Diverse Paths to Morphogen Gradient Robustness

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Summary

The patterning of many developing tissues is orchestrated by gradients of morphogens. Included among the molecular events that drive the formation of morphogen gradients are a variety of elaborate regulatory interactions. It is widely thought that the purpose of such interactions is to make gradients robust—i.e. resistant to change in the face of genetic or environmental perturbations—but precisely how this might come about is a major unanswered question. Here we identify two highly effective robustness strategies that can be exploited by any morphogen gradient in which morphogen degradation can occur through uptake by cell surface molecules other than signaling receptors. One strategy exploits the effects of non-linearity, i.e. saturability, in the binding of morphogens to receptors; the other exploits feedback inhibition of receptor synthesis. Interestingly, in the decapentaplegic gradient of the *Drosophila* wing disc, just such feedback inhibition occurs, and cell surface, non-receptor, morphogen-binding molecules (proteoglycans) are known to be key regulators of gradient shape. Thus, the robustness strategies identified here may help explain some of the regulatory architecture of one of the best studied gradient systems.

Introduction

The patterning of developing tissues requires cells to adopt fates according to their locations. The necessary positional information is often conveyed by morphogens, secreted signaling molecules that are transported away (e.g. by diffusion) from their site of production to form spatial gradients. Graded differences in morphogen receptor occupancy at different locations underlie the signaling differences that ultimately lead cells down different differentiation paths. Although the strategy of using morphogen gradients to create pattern is conceptually simple, the

need to build gradients of appropriate sizes, shapes and rates of formation imposes some constraints. For example, the tendency of signaling receptors to degrade their ligands can create a need for morphogen receptors to have slow association kinetics, and to be expressed at low levels [1].

A significant challenge for morphogen gradients is to produce patterns that are not easily altered by genetic or environmental fluctuations. The insensitivity of a system's output to variations in input or parameters is referred to as robustness, and a substantial number of recent studies have begun to investigate how robustness is achieved by morphogen gradients (e.g. [2-9]). A common approach has been to model the effects of heterozygous mutations in genes that encode morphogens, their receptors, or other molecules that participate in gradient formation. The fact that animal development tolerates heterozygosity for most such genes provides strong justification for the belief that morphogen gradients are highly robust.

Understanding how robustness arises is important not only to shed light on the reliability of developing systems, but also to help explain the ubiquitous presence of elaborate regulatory schemes in morphogen systems. These include feedback regulation of morphogen receptor synthesis by morphogen signaling (reviewed in [10]); modulation of gradient formation and morphogen signaling by cell surface proteoglycans (e.g. [11-20]); and the widespread utilization of secreted morphogen inhibitors (e.g. [21-27]).

An especially pressing question about morphogen gradient robustness is whether the strategies used by different gradients are generic or individualized. One recent mathematical and computational analysis identified a generic strategy for making gradients robust to alterations in the rate of morphogen production [6]. The crux of this strategy, termed "self-enhanced ligand degradation", is a feedback loop in which morphogen receptor synthesis is regulated by

morphogen signaling in such a way that morphogen degradation increases with the strength of signaling. Two of the morphogen gradients that operate in the *Drosophila* larval wing imaginal disc—those formed by the Hedgehog (Hh) and Wingless (Wg) morphogens—were shown to exhibit features consistent with the implementation of such a strategy: Hh upregulates its receptor Ptc, which increases Hh degradation, whereas Wg downregulates its receptor Dfz2, which also increases Wg degradation, because Dfz2 normally inhibits that degradation [6].

The *Drosophila* wing imaginal disc is patterned by a third morphogen, Dpp, a member of the bone morphogenetic protein branch of the transforming growth factor-β superfamily. Like Wg, Dpp downregulates its own receptor, the serine-threonine kinase Thick veins (Tkv). However, unlike Wg receptors, Tkv does not protect its ligand from degradation. On the contrary, overexpressing Tkv in wing discs shrinks the Dpp gradient [28]; this is just what calculations say a receptor that drives morphogen degradation should do [29, 30], and is precisely opposite to what happens to the Wg gradient when Dfz2 is overexpressed [31].

In the Dpp gradient, it would seem that feedback regulation of receptors can only cause self-repressed, never self-enhanced ligand degradation. Accordingly, either the Dpp gradient is not robust, or it uses robustness strategies fundamentally different from what has been proposed for other gradients. Here we address both issues. First, we provide experimental evidence that Dpp-mediated patterning of the *Drosophila* wing is substantially robust to Dpp dose. Second, we identify two novel strategies for achieving robustness, either of which may be employed by this gradient. One strategy exploits feedback regulation of receptor synthesis but, interestingly, the other does not. What is common to both strategies is that cell surface molecules besides signaling receptors must mediate a large proportion of overall morphogen degradation. Because these strategies are generic—i.e. they apply to any gradient in which non-receptor molecules

mediate morphogen degradation—they may help explain why cell-surface morphogen binding molecules other than receptors (e.g. heparan sulfate proteoglycans) are utilized almost universally in morphogen gradient systems.

Results

Dpp-mediated wing patterning is robust to *dpp* dose

Dpp is a crucial morphogen in the early *Drosophila* embryo, and animals heterozygous for *dpp* rarely survive embryogenesis. One might speculate that the wing disc Dpp gradient has no need to be robust to *dpp* dose because animals with abnormal levels of Dpp never reach larval stages. On the other hand, *dpp*-/+ flies occasionally develop to adulthood, and such "escapers" have not been noted to display abnormal wings [32, 33]. To more accurately assess the robustness of the wing disc Dpp gradient, we closely examined the wings of adult *dpp*-/+ flies (Figure 1), which were obtained by three different approaches: rare male escapers of the genotype dpp^{H46}/+; rescued dpp^{H46}/+ females in which *dpp* was provided embryonically by the eve2-*dpp* construct [34]; and rescued dpp^{H48}/+ females in which the embryonic dose of *sog* was reduced to one copy (*sog*-/+; *dpp*-/+). The effective dose of *dpp* in the wing discs of rescued individuals is expected to be 50% of wildtype since the eve2-*dpp* construct is expressed only briefly during early embryogenesis, and because *sog* counteracts *dpp* in dosage sensitive fashion during early embryonic dorsovental patterning [33-35] but plays no significant role in determining the spacing of wing vein primordia [36, 37].

As a quantitative measure of the larval wing disc Dpp gradient, we measured the spacing between adult wing veins (Fig. 1a). The locations of veins L2 and L5 are established by the domains of larval expression of *spalt, optomotor blind,* and *brinker*, each of which is specified

by the Dpp gradient. In contrast, the locations of L3 and L4, which mark the boundaries of the Dpp production region, are established by the Hh gradient, and are independent of Dpp [38, 39]. Thus, the inter-vein distances L2/3 and L4/5 should provide a direct read-out of the larval gradient of Dpp activity, whereas the L3/4 distance should serve as a Dpp-insensitive control measurement. Indeed, in *dpp*-/+ flies generated by any of the above strategies, we observe a statistically significant decrease in both the L2/3 and L4/5 intervals accompanied by little or no change in L3/4. In Fig. 1b-d, these measurements are presented normalized to wing length, but much the same conclusions may be reached if one compares absolute vein spacing, or normalizes L2/3 and L4/5 to L3/4 (Table S1).

These results indicate that loss of one copy of *dpp* produces a shift in the location of just those wing veins whose positions are controlled by the larval Dpp gradient. Interestingly, the magnitudes of the shifts are quite small, corresponding to decreases in spacing on the order of 10-15% (Fig. 1, Table S1). As described below (and mentioned elsewhere [6]), in the absence of strategies to improve robustness, simple morphogen gradients ought to display much more severe pattern disruptions when morphogen levels change by two-fold. Thus, there is good reason to believe that some kind of robustness strategy is implemented by the larval wing disc Dpp gradient.

Modeling the Dpp gradient

To explore how gradients such as the wing disc Dpp gradient might become robust, we developed a set of computational models. As in earlier work by ourselves [1] and others [6, 40], we model morphogen gradients in *Drosophila* wing imaginal discs as instances of reaction and diffusion in one-dimension, but differ from previous studies in three significant ways: First, we explicitly specify a region of morphogen production in which morphogen-producing cells can

also have morphogen receptors and responses (as is clearly the case for Dpp in the wing disc [13, 41]). Second, we allow cell surface molecules other than receptors to bind, internalize and degrade morphogens. Such molecules—which for brevity we call "non-receptors"—would include (but need not be limited to) the heparan sulfate proteoglycans, which are present in most morphogen gradients, and bind morphogens of the TGF-β, Wnt, Hh and FGF families (reviewed in [42]). 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 (Figure 2a) 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 2b outlines a minimal set of reactions among morphogens, receptors and non-receptors that might occur in a gradient such as the Dpp gradient of a wing disc. To facilitate analysis, we may break out subsets of these reactions into individual models of increasing complexity: For example, we begin by considering only the interactions of morphogens with receptors (black symbols), omitting non-receptors or any feedback regulation of receptor synthesis; we refer to this simple situation as model 1. Model 2 adds negative feedback regulation of receptor synthesis (red arrows, symbols); we consider only negative feedback here, since that is what occurs in the Dpp gradient. Model 3 adds non-receptors (blue arrows, symbols) to model 1. Model 4 combines the elements of models 2 and 3. Finally, model 5 adds to model 4 by allowing morphogen signaling to repress the synthesis of non-receptors (shown in green). This addition incorporates recent findings [13] that, in the wing disc, 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 all five models by random parameter set searches: Setting time rates to zero, the differential equations corresponding to each model (Figure S1) were reduced to their simplest forms. After identifying a minimal set of required parameters, we specified broad ranges for each, covering what appeared to be all biologically plausible values (see Supplemental Data). Numerical 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 3-9% of the solutions, depending on the model (Table 1).

We then calculated the sensitivity of each steady state gradient shape to the rate of morphogen production, by doubling the rate of morphogen synthesis, recalculating the solution, and measuring the average amount by which the gradient shifted (see Supplemental Data). 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*." Roughly, *E* is the distance (as a fraction of gradient length) by which the average threshold shifts when morphogen synthesis undergoes a twofold change. The closer *E* is to zero, the more robust the gradient. The changes in wing vein spacing showing in Figure 1 can be shown to correspond, approximately, to *E*-values of 0.15-0.19 (see Supplemental Data).

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 3, and summarized in Table 1. In models 1 and 2, *E* is frequently in the range of 0.43-0.45, and almost never lower. The median *E*-value for model 2 is slightly higher than for model

1, implying that feedback inhibition of receptor synthesis tends, on average, to make gradients less robust.

In the three remaining models, E <0.43 in many cases, i.e. some feature of the models allow gradients to be more robust (Fig. 3; Table 1). In model 3 (which introduces non-receptors), E <0.43 for 42.8% of gradients, and for quite a few of these, E <0.2. In model 4 (which adds feedback inhibition of receptor synthesis to model 3), robust cases are even more numerous (nearly half have E<0.43). In model 5, the distribution of E-values is much like that in model 4, with a slight shift toward higher values. From these results we infer that simply including non-receptors in a morphogen gradient system can promote robustness, and this can be further enhanced by allowing for feedback downregulation of receptor synthesis. In contrast, feedback downregulation of non-receptor synthesis seems not to provide any further improvement in morphogen gradient robustness.

Origins of robustness due to non-receptors

Exploring complex systems by solving them for large numbers of random parameter sets can be a powerful tool for producing a good qualitative picture of what such systems do (e.g. [2, 6, 8, 43]). However, one needs to interpret such data carefully, especially when parameter ranges are chosen generously (doing so can cause one to explore large regions of parameter space that are biologically inaccessible). Accordingly, it is always desirable to use analytical methods to justify the validity and generality of results obtained from numerical simulations.

Exact mathematical solutions are unavailable even for the simplest of the models in Fig. 2, but under appropriate conditions approximate solutions can be found. Finding such conditions is aided by taking a closer look at the numerical data. For example, in Figure 4a, the E-value of every gradient calculated using model 1 is plotted against the value of a parameter, Ω_R that

quantifies the degree of receptor saturation, measured at the start of that gradient (i.e. just next to the zone of morphogen production, a position we designate as x=0). It is immediately evident that low receptor saturation (Ω_R<<1; see Supplemental Data) is both necessary and sufficient for greatest robustness (i.e. $E \approx 0.43$). Conveniently, in the limit of negligible receptor saturation, model 1 can be solved exactly. Specifically, for all x>0, the steady state concentration of occupied morphogen receptors = $LR_0e^{-x\Lambda}$, where Λ is a decay length constant, and LR_0—the value of morphogen receptor occupancy at x=0—is given by an expression proportional to v, the rate of morphogen synthesis [29]. It is straightforward to show (see Supplemental Data) that for all such gradients, no matter what the values of LR_0 and Λ, the value of E will be $ln2/ln5 \approx 0.43$.

Likewise, if one makes the assumption that receptor saturation is high (Ω_R>>1) at x=0, approximate solutions to model 1 may be found that imply that E-values will always exceed $ln2/ln5$ by a factor that, for large enough v, will be proportional to v (see Supplemental Data). In short, by analyzing what happens at the extremes of receptor saturation, we gain an understanding of why robustness is distributed as it is among the universe of all possible solutions to model 1. In doing so, we learn that, in such a simple morphogen gradient system, the only possible robustness strategy is to keep receptor saturation low. Fig. 4b shows such a gradient. Clearly, the best robustness attainable by model 1 is not especially good (recall that the robustness observed in vivo [Fig. 1] translates to value of E<0.2 [see above]). Fig. 4c shows the even less robust result one obtains when receptor saturation is not low (note also the characteristic sigmoidal shape of such gradients).

In Figure 4d, numerical solutions of model 2 (which introduces regulated receptor synthesis) were analyzed in the same fashion as in Fig. 4a. Again we see a lower bound of $E \approx$ 0.43 that is reached only when receptor saturation is low. Now, however, we observe that low

receptor saturation is no longer sufficient to guarantee $E \approx 0.43$. In effect, what feedback regulation of receptor synthesis adds to model 2 is simply the opportunity for some gradients to be less robust than they otherwise would be.

In Figure 5, the same approach was used to analyze model 3 (which adds non-receptors to model 1). Now we see that the many robust cases (i.e. cases with $E<0.43$) that newly emerge in model 3 all have a common feature: they occur when receptor saturation is high ($\Omega_R>>1$) at the gradient's start (Fig. 5a, arrow). Not all gradients with high receptor saturation behave in this way, however (e.g. Fig. 5a, arrowhead). One can separate out the robust gradients to some extent, by looking at levels of saturation of the *non-receptors*, which we can quantify with an analogous parameter, Ω_N. In Fig. 5a, the data points corresponding to each calculated gradient have been colored so that those with substantial non-receptor saturation ($\Omega_N>1$) at $x=0$ are in red, with the remainder green. The results show that the combination of low non-receptor saturation and high initial receptor saturation is a necessary condition for $E<0.43$. Similar information is provided in Fig. 5b, in which E values are plotted as a function of non-receptor saturation and points are colored according to their degree of receptor saturation.

Not all gradients in model 3 with high receptor saturation and low non-receptor saturation are robust, however (e.g. arrow in Fig. 5b). Therefore we looked for other gradient features that might correlate with robustness. In Fig. 5c, we plot E-values versus the fraction of total morphogen degradation (at $x=0$) that is mediated by receptors (as opposed to non-receptors). Here, four colors are used to identify those gradients with different degrees of receptor and non-receptor saturation, with those cases in which receptor saturation is high and non-receptor saturation low depicted in yellow. For such gradients there is a nearly linear relationship between E-value and the fraction of morphogen degradation mediated by receptors. Thus, three

conditions are jointly necessary and sufficient for robustness (E<0.43) in model 3: High receptor saturation, low non-receptor saturation, and a large proportion of morphogen degradation carried out by non-receptors (arrow, Fig. 5c).

Knowing only two of these conditions allows for a direct solution of model 3 (see Supplemental Data). If non-receptor saturation is low enough, and morphogen degradation mediated by receptors negligible compared with degradation via non-receptors, then E is predicted to follow the simple relationship $E = ln2/ln(5+4\Omega_R)$, where is Ω_R measured at x=0. This states that $E \approx 0.43$ whenever receptor saturation is low; $E \approx 0.32$ when receptors are 50% saturated (Ω_R=1) at x=0; and E becomes arbitrarily small as receptor saturation at x=0 approaches 100%. As a test of how well this prediction applies to actual cases generated from the parameter sets studied, Fig. 5d plots E values from numerical solutions to model 3, color-coded by their degree of receptor saturation, against values of $ln2/ln(5+4\Omega_R)$, based on each set of parameter choices. The results show a good agreement between observed and expected E values. Fig. 5e depicts the behavior of a particular robust solution to model 3 (E=0.13).

The preceding analysis implies that, in principle, as long as non-receptors dominate the process of morphogen degradation and receptors are largely saturated at the start of such gradients, non-receptors can make gradients highly robust to variation in morphogen synthesis. It should be noted, however, that as one attempts to use such a strategy to achieve ever greater robustness, one generates gradients that are increasingly sigmoidal in shape (i.e. long plateaus of receptor occupancy followed by steep declines over very short distances). Such step-like gradients may be useful for defining a single position in space, but it would be difficult to use them to define more than one. Thus, a need to position gene expression thresholds at multiple

locations might place practical limits on the amount of robustness the above mechanism could provide.

Origins of robustness due to receptor feedback

Model 2 adds feedback inhibition of receptor synthesis to model 1; model 4 adds feedback inhibition of receptor synthesis to model 3. In the first case, this addition only decreased the average robustness of gradients; in the second case it improved it (Fig. 3). Here we seek to understand why.

In Figure 6 we analyze *E*-values for model 4 in the same fashion as we did for model 3, i.e. as a function of the amount of receptor saturation (Fig. 6a), non-receptor saturation (Fig. 6b), and degradative flux carried by receptors (Fig. 6c) at x=0. As before, we see that low non-receptor saturation and a low contribution of receptors to total degradative flux are essential for robustness. In contrast to Model 3, however, many robust gradients now exhibit low, rather than high, receptor saturation at x=0 (arrows, Fig. 6a).

Once again, the conditions of low non-receptor saturation and low degradative flux through receptors allow us to derive an approximate analytical solution to model 4 (see Supplemental Data). For those cases with low receptor saturation, it is straightforward to show that maximum robustness requires feedback to be potent (morphogen signaling should be able to suppress 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 should be on the order of the amount of receptor occupancy that occurs a x=0). These requirements can be confirmed in the numerical solutions by plotting *E*-values against the ratio of receptor synthesis (ω_R) at x=0 to receptor synthesis that would have occurred had there not been any feedback (ω_{Rmax}). As shown in Fig. 6d, for those cases with low saturation of both

receptors and non-receptors (green dots), low *E*-values require small ω_R/ω_{Rmax}, with the lowest *E*-values for such cases being around 0.21.

Assuming low saturation of both receptors and non-receptors; low contribution of receptors to morphogen degradation; and ignoring any cooperativity in the feedback, the approximate analytical solution to model 4 yields the relationship $E = ln2/(2ln5 - lnQ)$, where Q is a lumped parameter that combines the effects of feedback strength and sensitivity (see Supplemental Data). By its definition, Q varies between 1 and 5, constraining E to vary between $ln2/(2ln5) \approx 0.215$ and $ln2/ln5 \approx 0.43$. In Figure 6e, E is plotted as a function of $ln2/(2ln5 - lnQ)$ for all gradients in model 4 for which non-receptor saturation is <50% and less than 10% of moprhogen degradation at x=0 is mediated via receptors, with color-coding to distinguish among those with different degrees of receptor saturation. One can see excellent agreement between the results of simulation and analysis. Fig. 6f depicts the behavior of a typical robust solution to model 4.

Robustness and the effect of morphogen signaling on morphogen degradation.

It has been proposed elsewhere that self-enhanced ligand degradation is essential for robustness of morphogen gradients [6]. The structure of model 4 is such that increasing morphogen signaling can only decrease, not increase, morphogen degradation. Of course, since a lack of significant contribution of receptors to morphogen degradation is required for robustness in model 4, it follows that, for the robust gradients at least, the effects of regulation of receptor expression will usually be to neither increase nor decrease overall morphogen degradation. We wondered whether this *lack* of influence on morphogen degradation was important for robustness, or merely coincidental.

An answer came from studying 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. 3, Table 1), suggesting that feedback control of non-receptor synthesis does not create any additional strategies for enhancement of robustness. Indeed, an analysis of the cases in model 5 (Fig. 7a-d) shows that the conditions necessary for robustness—low saturation of non-receptors, high saturation of receptors, and low contribution of receptors to net degradation; OR low saturation of both receptors and non-receptors, low contribution of receptors to net degradation, 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. 7e).

In other words, selecting for robustness selects against strong non-receptor feedback. Nevertheless, among cases with high as well as low receptor saturation, one can find many gradients in which non-receptor synthesis is significantly suppressed ($\omega_N/\omega_{Nmax}<0.5$), yet *E*-values are very low. An example is shown in Fig. 7f. In such cases it is necessarily true that increased morphogen signaling substantially decreases net morphogen degradation. Thus, being robust not only doesn't require self-enhanced morphogen degradation, it is compatible even with "self-inhibited" morphogen degradation.

Robustness strategies and the dynamics of gradient formation.

The above results 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 [44]. 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 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 8a-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, or non-receptors and feedback, 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 low contribution of receptors to net morphogen degradation holds not just at steady state, but always (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 [29, 30, 45]), and is largely dictated by rate constants associated with dissociation from and degradation by non-receptors, i.e. $j_{degobs}+j_{off}$. One may obtain an upper bound on the rate of approach of the gradient of receptor occupancy 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 $ln2/(j_{degobs}+j_{off}) + ln2/(k_{degobs}+k_{off})$.

In Fig. 8d, *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 results in Fig. 8c suggest that feedback inhibition of receptor synthesis can accelerate gradient formation. Because receptors are more numerous before feedback has had time to suppress their synthesis, they initially accumulate morphogen much faster than would have been the case if receptor levels had started out at their final values. This effect—negative feedback increasing the rate of approach of a system to steady state—is a well-known principle in control theory [46]. In robust morphogen gradients, it is the requirement that receptors contribute little to overall morphogen degradation that allows high receptor levels to drive faster gradient evolution without simultaneously hindering net morphogen diffusion. Since this requirement can ony be met when there is 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 tie the details of patterning to precise amounts of receptor occupancy at different points in space, the ability of development to proceed normally in the face of genetic and non-genetic perturbations translates into a need for morphogen gradients to be highly robust.

As shown here (and pointed out elsewhere [e.g. [7]), 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 they fall to 20% of their starting values (Fig. 4a-b).

In the present study, we sequentially added additional processes to the "basic" morphogen gradient, identifying those that make gradients more robust to variations in morphogen production. The successful strategies that emerged shared a single common feature: non-receptor molecules were responsible for most of the morphogen degradation. Simply having non-receptors carrying out this role was sufficient to convert those gradients with a high degree of receptor saturation from being the least robust (Fig. 4a,c) to being the most (Fig. 5). Likewise, when morphogen-induced suppression of receptor synthesis was added to the basic model, it only diminished robustness (Fig. 4d), 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. 5-6 suggest that one strategy (high initial receptor saturation, no feedback), can achieve more robustness than the other (low receptor saturation, feedback suppression of receptor synthesis), this conclusion is unwarranted. As already mentioned, the first strategy achieves extreme robustness only at the expense of a step-like gradient shape. And although the second strategy confronts a lower limit at E=0.215 in the examples shown, this is 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 Supplemental Data).

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 [13, 16], and feedback downregulation of Dpp receptors by Dpp is well established [28, 47]. Unfortunately, it is difficult to infer from experimental data whether the majority of Dpp degradation is carried out by receptors or non-receptors, as the analysis is complicated by additional, cell-autonomous effects of non-receptors on Dpp responsiveness [13]. It is well established in mammalian cells, however, that cell surface heparan sulfate proteoglycans target bound molecules for uptake and degradation [48-53]. 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 [54]. 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* [13]. 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 [13], the molecular mechanism of which is still unknown.

The robustness strategy of self-enhanced ligand degradation that was recently proposed by Eldar et al. [6] is clearly very different from (and incompatible with) the strategies described here. Although data presented by these authors indicate that allowing morphogens to inhibit their own degradation invariably leads to a decrease in robustness, their analysis was predicated

on two assumptions not made here: first, that only receptors degrade morphogens and, second, that binding to receptors is linear, i.e. does not saturate. 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.

It is becoming increasingly apparent that much of the complexity in biological systems is concerned with *control phenomena*, such as robustness and homeostasis. As others have pointed out, straightforward experimental approaches for discovering and understanding control elements are hampered by the fact that disrupting such elements can lead to anything from no effect to catastrophic system failure [55]—neither consequence being particularly instructive to the experimentalist. A major goal of mathematical and computational explorations, such as those in the present study, is to provide a systematic way to identify control elements, and help experimentalists recognize control systems when they see them. To this end, the fact that there exist at least three mutually exclusive strategies for morphogen gradient robustness is sobering, informing us that in different systems the same mechanism (e.g. receptor-mediated inhibition of receptor synthesis) could achieve the same end (robustness) by a variety of different means. If nothing else, caution is called for in drawing inferences about biological control from limited experimental data.

Methods.

Drosophila mutants

sog^{6}/+; dpp^{H48}/+ females (N=100) were collected from a cross of female dpp^{H48}/CyO^{23}*wg-lacZ* flies to male sog^{6}/Dpsog^{+} flies. Males and females of the genotype w; dpp^{H46} Sp st2-*dpp*/+ were generated by crossing *yw*; dpp^{H46} *Sp st2-dpp* males to *w*-; +; + females. Only females (N=115)

were chosen for wing measurements. Rare escaper males of the genotype *w*-; dpp^{H46} *Sp*-/+ were pooled from a cross of Dpdpp; dpp^{H46} *Sp*/*Gla* males to *w*-; +; + females and from a cross of *w*-; +; + males to dpp^{H46} *Sp*/CyO^{23} females to provide a total of 25 males that were measured. *w*-; +; + females (N=100) and males (N=49) were also measured for use as controls. Molecular lesions associated with the null dpp^{H46} and sog^{6} alleles are described respectively in references [56] and [35].

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 (see Supplemental Data), the differential equation was numerically solved using the shooting method [57]. 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 [57].

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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Author contributions. ADL, QN, FW, and EB conceived the experiments. Numerical solutions were calculated by QN and analyzed by ADL and QN. Mathematical analysis was done by FW and ADL. *Drosophila* experiments were designed by EB and carried out by HE and CM. The paper was written by ADL with contributions from EB, QN and FW.

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Figure Legends

Figure 1. Dpp-mediated wing patterning is weakly sensitive to *Dpp* dosage. To examine the effects of decreased *dpp* gene dosage on wing patterning, three genetic strategies were used to produce flies heterozygous for either of two null mutations in *dpp* (see Methods). A) The distances between pairs of wing veins, as well as overall wing length and width, were measured at the locations shown for a large number of adult wings of each genotype. B-D) Mean values of L2/3 spacing (B), L3/4 spacing (C) and L4/5 spacing (D) are shown normalized to wing length, for mutant (hatched bars) and wildtype (solid bars) flies. Error bars represent standard deviations. (E-F) Photographs of representative wings from wildtype (E) and sog^{6}/+; dpp^{H48}/+ (F) flies. The data show that, as expected, the L2/3 and L4/5 spacings are *dpp*-dependent, but the overall effect of halving *dpp* dosage is small. Raw data and additional analysis can be found in Table S1 (Supplemental Data).

Figure 2. Models of morphogen gradient formation. A. Schematic depiction of interactions of morphogen ligands (L, lavender) with receptors (R, black) and non-receptors (N, blue), showing extracellular diffusion, intracellular trafficking, and influences of morphogen signaling (yellow arrow) on receptor and non-receptor synthesis. B. Representation of the interactions in A in the form of chemical equations: Morphogen (L) is produced in a discrete region at rate v, and can diffuse or bind receptors (R_{out}) or non-receptors (N_{out}) to form cell surface complexes LR_{out} and LN_{out}, respectively. These 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 regulate 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: 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 3. 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 4. 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 value of Ω_R at x=0. Ω_R is an expression that quantifies receptor saturation by bound morphogen (see Supplemental Data); Ω_R>1 indicates more than 50% saturation. The data show that a minimum value of E (≈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 (Ω_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 (Ω_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 receptor saturation approach the limiting *E*-value of 0.43.

Figure 5. Emergence of a robustness strategy with 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 Ω_R. Solutions with relatively low non-receptor saturation at x=0 (Ω_N<1) are represented by green dots; those with high non-receptor saturation ($\Omega_N \geq 1$) are red. Note the large collection of robust solutions for which, at x=0, Ω_R is high and Ω_N low (arrow). Not all solutions meeting this criterion are robust, however (arrowhead). B) The cases in A are plotted as a function of Ω_N, with green dots used to indicate solutions with low receptor saturation at x=0 (i.e. Ω_R<1), and red for those with high ($\Omega_R \geq 1$). One can easily identify the cluster of robust solutions for which Ω_R is high and Ω_N low, 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 at x=0 that is mediated by receptors. Solutions are depicted using four colors: Red dots for cases in which receptor and non-receptor saturation at x=0 are both high ($\Omega_R \geq 1$ and $\Omega_N \geq 1$); green dots for cases in which receptor and non-receptor saturation are both low (Ω_R<1 and Ω_N<1); blue dots for cases in which receptor saturation is low but non-receptor saturation is high (Ω_R<1 and $\Omega_N \geq 1$); and yellow dots for cases in which receptor saturation is high but non-receptor saturation is low ($\Omega_R \geq 1$ and Ω_N<1). 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, at x=0, $\Omega_N < 0.1$ and $\Phi_R < 0.1\Phi_{tot}$. For red dots, $\Omega_R > 10$; for green $1 \leq \Omega_R \leq 10$, and for blue Ω_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, Ω_R=58.4, whereas Ω_N=0.036, at x=0.

Figure 6. Emergence of a second robustness strategy with 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 Ω_R. Solutions with relatively low non-receptor saturation at x=0 (i.e. Ω_N<1) are represented by green dots; those with high non-receptor saturation ($\Omega_N \geq 1$) are red. Note the new collection of robust solutions for which Ω_R<1 (arrows). B) The cases in A are plotted as a function of Ω_N at x=0, with green dots used to indicate solutions with low receptor saturation (Ω_R<1), and red for those with high ($\Omega_R \geq 1$). 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 at x=0 that is mediated by receptors. Solutions are depicted using four colors: Red dots for cases in which receptor and non-receptor saturation at x=0 are both high ($\Omega_R \geq 1$ and $\Omega_N \geq 1$); green dots for cases in which receptor and non-receptor saturation are both low (Ω_R<1 and Ω_N<1); blue dots for cases in which receptor saturation is low but non-receptor saturation is high (Ω_R<1 and $\Omega_N \geq 1$); and yellow dots for cases in which receptor saturation is high but non-receptor saturation is low ($\Omega_R \geq 1$ and Ω_N<1). 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 ones predicted by an approximate solution for model 4. Dots represent only those gradients for which, at x=0, Ω_N<0.1, Ω_R< 0.1, and Φ_R<0.1Φ_{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=0.23) caused by a two fold increase in morphogen synthesis (red line). Parameters for this case are given in the appendix. In this example, Ω_R=0.156, Ω_N=0.005, and ω_R/ω_{Rmax} = 0.08, at x=0.

Figure 7. 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 Ω_R. Solutions with relatively low non-receptor saturation at x=0 ($\Omega_N<1$) are green; those with high non-receptor saturation ($\Omega_N\geq 1$) are red. B) The cases in A are plotted as a function of Ω_N at x=0, with green dots used to indicate solutions with low receptor saturation ($\Omega_R<1$), and red for those with high ($\Omega_R\geq 1$). C) E-values of the cases in A and B are plotted as a function of Φ_R/Φ_{tot}. Solutions are depicted using four colors as in Fig. 5c and 6c-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=0.25) caused by a two fold increase in morphogen synthesis (red line). Parameters for this case are given in the appendix. In this example, Ω_R=0.8616, $\Omega_N=4.676\times 10^{-6}$, ω_R/ω_{Rmax}=0.124 and ω_N/ω_{Nmax}=0.386 at x=0.

Figure 8. 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. 4a (model 1). B) Time evolution of the gradient shown in Fig. 5e (model 3). C) Time evolution of the gradient shown in Fig. 6f (model 4). Each curve in A-C represents one hour of elapsed time. Curves for the first two hours are labeled "1" and "2", and the curve representing steady state behavior is labeled "∞". 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. 7c-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*<0.43
		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.