REVIEW

Incredible journey: how do developmental signals travel through tissue?

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How developmental signaling proteins traverse tissue during animal development, through or around tightly packed cells, remains an incompletely resolved mystery. Signaling protein movement is regulated to create gradients, control amounts, impose barriers, or provide direction. Signaling can be controlled by the rate of signal production, modification, active transport, trapping along the path, or by the properties of the receptor apparatus. Signals may move by diffusion outside cells, attached to migrating cells, attached to carrier molecules, through cells by transcytosis, along cell extensions, or in released membrane packets. Recent findings about the movement of Hedgehog, Wntless (Wnt), and TGF- β signaling proteins have helped to clarify the molecular mechanisms used to ensure that developmental signals carry only good news.

The construction of an animal, with a single fertilized egg cell becoming thousands or tens of trillions of cells, requires exquisite coordination. Rather few types of protein signals, operating repeatedly, coordinate cell fate decisions and tissue and organ growth. These signals reliably guide growth and patterning despite variations between individuals in parameters such as temperature, body size, number of cells, and metabolic and nutritional status. How can developmental signals be so dependable? An equally severe problem emerges from examining the close apposition of cells in many tissues through which signals travel. Hormones can travel through blood, and some signals can penetrate cell membranes, but what about fairly large signaling proteins that appear to traverse tightly packed masses of cells?

The problem is less severe because, by and large, developmental signals do not have far to go. Many critical cell fate-determining events occur in small early embryos or when organ primordia are minuscule. The sig-

nals follow the political slogan: think globally, act locally. Signaling proteins generally have to work over distances of one to tens of cells, but the decisions made have enormous implications for the larger organs that grow from the primordia. The short-range action of most signals may reflect their evolution in organisms composed of one or a small number of cells.

In some cases signals form quantitative gradients that induce distinct cellular responses in a concentration-dependent manner. The concept was originally defined by Turing (1952), in reference to plant development: "The systems actually to be considered consist therefore of masses of tissues which are not growing, but within which certain substances are reacting chemically, and through which they are diffusing. These substances will be called morphogens, the word being intended to convey the idea of a form producer. It is not intended to have any very exact meaning." For a long time his last comment was rigorously and frustratingly honored, but in recent years evidence has accumulated for the existence of morphogens, both from observing protein gradients and from demonstrations that cells are capable of responding differently depending on the amount of a signal (e.g., see Nellen et al. 1996; Zecca et al. 1996; Gurdon et al. 1998; Briscoe et al. 2001; Chen and Schier 2001). Whether protein signals are acting in a concentration-dependent manner or not, the mechanisms underlying their travel to receiving cells remain obscure.

As we discuss further below, a great deal of hard work has been done on the problem of morphogen movement, but the central mysteries still remain. Much better ways of imaging functioning morphogens are needed. It would be particularly valuable to distinguish molecules that are actually in transit, or actually bound to a relevant cellular receptor, from bulk signaling proteins. We discuss movements of the signaling proteins Hedgehog (Hh; 257 amino acids), Wntless (Wg, a Wnt; 468 amino acids), and TGF- β family members such as Decapentaplegic (Dpp; 372 amino acids), bone morphogenetic proteins (BMPs; ~400 amino acids), and Activin (~370 amino acids) as examples, along with a few other relevant cases. Excellent reviews of morphogens (Tabata and Takei 2004), modifications and reception of Wnt and Hh signals

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(Nusse 2003), endocytosis in signaling (González-Gaitán 2003; Piddini and Vincent 2003), and signal trafficking (Vincent and Dubois 2002) provide additional information.

Central questions that this review address include (1) How far can proteins move through tissue? (2) Do signals travel through, around, or on the surfaces of cells, and if so how? (3) How do modifications of protein signals or associations with other proteins affect signaling processes? (4) What factors in, on, or around cells affect signal movements? (5) How do cell movements or the extensions of cell processes contribute to signaling? (6) How does processing of signals bound to receptors affect ongoing signaling? and (7) Does the same signaling protein use different mechanisms in traversing different tissues? In most cases the reason that a particular signaling protein is used to control a developmental process also remains mysterious. Why Wnt versus Hh versus TGF- β ? The choices may have been historical accidents, but greater understanding of how signals move may reveal why organisms evolved the use of certain signals for certain purposes. How fast a morphogen gradient is established may have dictated the choice of signal. Slower moving signals might have been unsuitable alternatives to fast ones, and vice versa. The most sophisticated studies of morphogen properties and functions in cell-cell signaling have been done in *Drosophila* imaginal discs (in particular, the precursors to wings and legs), in the vertebrate neural tube, and in the vertebrate limb; the arrangement of cells and signals in those tissues is shown in Figure 1.

Direct action of graded amounts of morphogen proteins at a distance from their source

The simplest mechanism for signal movement is passive diffusion: signal produced by localized cells diffuses freely through extracellular space (Fig. 2a). Accumulating evidence supports some type of diffusion mechanism for movement of Wg and Hh signals (Strigini and Cohen 2000; Han et al. 2004b), while Dpp appears to move both through extracellular spaces and by entering and leaving cells, a process called transcytosis (Entchev et al. 2000). Here we discuss data bearing on the range of direct action of a morphogen and how to distinguish direct from indirect influences of signals.

As a signal moves away from its source, it is diluted and/or degraded, generating a concentration gradient. Cells of the slime mold *Dictyostelium*, for example, emit cAMP, and it forms such a gradient; secretion of steroid hormones into the circulation is an example of relatively free movement. Erwin Schrödinger's influential book *What Is Life?* (Schrödinger 1944), a physicist's view of biology, includes a discussion of how the unpredictable and independent behaviors of individual molecules during diffusion collectively give rise to a statistically predictable behavior. However, that degree of order may be inadequate for developmental signals, and much thought is now given to mechanisms that help the signal to move and that influence the shape of the gradient.

Figure 1. Developing tissues for signaling studies. (a) *Drosophila* wing imaginal discs are one of the most intensively studied tissues for morphogen gradient formation. Wing discs are epithelial structures that eventually give rise to adult wing blade and thorax. Wing imaginal disc is divided into anterior (A) and posterior (P) compartments. Two morphogens, Hh and Dpp, operate to pattern the adult wing development. Hh protein produced by posterior disc cells (shown in red) reaches across the A–P boundary to activate Hh-responsive genes, including *en*, *dpp*, and *ptc* (shown in green) in a stripe in the A compartment. These three target genes are essential for the proper patterning of the adult wing blade. (b) The vertebrate neural tube provides another example of graded signaling protein activity. By embryonic day 10.5 (E10.5), Shh protein (shown in purple), produced from notochord (nc) and later on from the floor plate (fp), induces different neuronal cell fates along the dorsal–ventral axis, by establishing graded Hh target gene activity (as demonstrated here by a *ptc-lacZ* gradient shown in blue). (c,d) Shh is a key signal in the formation of the developing vertebrate limb bud. By E10.5, Shh (shown in purple) is produced in the posterior limb bud, a region known as the Zone of Polarizing Activity (ZPA). High concentrations of Shh promote posterior digit formation (digits 4 and 5), with successively lower amounts promoting more anterior digit patterning (digits 2 and 3). The most anterior digit (digit 1) is formed due to the absence of Shh signaling from the ZPA.

A signal could be produced in one cell and influence a neighboring cell, which produces another signal, and so on through a series of relays, or a signal could act at a distance on many cells, perhaps in a concentration-dependent manner. How does one tell that a signal is received at some distance from its source? About a decade ago, Gurdon et al. (1994) performed an elegant experiment using an assay in which parts of frog embryos are cultured and the cells are monitored for their response to a secreted protein. The signal source was restricted by injecting RNA encoding the signal into only a particular embryonic cell. In the first experiments the TGF- β -class protein Activin was used. Activin induced target gene activation in a dose-dependent manner at a distance up to 300 μ m from its source, through or around about a dozen fairly large cells. They showed convincingly that this gradient is formed via passive diffusion, rather than through a cell-by-cell relay mechanism (Gurdon et al. 1994), and that the signal can move this far in just a few hours. Wg is also a fast diffuser, traveling at least 50 μ m in 30 min (Strigini and Cohen 2000), a rate comparable to that of Activin. Using beads coated with TGF- β 1 and implanted in frog embryos, TGF- β 1 was found to form a

Figure 2. Models of the establishment of morphogen gradients. (a) Morphogens are signaling proteins that are secreted from a source (cells shown in blue). A functional morphogen gradient could be formed entirely by passive diffusion away from the source in extracellular space, either through dilution or degradation. As a consequence of the gradient, cells respond differently in activating downstream signal transduction cascades, as shown by the graded target gene transcription. (b) Alternatively, the morphogen gradient can be generated by some sort of active transport process, or diffusion influenced by molecules along the way. One example is planar transcytosis, a process that involves multiple rounds of receptor-mediated endocytosis, trafficking through the endocytic pathway, and exocytosis. (c) Another type of signal movement is regulated by heparin sulfate proteoglycans (HSPGs). GPI-anchored HSPGs could act in a "bucket brigade" fashion to pass morphogen molecules between neighboring cells. Alternatively, HSPGs may act as co-receptors to create a high concentration of morphogen, or morphogen in a certain conformation or modification state, that can bind to receptors nearby after release from the HSPGs.

gradient extending up to seven cell diameters (~150–200 μm) from its source (McDowell et al. 2001). Most of the TGF- β 1 was detected in extracellular spaces. Most importantly, internalization is not required for gradient formation, since the TGF- β 1 gradient forms at 4°C, a temperature at which endocytosis is inhibited. This experiment suggests that, at least in this artificial cultured embryo assay, a functional morphogen gradient can be formed entirely by a passive diffusion mechanism.

Genetic tests have also been used to find out whether a signal acts directly on cells at a distance. One way to distinguish direct from indirect action of a signaling protein is to measure the range of action of released versus tethered signaling proteins; the latter would still trigger a relay. This analysis was applied to Wg (Wnt) signaling in *Drosophila* imaginal discs. The results showed that tethered protein can act only over short distances (Zecca et al. 1996), whereas normal Wg can act in graded fashion at a distance. A second approach is to see whether a signal's receptor is required in all responding cells or just nearby ones. This logic was applied to Dpp signaling in *Drosophila* imaginal discs (Nellen et al. 1996). The Dpp signal was found to work directly on distant cells.

Similar logic was applied to analyze Hedgehog (Hh) signaling in the vertebrate neural tube (Fig. 1b). The experiments tested whether Hh effects on cells at a distance are mediated by Smoothened (Smo), a transmembrane protein that transduces the Hh signal. If Hh were to work by driving production of another signal, Smo would not be relevant in cells that receive only the other signal. This approach was used in studies of the vertebrate spinal cord (Wijgerde et al. 2003). Chimeric mice were constructed lacking Smo in cells in different dorsal to ventral positions in the spinal cord, and all ventral cell fates were found to depend on a direct signal from Sonic Hh (Shh) or Indian Hh. No evidence for a signal relay was found. A related study was done using a mutant form of the Patched (Ptc) receptor that is unable to bind Hh but is able to inhibit Smo (Briscoe et al. 2001). In both fly imaginal discs and in the vertebrate (chick) neural tube, such a Ptc protein blocks Hh signal reception and induction of target genes. In the neural tube, because cells making the altered Ptc fail to respond to a Hh signal even if they lie a long distance from the source, it is the Hh signal that matters and not some other relaying mediator. Therefore, Hh proteins move over many cell diameters to signal in the neural tube.

Shh is a key signal in the formation of the developing vertebrate limb bud (Fig. 1c,d). Shh is produced by mesoderm cells in the posterior bud, a region known from early transplantation studies as the Zone of Polarizing Activity (ZPA). If a ZPA, or beads containing Shh, is implanted in the anterior limb bud, the limb is repolarized so that duplicate posterior structures form in the anterior and the limb grows a set of approximately mirror-image digits. High concentrations of Shh promote posterior digit formation, with successively lower amounts promoting more anterior digit patterning (Yang et al. 1997). The range of action of Shh in this situation has raised longstanding questions: does Shh act directly on cells at a distance, as a morphogen, or is there a relay? Do cells always respond the same way to a given concentration or does their response change in time or space? Candidates for the relaying signal include members of the BMP class of TGF- β signals, which are produced in response to Shh. Direct observation of Shh protein has revealed its movement into more anterior limb bud regions (Lewis et al. 2001), a pattern reflected in the transcription patterns of two direct target genes, *ptc1* and *Gli1*.

Recent studies have used cells indelibly marked with *lacZ* expression to follow movements and fates of cells as the limb develops (Ahn and Joyner 2004; Harfe et al. 2004). In one study (Harfe et al. 2004), a Cre recombinase coding sequence was inserted into the Shh gene. In combination with a *lacZ* gene flanked by loxP sites, this allows all cells that have ever expressed the Shh gene, and their descendants, to become marked with β -gal. A further refinement was to use a form of Cre that is activated by the drug tamoxifen, so that the time of cell marking can be controlled. The authors found that cells derived from the ZPA in the posterior limb bud spread widely toward the anterior, something that was not ap-

parent from watching *Shh* transcription, since that remains confined to the posterior. The cell expansion is not sufficient, however, to explain the graded *Shh* distribution, since the *Shh* protein goes farther anteriorly than the spreading ZPA-derived cells. Many cells derived from the ZPA contribute to digits 3, 4, and 5, that is, the three most posterior of the five digits, so how are the different digit shapes controlled by *Shh*? There seems to be little difference in the amount of *Shh*. Instead, the authors show that the type of digit formed is due to the amount of time that cells have been exposed to *Shh*. Cells that move away first, toward their digit 3 destination, are exposed to *Shh* for a shorter time, while later departing cells see more *Shh* and form digit 4, and so forth. The cells have a type of memory, carrying out their fates according to *Shh* exposure that happened some time before. The time-of-exposure model helps to explain earlier data where a nondiffusing variant of *Shh* was nonetheless able to differentially pattern digits 4 and 5 (Lewis et al. 2001). That result can now be viewed as depending on the time of exposure of cells to *Shh* (Harfe et al. 2004). The authors conclude that the effect of *Shh* is controlled by both temporal and spatial gradients.

In a different application of the *lacZ* marker approach, a Cre recombinase gene was inserted into the *Shh* target gene *Gli1*, and this in combination with a *lacZ* gene flanked by loxP sites gave rise to β -gal expression in cells that received the *Shh* signal (Ahn and Joyner 2004). As the same tamoxifen-activated version of Cre was used, the reporter gene activation is controlled in time with the drug and in space by the *Shh* signal. The authors found that all posterior mesenchyme and ectoderm cells respond to *Shh* from the ZPA, but with time the most posterior cells stop responding to *Shh*. Cells all the way to the digit 2 primordium respond directly to *Shh*, thus the protein is, indeed, a long-distance signal.

The movement of signaling protein through tissue must be governed so that the receiving cell can properly interpret spatial gradients or temporal changes in protein concentration. There is some controversy about whether the amount of signal received by a cell is measured as an absolute, that is, the number of receptors occupied by ligand, or as an interpretation of the ratio of bound to unbound receptors. As in *Hh* signaling during limb development, the duration of contact between a cell and a signal source may also be important. Similarly in frog embryo experiments, 100–300 molecules of Activin, occupying 2%–6% of the total receptors on a receiving cell, are capable of triggering target gene expression changes. Most importantly, overexpressing Activin receptors does not change gene responses (Dyson and Gurdon 1998). These experiments showed that the absolute number of receptors occupied matters, rather than the ratio of bound to unbound receptors.

The interpretation of signals by receptors has been studied for *Hh* signaling in mammalian tissue culture and in fly imaginal discs, and the results have been rather different. *Hh* signal has a peculiar relationship with its receptor Ptc: *Hh* blocks Ptc receptor from repressing target gene transcription. In mammalian tissue

culture cells, the amount of unliganded *Hh*-free Ptc is what determines the amount of repression (Taipale et al. 2002).

A recent study of *Hh* signaling in imaginal discs (Casali and Struhl 2004) used modified Ptc proteins to control the ratio of *Hh*-bound to unbound Ptc. One modification was a covalent fusion of *Hh* to Ptc. If only unliganded Ptc is active in repression, a self-inactivating *Hh*-Ptc fusion should be unable to affect signaling. The second modified form was a partly deleted Ptc that cannot bind *Hh*; this form should repress regardless of the presence of *Hh*. The logic of the experiment was that if the amount of *Hh*-Ptc fusion has some influence on the activity of either wild-type protein or the non-*Hh*-binding Ptc, it is the ratio of bound to unbound forms that matters rather than the absolute amount of unliganded Ptc. This study found that the *Hh*-Ptc fusion inhibited the activity of both normal Ptc and the modified Ptc that cannot bind *Hh*. This implies an interaction between the liganded fusion protein and other Ptc molecules, or with other unknown molecules. Evidence in favor of Ptc multimerization comes from several dominant-negative forms of the protein (Johnson et al. 2000; Martin et al. 2001; Hime et al. 2004).

The activity of the *Hh*-Ptc fusion was interpreted as showing that the ratio of liganded to unliganded Ptc protein is important. How can this be reconciled with the results of the Activin experiments or earlier *Hh* experiments in cultured cells? First, Activin and *Hh* receptor systems may not read morphogen amounts in the same way, and different tissues may have distinct *Hh* response properties. Second, the lack of direct biochemical observation of the proteins may account for some of the discrepant results. Total protein levels measured, in the published work, with blots or microscopy, may or may not reflect the concentrations of active molecules. The biochemistry of the receptor system remains quite unclear, for example, in how other surface molecules interact with Ptc. Ptc (or *Hh*) may interact with other receptor components that are as yet invisible to us. The *Hh*-Ptc fusion might reduce repression by Ptc because the fusion protein competes for the attention of other proteins, such as coreceptors or modifying enzymes.

In summary, *Hh*, Wnt, and TGF- β proteins act at a distance after secretion, moving through or around cells, and the concentration of signal matters. How the concentration is accurately measured by cells is not yet clear. The amount of signal that reaches a recipient cell is profoundly influenced by cells and proteins along the way, events we will now examine.

Endocytosis and transcytosis can influence signal gradients and receptor abundance

Endocytosis is increasingly being scrutinized for its importance in signaling (González-Gaitán 2003; Piddini and Vincent 2003). If signals travel outside cells, endocytosis can influence gradient formation by serving as a sink for the signal, bound to its receptor. If signals travel through cells, endocytosis is one way to import a signal

for its passage across a cell; other uptake mechanisms could be equally or more important. Evidence for the importance of signal internalization comes from studies of a fusion of Wg (Wnt) signal to horseradish peroxidase (HRP), which allowed the fusion protein to be directly observed in fixed material. Using this tool it was found that Wg emerges from its source cells in the *Drosophila* embryo and moves both anterior and posterior, but the protein that moves toward the posterior is more rapidly degraded (Dubois et al. 2001). Components of the endocytosis pathway are required for Wg degradation. No differential degradation of secreted HRP is observed, thus the turnover mechanism recognizes the Wg part of the fusion protein.

Endocytosis has strong effects on receptor concentration and subcellular localization. In *Drosophila*, because mutations that eliminate the endosomal ubiquitin-binding protein Hrs cause the accumulation of Notch, TGF- β , Hh, and receptor tyrosine kinase (RTK) receptors, endocytosis of receptors could influence the dynamics of signaling (Lloyd et al. 2002; Jekely and Rørth 2003). In *hrs* mutants, RTK and Dpp signaling is facilitated. Endocytosis is used for the internalization of Frizzled4, a Wnt receptor, in cultured kidney cells (Chen et al. 2003b). The internalization and lysosomal destruction of Wnt and Frizzled is stimulated by the arrival of a Wnt signal and is facilitated by the action of Dishevelled2, a Wnt pathway component, in recruiting the adaptor protein β -arrestin2. Thus, cells carrying Wnt receptor will intercept and destroy the passing signal. A similar action of β -arrestin2 has been shown for TGF- β signaling (Chen et al. 2003a). β -arrestin2 can bind to type III TGF- β receptors, a single transmembrane domain protein that is distinct from the seven-transmembrane protein receptors found in the past to become linked to β -arrestin2. TGF- β receptors are constitutively internalized, whether or not ligand is present. Blocking receptor internalization by eliminating β -arrestin2 function caused increased TGF- β signaling.

Receptor–ligand interaction and endocytosis affect Hh protein gradient formation. Hh protein is secreted by posterior imaginal disc cells and is received by anterior disc cells within range of the Hh travel pattern (Fig. 1a). The anterior cells that receive sufficient Hh respond by producing a large amount of the Hh receptor, Ptc. The induced Ptc limits the range of the Hh signal by soaking it up (Chen and Struhl 1996; Burke et al. 1999). As is the case for many ligands, Hh protein is internalized by endocytosis and degraded after it associates with its Ptc receptor (Denef et al. 2000; Incardona et al. 2002). Hh and Ptc colocalize with endosome markers after Hh uptake, and they are both subsequently degraded. Therefore, the shape of the Hh gradient is affected by its the association with Ptc. However, surprisingly, blocking endocytosis in *Drosophila* imaginal wing discs with a Dynamin mutation (in *Drosophila*, *shibire*), or blocking Hh and Ptc protein degradation with a mutation in *deep orange* (*dor*; which encodes a protein required for normal delivery of proteins to lysosomes) has little effect on Hh target gene induction (Torroja et al. 2004). It appears, therefore, that

Hh signaling can do without the Dynamin and *dor*-mediated endocytosis and degradation pathway, and a receptor (Ptc)-independent mechanism may be used to modulate Hh signaling output.

The impact of endocytosis on Hh signaling was further tested using a mutant Ptc protein that cannot bind Hh or undergo endocytosis but can repress target gene transcription (Torroja et al. 2004). In imaginal disc cells containing only this form of Ptc, neither Ptc nor Hh efficiently reaches endosomes, and Hh and Ptc no longer colocalize. However, Hh does still move into cells in the mutant, as it does in the total absence of Ptc, thus not all Hh internalization depends on Ptc. The binding of Hh to Ptc affects the distance over which Hh moves, but events necessary for Hh importation and signal interpretation are, at least to a degree, separable from the endocytosis pathway.

A numerical analysis supports the significance of signal–receptor interactions in establishing gradients. Signaling proteins should decay rapidly close to their source but turn over at a much slower rate further away from the source (Eldar et al. 2003). Two network designs support robustness to fluctuations in signaling protein production rate. One design represents the Wg system in which signaling represses receptor expression and the receptor stabilizes free morphogen, as experimentally demonstrated by Cadigan et al. (1998) and Lecourtois et al. (2001). The other design fits very well with Hh signaling; signaling activates receptor expression, and receptor enhances the degradation of free signal (Chen and Struhl 1996; Incardona et al. 2002).

The importance of signals passing through cells emerged from studies of TGF- β signaling. Entchev et al. (2000) studied Dpp, a member of the TGF- β superfamily, in the context of wing development in *Drosophila*. Dpp signaling is essential to the formation of normal patterns of veins and bristles. Dpp emanates from a stripe of cells along the center of the disc (Fig. 1a) and controls cell fates at a distance of tens of cells, with cells nearer the source responding differently from those farther away. In wing discs, a gradient of Dpp stretching over 40 cells forms *in vivo* in ~8 h (Entchev et al. 2000). Concentration-dependent Dpp function was observed using a Dpp-green fluorescent protein (GFP) fusion. The Dpp-GFP spreads over a range of ~80 μ m, that is, beyond 40 cells away, up to and beyond the normal range of Dpp action. Dpp-GFP turns over rapidly; its movement cannot be accounted for by cell division (Teleman and Cohen 2000). Further experiments established that (1) the gradient of Dpp-GFP requires receptor-mediated endocytosis; (2) blocking endocytosis with a dynamin mutation impedes the movement of Dpp-GFP through target cells to more distant target cells; (3) the transmission of the signal is blocked by interfering with Rab 5, a GTPase necessary for formation of clathrin-coated vesicles; and (4) a gain-of-function Rab 7 protein caused increased destruction of the signal and consequently a shorter range of action of the signal (Entchev et al. 2000). Evidently, transmission of Dpp requires receptor-mediated movement into and through cells, “transcytosis” (Fig. 2b). A

key conclusion of the Dpp-GFP study is that receiving cells do not passively respond to a gradient. Instead, they actively shape the gradient.

The transcytosis model of the movement of Dpp and other proteins has been challenged by a mathematical study of gradient formation (Lander et al. 2002). They argue that the importance of endocytosis does not constitute an adequate test of whether movement of signals occurs by diffusion or transcytosis. Their calculations show that the inhibitory effects of endocytosis blockage can be consistent with some type of diffusion, if receptor kinetics are taken into consideration. Also, there are timing problems. The Dpp gradient is fully established within 8 h, thus the average time to cross a single cell is less than a minute. This is too fast to be compatible with estimated rates of transcytosis, which range from 20 min to 4 h for crossing one cell. The estimates are based on transcytotic rate constants for transferrin, EGF, and polymeric immunoglobulin receptors. Similarly, the “bucket brigade” relay (i.e., signal proteins are handed over from one cell to the next by translocating around the surface of the cell) proposed in modeling by Kerszberg and Wolpert (1998) would be too slow; at least in the case of TGF- β with its slow dissociation from its receptors. In a separate numerical simulation, Dillon et al. (2003) concluded that another secreted signal, Shh, travels through the chick limb by diffusion.

Although planar transcytosis appears to be important for long-distance Dpp transport, movement of Dpp can also take place in extracellular spaces. Within clones of imaginal disc cells lacking the Dpp receptor, Tkv, Dpp-GFP accumulates on the surfaces of the first several rows of cells closest to the signal source (Entchev et al. 2000). If Dpp-GFP moved solely by receptor-mediated transcytosis, it would accumulate only in the first row of mutant cells, thus either it moves outside cells or a receptor-independent transcytosis process occurs. High levels of Tkv can limit the distribution of Dpp in wing imaginal discs, probably by trapping extracellular Dpp (Lecuit and Cohen 1998; Tanimoto et al. 2000). Even in Gurdon’s animal cap experiments, Activin distribution is not established strictly through free diffusion. They showed that a high level of type II TGF- β receptor is necessary for detectable formation of the TGF- β 1 morphogen gradient, suggesting that the gradient forms in part owing to receptor–ligand interaction (McDowell et al. 2001).

Signal–inhibitor interactions and targeted degradation create signaling protein activity gradients

Receptors are not the only proteins that control signal abundance and activity. Interactions of a signaling protein with regulatory proteins can influence the location and level of signal activity. The amount of active protein need not correspond to the total amount of protein.

During dorsal–ventral patterning of early fly embryos, for example, two TGF- β molecules of the BMP class, Dpp and Screw (Scw), regulate cell fates along the dorsal-to-ventral axis (Fig. 3). Similar regulation occurs in ver-

Figure 3. Establishment of dorsal–ventral ectoderm cell fates in early *Drosophila* embryos. (a) Schematic drawing of a cross-section through a blastoderm-stage embryo showing mesoderm, ventral-lateral ectoderm, and dorsal-lateral ectoderm. While *scw* transcripts are ubiquitously distributed, *dpp* is uniformly expressed in the dorsal-lateral ectoderm, and *sog* expression is confined to the ventral-lateral ectoderm near ventral midline, their protein products have graded activities that generate cell fate decisions in early embryos along the dorsal–ventral axis. (b) The BMP antagonist, Sog protein, together with another protein called Tsg, forms an inhibitory complex with two BMP ligands, Dpp and Scw (D/S), preventing Dpp and Scw from binding to their receptors. In addition, Sog also plays a positive role in enhancing Dpp activity dorsally by facilitating the movement of Dpp protein from more lateral to more dorsal regions of the embryo. Sog protein diffuses dorsally and its activity gradient is regulated by proteolysis and endocytosis. Tld, a metalloprotease that is produced in the more dorsal region of the embryo, degrades Sog there in a Dpp-dependent manner, thus freeing Dpp to activate its receptors. In addition, Dynamin-mediated endocytosis plays a positive role in removing active Sog from the dorsal region, thus creating a positive feedback loop to generate a Dpp activity gradient.

tebrate development (Piccolo et al. 1997). *dpp* transcripts are at a high level in most dorsal and dorsal-lateral regions, while at this key stage *scw* is ubiquitous. Yet both molecules have graded effects on cell fates, because of an activity gradient that is established by interacting proteins. A molecule called Short Gastrulation (Sog) is produced near the ventral midline and moves dorsally in a graded fashion. Sog forms a complex with Scw or Dpp and another protein, Tsg. Because the complex prevents the BMP ligands from binding to their receptors, Dpp and Scw are inhibited in lateral and ventral regions. Curiously, Sog and Tsg have positive as well as negative roles. In addition to inhibiting BMP receptor binding to its receptor in these regions, they facilitate the movement of Dpp from more lateral to more dorsal cells, thus increasing the activity of Dpp in more dorsal cells. The

Sog gradient is generated by two mechanisms that destroy Sog in dorsal cells: specific proteolytic degradation (Marqués et al. 1997), and Dynamin-mediated endocytosis (Srinivasan et al. 2002). The degradation is done by the Tolloid (Tld) metalloprotease. Since Tld-mediated degradation of Sog is stimulated by Dpp itself, a sharp boundary of Dpp activity is created by a positive feedback loop (more active Dpp, more proteolysis, still more active Dpp) (Shimmi and O'Connor 2003). In wing discs, Sog interacts with integrin proteins that may help to transport different variant Sog proteins (Araujo et al. 2003). Computational simulations in combination with experimental data have shown that the stability of the Dpp morphogen gradient requires extensive diffusion of Dpp–Sog complexes in the perivitelline fluid that surrounds the embryo, together with restricted diffusion of Dpp-like ligands (Eldar et al. 2003).

Spatially regulated signal protein destruction is also used in Wnt signaling. Electron microscopic (EM) studies of Wg signaling in *Drosophila* embryos reveal the importance of degradation in controlling Wg distribution (Dubois et al. 2001). In a *Drosophila* embryo, Wg is made by the last row of cells in the anterior (A) part of each body segment. From there Wg spreads forward to form a gradient, thus providing positional information to the A cells in front of the Wg source. Wg also provides positional information to the posterior (P) cells behind the source. However, in this direction the spreading of Wg is dramatically restricted to the cells immediately adjacent to the source. Wg spreading in wing imaginal disc cells is nondirectional (Strigini and Cohen 2000), but the directional movement of Wg in the embryo is notably different. What keeps Wg low in the posterior (P) cells? A functional horseradish peroxidase (HRP)–Wg fusion protein was studied at the EM level (Dubois et al. 2001). HRP–Wg is found in degradative compartments including multivesicular (MVB) and multilamellar (MLB) bodies. Although Wg is degraded in both A and P cells, there are four times more HRP–Wg-containing MVB and MLB in P cells than in A cells. Reduced endocytosis in A cells increases the activity of Wg and its apparent range. Furthermore, the enhanced Wg degradation in P cells is initiated by Hh-induced EGF (epidermal growth factor) receptor signaling in P cells (Dubois et al. 2001). Intriguingly, most Wg (and Dpp) molecules are believed to be extracellular, based on assays using Proteinase K (Strigini and Cohen 2000; Teleman and Cohen 2000). Thus, Wg moves through extracellular space, with signal range and activity temporally and spatially regulated by ligand turnover.

Signal protein modifications

In addition to the formation of protein complexes or regulated degradation, covalent modifications of signaling protein have a powerful influence on their activities.

Newly produced Hh undergoes dual lipid modification: N-terminal palmitoylation and C-terminal cholesterylization. Palmitoylation of Hh is at least partly dependent on cholesterol modification, confounding to a

degree experiments designed to sort out the relative importance of each (for review, see Nusse 2003). A simple diffusion model was proposed for establishing the Hh morphogen gradient. A freely diffusible form of Shh, s-ShhNp, that is cholesterol-modified but not membrane-bound appeared sufficient to set up a long-range morphogen gradient across the anterior–posterior axis of the chick limb (Zeng et al. 2001), although how this relates to the normal situation is unclear. Mice expressing a form of Shh that does not have linked cholesterol have short-range Shh signaling but reduced long-range Hh signaling (Lewis et al. 2001). In contrast, in *Drosophila*, Hh-N lacking cholesterol modification can act over a longer distance than wild-type Hh (Burke et al. 1999).

How these greasy Hh molecules travel through the tissue is unknown. Experiments have shown that Hh travels ~50 μ m in *Drosophila* wing imaginal disc and ~300 μ m in vertebrate limb bud. If *Drosophila* embryos are engineered to produce extra Hh in its normal locations, in a stripe about two to three cells wide in the posterior of each body segment, little effect on segmentation is observed. If Hh that cannot be linked to cholesterol is produced in the same pattern, segmentation defects characteristic of *hh* ectopic expression are observed (Porter et al. 1996). The unmodified protein moves a few cell diameters farther from its source than normal protein. The normally modified protein is located in punctate structures in basal regions of the epithelial cells, while the protein without cholesterol appears instead in a diffuse pattern apically. Thus cholesterol modification changes the subcellular location of the protein and also prevents long-distance movement of the protein. Overexpressed *Drosophila* Hh lacking palmitoylation has a dominant-negative effect on Hh signaling (Lee et al. 2001). In vertebrate limb development, the N-terminal part of Shh with cholesterol attached (designated N-Shhp) was found to act over a few hundred microns (30 cell diameters) (Lewis et al. 2001). A truncated version of N-Shh that is not cholesterol-modified has biological activity similar to wild-type N-Shhp. However, while the unmodified Shh protein is capable of activating transcription of the target genes *ptc1* and *Gli1* in the limb bud, the signaling range is shortened to about one-third of its normal distance (Lewis et al. 2001). Shh lacking palmitoylation can induce ectopic Hh target gene activity in mouse forebrain and limb (Kohtz et al. 2001; Lee et al. 2001). Since many aspects of Hh signaling are well-conserved from *Drosophila* to vertebrates, these opposite effects of lipid modification are surprising. The difference may be due to the distance that Hh travels (50 μ m in wing and 300 μ m in limb), or due to the experimental differences such as the degree of protein overproduction.

A recent report by Gallet et al. (2003) shows that cholesterol-modified Hh (HhNp) as well as wild-type Hh is organized into large punctate structures (LPS) in *Drosophila* epithelia. These experiments were done in embryos with overexpressed, untagged Hh, with or without cholesterol or palmitate modification. Overexpressed cholesterol-modified HhN (HhNp) had the same LPS distribution as wild-type Hh. *Drosophila* Dispatched, a pro-

tein required in Hh-producing cells and proposed to be a processing or releasing factor (Burke et al. 1999), is responsible for the apical sorting of LPS. Conceivably, packets of Hh-containing LPS could be pinched off from apical membrane domains and moved through extracellular space. The possible role of Dispatched in Hh processing and release (Ma et al. 2002) has become more controversial with reports that Shh made in mouse cells is brought to the surface, secreted, and active in signaling even when the producing cells lack Dispatched1 function (Tian et al. 2004). Yet mice lacking Dispatched1 clearly have dramatic Hh-related phenotypes (Ma et al. 2002). These findings suggest that Dispatched may do something other than facilitate secretion, perhaps conveying Hh to a specific receptor, transporter, or compartment.

Another type of signaling molecule, Wnt proteins, are notorious for their insolubility. Like Hh proteins, Wnt proteins are palmitoylated (Willert et al. 2003); in addition, they are glycosylated. It is remarkable that they can move at all. Removing the palmitate group from Wnt3a (a vertebrate Wg homolog) using acyl-protein thioesterase-1 (APT-1) results in loss of signaling activity in cultured cells (Willert et al. 2003). Glycosylation facilitates the secretion and movement of Wg protein in *Drosophila* embryos (Tanaka et al. 2002). It is not yet clear how important the glycosylation is for signal function, since molecules lacking glycosylation have not been tested.

The studies discussed above show that modifications of signaling proteins can have potent effects on their movements and function. These modifications may influence how signals interact with cell surfaces along the way. Recent studies indicate the crucial importance of proteoglycans on cell surfaces for regulating development.

Proteoglycans in the creation of signaling surfaces

Heparin sulfate proteoglycans (HSPGs) play a key role in secreted protein gradient formation and signaling (Fig. 2c). HSPGs are large macromolecules, abundantly present at the cell surface and also components of the extracellular matrix. HSPGs consist of a protein core (including syndecans and glypicans) to which heparan sulfate glycosaminoglycan (HS GAG) chains are attached. GAGs have been implicated in binding signaling molecules. The nature of the protein cores and modification of HS GAG chain biosynthesis can determine the specificity and function of HSPGs (for review, see Filmus and Selleck 2001). Several HSPG protein cores and GAG biosynthetic enzymes affect signaling protein distribution and activity. HSPGs may be passive, providing a surface to which signaling proteins bind transiently. Alternatively, HSPGs may play an active role in facilitating protein movement, for example, with catalytic events involving active modification of signals or HSPGs that promote movement. There could be some type of "bucket brigade" mechanism, although no evidence exists at present for directional bias in HSPG action.

Transfer of GPI-linked proteins between neighboring cells has been suggested for proteins located in lipid

rafts. GPI-linked proteins are inserted into the outer leaflet of the lipid bilayers and might be transferred from one cell to another through a flip-flop mechanism between adjacent outer leaflets (Kooyman et al. 1995). Through such a mechanism HhNp could be passed between neighboring cells through GPI-linked HSPGs.

Several mutations involved in the biosynthesis of HS GAGs have been linked to the Wg and Hh signaling pathways. These include *sugarless* (*sgl*), *sulfateless* (*sfl*), *tout-velu* (*ttv*), *sister of ttv* (*sotv*), *brother of ttv* (*botv*), and *notum/wingful*. *sgl* encodes a protein with homology to UDP-glucose dehydrogenase, which generates a substrate for the biosynthesis of HS GAGs. Mutations in the *sgl* gene reduce the range of Wg and Dpp proteins (Binari et al. 1997; Hacker et al. 1997; Haerry et al. 1997). *Sfl* is a protein related to heparan sulfate N-deacetylase/N-sulfotransferase that is necessary for the modification of HS GAGs. Wg protein expression is diminished in *sfl* mutant cells (Lin et al. 1999; Baeg et al. 2001). Overexpression of *wg* partially rescues *sgl*- and *sfl*-null embryos, suggesting that *sgl* and *sfl* function in Wg signaling by restricting Wg protein diffusion and therefore facilitating the binding of Wg to its receptor (Hacker et al. 1997). *Ttv* and its related proteins *Sotv* and *Botv* are type II transmembrane HS polymerases that are essential for biosynthesis of HSPGs. They are required for shaping the Hh, Wg, and Dpp gradients; all three signals accumulate in front of the mutant cells (Bellaiche et al. 1998; The et al. 1999; Bornemann et al. 2004; Takei et al. 2004). In addition, *notum/wingful*, which encodes a member of the hydrolase superfamily, limits Wg protein distribution by destabilizing the HSPGs (Gerlitz and Basler 2002; Giraldez et al. 2002). *notum/wingful* is itself induced by Wg signaling, creating a feedback loop that reins in the effect of Wg. In vertebrate tissues, Hh gradients can be visualized by immunostaining only under conditions that allow HSPG preservation (Gritli-Linde et al. 2001).

division abnormally delayed (*dally*) encodes a GPI-linked glypican (a HSPG protein core), which has been implicated in the regulation of both Wg and Dpp morphogen gradient formation and signaling (Lin et al. 1999; Fujise et al. 2003). *Dally* could serve as a coreceptor for Wg or Dpp, and limit the free diffusion of Wg or Dpp by retaining it at the cell surface, thus contributing to shaping the Wg or Dpp morphogen gradient. In the absence of *dally* activity, Dpp (in the form of Dpp-GFP) fails to form an evident gradient across a field of Dpp-receiving cells. Conversely, when *dally* is overexpressed, Dpp trapped by binding to excessive *Dally* fails to distribute properly (Fujise et al. 2003). Most recently, Belenkaya et al. (2004) re-examined the role of endocytosis and proteoglycans in Dpp-GFP spreading and signaling. They found that Dpp is mainly extracellular, and that its movement is blocked along cells lacking *dally*. The information of the extracellular Dpp gradient is not inhibited by disruption of endocytosis with the *shibire* (Dynamin) mutation that blocks endocytosis, but this mutation does reduce Dpp signal transduction within cells. They conclude that Dpp moves by restricted extracellular diffusion that requires glypicans (HSPGs). Furthermore, a *Dally*-related

protein, Dally-like (Dlp), is required for Hh, Dpp, and possibly Wg signaling (Baeg et al. 2001; Desbordes and Sanson 2003; Belenkaya et al. 2004; Han et al. 2004a).² Overexpressing *dlp* leads to a massive accumulation of extracellular Wg in wing imaginal discs (Baeg et al. 2001; Giraldez et al. 2002). This may provide a high concentration of Wg protein that can bind to receptors on nearby cells after release from the HSPGs; in this case, HSPGs could act as a coreceptor for Wg signaling (Han et al. 2004a). However, the effects of *dlp* mutations on Wg signaling are subtle.

In addition to regulating Wg morphogen formation and activity, Dally and Dlp also contribute significantly to Hh movement (Bellaiche et al. 1998; The et al. 1999; Han et al. 2004b). Dlp protein colocalizes with and stabilizes Hh. Dlp emerged from a RNAi screen for Hh pathway components and was shown to be required for Hh signaling if the Hh was provided to cells in the medium but not if Hh was produced within cells (Lum et al. 2003). Therefore, Dlp seems to facilitate reception, processing, or transport of Hh. *sfl* clones in posterior, Hh-producing cells of wing imaginal discs eliminate functional Dally and Dlp. These clones cause the loss of Hh target gene (*dpp*) expression from nearby anterior wild-type cells. Blocking Dynamin-mediated endocytosis does not eliminate Hh movement, further implying that Hh travels along the cell surface using a HSPG-dependent cell-to-cell mechanism (Han et al. 2004b; Torroja et al. 2004). The exact mechanism of the movement remains mysterious, as the Hh signal must be continuously bound, released, and bound again.

Ttv, a GPI-anchored HSPG, is required for the proper diffusion of the cholesterol-modified, membrane-associated Hh (HhNp) (The et al. 1999; Gerlitz and Basler 2002). HhNp could be apically sorted into lipid rafts, proposed microdomains in the plasma membrane that are rich in sphingolipids, cholesterol, and GPI-anchored proteins. Ttv may be required to target HhNp to lipid rafts.

HSPGs have emerged as major modulators for shaping morphogen gradients, facilitating restricted movement of extracellular ligands from one cell surface to the next, when the cells are in contact. This facilitated diffusion seems likely to be at least partially responsible for bringing developmental signals to distant target cells.

Cell movements, emitted vesicles, and cytoplasmic processes as signaling mechanisms

Several cell-based mechanisms that do not involve diffusion have been proposed as a way to set up graded or restrained signaling. In the eye imaginal disc, a groove moves across the developing eye concomitant with Hh

signaling. This depression in the field of cells, called the morphogenetic furrow, is caused by a transient change in cell shape. The apical ends of cells are constricted, which reduces the apical surface area and limits the rate of active Hh emission and therefore presumably lessens long-range Hh movement (Benlali et al. 2000). In a rapidly changing, morphologically dynamic eye disc, cell constriction by controlling actin polymerization is probably a faster way to set up a Hh boundary than changes in the level of Ptc controlled at the transcriptional level.

Another mechanism is a special form of transcytosis, involving particular vesicles called argosomes. Argosomes bud off from basolateral membranes in wing disc cells and move through the disc epithelium (Greco et al. 2001). They travel through adjacent cells at a rate of up to 16 cells in 3 h, comparable to the speed at which Wg morphogen gradients are generated. A fraction of Wg-containing vesicles colocalize with argosomes in Wg-expressing cells, raising the intriguing possibility that argosomes may start as intracellular vesicles and then emerge to carry and spread intercellular signals like Wg. One inconsistency is that argosomes are derived from basolateral membrane domains, while Wg- and Dpp-containing endocytic vesicles are localized in the apical region of the cells, and apical localization of Wg protein is essential for signaling in embryonic ectoderm (Simmonds et al. 2001).

Transport through cell-cell contacts, perhaps involving argosomes, could be especially useful in tissues where the sheet of cells is highly convoluted, like imaginal discs. Problems could arise if a free molecule jumped past nearby cells to reach cells that are closer in space but farther along the epithelium, and this would be prevented by a system of cell-to-cell contacts and transfers. Another potential advantage of argosome transmission is the shipping of membranes along with the signal. Packages involving membranes and proteins may have useful properties that a free protein signal would not, such as stability or a controlled path of import.

Another possible mode of signal transmission depends on a special structure called the cytoneme, a long, actin-based cell extension first identified in wing imaginal discs (Ramirez-Weber and Kornberg 1999). Analogous processes at a somewhat different scale were described by Wiemann in 1910, as quoted by Wilson (1928): "[I]n the beetle . . . cells of the endchamber of the ovary elaborate basophilic granules that flow downward into the eggs through protoplasmic pedicles by which the latter are connected with the egg chamber."

Cytonemes extend from the disc periphery toward the anterior-posterior compartment border where Dpp signaling protein is produced. Many thousands of cytonemes converge on the central part of the discs. Dpp protein, or other signals, might travel from the source cells along cytonemes to cells elsewhere in the disc. Cytonemes can be induced, and the length of projections that grow between cells in culture can exceed the size of the entire wing disc. In addition, similar structures like cytonemes have been found in chick limb bud cells (Ramirez-Weber and Kornberg 1999). Cytonemes poten-

²Recent studies by Desbordes and Sanson (2003) and Han et al. (2004a) support an argument against a role for Dally or Dlp in Wg signaling. These results are in disagreement with earlier studies (e.g., Baeg et al. 2001; Gerlitz and Basler 2002; Takei et al. 2004). The discrepancy may arise from the different alleles, markers, or tissues used in the experiments (details can be found in Perrimon and Hacker 2004; Sanson 2004; Tabata 2004). Further studies will be required to settle the controversy.

tially provide a mechanism for rapid long-distance morphogen spreading, bypassing the requirement for movement through the extracellular environment. No direct evidence for cytoneme involvement in signaling has yet been obtained. One puzzle concerning possible cytoneme-mediated ligand transport is that ligands normally move in a nondirectional fashion in imaginal discs, while cytonemes have an intrinsic orientation toward the signal source.

In addition to cytonemes, two conceptually related signal transmission phenomena have been found in *Drosophila*. In the first case, Hh protein induces particular divisions of neuron precursors in the development of the *Drosophila* visual system. Hh protein travels through long retinal axon projections to directly initiate neurogenesis in the neural lamina (Huang and Kunes 1996), although the mechanism of transport is unknown. The second case comes from Delta/Notch signaling. In the development of *Drosophila* sensory organs, lateral inhibition mediated by the ligand Delta (a transmembrane protein) acting on the receptor Notch signaling ensures that only one cell from a field of proneural cells will be singled out to become a sensory organ precursor (SOP). SOPs promote the formation of filopodia that contain inhibitory, membrane-bound Delta protein. This actin-based structure can reach cells over a distance of ~120 μm , thus providing lateral inhibition up to 20 cell diameters away (de Jussieu et al. 2003).

Movements of cells can create graded signals without any need for secretion or for much cell-independent protein movement. Recent studies on fibroblast growth factor (FGF) signaling show how a gradient of protein signal concentration can form due to a gradual decay of mRNA. A graded distribution of FGF8 protein is required for the patterning and maturation of presomitic mesoderm (PSM) in early vertebrate embryos. Dubrulle and Pourquié (2004) found that *fgf8* mRNA is made only in tail bud cells of chick and mouse embryos. As cells move out from the tail bud, *fgf8* mRNA decays gradually, resulting in a RNA gradient higher in tail bud cells and progressively lower in more anterior cells. This *fgf8* RNA gradient is translated into a FGF8 protein gradient.

A novel, cell-movement-based mechanism for ligand transport has also been proposed for Wg signaling (Pfeiffer et al. 2000). In *Drosophila* embryos, an engineered membrane-tethered Wg is able to rescue many of the patterning functions that have been previously attributed to the action of secreted Wg at a distance. Pfeiffer et al. observed that some cells derived from Wg-producing cells move anteriorly, up to four cells away, and retain the inherited Wg in intracellular vesicles, which are shown to be secretory vesicles. Thus, this cell-based transport is, in principle, sufficient to account for the normal range of Wg morphogen in embryonic ectoderm. Wg distant from the source cells also can be stabilized by association with the Frizzled2 receptor (Cadigan et al. 1998). There are interesting parallels here with the movements of ZPA-derived cells in limb bud development (Ahn and Joyner 2004; Harfe et al. 2004). Inheritance of signal information by migrating cells may be

more frequent than has been appreciated, as careful tracing studies are needed to detect it.

Continuing the journey

We have summarized different ideas about how morphogen gradients are established. A complete understanding of signaling protein movement awaits improvements in live imaging of actively signaling tissues and a lot more biochemistry. It is difficult to definitely confirm or rule out passive diffusion mechanisms experimentally. Theoretical studies, like those applied to segment polarity gene functions by von Dassow et al. (2000) and Ingolia (2004), need to be more extensively applied to problems of morphogen gradient formation. Such studies would be energized by more quantitative data about signals and signal transduction, and by new ways to observe morphogen movements in real time. A valuable step forward would come from devising modified signaling proteins that emit a different signal when bound to a receptor or to a diffusion-facilitating partner protein. Fluorescence resonance energy transfer (FRET) offers such an approach, but it has not yet been widely applied to the problem of signaling proteins moving through tissue. FRET applications to signal transduction, for example, involving EGF receptor (for review, see Hayes et al. 2004) bode well for using FRET and other methods to follow signals as they move around, astride, and within cells. The mechanisms of protein signal movement are one example of a general problem in cell biology, relating biochemical observations to microscope images. For example, signal-receptor interactions measured in vitro may be affected in vivo by unknown cofactors, and the protein distributions observed using antibodies or fluorescent protein tags may or may not be indicative of where the active signaling molecules lie. Much of the accumulated protein so easily seen in the microscope could be inactive.

The range over which signaling proteins move often determines the place where particular differentiated cells form, and the size of organ and tissue primordia. Changes in the range of action of a signaling protein thus have profound influence on the eventual structure of an animal. It seems likely that signal ranges were altered during evolution to adapt to environmental changes and to allow, and indeed direct, the formation of new animal designs. Thus future quantitative, comparative studies of the properties of signaling proteins from different species may reveal how signaling range has been adapted to morphogenesis requirements. Since damage to any of the known developmental signaling pathway can cause human disease, better comprehension of how signals normally work and how they can be repaired or manipulated will surely lead to new opportunities in medicine.

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References

- Ahn, S. and Joyner, A.L. 2004. Dynamic changes in the response of cells to positive Hedgehog signaling during mouse limb patterning. *Cell* **118**: 505–516.
- Araujo, H., Negreiros, E., and Bier, E. 2003. Integrins modulate Sog activity in the *Drosophila* wing. *Development* **130**: 3851–3864.
- Baeg, G.H., Lin, X., Khare, N., Baumgartner, S., and Perrimon, N. 2001. Heparan sulfate proteoglycans are critical for the organization of the extracellular distribution of Wingless. *Development* **128**: 87–94.
- Bellaiche, Y., The, I., and Perrimon, N. 1998. *Tout-velu* is a *Drosophila* homologue of the putative tumour suppressor *EXT-1* and is needed for Hh diffusion. *Nature* **394**: 85–88.
- Belenkaya, T.Y., Han, C., Yan, D., Opoka, R.J., Khodoun, M., Liu, H., and Lin, X. 2004. *Drosophila* Dpp morphogen movement is independent of Dynamin-mediated endocytosis but regulated by the glypican members of heparan sulfate proteoglycans. *Cell* **119**: 231–244.
- Benlali, A., Draskovic, I., Hazelett, D.J., and Treisman, J.E. 2000. *act up* controls actin polymerization to alter cell shape and restrict Hedgehog signaling in the *Drosophila* eye disc. *Cell* **101**: 271–281.
- Binari, R.C., Staveley, B.E., Johnson, W.A., Godavarti, R., Saisekharan, R., and Manoukian, A.S. 1997. Genetic evidence that heparin-like glycosaminoglycans are involved in Wingless signaling. *Development* **124**: 2623–2632.
- Bornemann, D.J., Duncan, J.E., Staata, W., Selleck, S., and Warrior, R. 2004. Abrogation of heparan sulfate synthesis in *Drosophila* disrupts the Wingless, Hedgehog and Decapentaplegic signaling pathways. *Development* **131**: 1927–1938.
- Briscoe, J., Chen, Y., Jessell, T.M., and Struhl, G. 2001. A Hedgehog-insensitive form of Patched provides evidence for direct long-range morphogen activity of Sonic hedgehog in the neural tube. *Mol. Cell* **7**: 1279–1291.
- Burke, R., Nellen, D., Bellotto, M., Hafen, E., Senti, K.A., Dickson, B.J., and Basler, K. 1999. Dispatched, a novel sterol-sensing domain protein dedicated to the release of cholesterol-modified Hedgehog from signaling cells. *Cell* **99**: 803–815.
- Cadigan, K.M., Fish, M.P., Rulifson, E.J., and Nusse, R. 1998. Wingless repression of *Drosophila* Frizzled 2 expression shapes the Wingless morphogen gradient in the wing. *Cell* **93**: 767–777.
- Casali, A. and Struhl, G. 2004. Reading the Hedgehog morphogen gradient by measuring the ratio of bound to unbound Patched protein. *Nature* **431**: 76–80.
- Chen, Y. and Schier, A.F. 2001. The zebrafish Nodal signal Squint functions as a morphogen. *Nature* **411**: 607–610.
- Chen, Y. and Struhl, G. 1996. Dual roles for Patched in sequestering and transducing Hedgehog. *Cell* **87**: 553–563.
- Chen, W., Kirkbride, K.C., How, T., Nelson, C.D., Mo, J., Frederick, J.P., Wang, X.F., Lefkowitz, R.J., and Blobe, G.C. 2003a. β -Arrestin 2 mediates endocytosis of type III TGF- β receptor and down-regulation of its signaling. *Science* **301**: 1394–1397.
- Chen, W., ten Berge, D., Brown, J., Ahn, S., Hu, L.A., Miller, W.E., Caron, M.G., Barak, L.S., Nusse, R., and Lefkowitz, R.J. 2003b. Dishevelled 2 recruits β -arrestin 2 to mediate Wnt5A-stimulated endocytosis of Frizzled 4. *Science* **301**: 1391–1394.
- de Jousssineau, C., Soule, J., Martin, M., Anguille, C., Montcourrier, P., and Alexandre, D. 2003. Delta-promoted filopodia mediate long-range lateral inhibition in *Drosophila*. *Nature* **426**: 555–559.
- Denef, N., Neubuser, D., Perez, L., and Cohen, S.M. 2000. Hedgehog induces opposite changes in turnover and subcellular localization of Patched and Smoothened. *Cell* **102**: 521–531.
- Desbordes, S.C. and Sanson, B. 2003. The glypican Dally-like is required for Hedgehog signalling in the embryonic epidermis of *Drosophila*. *Development* **130**: 6245–6255.
- Dillon, R., Gadgil, C., and Othmer, H.G. 2003. Short- and long-range effects of Sonic hedgehog in limb development. *Proc. Natl. Acad. Sci.* **100**: 10152–10157.
- Dubois, L., Lecourtois, M., Alexandre, C., Hirst, E., and Vincent, J.-P. 2001. Regulated endocytic routing modulates Wingless signaling in *Drosophila* embryos. *Cell* **105**: 613–624.
- Dubrulle, J. and Pourqu, O. 2004. fgf8 mRNA decay establishes a gradient that couples axial elongation to patterning in the vertebrate embryo. *Nature* **427**: 419–422.
- Dyson, S. and Gurdon, J.B. 1998. The interpretation of position in a morphogen gradient as revealed by occupancy of activin receptors. *Cell* **93**: 557–568.
- Eldar, A., Rosin, D., Shilo, B.Z., and Barkai, N. 2003. Self-enhanced ligand degradation underlies robustness of morphogen gradients. *Dev. Cell* **5**: 635–646.
- Entchev, E.V., Schwabedissen, A., and Gonzlez-Gaitn, M. 2000. Gradient formation of the TGF- β homolog Dpp. *Cell* **103**: 981–991.
- Filmus, J. and Selleck, S.B. 2001. Glypicans: Proteoglycans with a surprise. *J. Clin. Invest.* **108**: 497–501.
- Fujise, M., Takeo, S., Kamimura, K., Matsuo, T., Aigaki, T., Izumi, S., and Nakato, H. 2003. Dally regulates Dpp morphogen gradient formation in the *Drosophila* wing. *Development* **130**: 1515–1522.
- Gallet, A., Rodriguez, R., Ruel, L., and Therond, P.P. 2003. Cholesterol modification of Hedgehog is required for trafficking and movement, revealing an asymmetric cellular response to Hedgehog. *Dev. Cell* **4**: 191–204.
- Gerlitz, O. and Basler, K. 2002. Wingful, an extracellular feedback inhibitor of Wingless. *Genes & Dev.* **16**: 1055–1059.
- Giraldez, A.J., Copley, R.R., and Cohen, S.M. 2002. HSPG modification by the secreted enzyme Notum shapes the Wingless morphogen gradient. *Dev. Cell* **2**: 667–676.
- Gonzlez-Gaitn, M. 2003. Signal dispersal and transduction through the endocytic pathway. *Nat. Rev. Mol. Cell. Biol.* **4**: 213–224.
- Greco, V., Hannus, M., and Eaton, S. 2001. Argosomes: A potential vehicle for the spread of morphogens through epithelia. *Cell* **106**: 633–645.
- Gritli-Linde, A., Lewis, P., McMahon, A.P., and Linde, A. 2001. The whereabouts of a morphogen: Direct evidence for short- and graded long-range activity of Hedgehog signaling peptides. *Dev. Biol.* **236**: 364–386.
- Gurdon, J.B., Harger, P., Mitchell, A., and Lemaire, P. 1994. Activin signalling and response to a morphogen gradient. *Nature* **371**: 487–492.
- Gurdon, J.B., Dyson, S., and St Johnston, D. 1998. Cells' perception of position in a concentration gradient. *Cell* **95**: 159–162.
- Hacker, U., Lin, X., and Perrimon, N. 1997. The *Drosophila* *sugarless* gene modulates Wingless signaling and encodes an enzyme involved in polysaccharide biosynthesis. *Development* **124**: 3565–3573.
- Haerry, T.E., Heslip, T.R., Marsh, J.L., and O'Connor, M.B. 1997. Defects in glucuronate biosynthesis disrupt Wingless signaling in *Drosophila*. *Development* **124**: 3055–3064.
- Han, C., Belenkaya, T.Y., Khodoun, M., Tauchi, M., and Lin, X.

- 2004a. Distinct and collaborative roles of *Drosophila* EXT family proteins in morphogen signalling and gradient formation. *Development* **131**: 1563–1575.
- Han, C., Belenkaya, T.Y., Wang, B., and Lin, X. 2004b. *Drosophila* glypicans control the cell-to-cell movement of Hedgehog by a Dynamin-independent process. *Development* **131**: 601–611.
- Harfe, B.D., Scherz, P.J., Nissim, S., Tian, H., McMahon, A.P., and Tabin, C.J. 2004. Evidence for an expansion-based temporal Shh gradient in specifying vertebrate digit identities. *Cell* **118**: 517–528.
- Hayes, N., Howard-Cofield, E., and Gullick, W. 2004. Green fluorescent protein as a tool to study epidermal growth factor receptor function. *Cancer Lett.* **206**: 129–135.
- Hime, G.R., Lada, H., Fietz, M.J., Gillies, S., Passmore, A., Wicking, C., and Wainwright, B.J. 2004. Functional analysis in *Drosophila* indicates that the NBCCS/PTCH1 mutation G509V results in activation of smoothened through a dominant-negative mechanism. *Dev. Dyn.* **229**: 780–790.
- Huang, Z. and Kunes, S. 1996. Hedgehog, transmitted along retinal axons, triggers neurogenesis in the developing visual centers of the *Drosophila* brain. *Cell* **86**: 411–422.
- Incardona, J.P., Gruenberg, J., and Roelink, H. 2002. Sonic hedgehog induces the segregation of Patched and Smoothed in endosomes. *Curr. Biol.* **12**: 983–995.
- Ingolia, N.T. 2004. Topology and robustness in the *Drosophila* segment polarity network. *PLoS Biol.* **2**: 805–815.
- Jekely, G. and Rørth, P. 2003. Hrs mediates downregulation of multiple signalling receptors in *Drosophila*. *EMBO Rep.* **4**: 1163–1168.
- Johnson, R.L., Milenkovic, L., and Scott, M.P. 2000. In vivo functions of the Patched protein: Requirement of the C terminus for target gene inactivation but not Hedgehog sequestration. *Mol. Cell* **6**: 467–478.
- Kerszberg, M. and Wolpert, L. 1998. Mechanisms for positional signaling by morphogen transport: A theoretical study. *J. Theor. Biol.* **191**: 103–114.
- Kohtz, J.D., Lee, H.Y., Gaiano, N., Segal, J., Ng, E., Larson, T., Baker, D.P., Garber, E.A., Williams, K.P., and Fishell, G. 2001. N-Terminal fatty-acylation of Sonic hedgehog enhances the induction of rodent ventral forebrain neurons. *Development* **128**: 2351–2363.
- Kooyman, D.L., Byrne, G.W., McClellan, S., Nielsen, D., Tone, M., Waldmann, H., Coffman, T.M., McCurry, K.R., Platt, J.L., and Logan, J.S. 1995. In vivo transfer of GPI-linked complement restriction factors from erythrocytes to the endothelium. *Science* **269**: 89–92.
- Lander, A.D., Nie, Q., and Wan, F.Y. 2002. Do morphogen gradients arise by diffusion? *Dev. Cell* **2**: 785–796.
- Lecourtois, M., Alexandre, C., Dubois, L., and Vincent, J.-P. 2001. Wingless capture by Frizzled and Frizzled2 in *Drosophila* embryos. *Dev. Biol.* **235**: 467–475.
- Lecuit, T. and Cohen, S.M. 1998. Dpp receptor levels contribute to shaping the Dpp morphogen gradient in the *Drosophila* wing imaginal disc. *Development* **125**: 4901–4907.
- Lee, J.D., Kraus, P., Gaiano, N., Nery, S., Kohtz, J., Fishell, G., Loomis, C.A., and Treisman, J.E. 2001. An acylatable residue of Hedgehog is differentially required in *Drosophila* and mouse limb development. *Dev. Biol.* **233**: 122–136.
- Lewis, P.M., Dunn, M.P., McMahon, J.A., Logan, M., Martin, J.F., St-Jacques, B., and McMahon, A.P. 2001. Cholesterol modification of Sonic hedgehog is required for long-range signaling activity and effective modulation of signaling by Ptc1. *Cell* **105**: 599–612.
- Lin, X., Buff, E.M., Perrimon, N., and Michelson, A.M. 1999. Heparan sulfate proteoglycans are essential for FGF receptor signaling during *Drosophila* embryonic development. *Development* **126**: 3715–3723.
- Lloyd, T.E., Atkinson, R., Wu, M.N., Zhou, Y., Pennetta, G., and Bellen, H.J. 2002. Hrs regulates endosome membrane invagination and tyrosine kinase receptor signaling in *Drosophila*. *Cell* **108**: 261–269.
- Lum, L., Yao, S., Mozer, B., Rovescalli, A., Von Kessler, D., Nirenberg, M., and Beachy, P.A. 2003. Identification of Hedgehog pathway components by RNAi in *Drosophila* cultured cells. *Science* **299**: 2039–2045.
- Ma, Y., Erkner, A., Gong, R., Yao, S., Taipale, J., Basler, K., and Beachy, P.A. 2002. Hedgehog-mediated patterning of the mammalian embryo requires transporter-like function of Dispatched. *Cell* **111**: 63–75.
- Marqués, G., Matuschewski, M., Shimell, M.J., Wunnenberg-Stapleton, K., Cho, K.W., and O'Connor, M.B. 1997. Production of a DPP activity gradient in the early *Drosophila* embryo through the opposing actions of the SOG and TLD proteins. *Cell* **91**: 417–426.
- Martin, V., Carrillo, G., Torroja, C., and Guerrero, I. 2001. The sterol-sensing domain of Patched protein seems to control Smoothed activity through Patched vesicular trafficking. *Curr. Biol.* **11**: 601–607.
- McDowell, N., Gurdon, J.B., and Grainger, D.J. 2001. Formation of a functional morphogen gradient by a passive process in tissue from the early *Xenopus* embryo. *Int. J. Dev. Biol.* **45**: 199–207.
- Nellen, D., Burke, R., Struhl, G., and Basler, K. 1996. Direct and long-range action of a DPP morphogen gradient. *Cell* **85**: 357–368.
- Nusse, R. 2003. Wnts and Hedgehogs: Lipid-modified proteins and similarities in signaling mechanisms at the cell surface. *Development* **130**: 5297–5305.
- Perrimon, N. and Hacker, U. 2004. Wingless, Hedgehog and heparan sulfate proteoglycans. *Development* **131**: 2509–2511.
- Pfeiffer, S., Alexandre, C., Calleja, M., and Vincent, J.-P. 2000. The progeny of wingless-expressing cells deliver the signal at a distance in *Drosophila* embryos. *Curr. Biol.* **10**: 321–324.
- Piccolo, S., Agius, E., Lu, B., Goodman, S., Dale, L., and De Robertis, E.M. 1997. Cleavage of Chordin by Xolloid metalloprotease suggests a role for proteolytic processing in the regulation of Spemann organizer activity. *Cell* **91**: 407–416.
- Piddini, E. and Vincent, J.-P. 2003. Modulation of developmental signals by endocytosis: Different means and many ends. *Curr. Opin. Cell Biol.* **15**: 474–481.
- Porter, J.A., Ekker, S.C., Park, W.J., von Kessler, D.P., Young, K.E., Chen, C.H., Ma, Y., Woods, A.S., Cotter, R.J., Koonin, E.V., et al. 1996. Hedgehog patterning activity: Role of a lipophilic modification mediated by the carboxy-terminal autoprocessing domain. *Cell* **86**: 21–34.
- Ramirez-Weber, F.A. and Kornberg, T.B. 1999. Cytonemes: Cellular processes that project to the principal signaling center in *Drosophila* imaginal discs. *Cell* **97**: 599–607.
- Sanson, B. 2004. Do glypicans play a role in Wingless signaling in *Drosophila*? *Development* **131**: 2511–2513.
- Schrödinger, E. 1944. *What is life?* Cambridge University Press, Cambridge, England.
- Shimmi, O. and O'Connor, M.B. 2003. Physical properties of Tld, Sog, Tsg and Dpp protein interactions are predicted to help create a sharp boundary in Bmp signals during dorsoventral patterning of the *Drosophila* embryo. *Development* **130**: 4673–4682.
- Simmonds, A.J., dosSantos, G., Livne-Bar, I., and Krause, H.M. 2001. Apical localization of wingless transcripts is required for Wingless signaling. *Cell* **105**: 197–207.

- Srinivasan, S., Rashka, K.E., and Bier, E. 2002. Creation of a Sog morphogen gradient in the *Drosophila* embryo. *Dev. Cell* **2**: 91–101.
- Strigini, M. and Cohen, S.M. 2000. Wingless gradient formation in the *Drosophila* wing. *Curr. Biol.* **10**: 293–300.
- Tabata, T. 2004. The role of Ttv in Wg signaling. *Development* **131**: 2513.
- Tabata, T. and Takei, Y. 2004. Morphogens, their identification and regulation. *Development* **131**: 703–712.
- Taipale, J., Cooper, M.K., Maiti, T., and Beachy, P.A. 2002. Patched acts catalytically to suppress the activity of Smoothed. *Nature* **418**: 892–897.
- Takei, Y., Ozawa, Y., Sato, M., Watanabe, A., and Tabata, T. 2004. Three *Drosophila* EXT genes shape morphogen gradients through synthesis of heparan sulfate proteoglycans. *Development* **131**: 73–82.
- Tanaka, K., Kitagawa, Y., and Kadowaki, T. 2002. *Drosophila* segment polarity gene product porcupine stimulates the posttranslational N-glycosylation of Wingless in the endoplasmic reticulum. *J. Biol. Chem.* **277**: 12816–12823.
- Tanimoto, H., Itoh, S., ten Dijke, P., and Tabata, T. 2000. Hedgehog creates a gradient of DPP activity in *Drosophila* wing imaginal discs. *Mol. Cell* **5**: 59–71.
- Teleman, A.A. and Cohen, S.M. 2000. Dpp gradient formation in the *Drosophila* wing imaginal disc. *Cell* **103**: 971–980.
- The, I., Bellaiche, Y., and Perrimon, N. 1999. Hedgehog movement is regulated through *tout velu*-dependent synthesis of a heparan sulfate proteoglycan. *Mol. Cell* **4**: 633–639.
- Tian, H., Tenzen, T., and McMahon, A.P. 2004. Dose dependency of Displ and genetic interaction between Displ and other Hedgehog signaling components in the mouse. *Development* **131**: 4021–4033.
- Torroja, C., Gorfinkiel, N., and Guerrero, I. 2004. Patched controls the Hedgehog gradient by endocytosis in a dynamin-dependent manner, but this internalization does not play a major role in signal transduction. *Development* **131**: 2395–2408.
- Turing, A.M. 1952. The chemical basis of morphogenesis. *Proc. R. Soc. B* **237**: 37–72.
- Vincent, J.-P. and Dubois, L. 2002. Morphogen transport along epithelia, an integrated trafficking problem. *Dev. Cell* **3**: 615–623.
- von Dassow, G., Meir, E., Munro, E.M., and Odell, G.M. 2000. The segment polarity network is a robust developmental module. *Nature* **406**: 188–192.
- Wijgerde, M., McMahon, J.A., Rule, M., and McMahon, A.P. 2003. A direct requirement for Hedgehog signaling for normal specification of all ventral progenitor domains in the presumptive mammalian spinal cord. *Genes & Dev.* **16**: 2849–2864.
- Willert, K., Brown, J.D., Danenberg, E., Duncan, A.W., Weissman, I.L., Reya, T., Yates III, J.R., and Nusse, R. 2003. Wnt proteins are lipid-modified and can act as stem cell growth factors. *Nature* **423**: 448–452.
- Wilson, E.B. 1928. *The cell in development and heredity*. MacMillan, New York.
- Yang, Y., Drossopoulou, G., Chuang, P.T., Duprez, D., Marti, E., Bumcrot, D., Vargesson, N., Clarke, J., Niswander, L., McMahon, A., et al. 1997. Relationship between dose, distance and time in Sonic hedgehog-mediated regulation of anteroposterior polarity in the chick limb. *Development* **124**: 4393–4404.
- Zecca, M., Basler, K., and Struhl, G. 1996. Direct and long-range action of a Wingless morphogen gradient. *Cell* **87**: 833–844.
- Zeng, X., Goetz, J.A., Suber, L.M., Scott, W.J.J., Schreiner, C.M., and Robbins, D.J. 2001. A freely diffusible form of Sonic hedgehog mediates long-range signalling. *Nature* **411**: 716–720.

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