Chapter 6

Future Directions

Besides our contributions discussed in previous chapters to the problem of developmental pattern formation, this work has also brought new questions that remain unanswered. The purpose of this Chapter is to highlight some problems that will likely provide new insights into the field and will be interesting to consider in follow-up studies.

6.1 Future Directions for Chapter 2

Genetic Characterization of the "Memory Module"

The Overshoot Model proposed in Chapter 2 to explain the interpretation of the Hh morphogen in the *Drosophila* wing disc depends on two network subcircuits or "modules" (see Fig. 2.7B). The Overshoot Module causes a delay in Hh-dependent *ptc* upregulation that results in a transient overshoot of the Hh gradient. The Hh overshoot is essential to expose a subset of cells transiently to the signal, but the Overshoot Module fails to explain patterning if this subset of cells is not able to "recall" its history of Hh signaling exposure. The maintenance of this transient state likely depends on a positive feedback loop on *dpp* transcription that maintains expression of *dpp* once the transient signal ceases. However, the genetic players that participate in this transcriptional subcircuit or "Memory Module" have not yet been identified.

One way to identify novel players in the Memory Module is to use a candidate approach, this is, to investigate if "memory" of *dpp* expression persists after some

candidates are impaired one at a time. For example, one natural candidate is *dpp* itself, as it encodes a signaling molecule that could enhance its own expression after it is activated by a transient Hh signal. However, in Chapter 2 we provided evidence against this possibility (see Supporting Figure 2.3). Other possible candidates include members of other signaling pathways that participate in wing disc patterning, such as Wingless and EGFR.

A more direct approach to identify potential transcriptional players in the "Memory Module" is through cis-regulatory analysis; if genetic players exist that determine memory of *dpp* expression through a transcriptional feedback loop, we should be able to identify the region in the *dpp* enhancer DNA where these players bind and activate transcription of *dpp*. Cis-regulatory studies on the *dpp* enhancer have identified a "minimal enhancer" (800 base pairs long) that is capable of reproducing the normal pattern of *dpp* in wing discs (Müller and Basler, 2000). Preliminary data suggest that this minimal element does support memory of *dpp* expression after the inactivation of Hh signaling using the hh^{ts} system described in Chapter 2 (data not shown). However, the confirmation and continuation of this work is left to immediate future studies.

Another possibility is that the "Memory Module" does not require other transcription factors, but instead employs regulation of the existing ones. For example, it is possible that the mechanism for *dpp* memory is provided by the differential responsiveness of Hh target genes to the activator (Ci155) and repressor (Ci75) forms of the transcription factor Ci. Unlike other Hh target genes, *dpp* does not require activation by Ci155 (as *ci* clones located anywhere within the anterior compartment of the wing disc express), but presence of the repressor form Ci75 is sufficient to abolish *dpp* expression

(Méthot and Basler, 1999). These data suggest the existence of (ubiquitous) transcription factors that activate *dpp* expression in the absence of Ci75 and propose a potential mechanism by which *dpp* expression may be maintained upon transient Hh signaling activation; perhaps there is a Hh-dependent factor (e.g., a ligase or a protease) that impairs the activity of Ci75 in the *dpp* domain. One potential factor is *roadkill* (*rdx*), a Hh-target gene that encodes a subunit of a E3 ubiquitin ligase that targets Ci to degradation via the proteasome ubiquitin pathway (Kent et al., 2006; Zhang et al., 2006). Ubiquitous Rdx upregulation causes loss of *ptc* expression and expansion of the *dpp* domain (Kent et al., 2006). However, some evidence argues against the role of rdx as a key player in the "Memory Module." First, Rdx preferentially promotes destruction of full length Ci than Ci75 (Kent et al., 2006). Moreover, *rdx* is normally expressed in a narrow domain abutting the AP boundary of the wing disc, a region of sustained Hh signaling activity (Kent et al., 2006; Zhang et al., 2006).

When Does the Hh Overshoot Occur *in vivo*?[*]

Our study in Chapter 2 provides evidence for the existence of a Hh gradient overshoot upon reinitialization of the gradient using a temperature-sensitive *hh* allele, but when such a dynamic shift in the gradient occurs during normal development remains to be identified. Alternatively, it is also possible that the overshoot occurs multiple times in wing disc development. Such oscillations in the range of the signal may occur if Hh-dependent Ptc upregulation becomes sufficiently strong so that Hh signaling is repressed

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completely, and thus that expression of *ptc* is interrupted - allowing for multiple rounds of Hh gradient expansion and refinement. However, this periodic behavior of Ptc expression has not been reported and, furthermore, we suggest that such regulation is unlikely to occur in a synchronized manner. These important aspects will require direct temporal examination of Hh gradient formation and Ptc expression in living tissues over a long period of time, but this remains technically challenging.

Integration of Patterning by the Overshoot Model with Tissue Growth[*]

Another important problem that will require further investigation is how this model accommodates tissue growth. In particular, if cells that experience a transient Hh signaling retain *dpp* expression by some 'memory' mechanism, whether or not this is retained after cell division, is still in question. Our data show that the time-scale of the overshoot (~6 hours) is shorter than the average cellular proliferation rate in the wing disc during the third instar (~8.5 hours; González-Gaitán et al., 1994); therefore, the dynamics of the gradient should not be directly affected by tissue growth. However, it remains unclear why all the cells derived from *dpp*-expressing progenitors do not retain *dpp* expression; a fraction of cells that are sufficiently close to the AP boundary (where Hh signaling is ON) may end up located farther away from it as a result of tissue growth (where Hh signaling is OFF). One possible explanation is that cells expressing *dpp* maintain their relative position in the wing disc as a result of cell affinity, but their progeny eventually lose the ability to maintain *dpp* expression and are pushed away from

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the anterior-posterior boundary. In fact, the hypothesis that *dpp*-expressing cells attempt to remain together during wing disc development is supported by a study that suggest that *dpp*-expressing cells may regulate a cell adhesion molecule that is necessary to avoid intermixing of anterior and posterior cells (Dahmann and Basler, 2000). However, it is unclear if the progenitors of *dpp*-expressing cells that are no longer exposed to Hh would lose their ability to maintain *dpp* expression. In summary, the relationship between patterns and growth, and particularly, how *dpp* 'memory' is affected by cell proliferation deserves further investigation as well.

The Overshoot Model in Other Patterning Systems[*]

Our model of Hh-dependent patterning in the *Drosophila* wing disc primarily depends on a particular gene network architecture, rather than on Hh concentration thresholds. Numerous studies in different developmental contexts have revealed that the Hh signaling gene network architecture is largely conserved from flies to humans. In particular, Hh-dependent *ptc* upregulation is a common feature in all the systems studied so far. Thus, an exciting question for the future is whether similar models of pattern formation hold for systems with equivalent network architectures, or the principles of developmental pattern formation evolved despite the conservation of gene network topologies. Interestingly, recent data from the vertebrate neural tube suggest that cells determine their fate by integrating the strength of Hh signaling over time (Dessaud et al., 2010), while another study in the same system reported that some positional information

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is lost when *ptc* is not upregulated in response to Hh signaling (Jeong and McMahon, 2005). These results are in close agreement with our model of Hh patterning in the *Drosophila* wing disc (Chapter 2), but additional studies will reveal if developmental patterning in other Hh-dependent systems employs similar principles.

6.2 Future Directions for Chapter 3

A technical challenge that deserves further discussion in the future is how to analyze and visualize steady-state invariant sets in realistic models of developmental patterning. This problem is common in practice because steady-state invariant sets are usually contained in high dimensional spaces. For example, the dimension of the parameter space of our highly simplified model of Hh signaling is 16. More realistic models may involve an even larger number of parameters making the resulting steady-state invariant set difficult to analyse and visualise. Our analysis in Chapter 3 reduces to the study of subsets [Equations (3.10) and (3.11)] that are contained in particular parameter subspaces, but an interesting future direction is to use theoretical tools (e.g., nonlinear extensions of Principal Component Analysis; see Kruger et al., 2007, for a review) to reduce high-dimensional steady-state invariant sets to sets of lower dimensions that are easier to visualise and more useful for experimental design.

6.3 Future Directions for Chapter 4

The work presented in Chapter 4 is an unfinished project that will soon be considered for publication. However, there are some points that require additional experimental support that are currently in progress. For example, the confidence of some of the results (based

on the value of the scaling percentage) is relatively low, partly because the number of embryos considered is not appropriate. In particular, we would like to determine with greater confidence if scaling of the nuclear dl gradient can be confirmed by adding more data to the sample. In addition, it will be useful to investigate the correlation between the width of the dl gradient and the other target genes under study.

Our conclusion that spatial scaling of DV patterns is gene-dependent raises questions about the scalability of other genes in the system. Some candidates include *twist*, *snail*, *rhomboid*, and *brinker*. For example, it would be useful to determine if there are genes whose scalability can be explained by the nuclear dl gradient and whether their scaling can be used to establish scaling of other genes. As DV genes affect each others patterns through a network of complex interactions (reviewed by Stathopoulos and Levine 2005), it is likely that they contribute to each other's ability to scale with respect to the length of the DV axis. Particularly, we would like to test our model that *snail* acts as an intermediate factor to establish scaling of *sog* (see Chapter 5).

We are trying to identify gene-specific scaling mechanisms using a candidate approach, in which scaling of *vnd*, *sog*, and *ind* is assayed in different mutant backgrounds. One limitation of this approach is that the process to screen for scaling using embryo cross sections is very slow, making it difficult to design a large screen that can help in the identification of molecular players that are required for scaling. The search for genetic players that affect scaling in this system is also technically difficult because it is likely that mutants that affect scaling also affect other aspects of patterning, such that the effects on scaling and patterning cannot be genetically separated. On the

technical side, it would be useful to improve our methods to screen faster and more reliably for factors that affect scaling.

There is no doubt that the use of multidisciplinary and quantitative tools will provide new insights into the mechanisms of pattern formation and promise a fruitful path to the future of developmental biology. But this approach is still in its infancy. Our ability to measure gene expression with high temporal and spatial resolution is still very limited even in well-characterized model systems. However, future studies will surely provide a more quantitative picture of animal development that will contribute to a better understanding of the relationship between positional signals and animal design.