our understanding of the genetic mechanisms underlying eyespot number differences between wings and how eyespot evolution proceeded in nymphalid butterflies.

MATERIALS AND METHODS

Butterfly husbandry

B. anynana, originally collected in Malawi, have been reared in the lab since 1988. Larvae were fed on young corn plans and adults on mashed banana. *B. anynana* were reared at 27°C and 60% humidity in a 12 h:12 h light:dark cycle.

Short guide RNA (sgRNA) design

sgRNA target sequences were selected based on their GC content (around 60%), and number of mismatch sequences relative to other sequences in the genome (at least four sites). In addition, we picked target sequences that started with a guanidine for subsequent *in vitro* transcription by T7 RNA polymerase.

sgRNA production

The template for *in vitro* transcription of sgRNA was made with the PCR method described by Matsuoka and Monteiro (2018). The forward primer contained the T7 RNA polymerase binding site and sgRNA target site (GAAATTAATACGACTCACTATAGNN$_{19}$ GTTTTAGAGCTAGAAAT-AGC). The reverse primer contained the remainder of sgRNA sequence (AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGAC-TAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC). PCR was performed with Q5 High-Fidelity DNA Polymerase (NEB) in 100 µl reaction volumes. After running PCR amplicon on a gel electrophoresis, the PCR product was purified with the Gene JET PCR purification kit (Thermo Fisher). *In vitro* transcription was performed overnight with T7 RNA polymerase (NEB) using 500 ng of purified PCR product as a template. After removal of template DNA with DNase I treatment, the RNA was purified by ethanol precipitation. The RNA was suspended to RNase-free water and stored at −80°C. sgRNA sequences were described previously (Matsuoka and Monteiro, 2021; Murugesan et al., 2022).

Cas9 mRNA production

Plasmid pT3TS-nCas9n (Addgene #46757) was linearized with XbaI and purified by phenol/chloroform and ethanol precipitation. *In vitro* transcription of mRNA was performed with the mMESSAGEmMACHINE T3 kit (Ambion) using 1 µg of linearized plasmid as a template, and a poly(A) tail was added to the synthesized mRNA by using the Poly(A) Tailing Kit (Thermo Fisher Scientific). The RNA was purified by lithium-chloride precipitation, and then suspended to RNase-free water and stored at −80°C.

Embryo microinjections

Butterflies were allowed to lay eggs on corn leaves for 30 min. We co-injected 0.5 µg/µl final concentration of sgRNA and 0.5 µg/µl final concentration of Cas9 mRNA into embryos within 2-3 h of egg laying. Eggs were sunk into PBS, and injection was performed into the PBS. Food dye was added to the injection solution for visualization. Injected eggs were incubated at 27°C in PBS, and transferred onto wet cotton the next day, and further incubated at 27°C. After hatching, larvae were moved to corn leaves, and reared at 27°C with a 12 h:12 h light:dark cycle and 60% relative humidity. See Table S1 for a summary of injections.

Immunohistochemistry for embryos and wing tissues

Fifth instar wing tissues were dissected in PBS buffer under the microscope. The samples were fixed in 4% formaldehyde in Fix buffer (0.1 M PIPES pH 6.9, 1 mM EGTA pH 6.9, 1.0% Triton X-100, 2 mM $MgSO_4$) for 30 min on ice. The samples were washed with 0.02% Tween 20 in PBS (PBSTw) three times, 10 min per wash, and then the samples were incubated in 5% bovine serum albumin (BSA)/PBSTw for 1 h, or further stored at 4°C.

The samples were replaced into a 5% BSA/PBSTw solution containing primary antibody, and incubated at 4°C overnight. We used rabbit polyclonal anti-Dll (1:200, a gift from Grace Boekhoff-Falk, University of Wisconsin–Madison, WI, USA), mouse monoclonal anti-Antp 4C3 (1:200; Developmental Studies Hybridoma Bank), rabbit anti-*Junonia coenia* Ubx antibody [1:500; a gift from L. Shashidhara, Indian Institute of Science Education and Research (IISER), Pashan Pune, India], rabbit anti-Sal (1:20,000; de Celis et al., 1999), mouse anti-UbdA (1:200; Developmental Studies Hybridoma Bank) and anti-Arm (1:1000; Banerjee and Monteiro, 2020). For double staining, we added two primary antibodies in the same tube. The wings were washed three times with PBSTw, 10 min per wash. Then, the PBSTw was replaced with 5% BSA/PBSTw as a blocking reaction for 1 h, and this solution was replaced with a 5% BSA/PBSTw solution with an appropriate secondary antibody (1:200), and incubated at 4°C for 2 h.

Secondary antibodies used were: anti-rabbit AF488 (Invitrogen, A-11008), anti-rabbit AF555 (Invitrogen, A-21428), anti-mouse AF488 (Invitrogen, A28175), anti-mouse AF555 (Invitrogen, A21422), anti-guinea pig AF555 (Invitrogen, A-21435) and anti-rat AF555 (Invitrogen, A21434). The wings were washed three times, 10 min per wash, and mounted in ProLong Gold mounting media (Thermo Fisher Scientific). The images were taken under an Olympus FV3000 fluorescence microscope.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: Y.M., A.M.; Methodology: Y.M.; Validation: Y.M.; Formal analysis: Y.M.; Investigation: Y.M.; Data curation: Y.M.; Writing - original draft: Y.M.; Writing - review & editing: Y.M., A.M.; Supervision: A.M.; Funding acquisition: A.M.

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