Theoretical and experimental approaches to understand morphogen gradients

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Morphogen gradients, which specify different fates for cells in a direct concentration-dependent manner, are a highly influential framework in which pattern formation processes in developmental biology can be characterized. A common analysis approach is combining experimental and theoretical strategies, thereby fostering relevant data on the dynamics and transduction of gradients. The mechanisms of morphogen transport and conversion from graded information to binary responses are some of the topics on which these combined strategies have shed light. Herein, we review these data, emphasizing, on the one hand, how theoretical approaches have been helpful and, on the other hand, how these have been combined with experimental strategies. In addition, we discuss those cases in which gradient formation and gradient interpretation at the molecular and/or cellular level may influence each other within a mutual feedback loop. To understand this interplay and the features it yields, it becomes essential to take system-level approaches that combine experimental and theoretical strategies.

Introduction

Embryonic development involves spatial and temporal patterns of cellular differentiation and the shaping of form. How do embryonic tissues organize in space and time such that a field of distinct cells emerges reliably? This question has fascinated developmental biologists for decades. Early in the last century, the existence of gradients that could signal over large distances was proposed to account for patterning (Morgan, 1901). Indeed, morphogen gradients, defined as graded distributions of secreted molecules that specify distinct fates for the cells in a concentration-dependent and direct manner (Wolpert, 1969), have become, over the last few decades, a highly influential framework to test and understand pattern formation processes during embryonic development (Figure 1A).

The concept that the fate of cells depends on their spatial position, enabling an organized pattern to arise, was formalized by Lewis Wolpert in his positional information model (Wolpert, 1969). According to this model, cells have their spatial position specified along specific directions with respect to one or more reference points and translate such positional information into specific cell behaviours, which depend, as well, on the developmental history of the cell. Which kind of signals could provide positional information to the cells? Wolpert envisaged spatial gradients of a chemical’s concentration over a field of cells as one of the potential signals: cells that sense a low amount of chemical are more distant from the reference point (i.e. the source of the chemical) than cells that sense a higher amount.

The first molecular demonstration of the concept of gradients specifying distinct fates in a direct manner took time to appear and was provided in the Drosophila syncytium (Driever and Nüsslein-Volhard, 1988a,b). The transcription factor Bicoid was shown to be distributed along a gradient expanding from the anterior pole to more than one-half of the embryo and to regulate the expression of downstream gap genes (for a review, see Ephrussi and Johnston, 2004). Afterwards, other signalling proteins such as Dpp, Wingless, Spitz, Hedgehog, Activin and Nodal have been described as morphogens in a wide variety of organisms (for reviews, see Green, 2002; Martinez Arias, 2003; Tabata and Takei, 2004; Schier and Talbot, 2005; Affolter and Basler, 2007). It is worth stressing, however, that the case of Bicoid is a rather unusual one. Whereas the above-mentioned morphogens correspond to secreted molecules that can form gradients extracellularly, Bicoid is a transcription factor that forms a gradient before cellularization in the Drosophila embryo, from a localized region of transcription in the anterior pole.

To check whether a gradient acts as a morphogen, it is important to unveil whether it specifies distinct fates over space in a direct manner. Accordingly, experimental designs that evaluate the direct action and, hence, the requirement of the morphogen molecule at long distances have been elaborated (for a review, see Tabata and Takei, 2004). In addition, the shape of gradients has been altered by...
changing the level of the morphogen signal (Shimizu and Gurdon, 1999; Ashe et al., 2000) to observe whether spatial shifts in the fates of cells and in the expression pattern of target genes arise (Ashe and Briscoe, 2006). Hence, as it is expected from a morphogen, changes in Bicoid concentration along its gradient elicit shifts in the expression domain of its downstream target gap genes and alter the fate of cells (Driever and Nüsslein-Volhard, 1988a; Driever et al., 1989a, c; Driever and Nüsslein-Volhard, 1989b; Struhl et al., 1989; St Johnston and Nüsslein-Volhard, 1992; Rivera-Pomar and Jäckle, 1996).

Although other frameworks for patterning processes have been proposed (Turing, 1952), morphogen gradients have been the most influential up to now, stimulating and promoting strategies to unveil the process of embryonic patterning. Hence, graded signals have been searched and computational strategies that quantify and characterize the formation of gradients as well as theoretical approaches that address open issues related to the properties of gradients have become a common approach (see, for instance, Kerszberg and Wolpert, 1998; Eldar et al., 2002, 2003, 2006; Lander et al., 2002, 2007; Jaeger et al., 2004; Kruse et al., 2004; Aegeter-Wilmsen et al., 2005; Bollenbach et al., 2005; England and Cardy, 2005; Houchmandzadeh et al., 2005; Howard and ten Wolde, 2005; Melen et al., 2005; Mizutani et al., 2005; Shimmi et al., 2005; Ibañes et al., 2006; McHale et al., 2006; Umulis et al., 2006; Bergmann et al., 2007; Gregor et al., 2007a, b; Kicheva et al., 2007).

However, as we have learned more about morphogen gradients and their role in shaping the embryo, new complexities have emerged. Herein, we examine these issues, highlighting those recent findings that unveil novel aspects of morphogen gradients with an emphasis on how theoretical and computational studies have contributed. Moreover, we discuss how these findings emphasize the need for taking new approaches that utilize experimental and theoretical strategies to integrate both the formation and interpretation of morphogen gradients into a single framework.

**A wide variety of molecular gradients**

During the last few decades, gradients of different kinds of molecules, with a wide variety of sizes and dynamics, have been uncovered, revealing that both the morphogen and the
The visualization of the protein gradient is the first step in detecting a morphogen. To this end, antibody staining and GFP fusion proteins, among others, have been used to provide a static image of the gradient on fixed tissue. Attaining more detailed measurements has allowed quantification of morphogen gradients. Specifically, imaging of functional fluorescent green protein-morphogen fusions over space has shown that Bicoid in the Drosophila syncytium and Dpp and Wingless in the fly’s wing form gradients with the same kind of decay characterized by an exponential shape. This kind of profile implies that the fraction of morphogen that decreases over space is the same all over the gradient. Accordingly, a single scale characterizes the spatial decay and it can be used as a measure of its size. By fitting an exponential profile to the fluorescent data, a characteristic length of the gradient can be obtained. This procedure has shown that the Bicoid gradient is much larger, with a characteristic length of 100 μm, than the Dpp and Wingless gradients, which have a characteristic length of 20 and 6 μm, respectively (Houchmandzadeh et al., 2002; Kicheva et al., 2007) (Figure 1B). Activity gradients have been monitored as well by measuring the amounts of downstream intracellular responses (e.g. kinase phosphorylation). This is the case of the BMP gradient, which patterns the dorsoventral axis in Drosophila embryos. This gradient shows a sharp profile that decays strongly over a field of five cells and is formed within 30 min (Eldar et al., 2002; Mizutani et al., 2005).

Once the gradient profile has been fitted, it remains to be elucidated which dynamic yields it. Mathematical and numerical modelling can be very helpful to this end (Box 1; Figure 2A and B). In addition, theoretical approaches can also enable the quantification of the dynamics taking place. For instance, an exponential gradient profile is the steady-state solution of a morphogen dynamic involving diffusion and linear degradation. Taking into account that diffusion rates have dimensions of surface over time and degradation rates of the inverse of time, a dimensional analysis of this dynamic readily reveals that the characteristic length of the gradient depends on these parameters as \( \sqrt{D/\beta} \), where \( D \) and \( \beta \) are the diffusion and degradation rates, respectively. Therefore, all those different values of \( D \) and \( \beta \) rates that yield the same ratio \( D/\beta \) elicit a steady-state gradient spanning the same spatial region (Figure 1C). Conversely, by quantifying the characteristic length of the gradient through the data on the gradient profile, we cannot infer which values of the diffusion and degradation rates underlie the morphogen dynamics. However, the transient dynamics may depend on the diffusion rate \( D \) on its own (Figure 1C and D). Thus, we can obtain the value of \( D \) by measuring this dynamic. This is the approach used by Kicheva et al. (2007) to characterize the kinetics of the Dpp and Wingless gradients. The authors measured the fluorescence recovery after photobleaching (FRAP) during a time interval of 1 h. FRAP experiments were modelled, taking into account both production and degradation processes, and the mathematical expression of the recovery dynamics, with their dependence on the kinetic morphogen parameters, was formulated. Importantly, the recovery dynamics depend on the diffusion rate \( D \) and not just on the characteristic length (Kicheva et al., 2007). By adjusting the mathematically derived expressions of the recovery dynamics with the measured FRAP data over time and space, Kicheva et al found the value of \( D \). Moreover, as the characteristic length was also known from the gradient profile data, the degradation rate \( \beta \) could be inferred (Kicheva et al., 2007). Note that for the sake of...
simplicity and clarity, herein we have sketched the approach that in the original work was much more complex and involved several parameters to be determined.

Assigning quantitative values to the kinetics of the Dpp and Wingless gradients allows pinpointing which element is responsible for the shorter spatial range of the Wingless gradient: does Wingless diffuse much less than Dpp or does it have a much shorter half-life? The study by Kicheva et al (2007) showed that the short half-life of Wingless (8 min) compared with the 45 min half-life of Dpp yields a Wingless gradient spanning much fewer cells, whereas the effective diffusion rates are rather similar between both morphogens ($D \approx 0.1 \mu m^2/s$ for Dpp and $D \approx 0.5 \mu m^2/s$ for Wingless).

The numerical solution for the dynamics of morphogen diffusion from a localized source of production and linear morphogen degradation shows that the morphogen gradient achieves a steady state over a broad spatial region on time periods one order of magnitude longer than the morphogen half-life (Bergmann et al, 2007). Thus, by using the above-mentioned molecular half-lives, it can be reasoned that the Dpp gradient takes around 450 min to be formed, whereas Wingless forms its gradient much more rapidly, in only 80 min. This is in agreement with the time of morphogen signal recovery after reversible blockade of the morphogen through temperature changes, which has also been used to infer how long a gradient takes to be formed (Entchev et al, 2000; Teleman and Cohen, 2000). Recently, in vivo optical imaging that allows the measurement of the whole gradient all along its formation has been implemented for the Bicoid gradient. Panels C and D use the same parameter values except for $p$. Profiles in panels A and B are computed from analytical expressions from Eldar et al (2003). Panels C and D are computed as in Figure 1.

**Figure 2** Gradient responses to perturbations. Responses of gradients to changes in the production rate $p$ at the source. (A) Steady-state gradient profiles for two types of gradients (green, black) and for two different production rates (lines for $p=1$ and circles for $p=5$). Gradients formed by diffusion and linear degradation are depicted in black (exponential profile), whereas those formed by diffusion and nonlinear (enhanced) degradation are depicted in green (power-law profile). Two quite similar steady-state gradient profiles (green and black lines) become much more distinct when the production rate is increased by a factor of 5 (green and black circles). (B) Steady-state morphogen level at the source as a function of the production rate $p$ for the two types of gradients analysed in panel A. The qualitative dependence is shown. Power 2 is used for nonlinear degradation. (C, D) Gradient profiles formed by diffusion and linear degradation for $p=1$ (black) and $p=5$ (grey) at a transient dynamical stage (C) and at the steady state (D). The dotted horizontal lines denote a threshold of morphogen level. Red arrows denote the spatial shift that is elicited when the production rate increases. Vertical dotted lines denote the spatial position where the threshold is located. The shift is much larger at the steady state than at a transient state. Also note that the spatial position is different at the transient state and at the steady state. See Bergmann et al (2007) for a study of these features on the Bicoid gradient. Panels C and D are computed from analytical expressions from Eldar et al (2003). Panels C and D are computed as in Figure 1.
and the homeobox gene *Hoxd13* in the chick limb, have been shown to form gradients over large fields of cells (Dubrulle and Pourquié, 2004; Ibañes et al, 2006). In the case of *Hoxd13*, the gradient spans more than 400 μm and takes several hours to be formed (Figure 3B). These graded distributions of mRNA necessarily elicit protein gradients (Ibañes et al, 2006). These protein gradients could potentially specify (in the case of secreted proteins) or control (in the case of transcription factors) distinct cell fates. Therefore, these novel observations raise the intriguing question of whether these gradients are acting in a morphogen-like manner (i.e. by instructing directly distinct cell fates). If future investigations support such a role, then the concept of morphogen could be extended to include non-secreted proteins.

**Box 2 Diffusion and the Bicoid gradient**

To demonstrate the actual participation of diffusion as a transport mechanism for morphogen gradients, several strategies have recently been used to evaluate the Bicoid diffusion rate. The data, however, have yielded strikingly different values (Gregor et al, 2006, 2007b). On the one hand, inert fluorescent dextran molecules were injected in the anterior pole of the *Drosophila* syncytium and the fluorescent intensity over 1 h at different spatial positions a few hundred microns from the injection point was measured (Gregor et al, 2005). All these time-evolution data curves were fitted by computationally derived time courses obtained from a 3D description of diffusive transport over a domain with geometry determined by two-photon images of embryos. Analysis of the fluorescent curves was made such that the single free parameter to be fitted was the diffusion rate. Dextran molecules of several molecular masses were used, in the range of the Bicoid molecular mass, uncovering that data adjusted to a modified Stokes–Einstein relationship (i.e. diffusion rate decreases as an inverse function of molecular radius) in which diffusion is uniformly increased (Gregor et al, 2005). The inferred diffusion rates were in the order of 10 μm²/s, which were in the range expected if the Bicoid gradient, with characteristic length of 100 μm, is assumed to reach the steady state within 1 h (see main text). However, direct measures of Bicoid motion have yielded a very different, much smaller, diffusion rate. By measuring Bicoid dynamic recovery 1 min after photobleaching at the cortical cytoplasm, and by fitting time-course curves obtained from a 3D diffusion-transport to these data, the Bicoid diffusion rate was inferred to be around 0.3 μm²/s, three orders of magnitude smaller than the diffusion rate of dextran molecules (Gregor et al, 2007b). Note that, due to technical issues, this measure could only be made for Bicoid motion within the cortical region and at times beyond 1 h after fertilization. Can this small diffusion coefficient account for the Bicoid gradient profile? If this is the rate of Bicoid diffusion all over the time period of gradient formation (around 2 h) and all through the cytoplasm (within the bulk as well), it would imply that the Bicoid gradient does not reach a steady state, as it has been proposed (Bergmann et al, 2007, 2008). However, criticisms have been raised against this proposal, which argue that the diffusion coefficient driving the long-time and large-scale dynamics must be higher, as the inferred diffusion rate is too small to account for the hundreds of microns Bicoid spans in just 2 h (Gregor et al, 2007b; Bialek et al, 2008). Different diffusion rates over space (at the bulk cytoplasm and the cortex) and time (during the first hour after fertilization and thereafter), or active transport mechanisms that may yield to faster diffusion at long times have been alternatively proposed to resolve this paradox (Gregor et al, 2007b; Bialek et al, 2008). Some of the experiments and analyses that could shed light on this puzzling issue, and which are challenging due to technical limitations and difficulties they involve, are measurements of the Bicoid dynamics at the bulk of the cytoplasm and during the first hour after fertilization, measurements of the overall level of Bicoid along time at a specific spatial position, the measurement of Bicoid lifetime or of changes in the gradient when this lifetime is altered, as well as theoretical analyses that can point out the range of plausible Bicoid diffusion rates when the gradient is transient, at the steady state or driven by additional transport mechanisms.

**Creating a molecular gradient**

Three main elements participate in the creation of a steady-state chemical gradient: the source of morphogen production, the sink and the transport of the morphogen through space. Regarding the source, focus has been mainly on local homogeneous sources of production, defined by a spatial domain where production is uniform over space and constant over time. However, non-uniform sources of protein production can also exist, arising from mRNA gradients, for instance, and which readily create protein gradients (Dubrulle and Pourquié, 2004; Ibañes et al, 2006).

Degradation of the molecule (or in general terms, the subtraction of the morphogen) facilitates the formation of gradients. Such degradation can be an active process in which specific proteins destroy the morphogen, or a passive dilution driven by cell division for rather stable proteins (Ibañes et al, 2006). The interaction of the morphogen with other molecules, for example, ligand morphogen binding and unbinding with receptors, can be re-interpreted in terms of degradation and source-like terms and, as expected, can also shape the
gradient. Recent studies have shown that morphogen degradation can be controlled by the morphogen signal, setting a feedback between gradient formation and signalling, and eliciting differential degradation over space. Invertebrate and vertebrate Hedgehog morphogen as well as retinoic acid in zebrafish embryos has been shown to make use of these feedbacks when creating morphogen gradients (Eldar et al., 2003; Dessaud et al., 2007; White et al., 2007). Hedgehog signalling induces the expression of its receptor, Patched, which in turn is endocytosed, thereby degrading the ligand. In the case of retinoic acid, its signal induces the expression of the retinoic acid-degrading enzyme Cyp26a1. Thus, the degradation of these morphogens is enhanced in those spatial regions of high morphogen activity. This kind of feedback between morphogen signalling and degradation (and hence, formation) can be modelled mathematically by setting the morphogen degradation as nonlinear (at least close to the source) (Eldar et al., 2003). The steady-state profile of a dynamic involving diffusion and nonlinear degradation can be obtained analytically and corresponds to a power-law shape (it decays more abruptly close to the source and less markedly on the tails than an exponential profile) (Eldar et al., 2003). But is this feedback relevant? Mathematical analysis of the gradient profiles when gene dosage is increased reveals that this differential degradation confers robustness to these changes (Eldar et al., 2003), indicating that this robustness may be a desired property of the morphogen gradient.

The transport of a morphogen

The concept of morphogen gradients is tightly related to long-range signalling. How can molecules span over large spatial regions to elicit direct responses? According to the values known for the diffusion rates of molecules, Francis Crick proposed that diffusion enables the formation of gradients over fields of 50 cells within a scale of a few hours (Crick, 1970). Since then, diffusion has taken the leading role as the transport mechanism for gradient formation. At present, diffusion is commonly named restricted or effective diffusion, to emphasize that the diffusion rate values in the extracellular medium are much smaller than those measured in aqueous media (Tabata and Takei, 2004; Strigini, 2005; Kicheva et al., 2007). The difference in such rates is thought to arise partially from the properties of the extracellular medium, a crowded environment with non-uniform matrix geometries and molecular distributions that interact with the morphogen. In addition, the effective diffusion can involve other non-directional random transports. For instance, the recently inferred that effective diffusion rate of Dpp, $D = 0.1 \mu m^2/s$, is much (three orders of magnitude) smaller than what it would be expected according to its size when diffusing freely in water (Kicheva et al., 2007). Note that the procedure involved in inferring this value took all kinds of Dpp transport as a single diffusive-like motion, as described above. Therefore, this rate corresponds to an effective motion in which other non-directional random transports can be involved, which potentially may strongly slow down the dynamics.

Diffusion arises from the random motion of molecules. Molecules perform a kind of random walk-like motion going in all, even opposite, directions. Thus, the mean displacement of molecules does not increase linearly with time, as in ballistic motion, but much more slowly, as the root square of time. This pure diffusion is not the only passive random motion molecules can trace within a biological medium. Indeed, in prokaryotes, large biological molecules such as mRNAs have been shown to perform an intracellular random motion slower than diffusion and named subdiffusion, that is, the mean displacement of mRNA molecules increases over time much more slowly as $\sqrt{t}$ with $\alpha < 1$ (it stands for the time) (Golding and Cox, 2006). Likely elements that underlie such behaviour are the random trapping and binding of the mRNA molecules with other molecules inside the crowded intracellular environment and, accordingly, smaller molecules such as proteins are expected to be less influenced (Elowitz et al., 1999; Golding and Cox, 2006). In addition, cytoskeletal dynamics in eukaryotic cells can elicit fast random molecular motions, in between diffusion and ballistic movement, called super-diffusive motions (Lau et al., 2003), that is, the mean squared displacement follows over time a power-law dynamic with an exponent greater than 1. Theoretical analysis evaluating the effect of subdiffusive random motions on the formation of gradients has started (Hornung et al., 2005). Future work is thus expected to elucidate how the random motion of proteins occurs extracellularly and within cells to form a morphogen gradient and how the gradient profile and dynamics depend on this motion.

In the last decade, driven by novel molecular data, new biological transport mechanisms for secreted morphogens that involve active processes have been proposed (for reviews, see Zhu and Scott, 2004; Strigini, 2005; Figure 3A). Vesicle-mediated transport mechanisms that can take place along the extracellular space have been shown (Greco et al., 2001; Panáková et al., 2005; Tanaka et al., 2005). Direct long-range interactions through long cellular protrusions have been suggested as well (Ramírez-Weber and Kornberg, 1999). In addition, transport through cells mediated by cycles of endocytosis and exocytosis (named ‘transcytosis’) has been proposed (reviewed by Vincent and Dubois, 2002; Gonzalez-Gaitan, 2003). Data on mosaic experiments in the fly’s wing, which set a patch of cells with impaired endocytosis near the Dpp morphogen source, raised the question of whether diffusion was the main mechanism of Dpp transport (Entchev et al., 2000). During gradient formation, the amount of Dpp morphogen decayed strongly behind the clone of cells (showing a so-called ‘shadow’), which suggested that the transport of Dpp requires endocytosis to reach those cells (Entchev et al., 2000). However, a mathematical and numerical analysis challenged this view (Lander et al., 2002). In the model that was formulated, Dpp transport was driven just by diffusion. Internalization and recycling of the free ligand and of bound receptors was considered, as well as degradation of these molecules inside cells. Impaired endocytosis was modelled as a reduction of the internalization rate and an increase of cell surface receptors. In this scenario, Dpp became trapped through its binding with the high amount of cell surface receptors and a shadow appeared behind the clone. Therefore, the appearance of ‘shadows’ could not be used to exclude diffusion as the transport mechanism (Lander et al., 2002). However, despite exhibiting a shadow, the morphogen
profile did not agree completely with the experimental data (Gonzalez-Gaitan, 2003). Another theoretical analysis formulated the same model, by extending it to two dimensions and setting a different parameter-dependent source of receptors and different boundary conditions, to name some of the changes. Impaired endocytosis was modelled as a reduction of the internalization rates, and the results showed that a natural strong and rapid accumulation of receptors occurred within the clone, which was essential to cause a shadow (Kruse et al., 2004). However, no such increase could be found experimentally, nor was the ligand profile totally consistent with mosaic data, pinpointing that diffusion could not be the single mechanism of Dpp transport (Kruse et al., 2004). Recent new theoretical modelling that takes into account Dpp transport through both transcytosis and diffusion at the scale of each cell has been able to obtain more proper ligand profiles as observed in mosaic experiments, even when the total amount of cell surface receptors is constant, supporting transcytosis as a mechanism for Dpp transport (Bollenbach et al., 2007). In addition to their relevance in addressing the issue of how Dpp is transported, all these studies exemplify how many challenges we also face from a theoretical point of view when trying to reject the plausibility of a mechanism.

The study of morphogen gradients has focused on secreted molecules, partially because these molecules can move extracellularly over large distances. However, molecular gradients along a cellular tissue can arise without requiring any dynamics on the extracellular space (Figure 3A). On the one hand, transport from cell to cell through gap junctions can occur (Esser et al., 2006). On the other hand, cells can be the transport vehicle of the molecule (Lecuit and Cohen, 1998; Tabata, 2001; Teleman et al., 2001). In the last years, experimental and theoretical evidence in favour of cellular-based transport mechanisms has been shown (Pfeiffer et al., 2000; Gaunt et al., 2003; Dubrulle and Pourquié, 2004; Ibañes et al., 2006). These kinds of transport enable the formation of gradients of non-secreted molecules. Two mechanisms, which can both take place for the same molecular gradient, have risen. In both cases, the source corresponds to a spatial region where cells divide and have the ability to produce the molecular component (e.g. the mRNA). Thus, when cells become placed outside this region, they cease to produce the molecule. If over time cells move or become displaced further away from the source while degrading their molecular content, a spatial gradient is formed (Gaunt et al., 2003; Dubrulle and Pourquié, 2004; Ibañes et al., 2006). In addition, molecular gradients can also be formed by the dilution of the molecular content on cells that continuously divide and become displaced away from the source, a mechanism termed cell-lineage transport (Ibañes et al., 2006).

Gradients of non-secreted molecules can in turn create graded distributions of secreted factors and other kinds of molecules. As indicated, gradients of mRNA provide a graded source for protein translation, which elicits a protein gradient (Dubrulle and Pourquié, 2004; Ibañes et al., 2006). In addition, gradients of non-secreted molecules can potentially convert a molecular uniform distribution into a morphogen or signalling gradient, by modulating its degradation or its transduction, respectively, on each cell. For instance, if the non-secreted molecule inhibits morphogen degradation, the morphogen will be less degraded in cells close to the source of the non-secreted molecule than in more distant cells. Thus, differential degradation along a field of cells could induce a morphogen gradient. Until now, differential degradation mediated by gradients of secreted molecules has been shown to shape and stabilize morphogen gradients. This is the case of the anterior-to-posterior gradient of retinoic acid in the developing nervous system of zebrafish embryos (White et al., 2007), which is shaped by a parallel gradient of the secreted factor Fgf8 that suppresses the degradation of retinoic acid by inhibiting the expression of the degrading enzyme Cyp26a1.

The formation of a single morphogen gradient can be driven by several of the transport mechanisms described so far, altogether enhancing the long-range transport of the morphogen. For instance, the FGF8 protein gradient from the tail bud to more anterior regions in vertebrate embryos might be driven by diffusion as well as by cell-based transport mechanisms. This is a very complex system that will require both challenging experimental and theoretical strategies to be fully characterized. As diffusion sets a much faster spatiotemporal dynamic than cell division in the tail bud region, increasing the degradation rate of Fgf8 to values in which tissue growth cannot drive protein transport could provide information on the range and rate of Fgf8 diffusion. Thus, theoretical predictions on the spatial range of the gradient based on diffusive transport alone could be compared with in vivo data. In addition, setting a framework that couples both transport mechanisms at the cellular level (i.e. at this scale, diffusive transport might be seen as a slave dynamic that quickly adapts to perturbations set by proliferating cells) could evaluate how single cells sense the gradient and act on it.

**Gradient dynamics**

Much work on morphogen gradients assumes that cells sense and interpret a steady morphogen concentration. Accordingly, focus has been set on modelling the formation of steady-state gradients. By analytically and numerically solving the gradient dynamics and the steady-state solution, it can be seen that the transient and steady-state gradients depend distinctly on the parameter values that characterize the transport, the degradation and the source rates (Figure 1C and D) and thus will respond to changes in a different manner (Bergmann et al., 2007; Lander, 2007). The different response transient and steady-state gradients will be relevant if we take into account that it is not always easy to know whether a morphogen gradient has reached its steady state in vivo (Bergmann et al., 2007, 2008; Gregor et al., 2007b; Bialek et al., 2008).

As ultimately cells sense the morphogen and interpret it accordingly, it is interesting to know how cells see the gradient over time. In addition, not all cells may respond simultaneously to the gradient, but alternatively each cell (or groups of cells) may respond to the gradient at a different time, as it has been described for BMP signalling (Tucker et al., 2008). While a gradient driven by diffusion or other molecular transport mechanism is being formed in a static field of cells, the cells are exposed to increasing levels of morphogen over time. Cells located close to the source are the ones to first sense the morphogen and to experience higher morphogen levels.
than cells located at farther distances. Once the steady state has been reached, cells sense a constant amount of morphogen, which depends on where the cell is located. But even if the gradient is in a steady state, its activity can be dynamical and transient. This is the case of the activity of Sonic hedgehog (a vertebrate homologue of invertebrate hedgehog) in vertebrate neural cells, which lasts a time period proportional to the amount of morphogen (Dessaud et al., 2007).

Gradients driven by cell-based transport involve a dynamic field of cells. In this case, the molecular content inside cells does not reach a steady state and cells sense a decreasing molecular amount over time, which is related to their increasing distance from the source (Ibanes et al., 2006). Therefore, the way cells sense the gradient and thus can subsequently interpret it strongly depends on the mechanism of gradient formation and signalling.

### Gradient interpretation is not just the final step

Three main questions are involved in gradient interpretation: which is the graded information that is interpreted? How does this interpretation occur? And what does this graded information specify? Over the last few decades, several groups have started addressing these questions, uncovering further complexities involved in morphogen gradients.

The study of different morphogens has shown that the graded information (or signal) that is interpreted may depend on the specific morphogen gradient. Thus, the graded signal can be the steady-state amount of morphogen around a cell, which might be measured by the number of bound receptors or, alternatively, by the ratio of bound to unbound receptors (Dyson and Gurdon, 1998; Gurdon and Bourillot, 2001; Casali and Struhl, 2004). It could also be the amount of morphogen in a transient dynamic state (Bergmann et al., 2007). Another option is that the graded signal that is interpreted is not the level of morphogen but instead it is the steepness of the gradient (Lawrence, 2001; Rogulja and Irvine, 2005). Alternatively, the signal that is interpreted could be the total amount of morphogen cells have been exposed to over time (Ahn and Joyner, 2004; Harfe et al., 2004; McGlenn and Tabin, 2006; Tarchini and Duboule, 2006; Tabin and Wolpert, 2007). Moreover, the morphogen level could be transduced into a graded time period of activity that is interpreted (Dessaud et al., 2007).

Experimental evidence indicates that morphogen gradients can direct more than two different cell fates. Commonly, the specification of distinct fates has been addressed in terms of the induction of downstream genes (for review, see Ashe and Briscoe, 2006). Accordingly, distinct cellular responses are characterized by which targets are active (Figures 1A and 4D). The graded signal coming from the morphogen is thus converted into roughly binary responses of each target, defining sharp response borders (Figure 4B and D). Different

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**Figure 4** Morphogen gradient interpretation. (A) The morphogen gradient elicits a signal (S, in blue) to which a cell (orange circle) responds. The signal induces (blue arrows) the expression of targets X, Y and Z. These targets have different sensitivity (denoted by open squared boxes) to the same signal S. X is weakly sensitive to S, Y is mildly sensitive and Z is very sensitive. (B) Binary response of target genes X, Y and Z to signal S. Low levels of S activate only Z, medium levels activate both Z and Y, and high levels activate all targets. (C) The signal induces the expression of targets X, Y and Z, which here have the same sensitivity but interact with each other. An example of plausible cell-autonomous interactions is depicted, in which Z represses both Y and X, and Y represses X (repression is shown by curves with line-end; arrows indicate induction). In this case, to elicit different responses along a gradient, different sensitivities are not required, but could also be participating. As X is repressed by Y and Z, the overall signal it perceives is smaller than the signal Y perceives, which, in turn, is smaller than the signal Z perceives. Thus, the binary response of the target genes to different values of signal S is also shown in (B). (D) X, Y and Z binary response (lines) to a graded signal (S, blue triangle) along a field of cells (orange circles). Three different spatial regions and fates are induced, which are characterized by those genes that are expressed. Expression inside cells is denoted by a coloured rectangle (violet for X, red for Y and orange for Z).
mechanisms ranging from positive feedback, cooperativity and zero-order ultrasensitivity have been shown to convert a graded signal into a binary response (for reviews, see Ferrell, 2002; Ashe and Briscoe, 2006). Theoretical approaches have been important to propose and characterize these mechanisms for switch-like behaviour by analysing the steady states of a specific cell-autonomous dynamic under an external input stimulus (Box 3).

Box 3 How to infer the mechanism of gradient interpretation? An example of a procedure

To address gradient interpretation, the morphogen gradient or its targets need to be altered and the shifts on cell fate and on downstream targets that are elicited accordingly need to be measured. The extent of the shift (both in space and time) will depend crucially on the mechanism of gradient interpretation taking place. Thus, data on the actual shifts can be used to potentially discard scenarios of gradient interpretation. An illustration of these concepts is provided by the study of how a continuous gradient can be converted into sharp developmental domains in Drosophila embryos (Melen et al., 2005).

The ventral ectoderm of Drosophila embryos is patterned by a gradient of the secreted ligand Spitz, a TGF-β homologue. Spitz is secreted by a single row of glial cells at the midline. On binding to epidermal growth factor receptor (EGFR), it activates mitogen-activated protein kinase (MAPK), inducing a gradient of EGFR activation and MAPK over five rows of cells on either side of the midline. MAPK in turn regulates several target genes. Specifically, it controls the degradation of the transcriptional repressor Yan. As a result, Yan protein is absent from the two cell rows that are closest to the midline and it is found at a uniform level on the remaining cell rows. Therefore, the gradient of MAPK is converted into a binary response of Yan protein levels (Melen et al., 2005). To decipher how this response is elicited, Melen et al. (2005) constructed a mathematical model that could analyse three mechanisms of sharp graded-to-binary conversion: cooperativity, positive feedback and zero-order ultrasensitivity (indeed, the model studied first-order conversion as well, which elicits more gradual responses and which we do not detail herein for simplicity). In the model, the amount of MAPK is the signal that is interpreted and it triggers a cell-autonomous dynamic that facilitates Yan degradation through its MAPK-mediated phosphorylation. By numerically finding the steady state of Yan expression in a single cell for different levels of MAPK, it was found that cooperativity (by multiple MAPK bindings), positive feedback (elicited when Yan dephosphorylation rate decreases with the level of phosphorylated Yan) and zero-order ultrasensitivity (the levels of MAPK and phosphatase enzyme are limiting whereas the substrate Yan is in excess) all elicit a sharp binary response, and a similar threshold of MAPK activity can be defined below which Yan is expressed. Therefore, by looking just at the wild-type Yan expression, none of the three mechanisms could be discarded and all seemed plausible. However, numerical analysis of the dynamics and steady state of Yan levels showed that responses to Yan overexpression are specific for each mechanism. Thus, when Yan is overexpressed, a shift towards the midline of steady-state Yan expression occurs if positive feedback is controlling the conversion. In contrast, no shifts are elicited when cooperative and zero-order ultrasensitivity mechanisms do the interpretation. In addition, the time to reach the steady state increases strongly, such that transient ectopic Yan expression in all cells should be noticeable only if zero-order ultrasensitivity is driving the process. Therefore, these analyses revealed that the actual mechanism of gradient interpretation could be deciphered if Yan was ectopically expressed and the transient and steady state patterns of Yan expression were measured. In vivo ectopic expression of Yan in Drosophila embryos showed no shift of Yan expression at long times (discarding positive feedback), and a long transient distribution of exogenous Yan. Therefore, these data on spatial and temporal shifts of Yan expression pointed to zero-order ultrasensitivity to be the mechanism converting MAPK graded signal into a Yan degradation binary response (Melen et al., 2005).

How does the morphogen gradient specify several distinct fates? On the one hand, the targets of the morphogen signal can be characterized by different levels of sensitivity (Figure 4A). This sensitivity can correspond to the affinities of the target genes to morphogen signal-binding sites. Each target will respond above a different threshold signal, which will depend on the affinity of the morphogen-binding site (i.e. those targets that exhibit low affinity will respond to only high levels of the signal, whereas those with high affinity will respond to lower levels of the signal; see Figure 4B). Thus, the level of morphogen signal will select which targets are induced, and a pattern over space of different activated targets will be elicited (Figure 4D). On the other hand, the morphogen signal can similarly activate or repress several targets, which, as a result of their interaction within a network, respond distinctly to the same signal (Figure 4B and C). Accordingly, for a graded signal over space, a pattern of different active targets will arise (Figure 4D). In this case, although the sensitivity of the targets might be the same, the response will differ depending on the amount of signal. Note that a huge landscape of different network topologies and dynamics can be envisaged that could elicit many diverse patterns. Importantly, the two strategies being described can be acting at the same time on a single morphogen to direct a response. Indeed, both mechanisms have been reported for the Bicoid gradient. The number and affinity of Bicoid-binding sites provide a different level of sensitivity to the Bicoid signal (Driever et al., 1989c; Struhl et al., 1989; Gao et al., 1996). In addition, downstream target gap genes, activated by Bicoid, repress each other and thus also interpret the gradient through their interaction (Jaeger et al., 2004; Jaeger and Reinitz, 2006; Bergmann et al., 2007). However, in addition, a bioinformatic analysis has found out that the combination of several additional activators that bind the promoter regions of Bicoid target genes can be relevant to control and specify the Bicoid gradient interpretation (Ochoa-Espinosa et al., 2005).

Distinct patterns of gene expression between cells are expected to elicit ultimately different cellular behaviours. Specifically, the Dpp gradient in the wing of Drosophila embryos has been shown to control cell proliferation (Rogulja and Irvine, 2005). In addition, the FGF8 gradient in the presomitic mesoderm of chick embryos is translated into a gradient of extracellular signal-regulated kinase (ERK), which promotes cell migration (Delfini et al., 2005). Can this induced cell behaviour in turn alter the morphogen gradient, setting a feedback between gradient formation and interpretation? Potentially yes, as cell dynamics can shape morphogen gradients (Dubrulle and Pourquié, 2004; Ibañes et al., 2006). Indeed, cell dynamics are essential to create the mRNA fgf8 gradient (Dubrulle and Pourquié, 2004). Thus, these cell dynamics can have an important role in shaping the protein and ERK signalling gradient as well. The relevance of such feedback, according to the timescale of gradient interpretation, cellular response and gradient formation, will require a careful evaluation aided by theoretical approaches. Moreover, molecular feedbacks between gradient signalling and formation (such as those eliciting enhanced degradation, as previously discussed) have been uncovered (Lander, 2007), which stress, as well, the importance of evaluating the process of formation and interpretation altogether as a whole system.
Future perspectives: a systems biology approach for morphogen gradients

Morphogen gradients have been an extremely useful framework to characterize pattern formation processes in developing embryos. Despite their apparent simplicity, novel data on morphogen gradients are stressing and expanding the complexities that this framework involves. Thus, morphogen gradients exemplify most of the complexities we have to tackle when studying embryonic development: the interplay between several and different levels of organization, time and spatial scales and components. Morphogen gradients pose two advantages, that is, the thorough knowledge we have acquired in the last decades on this topic and the additional knowledge we gain each time we use theoretical and computational strategies combined with experimental approaches to decipher their features.

New data have shown cell-based mechanisms for the transport of molecules that allow the formation of gradients of non-secreted molecules. If such gradients are shown to shape graded information to which cells respond, morphogens should be extended to include non-secreted molecules. In addition, these and other data have highlighted that knowing the dynamics of gradients, and not only their steady state, is becoming increasingly important. At present, we still know very little about how the dynamics of gradients participate in the overall process of gradient interpretation. To shed some light on this issue, mathematical approaches can be extremely useful by predicting the kind of response we might expect. In most cases, the shape and dynamics of morphogen gradient formation are analysed independent of gradient interpretation. Thus, gradient interpretation has been commonly neglected when studying gradient formation. However, some of the findings we have highlighted herein emphasize a very important issue that has been largely avoided: the gradient can depend on the response it elicits. As the response is directed by the gradient, a feedback between gradient and response can be present. At the molecular level, such feedback has already been taken into account: morphogen signalling can feedback to receptor or ligand production, modifying the profile of the gradient (Lander, 2007). However, such feedback does not necessarily occur just at the molecular level but can also involve the cell dynamics. We have seen that morphogen gradients can induce changes in the dynamics of cells, activating their proliferative and migratory state (Delfini et al., 2005; Rogulja and Irvine, 2005). As cell proliferation and spatiotemporal cell displacement can shape a gradient (Ibañes et al., 2006), such a cellular response can modify the gradient in return. Thus, as the responses at both the molecular and cellular levels can shape the morphogen gradient, it becomes necessary to study gradient formation and interpretation processes together (Figure 5).

Therefore, understanding pattern formation through morphogen gradients can require integrative approaches that take both gradient formation and interpretation into account in a common framework. Hence, we suggest system-level perspectives on morphogen gradients that address the problem of pattern formation from gradient formation to gradient interpretation and vice versa. Moreover, by defining the morphogen system as the molecules forming the gradient, the graded signal and the interpretation dynamics, new perspectives will be gained. Issues such as finding those reliable dynamics of the morphogen system, and how this reliability and performance is achieved can uncover vital insights (Eldar et al., 2003; Lander, 2007). By implicitly neglecting such feedback, we reduce the range of possible designs enabling morphogen reliability: the morphogen gradient itself is very reliable and such robustness is preserved during gradient interpretation; alternatively, the process of gradient interpretation is responsible for setting a reliable response from a variable graded input. Yet, a system approach will broaden the scope and another plausible answer could arise: reliability depends on the interaction between formation and interpretation dynamics.

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