Scaling of dorsal-ventral patterning in the *Xenopus laevis* embryo

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Scaling of pattern with size has been described and studied for over a century, yet its molecular basis is understood in only a few cases. In a recent, elegant study, Inomata and colleagues proposed a new model explaining how bone morphogenic protein (BMP) activity gradient scales with embryo size in the early *Xenopus laevis* embryo. We discuss their results in conjunction with an alternative model we proposed previously. The expansion-repression mechanism (ExR) provides a conceptual framework unifying both mechanisms. Results of Inomata and colleagues implicate the chordin-stabilizing protein sizzled as the expander molecule enabling scaling, while we attributed this role to the BMP ligand Admp. The two expanders may work in concert, as suggested by the mathematical model of Inomata et al. We discuss approaches for differentiating the contribution of sizzled and Admp to pattern scaling.

Keywords:
BMP; development; mathematical modeling; morphogen gradient; patterning; scaling; *Xenopus*

Scaling pattern with size during early embryonic patterning

One of the striking aspects of developmental processes is their ability to adjust the body pattern with the size of the individual. This adjustment, termed *scaling*, has been observed and studied by developmental biology pioneers at the end of the 19th century including Morgan, Driesch and Spemann [1]. In early embryos and throughout development, morphogen gradients are used repeatedly to pattern fields of cells in a concentration-dependent manner. How pattern scaling is achieved within this model represents a long standing problem, already observed by Wolpert's "French flag" analogy: morphogen gradients will divide the flag into three domains, blue, white, and red. Scaling requires that this same relative division will be maintained regardless of the size of the flag. This is the essence of scaling: the proportions within and between different tissues (the colors of the flag) remain constant, regardless of the size of the tissue or embryo [2]. Several experimental systems have been developed to study morphogen gradient scaling, most notably the *Drosophila* embryo anterior-posterior (AP) and dorso-ventral (DV) patterning, and the AP patterning along the Drosophila wing imaginal disc [3–10]. These studies confirm that scaling occurs already at the very initial stages of embryogenesis.

Different theoretical models have been put forward to explain scaling [11–15]. In particular, we recently found that scaling is naturally achieved by a feedback motif termed expansion-repression (ExR), in which expression of a stable and diffusible expander protein that increases morphogen spread is repressed by morphogen signaling [6, 16, 17] (Fig. 1A). In effect, the expander "measures" the size of the tissue as its levels accumulate: in larger embryos, expander levels continue to increase until the morphogen gradient is wide enough to repress expander expression in distal regions. Morphogen concentration at the distal border is therefore pinned to a fixed value, defined by the threshold for expander repression.

Patterning the dorso-ventral axis in *Xenopus* embryos: A model for morphogen gradient scaling

Seminal work established the amphibian embryo as a classical model for pattern scaling. Spemann [1] found that dorsal-halved newt embryos develop into normally patterned

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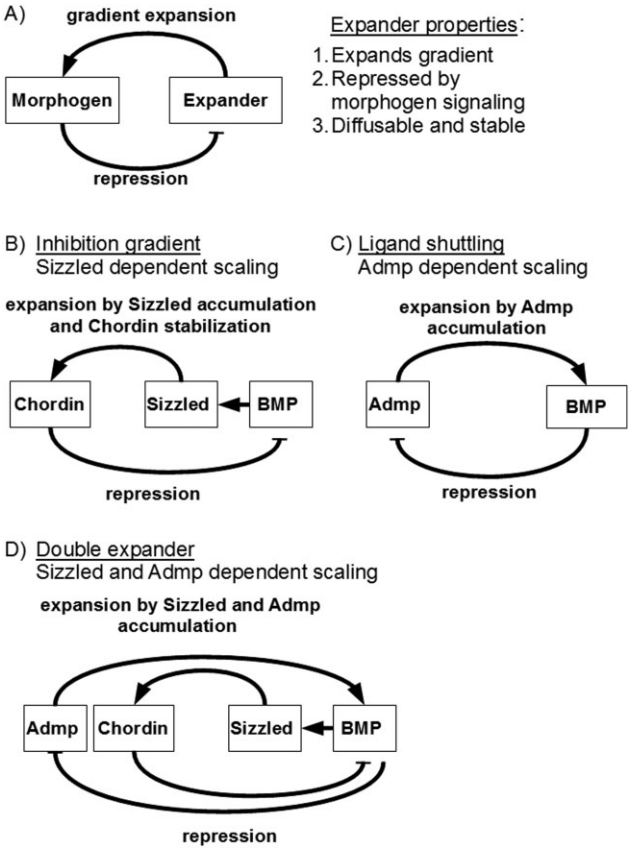

Figure 1. Proposed implementations of the expansion-repression model in *Xenopus laevis* dorso-ventral patterning. **A:** *The expansion-repression model*: morphogen signaling represses production of the expander, a diffusible and stable molecule that expands the morphogen gradient, e.g. by stabilizing it or increasing its diffusion. The gradient expands until the expander is repressed distally, pinning distal signaling level to its repression threshold. **B:** *Scaling by sizzled-dependent ExR motif*: sizzled can function as an expander of the BMP antagonist chordin gradient. It is stable and diffusible, stabilizes chordin by inhibiting its protease, and its expression requires high BMP signaling and is therefore repressed by chordin. **C:** *Scaling by Admp-dependent ExR motif*: Admp can function as an expander for BMP signaling. It is shuttled across the dorso-ventral axis, repressed by BMP signaling, and expands the gradient by competing with other BMP ligands over ventral shuttling by chordin. **D:** *A dual expander model*: the model suggested by Inomata and colleagues makes use of both the sizzled and Admp expander. The two expanders set the ventral and dorsal signaling levels by their induction and repression thresholds, accordingly.

tadpoles, an experiment that was later reproduced in *Xenopus* [18]. The fact that mesodermal tissue proportions are maintained by adjusting the pattern (rather than recovering normal size) was verified by Cooke [19, 20], who quantified the number of cells assigned to each tissue in size reduced embryos. Scaling of pattern was evident along both AP and DV axes.

In *Xenopus* embryos, as in many other metazoans, DV patterning is guided by a gradient of bone morphogenic protein (BMP) morphogen activity [21–25]. In vertebrates, and in particular in *Xenopus*, the BMP gradient peaks at the ventral pole [18, 26]. Four BMP ligands contribute to this gradient, three of which are initially broadly expressed, while expression of the fourth, *admp* is limited to the dorsal pole [27, 28]. Notably, *bmp2* is also expressed dorsally at later stages of gastrulation [29, 46]. An additional critical factor for gradient formation is chordin, a diffusible, evolutionary conserved BMP ligand inhibitor that is expressed at the dorsal pole [30, 31]. Chordin contributes to the formation of the BMP activity gradient in two ways. First, as it forms a concentration gradient that peaks at the dorsal pole, it inhibits BMP ligand activity preferentially on that side, leading to an inverse gradient of BMP activity. Second, as chordin binds to BMP ligands, its diffusive flux translocates ("shuttles") the ligands ventrally, establishing a concentration gradient of the BMP ligands themselves. The relative contributions of the chordin inhibition gradient versus ligand shuttling to the BMP activation gradient depend on kinetic parameters, including the degradation of free chordin, BMP ligand diffusion, and the BMP-chordin association rate [32].

We previously proposed a model, which explains this scaling of the BMP activation gradient. This model attributes scaling to Admp, a BMP ligand that is repressed by BMP signaling [32]. Admp protein spreads from dorsal to ventral regions, and effectively expands the BMP signaling gradient [27]. It can therefore function as an expander in this system, and scaling is indeed achieved through the ExR mechanisms (Fig. 1C). Quantitatively, scaling by the Admp-dependent ExR motif is most precise when shuttling is the dominant process establishing the BMP activity gradient, as in this case Admp competes with the other BMP ligands for the chordin-dependent shuttling and in this way modulates the gradient length scale, rather than merely its amplitude.

A sizzled-dependent ExR-based scaling

The scaling model we proposed can explain many properties of the system, yet questions were raised about some of its assumptions and in particular the ability of dorsal-halved embryos to accurately scale their pattern, and the dominant role of Admp as an expander [33]. Studying the same system, Inomata et al. confirmed that scaling indeed occurs in dorsal-halved embryos. They further propose alternative means by which scaling is achieved, based on an expander-like role of the secreted protein sizzled [29, 34] (Fig. 1B).

Sizzled is a secreted protein that was shown to inhibit the activity of Xolloid, a Tolloid class chordin protease. It is induced in the ventral region of the embryo in response to high BMP signaling [35, 36]. While those properties of sizzled were known, their possible role in scaling was not considered. To study sizzled function in more depth, Inomata et al. [29] have reconstituted the DV axis in the embryo by inhibiting the primary β-catenin organizer induction signal, and injecting *chordin* mRNA at the would-be new organizer. *chordin* mRNA was indeed sufficient to reconstitute the entire DV axis providing a controlled system where properties of the different proteins can be tested. Sizzled was shown to regulate the range of chordin activity by affecting its stability, rather than its diffusion. The induction of *sizzled* transcription by high BMP signaling in regions where chordin is low, was verified.

Together, these results suggested that chordin and sizzled form a long-range feedback loop constituting the ExR motif: chordin diffusion restricts the *sizzled* expression domain, while sizzled expands chordin distribution. Indeed, a system with only sizzled, chordin, and ventrally-expressed BMP ligand scales well, and is relatively robust to biochemical parameters, as expected from an ExR motif (Fig. 2A and B).

Testing this notion experimentally, Inomata et al. [29] reported that chordin levels and stability increase with time, in a manner that correlated with accumulation of sizzled, while *sizzled* transcript levels decreased in a chordin-dependent manner. Repression of *sizzled* led to lower levels of chordin and to the ventralization of the embryo. This ventralization was more prominent than the dorsalization observed following *admp* repression. Following Spemann's experiment, Inomata et al. showed that dorsal half embryos scale very well, and that *sizzled* expression is essential for this scaling. The results were complemented by a mathematical model that considered the roles of Admp, BMP ligands, chordin, and sizzled in patterning and scaling, and was indeed sufficient to explain scaling as well as other properties of DV axis patterning.

Distinguishing the sizzled versus Admp-based scaling models

Scaling of the early BMP gradient can be theoretically explained by at least two ExR-based models, which differ in the key protein playing the role of the expander (sizzled vs. Admp) (Fig. 1B and C). Which of those models functions in embryos? The key question is whether the BMP activity gradient is formed by a ligand shuttling mechanism (as required for Admp-mediated scaling), similar to *Drosophila* embryonic DV patterning [37–41], or if the gradient is reciprocal to a chordin inhibition gradient (as required for sizzled-mediated scaling). In addition to this global functional distinction, we note here three specific biochemical aspects that distinguish the models:

(1) The most fundamental aspect differentiating the two models is the degradation of chordin by proteases. Degradation of free chordin is central for the chordin-mediated scaling model as it enables sizzled to directly impact the length scale of free chordin, which in turn defines the width of the reciprocal BMP activity gradient. In contrast, the Admp-mediated scaling functions more effectively when chordin degradation is facilitated by BMP binding, as in this limit shuttling dominates, and Admp levels affect the width of the BMP activity gradient. Inomata et al. [29] and others [42] have shown that unlike the case in *Drosophila* [43], Tolloid class proteases can cleave chordin effectively when alone or in complex with BMP ligands. Moreover, when a vertebrate chordin-like construct is expressed in *Drosophila* instead of the endogenous ortholog, Sog, the shape of the BMP gradient changed significantly [44], indicating that the ligand-dependent cleavage of Sog is characteristic of *Drosophila* and not vertebrates. Other studies implied that simultaneous binding of Bmp4 to chordin and the adaptor protein xTsg significantly increases the affinity of the protease to the chordin-BMP complex [45]. Pin pointing the critical factors that affect chordin stability in vivo is particularly challenging since many proteins potentially affect chordin stability such as Xolloid Related, Bmp4, Admp, xTsg, and Ont1, a scaffold for the chordin-protease interaction [46], are targets of BMP activity.

(2) Another key parameter is the relative diffusion of BMP ligands when free or bound to chordin. Shuttling occurs when BMP ligands diffuse preferentially when bound to chordin. This happens when free BMP does not readily diffuse (e.g. when free ligand rapidly binds to membrane-bound receptors and additional modulators [47, 48]), or when chordin is highly abundant such that free BMP rapidly forms a complex with chordin [32, 37, 39], as is the case analyzed by Inomata et al. (Fig. 2C). Shuttling is central for the Admp-mediated scaling we proposed, and we provided evidence that diffusion of free Bmp4 is greatly facilitated by chordin in embryos [32].

(3) The final assumption required by Admp-mediated scaling but not by chordin-mediated scaling is the higher affinity of chordin to the broadly expressed BMP ligands versus lower affinity to Admp. This assumption is required for Admp-dependent modulation of the gradient length scale and is supported by experimental observations [27, 49].

A global scaling model depending on both sizzled and Admp

While either Admp or sizzled can provide scaling on their own through implementation of the ExR described above, one can envision a range of scaling models that make use of both expanders. In fact, the mathematical model presented by Inomata and colleagues, exploits the expander properties of both Admp and sizzled to explain scaling. In this model, Admp is shuttled ventrally by chordin, where it expands the BMP signaling gradient: ventral Admp complements the other BMPs in inducing *sizzled* expression, thereby expanding the chordin gradient, which further impacts the BMP signaling gradient in the entire embryo (Fig. 1D). Admp accumulates until the BMP activity gradient is wide enough to repress *admp* expression dorsally, at which point the system approaches a scaled steady state. Hence, Admp tunes signaling throughout most of the embryo indirectly, through *sizzled* regulation, and in particular at the dorsal region where its signaling is dominant. Notably, also in this model, transport of Admp depends on its shuttling by chordin (Fig. 2C): reducing the diffusion of free Admp/BMP by 10-fold has no effect on the gradient, whereas reducing the diffusion of the chordin-Admp/BMP complex abrogates the pattern. Consistent with Admp function as an expander, changing the threshold for *admp* repression alters the gradient length scale, so that its distal-most level is tuned to the new repression threshold (Fig. 2D), and Admp levels correlate with embryo size (Fig. 2E). Removal of Admp in this model abrogates pattern scaling and results in complete dorsalization of the system if allowed to

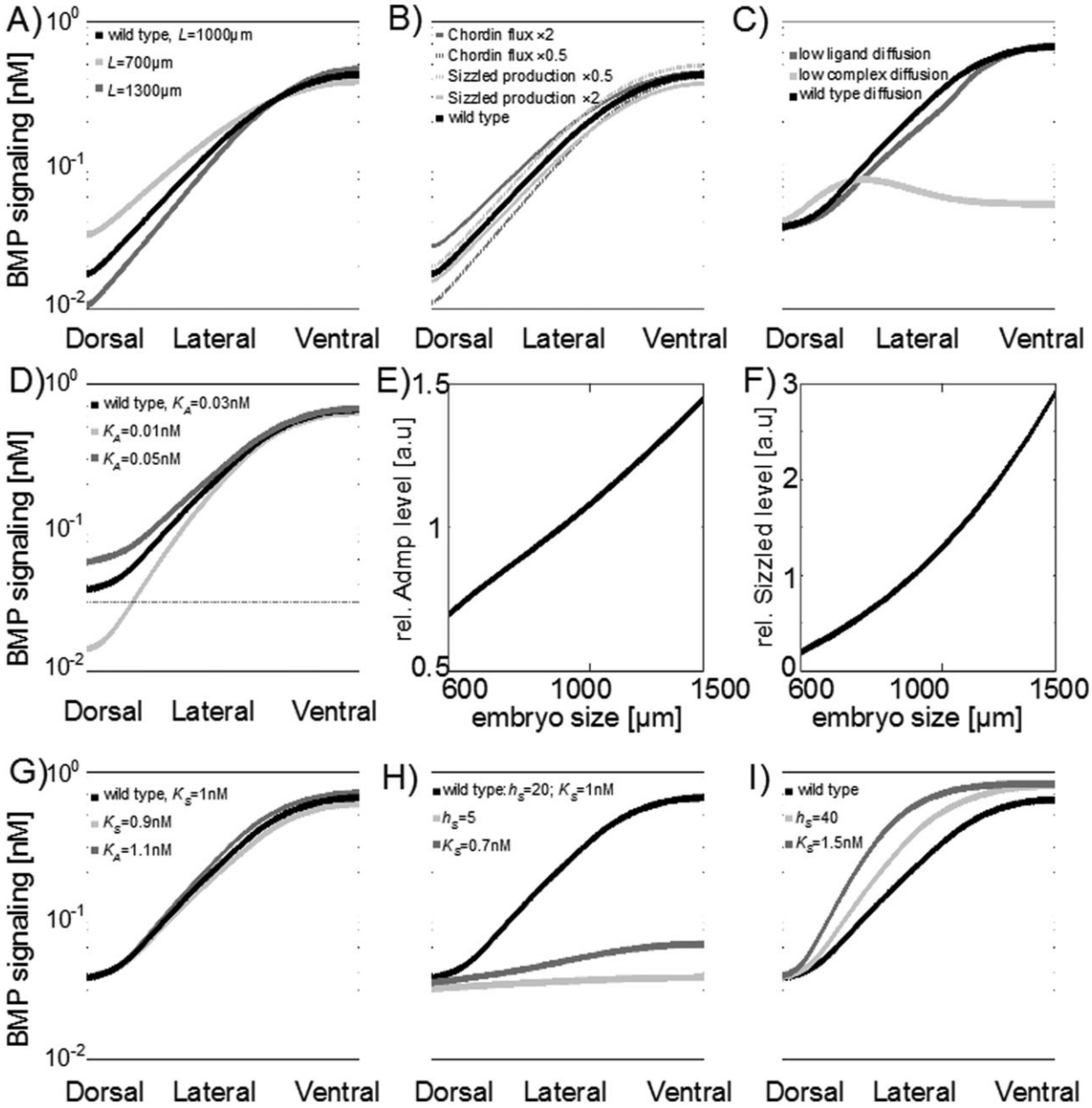

Figure 2. Simulation of sizzled-dependent scaling models. **A** and **B:** *Sizzled-dependent ExR motif* (Fig. 1B): the model is based on the full Inomata model (Fig. 1D) but does not include Admp, saturation of chordin degradation and regulation of chordin or BMP expression. A: *Scaling* is observed mostly in ventral regions. B: *Robustness* of the pattern to perturbations in the sizzled and chordin production rates. For the equations and parameters used for generating this model, see Box 1. **C–I**: *A dual expander model*: the simulations are based on the full Inomata model. In the case of wild-type the parameters correspond to the parameters provided in [1]. C: *Diffusion of free BMP/Admp does not contribute to the activation gradient*: BMP activation profile is shown in wild-type parameters, upon 10-fold reduction in ligand diffusion and upon 10-fold reduction in chordin-ligand complex diffusion. The profile is largely invariant to changes in free ligand diffusion, but fails to form when the diffusion of the ligand-chordin complex is reduced, highlighting the role of ligand shuttling by chordin. Therefore, ventral ligand accumulation is largely dependent on chordin-mediated ligand shuttling. D: *Dorsal activation level follows the admp repression threshold*: BMP activation profile in a range of *admp* repression thresholds, K_A (dashed). Dorsal signaling levels follow the *admp* repression threshold. E: *Admp levels in different size embryos*: average levels of Admp as function of embryo size in steady state. Admp levels are normalized to the level in the wild type system $L = 1{,}000\,\mu m$. Admp levels correlate with embryo size. F: *Sizzled levels in different size embryos*: average levels of sizzled as function of embryo size, L. Sizzled levels are normalized to the level in the wild type systems $L = 1{,}000\,\mu m$. Sizzled levels correlate with embryo size. G: *Ventral activation level follows the sizzled induction threshold*: BMP activation profile in a range of *sizzled* induction thresholds, K_S. Ventral signaling levels follow the *sizzled* induction threshold. Rapid sizzled production rates lead to lower ventral signaling than the *sizzled* induction threshold. H and I: *Sensitivity to the parameters controlling sizzled induction*: reduction of the *sizzled* induction Hill coefficient h_S, or its induction threshold K_S results in dorsalization (H), while increasing *sizzled* induction Hill coefficient h_S or its induction threshold K_S results in ventralization of the profile (I).

Box 1

Equations and parameters for the model in Fig. 2A and B:

$$\frac{\partial C}{\partial t} = D\nabla^2 C - \frac{\lambda_C}{1+(S/K_i)}C - kBC \quad (1)$$

$$\frac{\partial B}{\partial t} = D\nabla^2 B - \frac{\lambda_C}{1+(S/K_i)[\mathrm{CB}]} - kBC \quad (2)$$

$$\frac{\partial [\mathrm{CB}]}{\partial t} = D\nabla^2 [\mathrm{CB}] - \frac{\lambda_C}{1+(S/K_i)[\mathrm{CB}]} + kBC \quad (3)$$

$$\frac{\partial S}{\partial t} = D\nabla^2 S + V_S - \frac{B^h}{B^h + K_S^h} \quad (4)$$

C, B, [CB], and S denote chordin, BMP, chordin-BMP complex, and sizzled concentrations, accordingly. When possible, parameters were matched with those used by Inomata et al. $D = 15\,\mu m^2$/second, $\lambda_C = 10^{-3}$ second^{-1}, $K_i = 25$ nM, $k = 0.28 \times 10^{-3}$ nM^{-1} second^{-1}, $h = 5$, $K_S = 2$ nM, $V_S = 0.01$ nM/second. Initial conditions were zero concentration for all molecular species except initial BMP level of 1 nM. Boundary conditions were no flux in all cases, except a dorsal flux of chordin $J_C = 4.8\,\mu$m nM/second.

reach steady state, uncovering a role for Admp in stabilizing patterning.

sizzled regulation is critical for scaling; as shown by Inomata et al., sizzled levels correlate with embryo size and the span of the chordin and BMP gradients. Thus, sizzled levels "measure" embryo size (Fig. 2F), and BMP signaling in the ventral pole is set by the effective *sizzled* induction threshold (Fig. 2G). The significance of tight regulation over *sizzled* expression is demonstrated by the high sensitivity of the model to the "all or none" nature of *sizzled* induction (Hill coefficient = 20) and its fast production rate. Indeed, deviation from these parameters results in either contraction of the gradient (reduced sizzled levels leading to enhanced chordin degradation), or complete inhibition of BMP signaling (too much sizzled leading to a chordin gradient that is too broad; Fig. 2H and I). Together, the double expander system mediates scaling by pinning the BMP signaling values in the dorsal and ventral sides to the *admp* repression and *sizzled* induction thresholds, respectively.

Perspective and future directions

The relative contribution of Admp or sizzled to scaling is yet to be determined experimentally. Neither of the studies measured the scaling of the gradient in the absence of Admp or sizzled. Clearly, the sizzled-mediated inhibition gradient model predicts that scaling will be lost when sizzled is depleted, while the Admp-mediated shuttling model predicts the same when Admp is depleted. Admp was shown to be essential for patterning halved embryos [27], but this may represent an extreme case in which Admp is the dominant BMP ligand affecting earlier processes, and not merely a problem of scaling. It is important to distinguish between a patterning and a scaling phenotype: Inhibition of *sizzled* expression will lead to ventralization of the embryo since chordin will be degraded faster by its proteases. The question is whether this distorted pattern will scale with the size of the embryo. In the case of Admp, this question becomes even more complex as it also has a role in head induction and interaction with other signaling pathways [49, 50]. Again, *Xenopus* stands out as a highly suitable model to study this question, since it is amenable to experimental manipulation of embryo size, and it displays natural variability in oocyte sizes.

Similarly, neither of the studies rigorously measured the temporal signaling in size-perturbed embryos. More quantitative experiments are therefore required, using natural variability or induced changes in size to generate smaller and larger embryos. Another key experiment would be to measure the levels of sizzled and Admp in size-modulated embryos. Indeed, as both models predict the level of expander to increase with embryo size, quantifying their levels in different size embryos will therefore be important for distinguishing the underlying mechanisms. Alternatively, it will be interesting to ask if the shape of the activation gradient is sensitive to a moderate ectopic increase in the level of sizzled or Admp, since it is the accumulating amount of expander, which determines the final length scale. Finally, an experiment that is more challenging but may be highly informative involves modulating the thresholds for *sizzled* induction or *admp* repression, and measuring the predicted consequences on the shape of the gradient.

In conclusion, further experiments will reveal the regimens in which each of scaling mechanisms functions, and the possible conjunction between them. What becomes increasingly clear is that mathematical analysis will be indispensable in the process of pinpointing the patterning mechanism for defining how scaling occurs in this system and others. Any proposed mechanism should be consistent with available mutant phenotypes, to examine if it can correctly predict the dynamics of gradient formation, differentiate between steady state and pre-steady state properties and provide some robustness to parameters (e.g. Hill coefficients or reaction rate constants). As emphasized by the present analysis, qualitative intuition may need to be modified, once a quantitative description is attempted. This rich and exciting system promises a productive avenue for further systems-biology investigations that will likely reveal general design principles of patterning networks used in the development of multicellular organisms.

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