Transcriptome analysis reveals *wingless* regulates neural development and signaling genes in the region of wing pigmentation of a polka-dotted fruit fly.

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Data accessibility

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Abstract

How evolutionary novelties have arisen is one of the central questions in evolutionary biology. Pre-existing gene regulatory networks or signaling pathways have been shown to be co-opted for building novel traits in several organisms. However, the structure of entire gene regulatory networks and evolutionary events of gene co-option for emergence of a novel trait are poorly understood. In this study, to explore the genetic and molecular bases of the novel wing pigmentation pattern of a polka-dotted fruit fly (*Drosophila guttifera*), we performed *de novo* genome sequencing and transcriptome analyses. As a result, we comprehensively identified the genes associated with the pigmentation pattern. Furthermore, we revealed that 151 of these associated genes were positively or negatively regulated by *wingless*, a master regulator of wing pigmentation. Genes for neural development, Wnt signaling, Dpp signaling, and effectors (such as enzymes) for melanin pigmentation were included among these 151 genes. None of the known regulatory genes that regulate pigmentation pattern formation in other fruit fly species were included. Our results suggest that the novel pigmentation pattern of a polka-dotted fruit fly might have emerged through multi-step co-options of multiple gene regulatory networks, signaling pathways, and effector genes, rather than recruitment of one large gene circuit.

Introduction

How do evolutionary novelties emerge? Researchers have tried to unravel the developmental genetic program underlying traits in order to clarify the origins of evolutionary novelties [1]. Gene regulatory networks for producing novel traits have been supposed to be composed of a combination of genes forming other traits. One of the most significant current discussions regarding the production of evolutionary novelty is how pre-existing regulatory networks were utilized for this production [1,2].

So far, gene regulatory networks or signaling pathways involved in development of novel traits have been scrutinized in several animals. For example, in a horned dung beetle, limb and wing patterning genes are co-opted for horn formation [3,4]. In Nymphalid butterflies, components of the appendage-patterning gene regulatory network, such as *Distal-less*, *wingless*, and *decapentaplegic* signaling, contributed to development of eyespot, another representative novel trait [5-10]. In the fruit fly *Drosophila melanogaster*, it was shown experimentally that the gene regulatory network

for larval posterior spiracle development was re-used for the posterior lobe, a novel trait observed in male genitalia [11]. Many studies have shown or suggested which gene regulatory networks or signaling pathways are necessary for, or involved in, development of novel traits. However, the structure of entire gene regulatory networks and evolutionary events of gene co-option for emergence of a novel trait are poorly understood.

Fruit fly species have been used to study regulatory evolution of pigmentation pattern, and provided many examples of mechanisms underlying phenotypic evolution [12-15]. In a polka-dotted fruit fly (*Drosophila guttifera*), which has a novel polka-dotted pigmentation pattern on the wings, a melanin synthesis gene, *yellow*, was expressed in the polka-dotted pattern [16-18]. A Wnt signaling gene, *wingless*, was expressed in the centers of pigmentation areas, and positively regulated the expression of *yellow* through an enhancer (Fig. 1a, c, e) [16,17]. Ectopic expression of *wingless* induced ectopic wing pigmentation (Fig. 1b, d, f) [17]. The unique expression pattern of *wingless* seemed to be caused by evolutionary gain of novel enhancer activities [19,20]. In *Drosophila melanogaster*, however, there is no pigmentation around crossveins where *wingless* is expressed. If we assume the ancestral species had *wingless* expression and no pigmentation as in *D. melanogaster*, gain of novel expression pattern of *wingless* alone is not sufficient, for emergence of pigmentation pattern [17]. Also, expression of the melanin synthesis gene *yellow* is not sufficient to induce pigmentation in the *Drosophila melanogaster* wing [16,21], indicating that expression changes of multiple genes were required for the evolution of pigmentation. Therefore, comprehensive exploration of genes downstream of *wingless*, which include both regulatory and effector genes, is necessary for understanding the emergence of the novel wing pigmentation pattern.

In this study, we comprehensively identified the genes that are expressed in pigmentation areas and are also regulated by *wingless*, by *de novo* genome sequencing and two successive transcriptome analyses by Quartz-Seq, a highly sensitive method of RNA sequencing. In the first transcriptome analysis, we compared gene expression patterns between pigmentation areas and an unpigmented area and searched for differentially expressed genes (DEGs). In the second transcriptome analysis, we tested whether those DEGs were regulated by *wingless*, the master control gene for wing pigmentation.

Results

Genome sequencing

To obtain the genome sequence of *D. guttifera*, we constructed paired-end and mate pair libraries. The information about the constructed libraries (number of reads, total number of base pairs, average insert size) is summarized in Table 1. After sequencing, we assembled the reads *de novo* to obtain scaffolds. Characteristics of the scaffolds are summarized in Table 2.

Genes expressed in the polka-dotted pattern

We compared gene expression patterns between pigmentation areas and an unpigmented area, and searched differentially expressed genes (DEGs). These areas can be distinguished by GFP label using a transgenic line which carries *eGFP* connected with an enhancer of *yellow* [17] (Fig. 1e, f). We identified genes upregulated or downregulated commonly in Area 1 (pigmentation area around a campaniform sensillum, Fig. 2a) and Area 2 (vein tip, Fig. 2a), compared with Area 3 (unpigmented, Fig. 2b). Comparison of the gene expression between Area 1 and Area 3 showed that 2333 genes were differentially expressed. Among them, 1390 genes were upregulated (Fig. 3a) and 943 genes were downregulated (Fig. 3b) in Area 1 in comparison to Area 3. 2582 genes were differentially expressed between Area 2 and Area 3. Among them, 1593 genes were upregulated (Fig. 3a) and 989 genes were downregulated (Fig. 3b) in Area 2 in comparison to Area 3. Integrating these data, the number of common DEGs was 1035. Among them, 615 genes were upregulated both in Area 1 and Area 2 (Fig. 3a), while 420 genes were downregulated in Area 1 and Area 2 (Fig. 3b). Consistent with previously reported findings, *wingless* and *yellow* were expressed in the pigmentation areas [17,19], indicating the high sensitivity and accuracy of the present method of analysis. *wingless* and *yellow* were included in the 615 commonly upregulated DEGs (Fig. 4, Table 3, Table 4, (the complete table is available at https://figshare.com/articles/Genes_differentially_expressed_in_the_pigmentation_areas_of_Drosophila_guttifera_/11888898)).

Pigmentation pattern-associated genes regulated by *wingless*

Because ectopic expression of *wingless* is known to induce pigmentation, genes

sufficient for pigmentation formation in wings must be included in the gene network downstream of *wingless*. To identify the genes that are under the control of *wingless*, we identified genes upregulated or downregulated when *wingless* was ectopically expressed. Among the 615 common upregulated (Area 1 and 2) DEGs, 78 genes were upregulated by ectopic expression of *wingless* (Fig. 3a). In 420 common downregulated (Area 1 and 2) DEGs, *wingless* downregulated 73 genes (Fig. 3b). In total, 151 genes associated with the pigmentation pattern were regulated by *wingless* gene. These 151 genes were blasted against the protein database of *Drosophila melanogaster* and 131 genes were annotated. For these 131 genes, enrichment analysis with DAVID resulted 14 functional annotation clusters, and 6 of which were significant (Table 5). In the most significant cluster, Gene Ontology (GO) terms “Glycoprotein”, “Plasma membrane”, “Disulfide bond”, “Signal peptide” and “Receptor” were included (Table 5). GO terms such as “cuticle pigmentation” and “melanin biosynthetic process” were included in the 3rd significant cluster. 20 genes that could not be annotated were reanalyzed with Blast2GO. Four genes were annotated and remaining 16 genes did not match to any gene in the database.

Among pigmentation pattern associated genes regulated by *wingless*, six genes can be categorized as melanin synthesis-related genes [22-24]. Among them, *yellow*, *laccase2*, and *tan* were upregulated in the pigmentation areas (Fig. 4a, b, Table 3, Table 4) and also upregulated by *wingless* (Fig. 5a, Table 6). *yellow-e*, *yellow-h*, and *silver* (*svr*) were downregulated both in the pigmentation areas (Fig. 4a, b, c, d, Table 3, Table 4) and in the area where *wingless* was ectopically expressed (Fig. 5a, b, Table 6).

Regulatory genes, such as transcription factors and genes involved in signaling pathways are important to understand the regulatory network controlling the pigmentation pattern. Eight transcription factors were associated with pigmentation and regulated by *wingless*. *Zinc-finger protein interacting with CP190* (*ZIPIC*), *zinc finger protein 28-like*, and Enhancer of split complex genes such as *E(spl)m3-HLH*, *E(spl)m5-HLH*, *E(spl)m7-HLH*, and *lethal (3) malignant brain tumor* (*l(3)mbt*) were upregulated in the pigmentation areas (Fig. 4c, d, Table 3, Table 4) and by *wingless* (Fig. 5b, Table 6). *Mothers against dpp* (*Mad*), and *Mediator complex subunit 18* (*MED18*) were downregulated in the pigmentation areas and by *wingless*. Two signal ligands, *Delta* (*Dl*) and *Wnt oncogene analog 4* (*DWnt4*, ortholog of human *WNT9A/B*, [25]) were upregulated in the pigmentation areas and by *wingless* (Fig. 4c, d, Fig. 5b, Table 3, Table 4, Table 6). As receptors of ligands, *saxophone* (*sax*) was upregulated and *frizzled 2* (*fz2*)

was downregulated in the pigmentation areas and by *wingless* (Fig. 4c, d, Fig. 5b, Table 3, Table 4, Table 6). *wingless* itself was not detected in DEG analysis with ectopic *wingless* expression, which is reasonable in our experimental design. Our transgenic line ectopically drove the *wingless* gene originated from *D. melanogaster* [17] and its transcripts were not mapped on *D. guttifera* genome in the analysis.

Discussion

A large number of genes were specifically regulated in the area of pigmentation formation

Transcriptome analyses revealed that a large number of genes were specifically regulated in the area of pigmentation formation: 78 genes were upregulated commonly in the pigmentation area and by *wingless*, and 73 genes were downregulated commonly in the pigmentation area and by *wingless*. In the butterfly *Bicyclus anynana*, 132 genes were upregulated and 54 genes were downregulated in relation to eye spot formation [26]. In comparison with these numbers of genes in butterflies, the number of genes identified by our results seem reasonable. However, *Drosophila* pigmentation has been thought to be a simple trait, and researchers have tried to explain the evolution of pigmentation by changes of expression of a small number of genes [16,27-29]. In the abdominal tergite of *Drosophila melanogaster,* the combination of *ebony* mutation and ectopic expression of the *yellow* gene can induce ectopic pigmentation [30]. In *D. melanogaster* wings, however, the same combination resulted in scarcely any ectopic pigmentation [16,21]. Those findings are consistent with those of the present study, in which we found many genes that were specifically regulated in the areas of pigmentation formation. This also suggests that experimental reproduction of the gain of pigmentation patterns through overexpression of genes is not trivial.

Although ectopic expression of *wingless* can induce pigmentation, the intensity of induced pigmentation is weaker than that of the natural spotted pigmentation. 537 genes were upregulated and 347 genes were downregulated commonly in Area 1 and Area 2 but not regulated by *wingless* (Fig. 3). Thus, they were not essential to make pigmentation, but might have supplemental roles, or they might be unrelated to pigmentation but have a structural role unique to the pigmented area.

Known gene regulatory networks of *Drosophila* pigmentation were not responsible

for *D. guttifera* wing pigmentation

Regulation of pigmentation has been studied in multiple *Drosophila* models. The best-studied case was abdominal pigmentation in *D. melanogaster*. Male-specific pigmentation was controlled positively by *Abd-B* and negatively by *bab* genes, and pigmentation common to the two sexes was positively controlled by *omb* [31]. In *D. biarmipes* wings, the pigmentation is controlled positively by *Dll*, and negatively by *en* [13,16]. Neither of these genes was included in DEGs in our analysis. Thus, regulatory mechanisms of pigmentation in *D. guttifera* are not a simple co-option of the known gene regulatory network of pigmentation. Then, what kinds of genes are responsible for the emergence of the novel pigmentation pattern in *D. guttifera*?

***wingless* regulated neural development genes and other signaling genes specifically in the pigmentation areas**

We identified 12 regulatory genes (transcription factor genes and signaling factor genes) that were regulated by *wingless* in the pigmentation areas. Among them, four (*Dl*, *E(spl)m3-HLH*, *E(spl)m5-HLH* and *E(spl)m7-HLH*) genes belonged to neurogenesis genes of GO terms (Fig. 6). Because Area 2 (vein tip) did not include any tissue of neural origin, these genes were considered to be expressed in epidermal cells, which is the only cell type present in Area 2. In wing discs of *D. melanogaster*, *Dl* and *E(spl)* complex genes are involved in neurogenesis mediated by Notch signaling [32]. During this process, the expression of *wingless* is necessary and sufficient for the expression of *Dl* [33].

Decapentaplegic (Dpp) and Hedgehog (Hh) signaling can work cooperatively or antagonistically with Wingless signaling in various aspects of *Drosophila* development [34,35]. In the present study, genes involved in Dpp signaling, *saxophone* (*sax*) and *Mothers against dpp (Mad)*, were regulated by *wingless* in the pigmentation areas. *hh* and *patched* of the Hh signaling pathway were up-regulated in the area of pigmentation, but not regulated by *wingless* (Table 7).

Wnt signaling genes *fz2* and *DWnt4* were regulated in the pigmentation areas and by *wingless* (Fig. 6). Downregulation of *fz2* in the pigmentation areas of *D. guttifera* might contribute to achieving the proper gradient of Wingless protein, as it is known to do in *D. melanogaster* wing discs [36]. *DWnt4* was expressed in the area surrounding the *wingless* expression region in wings of *D. guttifera* [19], suggesting that it might play a

role in pigmentation pattern formation.

For the formation of the wing pigmentation pattern of *D. guttifera*, the necessity for a transcription factor gene or signaling factor gene other than *wingless* was suggested in a previous study. When putative binding sites of T-cell factor (Tcf) family proteins [37] in the *yellow* enhancer were artificially mutated, EGFP expression driven by the mutated enhancer was not altered, indicating the canonical Wnt signaling does not directly give input to the enhancer, and another factor does [17]. It is possible that the gene which regulates the expression of *yellow* downstream of *wingless* is included in the genes identified in this study. We also found multiple putative binding sites of Tcf family proteins around most of the genes in Table 6, both in *D. guttifera* and in *D. melanogaster* by using GenePalette [38], suggesting potential connections between *wingless* and these genes, although putative binding sites do not guarantee actual binding or function.

Known functions of pigmentation genes regulated by *wingless*

In *D. melanogaster*, *yellow*, *tan*, and *laccase2* are known to be effector genes for pigmentation and have been proven to promote melanin pigmentation in *D. melanogaster* [21,30,39]. Association of these genes with melanin pigmentation patterns was also reported in other insects [23,40,41]. Therefore it was not surprising to detect these three genes in the present study. The *svr* gene, which encodes a carboxypeptidase and is involved in metabolism of N-acetyl dopamine (NADA) in *D. melanogaster* [22,42], was downregulated in the area of pigmentation of *D. guttifera*. Expression of Yellow family protein genes such as *yellow-e* and *yellow-h* was also downregulated in the pigmentation areas on the wings of *D. guttifera*, but the molecular functions of these genes have not been identified in any insect. In wings of the butterflies (*Vanessa caudui* and *Heliconius* spp.), another Yellow family protein gene, *yellow-d*, was upregulated at red pigmentation areas compared with black pigmentation areas [41,43]. Our results together with these previously reported studies suggest that these Yellow protein family genes might play a role in inhibiting melanin pigmentation.

Evolutionary scenario of pigmentation pattern evolution

The genes co-opted for the pigmentation formation are highly likely to have been included in the 151 genes identified in this study. This raises the question: What kind

of genetic change enabled the evolution of pigmentation patterns? There is an ongoing discussion about whether a large gene circuit is recruited or many genes are individually recruited to form a circuit during the evolution of a novel trait [44,45]. Examples of co-option of pre-existing circuits consisting of multiple regulatory genes are known from animals and plants [11,26,46-49]. In the present case, we can ask whether the large gene network for pigmentation regulated by a master control gene, *wingless*, was co-opted all at the same time, or whether individual genes were co-opted one by one to form the current network. To our knowledge, there is no functional evidence of a pigmentation pattern mainly regulated by *wingless* in other *Drosophila* species. Therefore, a "large gene circuit model" does not seem reasonable to explain emergence of the novel pigmentation pattern in *Drosophila guttifera*.

Taking our findings altogether, we suggest that the novel pigmentation pattern of *D. guttifera* was not caused by co-options of a large gene circuit of regulatory genes and effector genes. To test whether the pigmentation pattern has evolved by multi-step co-option, we will have to compare multiple species with and without pigmentation, as well as to test individual gene functions in *D. guttifera*. These investigations will further our understanding of the evolution of pigmentation pattern formation in *D. guttifera*, as an example of the emergence of evolutionary novelties.

Materials and methods

Flies

Drosophila guttifera is a North American species that belongs to (or is closely related to) the *quinaria* group of subgenus *Drosophila* [50,51]. The inbred line (A5) was made by ten successive sibling crosses of a wildtype (stock no. 15130-1971.10) obtained from the *Drosophila* Species Stock Center at the University of California, San Diego. Two lines (transgenic lines No. 1 and No.2) of *D. guttifera* were used for transcriptome analyses. Transgenic line No. 1 was established by five successive backcrosses (introgression) of a transgenic line that carries *nuclear eGFP* connected with a *yellow* enhancer (*vein spot* CRE-*nuclear eGFP*, gut 1c+R GFP #12) [17] with the A5 inbred line. Transgenic line No. 2 was established by two successive backcrosses of a UAS-*wg* line [17] with transgenic line No. 1. These backcrosses aimed to unify the genetic backgrounds to improve the mapping efficiency in the transcriptome analyses. Flies were reared with standard cornmeal/sugar/yeast/agar food at 25 °C [52].

Genome sequencing and gene prediction

Genomic DNA was extracted from adults of the inbred line (A5) with a Gentra Puregene Tissue Kit (Qiagen). A paired-end library with an average insert size of 450 bp was constructed with a TruSeq DNA PCR-free Library Prep Kit (Illumina) and two different mate-pair libraries (3 kb and 5 kb) were prepared with a Nextera Mate Pair Library Prep Kit (Illumina). Sequencing libraries were run on the Illumina Hiseq 2500 sequencer with a read length of 150 bp. Paired-end and mate pair reads were *de novo* assembled using Platanus v1.2.1.1 [53] after removal of adaptors and error correction with SOAPec [54]. The assembly sequences had 767 scaffolds with a total length of 168 .4 Mb and a scaffold N50 length of 1.8 Mb.

Gene prediction was conducted with Augustus [55] on the scaffolds of the *D. guttifera* inbred line (A5). The option used in the analysis with Augustus was "--species=fly".

Collecting samples for transcriptome analysis

Pupae at stage P12 (i) or P12 (ii) were used for two successive transcriptome analyses. These stages are just after the stage when *yellow* expression and pigmentation process have started [56]. The transcriptomes were compared by performing the combination of utilization of fluorescence-marked tissues, repetitive microsurgical samplings, and a sensitive RNA sequencing technology. For the first experiment, individuals from the transgenic line No. 1 which carries *eGFP* connected with an enhancer of *yellow* were used. An EGFP-positive area around a campaniform sensillum on 3rd longitudinal vein (Area 1, Fig. 2a), an EGFP-positive area at the tip of 3rd longitudinal vein (Area 2, Fig. 2a), and an EGFP-negative area on 3rd longitudinal vein in wings of flies (Area 3, Fig. 2b) were separated with a surgical knife under a stereo microscope SZX-16 (Olympus). By using those areas on the same wing vein, we aimed to exclude gene expression changes in regions between wing veins. The width of these tissues was about 50 μm. For the second experiment, an EGFP-positive area and an EGFP-negative area with a width of about 75 μm at the same place in individuals from transgenic lines No. 1 and No. 2 were dissected (Fig. 2c, d). From dissected tissues, RNA was collected with an RNeasy Micro Kit (Qiagen) and stored at -80°C. For RNA extraction, 20 dissected tissues were used for one replicate. Five biological replicates for

each area were prepared (total: 20 x 5 = 100 tissues). The quality of extracted RNA was examined with an Agilent 2100 bioanalyzer (Agilent Technology).

RNA sequencing

The library for RNA sequencing was constructed according to the protocol of Quartz-Seq, a highly sensitive method of RNA sequencing [57]. This protocol includes two PCR steps. Twenty-one cycles were performed for the first PCR, and eight cycles were performed for the second PCR. RNA sequencing was performed with NextSeq 550 (Illumina).

Transcriptome analysis and enrichment analysis

The sequenced transcriptome was mapped to the genome of *D. guttifera* with HISAT2 [58]. Transcriptome assembly was conducted with StringTie [59]. Differentially expressed genes were identified with edgeR [60]. An FDR (false discovery rate) of 0.05 was chosen as the threshold to identify DEGs.

DEGs were blasted against the protein database of *D. melanogaster*, obtained from Ensembl [61,62]. BLAST analysis was performed with Blastx using an E-value < 1e-3. The top hit outcome for each gene was taken as the result of gene annotation. Based on the obtained gene annotation, enrichment analysis was conducted with DAVID [63]. Genes that could not be annotated was reanalyzed with Blast2GO (database: nr, E-value < 1e-3) [64].

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Tables

Table 1

The data of the libraries of *D. guttifera* constructed for genome sequencing. PE400, MP3kbp, and MP5kbp respectively indicate the paired-end library, the mate-pair library (3kb), and the mate-pair library (5kb).

Library	Number of reads	Total number of base pairs (bp)	Average insert size (bp)
PE400	151,389,704	22,708,455,600	450
MP3kbp	41,484,414	6,222,662,100	3,169
MP5kbp	30,474,280	4,571,142,000	5,227

Table 2

Characteristics of the *D. guttifera* genome sequence obtained by *de novo* assembly.

Characteristics	Measures
Total number of base pairs (bp)	168,421,893
Number of scaffolds	767
Scaffold N50 (bp)	1,784,351
Contig N50 (bp)	182,614

Table 3

Genes differentially expressed in a pigmentation area around a campaniform sensillum. FC, CPM, and FDR indicate fold change, counts per million, and false discovery rate,

respectively.

	logFC	logCPM	FDR
y	4.97989524	13.1819431	2.38E-36
t	2.76380354	12.0058893	3.29E-32
laccase2	2.49456691	11.8424645	2.15E-33
yellow-e	-1.6407385	10.3188765	1.81E-15
yelloe-h	-0.5731037	10.5879713	0.02343591
svr	-0.7948767	9.41379963	0.00438384
ZIPIC	4.53189996	0.48568358	0.0208437
zinc finger protein 28-like	6.75852343	1.85690936	0.0005392
E(spl)m3-HLH	1.23731928	8.47601255	0.0003062
E(spl)m5-HLH	6.77199195	3.84590398	6.05E-10
E(spl)m7-HLH	3.36253299	4.92651899	0.0000107
Mad	-1.5333172	5.94437046	0.02335105
Dl	1.70066765	8.69275453	3.05E-07
DWnt4	7.88039218	2.78881957	0.00610713
sax	1.84041069	5.74292161	0.04887547
fz2	-1.7268879	8.84471432	1.56E-08
wg	4.90156885	0.66703408	0.00181758
MED18	-7.5365154	2.49510051	0.00031397
l(3)mbt	4.03476224	4.54864458	0.00016924

Table 4

Genes differentially expressed in a pigmentation area at the tip of a vein. FC, CPM, and FDR indicate fold change, counts per million, and false discovery rate, respectively.

	logFC	logCPM	FDR
y	4.21635634	12.5960958	5.72E-28
t	3.17677306	12.5066497	3.28E-46

laccase2	1.9708477	11.544914	8.32E-28
yellow-e	-2.5486142	10.2771931	4.54E-32
yelloe-h	-0.6835507	10.671958	0.00134155
svr	-0.6447186	9.60117515	0.00423173
ZIPIC	4.73395417	0.69571295	0.0256575
zinc finger protein 28-like	5.82784953	1.30102744	0.00000242
E(spl)m3-HLH	1.3827603	8.72212278	0.0000125
E(spl)m5-HLH	5.06729935	2.44510302	0.00069965
E(spl)m7-HLH	3.60181175	5.29016258	0.00000958
Mad	-2.0456884	5.94769958	0.01764714
Dl	0.92496862	8.28076591	0.01422465
DWnt4	7.3910185	2.48248069	0.00010193
sax	2.05209481	6.05221034	0.01234576
fz2	-2.6177129	8.81769459	2.23E-19
wg	6.33393063	1.65109029	0.00062794
MED18	-7.5507612	2.61383665	0.00022139
l(3)mbt	3.71774754	4.41678381	0.03790313

Table 5

The results from enrichment analysis with DAVID, showing clusters with enrichment score > 1.3 and Gene ontology terms with *p* value < 0.05 in each cluster (Count indicates the number of genes for each Gene ontology term).

Cluster 1 **enrichment score: 2.42**	**Count**	***p* value**
Glycosylation site: N-linked	16	1.90E-05
Glycoprotein	16	4.90E-05
Plasma membrane	18	8.00E-04

Cell membrane	11	2.90E-03
Topological domain: Extracellular	10	6.10E-03
Disulfide bond	14	7.60E-03
Topological domain: Cytoplasmic	11	9.70E-03
Transmembrane region	13	1.70E-02
Signal peptide	10	4.20E-02
Receptor	9	4.60E-02
Cluster 2 **enrichment score: 2.38**	**Count**	***p* value**
Extracellular matrix	8	3.40E-04
Chitin-based cuticle development	7	2.60E-03
Structural constituent of cuticle	6	3.90E-03
Structural constituent of chitin-based larval cuticle	5	1.70E-02
Insect cuticle protein	5	2.10E-02
Cluster 3 **enrichment score: 2.18**	**Count**	***p* value**
Cuticle pigmentation	4	6.60E-04
Melanin biosynthetic process	3	7.40E-03

Major royal jelly	3	7.80E-03
Cluster 4 **enrichment score: 1.39**	**Count**	***p* value**
Membrane	42	1.20E-02
Cluster 5 **enrichment score: 1.34**	**Count**	***p* value**
ANK repeat	4	3.60E-02
Ankyrin repeat	4	4.70E-02
Cluster 6 **enrichment score: 1.33**	**Count**	***p* value**
Carbohydrate metabolic process	5	1.40E-02

Table 6

Genes differentially expressed in an area where *wingless* is ectopically expressed. FC, CPM, and FDR indicate fold change, counts per million, and false discovery rate, respectively.

	logFC	logCPM	FDR
y	4.54116336	12.8494004	3.6E-148
t	2.40367879	12.0035577	1.25E-74
laccase2	2.04478128	11.4946868	3.67E-39
yellow-e	-1.2740677	11.392683	1.96E-25
yelloe-h	-0.4516609	9.7843299	0.00750851
svr	-0.6215228	9.01017126	0.00382779
ZIPIC	5.0147075	-0.3005216	0.0284088
zinc finger protein 28-like	5.61711109	2.5793453	0.00012374

E(spl)m3-HLH	0.53145394	8.24183597	0.00380777
E(spl)m5-HLH	5.25665975	1.58014564	0.00043059
E(spl)m7-HLH	1.67127368	5.65074921	1.23E-15
Mad	-0.8277961	4.89916451	0.01393637
Dl	1.02257261	7.82859549	8.12E-11
DWnt4	8.32686641	2.16749637	4.35E-09
sax	0.91143441	5.58205955	0.00742031
fz2	-0.4058316	8.27179547	0.02026252
wg	0	-1.2863604	1
MED18	-6.8364613	0.89990996	0.00079404
l(3)mbt	1.93217694	4.83757955	0.01514216

Table 7

Data of expression of *hedgehog* (*hh*), *patched* (*ptc*), and *smoothened* (*smo*) in a pigmentation area around a campaniform sensillum, at the tip of a vein, and in an area where *wingless* is ectopically expressed.

		logFC	logCPM	FDR
hh	Campaniform sensillum	4.85624359	0.63602448	0.04417015
	Vein tip	7.03066917	2.18407496	0.01422465
	wingless overexpression	-4.2417977	-0.6349855	0.0705383
ptc	Campaniform sensillum	6.41499537	3.16486805	0.0012656
	Vein tip	5.79689401	2.74718533	0.02202927
	wingless overexpression	-0.6229317	3.57406688	1
smo	Campaniform sensillum	-1.4152405	5.26637498	0.51827634

smo	Vein tip	-6.8976128	4.95229132	1.01E-08
	wingless overexpression	0.52985214	4.07845493	0.95456002

Figure legends

Fig. 1

Wings of *Drosophila guttifera* and the expression pattern of *wingless*. **a**: A wing of *Drosophila guttifera*. This picture shows wild type pigmentation pattern. **b**: The wing pigmentation pattern of an individual in which *wingless* is ectopically expressed. **c**: The expression pattern of *wingless* (blue) in a wing of a wild type individual. **d**: The position where *wingless* is ectopically expressed (blue) in the fly shown in b (Modified from [17]. **e**: The expression pattern of EGFP driven by an enhancer of *yellow*. In this individual, the expression pattern of *wingless* is the same as wild type. **f**: The expression pattern of EGFP driven by an enhancer of *yellow* in an individual with ectopic *wingless* expression shown in d. Individuals shown in e and f were from transgenic lines No. 1 and No. 2 for each (see Materials and methods). For experiments, 100 individuals from No. 1 and No. 2 for each were used. The expression pattern of EGFP of 100 individuals from No. 1 and No. 2 were the same as the patterns shown in e and f for each. The scale bar indicates 250 μm.

Fig. 2

Pupal wing tissues used for transcriptome analyses. Dissected areas are indicated with white boxes. **a**: Area 1 (an EGFP-positive area around a campaniform sensillum on the 3rd longitudinal vein) and Area 2 (an EGFP-positive area at the tip of the 3rd longitudinal vein, which does not include bristles or other tissue of neural origin). **b**: Area 3 (an EGFP-negative area on the 3rd longitudinal vein). **c**: An EGFP-positive area where *wingless* is ectopically expressed. **d**: An EGFP-negative area where *wingless* is not ectopically expressed. For each area, 20 tissues were dissected for one replicate and 5 biological replicates were used for experiments. Scale bars indicate 250 μm.

Fig. 3

The number of differentially expressed genes (DEGs) detected in transcriptome analyses.

a: The number of DEGs upregulated at pigmentation areas and an area where *wingless* is ectopically expressed. **b**: The number of DEGs downregulated at the pigmentation areas and an area where *wingless* is ectopically expressed. The circle labeled with "Campaniform sensillum" indicates the result from the comparison of transcriptome between Area 1 (a pigmentation area around campaniform sensillum) and Area 3 (unpigmented). The circle labeled with "Vein tip" indicates the result from the comparison of transcriptome between Area 2 (a pigmentation area at the tip of 3rd longitudinal vein) and Area 3. The circle labeled with "*wingless* overexpression" indicates the number of genes differentially expressed where *wingless* is ectopically expressed.

Fig. 4

Volcano plots of the results from the first transcriptome analysis. The horizontal axis indicates fold changes and the vertical axis indicates significance calculated as FDR with edgeR. Orange points indicate upregulated DEGs and blue points indicate downregulated DEGs. **a**: This plot shows genes differentially expressed in Area 1 (a pigmentation area around a campaniform sensillum) compared with gene expression of Area 3 (unpigmented). **b**: This plot shows genes differentially expressed in Area 2 (a pigmentation area at the tip of 3rd longitudinal vein) compared with gene expression of Area 3. **c**: Genes with -log 10 (FDR) < 20 are extracted from a. **d**: Genes with -log 10 (FDR) < 20 are extracted from b. In c and d, *m3*, *m5*, and *m7* indicate *E(spl)m3-HLH*, *E(spl)m5-HLH*, and *E(spl)m7-HLH* respectively.

Fig. 5

Volcano plots of the results from the second transcriptome analysis. The horizontal axis indicates fold changes and the vertical axis indicates significance calculated as FDR with edgeR. Orange points indicate DEGs upregulated and blue points indicate downregulated DEGs. **a**: This plot shows genes differentially expressed where *wingless* is ectopically expressed. **b**: Genes with -log 10 (FDR) < 20 are extracted from **a**.

Fig. 6

The putative gene regulatory network for formation of the novel wing pigmentation pattern. Green box: Notch signaling gene, grey boxes: melanin synthesis genes, yellow

boxes: Wnt signaling genes, and blue boxes: Dpp signaling genes.

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Authors' contributions

Y.F., S.Kondo, S.S. and S.Koshikawa conceived of and designed the study; Y.F., S.Kondo, and A.T. collected data and conducted the analysis; Y.F., A.T. and S.Koshikawa drafted the manuscript, with input from S.Kondo and S.S.; Y.F. and S.Koshikawa edited and wrote the final version; all authors gave final approval for publication and agree to be held accountable for the work performed therein.

Competing interests

We declare we have no competing interests.

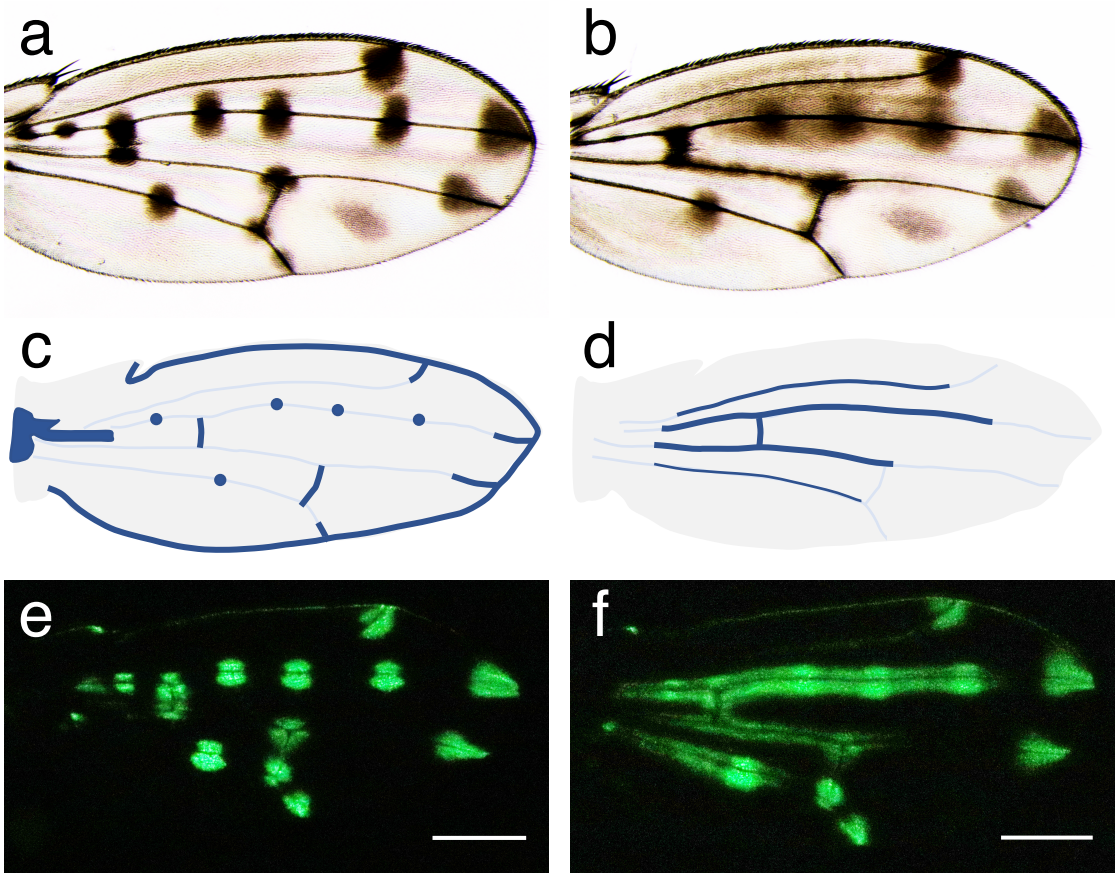

Fig. 1

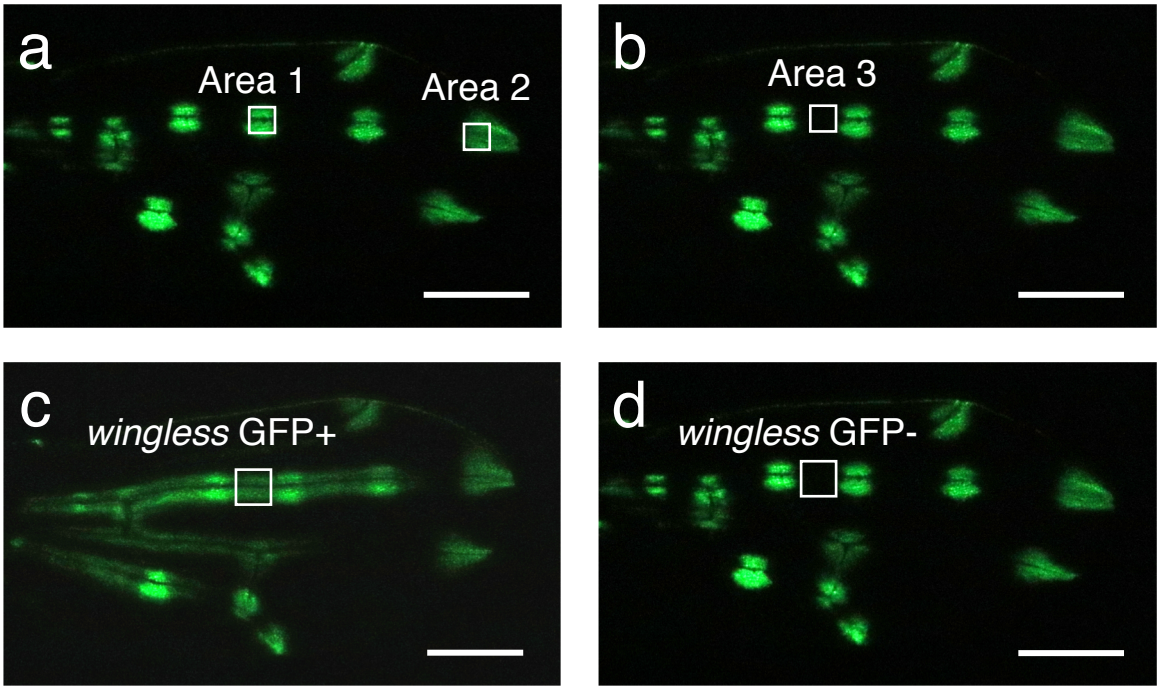

Fig. 2

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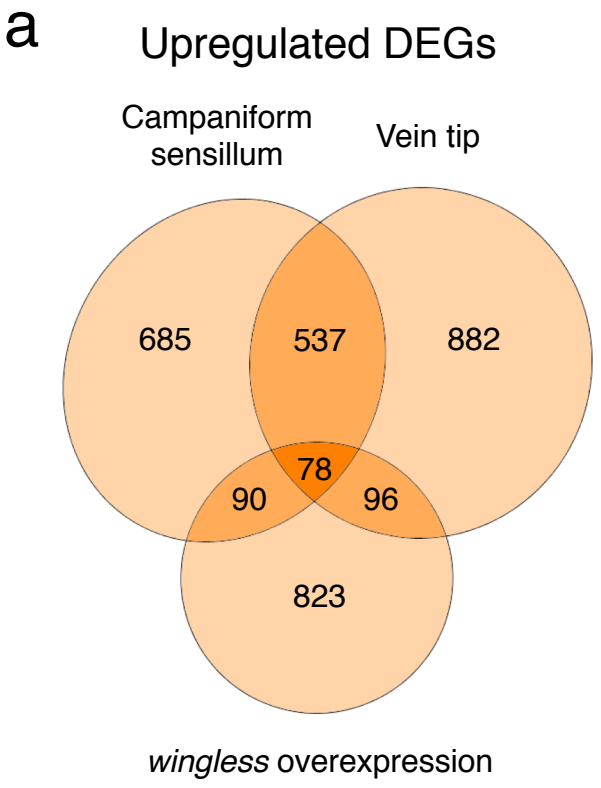
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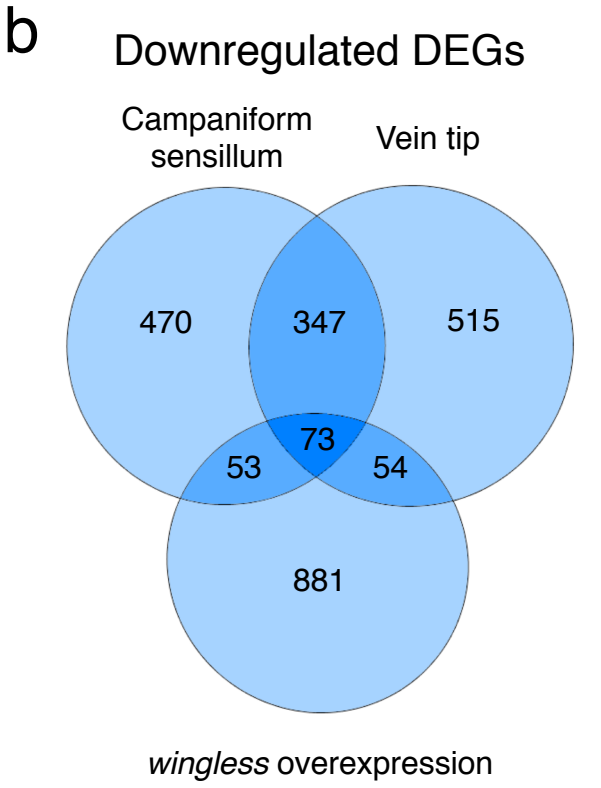
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881
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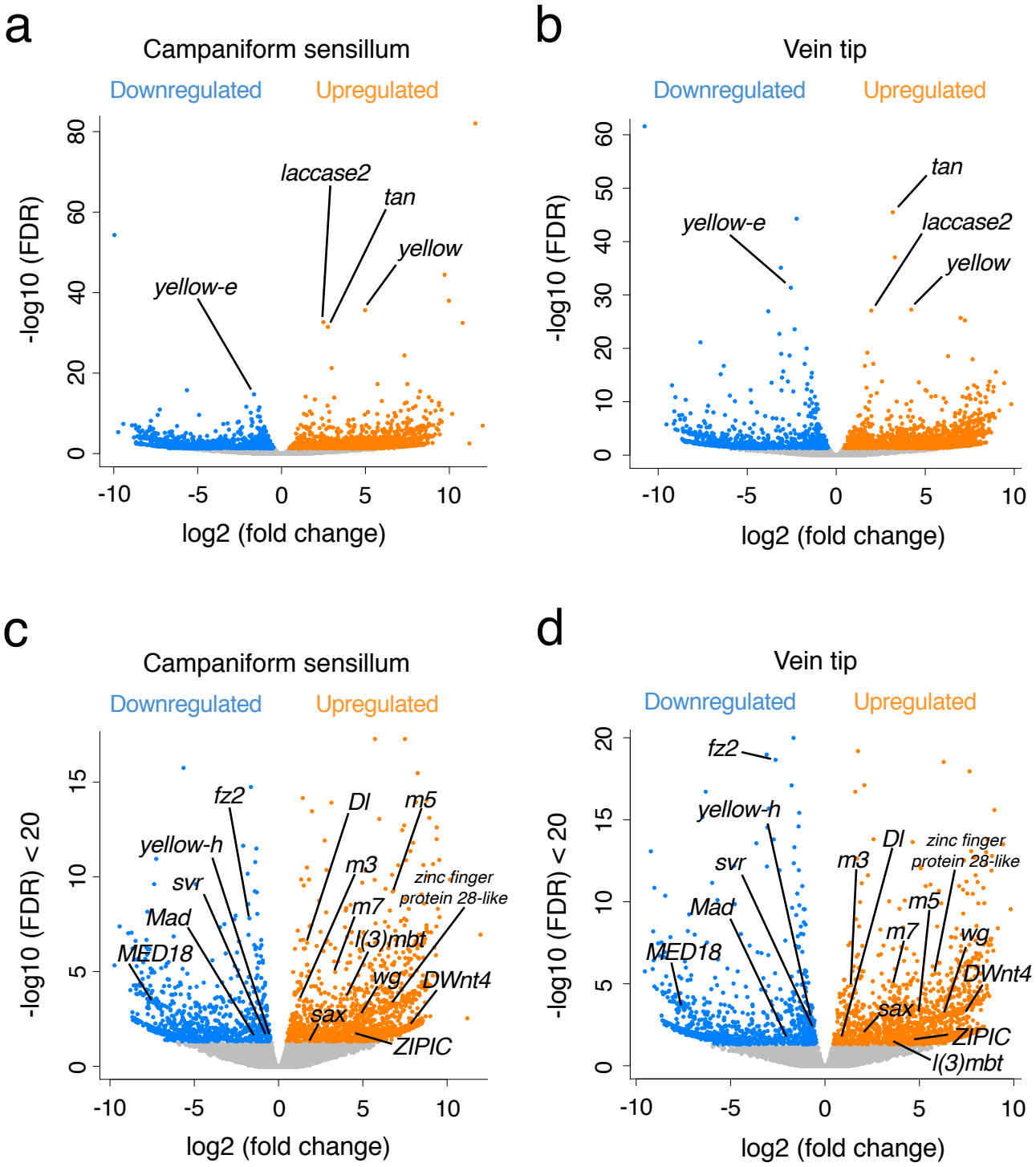
Fig. 3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Upregulated
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log2 (fold change)
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tan
yellow
yellow-e
b
Vein tip
Downregulated
Upregulated
-log10 (FDR)
log2 (fold change)
tan
yellow-e
laccase2
yellow
c
Campaniform sensillum
Downregulated
Upregulated
-log10 (FDR) < 20
log2 (fold change)
fz2
Dl
m5
yellow-h
m3
svr
zinc finger protein 28-like
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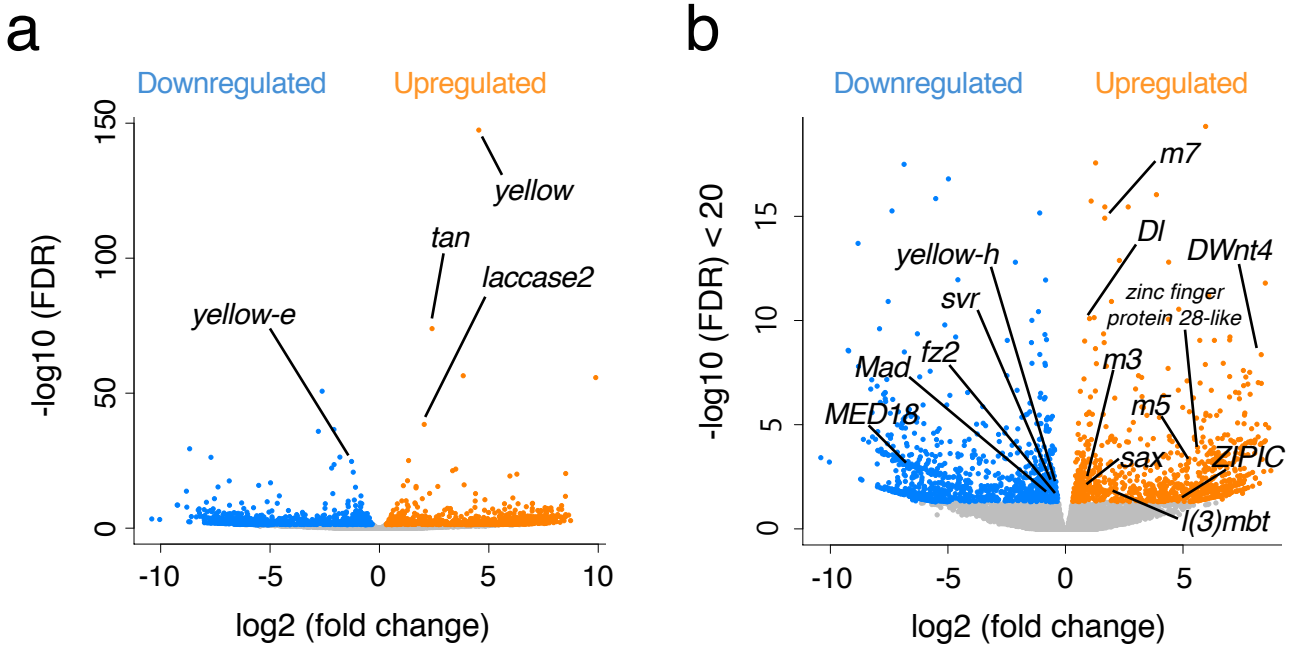
Fig. 4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Downregulated
Upregulated
-log10 (FDR) < 20
15
10
5
0
m7
Dl
DWnt4
yellow-h
svr
zinc finger
protein 28-like
fz2
Mad
m3
MED18
m5
sax
ZIPIC
l(3)mbt
-10
-5
0
5
log2 (fold change)

Fig. 5

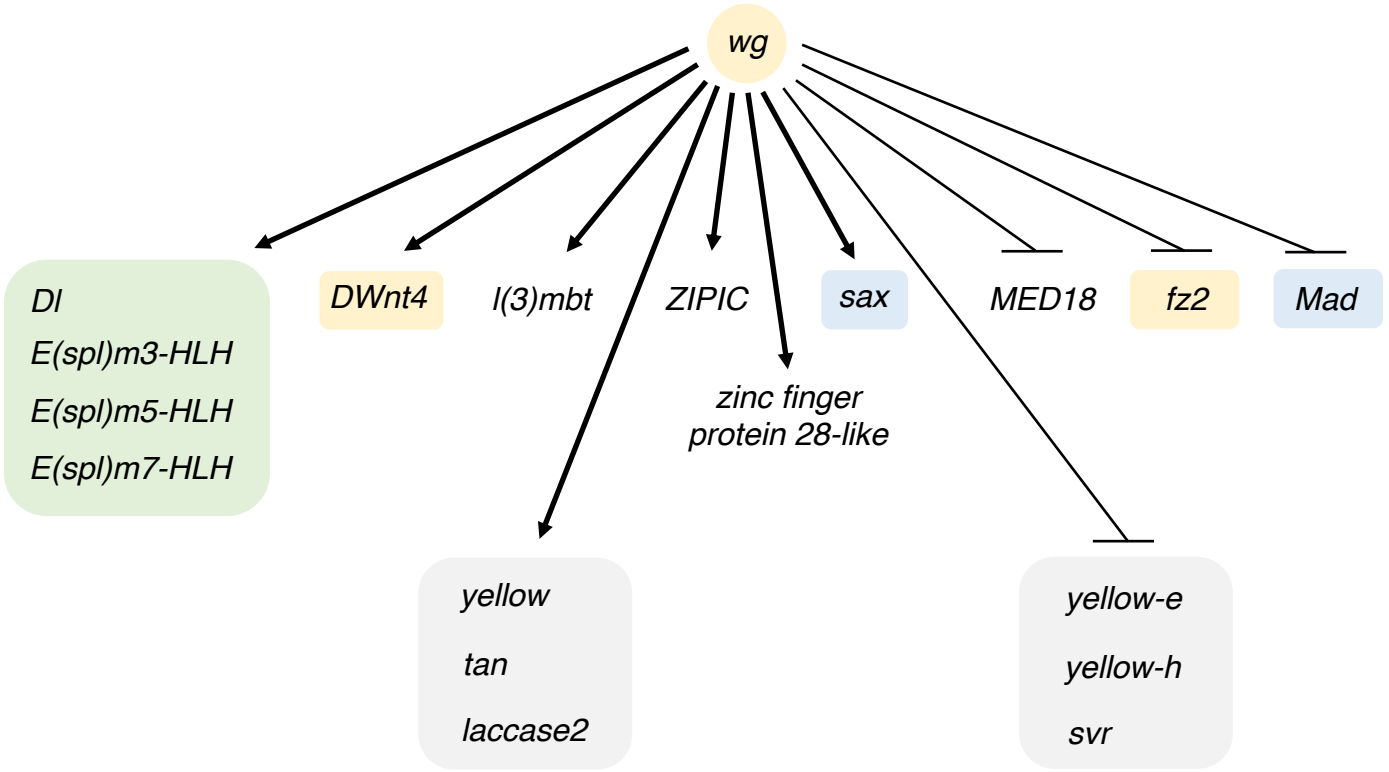
wg
Dl
E(spl)m3-HLH
E(spl)m5-HLH
E(spl)m7-HLH
DWnt4
l(3)mbt
ZIPIC
zinc finger
protein 28-like
sax
MED18
fz2
Mad
yellow
tan
laccase2
yellow-e
yellow-h
svr

Fig. 6