Chapter 1

Introduction

"Development starts off from a more or less spherical egg, and from it an animal develops that is anything but spherical; it has legs, a head, a tail, etc. and internal organs that also have determined constant forms…"

C. H. Waddington (The Nature of Life, 1961)

It is difficult to think of a natural process that displays a more elegant combination of efficiency, complexity, and self-organization than animal development. The species that today populate our planet are the result of the most spectacular experiment in natural history; through millions of generations, organisms have adapted their body plans to coexist with each other, often in unfavorable or hostile environments. The final product of this long evolutionary process is not only a plethora of living shapes and forms, but also the precise "instructions" of how they develop and come to be. These "instructions" or principles have only become available to us recently, thanks to the advancement of microscopy, molecular biology, and genetics. In fact, much of the research in modern developmental biology relates to finding and interpreting the principles underlying animal design (Carroll et al., 2005; Davidson, 2006; Lawrence, 1992). A central aspect to the understanding of animal development is the study of pattern formation, this is, the spatiotemporal specification and organization of cell types in a developing group of cells. Although there has been much scientific interest in developmental patterning during the past 40 years, the mechanisms by which cells interpret the signals that control gene expression patterns remain controversial. This chapter introduces the ideas and models

that have played a key role in trying to explain the formation of pattern in developing systems and highlights some fundamental problems that will be the focus of this work.

1.1 Historical Perspective

For centuries, scientists and naturalists have been fascinated by the complexity of animal development and have speculated about the origin of life forms and functions. While the importance of embryonic pattern formation was well recognized by embryologists in the late 19th century, the models of how cells in a developing embryo or tissue acquire a precise spatial organization have changed dramatically in the history of biology. Today we know that all cells in a developing animal carry the same genetic information and that the spatial organization of cells during development depends on their ability to control, in space and time, the expression of a set of key genes. But for much of the first half of the 20th century, the genetic basis of spatial order in development was a controversial subject. This brief historical remark does not intend to summarize the complex history of embryonic pattern formation, but tries to trace the origin of some of the concepts that are currently widely used in developmental patterning, such as the concepts of organizer, embryonic field, and morphogen gradient.

Much of the early efforts to explain pattern formation originate from regeneration experiments in insects (Morgan, 1897) and the concept of embryonic induction in vertebrates (Spemann, 1938). These experiments revealed the amazing ability of embryos to self-organize their developmental programs upon external manipulations. The global properties of spatial order were captured in the concept of embryonic field, first postulated by Boveri in 1910 (reviewed by Sander, 1994). In its simplest form, an

embryonic field refers to a system that, as long as it remains in contact with some substratum, is able to spatially reorganize its developmental program in response to changes in size or mass. Although the concept of embryonic field was not precisely defined and the mechanisms of action of the field were not known in the early 20th century, many sources of experimental evidence favored the existence of embryonic fields and the concept rapidly became a paradigm of experimental embryology.

Strong support for the embryonic field concept came from spectacular transplantation experiments in newts and salamanders. In 1924, Hans Spemann and Hilde Mangold performed a famous experiment using salamander eggs from two different species (Spemann and Mangold, 1924). They transplanted dorsal cells from one embryo (the donor) and introduced them into the ventral region of the other embryo (the host). As revealed by the difference in cell pigmentation between the two species, the donor cells were able to induce dorsal neural fates in the surrounding host tissue. The experiment suggested the presence of an "organizing" substance emanating from the donor tissue that acts at the distance to orchestrate patterning in the host. The discovery of the "Organizer" could have had an immediate impact on the problem of pattern formation, but the lack of success in identifying the organizing substance over many decades obscured the interest in the problem (Witkowski, 1985).

Almost at the same time that Spemann popularized the concept of induction, the field of genetics began to demonstrate that its tools had much to offer to the problem of pattern formation. The identification of mutants in fruit flies and mice with morphological defects and the discovery of genetic mosaics provided a fantastic opportunity to study the problem of developmental patterning. Thomas Hunt Morgan,

who established the modern school of genetics in the fruit fly *Drosophila melanogaster*, was also an influential embryologist. In 1934, Morgan wrote a review entitled *Embryology and Genetics* with the objective to set a ground of contact for the two disciplines (Morgan, 1934). However, only a few embryologists at that time believed that genes could have a developmental role and Morgan's attempt to initiate crosstalk between geneticists and embryologists was not very fruitful. The most significant efforts to integrate genetics and embryology came from Conrad Hal Waddington, a British paleontologist by training that was attracted to embryology by the Organizer problem. As many embryologists and biochemists that turned their attention to the Spemann Organizer, Waddington failed to find the chemical nature of the organizing substance, but in the late 1930s, he uncovered another aspect of the Organizer problem. In Spemann's work, too much attention was given to the inducer tissue (the Organizer) rather on the response of the surrounding cells to the organizing substance. Waddington realized that in order for ventral cells in the host tissue to develop into ectopic dorsal tissue in the Spemann and Mangold experiment, they must be competent to respond to the inducing signal. This idea prompted Waddington to propose that the development potential of cells to adopt a particular fate should be under the control of the genes. The idea that genes were under control of developmental patterning was not entirely new, but was very unpopular among embryologists who claimed that different patterns could not be explained by the action of genes whose DNA template was the same in every cell type of the embryo. By 1940, Waddington was able to gather a series of analogies to support the equivalence of organizers and genes (Waddington, 1940). A celebrated example was that of the gene *aristapedia* which is responsible for the transformation of legs into antennas

in *Drosophila* (since in *aristapedia* mutants, legs develop in place of the antennas). He argued that in the same way that host cells in the Organizer experiment adopt different fates in the presence or absence of the organizing substance, *aristapedia* was able to *induce* the "antenna" fate instead of the default "leg" fate. In 1956, Waddington presented a genetic model of embryonic cell differentiation suggesting that organizing substances in the cytoplasm influence the activation of genes in the nucleus (Waddington, 1956). With his ideas, Waddington founded the genetic basis of embryonic induction and pattern formation, but his work received full consideration by embryologists only after Jacob and Monod demonstrated that cell differentiation in bacteria was under the transcriptional control of gene activity (Jacob and Monod, 1961).

The synthesis of genetics and embryology opened a new research era in the problem of developmental patterning. During this new stage, organisms that were extensively used for the study of embryology, such as flat worms, salamanders, and frogs, were replaced by emerging genetic "model" organisms such as fruit flies and mice. Genetic screens prompted the discovery of new genes with developmental phenotypes, but the technology was still somewhat immature to reveal the molecular mechanisms underlying the spatial control of gene expression. These conditions favored the development of theoretical and mathematical models of pattern formation. The main challenge of these theoretical studies was to introduce a conceptual framework to explain the stable generation of patterns and their self-organizing properties. An almost universal feature of the theories of pattern formation (that prevails until today) was to postulate the existence of chemical gradients (or gradient-fields), which were considered particular cases of morphogenetic fields in the 1920s and 1930s. Gradients of different kinds were

originally considered in theories by Huxley and de Beer (1934), Dalcq (1938), and Child (1941); but perhaps the most influential theoretical contributions to the current understanding of pattern formation came in the 1950s and 1960s with Turing's reaction-diffusion model of morphogenesis (Turing, 1952), Stern's prepattern hypothesis (Stern, 1954), and Wolpert's positional information theory (Wolpert, 1969).

The use of reaction-diffusion dynamics in developmental patterning is due to the famous mathematician, Alan Turing, a pioneer in the development of mathematical models in embryonic pattern formation. In his 1952 seminal paper, Turing used partial differential equations to model the concentrations of two chemical species diffusing and reacting with one another. It was shown that under certain conditions on the parameters (e.g., assuming short-range activation and long-range repression) a broad variety of periodic patterns (such as "spots" or "stripes") could be generated using Turing's formalism. In his paper, Turing also coined the term "morphogen" to refer to the chemical species that work as "generators of form," a concept that is prevalent in the modern literature. Meinhardt and Gierer later expanded the efforts of Turing and applied them to patterning of *Hydra* (Meinhardt and Gierer, 1972). A recent application of Turing's concept is the case of Spätzle, a ligand that activates the Toll receptor in the early *Drosophila* egg. Spätzle is "freely" diffusible in the extracellular space surrounding the embryo known as the perivitelline fluid where it interacts with other factors that regulate its activity. These peculiar characteristics make the system suitable for modeling by using Turing's formalism (Meinhardt, 2004). In fact, reaction-diffusion models have successfully explained some intriguing properties of this system such as axis duplication when Spätzle is over-expressed (Morisato, 2001). Turing' reaction-diffusion models are

very influential today in mathematical biology and have also been shown to have experimental significance in the formation of skin and pigmentation patterns (Kondo and Asai, 1995).

On the other hand, one of the first attempts to explain pattern formation based on genetic evidence is the work on genetic mosaics in *Drosophila* and the theory of prepatterns proposed by Curt Stern (Stern, 1954). The theory postulates the existence of genetic prepatterns and suggests that final patterns in a tissue result from the competence of cells to respond to the prepattern cues (or 'singularities'). In a mutant clone, for example, mutant cells may not affect the prepattern, but may cause a change in their competence to respond to it. With the prepattern concept, Stern was able to explain the phenotypes resulting from genetic mosaics of homeotic mutants in *Drosophila* (i.e., mutants transform one tissue identity into another). Stern's prepattern hypothesis was very influential in the 1960s, especially on insect patterning (Sondhi, 1963; Lawrence, 1966; Stumpf, 1967). However, the prepattern hypothesis lost general applicability when it was found to be insufficient to explain bristle patterns in different *Drosophila* tissues (Bryant, 1969; Tokunaga, 1978). Nonetheless, Stern's theory had a profound influence on Wolpert's positional information theory and was also later proposed to apply to patterning of the *Drosophila* segmentation network (Akam, 1987).

1.2 Positional Information and Morphogen Gradients

The prevailing view about developmental pattern formation comes from Lewis Wolpert's theoretical work on positional information (Wolpert, 1969). In contrast to previous models of pattern formation, Wolpert's goal was to propose a general conceptual

framework in which the common properties of pattern formation could be explained. Wolpert reasoned that in the same way that universal principles govern the synthesis of proteins from a DNA template (cf. the central dogma of molecular biology), there should be general principles involved in the translation of genetic information into spatial patterns of cellular differentiation; if cells depend on their spatial location to determine their fate, what are the general principles by which cells acquire and interpret their "positional information"?

According to the theory of positional information, developmental patterning is the process by which each cell, based on its genetic information and developmental history, determines its pattern of molecular differentiation according to its location in the system. Hence, pattern formation is regarded as a sequential two-step process comprising the specification and the interpretation of positional information. The specification of positional information in an embryonic field is the equivalent of choosing a coordinate system (a notion proposed originally by Hans Driesch in 1894). Coordinate systems are defined with respect to reference points and depend on the polarity of the system, i.e., the direction in which positional information is to be measured. One mechanism (originally proposed by Stumpf in 1966) by which cells may acquire their positional coordinates is by measuring the concentration of a chemical substance that is present in a concentration gradient across the field of cells (Stumpf, 1966). A concentration gradient capable of specifying positional information in a concentration-dependent manner is referred to as a morphogen gradient in the modern literature. Although this is not the only way to specify positional information, the existence of morphogen gradients and their role in

developmental patterning was later strongly supported by many experimental studies and constitutes one of the paradigms of modern developmental biology.

The second step in the process of pattern formation is the interpretation of positional information, that is, how "positional values" are converted into discrete states of cellular differentiation. The interpretation step must depend on a regulatory code by which cells translate positional information signals into differentiation genes, but may also depend on cellular competence to positional signals, developmental history, and cell-cell interactions.

Perhaps the most important contribution of Wolpert's positional information theory is the postulate that the coordinate systems required for positional specification may be universal (at least within the same animal). For example, the specification of positional values in two developing tissues may be the same, but they give rise to two different adult parts because cells interpret positional coordinates in a different manner within each tissue. Evidence for this universality hypothesis was provided by genetic mosaics of homeotic mutants (Roberts, 1964) or from transplantation experiments in insect segments (Stumpf, 1967). For example, clones of cells lacking the gene *aristapedia* produce leg structures in the antenna that correlate with their corresponding position in the leg (Roberts, 1964). One corollary of the universality hypothesis of coordinate systems is that the process of positional specification is independent from its interpretation. In particular, the specification of positional information should be -in principle- independent of the field's genome or its developmental history.

The original motivation of the theory of positional information was to address the problem of size-dependent scale invariance in embryonic patterning. This problem can be

abstracted by the famous French flag problem, which Wolpert introduced a year earlier and can be stated as follows (Wolpert, 1968). Consider an embryonic field that is subdivided in three contiguous patterns such that the first third contains cells of "blue" type, the second third contains cells of "white" type, and the remaining third contains cells of "red" type (resembling the pattern of a French flag). The problem consists of explaining how the patterns maintain their proportions over large deviations in the original size of the field (i.e., after the field has been 'cut' into half or after two fields have been fused). It is evident that to generate a French flag pattern irrespective of the size of the field, each cell must poses information about its location, not only with respect to a reference point, but also relative to the length of the axis. Wolpert offered a solution in terms of a diffusible substance (morphogen) that is secreted at one end of the embryonic field (source) and destroyed at the other (sink) (Wolpert, 1968). This solution of the problem suggests the existence of two or more 'thresholds' in positional information values that cause any two adjacent cells to adopt the same pattern when their positional values are within the same threshold domain, but another pattern if they correspond to different threshold regions [an idea originally proposed by Dalcq and Pasteels (1937)]. For its simplicity, the formation of pattern as a result of concentration thresholds from a morphogen gradient rapidly became a classical textbook model of developmental patterning (popularly known as Wolpert's French flag model[*] or "Classical Morphogen" model). Subsequent theoretical work explored the biological

[*] The use of the name Wolpert's French Flag *model* in the modern literature is unfortunate because it does not necessarily refer to the solution of Wolpert's French Flag *problem* described above (i.e., the size-dependent scale invariance problem), but simply to the existence of morphogen concentration thresholds that define boundaries of gene expression patterns. Perhaps, the name "Dalcq-Pasteels Thresholds" model would have made more historical sense. In the rest of this work, I will use the term "classical" morphogens to distinguish morphogens that specify positional information in a concentration-dependent manner from (non-classical) morphogens that do not employ multiple thresholds (see below).

feasibility of morphogen gradients to be established by diffusion (Crick, 1970) and discussed the mechanisms in which concentration thresholds could lead to sharp boundaries of gene expression (Lewis et al., 1977). However, the existence of morphogens and their mechanisms of action remained a theoretical speculation until the first morphogen was discovered experimentally 20 years later.

The molecular identification of morphogen gradients in the late 80s and 90s provided the first opportunity to test their proposed role as carriers of positional information. The first genetic gradient to be observed experimentally was the maternal product, Bicoid (Bcd), in the early *Drosophila* embryo (Driever and Nusslein-Volhard, 1988a). In their studies, Driever and Nusslein-Volhard were able to show not only that the Bcd protein forms a concentration gradient that peaks at the anterior pole and decreases posteriorely in an exponential fashion, but they provided genetic evidence that patterning of the anterior-posterior axis in the embryo depends on Bcd concentrations (Driever and Nusslein-Volhard, 1988b). The news that Bcd apparently behaves as a classical morphogen provided strong support to Wolpert's positional information theory and initiated the "hunt" for additional morphogens in other systems. In the following 10 years, many other morphogen candidates were identified in different systems, including Activin in the frog embryo (Green and Smith, 1990), Decapentaplegic (Dpp) in the *Drosophila* embryo and developing wing (Ferguson and Anderson, 1992; Nellen et al., 1996), and Sonic Hedgehog in the chick limb and spinal cord (Riddle et al., 1993; Roelink et al., 1995). Moreover, comparative studies across different phyla have revealed that most morphogens belong to just a few families of signaling molecules, namely, the Hedgehogs, the Wnts, and the families of Transforming, Epithelial, and Fibroblast

Growth Factors (TGFs, EGFs, and FGFs, respectively). This remarkable fact shows that, in some sense, the specification of positional information is universal; despite the vast repertoire of animal body plans, positional information is established using essentially the same molecular machinery.

However, morphogens are by no means the only way to establish positional information. For example, in the sea urchin embryo, it is possible to explain cell differentiation solely from the dynamics of transcriptional networks (Smith et al., 2007; Bolouri, 2008). Another intriguing case of developmental patterning without morphogens is that of the slime mold *Dictyostelium discoideum* in which cells first differentiate at scattered locations and then sort themselves in the aggregate to form a reproducible "French flag" pattern (Thompson et al., 2004). Conversely, the role of morphogens is not limited to pattern formation. For example, morphogens are also recognized to participate in cell affinity, polarity, and tissue growth (Lawrence, 2001).

Recent advances in genetic, molecular, and microscopy tools have provided the opportunity to study the mechanisms underlying morphogen-mediated patterning in high resolution and have favored some experimental model systems over others. The experimental studies in this work are based on two systems that lead much of the current research in developmental patterning, the early *Drosophila* embryo and the developing *Drosophila* wing (or wing imaginal disc). One of the advantages of the first system as a model to study the role of morphogens is the fact that during the first ~4 hours (known as the syncytial blastoderm stage), the *Drosophila* embryo is a giant cell comprising nuclei that share a common cytoplasm and are encompassed by a single cell membrane. (Individual cell membranes form later during a stage known as cellularization). This

means that the early gradients in the *Drosophila* embryo are intracellular. In fact, the maternal factors Bcd and Dorsal (dl) that initiate patterning of the embryo are transcription factors, meaning that they directly bind DNA and control gene expression. This suggests that the interpretation of positional information in the early *Drosophila* embryo is much simpler than in other systems in which patterning is orchestrated by extracellular morphogens that depend on complex signal transduction pathways. Another advantage of the early *Drosophila* embryo is that its size does not change over time, that is, once an embryo is laid, it will retain its size during embryonic development; this provides the advantage that positional coordinates will not need to change over time as a result of embryo growth. On the other hand, the wing imaginal disc has proven to be one of the most convenient models to study extracellular morphogens because powerful genetic toolkits permit a precise spatiotemporal tuning of morphogen activity. One example is the increasing stock collection libraries employing the Gal4-UAS system (Fischer et al., 1988; Brand and Perrimon, 1993). Gal4 is a yeast protein that, when expressed in a particular pattern in flies, can activate transcription of any gene that is under the control of the "Upstream Activation Sequence" (UAS). The Gal4-UAS system can be combined with other genetic techniques such as mitotic recombination to express any desired gene in genetic mosaics, or can be enhanced for temporal control when expressed in conjunction with a temperature-sensitive form of Gal80 yeast protein which, at permissive temperature, binds Gal4 and blocks the transcription of the gene under UAS control (McGuire et al., 2004; see Chapter 2 for an example).

One important difference between morphogen-mediated patterning in the early *Drosophila* embryo and in imaginal discs is the origin of positional information. Unlike

in the early embryo in which the morphogens are established maternally (i.e., mother cells deposit *bcd* and *dl* RNA into the egg when it is still in the female's abdomen), the classical paradigm by which imaginal discs are patterned can be described as a three-step process (Lawrence and Struhl, 1996). In the first step, positional information from both the anterior-posterior (AP) axis and the dorsal-ventral (DV) axis of the embryo defines the location of a subset of cells that will become the disc primordium. The second step consists of forming 'compartments' within the disc primordium. For example, cells located at the posterior side of the disc primordium express the 'selector' gene *engrailed* (Morata and Lawrence, 1975) that distinguishes them from cells at the anterior side of the primordium that do not express it. In this case, the restriction of *engrailed* expression to posterior cells is defined earlier during the patterning of the embryo segments. The expression of selector genes is inherited after cells divide and also influences cell affinity to result in sharp boundaries between compartments. In this way, cells segregate into distinct compartments and maintain their identity as the tissue grows (García-Bellido, 1975). Third, cells in each compartmental boundary are instructed to serve as the source of morphogens, which establish positional information within the disc (Meinhardt, 1983).

In recent years, the study of how morphogen gradients are established and interpreted is at the very front of research in developmental biology. Yet, our progress on understanding their mechanisms of action as organizing molecules of pattern formation has been frustratingly slow. The study of morphogen-mediated patterning can be conceptually subdivided into two classes of problems: how morphogen gradients are established (specification of positional information; reviewed by Zhu and Scott, 2004; Kornberg and Guha, 2007), and how morphogen gradients specify patterns (interpretation

of positional information; reviewed by Gurdon and Bourillot, 2001; Ashe and Briscoe, 2006). In this thesis, I present the results of my investigations on three fundamental problems regarding how morphogen signals are interpreted to control developmental patterning. These problems, which are briefly discussed and justified in the following sections, are the driving force and motivation of my multidisciplinary research program.

1.3 The Problem of How Extracellular Morphogens are Interpreted

The advancement of molecular and genetic tools in developmental biology has left no doubt about the importance of organizing molecules or morphogens in the formation of patterns of gene expression. Although the progress regarding the identification and localization of the relevant morphogens that orchestrate patterning in different experimental systems has been spectacular in the past 10 years, the problem of how morphogens act, that is, how signaling gradients give rise to different cellular responses, still deserves much additional consideration.

The Classical Morphogen model provides a simple and elegant mechanism by which graded spatial information can be converted into discrete patterns of gene expression. This mechanism depends on the existence and interpretation of morphogen concentration thresholds, but the molecular basis of how thresholds from a graded input give rise to multiple discrete responses remains poorly understood. Theoretical models can mimic concentration thresholds by assuming high cooperative binding of the morphogen or imposing positive feedback on gene responses (Lewis et al., 1977; Meinhardt, 1978). On the other hand, experimental studies in many systems have shown that chemical gradients often correlate with the position of gene expression patterns.

These observations provide support to the Classical Morphogen model, but do not conclusively demonstrate that patterning is a concentration-dependent process. In fact, with the exception of a few cases in which the interpretation of the morphogen signal has been studied in detail, it is largely unclear if the position of different patterns actually corresponds to different concentration thresholds. One such exception is the case of Activin in the frog embryo (Green and Smith, 1990; Gurdon et al., 1998). Activin is a member of the TGF-β family that patterns the dorsal mesoderm in the frog embryo. Through a series of elegant studies, John Gurdon and his colleagues have been able to convincingly show that Activin works as a classical morphogen by directly activating the genes *goosecoid* (*gsc*) and *Xenopus brachyury* (*Xbra*) in a dose-dependent manner. First, Gurdon et al. observed that glass beads soaked in Activin transplanted into the embryo result in the expression of *gsc* next to the beads followed by an expression domain of *Xbra* (Gurdon et al., 1994; Gurdon et al., 1995). Second, using radioactively labeled Activin protein, it was shown that signal responses depend on absolute receptor occupancy (in particular, about 100 Activin-bound receptors are sufficient to activate *Xbra*, while approximately 300 are required for *gsc*; Dyson and Gurdon, 1998). Third, changes in Activin dosage correspond to similar concentration changes in the intracellular transducer of the signal, Smad2 (Shimizu and Gurdon, 1999). Furthermore, Gurdon and collegues ruled out that other mechanisms played a role in Activin patterning. For example, they showed that once activin signaling is stabilized, increased temporal exposure to the signal does not affect the patterning outcome (Dyson and Gurdon, 1998). In addition, cells appear to respond to Activin signaling levels independent of the levels of their neighbors (Gurdon et al., 1999). Taken together, this

detailed body of data provides strong evidence that Activin acts as a classical morphogen in the frog embryo.

However, not all the gradients that have been studied in detail support the Classical Morphogen model. For example, the specification of digits in the developing vertebrate limb depends on the temporal exposure to Sonic Hedgehog (Shh) signaling. A group of cells in the posterior limb bud produces Shh, which forms a gradient along the AP axis of the limb. In vivo studies in which the fate of Shh producing cells is genetically marked have demonstrated that many of the cells that initially express Shh are displaced anteriorely as a result of tissue growth away from the region of Shh-expressing cells. This process results in populations of cells that experience different temporal exposures to Shh and suggests that the identity of posterior digits depends on their temporal exposure to Sonic Hedgehog signaling (Ahn and Joyner, 2004; Harfe et al., 2004). Follow up studies confirmed that temporal exposure rather than Shh signaling "strength" is the predominant factor in the specification of posterior digits (Scherz et al., 2007; Towers et al., 2008) and concluded that Shh acts as a "non-classical" morphogen in the vertebrate limb.

Although the Classical Morphogen model remains as a paradigm of modern developmental biology, the importance of concentration thresholds remains doubtful in many systems that are often considered "classical." Regardless of whether or not the Classical Morphogen model can be verified or ruled out in other systems, a more detailed study of morphogen signaling will provide the opportunity to uncover the precise mechanisms in which positional information give rise to form and pattern in developing systems. In Chapter 2, I present our study of how a gradient of Hedgehog (Hh) is interpreted to give rise to at least three patterns of gene expression in the *Drosophila*

wing disc. Hh belongs to a family of widely conserved signaling molecules that organize patterning in different developmental contexts (reviewed by Ingham and McMahon 2001, Jiang and Hui, 2008; Varjosalo and Taipale, 2008; see Box 1.1). We demonstrate that patterning by Hh in the *Drosophila* wing disc largely depends on the properties of the Hh gene network architecture rather than in the "shape" of the Hh concentration. Our results suggest that, contrary to what was previously thought, Hh does not seem to act as a classical morphogen in this system and we introduce a novel mechanism in which patterns of gene expression can be established during development (Nahmad and Stathopoulos, 2009).

1.4 The Problem of How Gradient Dynamics Affect the Interpretation of Morphogen Signals

Developing systems exhibit various sorts of dynamic behaviors. For example, some cells grow and divide, while other cells die prior to reaching maturity; some cells remain in their original locations while others migrate several cell diameters away from the site from which they originated. Yet, the resulting morphogenetic pattern in adult animals is usually invariant and highly reproducible from individual to individual suggesting that the interpretation of positional information takes into account these dynamical processes. One idealization of the Classical Morphogen model, at least in its naïve "textbook" version, is its static nature. In this view, patterns correspond to the read out of concentration thresholds from a stable morphogenetic landscape. However, morphogen gradients are more likely dynamic entities rather than fixed systems of coordinates. In fact, several experimental studies in many systems have shown that morphogen gradients remain changing in time during and even after the process of pattern formation takes

Box 1.1. Brief overview of the Hedgehog signaling pathway.

hegehog (*hh*) was discovered in genetic screens that affect early *Drosophila* patterning (Nusslein-Volhard and Wieschaus, 1980). Since its discovery, the Hh pathway has been central in the study of development and disease. Unlike other signaling pathways, activation of Hh signaling depends on the interruption of an inhibitory signaling cascade, rather than on leading the activation of a pathway. In the absence of Hh, the 12-span receptor Patched (Ptc) maintains the signaling pathway inactive by repressing phosphorylation of the 7-transmembrane effector Smoothened (Smo). Although the precise mechanism of how Ptc inhibits Smo activation remains unclear, Ptc and Smo do not seem to interact directly (Denef et al., 2000). Instead, Ptc seems to repress Smo catalytically (Taipale et al., 2003), probably by secreting a small molecule that results in Smo repression (Bijlsma et al., 2006). Upon Hh binding to Ptc, the Hh-Ptc complex is internalized via endocytosis and degraded in lysosomes (Torroja et al., 2004). This leads to removal of Ptc and activation of the pathway. However, activation of Hh signal does not seem to depend on the absolute levels of unliganded Ptc, but also on the levels of the Hh-Ptc complex which can activate the signal by titrating unliganded Ptc molecules (Casali and Struhl, 2004). Hh signal activation induces phosphorylation of Smo by Protein Kinase A (PKA) (Jia et al., 2004; Zhao et al., 2007) which enhances the recruitment of a series of kinases that include the kinesin-like molecule Costal2 (Cos2) and the kinase Fused that prevents PKA-dependent phosphorlylation and cleavage of the zinc-finger transcription factor *cubitus interruptus* (*ci*). In the absence of Hh signaling, the cleaved Ci fragment (known as Ci75) acts as a repressor of Hh target genes. Activation of the Hh pathway inhibits the processing of Ci into Ci75 and permits full-length Ci to enter the nucleus as a transcriptional activator of Hh target genes (Vervoort, 2000). A key feature of Hh signaling is that *ptc*, the gene that encodes the Hh receptor, is transcriptionally activated by Hh signaling. This feedback gives Ptc the dual function of receiving the signal and limiting its range of action (Chen and Struhl, 1996). Although most key aspects Hh signaling are generally conserved from flies to vertebrates, there are some differences. For example, vertebrates have three Hh ligands and transcriptional activation depends on the balance of three *ci* homologs, known as Gli's. Moreover, unlike in *Drosophila,* Hh signaling in mammals depends on a cellular structure called the primary cilium (Huangfu, et al. 2003). In addition to its role as a morphogen in many developmental contexts, Hh has also been implicated in tissue homeostasis, stem cell maintenance, and growth control (reviewed by Jiang and Hui, 2008).

place (Bergmann et al., 2007; Harvey and Smith, 2009; Kanodia et al., 2009; Liberman et al., 2009). The problem of how the dynamics of morphogen gradients contributes to the establishment and interpretation of pattern is little understood and only recently has received full consideration (Jaeger and Reinitz, 2006; Kutejova et al., 2009).

How is a stable pattern established from a gradient that changes in time? And moreover, how do morphogen dynamics contribute to the interpretation of positional information? The first question has been recently studied in detail in some systems. For example, temporal changes in Activin concentrations in the frog embryo are rapidly reflected in concentration changes of the transducer, Smad2 (Bourillot et al., 2002). In this case, cells may switch from *Xbra* to *gsc* expression as Activin levels are temporally established. Therefore, Activin signaling is continuously transduced; a transient signal corresponding to transient pattern of gene expression that converge to stable patterns once the Activin gradient reaches a steady-state distribution. Furthermore, the role of transient morphogen gradients is especially relevant in systems in which patterning takes place within a short window of time. For instance, in the early *Drosophila* embryo, patterning along the AP axis takes about 90 minutes, while the rates of Bcd gradient formation in diffusion models is estimated to take on the order of a few hours. This has led to the proposal that the interpretation of the Bcd gradient takes place prior to reaching its steady state (Bergmann et al., 2007).

However, the problem of whether or not morphogen gradient dynamics *per se* encodes some sort of positional information has not been carefully explored experimentally. For example, it is conceivable that cells respond to the rate of change of a morphogen signal or that a transient exposure to the signal changes the context in which

cells respond to the morphogen at later times (Pages and Kerridge, 2000). In our study of Hh signaling in the *Drosophila* wing disc (presented in Chapter 2), we provide experimental evidence that changes in the distribution of the Hh gradient are essential for the formation of different patterns of gene expression and that the establishment of Hh-dependent gene expression patterns depends on the history of Hh signaling exposure, rather on the steady-state profile of the gradient (Nahmad and Stathopoulos, 2009).

In general, the study of the role of morphogen dynamics in pattern formation remains technically challenging because genetic manipulations cannot be finely tuned enough to selectively perturb transient gradients in a reproducible way, without affecting the steady-state distribution of the morphogen. In Chapter 3, I present a modeling-based experimental design framework to isolate the roles of transient and equilibrium dynamics in morphogen-mediated patterning. Although the experimental implementation of this idea is left for future studies, these theoretical tools are not limited to the study of morphogen dynamics, in particular, and might be applicable to a wide variety of problems.

1.5 The Problem of Size-Dependent Scale Invariance of Pattern

Many animals exhibit an extraordinary ability to re-organize their developmental programs in response to changes in embryo size. For example, sea urchins embryos are subdivided along their animal-vegetal axis into three germ layers (mesenchyme, endoderm, and ectoderm) and several embryological studies show that the proportions of this pattern are preserved when the size of the embryo is manipulated up to 8-fold

(Hörstadius, 1939; Gustafson, 1965). This ability to adapt pattern to the size of the embryo is commonly referred as size-dependent scale invariance or, simply, scaling.

The problem of scaling can naturally be stated in terms of positional information (Wolpert, 1969), this is, how do cells acquire information about their *relative* location in a developing field? One way to illustrate the concept of establishing positional information in relative rather than absolute coordinates is provided by the famous French flag problem introduced above (Wolpert, 1968): Is it possible to establish a pattern in which three sequential states can be established in equal proportions independently of the size of the system? A simple solution of the French flag problem is to postulate the existence of a morphogen gradient established by diffusion that is produced at one end of the embryo and destroyed at the other (Wolpert, 1968). However, this solution assumes that the distal end of the embryo works as a "perfect sink", a scenario that is likely unrealistic. Other theoretical and empirical models that have been proposed to explain the problem of scaling include the double gradient theory of Dalcq (Dalcq, 1938; Hörstadius, 1939; Needham, 1942; Wolpert, 1969) that suggests that patterning depends on the concentration of two gradients emanating from opposing ends of the embryo (an idea that was recently explored further using mathematical modeling by McHale et al., 2006), and the "short-range activator, long-range inhibition" model of Gierer and Meinhardt (1972). Although these models provide plausible solutions to the scaling problem in general, they lack experimental significance because they were based on hypothetical gradients rather than on real morphogens. Since the spatial distribution of different cell types ultimately depends on patterns of gene expression, the problem of scaling is equivalent to the

question of how morphogen gradients determine patterns that correlate with changes in the size of the system.

The most extreme manifestation of scaling is observed in experiments in which embryo size is externally manipulated resulting in well-proportioned animals that are much smaller or much bigger than the average adult. For instance, classical experiments in amphibians reveal that bisected embryos that contain a portion of the dorsal lip result in smaller, but anatomically normal tadpoles (Spemann, 1938). Conversely, adding extra tissue to a frog embryo results in a bigger animal but its overall anatomy is preserved (Waddington, 1938). Recent progress on the problem of scaling in frogs revealed that the mechanisms of scaling depend on protein-protein interactions of morphogens from the family of Bone Morphogenetic Proteins (BMPs) and their inhibitors (reviewed by De Robertis, 2006). In particular, it was found that Admp, a BMP ligand whose expression is inhibited by BMP signaling, is essential for scaling (Reversade and De Robertis, 2005). Further, mathematical modeling suggest that scaling in this system depends on shuttling of BMPs to the ventral midline by binding the inhibitor Chordin, coupled with Admp auto-repression (Ben-Zvi et al., 2008).

A different aspect of scaling is exhibited across a population of animals in which the proportions of gene expression patterns are maintained despite natural variations in embryo size. In Chapter 4, I present results on the problem of scaling due to natural variations in embryo size along the DV axis in *Drosophila*. Patterning of the DV axis in *Drosophila* depends on the nuclear distribution of the maternal factor Dorsal (dl), a homolog of vertebrate NF-κB (reviewed by Moussian and Roth, 2005; Reeves and Stathopoulos, 2009; see Box 1.2). Our results show that the dorsal borders of DV patterns

Box 1.2. Brief overview of dorsal-ventral patterning in the *Drosophila* embryo.

The establishment of dorsal-ventral (DV) positional information in the early *Drosophila* embryo is largely under maternal control and originates early during oogenesis.
The signaling cascade that provides DV asymmetry in the oocyte arises from the dorsally restricted activation of EGFR in the follicular epithelium, a group of somatic cells that cover the surface of the developing egg (Price et al., 1989; Schejter and Shilo, 1989). This asymmetry restricts the transcription of *pipe*, a gene that encodes a glycosaminoglycan-modifying enzyme, to the ventral side of the egg chamber (Sen et al., 1998). Pipe localization initiates a cascade of reactions that involve complex interactions of proteases in the perivitelline fluid, a space that separates the embryo from the follicular epithelium (reviewed by Moussian and Roth, 2005). The protease cascade results in the formation of a gradient of Spätzle in the perivitelline space. The process of translating maternal signals into the embryo is afforded by the Spätzle-dependent activation of the Toll signaling pathway, which also plays a pivotal role in the immune response of insects and vertebrates (reviewed by Imler and Zheng, 2004). Toll activation recruits a complex that includes the serine/threonine kinase Pelle (Towb et al., 1998). This process leads to Pelle accumulation and auto-phosphorylation and results in a gradient of activated Pelle in the ventral region of the embryo that is known to be sufficient for DV patterning of the embryo (Towb et al., 2001; Stathopoulos and Levine, 2002). In the cytoplasm, Pelle likely phosphorylates and promotes the proteolysis of the IκB homolog, Cactus (Edwards et al., 1997; Drier et al., 1999). Destruction of Cactus releases the NF-κB homolog, Dorsal (dl) and permits its nuclear import where it acts as a transcription factor (Belvin et al., 1995; Bergmann et al., 1996; Reach et al., 1996). The end result of the pathway is a graded nuclear distribution of dl that organizes the subdivision of the DV axis in distinct domains of gene expression (Roth et al., 1989; Rushlow et al., 1989; Steward, 1989). High levels of dl lead to transcription of *twist* and *snail* that define mesodermal precursor cells in the ventral-most region of the embryo (Jiang et al., 1991; Pan et al., 1991). Intermediate to low levels of nuclear dl activate *short of gastrulation* (*sog*) and *rhomboid* in ventrolateral regions that define the neurogenic ectoderm (Bier et al., 1990; François et al., 1994; Ip et al., 1992). Finally, *decapentaplegic* (*dpp*) and *zerknült* (*zen*) are activated in the dorsal and dorso-lateral regions of the embryo that correspond to the precursors of the amnioscerosa and the dorsal ectoderm (Rushlow et al., 1987). Although this view fits well with the hypothesis that dl acts as a classical morphogen, patterning of the DV axis depends on a complex transcriptional network in which dl-target genes interact with each other to establish precise domains of gene expression (Stathopoulos and Levine, 2005).

that depend on the maternal factor dl scale with respect to embryo size. Furthermore, we provide evidence that scaling in this system depends on dl but not in a direct manner. We propose that additional factors downstream of dl are required to determine relative coordinates along the DV axis.

1.6 A Multidisciplinary Approach to the Study of Developmental Pattern Formation

In his famous Lectures on Physics, the great Caltech physicist Richard Feynman wrote:

If our small minds, for some convenience, divide a glass of wine, this universe, into parts –physics, biology, geology, astronomy, psychology, and so on- remember that nature does not know it!

Feynman was obviously not talking about multidisciplinary "wine" science, but he was referring to the human obsession of classifying knowledge into categories. Scientific questions are "traditionally" studied using the tools and views of the discipline that they were classified into, rather than using a broad, unbiased perspective. The multidisciplinary philosophy followed in this work is, as much as possible, interest-driven towards the fundamental question of how cells interpret their positional information in order to acquire a specific pattern or fate, rather than limited by the tools or concepts from a certain discipline. The history of the problem of pattern formation has facilitated its study using both theoretical and experimental viewpoints (Ibañez and Izpizúa-Belmonte, 2008; Green, 2002; Lander, 2007), but the interplay between theory and experiment is often forced to exhibit its usefulness instead of being truly motivated by scientific questions.

We are now witnessing a second major revolution in developmental biology (the first one was the synthesis of embryology and genetics in the first half of the 20th

century). I am referring to the post-genomic revolution. This new era is not only facilitating whole-genome tools for the study of biological systems and expanding the availability of unexplored experimental systems, but is changing the way we think about developmental mechanisms in terms of gene regulatory networks (Levine and Davidson, 2005). Furthermore, the post-genomic revolution is also bringing a new multidisciplinary viewpoint to the study of biological systems that promotes the use of tools from different disciplines. This viewpoint is already affecting the way we study the developing embryo and will hopefully bring new light to the understanding of the "building blocks" underlying animal design.