Morphogens, their identification and regulation

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Summary

During the course of development, cells of many tissues differentiate according to the positional information that is set by the concentration gradients of morphogens. Morphogens are signaling molecules that emanate from a restricted region of a tissue and spread away from their source to form a concentration gradient. As the fate of each cell in the field depends on the concentration of the morphogen signal, the gradient prefigures the pattern of development. In this article, we describe how morphogens and their functions have been identified and analyzed, focusing on model systems that have been extensively studied.

Introduction

The term morphogen is used rigorously to describe a particular type of signaling molecule that acts on cells directly to induce distinct cellular responses in a concentration-dependent manner. Although there is abundant evidence for concentration-dependent activity of secreted signaling molecules, evidence for their direct action on cells has been lacking in many cases and, so far, only a few such molecules fulfill the criteria of morphogens. Nevertheless, the roles of morphogens during the development of *Drosophila* appendages have been extensively studied, and a few examples of morphogens have recently been identified in vertebrate development. These include members of the Hedgehog (Hh) family, for example, Hh in *Drosophila* appendage development (Mullor et al., 1997; Strigini and Cohen, 1997) and Sonic hedgehog (Shh) in chick neural tube development (Briscoe et al., 2001); the Wnt family member Wingless (Wg), which acts during *Drosophila* appendage development (Neumann and Cohen, 1997; Zecca et al., 1996); and some members of the TGFβ family, including Decapentaplegic (Dpp) in *Drosophila* appendage development (Lecuit et al., 1996; Nellen et al., 1996) and Squint in Zebrafish early embryonic development (Chen and Schier, 2001). Activin, another member of TGFβ family, has been well studied in the *Xenopus* model system, in which the exogenously supplied signaling molecule activates target genes in a dose-dependent manner, and this has already been well described in a comprehensive review (Gurdon and Bourillot, 2001). In this primer, we will give a brief history of strategies adopted for identifying secreted morphogens, taking the development of *Drosophila* appendages as a model system, and we discuss how these strategies could be applied to the study in vertebrates.

However, even for those morphogens that have been identified, we still do not understand crucial issues such as how morphogens are moved through a tissue, how a gradient is maintained, and how morphogens coordinate growth and patterning. In addition, the morphogen gradient must be invariable despite genetic or environmental fluctuations. Recent studies have revealed significant roles for cell surface molecules in shaping morphogen gradients, and these include morphogen receptors and heparan sulfate proteoglycans (HSPGs). Several reports have suggested the involvement of transcytosis, cytonemes and argosomes in morphogen transport, whereas numerical analyses have favored restricted diffusion as a mechanism for the formation of morphogen gradients. More cell biological and biochemical studies will be needed to evaluate the role of these activities and structures in gradient formation.

Morphogens in *Drosophila* wing development

Hh: a short range morphogen in wing development

The first step towards identifying and analyzing a morphogen is to determine whether a signaling molecule fulfills the criteria required to qualify as a morphogen, i.e. whether the molecule induces distinct cellular responses in a concentration-dependent manner and whether it acts directly on cells at a distance from its source. The *Drosophila* adult wing arises from the larval imaginal wing disc. An imaginal disc is a two-sided sac comprising a columnar cell layer (which gives rise to eye, antennae, wing or leg) and overlying squamous epithelium, known as the peripodial membrane (Fig. 1B). The wing imaginal disc is subdivided into non-intermingling anterior (A) and posterior (P) compartments along the anteroposterior axis. The identity of cells in the P compartment is imparted by the expression of the gene *engrailed* (*en*) (Guillen et al., 1995; Simmonds et al., 1995; Tabata et al., 1995). Under the control of En, cells of the posterior compartment synthesize Hh, which is secreted into the A compartment (Tabata and Kornberg, 1994) (Fig. 1C). There, Hh induces several target genes, including *patched*, *en* and *dpp*, and patterns the central domain of the wing (Fig. 2).

Does Hh act as a morphogen? The morphogen model predicts that an ectopic source of morphogen can induce a mirror image duplication of the developing field that is patterned by that morphogen (Fig. 1A). Ectopic production of Hh in the *Drosophila* wing (Fig. 1C) can induce mirror image

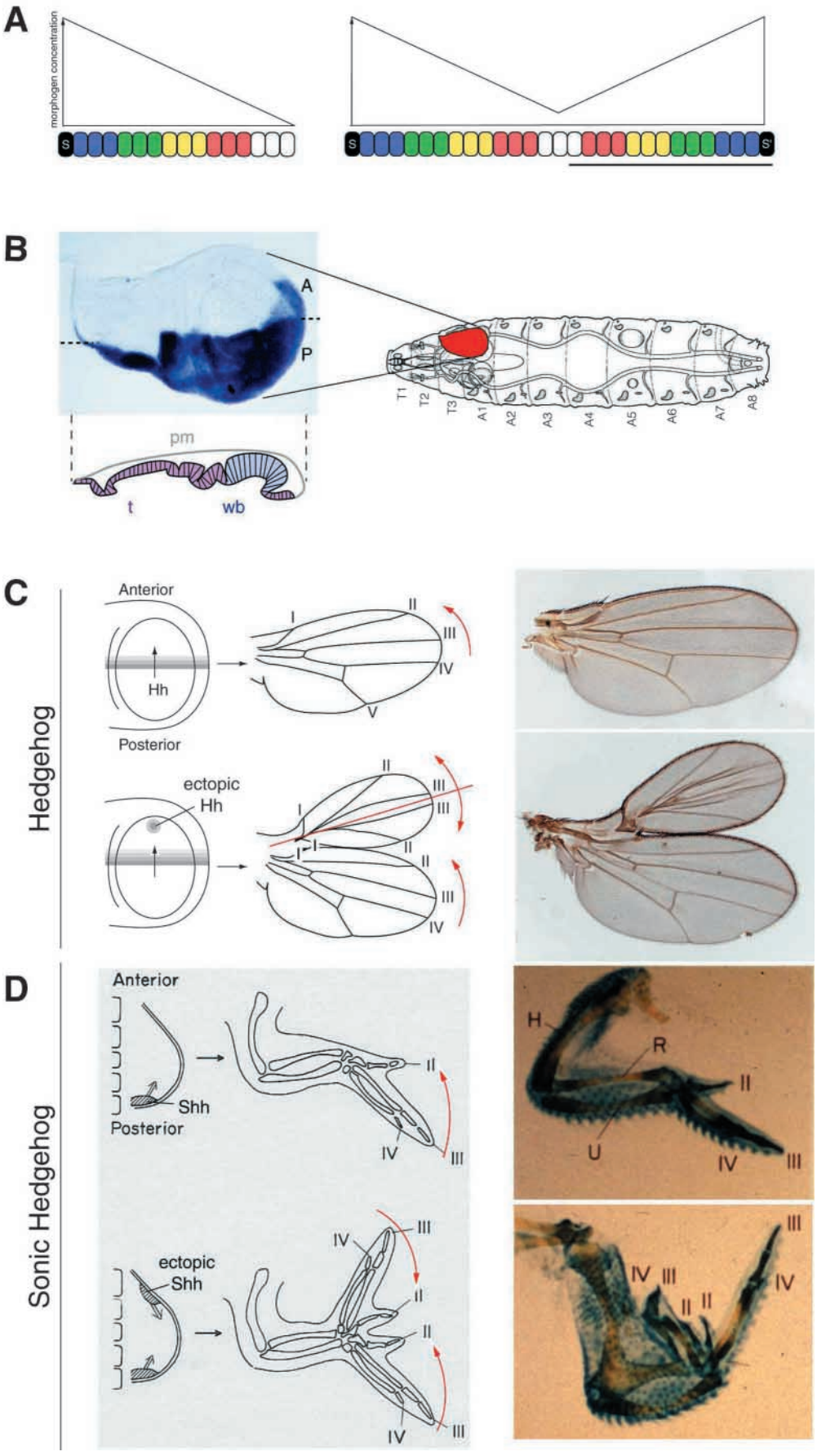

Fig. 1. Mirror image duplication can be induced by an ectopic source of the morphogen molecule. (A) A model for morphogen signaling. A morphogen emanating from the expressing cell (S) sets the positional value of a cell by forming a concentration gradient across the developmental field in which the cell resides; the value of the gradient at each point in the field is a function of the distance of the receiving cell from the morphogen-secreting cells (left). Introduction of an ectopic source (S′) of morphogen can induce mirror image duplication (underline; right). (B) Wing imaginal disc (red) of third instar *Drosophila* larva. The imaginal disc is a two-sided sac comprising a columnar cell layer that contains presumptive wing blade (wb) and thorax (t) regions, and an overlying squamous peripodial membrane (pm); it is set aside from the embryonic epidermis and develops at the larval stage. The imaginal disc is subdivided into anterior (A) and posterior (P) compartments along the anteroposterior axis. *hedgehog* (*hh*) is expressed in the posterior compartment; *hh* mRNA is visualized with in situ hybridization (left). Schematic on right modified with permission from Bryant and Levinson (Bryant and Levinson, 1985). (C) Ectopic expression of *hh*, by making a clone of cells expressing *hh*, induces a mirror image duplication of the anterior wing structure. Hh produced in the P compartment is secreted into the A compartment (top). A clone of cells ectopically expressing *hh* in the A compartment induces a complete mirror image duplication of the A compartment (bottom). Wing veins I-V are indicated. Reproduced with permission from Tabata (Tabata, 2001). (D) Ectopic production of Shh, induced by implanting *shh*-expressing cells into the anterior limb bud, induces a mirror image duplication of the wing structure. *shh* is expressed in the region corresponding to the ZPA in the wing bud (top). Implanted cells that ectopically produce Shh in the anterior of the limb bud induce a mirror image duplication of the wing structure (bottom). Digits (II, III and IV) are labeled on the schematic, and radius (R), ulna (U) and humerus (H) are labeled in the photographs on the right. Reproduced with permission from Riddle et al. (Riddle et al., 1993). Photographs courtesy of C. Tabin.

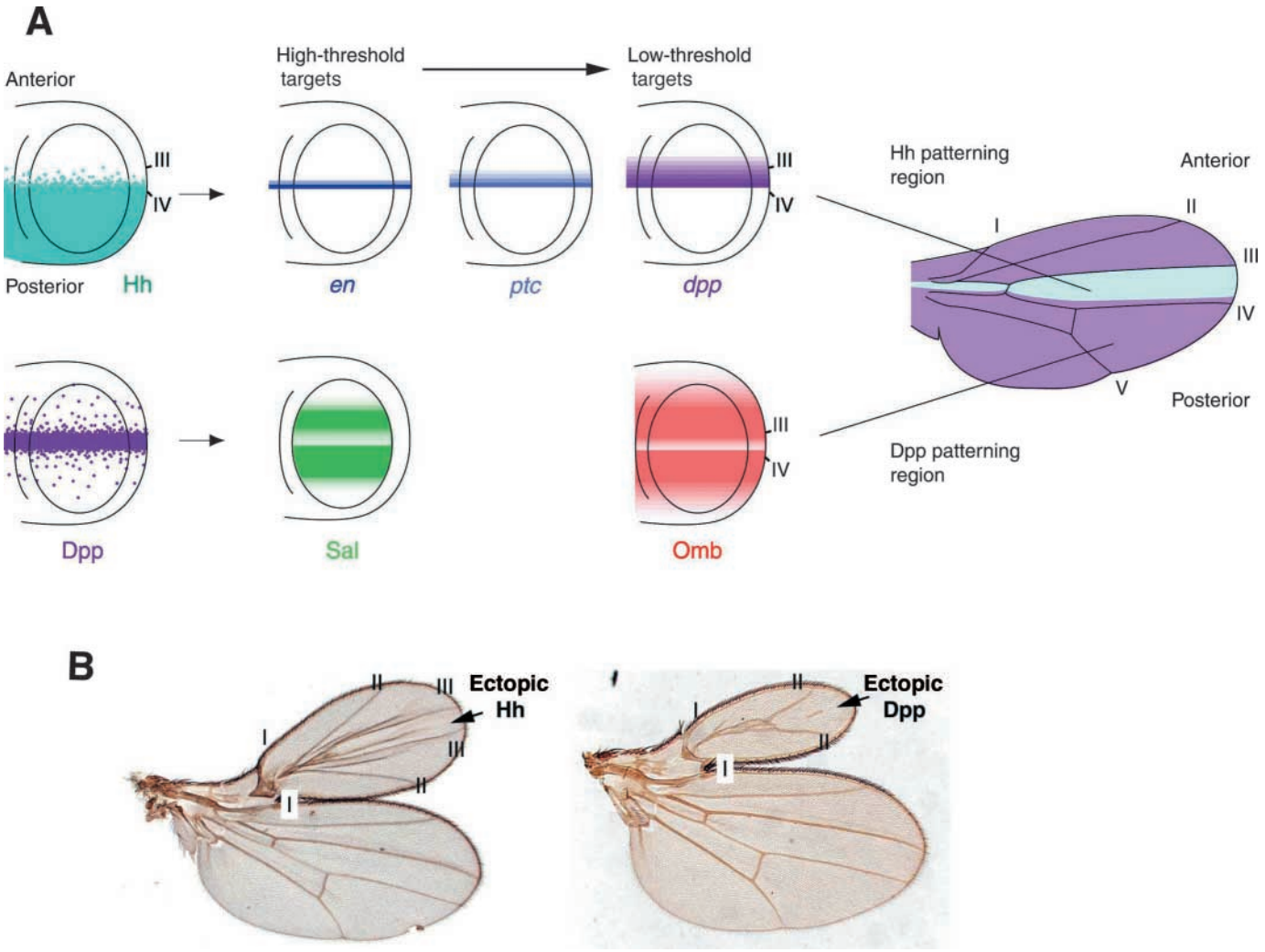

Fig. 2. The wing is patterned by two morphogens, Hh and Decapentaplegic (Dpp). (A) Hh produced in the posterior (P) compartment generates a short range gradient of Hh in the anterior (A) compartment. Hh both patterns the central domain of the wing and induces the expression of *en*, *ptc* and *dpp*, at high, middle and low thresholds, respectively, in a stripe of cells adjacent to the AP compartment boundary. Note that *en* is induced by Hh in the anterior compartment in late larval development. Dpp induces expression of *sal* and *omb* at high and low thresholds, respectively, and patterns the wing beyond the central domain. (B) Ectopic expression of *hh* results in a mirror image duplication of the entire A compartment while Dpp induces a mirror image duplication of the A compartment lacking the central domain (Zecca et al., 1995). Reproduced with permission from Tabata (Tabata, 2001).

duplications of the relevant tissues of the wing (Basler and Struhl, 1994). By making use of a temperature-sensitive *hh* allele, it has been shown that *dpp* expression can be activated by levels of Hh activity that are below the minimal levels required to activate *en* (Strigini and Cohen, 1997). This suggests that the different spatial domains of *dpp* and *en* expression are defined by the same local concentration gradient of Hh. Evidence that Hh acts directly on cells has come from experiments comparing the activities of the wild-type secreted form of Hh with a membrane-tethered form of the protein (Strigini and Cohen, 1997). Secreted Hh protein activates target genes in nearby cells over a range of 10 cells, whereas the membrane-tethered Hh only activates target genes in cells immediately adjacent to the Hh source. This demonstrates that Hh activates target genes directly and, together, these experiments show that Hh functions as a morphogen.

Dpp: a long range morphogen in wing development

One of the targets of Hh regulation, Dpp, then functions as a second morphogen that patterns the wing beyond the central domain (Lecuit et al., 1996; Nellen et al., 1996) (Fig. 2A). Thus, the wing is patterned by two different types of morphogens, and this has been well illustrated in experiments that ectopically express these proteins: Hh induces the mirror image duplication of the entire A compartment, whereas the duplication caused by an ectopic source of Dpp lacks the central domain (Zecca et al., 1995) (Fig. 2B). Dpp is expressed along the border between the A and P compartments, and induces several target genes including *sal* and *omb* (*bifid* – FlyBase), with *omb* being expressed in a wider domain than *sal* (Figs 2, 3). Dpp distribution in the columnar cell layer can be monitored using an antibody against Dpp (Gibson et al., 2002) or by fusing Dpp with green fluorescent protein (Dpp-GFP) (Entchev et al., 2000;

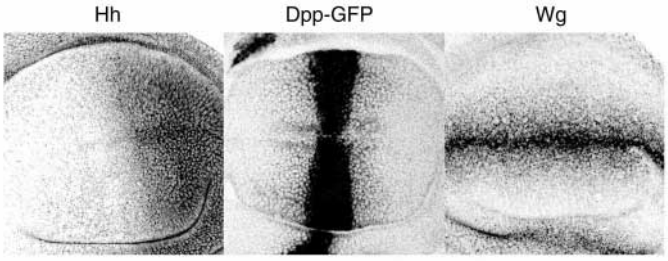

Fig. 3. Distribution of Hh, Dpp-GFP and Wg in the wing imaginal disc. Note the graded distribution away from the expressing domain.

Teleman and Cohen, 2000) (Fig. 3). The space between the two cell layers of the imaginal disc is called the disc lumen. Dpp is also detected uniformly in the disc lumen and is thought to be required mainly for cell survival (Gibson et al., 2002).

Evidence showing that Dpp acts in a concentration-dependent manner and acts directly on cells, rather than by acting through a signal relay mechanism, comes from experiments that used a constitutively active form of the Dpp receptor (Lecuit et al., 1996; Nellen et al., 1996). The pathway that transduces the Dpp signal involves a combination of two types of serine/threonine kinase receptors, type I and type II. The activated type I receptor phosphorylates cytoplasmic transducers, so-called receptor-regulated Smads (named after the first-identified members of this family: Sma in *C. elegans* and Mad in *Drosophila*), which, upon phosphorylation, translocate into the nucleus and regulate the expression of target genes (Fig. 4A). In *Drosophila* wing development, Thickveins (Tkv) acts as a type I receptor; its constitutively active form (Tkv*), when ectopically expressed, can induce the expression of the target genes *sal* and *omb* (Fig. 4). The key to showing whether Dpp acts directly on cells lies in determining whether the effect of expressing activated Tkv is cell-autonomous. If Dpp functions as a morphogen, the effects of Tkv* should be strictly cell-autonomous, because a second signal would not be secreted from the Tkv*-expressing cells. By contrast, if Dpp triggers a signaling relay mechanism, the effects of overexpressing *Tkv** should be non-autonomous because a second signal emanating from the cells overexpressing *Tkv** would also affect surrounding cells. The unambiguously cell-autonomous effects of Tkv* in inducing expression of *sal* and *omb*, indicate that Dpp functions directly on target cells (Fig. 4B,C). In addition, different levels of Dpp upregulate the different targets, Omb and Sal (Lecuit et al., 1996; Nellen et al., 1996).

Wg: the ventrodorsal axis of the imaginal disc

After being subdivided into A and P compartments, the wing imaginal disc is subsequently subdivided into dorsal (D) and ventral (V) compartments along the dorsoventral axis, and later the DV border develops into the wing margin (Fig. 5E). The protein Apterous acts on cells in the dorsal compartment (Diaz-Benjumea and Cohen, 1993), inducing expression of the gene *fringe* (Irvine and Wieschaus, 1994), which results in activation of the Notch receptor pathway at the DV border (Kim et al., 1995). Activated Notch induces Wg synthesis at the DV border (Doherty et al., 1996; Neumann and Cohen, 1996) (Fig. 4) where it functions as a morphogen to induce the expression of target genes, such as *achaete* (*ac*) *Distal-less* (*Dll*) and *vestigial* (*vg*), and organizes wing patterning (Fig. 5). Wg has been shown to function as a morphogen by experiments similar to those that provided evidence that Hh functions as a morphogen; although the wild-type secreted Wg activates target genes over a distance, a membrane-tethered form upregulates Wg-target genes only in its immediate neighbors (Zecca et al., 1996). In addition, the expression of Wg-target genes is cell-autonomously disrupted in clones of cells mutant either for *dishevelled* or *armadillo*, each of which encode a component of the Wg signal transduction pathway (Neumann and Cohen, 1997; Zecca et al., 1996). Furthermore, an experiment using a temperature-sensitive allele of *wg* has shown that the level of Wg activity minimally required to activate synthesis of Dll is higher than that required to activate synthesis of Vg (Neumann and Cohen, 1997). Together, these experiments indicate that Wg functions as a morphogen.

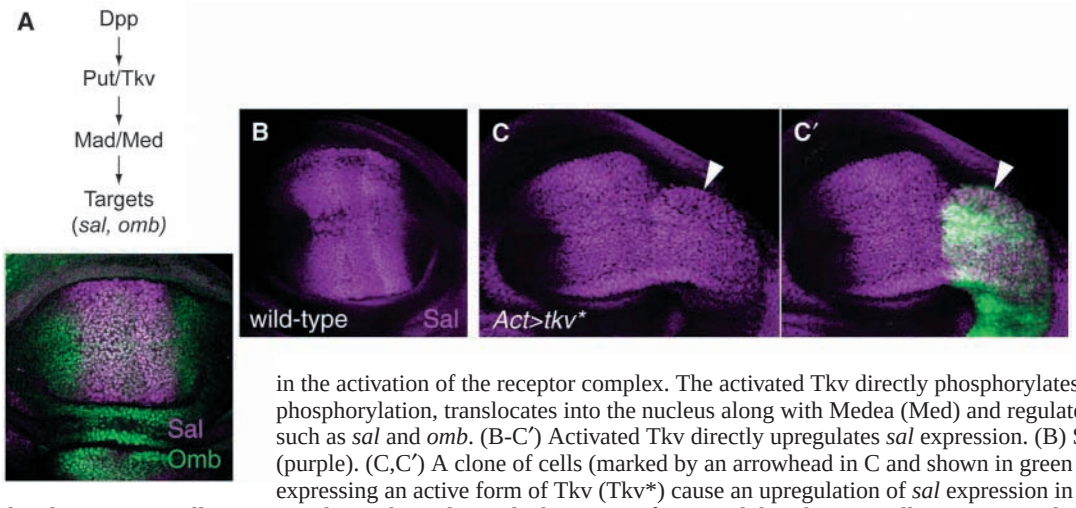

Fig. 4. Dpp signals through the Tkv receptor and directly activates target genes. (A) Signaling pathway of Dpp. Dpp is received by a complex of type I (Tkv or Sax) and type II (Put) serine/threonine kinase receptors. Binding of Dpp to this receptor complex results in the activation of the receptor complex. The activated Tkv directly phosphorylates Mad, which, upon phosphorylation, translocates into the nucleus along with Medea (Med) and regulates transcription of target genes, such as *sal* and *omb*. (B-C′) Activated Tkv directly upregulates *sal* expression. (B) Sal in the wild-type wing disc (purple). (C,C′) A clone of cells (marked by an arrowhead in C and shown in green in the merged image C′) expressing an active form of Tkv (Tkv*) cause an upregulation of *sal* expression in a region of the imaginal disc that does not normally express *sal*, seen here alongside the region of imaginal disc that normally expresses *sal*. Note the autonomy of the upregulation of *sal* expression by Tkv (C′).

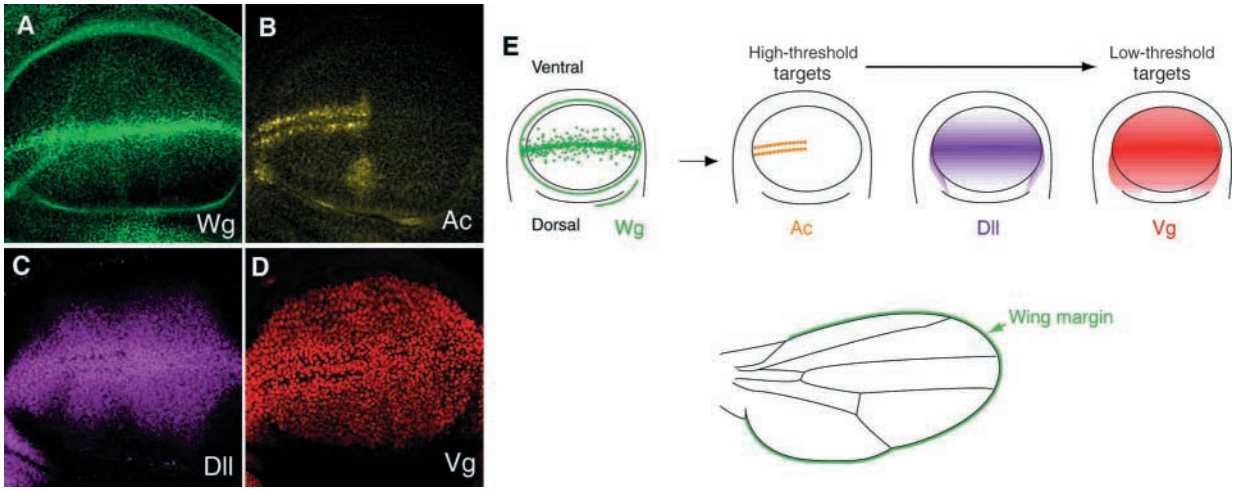

Fig. 5. Wg and its target genes. (A-D) Wg (A; shown in green) is produced along the DV border and induces the expression of target genes, such as *Ac* (B; yellow, and expressed only in the A compartment), *Dll* (C; purple) and *Vg* (D; red), at high, middle and low thresholds, respectively. Anterior is to the left. (E) Schematics showing the domains of target gene expression. Reproduced with permission from Briscoe et al. (Briscoe et al., 2001).

Morphogens in vertebrate development

Shh during limb bud development

Anterior/posterior polarity of the vertebrate limb is regulated by a posteriorly localized signaling center called the zone of polarizing activity (ZPA). Shh mirrors the properties of the ZPA; ectopic Shh activity induces digit duplications, with higher concentrations specifying increasingly more posterior digits (Riddle et al., 1993; Yang et al., 1997) (Fig. 1D). Furthermore, an ectopic source of Shh induces a mirror image duplication of the limb (Fig. 1C). Shh induces dose-dependent production of a Dpp ortholog, BMP2 (Yang et al., 1997), and misexpressed BMP2 can induce ectopic formation of the most anterior digit (Duprez et al., 1996). These observations readily prompt us to see the analogy between patterning by Shh of the chick limb bud and the role of Hh in *Drosophila* wing (Fig. 1C,D). However, no evidence for the direct action of Shh in regulating different target genes has yet been demonstrated, suspending the conclusion that Shh functions as a morphogen in the limb development.

Shh in neural tube development

During development of the chick neural tube, however, Shh does function as a morphogen. Shh emanates from the notochord to induce formation of the floor plate. Subsequent *Shh* expression in the floor plate generates a ventral-dorsal activity gradient of Shh that promotes the specification of a series of ventral cell types (Ericson et al., 1997). Furthermore, ectopic expression of a mutated form of the Shh receptor, Patched (Ptc), which does not bind Shh but does antagonize its signaling, causes cell-autonomous ventral-to-dorsal switches in neural progenitor identity (Briscoe et al., 2001) (Fig. 6), clearly indicating that Shh functions by acting on cells directly.

Squint in early embryogenesis

Evidence for another morphogen in vertebrate development has come from a study in Zebrafish. Squint is a member of the TGFβ family of signaling molecules, which induces formation of mesoderm and endoderm in embryos, and regulates different target genes in a concentration-dependent manner. Its direct action was revealed using a mutant embryo in which the Squint signal is not transduced because it lacks the activity of the protein One-eyed pinhead (which is required cell-autonomously for the reception of Squint). When *squint* RNA is injected into a single cell of the mutant embryo, the target genes are induced in wild-type cells implanted distantly from the squint-injected cell. Thus, although the mutant cells cannot

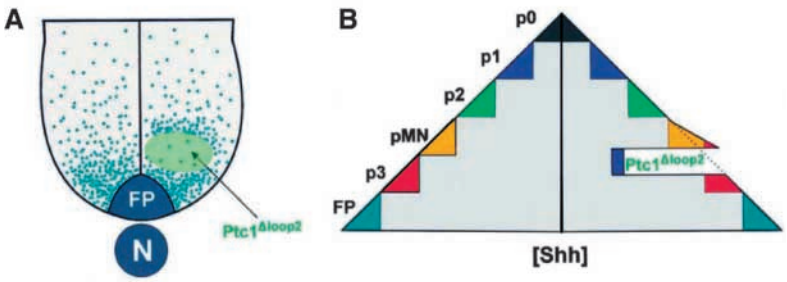

Fig. 6. A model for effects of Patched (Ptc) on neural patterning. (A; left half) Shh emanating from the notochord (N) induces formation of the floor plate (FP), and subsequent *shh* expression in the FP generates a ventral-dorsal activity gradient of Shh (as indicated by the density of the blue dots). (B; left half) The activity gradient of Shh promotes the specification of a series of ventral cell types: p0, p1, p2, pMN and p3, which are progenitor domains from which distinct V0 neurons, V1 neurons, V2 neurons, motoneurons and V3 neurons are generated respectively. Production of a mutated form of the Shh receptor Ptc ($Ptc1^{\Delta loop2}$; A; right half; light green), which does not bind Shh but antagonizes its signaling, causes cell-autonomous abnormal dorsal spread of Shh and (B; right half) ventral-to-dorsal switches in neural progenitor identity. Modified with permission from Briscoe et al. (Briscoe et al., 2001).

transduce the squint signal, wild-type cells placed at a distance from the source of squint still respond to squint (Chen and Schier, 2001). This excludes the possibility that relay mechanisms carry the squint signal and shows instead that squint acts directly on cells.

Cell surface molecules and morphogen gradients

Concentration and activity gradients of morphogens

Although morphogens form concentration gradients, the concentration of the protein is not necessarily directly proportional to the gradient of the signaling activity in morphogen-receiving cells. Dpp signaling activity can be visualized in situ in the Dpp-receiving cells of the wing imaginal disc. The Dpp signal is transduced by phosphorylation of the transducer Mad (Mothers against dpp) (Fig. 4A). The phosphorylated form of Mad (p-Mad) can be used as an intracellular marker to monitor Dpp morphogen activity. To this end, p-Mad levels are visualized using an antibody that specifically recognizes p-Mad (Tanimoto et al., 2000). In the wing imaginal disc, the amount of p-Mad is high in cells near the AP border, where Dpp concentration is high, but is severely reduced in cells along the AP border that express *dpp*, where the level of Dpp is also very high (Fig. 7). This reduction in p-Mad levels along the AP border, despite the high concentration of Dpp, is a result of the direct repressive action of Hh. Hh also directly organizes patterning in the region where it attenuates Dpp signaling, and it is possible that Hh downregulates Dpp signaling in this region to prevent it from interfering with patterning by Hh.

Morphogen receptors

It is possible that the concentration gradient of Dpp is modulated by the distribution of the Dpp receptor itself. Dpp preferentially signals through the Tkv receptor in the wing disc and also negatively regulates *tkv* expression (Lecuit and Cohen, 1998). The level of *tkv* expression is higher in cells located at the periphery of the disc and is lower in the central region (Tanimoto et al., 2000). In addition, *tkv* expression is lowest at the AP border (Fig. 7). We refer to the level of *tkv* expression in the area between the peripheral regions and the AP border as 'basal' and, interestingly, the basal level of *tkv* expression in the P compartment is higher than it is in the A compartment (Fig. 7). This might account for a steeper gradient of p-Mad in the P compartment compared with in the A compartment. The Dpp gradient is expected to be steeper in tissue with more receptors, because high levels of Tkv limit the distribution of Dpp. Thus, the distribution of Tkv modulates the distribution of Dpp itself.

The distribution of Tkv may also regulate Dpp activity, rather than just Dpp concentration. Hh-dependent reduction of p-Mad levels at the AP border occurs largely by repressing transcription of the *tkv* gene. Conversely, the higher Tkv level in the P compartment than in the A compartment is maintained by the activity of the transcription factor Engrailed. Both the Hh and En activities that regulate *tkv* levels are mediated by the gene *mtv* (*master of thickveins*), which encodes a putative nuclear protein (Funakoshi et al., 2001).

The ability of receptor levels to regulate the distribution of receptor ligands is not restricted to the Dpp morphogen, but is also seen for Hh. The Hh receptor Ptc is expressed in the A compartment at low levels and is strongly induced by Hh at the AP border. Here, Hh induces a high level of Ptc to limit the range of the Hh distribution gradient (Chen and Struhl, 1996).

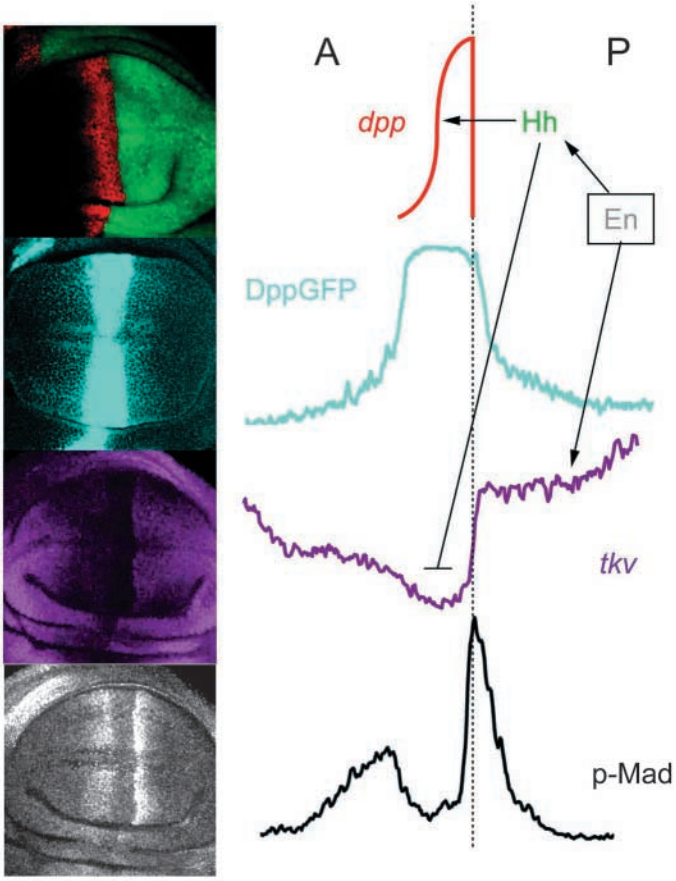

Fig. 7. Ligand and activity gradient of Dpp. Confocal microscopy images (left) and schematic drawings (right) of the part of the wing imaginal disc that gives rise to the adult wing. Hh (green), the synthesis of which is maintained by Engrailed (En) in P-compartment cells, induces Dpp expression (red) along the AP border. Dpp diffuses in both A and P directions and forms a gradient, which can be visualized by the distribution of the chimeric Dpp-GFP protein (blue). The level of Tkv, the Dpp receptor (purple), is very low along the AP border because Hh downregulates its expression. In the middle of the wing disc, abutting the AP border, the level of Tkv in the P compartment is higher than it is in the A compartment, which causes a steeper Dpp gradient to be present in the P compartment than in the A compartment. This dynamic Tkv pattern accounts well for the shape of the activity gradient of Dpp signaling, as shown by the levels of phosphorylated Mad (p-Mad).

Heparan sulfate proteoglycans

Recently, several reports have suggested that heparan sulfate proteoglycans (HSPGs) play a key role in morphogen transport and/or signaling. HSPGs are abundant cell surface molecules and are a part of the extracellular matrix. HSPGs consist of a protein core (such as syndecan and glypican) to which heparan sulfate glycosaminoglycan (HS GAG) chains are attached. GAG chains are long unbranched polymers consisting of many sulfated disaccharides. Genetic screens for mutations that affect morphogen signaling pathways in *Drosophila* have identified genes that have sequence homologies to genes that encode vertebrate GAG biosynthetic enzymes. These putative enzymes are encoded by *sugarless* (*sgl*), *sulfateless* (*sfl*), and members of the *Drosophila* EXT gene family consisting of

tout-velu (*ttv*), *brother of ttv* (*botv*) and *sister of ttv* (*sotv*; *Ext2* – FlyBase), which encode proteins with homology to UDP-glucose dehydrogenase, N-deacetylase/N-sulfotransferase and HS copolymerase, respectively. Mutations in *sgl* compromise signaling mediated by Wg (Binari et al., 1997; Hacker et al., 1997; Haerry et al., 1997) and Dpp (Haerry et al., 1997). Similarly, the *sfl* mutation affects Wg and Hh signaling (Lin et al., 1999; The et al., 1999), and in somatic *sfl* mutant clones Wg protein levels are diminished (Baeg et al., 2001). *ttv*, *botv* and *sotv* mutants affect Hh, Dpp and Wg signaling (Bellaiche et al., 1998; The et al., 1999; Takei et al., 2004). In addition, *Notum*, a gene that encodes a member of the α/β-hydrolase superfamily, influences Wg protein distribution by destabilizing the HSPGs (Gerlitz and Basler, 2002; Giraldez et al., 2002). Lastly, *dally* is proposed to encode a HSPG protein core, and is required for Wg and Dpp activity. Dally, and the related Dally-like protein (Dlp), bind and stabilize Wg at the cell surface (Baeg et al., 2001; Strigini and Cohen, 2000), and may provide a pool of Wg protein that can become available for receptor binding upon its release from HSPGs. Both *dally* and *tkv* expression are regulated by Hh and Engrailed. In addition, elevated levels of Dally increase the sensitivity of cells to Dpp, and alterations in the levels of Dally affect formation of both Dpp ligand and activity gradients (Fujise et al., 2003). Together, these findings indicate that HSPGs are major regulators of morphogen gradients.

Hh protein levels are significantly decreased in clones of cells mutant for the EXT genes *ttv*, *botv* and *sotv* (for Hh; Fig. 8C) when these clones are generated in the Hh-expressing cells of the wing. This indicates that HSPGs are required for stable retention of Hh on the cell surface. In wild-type imaginal discs, Hh protein synthesized in the P compartment appears to flow into the A compartment, with a moderate concentration gradient starting from the middle of the posterior compartment (Fig. 8A). However, when the EXT mutant clone is created in the A compartment along the AP boundary, Hh accumulates abnormally in the P compartment (Fig. 8B). This indicates that, because of a lack of appropriate HSPG, Hh fails to move into the mutant cells and, as a consequence, accumulates in posterior cells instead. Dpp-GFP and Wg also accumulate abnormally in the cells near EXT mutant clones, probably because these proteins cannot move into the cells mutant for EXT genes (Takei et al., 2004). These observations indicate that the HSPG-dependent diffusion is a common mechanism for the distribution of the three morphogens, Hh, Dpp and Wg.

Mechanisms by which morphogens traffic through tissues

We have witnessed significant progress in understanding the mechanisms by which morphogen signals regulate pattern formation in various contexts of development. Nevertheless, we still do not know the answer to the simple and fundamental question of how morphogens are propagated through tissues. Movement by free diffusion alone cannot explain the graded pattern of a morphogen because a secreted GFP fusion protein composed of GFP and the secretory transport domains of Dpp (i.e. lacking the mature Dpp peptide) fails to form a gradient (Entchev et al., 2000). We are beginning to see cell-biological findings that postulate mechanisms by which the morphogen

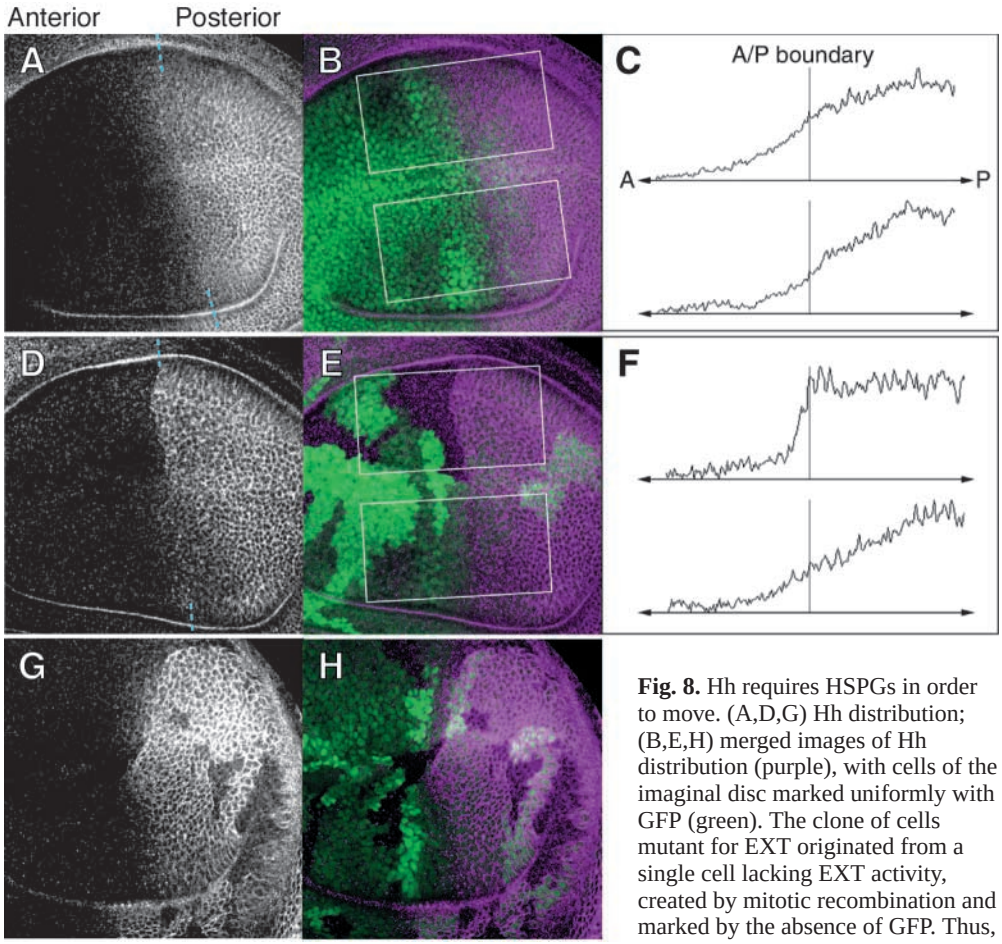

Fig. 8. Hh requires HSPGs in order to move. (A,D,G) Hh distribution; (B,E,H) merged images of Hh distribution (purple), with cells of the imaginal disc marked uniformly with GFP (green). The clone of cells mutant for EXT originated from a single cell lacking EXT activity, created by mitotic recombination and marked by the absence of GFP. Thus, cells homozygous for the EXT mutant are marked by an absence of GFP and cells heterozygous for the EXT mutant are pale green. The staining intensity of Hh in the selected area (white boxes in B and E) was integrated along AP axis, plotted using NIH Image software and presented schematically (C,F). (A-C) Hh protein synthesized in the posterior compartment appears to flow into the anterior compartment, with a moderate concentration gradient starting from the middle of the posterior compartment. (D-F) Hh accumulates abnormally in the posterior compartment when the EXT mutant clone is in the anterior compartment along the AP boundary. The A/P boundary is depicted with blue lines (A,D). (G,H) When the EXT mutant clone is produced in the posterior compartment, Hh accumulation is reduced.

molecules might traffic through a developing tissue. These mechanisms involve planar transcytosis, cytonemes and argosomes observed in imaginal disc development. Theoretical studies, however, favor restricted diffusion in extracellular spaces as a mechanism by which morphogens traffic through tissues. The term 'restricted diffusion' is used here to distinguish it from more elaborated mechanisms such as transcytosis. It is also different from 'free diffusion' in that 'restricted diffusion' implies interaction between the morphogen molecules and cell surface molecules such as the receptors and HSPGs. We will return to restricted diffusion at the last part of this section.

Planar transcytosis

One way to explain morphogen transport is that morphogens are transported through the tissue by repeated cycles of endocytosis and resecretion – known as planer transcytosis. The requirement for endocytosis in Dpp function was indicated by an experiment using Dpp-GFP and a mutation in the gene *shibire* (*shi*), which encodes Dynamin, a GTPase required for clathrin-dependent endocytosis. When a *shi* clone is made shortly after a short burst of Dpp-GFP expression, Dpp-GFP-positive endosomes are not present in the area behind the *shi* clone (Entchev et al., 2000). Furthermore, the activity of the small GTPase Rab5 is required for the fusion between endocytotic vesicles and early endosomes, and is thought to be rate limiting in the early endocytic pathway. When a dominant-negative mutant of Rab5 is expressed in the wing imaginal disc of wild-type flies, target gene expression is restricted to the cells adjacent to the Dpp source (Entchev et al., 2000). By contrast, overexpression of Rab5 broadens the expression domain of target cells. In addition, another small GTPase, Rab7, targets endocytic cargo from the early to the late endosome, and then to the lysosome for degradation. Expression of a dominant gain-of-function mutant of Rab7 decreases the levels of GFP-Dpp that are internalized and reduces the range of Dpp signaling (Entchev et al., 2000).

Nevertheless, Dpp could also be propagated in part by its diffusion in the extracellular space; the digestion of the extracellular proteins of the intact wing disc with proteinase K demonstrates that most of the Dpp-GFP signal appears to be in the extracellular space (Teleman and Cohen, 2000). Thus, more careful genetic and cell biological studies will be required to determine how much of the Dpp trafficking can be ascribed to the endocytotic mechanism, extracellular movement or other mechanisms.

In contrast to the role of planer transcytosis in the transport of Dpp, a report has argued against transcytosis as a mechanism of Wg trafficking in wing imaginal disc development (Strigini and Cohen, 2000). By devising a new antibody-staining protocol to detect extracellular Wg protein, the authors revealed that the Wg protein makes a shallow extracellular gradient (Strigini and Cohen, 2000). Wg does not localize to punctate structures in the *shi* mutant clones (as is the case for Dpp), and, in contrast with Dpp, Wg is internalized by wild-type cells behind the *shi* clone. This shows that Wg can move across the *shi* mutant tissue and is internalized by the adjacent wild-type cells. In fact, higher levels of extracellular Wg protein are present in *shi* mutant clones than in wild-type cells. (Strigini and Cohen, 2000). Recent reviews discuss the problems of morphogen transport that we have not been able to detail in this article (Teleman et al., 2001; Vincent and Dubois, 2002).

Cytonemes

Cells at the periphery of the imaginal disc have been found to extend actin-based long processes, called cytonemes, towards the AP border where Dpp is expressed (Ramirez and Kornberg, 1999). By using cytonemes, even cells far from the source of morphogen can make direct contact with cells that express the morphogen (Fig. 9). Although they await functional analysis before their role in gradient formation becomes clear, imaginal disc cells also extend other types of processes. It was proposed that these processes have roles in morphogen transport. As discussed earlier, the wing imaginal disc consists of a columnar cell layer, which gives rise to the wing, as well as an overlying peripodial epithelium (Fig. 1B). Inhibition of Dpp signaling only in the peripodial cells nevertheless disrupts growth and patterning of the wing (Gibson et al., 2002), suggesting that mechanisms that govern the growth and patterning of peripodial cells coordinate with those of columnar cells. The peripodial cells extend long cellular processes that traverse the acellular space between these layers and terminate on the surface of the columnar cells (Gibson and Schubiger, 2000). These processes may function to transmit the signal between the two layers of cells.

Argosomes

When parts of the membranes of cells of the imaginal disc are labeled with GFP linked to glycophosphatidylinositol (gpi), GFP-gpi localizes predominantly to the basolateral membrane. However, strikingly, GFP-gpi is also rapidly detected in nearby cells, suggesting that the basolateral membranes of disc cells can vesiculate and travel thoughout the disc epithelium (Greco et al., 2001) (Fig. 9). These membrane fragments, named argosomes, are produced by *wg*-expressing cells and co-localize with the Wg protein, suggesting that argosomes may provide a vehicle for the movement of Wg protein. The existence of argosomes may also have implications for the transport of other morphogens that have high membrane affinity.

Restricted diffusion and mathematical studies

The striking observation that blockage of endocytosis in clones of cells causes defects in Dpp transport (Entchev et al., 2000) led to the proposal that transcytosis can propagate Dpp through tissue, and seems to present the strongest argument against the restricted diffusion mechanism (the extracellular transport of functional morphogen interacting with cell surface molecules). A numerical analysis, however, has since shown that the observation in that experiment could also be explained by the restricted diffusion mechanism, if the internalization of the receptors by endocytosis is accounted for. The blockage of endocytosis is known to cause increased cell surface receptor levels. As gradient shape depends on cell surface receptor concentration (whereby the gradient becomes steeper in the tissue with more receptors), a numerical analysis predicts gradients to fall through such clones steeply enough to see the shadows behind the clones (Lander et al., 2002). This could be tested experimentally in *shi* mutant clones when antibodies against the morphogen and receptor become available. Furthermore, Lander et al. also suggest that, because Dpp gradient in the wing disc is almost fully established within 7

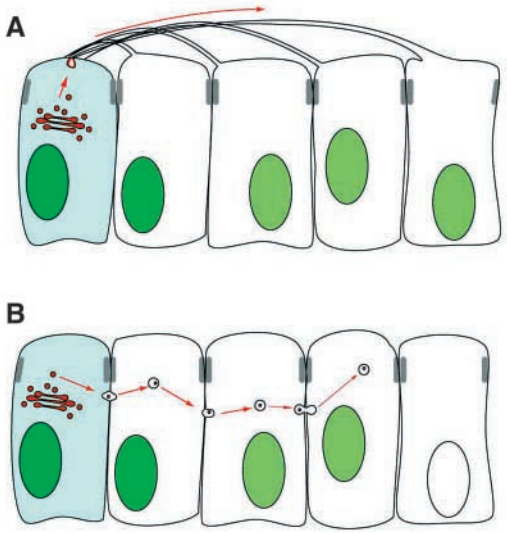

Fig. 9. Models for morphogen transport. (A) A model for cytonemes. Cells at the periphery of the imaginal disc extend long processes, cytonemes, towards the AP border, where Dpp is expressed (light blue). (B) A model for argosomes. The basolateral membranes of imaginal disc cells vesiculate and travel throughout the disc epithelium.

hours of the onset of Dpp expression, transcytosis would have to occur at implausibly fast rates (Lander et al., 2002).

If morphogens are transported by restricted diffusion, another numerical analysis also predicts the significance of morphogen receptors in establishing robust gradients. It has been proposed that morphogens are decayed rapidly close to the source and more gradually further away from the source in order to ensure their robustness and long-range action (Eldar et al., 2003). These authors proposed two models: (1) morphogen signaling represses receptor expression, while the receptor stabilizes the free morphogen; and (2) morphogen signaling activates receptor expression, while the receptor enhances the degradation of the free morphogen. They suggested that the former model represents the Wg system, and the latter the Hh system, as Wg downregulates its own recepter levels whereas Hh upregulates them, and they also showed that the Wg receptor Dfz2 stabilizes free Wg (Eldar et al., 2003). The models will be tested by experimental characterization of the receptor-dependent regulation of degradation of free morphogens.

Future perspectives

In 1924, Hans Spemann and Hilde Mangold carried out a spectacular experiment in which they transplanted the dorsal rip from an early gastrula of a newt into another early gastrula in the region that would become ventral epidermis. This resulted in a complete mirror image duplication of the whole body (Spemann and Mangold, 1924) (Fig. 10). We now know that many signaling molecules and their antagonists are involved in this process. When the whole process driven by the organizer is analyzed at the molecular level, it is probable that we will see successive rounds of regulation by both morphogens and signal relay mechanisms.

The potency of morphogens is impressive; when applied, they can organize the whole pattern of adult structures and

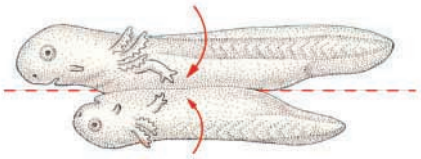

Fig. 10. Mirror image duplication reported by Hans Spemann and Hilde Mangold. Transplantation of dorsal rip tissue from an early gastrula of newt into another early gastrula in the region that would become ventral epidermis causes a mirror image duplication of the body axis (Spemann and Mangold, 1924). Reproduced with permission from Gilbert (Gilbert, 1997).

sometimes induce complete mirror image duplications. Although there are many secreted signaling molecules that seem to organize these patterns, only a small number of them are known to function as morphogens. Other candidates for morphogen molecules include members of the Fgf (Fibroblast growth factor) family of secreted signaling molecules, which have long been known to regulate many aspects of development. For example, Fgf8 is expressed at the junction between the midbrain and the hindbrain, known as the isthmic organizer, and it is thought to mediate, at least in part, the activity of the isthmic organizer that patterns the midbrain and rostral hindbrain (Martinez et al., 1999; Irving and Mason, 2000). The finding that varying amounts of Fgfs can induce different classes of spinal motoneurons indicates that Fgfs may function as morphogens (Liu et al., 2001).

Much of the knowledge about morphogens has come from the studies of imaginal disc development, mainly because of the simple structure of the disc and the powerful tools of analysis now available, especially mosaic clones (Blair, 2003). Advances in similar techniques will reveal more morphogens in vertebrates and will allow us to determine how their gradients are regulated.

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