Analysis of gene network for color pattern formation and the mechanism of the prepattern determination in *Drosophila guttifera*

（ミズタマショウジョウバエを用いた模様形成遺伝子ネットワークとプレパターン決定機構の解析）

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Introduction

Various color patterns have evolved in a large number of animal taxa. Some of those play roles in mimicry and aposematism, and others have a physiological function such as thermoregulation (Cott 1940; Ruxton et al. 2004). What kind of developmental mechanisms underlie the generation of various color patterns? Construction of mathematical models and transplantation of tissues led to the presumption that, in the case of color patterns on mammal furs and butterfly wings, the patterns were determined by diffusion of intercellular signal transduction molecules such as morphogens (molecules which determines the pattern of animal body plans by diffusion in ontogeny; Wolpert 1969) (Turing 1952; Murray 1981; Nijhout 1980; Kondo and Shirota 2009). Thereafter, gene overexpression and knock-out experiments on fruit flies and lepidopterans showed that diffusible intercellular signal transduction molecules have function in color pattern formation (Werner et al. 2010; Yamaguchi et al. 2013; Mazo-Vargas et al. 2017). However, it is unclear whether diffusion is essential to determine the patterns in such organisms. In color pattern formation, not only intercellular signal transduction molecules but products of pigmentation genes are involved (Arnoult et al. 2013; Zhang et al. 2017). Understanding of developmental mechanisms of color pattern formation requires revealing pattern determination and regulatory relationships among genes involved in the formation.

Drosophila guttifera (Insecta, Diptera) has a polka-dotted pattern of melanin pigmentation on wings (Fig. 1). *wingless* gene (a gene coding one of morphogens, a homologue of vertebrate *Wnt-1*) is known to control formation of the pigmentation pattern. *D. guttifera* can be a model organism for studying how morphogen controls color pattern formation (Martin and Courtier-Orgogozo 2017), but knowledge about the developmental mechanism is limited. Diffusion of Wingless protein from its source was assumed to

determine the prepattern (the area where pigmentation pattern forms in future) in the pupal period. Ectopic expression of *wingless* on wings induced ectopic pigmentation, accompanied by upregulating the expression of *yellow*, which is necessary for melanin synthesis (Gompel et al. 2005; Werner et al. 2010).

Chapter 1

When I started the research, there was a lack of fundamental information about the process of pigmentation pattern formation in *D. guttifera*. It was unclear when the expression of *yellow* starts, and when wing pigmentation starts and ends. Therefore, I analyzed the process of formation of wing pigmentation. In this study, I defined the pupal stages of *D. guttifera* and measured the pigment content of wing spots from the pupal period to the period after eclosion. Using a transgenic line which carries *eGFP* connected with an enhancer of *yellow*, a gene necessary for melanin synthesis, I analyzed the timing at which the *yellow* enhancer starts to drive *eGFP*. I also analyzed the distribution of Yellow-producing cells, as indicated by the expression of *eGFP* during pupal and young adult periods. The results suggested that Yellow-producing cells were removed from wings within 3 h after eclosion, and wing pigmentation continued without epithelial cells (Fig. 2). Furthermore, the results of vein cutting experiments showed that the transport of melanin precursors through veins was necessary for wing pigmentation. These results showed the importance of melanin precursors transported through veins and of extracellular factors which were secreted from epithelial cells and left in the cuticle.

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Chapter 2

To understand the mechanism of formation of the wing pigmentation pattern, it is necessary to know which genes are regulated downstream of *wingless*. In *D. guttifera* and *Drosophila melanogaster* (common fruit fly), *wingless* is expressed on wings. However, there is no wing pigmentation pattern in *D. melanogaster*. Genes regulated downstream of *wingless* in *D. guttifera* were assumed to be responsible for formation of wing pigmentation (Werner et al. 2010). Therefore, I comprehensively detected genes differentially expressed in pigmentation areas on wings by transcriptome analysis. For

those genes, I also tested whether their expression was regulated by *wingless*. As a result, 151 of genes expressed in pigmentation areas were positively or negatively regulated by *wingless*. Genes for neural development, Wnt signaling, Dpp signaling, and effectors (such as enzymes) for melanin pigmentation were included among these 151 genes (Fig. 3). None of the known regulatory genes that regulate pigmentation pattern formation in other fruit fly species were included. My results suggest that the novel pigmentation pattern of *D. guttifera* 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.

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Chapter 3

Furthermore, we have to reconsider how Wingless protein determines the prepattern in *D. guttifera*. In *D. melanogaster*, when Wingless protein was artificially tethered to the cell membrane, the body plan was normal (Alexandre et al. 2014). Therefore, some researchers argued that diffusion of Wingless protein might be unnecessary for determining the pattern of body plans. I estimated the necessity of Wingless diffusion for determining the prepattern of wing pigmentation of *D. guttifera*, from an experiment on *D. melanogaster*. When *eGFP* gene connected with a *yellow* enhancer of *D. guttifera* is introduced to *D. melanogaster*, a part of the wing pigmentation pattern of *D. guttifera* is reconstructed as the expression of EGFP on wings of *D. melanogaster* (Werner et al. 2010). I made individuals of *D. melanogaster* whose Wingless was artificially tethered to the cell membrane and which had *eGFP* gene connected with a *yellow* enhancer of *D. guttifera*.

Based on the above, in the section of General Discussion, I will discuss how my study of the wing pigmentation pattern of *D. guttifera* contributes to understanding developmental mechanisms underlying evolution of color patterns.

Discussion

In this research, I revealed the process of formation of wing pigmentation in *D. guttifera* (Chapter 1) and comprehensively analyzed genes regulated downstream of *wingless* (Chapter 2). I tested the necessity of Wingless diffusion for the prepattern formation in wing pigmentation of *D. guttifera* (Chapter 3). In Chapter 1, it was suggested

that Yellow protein and other extracellular factors contributes to continuing formation of wing pigmentation after Yellow producing cells disappeared. This is consistent with a result mentioned in Chapter 2. Enrichment analysis for GO terms of genes differentially expressed in pigmentation areas downstream of *wingless* showed that GO terms such as "Topological domain: Extracellular" and "Extracellular matrix" were enriched (Table 2-5). The result of enrichment analysis also suggested that extracellular factors contributes to formation of wing pigmentation. Based on the results in Chapter 1 to 3, the mechanism of wing pigmentation pattern formation in *D. guttifera* is estimated as follows: First, expression of *wingless* gene determines the prepattern. Second, the expression of genes for neural development, genes involved in Wnt and Dpp signaling, and genes for melanin synthesis are regulated by *wingless* and wing pigmentation starts in the later pupal stage (P12 (i)). Third, after eclosion, epithelial cells in pigmentation areas is retrieved from wings and wing pigmentation completes with Yellow protein, other extracellular factors, and substances transported through wing veins.

Then, how does the study of wing pigmentation pattern of *D. guttifera* contribute to understanding developmental mechanisms underlying evolution of color patterns? As mentioned in Chapter 2, *wingless* gene is expressed at crossveins on wings of *D. guttifera* (with wing pigmentation) and *D. melanogaster* (without wing pigmentation). For emergence of wing pigmentation, it might be necessary that genes for neural development, genes involved in Wnt and Dpp signaling, and genes for melanin synthesis become downstream of *wingless* (Chapter 2) (Fukutomi et al. 2020). Another *Drosophila* species, *Drosophila deflecta*, also has wing pigmentation around crossveins and the expression of *wingless* was also observed at crossveins of the species (Werner et al. 2010). In *D. deflecta*, same genes as the case of *D. guttifera* might be regulated downstream of *wingless* in pigmentation areas. Furthermore, the shape of pigmentation areas around a posterior crossvein are different between *D. guttifera* and *D. deflecta*. This difference might be able to be explained by the differences in distribution of Wingless protein, but whether *wingless* controls formation of wing pigmentation in *D. deflecta* has to be tested at first. Other factors might be responsible for the difference of wing pigmentation patterns. As it was suggested that transportation of substances through wing veins could affect intensity and area of pigmentation (Chapter 1) (True et al. 1999; Fukutomi et al. 2017), the difference of wing pigmentation patterns might be explained by this factor. Not only in *Drosophila* species, studying wing pigmentation pattern of *D.*

guttifera might contribute to understanding mechanisms of color pattern formation in other insects. Wingless diffusion was assumed to determine the prepattern of twin spots on the dorsum of a silkworm (*Bombyx mori*) and eyespots on wings of a butterfly (*Bicyclus anynana*), but the necessity of Wingless diffusion in prepattern determination has not been tested (Yamaguchi et al. 2013; Özsu et al. 2017).

As a future perspective, to elucidate how morphogen is transported and what concentration of morphogen is necessary to induce expression of downstream genes will be the key point to reveal developmental mechanisms of color pattern formation. As the necessity of Wingless diffusion and the downstream genes of *wingless* was analyzed in the wing pigmentation pattern of *D. guttifera*, it can be an appropriate model to solve these questions. If these two questions are studied in color patterns of many species of insects, that will provide novel insights into the understanding of color pattern evolution.

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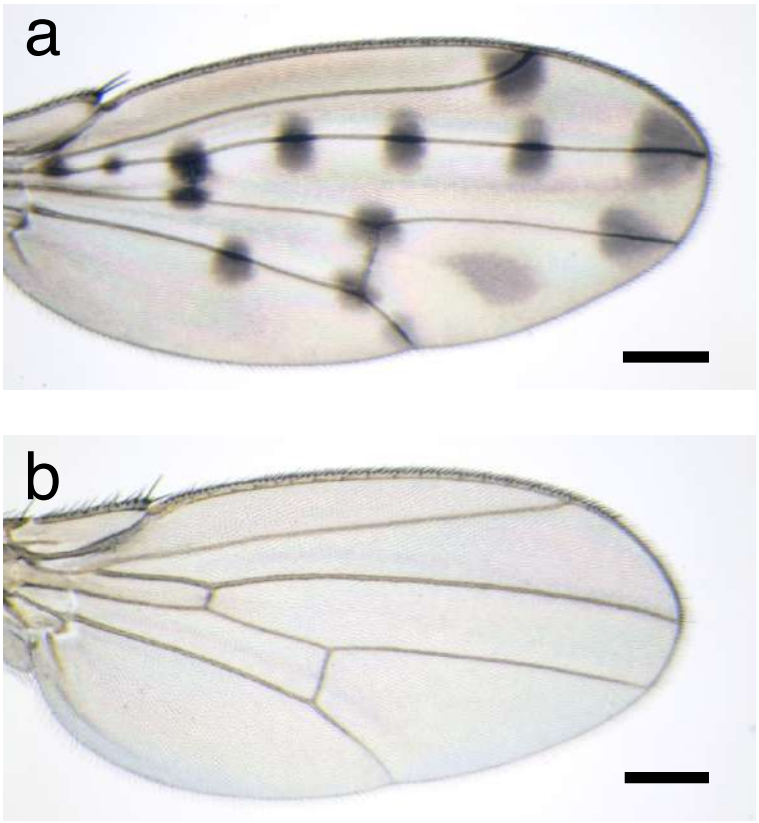

Fig. 1
Wings of *Drosophila guttifera* and *Drosophila melanogaster*. **a** A wing of *D. guttifera*. This species has a polka-dotted pigmentation pattern on its wings. **b** A wing of *D. melanogaster*. This species does not have a pigmentation pattern on its wings. Scale bars indicate 250 μm.

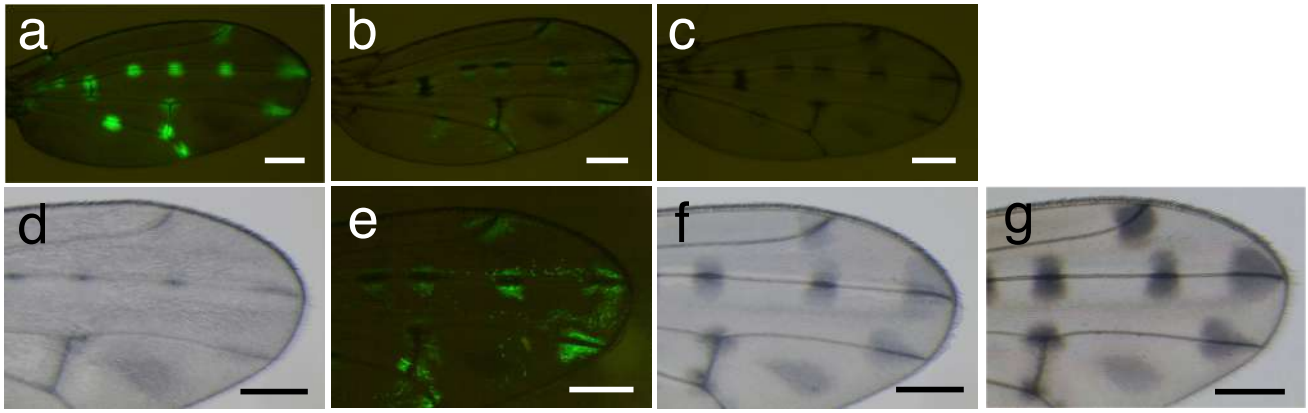

Fig. 2
Yellow-producing cells were detached from the original positions and migrated towards the thorax. Flies in **a**, **b**, **c**, and **e** carry *nuclear eGFP* connected with the *yellow* enhancer (*vein spot* CRE). **a** Distribution of EGFP in the wing of an adult fly just after expansion of the wings (green channel). In this stage, the cells with EGFP expression were strictly located in the positions of future spots. **b** A wing of an adult fly 1.5 h after eclosion (green channel). Epithelial cells started to migrate in this stage. **c** A wing of an adult fly 3 h after eclosion (green channel). Almost all the EGFP-labeled cells are retrieved. **d** A wing of an adult fly just after expansion of the wings (bright field). **e** A magnified view of **b**. 1.5 h after eclosion (green channel). **f** A wing of an adult fly 3 h after eclosion (bright field). **g** A wing of an adult fly 1 day after eclosion (bright field). Spots are darker than those at 3 h after eclosion. Scale bars indicate 250 μm.

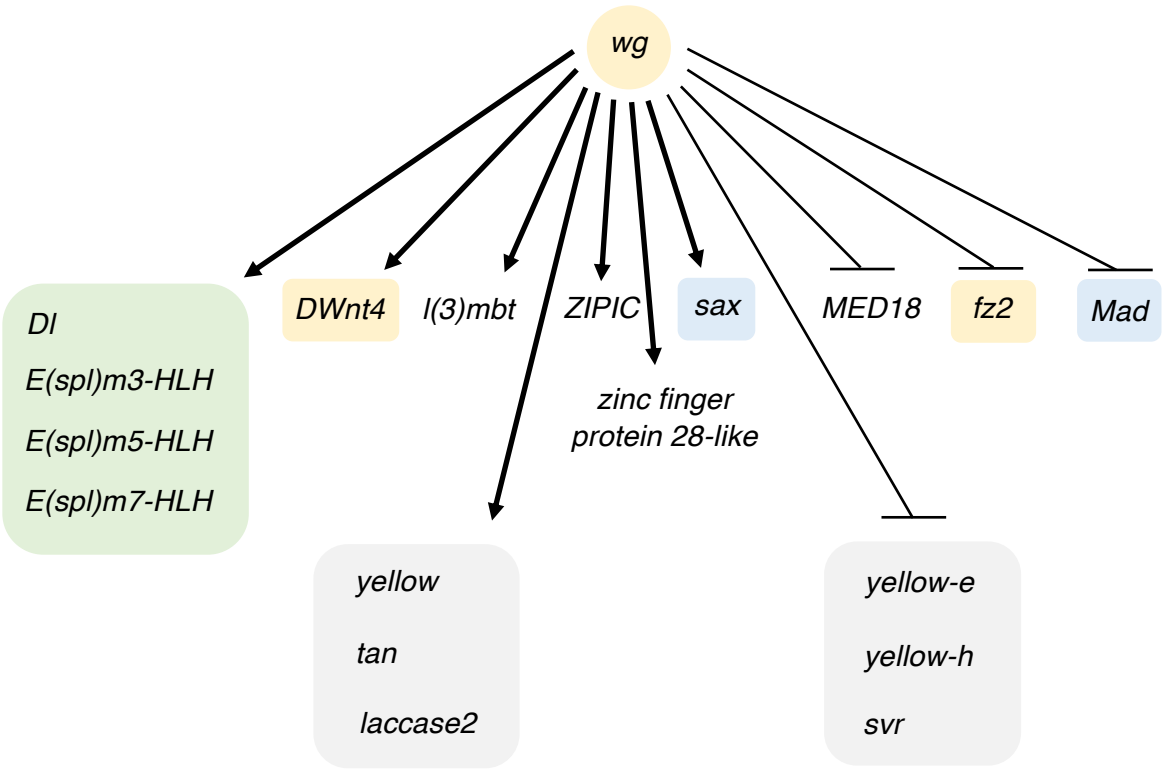

Fig. 3
The putative gene regulatory network for formation of the novel wing pigmentation pattern. Green box: Notch signaling gene, grey boxes: melanin synthesis genes, yellow boxes: Wnt signaling genes, and blue boxes: Dpp signaling genes.