OPINION

Wnts as morphogens? The view from the wing of *Drosophila*

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Morphogens are diffusible signalling molecules that pattern cellular fields by setting up differential gene expression in a concentration-dependent manner. Members of the Wnt family of signalling molecules are generally considered to be classical morphogens. However, a close analysis of their activity indicates that they do not fulfil all of the critera that are associated with the classical definition.

Pattern formation is a central issue in developmental biology. It refers to the process that leads to the spatial arrangement of different kinds of cells in ways that make sense either functionally (for example, the spacing of different sense organs or the scales on an epithelium, or the arrangement of different kinds of mesoderm in an early embryo) or visually (for example, the stripes of a zebra or the spots of a butterfly). From a mechanistic point of view, these patterns raise some important questions. How are such complex cellular arrangements specified? What are the molecules that provide the information for this process? The concept of morphogens (BOX 1), and in particular their linkage to the idea of positional information by Lewis Wolpert[1], provides a powerful, simple and elegant solution to the problem.

In its simplest definition, a morphogen is a diffusible molecule that elicits direct long-range concentration-dependent changes in gene expression and cellular behaviour[1–3]. This idea is seductive because it provides a simple correlation between specific substances (morphogens), an input (their concentration) and an output (the response of the cells, which is expressed as a pattern). The past ten years have bestowed the accolade of morphogen on several secreted molecules that are involved in cell interactions (BOX 1) and, in some cases, have led to proof-of-concept (for examples, see REF. 3).

Members of the Wnt (Wingless/Int-1) family of signalling molecules have often been associated with the classical idea of a morphogen[2–3]. However, the case for these molecules might not be as clear cut as it is for other molecules such as Hedgehog (Hh) and bone morphogenetic proteins (BMPs). Here, I discuss the evidence that Wingless — a *Drosophila melanogaster* Wnt — is a candidate classical morphogen, which is a concept that came mainly from the study of Wingless activity in the patterning of the wing during larval development. I believe that the conclusion that Wnt is a classical morphogen has not taken into account important biological parameters of the system under study (for example, the order of the onset of gene expression and the growth of the wing). The consideration of these parameters raises the possibility that, in many instances, Wingless does not function as a classical inductive morphogen, and that its concentration-dependent responses have more to do with an important role for Wingless in modulating the effects of inductive molecules.

Wingless as a classical morphogen

Wingless is a founder member of the Wnt family of signalling molecules[5]. Its gene, *wingless* (*wg*), was first identified as a mutation, *wg*^{1} (REF. 6), that removes the wing of *Drosophila*. *wg*^{1} is a regulatory mutation that

Box 1 | **Morphogens**

The key attributes of morphogens — on the basis of the classical definition and on present perceptions — are:

- **They are secreted, diffusible molecules that come to be distributed in a concentration gradient from a fixed spatial source.**
- **They generate several (at least three) discrete cellular states in response to different thresholds of the concentration gradient. These cell states are usually associated with differential gene expression.**
- **They are instructive (that is, they function as a determinant of the cell state) in a direct manner (that is, without intermediates; the response of the cells does not depend on the cell changing states first).**

Examples of molecules and situations that fulfil the criteria above are:

***Spatzle*, specifying the dorsoventral axis of the *Drosophila melanogaster* embryo.**

***Decapentaplegic*, patterning the *Drosophila* wing.**

***Activins*, specifying and patterning the mesoderm during the early development of amphibians and other vertebrates.**

***Bone morphogenetic proteins 2 and 4*, mediating the activity of Spemann's organizer in *Xenopus laevis*.**

***Sonic Hedegehog*, specifying motorneuron pools in the vertebrate central nervous system.**

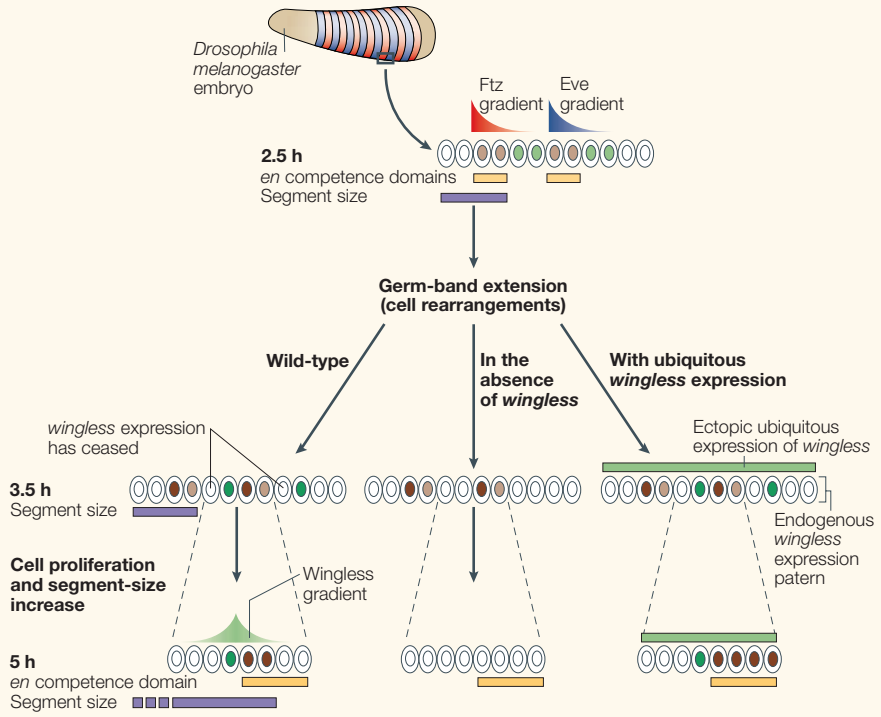
identified an essential function for Wingless in the initial specification of the wing[7], and null alleles of *wg* have more widespread effects on fly development[8].

The *wg* gene encodes a secreted protein that can function at a distance from its source[9,10]. In the *Drosophila* embryo, there is some evidence for concentration-dependent responses to Wingless. However, these responses are not multiple (that is, for a given pattern of Wingless expression, different concentrations do not elicit different responses) and there is no evidence that the establishment of these responses is a direct consequence of Wingless activity or that the concentration of Wingless is important for them[11,12]. The regulation of *engrailed* (*en*) expression in the *Drosophila* embryo provides an example of this. During the patterning of the blastoderm, the pair-rule genes — each of which specify a simple alternation with a repeat distance of two segments — establish domains of competence, in which defined groups of cells have the ability to express *en*[13] (BOX 2). However, *en* expression is only stabilized in a subset of these cells, that is, those that are exposed to 'enough' Wingless for a certain amount of time. The initiation of *en* expression is independent of Wingless, which only determines the maintenance of this expression[14]. The expression of *wg* is restricted to a one-cell-wide stripe per embryo segment and the extension of this expression to the whole of the segment does not result in a related expansion in the expression of *en*. Instead, *en* simply enlarges its domain slightly to occupy the region where the pair-rule genes activated its expression at the blastoderm stage[15] (BOX 2).

The specification of naked cuticle is a second example of Wingless function in the embryo[11,16]. In the absence of Wingless, the ventral epidermis develops a 'lawn' of denticles from anterior to posterior. In the wild type, however, periodically arranged sources of Wingless generate gaps in this lawn. The extent of these gaps is determined by the extent of Wingless diffusion and these gaps are achieved by repression of the gene *ovo/shaven-baby* (*svb*)[16]. As in the case of *en* expression, this repression and the ensuing patch of naked cuticle are the simple and unique response to a varying concentration of Wingless. So, for the repression of *ovo/svb*, as well as for *en* expression, all that is needed is Wingless signalling above a certain threshold, which is probably very low, and this is sufficient to elicit a unique response.

During larval development, Wingless has important functions in the development and patterning of the different elements of the adult fly. The legs and wings have been particularly well studied and it is in these contexts that the idea that Wingless is a classical morphogen has been proposed[17–19]. In particular, many of the arguments for Wingless as a classical morphogen are derived from the effects of Wingless on wing patterning. In the late third larval instar, which is the late phase of growth of the adult tissues (~100 h after egg laying), the developing wing is bisected by a narrow stripe of *wg* expression, which is the source of a steep symmetric gradient of the Wingless protein (FIG. 1a). This stripe of Wingless happens to coincide with a developmental landmark — the

Box 2 | Initiation versus maintenance of *engrailed* expression



The 'permissive' function of Wingless signalling is illustrated by its effect on the expression of *engrailed* (*en*) in the early *Drosophila melanogaster* embryo, which can be generalized to its effects on other targets. Early in the development of *Drosophila*, the embryo is subdivided into segments along the anterior/posterior axis by the transcription factors Fushi tarazu (Ftz; red in the figure) and Even skipped (Eve; blue). The genes *ftz* and *eve* are pair-rule genes. Ftz and Eve function as activators of *en* expression (light and dark brown shading in the figure highlight the levels of *en* expression (low and high expression, respectively)) and repressors of *wingless* (*wg*) expression (light and dark green shading in the figure highlight the levels of *wg* expression (low and high expression, respectively)), and they are deployed in alternative gradients that define regions of high and low activity across each segment. As a result of these patterns of expression, *en* and *wg* come to be expressed in adjacent single-row stripes of cells, which are determined by the highest (*en*) and lowest (*wg*) activity of Ftz and Eve. So, above a certain threshold, high levels of Ftz and Eve endow cells with the competence to express *en* (*en* competence domains are highlighted by yellow rectangles), perhaps by opening up chromatin and recruiting the basal transcriptional machinery to its promoter. Wingless signalling is necessary to make this pattern of gene expression stable.

As cells begin to proliferate and the segments begin to grow (3.5–5 h), Wingless diffuses from its source and creates a concentration gradient across the 'domains of competence' that were set up by Ftz and Eve. Above a certain threshold, Wingless maintains the expression of *en*, perhaps by stabilizing the structure of the chromatin. In the absence of Wingless, *en* expression is not compromised initially, but decays quickly. On the other hand, ectopic ubiquitous expression of *wingless* across the whole developing segment only allows *en* to be expressed in cells that are already competent to express it. This effect can be best seen when Wingless is ubiquitously provided in a *wg*-mutant embryo. In this case, the expression of *en* is identical to that of wild-type embryos in which *wg* is ubiquitously expressed. This experiment highlights the fact that, with regard to *en* expression, Wingless is a maintenance factor for the initial activity of the pair-rule genes.

appearance of the dorsoventral (DV) compartment boundary — and has therefore been endowed with organizing properties[18,19]. At this stage, and centered around the source of Wingless, there are three concentric domains of gene expression: a broad domain that expresses *vestigial* (*vg*); a narrower domain that expresses *Distal-less* (*Dll*); and a very narrow domain, which is adjacent to the source of Wingless, that expresses *achaete* (*ac*) (FIG.1b,c). Because alterations in the concentration of Wingless (for example, by the creation of ectopic sources in the developing wing) have subtle effects on the levels of expression of these genes and, in the case of *ac*, also on its domains of expression, it has been suggested that Wingless functions as a classical morphogen to pattern the wing[18,19]. As for any other putative classical morphogen, the most important element in the argument is that the three concentric domains of gene expression represent direct threshold responses to the gradient of Wingless. If this is not the case, Wingless cannot be said to function as a classical morphogen.

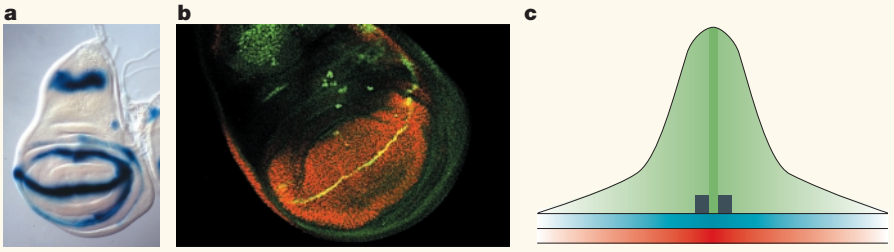


Figure 1 | **Wingless signalling in the wing primordium of a third larval instar wing disc of *Drosophila melanogaster*. a** | The pattern of *wingless* (*wg*) expression (blue) is shown by a β-galactosidase reporter that was inserted at the position of the *wg* gene. Notice a circle that delimits the wing tissue, and a stripe of expression that bisects this and delimits the dorsoventral (DV) boundary. **b** | The wing disc showing the expression of the Vestigial protein (red) relative to the source of Wingless at the DV boundary (yellow). **c** | A schematic of the gradient of the Wingless protein (green) and the observed patterns of response in the third larval instar wing. Achaete (black) is found in cells that are adjacent to the source of Wingless (dark green), and Distal-less (red) and Vestigial (blue) have similar graded responses. For further details, see REFS 18,19. The photograph in part **b** was kindly provided by S. Carroll and J. Paddock (University of Wisconsin, Madison, USA).

Wingless and the patterning of the wing

The patterns of expression of *vg*, *Dll* and *ac* in the third instar disc relative to the DV stripe of *wg* expression are indeed very indicative of the function of a classical morphogen. However, there are some problems with this interpretation because, throughout the development of the wing, *wg* expression is not fixed but undergoes several transitions between different patterns[7,20] (FIG. 2). Therefore, to evaluate the possibility that Wingless functions as a classical morphogen, it is important to consider the way the wing grows, the way the three genes are activated and, as the DV stripe of *wg* expression is only one of several patterns of *wg* expression during wing development, the relationship between the initial expression of each of these genes and the pattern of *wg* expression at that time. The consideration of these variables leads to a very different picture of how the domains of *vg*, *Dll* and *ac* expression arise and, more importantly, of the contribution of Wingless to this process[20–22].

The wing of *Drosophila* develops from a small group of cells that is specified by Wingless at the beginning of the second larval instar (~48 h after egg laying)[7,23]. From this moment onwards, a series of successive and mutually-dependent interactions between transcription factors and signalling molecules leads to the pattern that is visible in the third larval instar[4,20] (FIG. 1). In general, these interactions involve the activity of signalling centres along the anterior/posterior and the DV axes, and the activity of Wingless is associated with the DV axis.

At the onset of wing development, *wg* is expressed over the ventral side of the wing disc. From this moment, and until the third larval instar, it is expressed in a sequence of dynamic patterns that culminate in the formation of the DV stripe during the first half of the third instar (FIG. 2). By this time, both *vg* and *Dll* have been activated and are expressed in particular domains of the developing wing. The first gene to be activated is *vg*, early in the second instar, when *wg* is expressed in a rapid sequence of patterns that, for the most part, always overlap with the expression of *vg* (FIG. 2). At this stage, Wingless cannot elicit the ectopic expression of *vg*, but Delta, a ligand for the Notch receptor, can. Furthermore, when Wingless is provided together with Delta, it is possible to see a stronger effect of Delta, that is, the activity of Delta shows that there is a function of Wingless with which it works synergistically[21,22]. This led to the suggestion that the initiation of *vg* expression depends on the combination of Delta and Wingless, with Delta providing the initial input and Wingless providing a modulatory and stabilizing influence[21,22].

The expression of *Dll* is activated after that of *vg*, but it also occurs before *wg* is expressed in a symmetric DV stripe and at the time when *wg* is expressed in a very complex pattern over the developing wing. Again, Wingless is not able to elicit new expression of a target, in this case *Dll*. However, Vestigial can do this and, in a situation reminiscent to that of the earlier stage, Wingless functions synergistically, this time with Vestigial, to regulate the expression of *Dll*[21]. So, it would seem that, once activated, Vestigial induces the expression of *Dll*, the expression of which is only maintained in a domain that is determined by the activity of Wingless. Both of these situations are reminiscent of the regulation of *en* expression by Wingless in the embryo (BOX 2); a regulatory event (Delta signalling in the case of *vg* and Vestigial activity in the case of *Dll*) provides an input that defines a competence domain (as the pair-rule proteins do for *en*) and Wingless then stabilizes gene expression in a subdomain that is determined by its range of action and the responsiveness of the genes. By the time the DV stripe of Wingless appears, the expression of *vg* and *Dll* is well established and the function of Wingless is to modulate and maintain it with reference to the DV boundary.

At first sight, the case of *ac* seems different, because the onset of its expression is coincident with the DV stripe of *wg* expression. However, it might not be that different, because *ac* expression could be elicited by something other than Wingless (perhaps by Distal-less itself) and then be maintained where there are high levels of Wingless. It might well be that most of the wing is primed to express *ac*, and that only those regions that are exposed to high levels of Wingless do so stably. In the wild type, the Wingless gradient is very steep (FIG. 1c) and this, coupled to a very high response threshold, might be the simple explanation for the very narrow pattern of *ac* expression. This possibility is supported by observations of the function of Wingless in the regulation of *ac* in the notum — the main thoracic body part of the adult fly. In this case, ectopic expression of Wingless has little effect on the pattern; that is, Wingless can only elicit bristles, the associated sensory organs and *ac* expression where the 'pre-pattern' allows it to

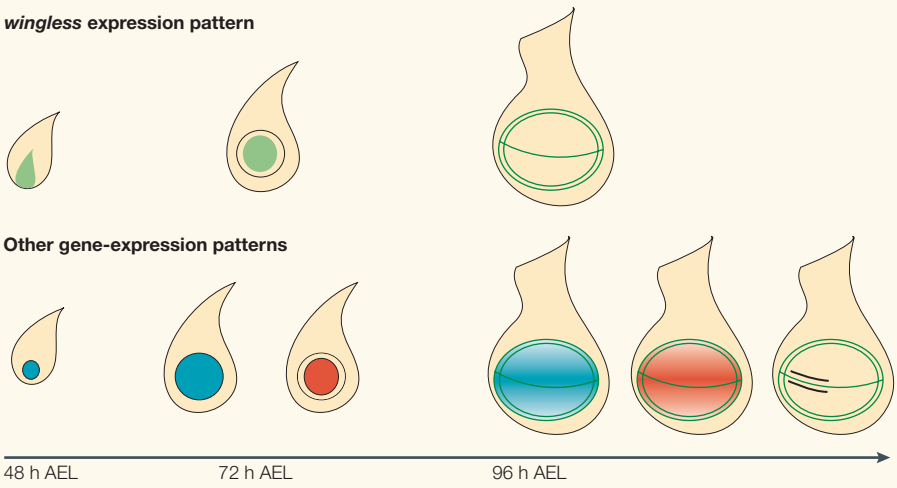


Figure 2 | **Temporal relationship between different patterns of *wingless* expression and the onset and modulation of gene expression during wing development.** During the second larval instar (about 48 h after egg laying (AEL)), *wingless* (*wg*; green) is expressed over the whole of the ventral region of the wing disc from which the wing primordium emerges, as identified by the expression of *vestigial* (*vg*) (blue). At the beginning of the third instar (about 72 h AEL), *wg* expression begins to be modulated by a pattern that occupies the whole of the wing primordium. At this time, *vg* is already expressed in this domain and *Distal-less* (*Dll*) expression (red) is initiated in a similar domain. By mid or late third instar (~96–100 h AEL), *wg* is expressed in the dorsoventral (DV) stripe and in a series of rings that outline the wing pouch. At this stage, the expression of *vg* and *Dll* is modulated with reference to the DV source of Wingless, and *achaete* expression (black) is initiated in cells that are adjacent to those producing Wingless. For further details of this process, see REFS 20–22.

do so[24,25]. Another important issue of the relationship between *ac* and Wingless is that only cells that are adjacent to the source of Wingless express *ac*[26]. This is not an effect at a distance.

Wingless is not a classical morphogen

An essential issue in assigning the qualities of a classical morphogen to a signalling molecule is the requirement for the candidate molecule to induce gene expression over a distance directly and *de novo*, that is, to be instructive[1–3]. The observations and experiments summarized above (also see REF. 22) indicate that Wingless does not initiate the expression of *vg* or *Dll* and this is supported by some experiments that tested this point directly[20,21].

The case for Wingless as a classical morphogen rests on the assumption that it is the diffusion of Wingless from the DV stripe that directly establishes the patterns of *vg*, *Dll* and *ac* expression. In the best of cases, it is difficult to devise experiments that test whether a molecule fulfils the criteria of a classical morphogen or not. The situation of the wing of *Drosophila* is particularly complex, because not only is there always an endogenous source of the molecule being tested, which complicates the analysis of the responses, but this source has a dynamic pattern of expression. The latter point is important because it means that it is always difficult to be sure which pattern of Wingless is responsible for a particular effect.

An approach that can be used to get around this problem is to take a mutant in which the DV stripe of *wg* is missing, and to look at the effects of reintroducing Wingless under these circumstances. This experimental situation is provided by wing discs with impaired Notch signalling. Mutants for *Suppressor of Hairless* or *apterous* lack the DV stripe of *wg*, as well as expression of *vg*, *Dll* or *ac*. Furthermore, these mutants lack wings. So, based on the assumption that Wingless functions as a classical morphogen to mediate the development and patterning of the wing, reintroducing Wingless to these mutants should, at the very least, restore wing development. However, this was not found to be the case[20,21], which confirms the impression that is gained from other studies, which is that Wingless cannot trigger the expression of the genes that mediate wing development and patterning. Interestingly, expression of *vg* in these mutants can rescue wing development[21], which underscores the fact that something else, and not Wingless, triggers the expression of *vg*. This result indicates that Wingless is not essential for wing development and also argues against the idea that it functions as a classical morphogen.

> "…although it is not possible to say that Wingless functions as a morphogen in the classical sense, its concentration-dependent effects on some aspects of gene expression highlight the need to reconsider the idea of a morphogen."

Cell fate: initiation or maintenance?

The idea that Wingless functions as a classical morphogen during wing development has influenced our way of looking at other Wnt proteins. The essence of a classical morphogen is its ability to induce different cell states in a concentration-dependent, direct and instructive manner. If any of these criteria are not met, the candidate should not be deemed a classical morphogen. During the patterning of the wing, Wingless fails to meet these criteria (also see REF. 22). However, one property of morphogens — that is, the concentration-dependent responses — should be looked at carefully. In the classical definition, this parameter determines the initiation of gene expression. In the context of Wingless signalling, it is clear that the maintenance, rather than the initiation, of the expression of different genes is sensitive to the concentration of Wingless and this might well be a general feature of Wnt-protein function (for examples, see REFS 21,22,27; see REF. 28 for an example from vertebrates).

So, although it is not possible to say that Wingless functions as a morphogen in the classical sense, its concentration-dependent effects on some aspects of gene expression highlight the need to reconsider the idea of a morphogen. It might be necessary to distinguish between functionally different kinds of morphogens (FIG. 3). The first kind of morphogen is the classical one, which fulfils the strict definition and includes, for example, BMPs and members of the Hh family[3]. The second kind of morphogen would encompass molecules that, rather than being instructive, have a secondary, but essential, role in the maintenance of cell fates. Wingless is the model example of this class. In all the cases that have been analysed in detail, the absence of Wingless does not affect the initial adoption of a fate, but affects only its maintenance and stability

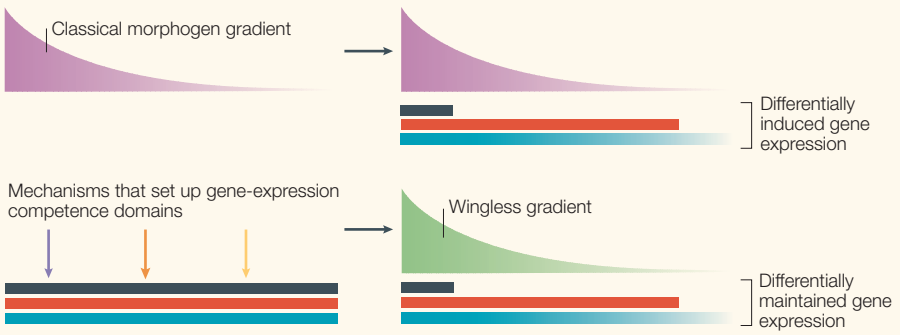


Figure 3 | **Different outcomes of concentration-dependent initiation or maintenance of gene expression. a** | A classical morphogen (pink) elicits differential gene expression (represented by the black, red and blue bars) by activating different genes at different concentrations. **b** | Molecules like Wingless (green), and possibly other Wnt proteins, function by modulating gene expression in a concentration-dependent manner. Genes with broad domains of potential expression are set up by various mechanisms (as indicated by the coloured arrows), which include the genetic history of the cell or intercellular signalling. Each of these genes has a different response threshold to Wingless. So, a gradient of Wingless results in the stable differential expression of the different genes and the patterning of the field of cells simply by determining their domains of maintenance. One way in which this can be achieved is by modulating chromatin structure.

(for a review, see REF. 22). The differential response of genes to this maintenance function results in a pattern. For example, there is very little difference between the domains of *Dll* and *vg* expression, and whatever difference there is might be due to subtly different responses to the maintenance activity of Wingless.

Conclusions and perspectives

One problem with the idea of a classical morphogen is that it lacks a well-defined mechanistic element. The original concept was formulated at a time when little was known about the molecular mechanisms of pattern formation and was, out of necessity, vague. Perhaps this is the reason why we often encounter hair-splitting debates on whether something is or is not a classical morphogen. For example, does Sonic hedgehog function as a classical morphogen during the patterning of the vertebrate limb? In the course of time, the original concept of a classical morphogen has evolved to accommodate molecules that are involved in pattern formation at a distance, but it might have to evolve more and become more precise if we do not want to become arbitrary in what we call, in the classical sense, a morphogen.

The distinction between two kinds of morphogens in terms of their molecular function might not just be an issue of semantics. The initiation and maintenance of gene expression are two mechanistically different steps[27,29] that have to be linked for cells to adopt fates stably. Classical morphogens are one way of activating the first step[1–4], and Wnt molecules might be a way of regulating the second step 'from the outside'. It is well known that chromatin-remodelling proteins have a role in stabilizing the gene expression that is associated with cell fates; Wnt signalling might be a way of regulating this process through intercellular signalling. The effects of molecules such as Wnt proteins therefore sharpen and refine the more 'coarse-grain' patterns that are laid out by classical morphogens or, in other circumstances, by lineage-related mechanisms[21,22,27]. It will be interesting to see if, in the complex context of growing fields of cells (such as the wing discs), the classical concept of a morphogen can be sustained, or whether, from a mechanistic point of view, allowances will need to be made for the different roles of other signalling molecules.

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