

Supplementary materials

Shared regulatory networks link vein positioning and eyespot ring formation in butterflies

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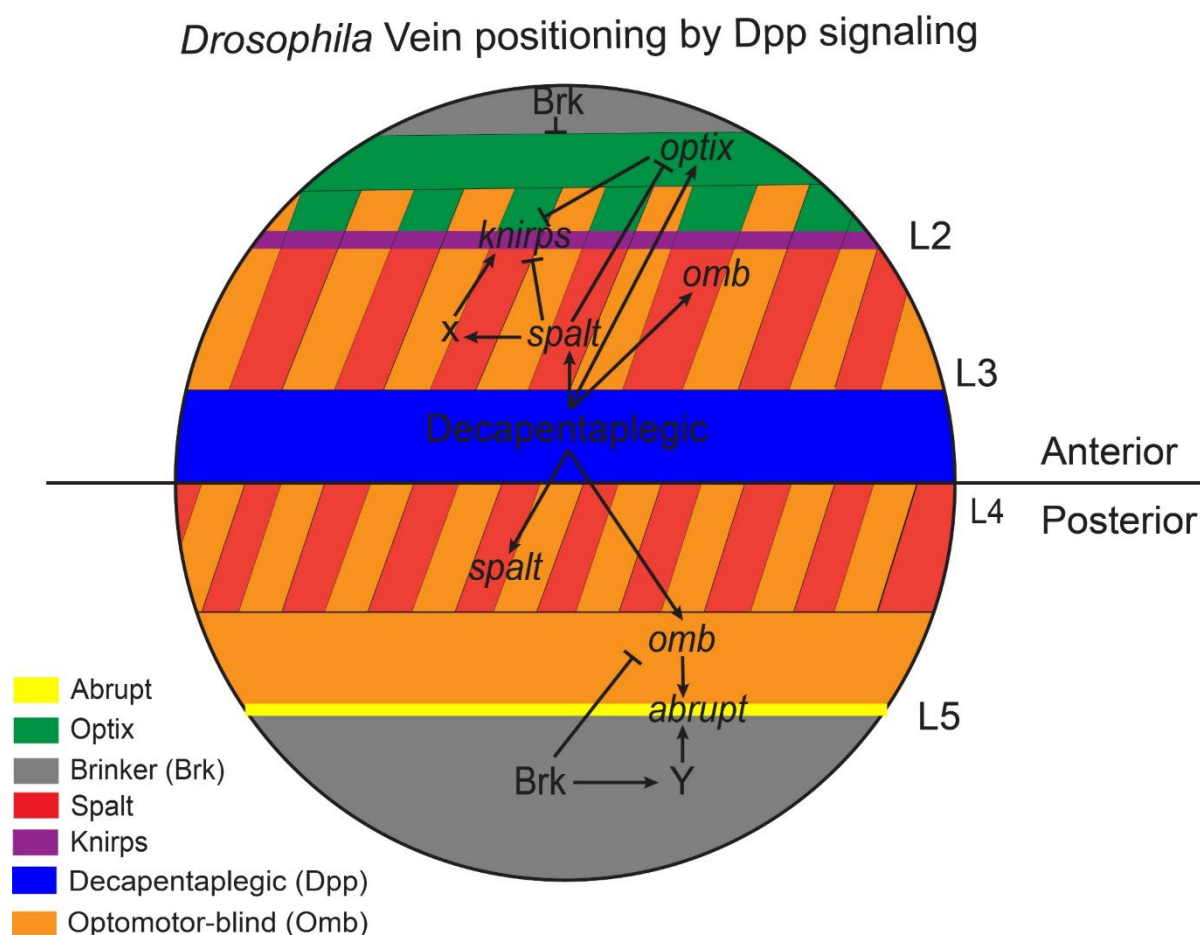
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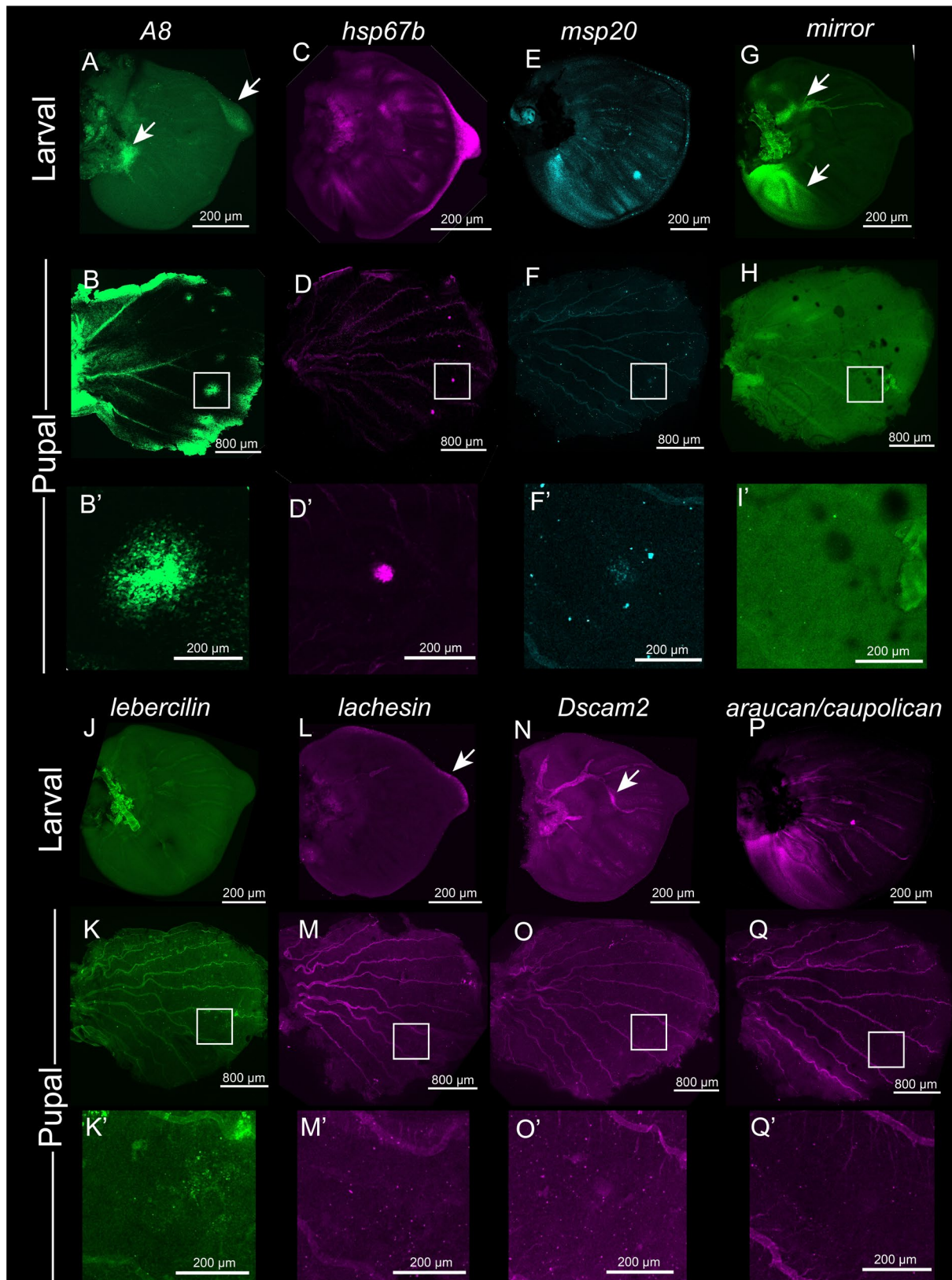
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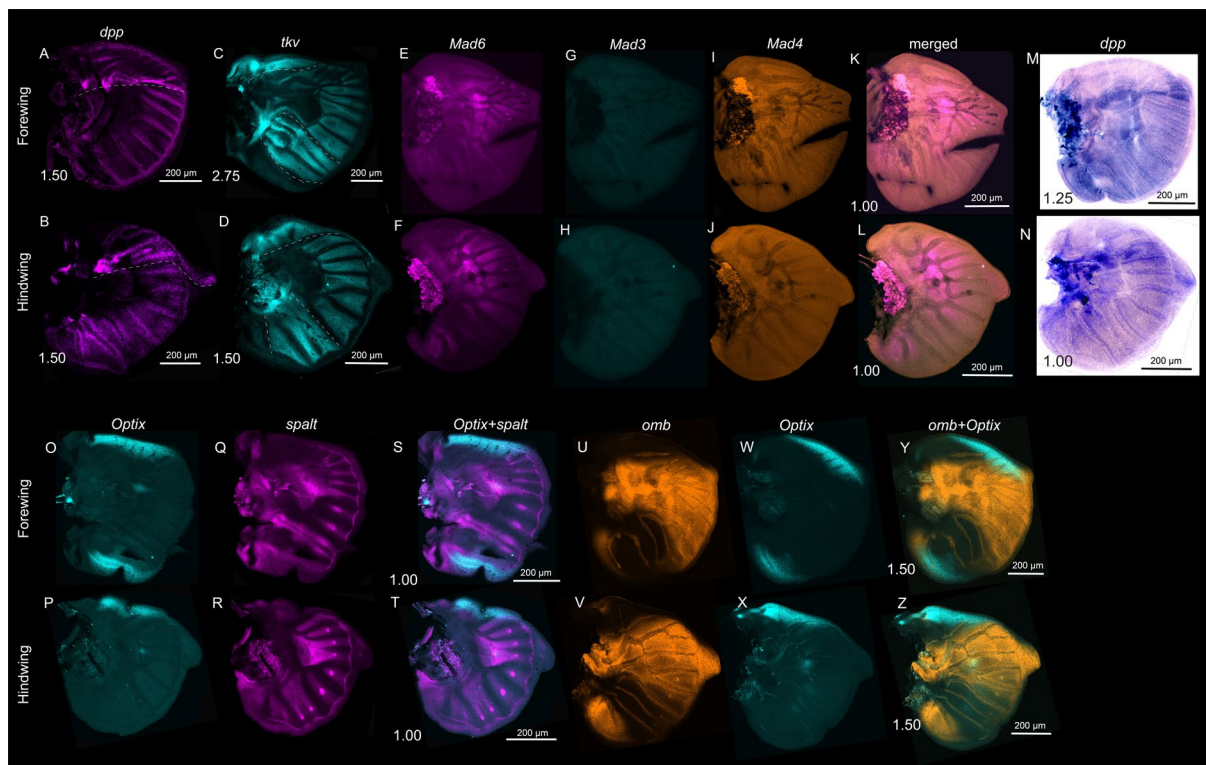
Supplementary Figure 1: Venation patterning in *Drosophila melanogaster* via Dpp signaling. High levels of Dpp activated at the AP boundary (between L3 and L4 veins) is capable of diffusing to the most anterior part of the larval wing disc where it activates key genes such as *Optix*, *omb* and *spalt* at different concentration thresholds, and is repressed by the transcription factor Brinker¹⁻⁴. Spalt protein represses the expression of *Optix* in the anterior compartment⁵. Spalt also activates the transcription factor *knirps* via a hypothetical

protein X⁶, while both Spalt and Optix repress *knirps*⁵ which defines the fate of the L2 vein⁵. In the posterior compartment, Omb activates the transcription factor *abrupt*⁷ while Brinker represses *omb*^{8,9}. Brinker is also responsible for the activation of *abrupt*⁷. Abrupt protein is responsible for the formation of the L5 vein⁷.

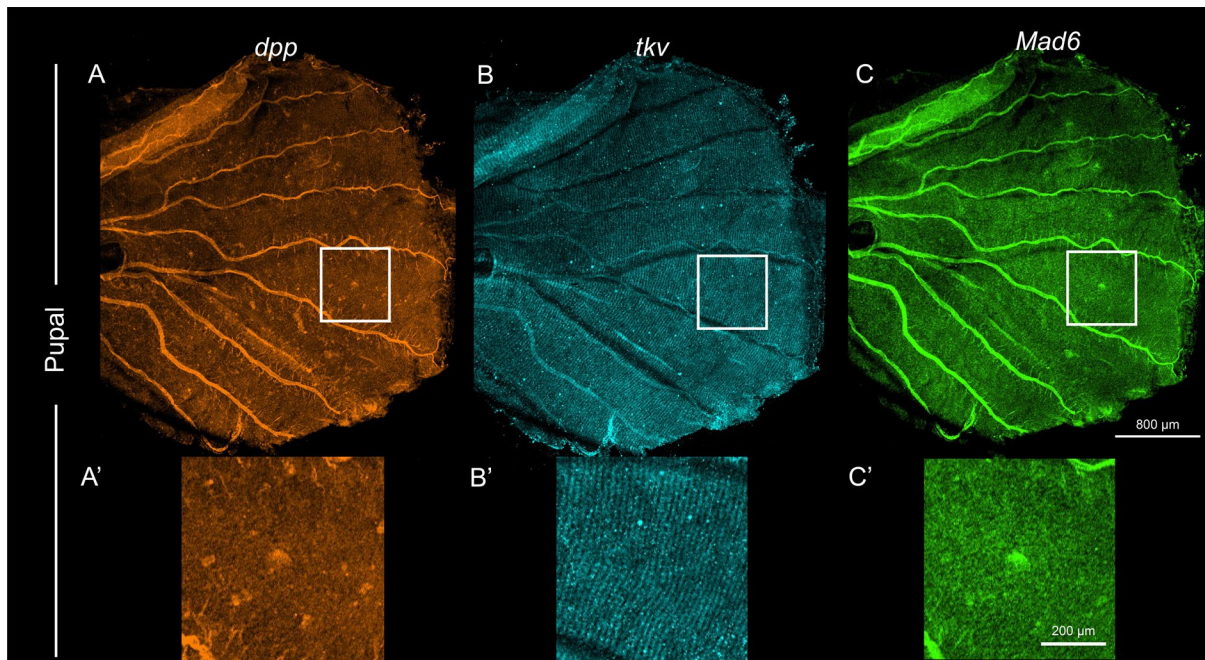


Supplementary Figure 2. Expression of *A8*, *hsp67b*, *msp20*, *mirror*, *lebercilin*, *lachesin*, *Dscam2*, and *araucan/caupolican* during larval and pupal wing development. (A) During the larval stage *A8* was expressed in two domains (white arrow). (B) During the pupal stage *A8* expression was observed strongly in the wing margin, in the eyespot center and the black scale cell domains. (C) *hsp67b* expression was observed in a complex domain during the

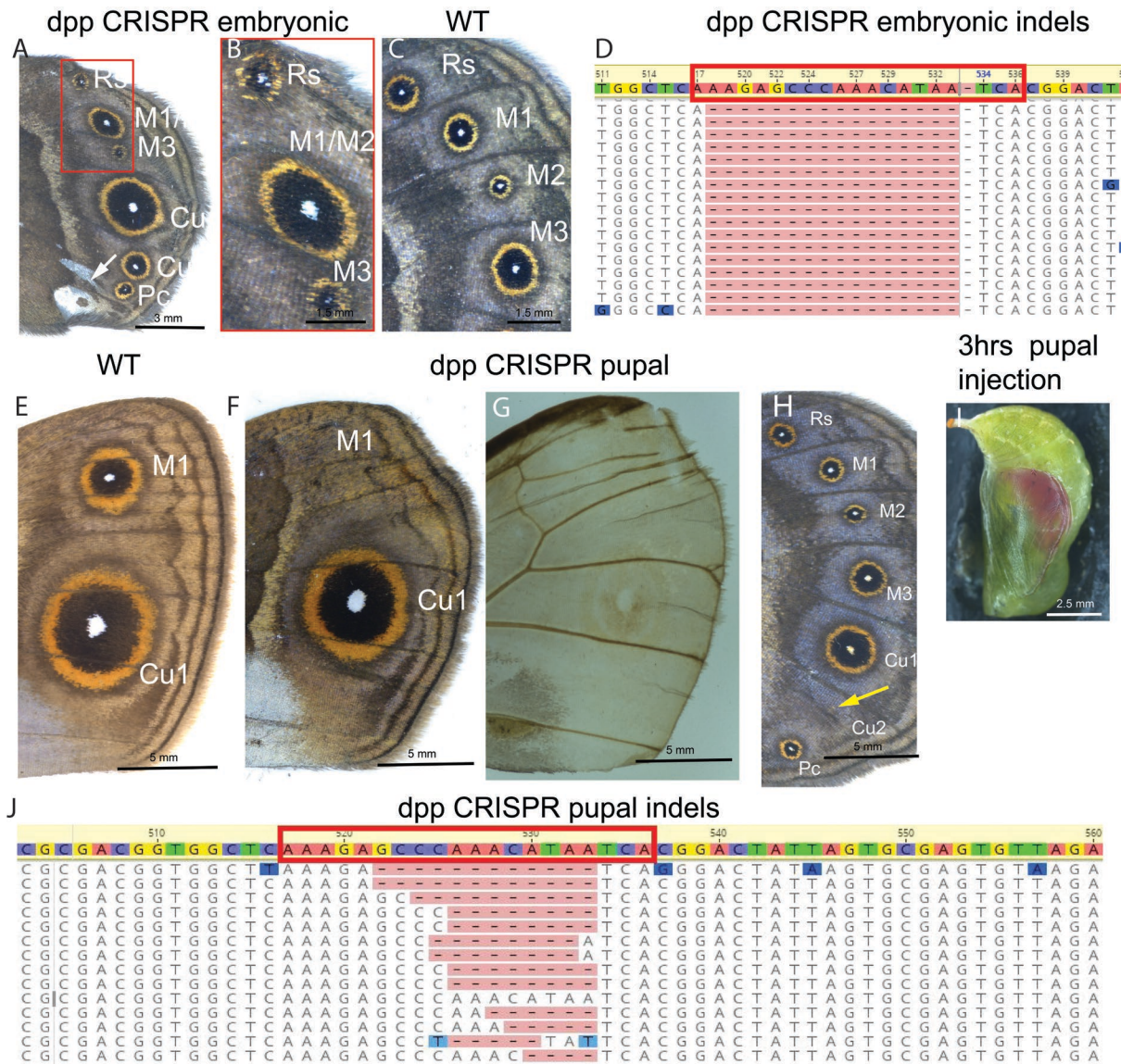
larval stage. (D) During the pupal stage, *hsp67b* expression was strongly observed in the center of the eyespots. (E) *msp20* expression was strongly observed in a single eyespot (Cu1) during the larval stage, along with stronger expression in the lower posterior compartment. (F) During the pupal stage, *msp20* expression continues in the Cu1 eyespot. (G) *mirror* expression was observed in the lower posterior compartment and in a proximal domain along the AP boundary. (H) During the pupal stage, no specific domain of *mirror* was observed. *lebercilin* didn't show any specific expression during the (J) larval stage and (K) pupal. (L) During the larval stage, *lachesin* was expressed in the anterior compartment along the wing margin (white arrow). (M) During the pupal stage, *lachesin* didn't show any specific expression domain. (N) During the larval stage, *Dscam3* was expressed in the discal spot. (O) During the pupal stage, *Dscam2* didn't show any eyespot specific expression. (P) During the larval stage, *araucan/caupolican* was expressed strongly in the lower posterior compartment. (Q) During the pupal stage, slightly elevated levels of *araucan/caupolican* were observed in the lower posterior compartment with no eyespot specific expression.



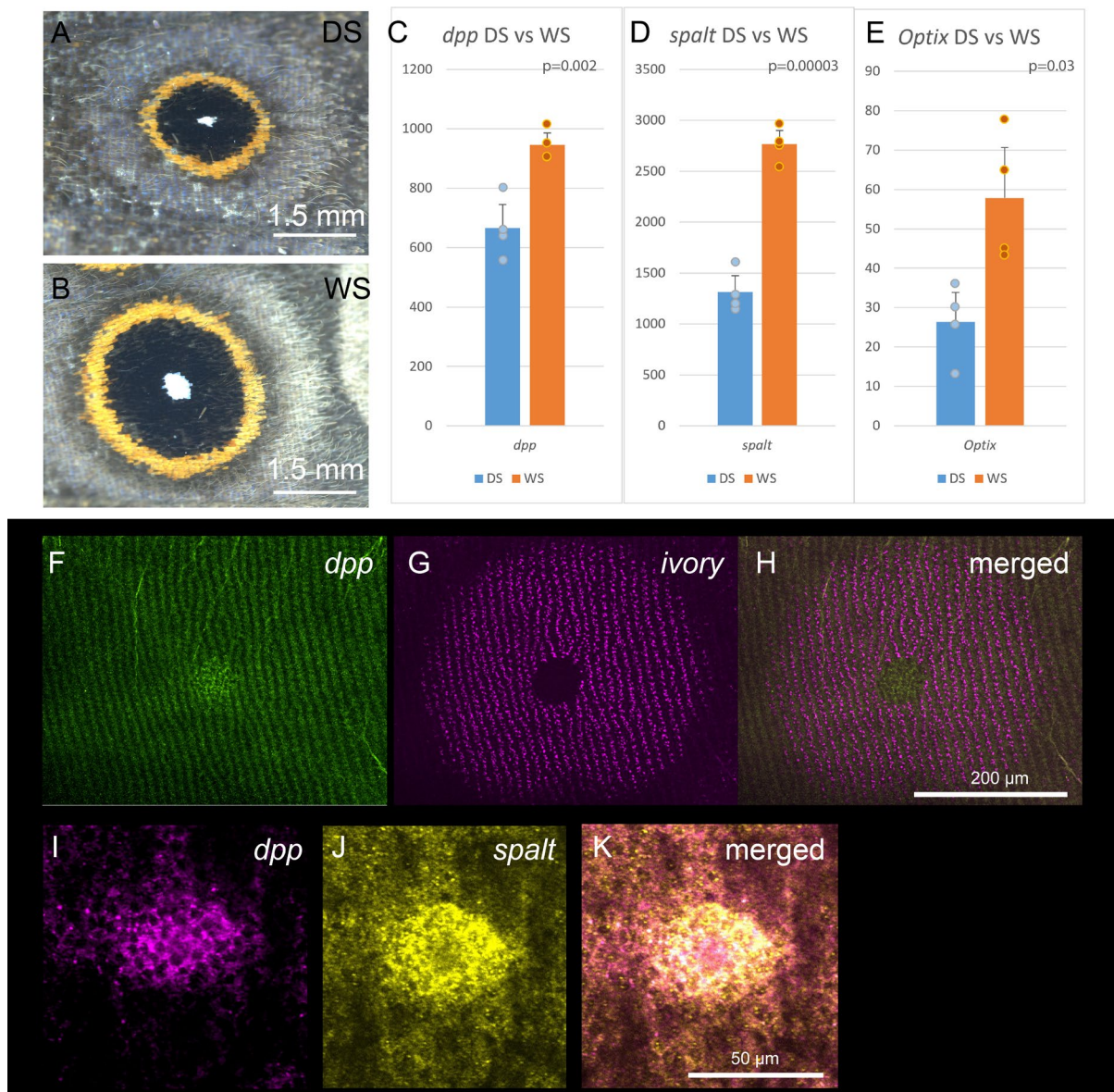
Supplementary Figure 3. Expression of *dpp*, *tkv*, *Mad6*, *Mad3*, *Mad4*, *Optix*, *spalt*, and *omb*. (A-Z) Expression of *dpp*, *tkv*, *Mad6*, *Mad3*, *Mad4*, *Optix*, *spalt*, and *omb* in the larval forewing and hindwing. No expression of *Mad3* and *Mad4* was observed.



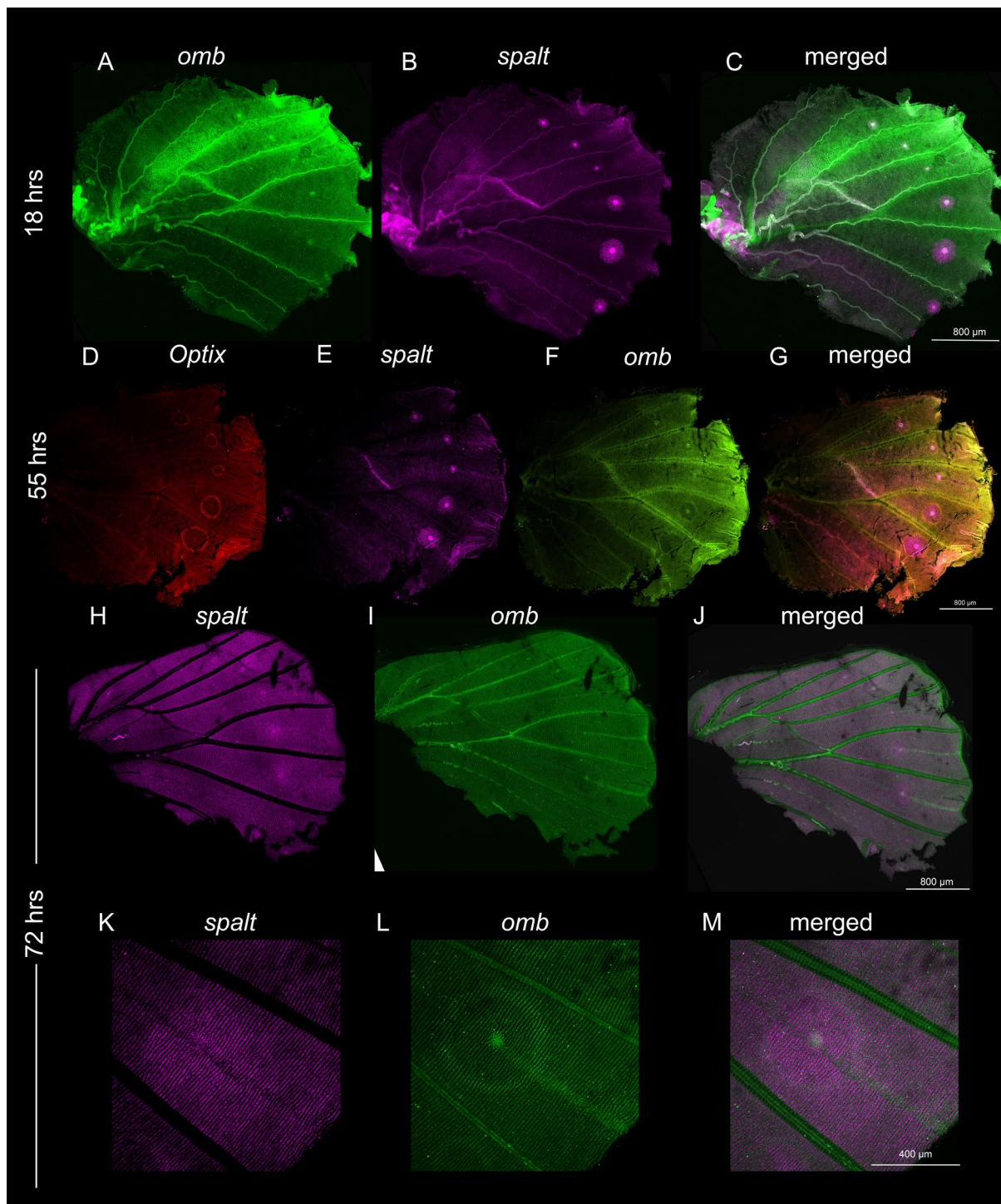
Supplementary Figure 4. Expression of *dpp*, *tkv*, and *Mad6* in a pupal wing of *B. anynana*. Expression of (A) *dpp* in the center of the eyespots, (B) *tkv* in the intervein cells, and (C) *Mad6* in the eyespot center and in the surrounding cells.



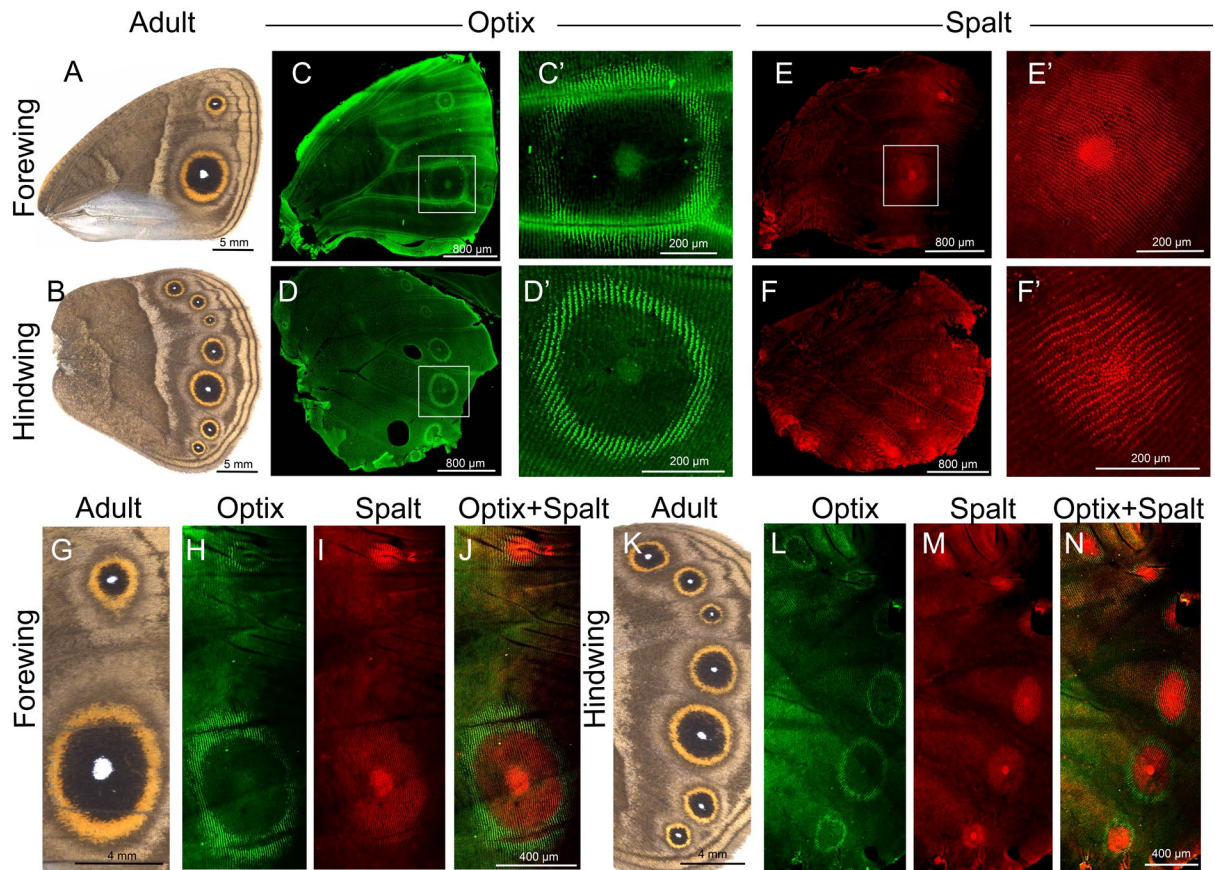
Supplementary Figure 5: Effect of *dpp* CRISPR on venation and eyespot development. (A and B) CRISPR-Cas9 injections of *dpp* during late embryonic development (6 hrs) resulted in the loss of either the M1 or M2 eyespot, increases in size of two eyespots M1 (or M2) and Cu1, and ectopic appearance of silver scales (white arrow) in a single individual (out of ~5000). (C) Adult WT hindwing. (D) Deletions (red box) 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 A and B. (E) WT adult forewing. (F-H) Pupal CRISPR-Cas9 injections using CRISPRMAX carrier resulted in a crispant showing loss of the anterior eyespot and disruptions in the M1 vein; and an individual with loss of Cu2 vein. (I) A 3 hr old pupa showing the injected medium (in red). (J) Indels at the site of *dpp* CRISPR from the wing in panel F. Embryonic injections resulted in high embryonic mortality. This is likely due to *dpp* being involved in patterning the early embryos. The red box indicate the CIRISPR target site.



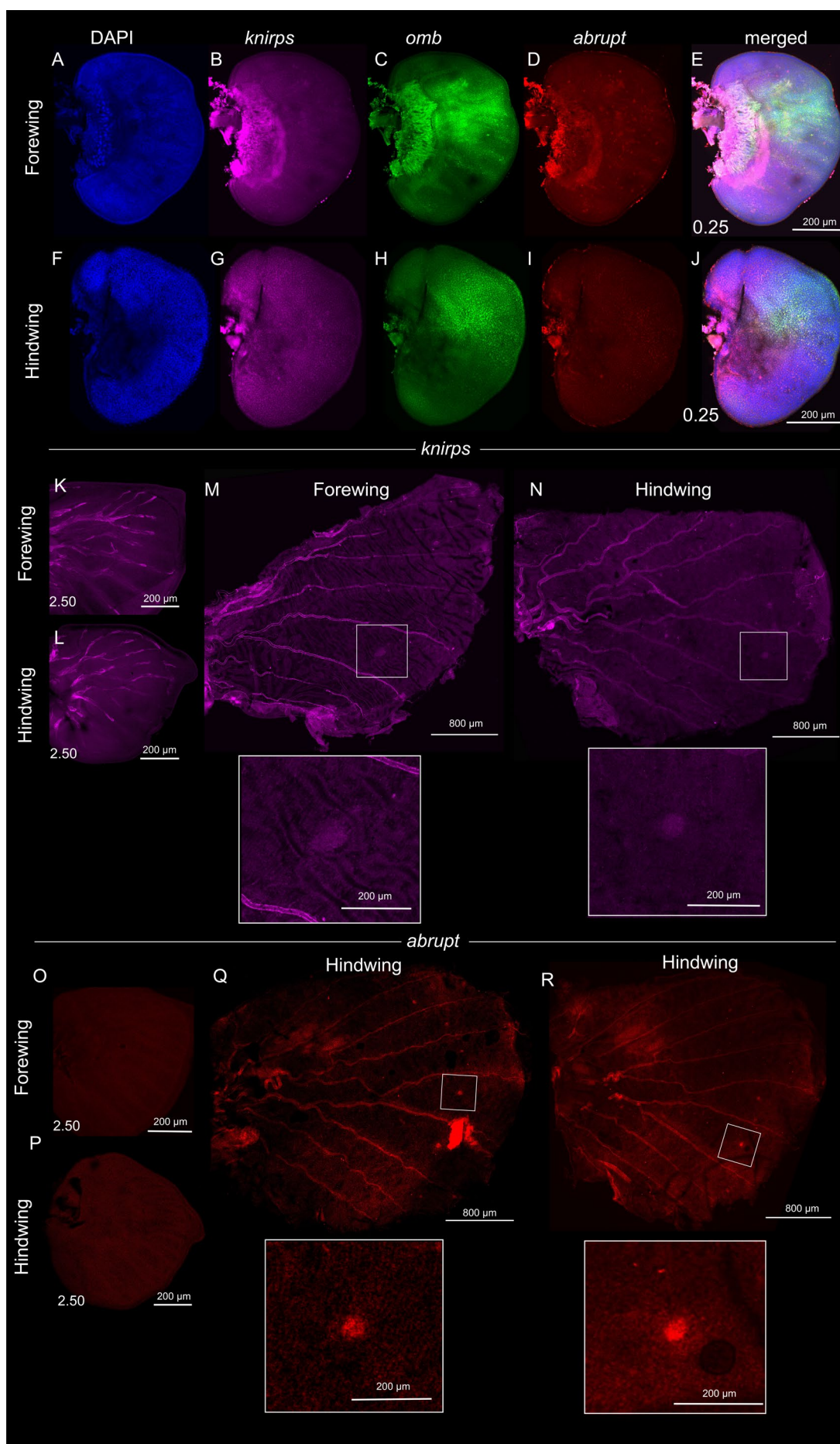
Supplementary Figure 6. Differential expression of *dpp*, *spalt*, and *Optix* in dry season (DS) and wet season (WS) forms, and the expression of *dpp*, *ivory*, and *spalt* in 20-24 hrs pupal wings. WT (A) DS and (B) WS eyespots. Eyespots are smaller in DS season forms reared at a lower temperature. Differential expression of (C) *dpp*, (D) *spalt*, and (E) *Optix* in the DS and WS forms (data from ¹⁰). Smaller domains of *spalt* and *Optix* are also observed in DS forms compared to WS forms ¹⁰. For the RNAseq methodology for DS and WS eyespot data, please check ¹⁰. Sample size for each type: 4 (Laser microdissection mediated RNAseq); Statistical test: ANOVA single factor; Error bars are standard deviation. (F-K) Expression of *dpp*, *spalt*, and *ivory* shows that the expression of *dpp* is restricted to the white center of the eyespots. *ivory* is only expressed in the black scales of the eyespots and is absent in the eyespot white centers ¹¹.



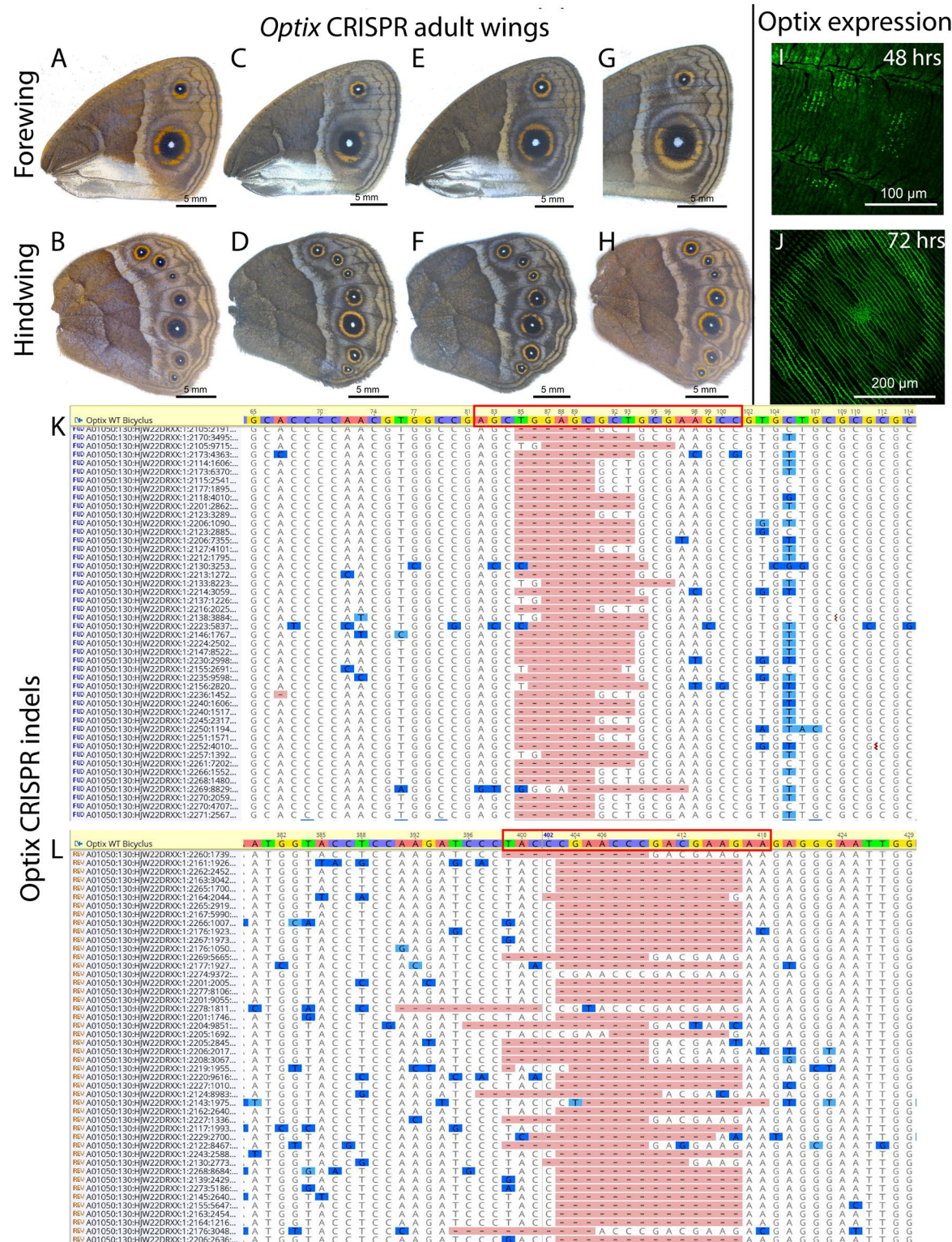
Supplementary Figure 7. Expression of *Optix*, *spalt*, and *omb* in 18-55 hrs pupal wings. (A-C) Expression of *spalt* and *Optix* in the 18 hrs pupal wings. (D-G) Expression of *Optix*, *spalt*, and *omb* in 55 hrs pupal wings. (H-M) Expression of *Optix* and *omb* in 72 hrs pupal wings. During the pupal wing development *omb* is expressed in the eyespot center, orange ring and along the conserved expression spanning the AP boundary, *spalt* is expressed in the eyespot center and the black disc cells, and *Optix* in the orange rings and along bands spanning the proximal-distal axis.



Supplementary Figure 8. *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.

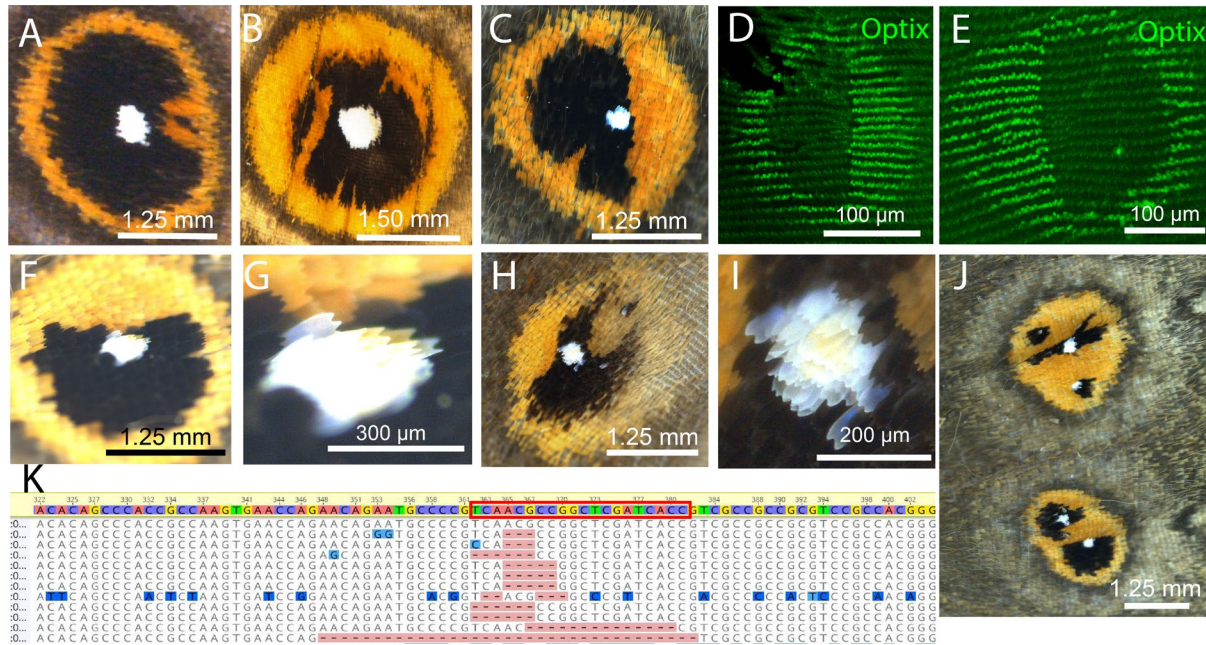


Supplementary Figure 9. Expression of *knirps*, *abrupt*, and *omb*. (A-J) Co-expression of DAPI, *knirps*, *omb*, and *abrupt* in early larval forewing and hindwing. *knirps* and *abrupt* didn't show any specific expression while *omb* was expressed in a broad AP domain and in the lower posterior domain. *knirps* didn't show any expression domain in the (K, L) late larval and (M, N) pupal wings. Expression of *abrupt* in the (O, P) late larval wing showing no specific expression domain and in the (Q, R) pupal wing showing expression in the eyespot centers.



Supplementary Figure 10. Function of *Optix* in *Bicyclus anynana* butterflies and localization of *Optix* protein in pupal wings. (A-H) *Optix* crisprants adult wings. *Optix*

knockouts result in the conversion of orange scales into brown scales in the eyespots. **(I and J)** Antibody staining of Optix proteins in an *Optix* crispant individual at 48 hrs and a WT 72 hrs old pupal wing. **(K and L)** Deletions at the two sites targeted for *Optix* CRISPR. The red boxes indicate the CIRISPR target site.



Supplementary Figure 11: 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). The red box indicate the CIRISPR target site.

Supplementary Table 1: 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	0.00064 6	0.20311 5
LOC112047337	toll-like receptor 7	0.78013	0.00070 2	0.21436 5
LOC112058222	lebercilin-like protein	7.045822	0.00074 1	0.22034 4
LOC112043001	uncharacterized protein LOC112043001	1.07768	0.00077 6	0.22448 2
LOC112048768	POU domain, class 6, transcription factor 2 isoform X1	-1.24136	0.00085 4	0.23696 7
LOC112054415	uncharacterized protein LOC112054415	0.83795	0.00086 2	0.23696 7
LOC112048478	protein mab-21	1.079178	0.00096 9	0.25985 9
LOC112050186	zinc finger protein 177-like	0.7199	0.00109 5	0.28658 6
LOC112051041	endocuticle structural glycoprotein ABD-5-like	2.864416	0.00112 6	0.28791 1
LOC112052161	protein patched isoform X1	0.902902	0.00116 2	0.29037 5
LOC112050109	probable chitinase 10	-1.04185	0.00126	0.30802 8
LOC112053786	uncharacterized protein LOC112053786	1.102876	0.00133 8	0.31975 5
LOC112055429	alaserpin-like isoform X1	0.69989	0.00153 4	0.35887 6
LOC112048344	cholinesterase 2-like	0.728368	0.00161	0.36884 4
LOC112043999	large neutral amino acids transporter small subunit 1	-0.5963	0.00208 5	0.46783 1

Supplementary Table 2: 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

Supplementary Table 3: Primer and CRISPR guide 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.	optix_CRISPR_1	GAAATTAATACGACTCACTATAGGGGCTTCGCAG CGCTCCAGCTGTTTTAGAGCTAGAAATAGC
10.	optix_CRISPR_2	GAAATTAATACGACTCACTATAGGTTCTTCGTCGG GTTCCGGTAGTTTTAGAGCTAGAAATAGC
11.	spalt_CRISPR_1	GAAATTAATACGACTCACTATAGGTGATCGAGCC GGCGTTGAGTTTTAGAGCTAGAAATAGC
12.	CRISPR_reverse	AAAAGCACCGACTCGGTGCCACTTTTT CAAGTTGATAACGGACTAGCCTTATTT TAACTTGCTATTTCTAGCTCTAAAC

Note: Synthetic guide was ordered for dpp CRISPR.

Supplementary Table 4. 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 ₂ O	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
	H ₂ O	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 ₂ O	179 ml
Mounting media	Tris-HCl (pH 9.2)	20 mM
	N-propyl gallate	0.5%
	Glycerol	60%

Supplementary Table 5. Enzyme based *in-situ* hybridization Buffers.

Buffers	Chemicals	Amount
10X PBS (500 ml) * Sterilize by autoclaving.	K ₂ HPO ₄	5.34 g
	KH ₂ PO ₄	2.64 g
	NaCl	40.9 g
	DEPC treated H ₂ O	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 ₂ O	Till 1000 ml
	Formamide	20 ml

Pre-hybridization buffer (40 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

Supplementary Note 1: Sequences of genes and oligos

The section contains the gene and probe sequences for in-situs and CRISPR.

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

GTTCTTCAACGTAAGCGGCGTACCGGCCGACGAGGTGGCGCGCGGCCGACCTCTCGTT
 CCAACGAGCCGTCGGCACCACCGGCAGACAGAGACTGTTGTTGTACGACGTGGTGCGCC
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 GCCCAAACATAATCACGGAATATTAGTGCAGTGTAGAGAAGAAGACGCCGCGAGTGCGA
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 GGAGTGGCGGACGGCGCAGCCGCTGCTCATGCTGTACACGGAGGACGAGCGCGCGCGCG
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 TCGTCGACTTCGCGGACGTGGGCTGGAGCGACTGGATCGTGGCCCCGCACGGCTACGAC
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CTGCGTGCCGACGCAACTCTCATCTATATCTATGTTATATATGGACGAAGTGAACAATGT
GGTGCTTAAAACTATCAGGACATGATGGTGGTAGGCTGTGG

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

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GTTGTTGTACGACGTGGTGCGCCCTGGCCGCCGCGGCCACTCCGAGCCGATCCTG
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GCTCTGGGAGCGGCGCGACGGTGGCTC **AAAGAGCCCCAAACATAATCA** CGGACTA
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GGCGCAGCCGCTGCTCATGCTGTACACGGAGGACGAGCGCGCGCGCGCTCGCG
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TATATGGACGAAGTGAACAATGTGGTGGCTTAAAACTATCAGGACATGATGGTG
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Region of *Optix* used for CRISPR-Cas9 (Highlighted in red)

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GCGCCTGGCGCGCTTCTTGTGGTGCCTGCCCCGTGGCGCACCCCAACGTGGCCG **AGCTGGA**
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GGCGAGCAGAAGACGCACTGTTTCAAGGAGCGGACGCGATCTCTACTCCGAGAATGGTA
CCTCCAAGATCCC **TACCCGAACCCGACGAAGAA** GAGGGAATTGGCGGCGGCGACGGGTC
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CGCCGCCAAGAACCCTCCGCCGTGCTGGGCAGAGGATAA

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

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

decapentaplegic (dpp)

>decapentaplegic_B1_XM_052883655.1

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Dpp2_HCR_P2B1: GCCAGGGCGCACCACGTCGTACAACAgAAgAgTCTTCCTTTACg

Dpp3_HCR_P1B1: gAggAgggCagCAAACggAACAGCCGACGATCGGCTCGGAGTGG

Dpp3_HCR_P2B1: CCCGGGCCGGAGCGGAACGGAGTCCTAgAAgAgTCTTCCTTTACg

Dpp4_HCR_P1B1: gAggAgggCagCAAACggAAGCCGCTCCCAGAGCGTCGGCGTTGA

Dpp4_HCR_P2B1: TGTTTGGGCTCTTTGAGCCACCGTCTAgAAgAgTCTTCCTTTACg

Dpp5_HCR_P1B1: gAggAgggCagCAAACggAACGCCACTCCTCCTCGTCCGTGA

Dpp5_HCR_P2B1: TACAGCATGAGCAGCGGCTGCGCCGTAgAAgAgTCTTCCTTTACg

Dpp6_HCR_P1B1: gAggAgggCagCAAACggAATCTCCGCGACGCGCGCGCGCTC

Dpp6_HCR_P2B1: GCTTGCTGCGCGTCAGCCGCTCGTTAgAAgAgTCTTCCTTTACg

Dpp7_HCR_P1B1: gAggAgggCagCAAACggAAGCCCACGTCCGCGAAGTCGACGAAC

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Dpp9_HCR_P1B1: gAggAgggCagCAAACggAACACTGAGTTGACCAGAGTCTGCACT

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optomotor-blind (omb)

>optomotor-blind_B1_XM_052882932.1

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spalt

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spalt7_HCR_P2B1: TGTGTTTGGTTCAATTAAGGACGGTTAgAAgAgTCTTCCTTTACg

spalt8_HCR_P1B1: gAggAgggCAgCAAACggAAATTTTTCCGGATTTTCGAAAAGCTA

spalt8_HCR_P2B1: CCACCACTTTTTCCATCATAAGGTGTAgAAgAgTCTTCCTTTACg

Optix

>Optix_B2_XM_024080404.2

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 GACGATCTGGGACGGCGAGCAGAAGACGCACTGTTTCAAGGAGCGGACGCGATCTCTACTCCGAG
 AATGGTACCTCCAAGATCCCTACCCGAACCCGACGAAGAAGAGGGAATTGGCGGCGGCGACGGGT
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 CCAAGAACCGCTCCGCCGTGCTGGGCAGAGGATAA

Optix1_HCR_P1B2: CCTCgTAAATCCTCATCAAACGCCGTCGTGGACTCGTCCCAG
 Optix1_HCR_P2B2: CGCCTCCAGGATGCGCGCGTGCAGCAAATCATCCAgTAAACCgCC
 Optix2_HCR_P1B2: CCTCgTAAATCCTCATCAAAGGCTCGGCGCGGTCTGGGCGCGGCGG
 Optix2_HCR_P2B2: AGCGTCAGCGGCGGAGGCTCGCACGAAATCATCCAgTAAACCgCC
 Optix3_HCR_P1B2: CCTCgTAAATCCTCATCAAAGGCAGCAGCGGCGTGGGCGCCGCCA
 Optix3_HCR_P2B2: GCGGCGCTGAAGCTCAGCGTGGGCAAATCATCCAgTAAACCgCC
 Optix4_HCR_P1B2: CCTCgTAAATCCTCATCAAACCGCTCTCCTCCAGCGTCTCGCACA
 Optix4_HCR_P2B2: AAGAAGCGCGCCAGGCGCTCCACGTAAATCATCCAgTAAACCgCC
 Optix5_HCR_P1B2: CCTCgTAAATCCTCATCAAAGCGGTGGCGGCCGGCGTGGAAAGGCG
 Optix5_HCR_P2B2: GTGGCGCTCGAGGATGGCGTACAGCAAATCATCCAgTAAACCgCC
 Optix6_HCR_P1B2: CCTCgTAAATCCTCATCAAAACAGCGCTTGCAGCTTGGCGTGGCT
 Optix6_HCR_P2B2: CAGCCTCCTGGTAGTGCGCCTCCAGAAATCATCCAgTAAACCgCC
 Optix7_HCR_P1B2: CCTCgTAAATCCTCATCAAAACAGTGCCTTCTGCTCGCCGTCC
 Optix7_HCR_P2B2: GAGTAGAGATCGCGTCCGCTCCTTGAAATCATCCAgTAAACCgCC
 Optix8_HCR_P1B2: CCTCgTAAATCCTCATCAAACGTCGGGTTCGGGTAGGGATCTTGG
 Optix8_HCR_P2B2: CGTCGCCGCCGCCAATTCCTCTTCAAATCATCCAgTAAACCgCC
 Optix9_HCR_P1B2: CCTCgTAAATCCTCATCAAATTTGAACCAGTTGCCGACTTGCGTC
 Optix9_HCR_P2B2: GGCCGCTCGGTCTCTTTGCCGTCGGAAATCATCCAgTAAACCgCC

thickvein (tkv)

>thickvein_B2_XM_024079796.2

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tkv1_HCR_P1B2: CCTCgTAAATCCTCATCAAACGTGCACTCGCACACGATGCCCCGA

tkv1_HCR_P2B2: CGCGCCGCCGGGGCACATGCCCCGCGAAATCATCCAgTAAACCgCC

tkv2_HCR_P1B2: CCTCgTAAATCCTCATCAAAGCGGTGCGCTCCAGCACCACGAGCC

tkv2_HCR_P2B2: CCCGACTCGTCGGGCGGCAGGCAGCAAATCATCCAgTAAACCgCC

tkv3_HCR_P1B2: CCTCgTAAATCCTCATCAAAGGGGCTCCGGCAGCTGCGGCCGCGAG

tkv3_HCR_P2B2: GGCCGGGGGTCTCGGTGACGTCGGGAAATCATCCAgTAAACCgCC

tkv4_HCR_P1B2: CCTCgTAAATCCTCATCAAACGCGCATCCTGAAGAGCAGCCAGA

tkv4_HCR_P2B2: GGGGAAGGCGGTGCGTTGCATCCTCAAATCATCCAgTAAACCgCC

tkv5_HCR_P1B2: CCTCgTAAATCCTCATCAAACGATCGACTCGACCATTTGTATCTG

tkv5_HCR_P2B2: ACCAGACTTCGCCGTAGCGACCTTTAAATCATCCAgTAAACCgCC

tkv6_HCR_P1B2: CCTCgTAAATCCTCATCAAATCGCATGAGAACCGTCTGGTATATC

tkv6_HCR_P2B2: CGCGATGAAGCCGAGGATGTTTTCGAAATCATCCAgTAAACCgCC

Mother against decapentaplegic 6 (Mad6)

>Mother_against_decapentaplegic_homolog_6 (Mad6)_Bany_10682-RB_B1

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GAATCTGATGGCGTCTGGCTCTACAATAGAAGCCAAGAACCCGTGTTTCGTCAGCTCCCCCGCGTTA
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GACCCCTCGTCGCCCCCGCCGCTGTGTGCTACCCACGTCGGGGCCAGTTGACCCAGATCTGTG
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Mad61_P1_B1 gAggAgggCAgCAAACggAACGTCTTGTGTAATTTGCGCCGTGTTC

Mad61_P2_B1 CAAGCCGCTCATGATTCGTCCTCCTTAgAAgAgTCTTCCTTTACg

Mad62_P1_B1 gAggAgggCAgCAAACggAACCCAGCTCTGCCGCTCTTCGCCATC

Mad62_P2_B1 AGTACGCCAGCCTGCACCACTCGGTTAgAAgAgTCTTCCTTTACg

Mad63_P1_B1 gAggAgggCAgCAAACggAAGCCAATCTTCGCTCGAGTTTTTCGC

Mad63_P2_B1 AGATTCTAAGGACAGTGTGACACCTTAgAAgAgTCTTCCTTTACg

Mad64_P1_B1 gAggAgggCAgCAAACggAACAGAGACAGTGTCTGTTGCAACCC

Mad64_P2_B1 GGCGGGGGCGACGAGGGGTCGAAGATAgAAgAgTCTTCCTTTACg

Mad65_P1_B1 gAggAgggCAgCAAACggAATCACAGATCTGGGGTCAACTGGCCC

Mad65_P2_B1 GGCCCCAGCCTTTTCGCGAACGATATTAgAAgAgTCTTCCTTTACg

Mother against decapentaplegic 3 (Mad3)

>XM_024082355.2 PREDICTED: *Bicyclus anynana* mothers against decapentaplegic homolog 3 (LOC112045951), transcript variant X2, mRNA

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Mad31_P1_B2	CCTCgTAAATCCTCATCAAAGCGGCGTGGTGGTGTTGCCGGGCAC
Mad31_P2_B2	CGCCGTCTCGCTCATGTAGCCCGGAAATCATCCAgTAAACCgCC
Mad32_P1_B2	CCTCgTAAATCCTCATCAAATGATGCTGCACCAGAAGGCGGGCTC
Mad32_P2_B2	CCACGCGCGTGTTTCAGCTCGTAGTAAATCATCCAgTAAACCgCC
Mad33_P1_B2	CCTCgTAAATCCTCATCAAACCTCCACCACCTCGTTTCTGTTACG
Mad33_P2_B2	CGCCCTTGCCGATGTGTGCGGCGGTCAAATCATCCAgTAAACCgCC
Mad34_P1_B2	CCTCgTAAATCCTCATCAAAAACCTCGGTGGCGGGGTGCCAGCCGT
Mad34_P2_B2	GACACCGACTGCGACAGCAGCGCCGAAATCATCCAgTAAACCgCC
Mad35_P1_B2	CCTCgTAAATCCTCATCAAAATCCAGCATGGCGTTCGACGTCACCG
Mad35_P2_B2	TGCAGCGGCCCCGTTTCAGGTGCAGCTAAATCATCCAgTAAACCgCC

Mother against decapentaplegic 4 (Mad4)

>Motherss_against_decapentaplegic_homolog_4 (Mad4) _Bany_17230-RA_B3

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GACCGGGAGCGCCATTGGTGCCGCCACAAATGGTGCCTCGCCTGGCGCGCAAAATGCTAAACCAC
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 GTAG

Mad41_P1_B3	gTCCCTgCCTCTATATCTTTGCTCTCTTGGAGAAACCCTCGCTC
Mad41_P2_B3	TTCAACTTCTTGACTAGTGACTCAATTCCACTCAACTTTAACCCg
Mad42_P1_B3	gTCCCTgCCTCTATATCTTTTTCTTCCTGCTACCTGTAACCGACC
Mad42_P2_B3	GGGCGTAGATGACGTGAGGAAAACCTTCCACTCAACTTTAACCCg
Mad43_P1_B3	gTCCCTgCCTCTATATCTTTACAACCTCTCTCATAGTGGTAAGGGT
Mad43_P2_B3	TTGCAACGCCATCTGGCGCTGGAGTTCCACTCAACTTTAACCCg
Mad44_P1_B3	gTCCCTgCCTCTATATCTTTCTGGCATCTGATTGACTGGTGGTT
Mad44_P2_B3	GTGTCCCCGGCCCCATTTGCGGTGTTTCCACTCAACTTTAACCCg
Mad45_P1_B3	gTCCCTgCCTCTATATCTTTCCCATCTGAGGCGGCCCTGGTCCCA
Mad45_P2_B3	TTTGTCCCCATCTGCGGTGTCCCCGTTCCACTCAACTTTAACCCg

abrupt

>|cl|XM_024085182.2_cds_XP_023940950.1_1 [gene=LOC112047886] [db_xref=GeneID:112047886]
 [protein=protein abrupt isoform X2] [protein_id=XP_023940950.1] [location=231..1376] [gbkey=CDS]

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 AATAAATGGCGCCCGGATCCCAACACTGAACTGCGAGATGTCGATAATTACCCAGCGTCAGGAGC
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B2_P1 (abrupt)_1 CCTCGTAAATCCTCATCAAAAAATAGGGACTGCATACTGAGAGGA

B2_P1 (abrupt)_2 CCTCGTAAATCCTCATCAAATTCCTGGTGAGCTACATCCTTTAGT

B2_P1 (abrupt)_3 CCTCGTAAATCCTCATCAAAGAAAAGCAGACAACTCTTGCTGTCT

B2_P1 (abrupt)_4 CCTCGTAAATCCTCATCAAAGCAGGAGGTGATTCACTTCTCTCTC

B2_P1 (abrupt)_5 CCTCGTAAATCCTCATCAAACCATTCAGGCAAACCTATCACCAATA

B2_P1 (abrupt)_6 CCTCGTAAATCCTCATCAAACCTTTAGGGACTAGAGAGCTAGCAGA

B2_P2 (abrupt)_1 CAGGGATTCATCTTAAATAGCTCCTAAATCATCCAGTAAACCGCC

B2_P2 (abrupt)_2 GTACATAAATTGCAAAAGCTGTCGCAAAATCATCCAGTAAACCGCC

B2_P2 (abrupt)_3 TGACTTGCAGCAGCTCTGCTGTGTGAAATCATCCAGTAAACCGCC
 B2_P2 (abrupt)_4 GCATTTAGTATATCTTCTTCCACTGAAATCATCCAGTAAACCGCC
 B2_P2 (abrupt)_5 AGGGATCGCTTCATCATTTGATGGTAAATCATCCAGTAAACCGCC
 B2_P2 (abrupt)_6 TTTTCAAAGGGCTCCGAGCAGCTTCAAATCATCCAGTAAACCGCC

Bicyclus anynana testis-specific gene A8 protein-like (A8)

>lcl|XM_052885974.1_cds_XP_052741934.1_1 [gene=LOC128198825] [db_xref=GeneID:128198825]
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B2_P1 (A8)_1 CCTCGTAAATCCTCATCAAAATGTCAGTCTTGACAATAGCTGGCG
 B2_P1 (A8)_2 CCTCGTAAATCCTCATCAAAGGGGGCGACAGCTAATGGCGCTGAG
 B2_P1 (A8)_3 CCTCGTAAATCCTCATCAAACAGCGGCGTATGTGCTGATTACACC
 B2_P1 (A8)_4 CCTCGTAAATCCTCATCAAATACGCTAATATGGGAGCACCATAAC
 B2_P1 (A8)_5 CCTCGTAAATCCTCATCAAACGCTTGAGATGGGATAGCGATGGAC
 B2_P1 (A8)_6 CCTCGTAAATCCTCATCAAACCTCAGGAGTTTCAGTAGGCGCAAC
 B2_P2 (A8)_1 ACTACAGGTGCGATTGCTGGAGCTAAAAATCATCCAGTAAACCGCC
 B2_P2 (A8)_2 GATGCGTGATTGAGTCGACACTGCGAAATCATCCAGTAAACCGCC
 B2_P2 (A8)_3 CCAGGGGCGCAGTGTACTCCAGGGGAAATCATCCAGTAAACCGCC
 B2_P2 (A8)_4 ATGGCGGCAGCGTAGATGGATGGACAAATCATCCAGTAAACCGCC
 B2_P2 (A8)_5 GGCCGCAGGGCTTTTCAGGTGCAGGCAAATCATCCAGTAAACCGCC
 B2_P2 (A8)_6 CTAGATGAGCGGCGCGCGCGGCGGCAAATCATCCAGTAAACCGCC

heat shock protein 67B1-like (hsp67B1)

>lcl|XM_024094531.2_cds_XP_023950299.2_1 [gene=LOC112054669] [db_xref=GeneID:112054669]
 [protein=heat shock protein 67B1-like] [protein_id=XP_023950299.2] [location=57..1211] [gbkey=CDS]

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B1_P1 (hsp67B1)_1	GAGGAGGGCAGCAAACGGAAGTTTACGATGACACGGAATTTGTCT
B1_P1 (hsp67B1)_2	GAGGAGGGCAGCAAACGGAAGCGCTCCTTGGACGAGGATGTAATC
B1_P1 (hsp67B1)_3	GAGGAGGGCAGCAAACGGAATTCAACGGAAGGCTGTACGTATGGA
B1_P1 (hsp67B1)_4	GAGGAGGGCAGCAAACGGAATTCACAGGAGCGGTTACATCCAAA
B1_P1 (hsp67B1)_5	GAGGAGGGCAGCAAACGGAACCTTTCACGGCTTGCCACTTTC AAC
B1_P1 (hsp67B1)_6	GAGGAGGGCAGCAAACGGAAGTCTTATCAACAGTTTCAACTGGGT
B1_P2 (hsp67B1)_1	CAGATCTTTCTTCTTGTAATCCTTATAGAAGAGTCTTCCTTTACG
B1_P2 (hsp67B1)_2	AGTCGTGGTCGTCATGCTTAGCCTCTAGAAGAGTCTTCCTTTACG
B1_P2 (hsp67B1)_3	GTAGCAGTGACATCTGATGCACTAGTAGAAGAGTCTTCCTTTACG
B1_P2 (hsp67B1)_4	AACAACCTTGTTCTTGTCATCGACATAGAAGAGTCTTCCTTTACG
B1_P2 (hsp67B1)_5	CAACAGGTTCTCTATCTTCTTTATCTAGAAGAGTCTTCCTTTACG
B1_P2 (hsp67B1)_6	AAAGTTTCGATTTTATCAACATTTTTAGAAGAGTCTTCCTTTACG

muscle-specific protein 20-like (msp20)

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lachesin

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Dscam2

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ivory

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Supplementary References

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