

Asymmetric flies

The control of developmental noise in *Drosophila*

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What are the sources of phenotypic variation and which factors shape this variation are fundamental questions of developmental and evolutionary biology. Despite this simple formulation and intense research, controversy remains. Three points are particularly discussed: (1) whether adaptive developmental mechanisms buffering variation exist at all; (2) if yes, do they involve specific genes and processes, i.e., different from those involved in the development of the traits that are buffered?; and (3) whether different mechanisms specifically buffer the various sources of variation, i.e., genetic, environmental and stochastic, or whether a generalist process buffers them all at once. We advocate that experimental work integrating different levels of analysis will improve our understanding of the origin of phenotypic variation and thus help answering these contentious questions. In this paper, we first survey the current views on these issues, highlighting potential sources of controversy. We then focus on the stochastic part of phenotypic variation, as measured by fluctuating asymmetry, and on current knowledge about the genetic basis of developmental stability. We report our recent discovery that an individual gene, *Cyclin G*, plays a central role—adaptive or not—in developmental stability in *Drosophila*.¹ We discuss the implications of this discovery on the regulation of organ size and shape and finally point out open questions.

Origin of Variation and Nature of Robustness

Phenotypes are remarkably stable and stereotyped given the extremely heterogeneous conditions under which organisms develop and the omnipresence of genetic variation. This stability shows that buffering processes are at play during development. The fact that major mutations or extreme environmental treatments are generally accompanied by a burst of phenotypic variation also indicates that this buffering can be overcome by sufficiently strong perturbations.^{2,3} The origin of this phenotypic stability, or robustness, has attracted a great deal of interest over the past 50 years but remains nevertheless a controversial issue.

In addition to the terminological confusion that has been discussed in the literature,^{4–6} this situation is also likely influenced by the diversity of approaches used: evolutionary biology, molecular developmental biology and more recently, systems biology. The different focus of these disciplines (i.e., population parameters vs. properties of individual molecules vs. network structure), as well as their contrasted conceptual traditions (i.e., functionalism vs. structuralism), has led each one to propose a different view of variation and its sources as well as of the processes regulating variation.

Evolutionary biology. Paradoxically, some of the most influential ideas on the evolution and control of phenotypic variation were proposed by Waddington, himself a developmental biologist. He suggested

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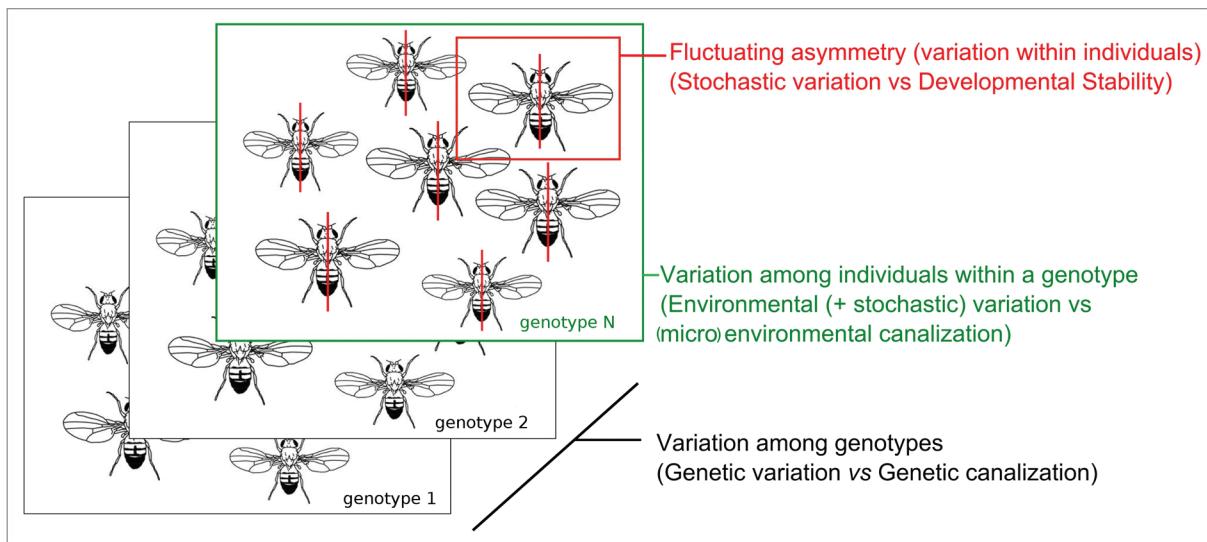


Figure 1. Sources of phenotypic variation (stochastic, environmental and genetic) vs. developmental buffering processes (developmental stability, environmental and genetic canalization) and the different levels at which they can be experimentally estimated.

that phenotypic variation is buffered by dedicated developmental processes—namely canalization and developmental stability.⁷ His concept of canalization is implicitly adaptive. It is noteworthy that such an adaptationist (functionalist) view was rather discordant with the structuralist views that prevailed among developmental biologists but quite in tune with those of contemporary evolutionary biologists. The notion of canalization is appealing to evolutionary biologists since an environmentally induced decanalization could reveal the normally buffered (and therefore cryptic) genetic variation, thereby increasing the population's adaptive potential and speeding up the tempo of phenotypic evolution,⁷ an idea endorsed by Rutherford and Lindquist in 1998.⁸ Waddington's views were nevertheless not included in the evolutionary synthesis of the 40s, which was too strongly centered on genes to accommodate developmental considerations, and only recently regained full respectability, particularly owing to the advent of evo-devo.⁹

The evolution of canalization and developmental stability has been studied in the context of quantitative genetics, both experimentally and theoretically.^{10,11} Both approaches rely on a precise decomposition of phenotypic variation and have in common that they do not require any precise understanding of the proximal buffering processes (conform to the functionalist tradition).

Variation is studied at the level of populations, and the sources of variation are clearly identified (Fig. 1).¹² Genetic variation can be measured as the variation among families of related individuals placed in controlled environmental conditions. Similarly, environmental variation can be measured as the variation among groups of genetically identical individuals placed in different conditions (then referred to as phenotypic plasticity). The remaining variation, i.e., the variation within a group of genetically identical individuals in the same general environment, is more difficult to assign to any definite source, as environmental heterogeneity can never be completely removed. For example, individual fly larvae within a vial will each encounter unique local conditions, which may affect their development. Micro-environmental variation thus occurs even in the most carefully controlled conditions. However, part of the within group variation cannot be attributed to environmental differences, but to small, random errors that affect all levels of biological organization during development, a phenomenon usually referred to as developmental noise (or stochastic variation).^{13,14} This clear distinction at the level of experimental groups of individuals, between genetic, environmental and stochastic variation, possibly combined with the remnant adaptationist tradition tending to consider every trait as

the result of natural selection, has led to the hypothesis that these different sources of variation are buffered by (undefined) developmental processes that have been selected for this function.⁵ Genetic variation is supposed to be buffered by genetic canalization, environmental variation by environmental canalization and developmental noise by developmental stability (Fig. 1). This view, which implicitly reifies canalization as a mechanism separate from the developing traits themselves and suggests that different processes buffer the different sources of variation, has been discussed—and often criticized—in both experimental and theoretical studies (see Flatt, 2005).³

Developmental genetics. Waddington's ideas had only a limited impact on developmental biology, as discussed by Scharloo (1991).¹⁵ The study of variation was long at odds with the very aim of developmental genetics, centered on identification of repeatable patterns required to firmly establish its principles (and in accordance with its structuralist premises). Deviations from the norm were thus considered a nuisance and buffering processes were thus for a long time without clear developmental or molecular support. Although genetic redundancy and dominance can be considered as canalizing processes,³ and both heterozygosity and inbreeding are known to affect phenotypic variability,¹⁶ the lack of molecularly well-characterized

buffering mechanisms has long weakened the concept of canalization. This is probably because developmental biology has not focused on variation among individuals, but on discrete variation among cells and tissue types (for which Waddington originally proposed the concept of canalization) and on macromutations that generate extreme phenotypes. Separation of genetic, environmental and stochastic variation becomes difficult in such a context. Every trait simultaneously depends on genetic and environmental inputs that influence each other. Unravelling the effects of genes from those of the environment becomes impossible and even meaningless at the individual level.¹⁷ Similarly, at the molecular level, stochastic variation affects genetic processes (e.g., levels of gene expression, morphogen diffusion ranges, etc.) as well as environmental parameters (e.g., light, food, pH, etc.). Moreover, a random process at one level can translate into determined patterns at an upper level. For example, selection among cells typically generates order from stochastic variation, a phenomenon that has lately attracted much attention in developmental biology.¹⁸⁻²¹

Recently, molecular developmental biology started to consider variation among individuals as of interest, leading to the first discoveries of molecular processes involved in canalization. The chaperone protein Hsp90 was shown to buffer genetic variation by stabilizing proteins impaired by mutation^{8,22} and was thus termed the first genetic canalization factor.²³ Hsp90 was originally presented as an “evolutionary capacitor,” which was implicated in an adaptive mechanism promoting evolvability. This view generated both high enthusiasm and criticisms and boosted experimental and theoretical work on canalization/robustness. The finding that the heat-shock protein GroEL protects endosymbiotic bacteria against the harmful effects of accumulated mutations suggested that molecular chaperones are also involved in canalization in prokaryotes.²⁴ Further molecules and mechanisms buffering genetic variation were then discovered. Among factors thought to play key roles in canalization are *microRNAs* due to their multiple targets and the many feedback regulations they generate,

as well as the yeast prion [PSI+], which reveals cryptic genetic variation beyond stop codons by impairing translation termination.²⁵⁻²⁷ Furthermore, comparative genomic analyses suggested that alternative splicing, which permits cryptic genetic variation to accumulate, also exhibits properties of a “canalizing agent.”^{27,28}

Systems biology. Parallel to this renewed interest of developmental genetics for canalization and stability, these concepts became objects for systems biology and cybernetics, partly as a reaction against the Waddingtonian idea—endorsed by Rutherford and Lindquist (1998)⁸—that canalization would be an evolved adaptive property.²⁹ These studies modeled phenotypes as the outcome of complex networks of interacting genes and proteins and suggested that canalization (usually referred to as robustness in this context) is an emergent property of these systems.³⁰ There would be no need of specific mechanisms devoted to buffering variation, since the system is inherently robust to perturbation.²⁹⁻³² The classical opposition between functionalism and structuralism clearly shows up in this opposition, as systems biology does not focus on individual genes but on the structure of the regulatory network. Congruent data arose from evolutionary genetics, with models showing that epistasis is sufficient to explain the increased variation often detected either in mutants or in extreme environments; no specialized canalization mechanism would thus be required.³³

Combining these opposite points of view. Existence of specific mechanisms buffering variation and dependence of robustness on genetic network architecture and complexity need not be mutually exclusive points of view.³ Rutherford et al.³⁴ suggested that the broad canalizing effect of Hsp90 could be related to the central position of this protein in a network of interactions where it would act as a hub.³⁵ This idea was documented in *Saccharomyces cerevisiae*, where about 300 environmental phenotypic capacitors were identified, notably transcription regulation and chromatin remodelling complexes that displayed similar buffering effects as Hsp90 and tended to be network hubs.³⁶

Specific or shared robustness mechanisms? Regardless of the mechanistic basis of robustness, whether it stabilizes the phenotype against all sources of variation by a single mechanism or whether specific processes are involved depending on the nature of the perturbation remains a contentious question. Given the relative rarity of mutations and the universal heterogeneity of the environment, genetic canalization might likely have evolved as a by-product of selection for environmental canalization.³⁷ According to this view, robustness would be general and buffer against all different sources of variation.³⁸ Neither simulation studies nor experimental work have to date provided clear results confirming this point of view.^{8,37} Indeed, a study in *Escherichia coli* suggested that network hubs are likely involved in environmental but not in genetic robustness.³⁹

In this context, the status of stochastic variation has been particularly unclear. Whether developmental stability, the process proposed to buffer developmental noise, is indeed different from canalization, either environmental or genetic, remains unknown.

Fluctuating Asymmetry and the Genetic Bases of Developmental Stability

Variation among clones raised under fully homogeneous environmental conditions has been considered as evidence for developmental noise. This, however, is problematic as micro-environmental variation always occurs, as mentioned above, even in the most carefully controlled experimental conditions. To avoid this and because genetic identity cannot be guaranteed in most cases, fluctuating asymmetry (FA), generally measured as the variance of the difference between right and left values of bilateral traits, has been proposed as an alternative to assess stochastic variation in metazoans. Both sides are considered replicates of the same genotype in identical conditions, and bilateral differences are thus expected to reflect random variation.^{40,41} FA manifests itself as subtle, random deviations from perfect symmetry, leading to a normal distribution of individual asymmetry values centered on zero (the mean phenotype is symmetrical). The

measured FA results from the interplay between developmental noise, pushing the phenotype away from perfect symmetry, and developmental stability, opposing or buffering such an effect. Under given conditions, the higher the FA and the lower the developmental stability.

Importantly, FA is distinct from overall morphological asymmetry, as displayed, for example, by internal organs in vertebrates. FA relates only to developmental imprecision and its buffering and thus does not bear on processes involved in laterality.

To understand developmental stability and its genetic bases requires ultimately to decipher the processes generating as well as buffering developmental noise (the latter being developmental stability *per se*). The distinction between noise and buffering may not always be justified, since noisiness at one level (e.g., noisiness in gene expression) does not necessarily translate into noisiness at another (e.g., at the organ level). This raises the question whether all mechanisms stabilizing gene expression should be considered as developmental stability processes. To answer this, noisiness and developmental stability mechanisms should be investigated across biological levels.⁴²

Genetics of FA have mainly been investigated by evolutionary biologists. A popular, although very controversial view is indeed that FA would reflect Darwinian fitness and the quality of environmental conditions.^{43,44} Most studies applied quantitative genetics designs to quantify FA heritability. The general picture is that FA heritability is typically very low, suggesting that FA has no, or a very low, additive genetic basis. In agreement with this hypothesis, QTL analyses performed on mouse teeth, mandible and skull found evidence for an epistatic genetic basis for FA (a dozen QTL detected).⁴⁵⁻⁴⁷ Furthermore, in *Drosophila* wings, Gomez and Norry⁴⁸ found that the number of FA QTLs was low but increased with temperature, also suggesting a non-purely additive genetic basis. Still, low values of FA heritability need not indicate a low heritability of developmental stability, as emphasized,⁴⁹⁻⁵¹ making our knowledge on developmental stability heritability very limited.⁴⁶

Recently, several studies have tried to identify genomic regions involved in developmental stability in *Drosophila*, scanning the genome using adjacent deletions.^{52,53} The latter study detected 89 regions with an effect on wing shape FA, 0 affected wing size FA and 5 affected bristle FA. These studies did not identify candidate genes, but nevertheless confirmed that FA determinism would be polygenic, as suggested by QTL analyses. This agrees with the fact that developmental stability can be strongly affected by general heterozygosity.⁴⁶ A recent study in the house sparrow showed that developmental stability depends both on overall and local heterozygosity, suggesting that “the molecular basis of developmental stability may involve complex interactions between local and genome-wide effects.”⁵⁴ Interpreting results of these genetic studies is often difficult. The magnitude of FA is typically very low; therefore most studies are affected by power issues, decreasing the likelihood of detecting any signal.^{50,51}

Besides marginal references,⁵⁵ FA has not been investigated in developmental biology, where the interest has generally been on large effects and not on subtle variation. Very few studies have focused directly on individual genes, and molecularly identified buffering mechanisms are rare. *Hsp90* was tested for FA in *Drosophila* wings and had only a limited effect.^{56,57} *Hsp70* and genes encoding small Hsp were also investigated, but their effect on FA was not clear.^{58,59} For long, one of the only molecularly identified gene that clearly alters FA was detected in the sheep blowfly *Lucilia cuprina*. *rbel*, the homolog of *Notch*, was shown to modify bristle FA via interaction with another gene, *Rop1*. This epistatic effect reinforced the hypothesis of a complex genetic basis for developmental stability.⁶⁰ As *Notch* is known to control bristle development, the data also suggest that this genetic basis for FA could be trait-dependent.

Cyclin G is an Important Determinant of Developmental Stability in *Drosophila*

We recently discovered that the *Cyclin G* gene (*CycG*), which encodes a protein

involved both in transcriptional regulation and in the cell cycle,⁶¹⁻⁶³ is essential for developmental stability in *Drosophila*.¹ Deregulating *CycG* expression—by overexpression or RNAi-mediated inactivation using a Gal4 driver—we generated extremely high levels of FA in the wing, i.e., an increase by a factor of 40 when *CycG* was overexpressed, which is an unprecedented effect in any system or trait.

We checked that this effect was really due to *CycG* in three ways: (1) We repeated the experiment in two different genetic backgrounds (i.e., *yw*^{67c23} and *w*¹¹¹⁸) and observed similar effects; this suggested that the asymmetry was not due to a specific interaction with the genetic background. (2) We overexpressed various genes involved in growth or cell cycle control (e.g., *dS6K*, *diminutive*, *CycD*, *Cdk4*, etc.) using the same Gal4 driver, but failed to generate effects on asymmetry; this indicated that *CycG* deregulation was special in its effects on FA and that our results did not illustrate a generic effect of important gene deregulation. (3) We deregulated *CycG* by using drivers of various strengths and found that amplitude of the FA phenotype was directly correlated with the level of *CycG* expression.

The use of nearly isogenic strains and carefully controlled environmental conditions allowed us to rule out that genetic or environmental variation were altering symmetry, indicating that developmental noise was responsible for FA increases. We then investigated the asymmetry phenotype in detail. To do so, we combined genetic manipulations with two levels of phenotypic analysis: (1) We assessed wing asymmetry using a geometric morphometric approach, which allows an extremely precise quantification of the patterns of size and shape variation.⁶⁴ (2) We investigated the cellular bases of the asymmetry, measuring both cell size and cell number.

The morphometric analysis lead to the conclusion that the *CycG*-induced asymmetry in wing shape was qualitatively very similar to that observed, although at much lower levels, in wild type flies. Hence, developmental processes impaired in our experiments are likely those that normally ensure stable development. *CycG*

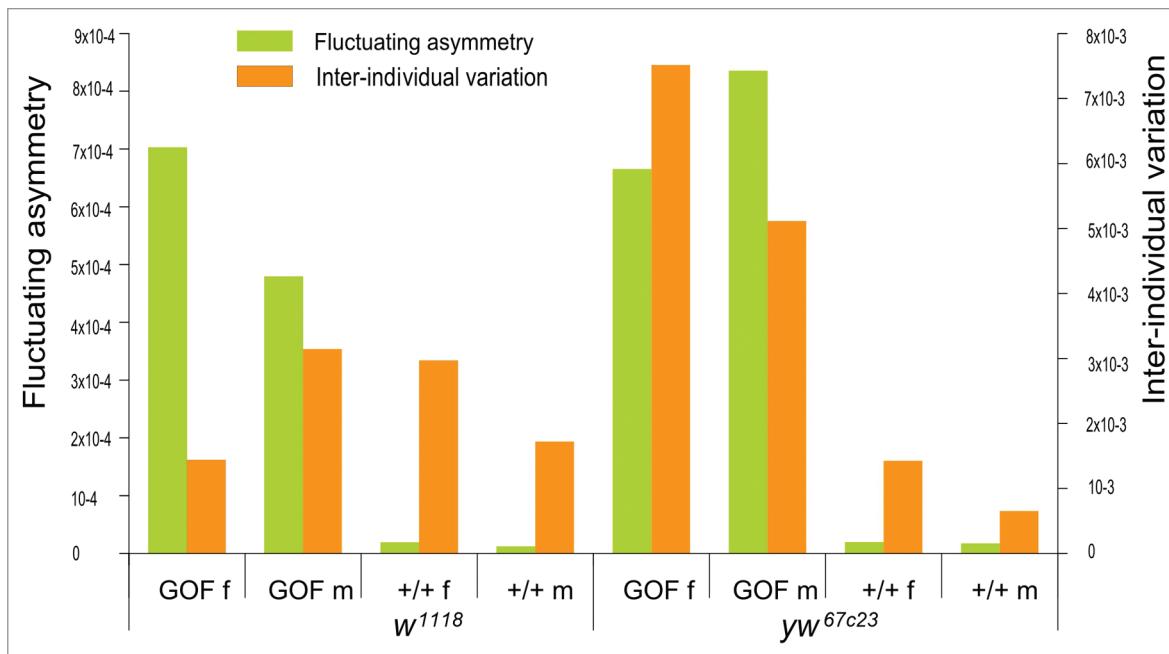


Figure 2. Effects of overexpressing *Cyclin G* on fluctuating asymmetry (green) and on inter-individual variation (orange) in two genetic backgrounds (*w¹¹¹⁸*, left; *yw^{67c23}*, right). The extreme increase in fluctuating asymmetry is found in both backgrounds, while the effect on inter-individual variation is limited to *yw^{67c23}*. GOF, Gain of function; *+/+*, wild type; f, females; m, males.

deregulation not only altered wing but also femur FA. Therefore the developmental stability mechanism mediated by *CycG* might be global. However, further preliminary data suggest that *CycG*-induced FA would not extend to bristles. As wing size and shape as well as femur length are directly influenced by quantitative growth variations, while bristle number mostly depends on patterning mechanisms,⁶⁵ this discrepancy could indicate that different stabilizing processes are activated depending on trait development.⁶⁶ Additional investigation of this issue will help to determine whether developmental stability is a general mechanism or rather is trait-dependent, as has indeed been proposed.⁶⁷

CycG expression level is fundamental for the buffering of stochastic variation as measured by FA, but does it also affect buffering of genetic or environmental variation? In other words, is *CycG* as important for environmental and genetic canalization as for developmental stability? In our experiments, genetic and environmental variations were reduced to a minimum: heterozygosity was very low and environmental conditions (temperature, food quality, larval density) were carefully standardized. Variation

among individuals (inter-individual variation; measured as the variance of the mean between left and right wing size) nevertheless included both a genetic (the remaining, low, within line genetic variation) and an environmental component (e.g., local individual positioning in a vial, local differences in temperature or food, etc.), in addition to the stochastic developmental variation. To focus on the non-stochastic part, we corrected the inter-individual variation by subtracting the intra-individual variation (i.e., the FA). Our measure of inter-individual variation thus only reflects differences in genetic and environmental sensitivity (i.e., canalization). Surprisingly, and contrasting with the effect on FA, we found that the effects of *CycG* deregulation on inter-individual variation were largely dependent on the genetic background. Indeed, in the *w¹¹¹⁸* background, inter-individual variation was not higher in transgenic flies than in controls (Fig. 2), suggesting a specific effect of *CycG* on developmental stability but not on canalization. On the other hand, in the *yw^{67c23}* background, *CycG* deregulation was associated with a very strong increase in inter-individual variation (Fig. 2), suggesting that both developmental stability and canalization

were impaired. This should obviously be confirmed using more than two backgrounds and an experimental setup allowing separate quantification of the genetic and environmental components of variation. However, our data suggest that FA and inter-individual variation are not necessarily coupled⁶⁸ and that the effect of *CycG* on canalization involves epistatic interactions.

Insights into the Regulation of Organ Size and Shape

At the cellular level, we identified a process by which wing size is stably maintained in wild-type flies: Cell size and cell number are indeed normally under tight negative correlation and compensate for each other. This compensation was lost when *CycG* was deregulated—either over-expressed or downregulated—thereby allowing random variation in both cell growth and proliferation to translate into changes at the level of wing size and shape. Altogether these results prompted the suggestion that *CycG* plays a major role in *Drosophila* developmental stability by coupling cell growth and proliferation. Strikingly, *CycG* deregulation leads to high variability of cell size in both wing

imaginal discs and adult wings, suggesting that growth might be the noisy process that causes increased FA.^{1,63}

Growth of developing organs is basically controlled by extrinsic nutrient-sensitive signals—IGF-1 (insulin-like growth factor 1) and Insulin-like peptides (ILP) activating the InR/TOR pathway—and intrinsic signals, i.e., morphogen gradients, orchestrating cell proliferation.⁶⁹ Interactions between neighboring cells, anisotropic distribution of receptors in the cell membrane and orientation of cell division all contribute to homeostasis of tissue growth and organ shaping (Garcia-Bellido's Entelechia model).⁷⁰ Recent studies have shown that alteration of extrinsic as well as intrinsic signals can affect developmental stability.

In response to localized growth perturbation (for instance injury of an imaginal disc), duration of the larval period is extended, allowing damaged tissue to be repaired and larval tissue growth to be synchronized.⁷¹ Two exciting recent studies showed that *Drosophila* ILP8 (DILP8), which is produced by the damaged disc and secreted into the hemolymph, mediates this growth retardation and suppresses production of ecdysone by the ring gland.^{72,73} Interestingly, DILP8 mutants display high FA, suggesting that DILP8 could also be necessary to maintain developmental stability during normal development.⁷² Whether DILP8, which is expressed during a short period between late second and early third larval instar, would maintain developmental stability by controlling ecdysone production or via another activity remains unknown. Interestingly, a sizeable percentage of larvae inactivating *CycG* fails to pupariate (our unpublished observations), which could argue for the idea that alteration in ecdysone production might increase FA.

During imaginal disc growth, metabolically disadvantaged cells are eliminated by their healthier neighbors by a phenomenon known as cell competition.^{74,75} For example, *Minute*/⁺ (*M*/⁺) clones (*M* mutants affect ribosomal protein encoding genes) or *diminutive* (*dm*) hypomorphic clones (*dm* encodes the protooncogene Myc)—called loser cells—are rapidly eliminated by surrounding wild type cells—called winner cells. Winner

cells activate the JNK stress pathway in loser cells, which induces the proapoptotic gene *hid*.⁷⁶ Interestingly, impairing cell competition (in *hid* mutants) induces stochastic variation of wing disc size⁷⁷ and high wing FA in adults.⁷⁶ As little apoptosis occurs during normal wing disc development, cell competition would rather be a safeguard mechanism to eliminate abnormal cells.^{78,79} Whether cell competition per se or *hid* acting in a still unknown pathway underlies developmental stability is not known. No extra apoptosis was observed when deregulating *CycG*, but overexpression of *CycG* facilitates apoptosis after DNA damage (our unpublished results). In this respect, *Drosophila CycG* is very similar to mammalian *Cyclin G1* (*CCNG1*), a transcriptional target of P53. Importantly, recent work in mammals has shown that *p53* mediates cell competition in hematopoietic stem and progenitor cells, but without apoptosis of loser cells,⁸⁰ and *Drosophila p53* is involved in coordinating tissue growth.⁸¹ It will be interesting to decipher the relationship between *Drosophila p53* and *CycG* and to test their potential common role in maintaining developmental stability.

Our recent studies have suggested another mechanism by which *CycG* could participate in regulation of cell growth. Cyclin G is a transcriptional regulator and interacts with the Enhancer of Polycomb and Trithorax Corto, suggesting that they co-regulate the transcription of numerous genes.^{61,62} Corto also interacts with ribosomal protein RPL12, which likewise binds chromatin and regulates transcription.⁸² Genome-wide analyses of transcripts from wing imaginal discs with deregulated *corto* or *RPL12* revealed that they control transcription of ribosomal protein genes. Hence, they could ensure tissue growth homeostasis by dynamically coordinating ribosome biogenesis.⁸² It is tempting to speculate that Cyclin G, which directly binds Corto, could also be involved in this process.

Open Questions

Beyond these findings, our work opens up many questions: (1) Are *CycG* effects on developmental stability adaptive? This is a difficult question. It is possible that *CycG*

(and/or the *CycG* network) was selected, at least partly, for its stabilizing effects. We might also discover that other genes affecting the cell size to cell number relationship have similar effects, suggesting that the selected trait would be proliferation/growth control, leaving stability as an emergent trait. This was nevertheless not the case for any other candidate gene investigated so far, suggesting that *CycG* is special in this respect. Our ongoing work should help clarifying this fundamental issue. (2) What is the exact mechanism by which *CycG* uncouples cell growth and proliferation? In this process, *CycG* likely interacts with other genes, which need to be identified. In the context of systems biology, we can wonder whether *CycG* is a hub in a network of interacting genes involved in maintaining tissue growth homeostasis and thus developmental stability—adaptively or not. (3) A further question is how deregulation of *CycG* induces noisiness of cell growth? As *CycG* is involved in transcriptional regulation, it is tempting to speculate that alteration of this function disrupts developmental stability. Indeed, gene expression is a noisy process that can induce non-genetic heterogeneity in prokaryotes as well as in eukaryotes.⁸³ (4) *CycG* is involved in buffering stochastic variation of organ size and shape, but what role, if any, does *CycG* play in the buffering of environmental and genetic variation? Repeating our experiment in a variety of genetic backgrounds and environmental contexts would allow to test for a role of *CycG* in genetic and environmental canalization.⁸⁴

Answering these questions will require further investigations. Our experiments reported here are nevertheless very promising in that they have allowed to identify some aspects of the genetic and developmental basis of developmental stability in *Drosophila*. This was made possible by coupling different methodologies that each focuses on a different level of biological integration. Transgenic lines allowed direct investigation of the effect of a single gene on FA, cell analyses allowed identification of a process by which wing size and shape are stabilized and geometric morphometrics allowed precise quantification of wing shape FA. While independent use of different approaches is likely to cause

confusion, their integration is thus very promising to gain real understanding of the processes generating and buffering variation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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