Multiple Paths to Morphogen Gradient Robustness

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Summary

Much recent attention has focused on the robustness of morphogen gradients, i.e. the ability of such gradients to resist change in the face of genetic and environmental perturbations. It has been suggested that some of the complex regulatory interactions found in morphogen gradients—feedback control of morphogen responsiveness, countergradients of secreted inhibitors, etc.—exist to enhance robustness, but how this occurs is only partly understood. Here we identify two strategies that make gradients robust to changes in morphogen synthesis rate. Both utilize non-receptor cell surface molecules to mediate a substantial fraction of morphogen degradation. One exploits the non-linear, i.e. saturable, nature of binding of morphogens to receptors; the other takes advantage of feedback inhibition of receptor synthesis by morphogen signaling. Such strategies may play a role in the Decapentaplegic gradient in the *Drosophila* wing disc, in which just such feedback is present, and in which cell surface non-receptor molecules (e.g. proteoglycans) are known to regulate gradient shape.

Introduction

Highly patterned arrangements of organs and tissues arise during development because groups of cells adopt different fates depending on the their precise location in space. This process is commonly orchestrated by secreted signaling molecules known as morphogens. Morphogens diffuse (or, as proposed by some investigators, are actively transported) randomly away from their site of production to form spatial gradients. At different distances, cells experience different levels of occupancy of morphogen receptors. The resulting graded differences in signaling ultimately give rise sharp differences in gene expression, from which follow stable differences in cell fate.

While the strategy of using morphogen gradients to create patterns is simple, implementing it poses challenges. For example, the tendency of receptors not only to signal but also degrade their ligands creates a need for morphogen receptors that have slow association kinetics, and are expressed at low levels (Lander et al., 2002). Recent studies have pointed out that some of the simplest strategies for generating morphogen gradients yield gradients that are not particularly robust: small changes in parameters—e.g. the levels of expression of morphogens or receptors—cause large shifts in gradient shape. In contrast, embryonic patterning is usually highly robust, resisting not only substantial changes (e.g. twofold) in the expression level of individual genes, but also fluctuating environmental conditions (e.g. varying temperature). Several groups have sought to identify ways of producing morphogen gradients that possess such robustness, applying both experimental and theoretical approaches to this task (Eldar et al., 2002; Eldar et al., 2003; Eldar et al., 2004; Houchmandzadeh et al., 2002; Ingolia, 2004; von Dassow et al., 2000; Von Dassow and Odell, 2002).

In many morphogen gradients, the amount of signal a cell receives influences the amount of morphogen receptor it makes. One group has proposed that this type of feedback effect can make gradients robust to changes in morphogen synthesis rate, but only if the feedback causes increased morphogen degradation (Eldar et al., 2003). To illustrate their point, they modeled the gradients formed by two of the morphogens that

operate in the Drosophila wing imaginal disc, Hedgehog (Hh) and Wingless (Wg). Hh signaling induces expression of the Hh receptor Ptc, which drives Hh degradation. In contrast, Wg represses expression of its receptor DFz2, but signaling mediated by DFz2 leads to stabilization, rather than degradation, of Wg. Thus, even though Wg and Hh have opposite effects on the synthesis of their receptors, they have similar effects on their own degradation. Accordingly, simulations predicted that both Wg and Hh gradients should be more robust to changes in morphogen synthesis than gradients in which receptor synthesis is not under feedback control.

The *Drosophila* wing imaginal disc is patterned by a third morphogen gradient, formed by Dpp, a member of the bone morphogenetic protein branch of the transforming growth factor-β superfamily. Like Wg, Dpp represses synthesis of its receptors. However, unlike Wg receptors, Dpp receptors enhance Dpp destruction, a point illustrated by the opposite effects of receptor overexpression, in Dpp vs. Wg gradients, on morphogen range of action (Cadigan et al., 1998; Lecuit and Cohen, 1998). This led us to wonder whether there might be mechanisms for achieving morphogen gradient robustness other than the one considered by Eldar et al. (2003). Here we show this to be the case: Provided that morphogens are bound and degraded not only by receptors, but also by non-signaling cell surface molecules ("non-receptors"), robustness can be achieved through either of two additional strategies: one requires no regulation of receptor expression, the other requires feedback repression of receptor expression. In neither case need morphogens enhance their own degradation. That non-receptor molecules can be key to several robustness strategies may explain, in part, growing evidence that molecules such as cell surface heparan sulfate proteoglycans play a major role in morphogen gradient formation.

Results

We treat the morphogen gradients in Drosophila imaginal discs as an instance of reaction and diffusion in one-dimension; this works well given that the wing disc is largely two-dimensional and morphogens in the disc tend to be synthesized by extended linear or rectangular cell arrays (XX). We differ from previous work (e.g. Eldar et al. 2003) in three significant ways: First, we explicitly include a region of morphogen production, allowing morphogen-producing cells to have morphogen receptors and responses (as is clearly the case for Dpp the wing disc [Fujise et al., 2003; Funakoshi et al., 2001]). Second, we include the possibility that cell surface molecules other than receptors might bind morphogens and mediate, in part, their internalization and degradation. Such molecules—which for brevity we simply call "non-receptors"—are certainly present in wing discs, the best studied being the heparan sulfate proteoglycans. Experiments show that changes in non-receptor expression can have dramatic effects on morphogen gradients (Baeg and Perrimon, 2000; Baeg et al., 2004; Belenkaya et al., 2004; Bornemann et al., 2004; Fujise et al., 2003; Han et al., 2004; Kirkpatrick et al., 2004; Kreuger et al., 2004; Ohkawara et al., 2002; Strigini and Cohen, 2000; The et al., 1999). Third, we explicitly include the production of receptors and non-receptors, and the formation, dissociation, endocytosis, recycling, and degradation of morphogen-receptor complexes as discrete events controlled by appropriate rate equations. Among other

things, this allows us to deal with the nonlinear, i.e. saturable, nature of morphogen-receptor binding. We shall see that is essential for obtaining correct results.

Figure 1 outlines a minimal set of reactions meant to capture the interactions among morphogens, receptors and non-receptors that might occur in a Drosophila wing disc. We can break out subsets of these reactions into individual models of increasing complexity. For example, Model 1 ("basic model") includes only the interactions of morphogens with receptors (black symbols), neglecting non-receptors and regulated receptor synthesis. Model 2 ("R-feedback") adds feedback regulation of receptor synthesis (shown in red) to model 1. Here we shall consider only negative feedback, as occurs in the Dpp and Wg gradients in the wing disc (reviewed by Cadigan, 2002). Model 3 ("non-receptors") includes morphogen interactions with non-receptors (shown in blue) but not regulated receptor expression. Like receptors, non-receptors are allowed to internalize and undergo degradation both when bound to morphogen and when free. Model 4 ("non-receptors + R-feedback") combines the elements of Models 2 and 3. Model 5 ("non-receptors, R- and N-feedback") adds to Model 4 by allowing morphogen signaling to repress the synthesis of non-receptors (shown in green). This addition incorporates the findings of Fujise et al. (Fujise et al., 2003) showing, in the wing disc, that Dpp signaling downregulates not only the Dpp receptor (*tkv*) but also *dally*, a major proteoglycan non-receptor for Dpp.

We explored the steady state solutions of these five models by random parameter set searches: Setting time rates to zero, the equations for each model were reduced to their simplest forms. After identifying a minimal set of parameters that determine the solutions, we specified broad ranges for each parameter, covering what appeared to be all biologically plausible values (see Appendix). Finite difference methods were used to calculate gradient shapes for $>10^6$ random parameter sets for each model. We then selected for further analysis those solutions that corresponded to gradients with generally appropriate sizes and shapes for the wing disc Dpp gradient (see Methods). These represented between 3 and 9% of the solutions, depending on the model (Table 1)

For all such gradients, we calculated the sensitivity of steady state gradient shape to the rate of morphogen production. This was done by doubling the rate of morphogen synthesis, recalculating the solution, and measuring the average amount by which the gradient shifted (see Appendix). This was normalized to the distance over which the initial gradient fell to 20% of its starting value, producing a unitless number we refer to as the induced relative error, "*E*." The lower the value of *E*, the more robust the gradient, with $E=0$ representing perfect robustness.

Non-receptors and feedback enhance gradient robustness

The frequency of occurrence of gradients with different *E*-values is shown for each of the models in Figure 2, and summarized in Table 1. In models 1 and 2, *E* is frequently in the range of 0.43-0.45, almost never falling below that value. The median *E*-value for model 2 is slightly higher than for model 1, implying that the addition of R-feedback to the basic model tends to decrease the robustness of the solutions.

In the three remaining models, many cases have E <0.43 (Fig. 2, Table 1). For example, in model 3 (non-receptors), 42.8% of gradients meet this criterion, i.e. are more robust than those produced by the basic model. There are even a reasonable number of cases for which E < 0.2, i.e. a doubling in morphogen synthesis causes a shift in patterning of <20% of the size of the overall patterned region. Apparently, simply adding non-receptors into the basic model allows highly robust gradients to be produced.

In model 4 (non-receptors, R-feedback), robust cases are even more numerous (nearly 50% have E<0.43). Thus, robustness is also enhanced by feedback regulation of receptor synthesis but, interestingly, only if non-receptors are present. In model 5, the distribution of E-values is much like that in model 4, with a slight shift toward higher values. Thus, feedback regulation of non-receptor synthesis does not further enhance robustness, and to some extent, diminishes it.

Origins of robustness due to non-receptors

Comparing solutions obtained from large numbers of random parameter sets can be a powerful tool for identifying general, qualitative features of a model's output (XX). However, one needs to interpret such data carefully, particularly when generous parameter ranges force one to explore large amounts of parameter space that may not necessarily be relevant. Accordingly, it is always desirable to use analytical methods to justify the validity and generality of results obtained from numerical simulations such as those described above.

General analytical solutions cannot be obtained (at least not useful ones) for any of the models in Fig. 1, but under appropriate conditions, good approximate solutions can be found. For example, if the concentration of free morphogen is sufficiently low that the degree of saturation of receptors is low at every point in the gradient, binding can be treated as a linear process, and the concentration of occupied receptors as a function of distance from the morphogen source can be shown to equal $LR_0e^{-x\Lambda}$, where Λ is a decay length constant, and LR_0—the value of morphogen receptor occupancy at x=0 (the boundary where morphogen production leaves off and the gradient can be considered to "start")—is given by an expression proportional to v, the rate of morphogen synthesis. It is straightforward to show (see Appendix) that E-values for all such gradients should equal $ln2/ln5 \approx 0.43$, regardless of the values of LR_0 and Λ.

The necessary and sufficient condition for low receptor saturation is $[L]<K_m\eta_R$, where [L] is the concentration of free morphogen, K_m is a modified receptor affinity constant, and η_R is a parameter that captures the degree to which bound receptors are degraded more or less quickly than free ones. In Figure 3a, we observe the relationship, for model 1, between E-values that were calculated from random parameter sets, and $L_0/(K_m\eta_R)$, where L_0 is the value of [L] at x=0. Notice that, for $L_0<<K_m\eta_R$, all E -values converge upon a single value of ≈ 0.43. Fig. 3b illustrates the behavior of a representative solution to Model 1, in which receptor saturation is low. Clearly E=0.43 corresponds to a rather substantial change in gradient shape in response to a two-fold change in morphogen synthesis.

We also see in Fig. 3a that, as receptor saturation becomes significant ($L_0 > K_m\eta_R$), gradients always become less robust ($E > 0.43$). Figure 3c illustrates the behavior of such a solution to Model 1. Note the sigmoidal gradient shape that results when receptor saturation is relatively high. Obtaining a good approximate analytical solution for such cases is difficult, but analysis suggests that their E-values always exceed $ln2/ln5$ by a factor that, for large enough v, is proportional to v (see Appendix). Thus, much of the behavior of the basic model can be explained by a fairly simple set of relationships between morphogen production and the degree to which receptors are saturated.

In Figure 3d, numerical solutions of model 2 (R-feedback) were analyzed in the same fashion as was done in Fig. 3a. We see that introduction of feedback does not alter the fact that optimum robustness requires $L_0 << K_m\eta_R$, and E is never lower than $ln2/ln5$. What it does alter is that it is no longer true that all gradients with very low receptor saturation have near-optimum robustness (arrow). In effect, feedback of the type used in model 2 provides only the opportunity to be less robust, not more.

In Fig. 4a, we analyze model 3 (non-receptors) in the same fashion as models 1 and 2, relating robustness to receptor saturation. Immediately we see a new trend: There are now many cases with $E < 0.43$ (arrow), and all of these have high levels of receptor saturation ($L_0 > K_m\eta_R$). However, not all gradients with high receptor saturation are unusually robust. Some are even less robust than the least robust cases in models 1-2 (arrowhead). One can separate out these groups to some extent, by looking at levels of saturation of the *non-receptors*. The necessary and sufficient condition for non-receptor saturation is $[L] > J_m\eta_N$, where J_m and η_N are defined analogously to $K_m$and η_R. In Fig. 4a, the data points have been colored so that those with substantial non-receptor saturation ($L_0 > J_m\eta_N$) are in red, with the remainder green. The results show that low non-receptor saturation is a necessary condition for strong robustness. Similar information is provided by Fig. 4b, in which E values are plotted as a function of $L_0/(J_m\eta_N)$ and points are colored according to their degree of receptor saturation.

Although the combination of high receptor saturation and low non-receptor saturation is a necessary condition for good robustness in model 3, it is not a sufficient one, as some gradients in Fig. 4a-b fit both criteria but are poorly robust (arrow in Fig. 4B). Therefore we next examined an additional characteristic of each gradient, the relative contribution of receptors *vs*. non-receptors to the total degradative flux, i.e. the total rate at which morphogens are degraded at any point in space. The total degradative flux may be expressed as the sum of the degradative flux through receptors (Φ_R) and that through non-receptors (Φ_N), each being the product of an effective degradation rate constant and the concentration of occupied receptors or non-receptors, respectively (see Appendix). As shown in Figure 4c, in which four colors are used to identify those gradients with different degrees of receptor and non-receptor saturation, there is a direct, nearly linear relationship between the E-value and the fraction of total degradative flux at x=0 that is mediated by receptors. In effect, all the highly robust gradients have very low Φ_R/Φ_{total} (arrow).

Knowing these pre-conditions for robustness facilitates the analysis of Model 3. As long as $[L] << J_m\eta_N$ and $\Phi_R << \Phi_{total}$, a very good approximate solution for Model 3 (see Appendix) yields the relationship

$$E = ln2/ln[5 + \frac{4L_0}{K_m\eta_R}],$$

where L_0, equal to [L] at x=0, is given by

$$L_0 = \frac{vJ_m}{j_{degobs}\theta_N N_0(1+\frac{coth(\sqrt{\theta_N}d_0\Lambda_N)}{\sqrt{\theta_N}})}$$

Note that, for $L_0 << K_m\eta_R$, E approaches $ln2/ln5$, our earlier result for cases without non-receptors. For cases with $L_0 > K_m\eta_R$, figure 4d shows that there is good agreement between the numerical solutions and the analytical result. Fig. 4e depicts the behavior of a particular robust solution to model 3.

The preceding analysis tells us that, in principle, provided that non-receptors dominate the process of morphogen degradation and receptors are largely saturated at the start of such gradients, non-receptors can make gradients arbitrarily robust to variation in morphogen synthesis. However, if one constrains the field of cells patterned by a gradient to a maximum width, then achieving ever greater robustness requires gradients to consist of ever longer regions of full receptor occupancy followed by ever shorter regions of rapid decline in occupancy. While such gradients can easily specify a single location in space, their steep declines make it difficult to specify more than one. Thus, a need to specify multiple states of gene expression would place practical limits on the ability of the above mechanism to provide robustness.

Origins of robustness due to receptor feedback

When added to the basic model, feedback repression of receptor synthesis only decreases robustness, but when added to the model with non-receptors, the number and proportion of robust gradients increases (Fig. 2). Here we seek to understand the origin of this effect.

In Figure 5a-c we plot E-values for model 4 (non-receptors, R-feedback) as a function of $L_0/(K_m\eta_R)$, $L_0/(J_m\eta_N)$, and Φ_R/Φ_{total} at x=0. As with model 3, low non-receptor saturation and a low contribution of receptors to total degradative flux appear to be necessary conditions for robustness. In contrast to Model 3, however, robust gradients are no longer limited to cases of high receptor saturation, with many robust gradients now having values of $L_0/(K_m\eta_R)$ that are low (arrows, Fig. 5a, c).

As with model 3, the conditions $L_0 << J_m\eta_N$ and $\Phi_R << \Phi_{total}$ allow us to derive an analytical expression for morphogen receptor occupancy, and from this to estimate E-values (see Appendix). For those cases with low receptor saturation, it is straightforward to show that maximum robustness should occur when feedback is potent (maximal morphogen

signaling suppresses receptor synthesis to a small fraction of its maximal level), and appropriately sensitive (the amount of morphogen receptor occupancy required to half-maximally suppress receptor synthesis is on the order of the amount of receptor occupancy that occurs a x=0). This relationship can also be revealed in the numerical solutions by plotting E-values against the ratio of receptor synthesis (ω_R) at x=0 to receptor synthesis in the absence of feedback (ω_{Rmax}). As shown in Fig. 5d, for those cases with low saturation of both receptors and non-receptors ($L_0<K_m\eta_R$, $L_0<J_m\eta_N$; green dots), lower E-values require small ω_R/ω_{Rmax}, with the lowest E-values for such cases being around 0.21.

Assuming that $L_0<< J_m\eta_N$, $L_0<< K_m\eta_R$ and $\Phi_R<<\Phi_{total}$, we can show that

$$E = ln2/(2ln5 - lnQ)$$

where Q stands for $\dfrac{100+5\zeta(5\rho_R-1)}{10-5\zeta-3\zeta\rho_R-2\zeta^2\rho_R{}^2+(10+2\zeta\rho_R)\sqrt{4\zeta+(\zeta\rho_R-1)^2}}$.

Here ρ_R is the ratio of the minimum receptor synthesis rate (i.e. the rate when morphogen signaling is arbitrarily high) to its maximum rate, while $\zeta = L_0R_0\gamma_{mod}/K_m$, with L_0 defined as above, and γ_{mod} a parameter proportional to feedback sensitivity (see Appendix). Q varies between 1 and 5 for all possible ρ_R and ζ, causing E to vary between $ln2/(2ln5) \approx 0.215$ and $ln2/ln5 \approx 0.43$.

According to this expression, E is smallest when Q is minimized, which tends to occur when ρ_R is small (i.e. when feedback is potent). If ρ_R=0, then as ζ grows (i.e. the more sensitive the feedback), Q approaches a minimum asymptotically; for nonzero ρ_R however, Q is minimized when $\zeta=\dfrac{5-4\sqrt{5\rho_R}-5\rho_R}{\rho_R-5\rho_R{}^2}$. In Figure 5e, E is plotted as a function of $ln2/(2ln5 - lnQ)$ for all gradients in Model 4 for which $\Phi_R/\Phi_{TOT}<0.1$ and $L_0<J_m\eta_N$, with color-coding to distinguish among those with different ranges of $L_0/(K_m\eta_R)$. One can see excellent agreement between the results of simulation and analysis. Fig. 5F depicts the behavior of a typical robust solution to model 4.

Robustness and the effects of morphogens on net morphogen degradation.
Because $\Phi_R<<\Phi_{TOT}$ is required for robustness in Model 4, it follows that, in the most robust gradients, feedback regulation has little impact on the overall rate of morphogen degradation. We wondered whether this was essential for robustness, or simply a consequence of the fact that, in Model 4, only receptors are subject to feedback regulation.

We therefore turned to the analysis of model 5, in which both receptor and non-receptor synthesis are suppressed by morphogen signaling. This model produces slightly fewer robust gradients than model 4 (Fig. 2, Table 1), suggesting that feedback control of non-receptor synthesis does not make available any additional strategies for enhancement of robustness. Indeed, an analysis of the individual cases in model 5 (Fig. 6a-d) shows that

the conditions necessary for robustness—small Φ_R/Φ_{tot}, small $L_0/J_m\eta_N$, and large $L_0/K_m\eta_R$; or small Φ_R/Φ_{tot}, small $L_0/J_m\eta_N$, small $L_0/K_m\eta_R$ and potent receptor feedback—are the same as in model 4. In addition, one observes that a majority of robust cases exhibit only minimal suppression of non-receptor synthesis by morphogen signaling (i.e. for most robust cases, ω_N/ω_{Nmax} is close to 1; Fig. 6e).

In other words, selecting for robustness selects against strong non-receptor feedback. Nevertheless, among both cases with small and large $L_0/K_m\eta_R$, one can find many gradients in which non-receptor synthesis is suppressed strongly ($\omega_N/\omega_{Nmax}<<1$), yet the *E*-values are very low. An example of such a case is shown in Fig. 6f.

Given that $\omega_N/\omega_{Nmax} \approx 1$ is not a requirement for robustness, there must be robust cases in which Φ_{tot} is substantially decreased by morphogen signaling (for example, this is true for the case in Fig. 6f). Accordingly, robustness can be achieved even when morphogen signaling suppresses net morphogen degradation.

Robustness strategies and the dynamics of gradient formation.
The results described so far come from numerical simulation or analysis of gradients under steady state conditions. For wing disc morphogen gradients this is a reasonable approach, as such gradients appear to be stable for days. However, experimental data suggest that the dynamics of formation of such gradients is moderately fast, requiring about 7-10 hours in the case of Dpp (Teleman and Cohen, 2000). Thus, for any of the mechanisms for achieving robustness that have been described here to be biologically relevant, they must be compatible with gradient formation on such a time scale.

It was not computationally practicable to generate time-dependent numerical solutions for tens of thousands of random parameter sets. However, we were able to examine selected robust cases from each of the models. As shown in Figure 7a-c, using parameters that generally lie within the middle of the ranges that were explored for the steady state solutions, examples can be found in which robustness may be enhanced by non-receptors alone (with $L_0> K_m\eta_R$), or non-receptors and feedback (with $L_0< K_m\eta_R$), and in which gradients of receptor occupancy reach nearly their final form within 7-10 hours.

A full dynamic analysis of each model is beyond the scope of the present study, but if we assume that the condition $\Phi_R<<\Phi_{tot}$ holds at all times (which should be true in many cases), then the rate at which free and non-receptor-bound morphogen approach steady state can be estimated (see XX), and is largely dictated by the rate constant of non-receptor-mediated degradation, $j_{degobs}+j_{off}$. One may obtain an upper bound on the rate of approach of the gradient of receptor-bound morphogen to steady state, by treating gradient formation as a two stage process: a first stage in which a fixed gradient of free morphogen forms (with no receptor binding), followed by a stage in which free morphogen binds receptors. The rate constant for the second stage will be slowest at the lowest levels of free morphogen, where it should asymptotically approach $k_{degobs}+k_{off}$. Thus, an overestimate of the time for a receptor occupancy gradient to evolve half way to steady state should be:

$$ln2\left(\frac{1}{j_{degobs}+j_{off}}+\frac{1}{k_{degobs}+k_{off}}\right)$$

In Fig. 7d, *E*-values have been plotted against this parameter (expressed in units of hours) for Model 4, with color coding use to distinguish among cases with different degrees of receptor- and non-receptor saturation. The results support the assertion that the robustness strategies described here do not generally interfere with rapid gradient formation.

In fact, the simulation results in Fig. 7c suggest that feedback inhibition of receptor synthesis can accelerate gradient formation. Because receptor levels are higher at early times (before feedback alters their synthesis) than at later ones, they accumulate morphogen rapidly, even overshooting the levels they will ultimately attain. This effect—negative feedback increasing the rate of approach of a system to steady state—is a well-known principle in control theory (Franklin et al., 1994). In morphogen gradients, it is the condition $\Phi_R << \Phi_{tot}$ that allows high receptor levels to drive faster gradient evolution without simultaneously hindering net morphogen diffusion. Since the condition $\Phi_R << \Phi_{tot}$ depends upon a degradative pathway for morphogen that bypasses receptors, we infer that the combination of non-receptors with feedback not only enhances gradient robustness, it can also speed gradient formation.

Discussion

Morphogen gradients provide a simple, yet elegant solution to the problem of specifying positional information during development. Because morphogen gradients closely tie the details of patterning to precise levels of receptor occupancy at different points in space, the need for development to occur normally in the face of various genetic and non-genetic perturbations translates into a need for morphogen gradients to be robust.

As shown here (and pointed out elsewhere [e.g. Eldar et al., 2004]), the basic processes of morphogen diffusion, receptor binding, and receptor-mediated degradation are sufficient to generate morphogen gradients of adequate shapes and rates of formation, but not sufficient to provide much robustness. When morphogen synthesis rates are varied by a factor of two, the most robust of such gradients still shift by 43% of the distance over which such gradients fall to 20% of their starting values (Fig. 3a-b).

In the present study, we sequentially added additional processes to the "basic" morphogen gradient, identifying those that made gradient shapes more robust to variations in morphogen production. The successful strategies that emerged shared a single common feature: non-receptors were responsible for most of the morphogen degradation. Simply having non-receptor molecules carrying out this role was sufficient to convert those gradients with a high degree of receptor saturation from being the least robust (Fig. 3a,c) to being the most (Fig. 4). Likewise, when morphogen-induced suppression of receptor synthesis was added to the basic model, it only diminished robustness (Fig. 3d), but when non-receptors were allowed to carry out most of the morphogen degradation, the same feedback greatly increased robustness. In both cases, the reason why non-receptors had such a dramatic effect is that they de-coupled the fixed relationship between morphogen sensing and morphogen destruction that occurs whenever receptors are the only molecules capable of destroying morphogens.

Although the data in Fig. 4-5 give the impression that one strategy (high receptor saturation, no feedback), can achieve a higher degree of robustness than the other (low receptor saturation, R-feedback), this is an artifact of the parameter ranges examined. As already mentioned, the first strategy can, in principle, achieve arbitrary robustness (but at the expense of a very shallow-then-steep gradient shape). The second strategy confronts a lower limit at $E=ln2/(2ln5)$ in the examples shown, but only because a Hill coefficient of 1 was assumed in the feedback function (i.e. feedback was not cooperative). For any Hill coefficient $n>1$, the general lower limit is $E=ln2/((n+1)ln5)$. Thus, for a Hill coefficient of 2, the lower bound on E would be 0.15 (see Appendix).

The results described here are of obvious relevance to the Dpp gradient in the *Drosophila* larval wing disc, as substantial evidence points to a role for non-receptors (heparan sulfate proteoglycans) in controlling the shape of that gradient, and feedback downregulation of Dpp receptors by Dpp is well established. Unfortunately, it is difficult to infer from existing data whether the majority of morphogen degradative flux is carried by receptors or non-receptors, as the analysis is complicated by the cell-autonomous effects of non-receptors on Dpp responsiveness (i.e. non-receptors also act as co-receptors [Fujise et al., 2003]). It is well established in mammalian cells, however, that cell surface heparan sulfate proteoglycans can target bound molecules for uptake and degradation (Belting, 2003; Berrou et al., 1995; Fuki et al., 1997; Reiland and Rapraeger, 1993; Sperinde and Nugent, 1998; Tyagi et al., 2001). Furthermore, in *Xenopus* embryos it has been shown that removing the heparan sulfate binding domain from BMP4 (an orthologue of Dpp) greatly increases its range of action as a morphogen (Ohkawara et al., 2002). This strongly suggests that, in at least one system, preventing interactions with heparan sulfate proteoglycans decreases the overall degradation of BMPs.

It is interesting that the present study found no evidence that robustness is ever improved by allowing morphogen signaling to suppress non-receptor synthesis (as in model 5), even though experiments show that Dpp strongly inhibits the expression of the non-receptor *dally* (Fujise et al., 2003). While it is possible that such feedback plays a role in gradient robustness to changes in parameters other than morphogen production rate, it is likely that a full understanding will require a better understanding of *dally's* activity as a co-receptor (Fujise et al., 2003), the molecular mechanism of which is still unknown.

The strategy recently proposed by Eldar et al. (Eldar et al., 2003) for achieving morphogen gradient robustness is clearly very different from the ones described here, as it is based upon the use of feedback regulation of receptor synthesis to increase morphogen degradation. This is not a feature of any of the models described here, and indeed, in model 5, morphogen signaling has the opposite effect on net morphogen degradation. Although the data presented by Eldar et al. (Eldar et al., 2003) argued that robustness was invariably diminished when morphogens inhibit their own degradation, this conclusion depends upon two assumptions not made here: first, that non-receptors are either absent or mediate no morphogen degradation and, second, that morphogen-receptor binding is linear, i.e. does not become saturated.

As the present study shows, a more inclusive treatment of how morphogen gradients form reveals the presence of multiple alternative paths by which robustness can be achieved. The flexibility with which complex biological systems, such as morphogen gradients, can achieve performance objectives creates obvious challenges for the experimentalist: Experiments that elucidate, in only a qualitative way, any number of detailed molecular interactions can still leave open too many options for what each interaction contributes to the larger system. At the same time, common genetic approaches (e.g. analyzing loss-of-function mutations) are limited in their ability even to identify control elements as such (as Doyle has pointed out, removing control elements from control systems tends either to have little effect under most circumstances, or to cause spectacular system crashes—neither result being terribly specific for control elements [Csete and Doyle, 2002]). Computational explorations, such as those in the present study, provide an increasingly valuable service to the experimentalist by identifying those features whose quantitative properties must be known if the function of a complex biological system is to be adequately understood.

Methods.

Numerical methods.

To obtain steady-state solutions, the equations for each system were reduced to a second-order ordinary differential equation for [L], and algebraic expressions for each of the variables in terms of [L]. After selecting random sets of parameters, the differential equation was numerically solved using the shooting method (Stoer and Burlirsch, 2002). The total number of steady state solutions calculated for each model was 2^{20} = 1,048,576. The number of cases in which the solution failed to converge varied between 148 and 175, depending upon the model (i.e. always less than 0.017% of cases).

The temporal evolution of individual solutions was calculated using the finite difference method, with a second-order central difference scheme for space and a fourth-order Adams-Moulton predictor-corrector method in time (Stoer and Burlirsch, 2002).

Analysis of numerical results

In order to focus our analysis on gradients of appropriate size and shape for the Dpp gradient in the Drosophila wing disc, we discarded numerical solutions with the following characteristics: (1) [LR] declines to < 0.01 LR_0 (where LR_0 stands for [LR] at $x=0^+$) in less than 40 μm (gradient too narrow); (2) [LR] exceeds 0.01 LR_0 at 160 μm (gradient too wide); (3) At a distance halfway between x=0 and the point where [LR] = 0.01 LR_0, [LR] > 0.5 LR_0 (gradient too convex); (4) In the vicinity of x=0, [LR] in the morphogen production region is higher than in the immediately adjacent gradient region (discontinuity in [LR] at x=0 opposite to that observed *in vivo*). Note that condition #2

justifies the appropriateness in generating the numerical solutions of using a boundary condition of [L]=0 at x=200 μm instead of at x=∞.

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Figure Legends

Figure 1. Models of morphogen gradient formation. In the most complete model, morphogen ligand (L) is produced in a discrete region at rate v, and is able to diffuse or bind to cell surface receptors (R_{out}) or non-receptors (N_{out}) to form cell surface complexes LR_{out} and LN_{out}, respectively. These complexes undergo reversible internalization to yield LR_{in} and LN_{in}, each of which is subject to degradation. Receptors and non-receptors are synthesized intracellularly at rates ω_R and ω_N, repectively, and undergo either reversible trafficking to the cell surface, or degradation. Morphogen signaling is considered to be proportional to $LR_{out} + LR_{in}$, and acts to negatively regulated both ω_R and ω_N. Of the five distinct models considered here, the first four each include only a subset of the interactions shown here: Model 1 includes only those symbols shown in black; model 2 includes those in black and red; model 3 includes black and blue; and model 4 black, blue and red.

Figure 2. Distributions of induced relative errors (*E*) for models 1-5. Morphogen gradients were calculated for each of the five models using random parameter sets as outlined in Methods. For all gradients meeting appropriate size and shape criteria, the relative effect of a twofold increase in morphogen synthesis rate was determined, and quantified as the induced relative error (E). The distribution of E-values was then plotted as a histogram (data are represented as a percentage of the total number of gradients originally calculated for each model; bin size = 0.01). The inset shows a higher magnification of the region between E=0.3 and E=0.5. Additional statistics may be found in Table 1.

Figure 3. Relationship between robustness and receptor saturation in models 1-2. A) Each of the gradients from model 1 for which E was calculated are represented as individual dots, showing the relationship between E-values and the expression $L_0/K_m\eta_R$. This ratio quantifies the degree of receptor saturation by bound morphogen at x=0; values greater than 1 indicate more than 50% saturation. The data show that a minimum value of E ($\approx$0.43) is reached, and shared by all gradients, as receptor saturation becomes very low. B) An example of a gradient (green line) with low receptor saturation at x=0 ($L_0/K_m\eta_R$=0.0056), and the shift caused by a two fold increase in morphogen synthesis (red line). $[LR]_{tot}$ stands for $[LR]_{out}+[LR]_{in}$. For parameter values, see Appendix. C) An example of a gradient (green line) with high receptor saturation at x=0 ($L_0/K_m\eta_R$=5.55), and the shift caused by a two fold increase in morphogen synthesis (red line). Note both the sigmoidal gradient shape, and the substantially greater green-to-red shift than in panel B. For parameter values, see Appendix. D) The gradients in model 2 (which adds receptor feedback to model 1) were analyzed as in panel A. The only noticeable difference from panel A is that not all gradients with low $L_0/K_m\eta_R$ approach the limiting E-value of 0.43.

Figure 4. Robustness-enhancing features of model 3. A) E-values associated with gradients calculated from model 3, which adds the presence of non-receptors to model 1 are plotted as a function of $L_0/K_m\eta_R$. Solutions with relatively low non-receptor saturation at x=0 (i.e. $L_0<J_m\eta_N$) are represented by green dots; those with high non-

receptor saturation ($L_0 > J_m\eta_N$) are red. Note the large collection of robust solutions for which $J_m\eta_N > L_0 > K_m\eta_R$ (arrow). Not all solutions meeting this criterion are robust, however (arrowhead). B) The cases in A are plotted as a function of $L_0/J_m\eta_N$, with green dots used to indicate solutions with low receptor saturation at x=0 (i.e. $L_0 < K_m\eta_R$), and red for those with high ($L_0 > K_m\eta_R$). One can easily identify the cluster or robust solutions for which $J_m\eta_N > L_0 > K_m\eta_R$, as well as those that meet the same criterion but are not robust (arrow). C) E-values of the cases in A and B are plotted as a function of Φ_R/Φ_{tot}, the fraction of morphogen degradation that is mediated by receptors at x=0. Solutions are represented with four colors: Red dots show cases for which receptor and non-receptor saturation are both high ($L_0 > K_m\eta_R$ and $L_0 > J_m\eta_N$); green dots indicate cases for which receptor and non-receptor saturation are both low ($L_0 < K_m\eta_R$ and $L_0 < J_m\eta_N$); blue dots depict cases for which receptor saturation is low but non-receptor saturation is high ($K_m\eta_R > L_0 > J_m\eta_N$); and yellow dots show cases for which receptor saturation is high but non-receptor saturation is low ($J_m\eta_N > L_0 > K_m\eta_R$). Nearly all of the robust cases (arrow) are found among the yellow dots, and are clustered toward the lowest values of Φ_R/Φ_{tot}. D) Comparison between calculated E-values and E-values predicted by an approximate solution for model 3. Dots represent only those gradients for which $L_0 < 0.1J_m\eta_N$ and $\Phi_R < 0.1\Phi_{tot}$. For red dots, $L_0/K_m\eta_R > 10$; for green $1 \leq L_0/K_m\eta_R \leq 10$, and for blue $L_0/K_m\eta_R < 1$. The clustering of the solutions above the dashed line indicates good agreement with the prediction. E) An example of a relatively robust gradient generated by model 3 (green line), and the relatively small shift (E=0.13) caused by a two fold increase in morphogen synthesis (red line). Parameters for this case are given in the appendix. In this example, $L_0/K_m\eta_R$=58.4, whereas $L_0/J_m\eta_N$=0.036.

Figure 5. Robustness-enhancing features of model 4. A) E-values associated with gradients calculated from model 4, which adds feedback control of receptor synthesis to model 3, are plotted as a function of $L_0/K_m\eta_R$. Solutions with relatively low non-receptor saturation at x=0 (i.e. $L_0 < J_m\eta_N$) are represented by green dots; those with high non-receptor saturation ($L_0 > J_m\eta_N$) are red. Note the new collection of robust solutions for which $L_0 < K_m\eta_R$ (arrows). B) The cases in A are plotted as a function of $L_0/J_m\eta_N$, with green dots used to indicate solutions with low receptor saturation at x=0 (i.e. $L_0 < K_m\eta_R$), and red for those with high ($L_0 > K_m\eta_R$). C) E-values of the cases in A and B are plotted as a function of Φ_R/Φ_{tot}, the fraction of morphogen degradation that is mediated by receptors at x=0. Solutions are represented with four colors: Red dots show cases for which receptor and non-receptor saturation are both high ($L_0 > K_m\eta_R$ and $L_0 > J_m\eta_N$); green dots indicate cases for which receptor and non-receptor saturation are both low ($L_0 < K_m\eta_R$ and $L_0 < J_m\eta_N$); blue dots depict cases for which receptor saturation is low but non-receptor saturation is high ($K_m\eta_R > L_0 > J_m\eta_N$); and yellow dots show cases for which receptor saturation is high but non-receptor saturation is low ($J_m\eta_N > L_0 > K_m\eta_R$). The robust cases that are added by introducing feedback (arrow; green dots) are strongly clustered toward the lowest values of Φ_R/Φ_{tot}. D) E-values of the cases in A-C are plotted as a function of ω_R/ω_{Rmax}, the degree to which receptor synthesis is suppressed, in the steady state, at x=0. Cases are color-coded as in panel C. Note that, among the green dots, robust cases require ω_R/ω_{Rmax} to be low, i.e. feedback must be potent. E) Comparison between calculated E-values and E-values predicted by an approximate solution for model 4. Dots

represent only those gradients for which $L_0 < 0.1 J_m \eta_N$, $L_0 < 0.1 K_m \eta_R$ and $\Phi_R < 0.1 \Phi_{tot}$. For red dots, $\omega_R/\omega_{Rmax} < 0.2$; for green $0.2 \leq \omega_R/\omega_{Rmax} \leq 0.6$, and for blue $\omega_R/\omega_{Rmax} > 0.6$. The clustering of the solutions around the dashed line indicates good agreement with the prediction. F) An example of a relatively robust gradient generated by model 4 (green line), and the relatively small shift (E=0.24) caused by a two fold increase in morphogen synthesis (red line). Parameters for this case are given in the appendix. In this example, $L_0/K_m\eta_R$=XX, whereas $L_0/J_m\eta_N$=XX.

Figure 6. Features correlating with robustness in model 5. A) E-values associated with gradients calculated from model 5, which adds feedback control of non-receptor synthesis to model 4, are plotted as a function of $L_0/K_m\eta_R$. Solutions with relatively low non-receptor saturation at x=0 (i.e. $L_0 < J_m\eta_N$) are green; those with high non-receptor saturation ($L_0 > J_m\eta_N$) are red. B) The cases in A are plotted as a function of $L_0/J_m\eta_N$, with green dots used to indicate solutions with low receptor saturation at x=0 (i.e. $L_0 < K_m\eta_R$), and red for those with high ($L_0 > K_m\eta_R$). C) E-values of the cases in A and B are plotted as a function of Φ_R/Φ_{tot}. Solutions are represented with four colors as in Fig. 5c-d. D) E-values of the cases in A-C are plotted as a function of ω_R/ω_{Rmax}. Cases are color-coded as in panel C. E) E-values of the cases in A-D are plotted as a function of ω_N/ω_{Nmax}. Cases are color-coded as in panel C. Note that, among the green dots, robust cases are more numerous when ω_N/ω_{Nmax} is high, but are not restricted to that range. F) An example of a relatively robust gradient generated by model 5 (green line), and the relatively small shift (E=0.24) caused by a two fold increase in morphogen synthesis (red line). In this example, ω_N/ω_{Nmax}=XX. Parameters for this case are given in the appendix. In this example, $L_0/K_m\eta_R$=XX, whereas $L_0/J_m\eta_N$=XX.

Figure 7. Dynamics of gradient formation. Time dependent solutions are shown for the formation of steady state gradients produced by models 1, 3 and 4. A) Time evolution of the gradient shown in Fig. 3a (model 1). B) Time evolution of the gradient shown in Fig. 4e (model 3). C) Time evolution of the gradient shown in Fig. 5f (model 4). Each curve in A-C represents one hour of elapsed time. D) E-values associated with gradients calculated from model 4, plotted as a function of $ln2/(j_{degobs}+j_{off})+ln2/(k_{degobs}+k_{off})$, which estimates the maximum time to reach 50% of steady state levels. Cases are color-coded as in Fig. 6c-e. The percentage of cases for which the this time is less than 7 hours varies between 90% (yellow dots) and 98% (blue dots).

Model	Gradients of appropriate size and shape	*E*-values of appropriate gradients		% of appropriate gradients with $E<ln2/ln5$
		Median	Smallest	
1 (Basic)	39,064 (3.73%)	0.478	0.424	0.1
2 (R-feedback)	31,344 (2.99%)	0.487	0.399	0.1
3 (Non-receptors)	92,052 (8.78%)	0.434	0.121	42.8
4 (Non-receptors; R-feedback)	85,704 (8.17%)	0.431	0.107	49.8
5 (Non-receptors; R- and N-feedback)	83,598 (7.97%)	0.438	0.116	40.4

Table 1. Statistics on the robustness of numerical solutions of each model. Numerical solutions were obtained for $>10^6$ random parameter sets for each model. Values in the table quantify the degree to which introduction of non-receptors (model 3) and non-receptors plus receptor-feedback (model 4) increase the proportion or robust solutions. For criteria for selection of gradients of "appropriate" size and shape, see Methods.