GENETICS OF MORPHOGEN GRADIENTS

Tetsuya Tabata

The organization of cells and tissues is controlled by the action of 'form-giving' signalling molecules, or morphogens, which pattern a developmental field in a concentration-dependent manner. As the fate of each cell in the field depends on the level of the morphogen signal, the concentration gradient of the morphogen prefigures the pattern of development. In recent years, molecular genetic studies in *Drosophila melanogaster* have allowed tremendous progress in understanding how morphogen gradients are formed and maintained, and the mechanism by which receiving cells respond to the gradient.

MESODERM
The third germ layer in the embryo, formed during the process of gastrulation.

Institute of Molecular and Cellular Biosciences, University of Tokyo, Yayoi 1-1-1, Tokyo 113-0032, Japan. e-mail: ttabata@ims.u-tokyo.ac.jp

"A frog breeds a frog", or like breeds like, as the Japanese proverb goes. Although organisms develop from a single undifferentiated cell, the body pattern is genetically programmed in great detail, right down to the shape of the fingers. During the past 20 years, there has been substantial progress in understanding the basic mechanisms of development. It has emerged that only a relatively small number of genetic networks are essential for designing the body pattern during development. Signalling molecules are key to such networks. Some of these molecules have been shown to function as morphogens. Here, the term 'morphogen' (literally 'form-giving') is used rigorously to indicate a particular type of signalling molecule that 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[1]. Morphogen gradients are not the only available method used to pattern a developmental field. The same patterning effect could be achieved through the sequential inductive signalling that is relayed between adjacent cells in the field (FIG. 1). However, it is more economical for organisms to use a single signalling system to produce the various cell types that depend on their position in a molecular concentration gradient than to develop a different type of signalling system for each cell type. Indeed, many patterning processes in vertebrates and invertebrates have been attributed to the graded distribution of just one molecule. The concept of a morphogen is a very old one; morphogens were speculated to exist before there was any molecular evidence for them[2]. The molecular details of morphogen function have therefore been one of the fundamental issues in the field of developmental biology — one that molecular genetic studies, primarily in *Drosophila melanogaster*, are beginning to unravel.

Secreted signalling molecules have been implicated as organizers of pattern and growth in many developing systems, both in embryogenesis and in organogenesis. Some of these signalling molecules, which include members of the transforming growth factor-β (TGF-β) superfamily, and the Hedgehog (Hh) and Wingless (Wg)/Wnt proteins, are thought to function as morphogens. Activin and bone morphogenetic protein (BMP), two members of the TGF-β superfamily, are known to specify the MESODERMAL cell fates of early *Xenopus* embryos in a concentration-dependent manner[3–5]. A concentration gradient of Sonic hedgehog (Shh) — a vertebrate member of the Hh family — has been shown to organize the ventral half of the developing neural tube[6] (FIG. 2). Shh is also expressed in the posterior edge of the developing limb bud, from which it organizes the patterning of the limb bud[6]. Although their mode of action remains elusive, several BMP and Wnt family members are expressed in the dorsal edge of the neural tube — the floor plate — which controls the identity and pattern of dorsal neural cell types[7,8].

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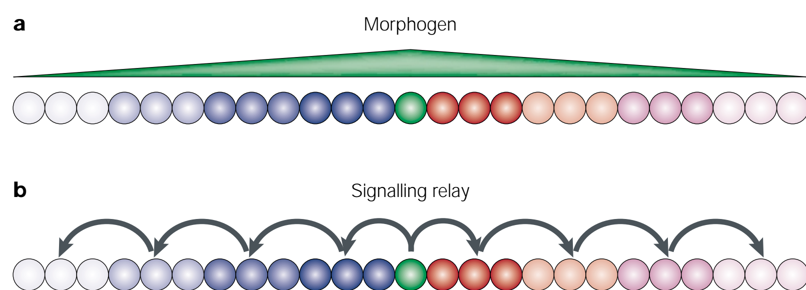

Figure 1 | **Patterning a developmental field by long-range signalling. a** | Morphogen signalling. A morphogen sets the positional value of a cell by forming a concentration gradient across the developmental field in which the cell resides; the value of the gradient at each point in the field is a function of the distance of the receiving cell from the morphogen-secreting cells (shown in green). **b** | Relay signalling. The positional value of each cell is set through the sequential inductive signalling events that are relayed between adjacent cells in the field.

SELECTOR GENES
A class of transcription factor, the products of which control the formation and identity of various morphogenetic fields.

Another example of a secreted signalling molecule is fibroblast growth factor 8 (FGF8), which is expressed at the junction between the midbrain and the hindbrain, known as the isthmic organizer (IsO). Although FGF8 is thought to mediate, at least in part, the activity of the IsO that patterns the midbrain and rostral hindbrain area[9–11], it is not yet clear whether FGF8 functions as a morphogen. These are just some of the cases in vertebrate development in which signalling molecules are likely to function as morphogens in various contexts. However, the mode of action of morphogens has never been challenged more rigorously by genetic analysis than in the development of insect appendages. The most notable examples of morphogens that have been extensively illustrated by genetic analysis are Decapentaplegic (Dpp) (of the TGF-β superfamily), Hh and Wg in patterning the adult appendages of the fruitfly, *D. melanogaster*. Out of all the secreted signalling molecules, Dpp, which is expressed in the developing fly wing, has been the most extensively studied morphogen. Recent work has investigated how both Dpp ligand and Dpp activity gradients are established and maintained. In this article, I therefore focus on the mechanisms that regulate the Dpp morphogen gradient in *Drosophila* wing development as a model system and, in less detail, the evidence for how Wg and Hh morphogen gradients might arise.

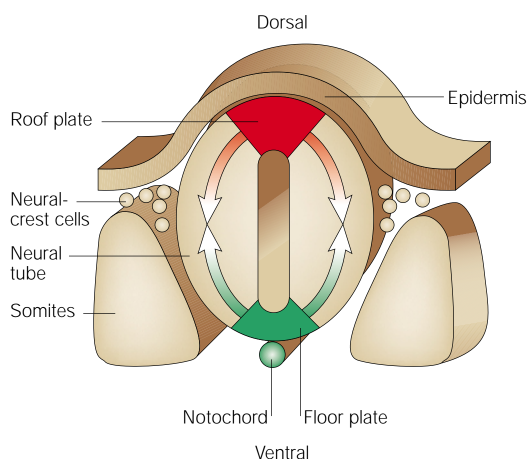

Figure 2 | **Inductive signals organize neuronal cell identity along the dorsoventral axis of the neural tube.** The expression of Sonic hedgehog (green) by floor-plate cells at the ventral midline patterns the ventral neural tube of vertebrate embryos. Signalling molecules, such as BMPs (red), which are expressed by roof-plate cells at the dorsal midline, pattern the dorsal neural tube.

Drosophila wing development

The *Drosophila* wing is an ideal model to study pattern formation, as a large body of knowledge exists on the regulatory networks that control gene function in this appendage. The adult wing arises from the larval imaginal disc — a single-layered sac of polarized epithelial cells. Imaginal discs are subdivided into non-intermingling sets of cells called compartments[12]: the wing imaginal disc is subdivided into anterior (A) and posterior (P) compartments along the A/P axis and is further subdivided into dorsal (D) and ventral (V) compartments along the D/V axis[12,13] (FIG. 3a). The source of morphogen lies at the border between adjacent compartments, which is stably maintained because cells in different compartments have a selective affinity for cells within their own compartment[14]. The identity of cells in the P compartment is imparted by the expression of the SELECTOR GENE *engrailed* (*en*)[15–17]. As a result, P cells secrete Hh (REF. 18), which acts as a morphogen to signal to A-compartment cells, and both patterns the central domain of the wing blade primordium[19,20] (FIG. 4) and induces *dpp* in a stripe of cells adjacent and anterior to the A/P compartment boundary[18,21,22] (FIG. 3b). Dpp is essential for the growth of wing cells and is responsible for patterning the wing beyond the central domain[21,23] (FIG. 4), using a concentration-dependent mechanism to induce target genes, such as *spalt* (*sal*) and *optomotor-blind* (*omb*, also known as *bifid*), at different distances from the A/P compartment border[24,25] (FIG. 5). Similarly, the expression of *fringe*[26] (which modulates Notch signalling) by cells in the D compartment, which are programmed by the gene *apterous*[27], results in activation of the Notch pathway at the D/V border[28]. Activated Notch induces *wg* expression at the D/V border[29,30] (FIG. 3c); here, Wg functions as a morphogen and organizes wing patterning by inducing target genes such as *Distal-less* (*Dll*) and *vestigial* (*vg*)[31,32].

Morphogens in the wing

Genetic evidence that Dpp functions as a morphogen comes from elegant experiments using a constitutively active form of the Dpp receptor. The pathway that transduces the TGF-β signals involves a combination of two types of serine/threonine kinase receptors (type I and type II)[33]. The activated type I receptor phosphorylates a specific member of cytoplasmic transducers, so-called 'receptor-regulated Smads', which, upon phosphorylation, translocate into the nucleus and regulate the expression of target genes (FIG. 5). In wing development, Thickveins (Tkv) and Saxophone (Sax) are the type I receptors, and Punt (Put) is the type II receptor. Tkv is crucial for wing development and its constitutively active form (Tkv*) can induce the expression of the target genes *sal* and *omb* when ectopically expressed[24,25]. This

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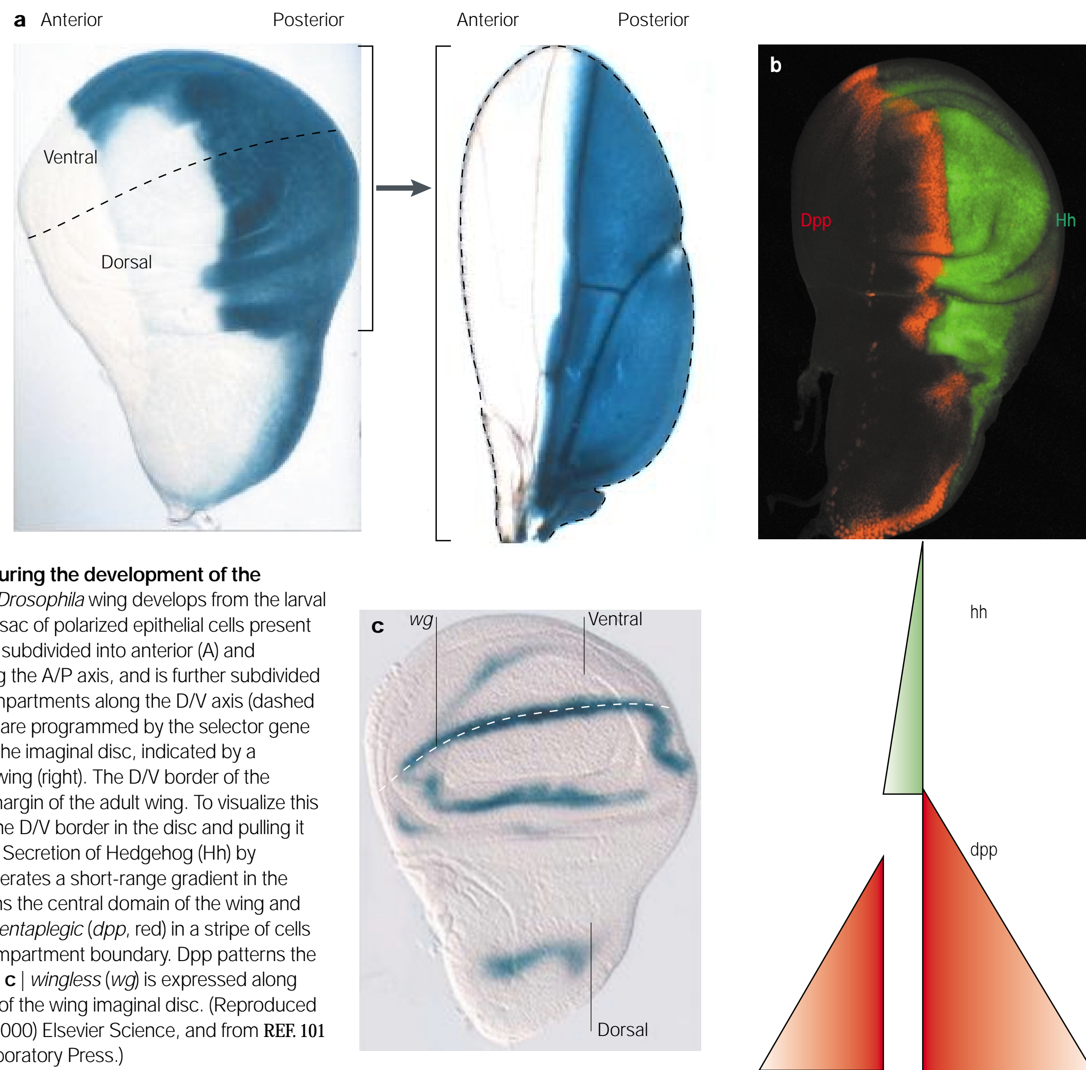

Figure 3 | **Inductive activities during the development of the *Drosophila* wing. a** | The adult *Drosophila* wing develops from the larval imaginal disc — a single-layered sac of polarized epithelial cells present in the larva (left). The wing disc is subdivided into anterior (A) and posterior (P) compartments along the A/P axis, and is further subdivided into dorsal (D) and ventral (V) compartments along the D/V axis (dashed line). Cells in the P compartment are programmed by the selector gene *engrailed* (*en*, blue). Only part of the imaginal disc, indicated by a bracket, develops into the adult wing (right). The D/V border of the imaginal disc develops into the margin of the adult wing. To visualize this movement, imagine picking up the D/V border in the disc and pulling it towards you, out of the page. **b** | Secretion of Hedgehog (Hh) by P-compartment cells (green) generates a short-range gradient in the A compartment and both patterns the central domain of the wing and induces the expression of *decapentaplegic* (*dpp*, red) in a stripe of cells adjacent anteriorly to the A/P compartment boundary. Dpp patterns the wing beyond the central domain. **c** | *wingless* (*wg*) is expressed along the D/V compartment boundary of the wing imaginal disc. (Reproduced with permission from REF. 70 © (2000) Elsevier Science, and from REF. 101 © (1990) Cold Spring Harbor Laboratory Press.)

information can be used to determine whether Dpp functions as a morphogen. The key to interpreting this experiment lies in determining whether the effect of expressing Tkv* is CELL AUTONOMOUS. If Dpp functions as a morphogen, the effects of Tkv* should be strictly cell autonomous; by contrast, if Dpp triggers a signalling relay mechanism, the effects of overexpressing Tkv* should be non-autonomous, because the second signal emanating from the cells that overexpress Tkv* would also affect surrounding (non-Tkv*-expressing) cells. The unambiguously cell-autonomous effects of expressing Tkv* indicate that Dpp functions directly at a distance[24,25]. Taken together with the observation that Dpp upregulates different sets of target genes at different concentration thresholds[25], it is likely that Dpp functions as a morphogen in the fly wing.

Similar experiments also indicate that Wg functions as a morphogen. Although the wild-type, secreted Wg activates target genes over a distance, a membrane-tethered form that was generated by fusing Wg to the carboxyl terminus of *Drosophila* Neurotactin (Nrt) — a type II transmembrane protein — upregulates Wg-target genes only in its immediate neighbours[31]. In addition, the expression of target genes is autonomously regulated in the mitotic clones of cells (BOX 1) that are mutant either for *dishevelled* or *armadillo*, which encode downstream components of the Wg signal transduction pathway[31,32]. Evidence that Hh functions as a morphogen has been provided in a similar way by comparing the activities of the wild-type, secreted form of Hh to a membrane-tethered form of the protein (HhCd2), which was generated by fusing the amino-terminal signalling domain of Hh to rat Cd2, a type I transmembrane protein[20]. Secreted Hh protein activates target genes in nearby cells over a range of ten cells, whereas the membrane-tethered Hh only activates target genes in cells immediately adjacent to the Hh source. This experiment shows that activation of Hh target genes at long range relies on the ability of Hh to move some distance from the cells in which it is expressed.

Vertebrate members of the same family of molecules probably also function as morphogens. Shh organizes the ventral half of the developing neural tube (FIG. 2); eliminating Shh activity through gene targeting in mice, for example, prevents the differentiation of ventral cells[34]. Moreover, ectopic expression of a mutated form of the Shh receptor, Patched (Ptc), which does

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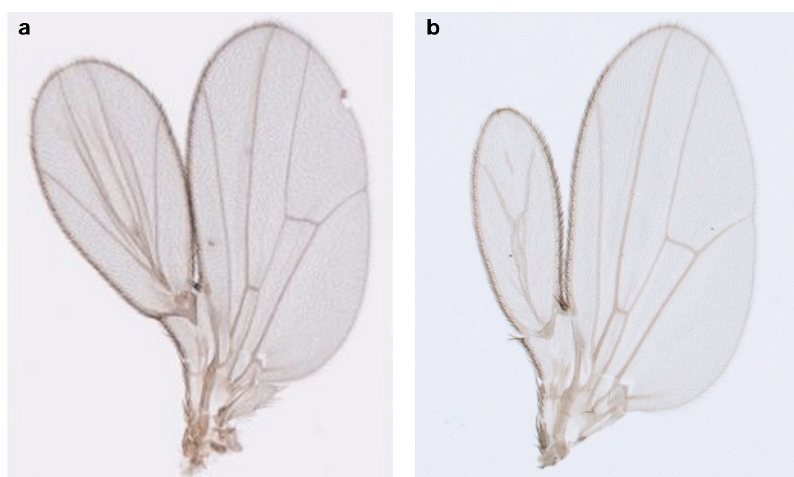

Figure 4 | **Inductive activities of Hedgehog and Decapentaplegic. a** | Ectopic expression of Hedgehog in a clone of cells causes a mirror-image duplication of the entire anterior compartment[22]. **b** | Ectopic expression of Decapentaplegic causes a mirror-image duplication of the anterior compartment that lacks the central domain[23]. Anterior is to the left.

not bind Shh but does antagonize its signalling, causes cell-autonomous V–D switches in neural progenitor identity in the chick neural tube, clearly indicating that Shh functions as a morphogen[35]. Shh is also known to organize the limb bud along the A/P axis and it has been shown recently that a cholesterol modification of Shh is required for the long-range action of Shh in the mouse developing limb bud[36]. During early vertebrate development, members of the TGF-β superfamily and their receptors are implicated in mesoderm formation. Although the extent to which they are essential for cell-type specification has not yet been fully understood, one TGF-β family member, Activin, has a morphogen-like capacity for inducing distinct mesodermal cell fates in *Xenopus*. The effects of constitutively active Activin receptors are shown to be strictly cell autonomous[4]. In zebrafish, Squint (Sqt) — another member of the same family — can induce different target genes at different concentration thresholds. Squint was recently shown to function as a morphogen: single-cell injection of *sqt* RNA into mutant embryos in which Sqt signalling has been genetically disabled can induce target genes in the wild-type cells that have been implanted far away from the Sqt-injected cell, indicating that Sqt acts directly at a distance[37].

The analysis on Shh and Sqt shown above are almost as rigorous as those done in *Drosophila*. However, in general, rigorous genetic analyses are not always possible in vertebrates and, therefore, the results in vertebrates should be interpreted with some caution.

CELL AUTONOMOUS
If the gene activity causes the effects only in the cells that express it, its function is cell autonomous; if it causes the effects in cells other than (or in addition to) those expressing it, its function is cell non-autonomous.

PLANAR TRANSCYTOSIS
A mechanism of transcellular transport within the plane of epithelium by which the molecule is internalized by endocytosis, transports intracellularly and is released to signal in the adjacent cells.

DYNAMIN
A GTPase required for clathrin-mediated endocytosis.

The Dpp morphogen gradient

One important question still remains: does Dpp really form a concentration gradient and, if so, what does it look like? Answering this question relied on being able to detect the Dpp protein *in vivo*, which, as the available antibody against Dpp fails to detect low levels of Dpp signal in the cells surrounding the Dpp source, meant having to overcome an experimental difficulty. Two groups dealt successfully with this problem by making a chimeric protein of Dpp and green fluorescent protein (GFP)[38,39]. The expression of Dpp–GFP in the endogenous Dpp expression pattern almost completely rescued the phenotype of a *dpp* mutant wing, indicating that Dpp–GFP functions in a manner similar to endogenous Dpp. The Dpp–GFP chimeric protein also allows researchers to visualize the functional Dpp gradient by monitoring GFP fluorescence. As shown in FIG. 6, fluorescence intensity is highest in cells in which Dpp–GFP is produced and forms a broad, shallow gradient on both sides of the endogenous Dpp expression domain. Movement by diffusion alone cannot explain the graded expression pattern, because a secreted GFP fusion protein that is composed of GFP and only the secretory transport domains of Dpp fails to form a gradient[39]. Having established that a Dpp gradient exists, the question that remains to be addressed is how the stable gradient is generated and maintained.

Generating the Dpp gradient. Several models have been proposed to explain the formation of a stable morphogen gradient. These models include the simple diffusion of molecules through the extracellular space, PLANAR TRANSCYTOSIS and displacement during growth (FIG. 7a–c). This last model predicts that the gradient might be formed upon cell proliferation: cells receiving Dpp could carry it away from the source as they are displaced by the addition of new cells (their descendants) during proliferation, thereby expanding the gradient[40]. There are two reasons why the displacement model does not explain convincingly how the Dpp gradient is formed. First, the model depends on the high stability of the Dpp molecule; however, the secreted Dpp–GFP protein is turned over rapidly (in under 3 h). Second, the time required to form a Dpp gradient in the wing imaginal disc takes only several hours, which is less than the average doubling time (8 h) of cells in the imaginal disc[38,39].

An analysis of the endocytosis requirements of Dpp–GFP supports the planar transcytosis model[39], although it does not exclude that the molecule moves through the extracellular space by diffusion. Signalling through Notch, the epidermal growth factor (EGF) and Wg requires DYNAMIN-mediated endocytosis[41–43]. That Dpp function requires endocytosis was indicated by the reduced expression domain of a Dpp target gene (***sal***) in endocytosis-defective mutants[44]. Dpp–GFP is normally found in the endocytic compartment situated close to the apical surface of the wing disc[39], but when endocysis is abolished using a Dynamin (***shibire***, ***shi***) mutant, no Dpp–GFP is internalized in endosomes[39]. Moreover, when a ***shi***$^-$ clone (BOX 1) 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 (that is, further away from the Dpp–GFP source) (FIG. 8). Cells that lie behind the ***shi*** mutant clone would be expected to receive Dpp–GFP from the upstream mutant cells when the leading edge of the Dpp–GFP wave passes through the clone. The absence of Dpp–GFP in these cells indicates that Dpp transport through the endocytic pathway might be essential for gradient formation.

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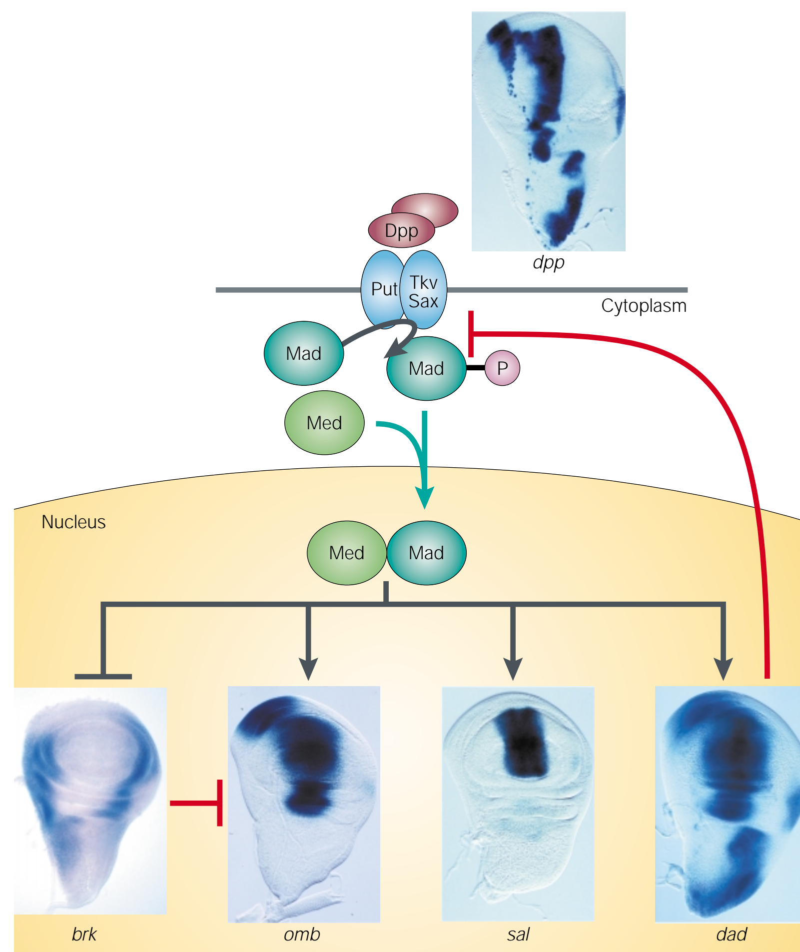

Figure 5 | **The Decapentaplegic signalling pathway.** The receptor for Dpp consists of a complex of type I (Tkv or Sax) and type II (Put) serine/threonine kinase receptors. Binding of Dpp to this receptor complex results in the activation of the receptor. The activated type I receptor directly phosphorylates Mad, which, upon phosphorylation, translocates into the nucleus along with Med and regulates the transcription of target genes. As a result, *sal*, *omb* and *dad* are upregulated, and *brk* is downregulated. Dad and Brk function as negative regulators of the pathway: Dad antagonizes Tkv-dependent phosphorylation of Mad, and Brk represses transcription of the target genes *sal*, *omb* and *dad*. The expression pattern of the Dpp target genes in the wing imaginal disc is also shown. *brk*, *brinker*; *dad*, *daughters against dpp*; Dpp, Decapentaplegic; Mad, Mothers against dpp; Med, Medea; *omb*, *optomotor-blind*; Put, Punt; *sal*, *spalt*; Tkv, Thickveins. (Reproduced with permission from REF. 82 © (1999) Macmillan Magazines Ltd.)

The fact that Dynamin is known to regulate receptor internalization and that Dpp–GFP colocalizes with its receptor, Tkv, in endosomes, raises the question of whether Dpp internalization involves receptor-mediated endocytosis. To address this question, Dpp–GFP localization was monitored in *tkv*$^-$ mutant clones. Dpp–GFP accumulates around the mutant cells in the *tkv*$^-$ clone that face the Dpp–GFP source, but it is found at much lower levels in both intra- and extracellular spaces in mutant cells behind them[39]. This implies that Dpp internalization is Tkv dependent and confirms that endocytosis is required for the long-range gradient formation of Dpp.

If Dpp is propagated through the endocytic transport pathway, then the components that regulate endocytic transport would be expected to control gradient formation. The activity of the small GTPase Rab5 is thought to be rate limiting in the early endocytic pathway. When a dominant-negative mutant of Rab5 is expressed in the wing disc, the expression of a Dpp target gene (*sal*) is restricted to the cells adjacent to the Dpp source[39]. Conversely, overexpression of Rab5 broadens the expression domain of Dpp target genes. Although Dpp–GFP could not be observed directly in these experiments, the results indicate that endocytic transport is rate limiting for determining the range of Dpp signalling. The authors also speculate that the gradient would remain stable if part of the internalized Dpp was degraded by the endocytic pathway[39]. Another small GTPase, Rab7, is known to target 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 signalling[39]. Taken together, these experiments indicate that transcytosis might be crucial for forming a long-range Dpp gradient, and that the balance between transcytosis and degradation can regulate that gradient. These conclusions were made despite the fact that, in experiments in which endocytosis was disrupted, the distribution of Dpp–GFP could not be observed directly. The experiments described above indicate that Dpp is propagated through the endocytic transport pathway. However, it is possible that Dpp is also propagated, in part, by diffusion in the extracellular space, because surface-labelling assays show that most of the Dpp–GFP signal appears to be in the extracellular space. So, more careful genetic and cell-biological studies will be required to determine the extent to which Dpp transport can be ascribed to endocytic mechanisms, to extracellular movement or to other mechanisms.

It has been suggested that transcytosis is required for the transport of Wg in embryonic development[41,45]. The lysosomal targeting and subsequent degradation of endocytosed Wg has been shown recently to be involved in generating the asymmetric distribution of Wg in the embryonic epidermis. A new antibody-staining protocol to detect extracellular Wg protein showed that the Wg protein forms a shallow extracellular gradient[46]. Although Wg was seen in punctate structures within cells, probably in endosomes, the following experiments indicate that endocytosis has no role in transporting the Wg protein in wing development. Wg does not localize to punctate structures in the *shi* mutant clones (as is the case for Dpp), and, in contrast to Dpp, Wg is internalized by wild-type cells behind the *shi*$^-$ clone[46]. Therefore, Wg can move across the *shi* mutant tissue and is internalized by the adjacent wild-type cells. In fact, Wg levels are at their highest in the *shi* mutant clones. It should be noted, however, that the Dpp and Wg experiments were done under different conditions: in the case of Dpp, the area lacking Dpp–GFP behind the *shi*$^-$ clone was only observed under one condition; that is, shortly after the leading edge of the Dpp–GFP wave had passed through disc clones in which pre-existing Dpp–GFP had been replaced by a pulse of Dpp–GFP[39] (FIG. 8). This precaution is necessary to prevent Dpp, which travels in all directions, from moving into the area behind the clone from the surrounding wild-type cells. The same model could easily apply to Wg. Therefore, an analysis of the

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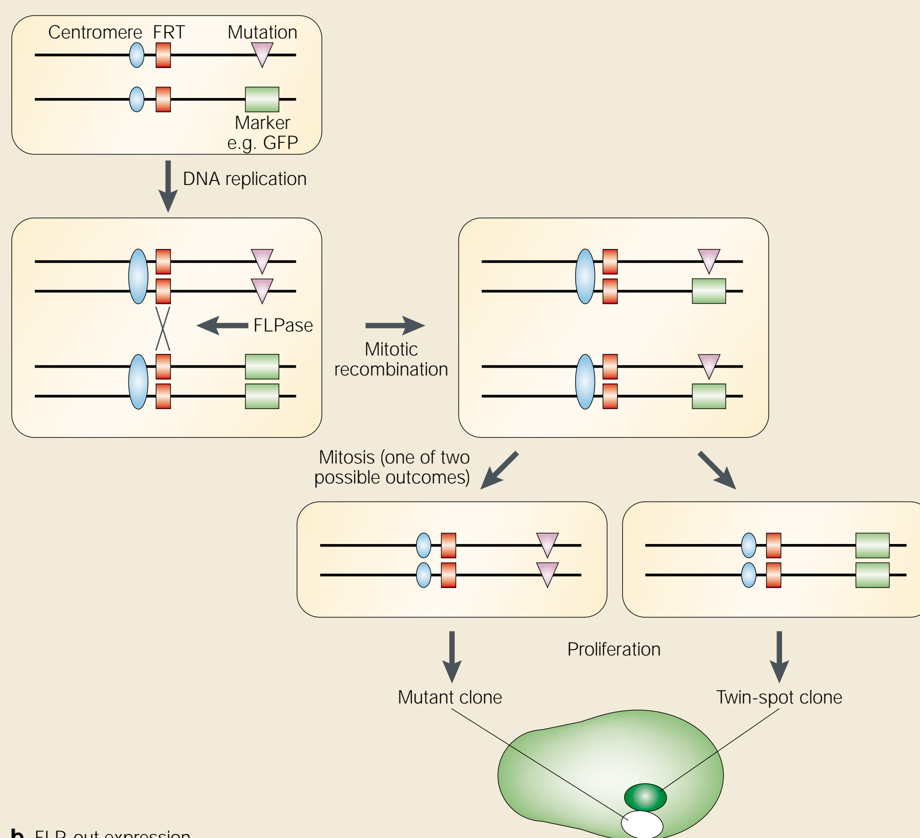
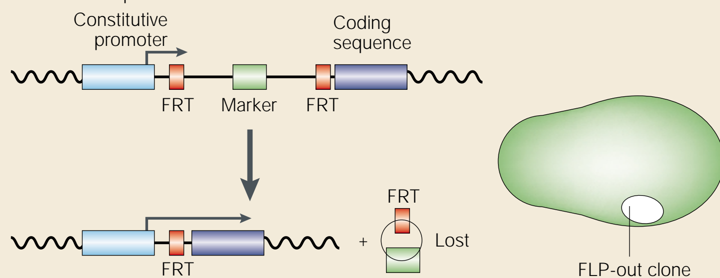
Box 1 | **Generating mitotic clones of cells in *Drosophila* imaginal discs**

A mitotically dividing cell normally gives rise to daughter cells that are genotypically identical to itself. However, if the exchange of sister chromatids (mitotic recombination) occurs, the resulting daughter cells can be genotypically different provided that the mother cell is heterozygous for the marker under study. Mitotic recombination can occur naturally, or be induced by DNA-damaging agents (X-rays, for example); however, a more controlled way of inducing mitotic recombination is to use site-specific recombination, as shown here. By creating *Drosophila* strains that contain a yeast (*Saccharomyces cerevisiae*) site-specific recombinase, FLPase, and its target site, FRT, clones of cells that are mutant for a gene[99,100] can be generated during imaginal development. A modification of this approach can be used to create cells that ectopically express a gene[22] (see figure). The clones can be induced at chosen stages of development by placing the *FLPase* gene under the control of a heat-shock promoter. The clones can be followed in imaginal discs or in adult structures by using various cellular or morphological markers.

Mutant clones

After the induction of FLPase by heat shock, homologous chromosome arms undergo site-specific recombination at FRT sites (a). If somatic recombination occurs in a cell that is heterozygous for a mutant gene of interest, then the ensuing cell divisions can give rise to two populations of daughter cells, one homozygous for the mutant gene (mutant clone) and the other homozygous for its wild-type gene (twin-spot clone). If a cellular marker (for example, GFP) is placed *in trans* to the mutant gene in the parent cell, then the homozygous mutant clone can be identified by the absence of the marker.





Creating clones that express a gene

Sometimes it is useful to induce the expression of a gene in a cell clone, rather than to remove its function (b). In flies that are transgenic for an FLP-out cassette, the coding sequence of a gene is separated from the promoter sequence by the insertion of a marker gene that is flanked by FRT sequences. In this construct, the coding sequence of the gene is not expressed, whereas the marker gene is expressed. After heat-shock induction, FLPase removes the marker gene by promoting site-specific recombination between the FRT sites and, in so doing, positions the coding sequence immediately downstream to the promoter, from which the coding sequence is expressed. The expressing clones can be identified by the absence of the marker.

Animated online

effects of disrupting and enhancing endocytosis will be required to establish whether transcytosis has a key role in Wg transport. M. Strigini and S. Cohen have shown that *shi* activity is required for Wg secretion[46]. This contrasts with the results described above that Shi does not affect Dpp–GFP secretion.

Compared with Dpp and Wg, Hh forms a relatively short-range gradient in imaginal development. Two transmembrane proteins, Smoothened (Smo) and Ptc, constitute the receptor complex for Hh: Smo activity is required to activate the Hh signal transduction pathway, whereas Ptc constitutively represses Smo activity[47]. Hh is thought to bind directly to Ptc and to liberate Smo from repression by Ptc. Although the underlying mechanism is not understood, the balance between Ptc and Smo proteins is thought to determine the activation state of the pathway (whether it is active or repressed)[48,49]. Hh and Ptc, but not Smo, have been shown to colocalize in endosomes[50]. Although Hh distribution is altered in *shi* mutant embryos (more Hh is apically localized[18]), it is not clear if endocytosis is crucial for forming a Hh gradient.

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Regulating morphogen gradients. Considerable progress has been made in recent years to identify the extracellular components that are required for the distribution of morphogen molecules. These molecules are collectively referred to as heparan sulphate proteoglycans (HSPGs), which form part of the extracellular matrix. HSPGs consist of a protein core to which heparan sulphate glycosaminoglycan (HS-GAG) chains are attached[51]. Genetic studies in *Drosophila* show clearly that HSPGs — which have been implicated in several signalling pathways, including those of Wnt, Hh, TGF-β and FGF — have crucial roles in these signalling pathways, both in embryonic and in imaginal development. The first indication came from the identification of mutations in two genes that are required for HS-GAG biosynthesis: *sugarless* (*sgl*)[52–54] and *sulfateless* (*sfl*). Mutations in *sgl* and *sfl* are defective in FGF and Wg signalling in embryonic development. Moreover, *dally* (division abnormally delayed), which encodes Glypican (the protein core of the HSPGs) has been implicated in Wg signalling[55,56]. HSPGs have also been proposed recently to regulate directly Wg distribution in the wing imaginal disc. A decrease in extracellular Wg is observed in *sfl* mutant cells and, conversely, overexpression of another Glypican, *dally-like,* sequesters Wg, which indicates that the HSPGs might be involved in the extracellular accumulation of Wg[57].

Hedgehog is unique in that it undergoes autoproteolysis to yield the functional amino-terminal half (HhNp)[58], to which cholesterol is covalently attached[59]. The movement of HhNp across the imaginal tissue is more restricted than that of a genetically engineered HhN that does not have a cholesterol moiety, which indicates that cholesterol modification of Hh regulates Hh transport by facilitating its diffusion[60]. Two genes have been identified that regulate Hh transport in a cholesterol-dependent manner. One of them encodes a novel Ptc-like transmembrane protein, Dispatched (Disp), which functions in Hh-secreting cells to liberate HhNp from the cells that express it[61]. The second gene, *tout-velu* (*ttv*)[62,63], has significant homology to the vertebrate exostoses (EXT) gene family that encodes the glycosyltransferase in heparan sulphate biosynthesis[64]. Ttv-modified HSPG is thought to be required for the proper distribution of HhNp because Hh is not detected in the anterior *ttv* mutant clones that abut the A/P border[62] and movement of HhNp, but not of HhN, is restricted in *ttv* mutant embryos[63]. So, movement of Hh is tightly controlled through its cholesterol moiety. Two lines of evidence support the role of EXT in vertebrate Hh transport. First, the EXT gene family is implicated in the inherited bone disorder, human multiple exostoses (EXT) syndrome[65,66], which is characterized by bone outgrowths and a high incidence of bone tumours. Second, Indian hedgehog (Ihh) — a member of the vertebrate Hh family that has been shown to regulate cartilage differentiation[67,68] — does not associate with the surface of cells that are deficient for the *Ext1* gene. Although Ihh is present around the surface of visceral endoderm cells of wild-type mice, no signal is detected in the same tissue of gene-targeted mice that are deficient for Ext1, despite the fact that Ihh protein and mRNA are expressed in mutant embryos at levels similar to those of wild-type embryos[69].

Although there has been no direct evidence for the extracellular movement of Dpp or the involvement of

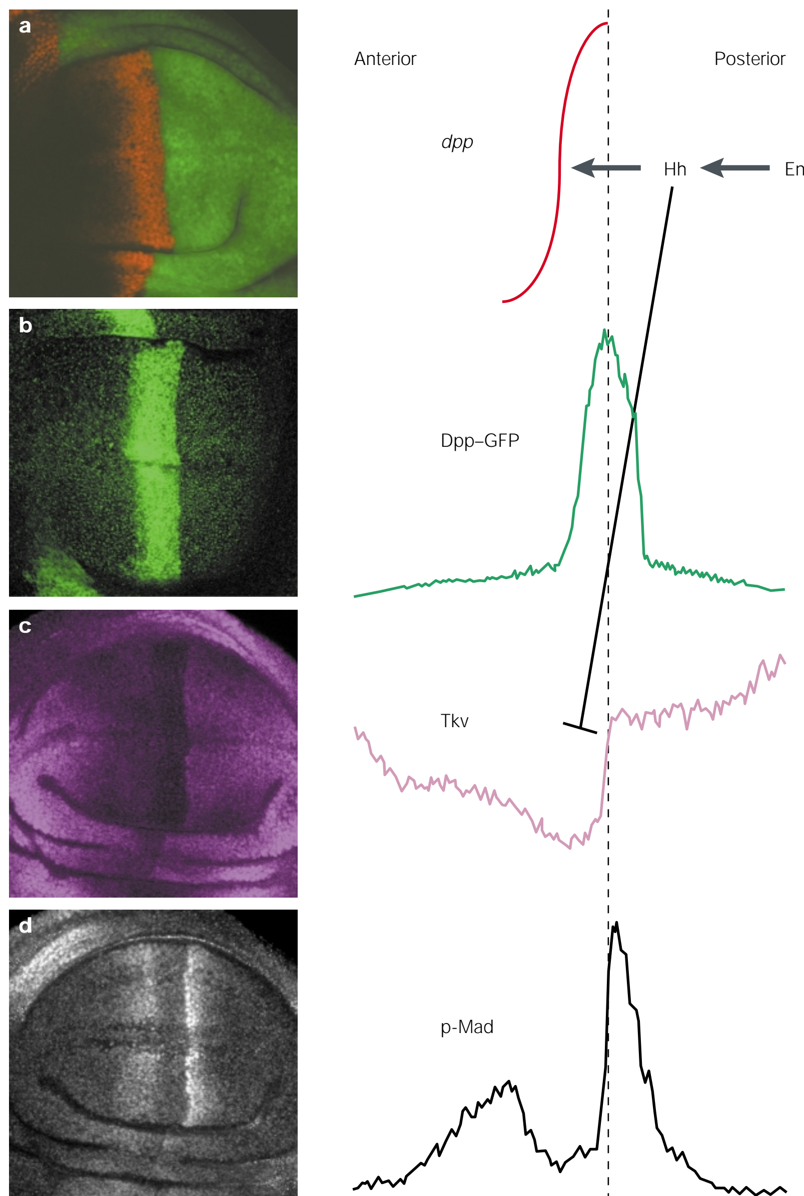

Figure 6 | **Ligand and activity gradient of the Decapentaplegic morphogen.** Confocal microscopy images (left) and schematic drawings (right) of a part of the wing imaginal disc that gives rise to the adult wing. **a** | Hedgehog (Hh, green), the expression of which is maintained by Engrailed (En) in the posterior (P)-compartment cells, induces *decapentaplegic* (*dpp*) expression (red) along the antero(A)/P border. **b** | Dpp diffuses in both A and P directions and forms a gradient, which can be visualized by the distribution of the chimeric protein Dpp–GFP (green). **c** | The expression level of Thickveins (Tkv), the Dpp receptor (purple), is very low along the A/P border because Hh downregulates its expression. In the middle of the wing disc, abutting the A/P border, the expression 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. This dynamic Tkv pattern accounts well for the shape of the activity gradient of Dpp signalling, as shown by **d** | the levels of phosphorylated Mothers against Dpp (p-Mad, grey), a downstream component of the Dpp signalling cascade. (Reproduced with permission from REF. 38 © (2000) Elsevier Science, and REF. 72 © (2001) Company of Biologists Ltd.)

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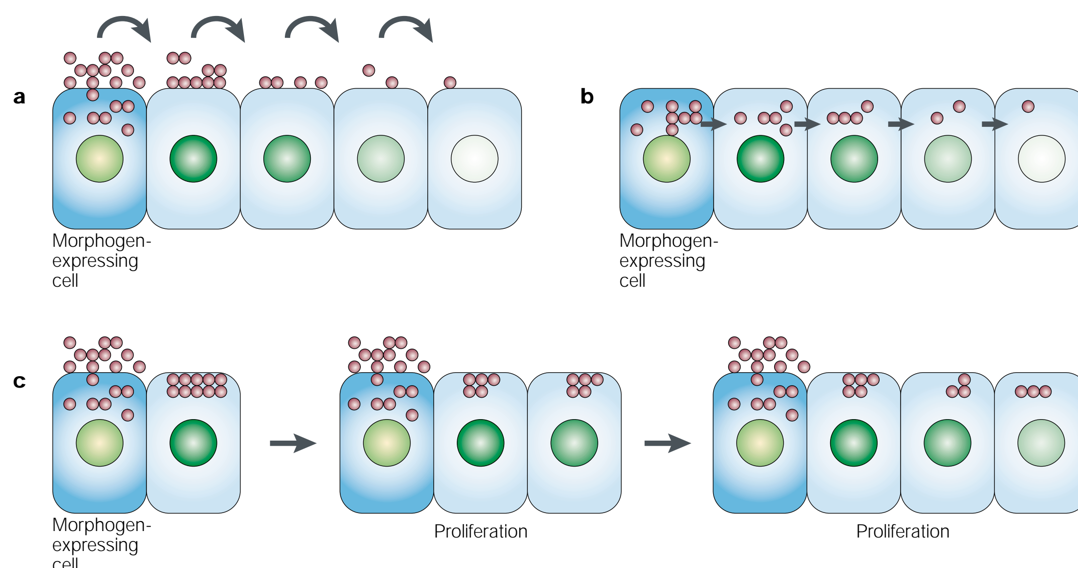

Figure 7 | **Models for movement of the morphogen molecule. a** | Diffusion through the extracellular space. **b** | Planar transcytosis. **c** | Displacement during growth.

HSPGs in Dpp transport, some extracellular molecule might restrict Dpp movement because Dpp cannot move freely across the tissue, even in the absence of the Tkv receptor[39].

Activity gradient of Dpp. Are the observed ligand gradients directly reflected in the activity gradient of the receiving cells? To address this question, Dpp signalling activity has been visualized *in situ* in the Dpp-receiving cells in the wing. As described above, the Dpp signal is transduced by phosphorylating a receptor-regulated Smad, Mothers against Dpp (Mad). Therefore, the phosphorylated version of Mad (p-Mad) can be used as an intracellular marker to monitor Dpp morphogen activity using a p-Mad-specific antibody[70]. The relative amount of p-Mad is higher in the cells near the A/P border, as expected; however, it is severely reduced in cells that express *dpp* (FIG. 6), as a result of the direct repressive action of Hh[70]. It is probably relevant to the significance of this complex regulatory interaction that Hh also directly organizes patterning in the region in which it attenuates Dpp signalling. Hh might need to downregulate Dpp signalling in this region to prevent Dpp signalling from interfering with its own patterning activities.

A Dpp target, Daughters against Dpp (Dad), has been shown to regulate the Dpp activity gradient; Dad competes with Mad for binding to the Tkv receptor and antagonizes Mad phosphorylation[71] (FIG. 5). So, the induction of Dad regulates the Dpp activity gradient by creating a negative-feedback loop that limits the domain of p-Mad activation.

Apart from the dip in the level of Dpp activity seen at the A/P border, the Dpp activity gradient also differs in the two compartments: it is steeper in the P compartment than in the A compartment (FIG. 6). This can be attributed to the differing levels of Dpp-receptor expression, as described below.

Receptor levels shape the morphogen gradient

The Dpp activity gradient shown by p-Mad levels differs from its ligand gradient, which raises the possibility that the Dpp activity gradient might be regulated between the Dpp ligand and a cytoplasmic signal transducer, probably at the level of the receptor. Dpp preferentially signals through the Tkv receptor in the wing disc and also negatively regulates *tkv* expression[40]. The level of *tkv* expression is higher in cells at the periphery of the wing disc and is lower in the central region (FIG. 6). In addition, a sharp reduction in *tkv* expression is seen at the A/P border of normal wing discs, a pattern very similar to that of p-Mad. I refer to the level of *tkv* expression in the area between the periphery and the A/P border as 'basal'. Interestingly, the basal level of *tkv* expression in the P compartment is higher than in the A compartment (FIG. 6). This might account for the steeper gradient of p-Mad in the P compartment; as high levels of Tkv limit the movement of Dpp, Dpp would not spread as far in the P compartment, leading to a steeper gradient of activity. In fact, the Dpp–GFP gradient is also steeper in the P compartment (FIG. 6). The Hh-dependent reduction of the p-Mad level at the A/P border discussed above has been shown to occur largely by repressing the transcription of the *tkv* receptor gene[70]. The higher Tkv level in the P compartment is maintained by the activity of the transcription factor En. Both the Hh and En activities that regulate *tkv* levels are mediated by the gene *master of thickveins* (*mtv*, also known as *scribbler*), which encodes a putative nuclear protein[72].

The ability of receptor levels to regulate the distribution of receptor ligands is not restricted to the Dpp morphogen. The same has also been reported for Hh. Ptc is expressed in the A compartment at low levels — thus repressing target genes of Hh signalling — and is highly induced by Hh at the A/P border. So, Hh upregulates the expression of its own repressor. This paradoxical property can be interpreted in the light of the other role of Ptc,

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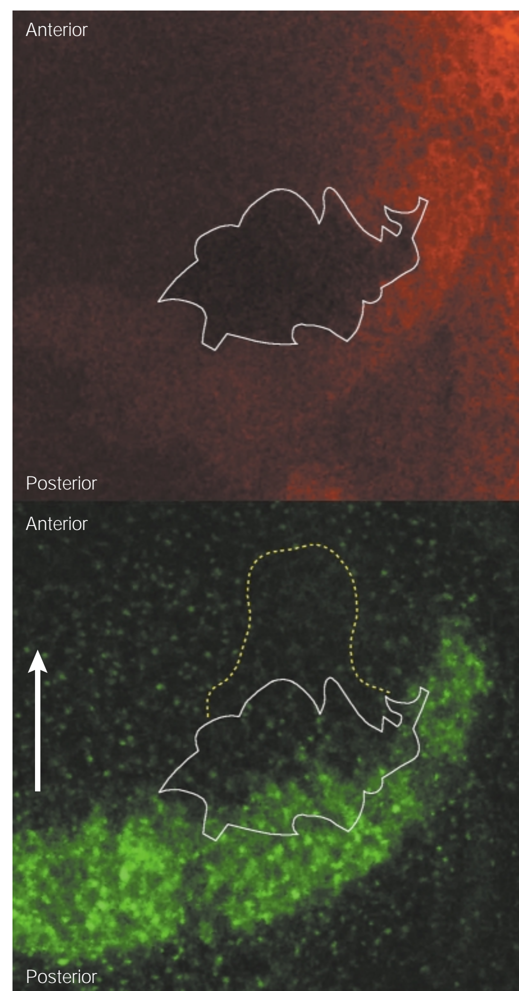

Figure 8 | **Evidence for planar transcytosis of Decapentaplegic in the wing imaginal disc.** Expression of Decapentaplegic (Dpp)–GFP in Dynamin (encoded by *shibire, shi*) mitotic clones in the *Drosophila* wing imaginal disc indicates that the gradient of Dpp is distributed by planar transcytosis. Mitotic clones of *shi*⁻ are marked by the absence of the Nmyc marker (area within the white line in both panels). Dpp–GFP (green) is expressed along the anteroposterior border of the disc and diffuses towards the periphery (arrow). Fewer or no Dpp–GFP vesicular structures are detected in the area (dotted yellow line) distal to the *shi* mutant clone, indicating that endocytosis is required for Dpp–GFP to reach those cells. (Reproduced with permission from REF. 39 © (2000) Elsevier Science.)

which is to prevent Hh from spreading too far into the A compartment[73]. The low level of Ptc in the A compartment is sufficient to suppress ectopic Hh signalling, but is insufficient to restrict Hh movement. Therefore, Hh induces a high level of Ptc to limit the range of its own distribution gradient.

For Wg, the situation is not so straightforward. Three members of the Frizzled (Fz) family of seven-pass transmembrane proteins — Fz, Dfz2 (REF. 74) and Dfz3 (REF. 75) — have been identified as receptors for Wg. Their functions are redundant; a single mutation in any of them impairs Wg signalling, whereas in cells that are doubly mutant for *fz* and *dfz2*, Wg signalling is abolished[76]. Expression of Dfz2 is negatively regulated by Wg and, therefore, the expression pattern of Dfz2 is complementary to the distribution of its ligand. This dynamically regulated distribution of Dfz2, and the fact that ectopically expressed Dfz2 leads to the accumulation of Wg, have led to the proposal that the Dfz2 level is crucial in determining the Wg gradient[77]. However, the absence of any phenotypes in Dfz2 mutants questions the significance of the Dfz2 distribution in shaping the Wg gradient[76]. A further complication is that Dfz3, the third member of the family, is induced by Wg signalling, although its binding affinity to Wg is much less than that of Dfz2 (REF. 75). So, regulation of receptor levels might not be crucial for the distribution of all morphogens.

Regulation of the receptor levels is poorly understood in vertebrates except for Ptc, which is always upregulated by Shh[78,79] and probably functions in the same way as in *Drosophila*.

Regulating target gene expression

A morphogen signal is transduced through an intracellular signalling cascade to the nucleus, where it regulates the transcription of target genes in the receiving cells. Because the activity gradient can be modulated at every step of the signal transduction pathway, the ultimate output of the morphogen gradient should be represented by the transcriptional activity that it induces. Therefore, to understand how the levels of a morphogen are modulated in the receiving cell, we must first understand how transcriptional activity is correlated with the morphogen gradient. One of the simplest explanations would be that transcription factor activity in target cells is a direct readout of the activity of the morphogen. Although this seems to be applicable to the Dpp, Hh and Wg morphogens, another layer of regulatory mechanism has begun to be elucidated.

In Dpp signalling, p-Mad translocates to the nucleus, where it functions as a component of the transcriptional complex and regulates the expression of target genes such as *sal, omb* and *dad*. We could, therefore, use the level of p-Mad as a measure of the ultimate determinant. However, there is another complexity in the Dpp morphogen system. The transcription factor Brinker (Brk) is known to repress Dpp target genes[80–82]. Furthermore, *brk* expression is downregulated by the Dpp signal, which leads to the formation of a counter-gradient of repressor to the Dpp morphogen (FIG. 5). So, Dpp can also indirectly regulate gene expression by downregulating the expression of *brk*. The regulatory sequences of several Dpp target genes have many Mad and Brk binding sites of which several overlap. Competition between the binding of Mad and Brk to overlapping sites could determine spatially restricted domains of expression of Dpp target genes[83–85]. Although direct evidence for this is lacking, data indicating that it occurs are now available. By comparing the Mad/Brk binding sites of *Ultrabithorax* (*Ubx*, a target of short-range activation in the visceral mesoderm) and those of *vg* (a target of long-range activation in the wing disc), a recent report showed that *Ubx* is more sensitive to repression by Brk. This indicates that Brk binding sites might be crucial in limiting thresholds for activation by Dpp[85].

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In both the Hh and the Wg pathways, a component of the signalling cascade translocates into the nucleus on reception of the signal and functions as a subunit of a transcriptional complex. Furthermore, balancing between the activator and repressor is also important in regulating transcription at the end of the Hh[86–89] and the Wg[90–92] signalling cascades. These features have been shown to be in large part conserved in vertebrates[93–96].

Conclusions

Is it possible to devise general principles to explain how morphogen gradients are formed and maintained? Our current knowledge is still incomplete. We do not know, for instance, whether endocytosis is a general way of transporting morphogens or whether regulation of receptor levels is a common mechanism for shaping the gradient. A morphogen might behave differently in separate developmental contexts. A relevant example exists for Dpp signalling: the secreted antagonist of Dpp, Short gastrulation (Sog), is important in embryonic[97], but not imaginal, development. The mode of action of a morphogen might depend on many other factors, such as the type of cell or the size of the field in which it is operating, or the length of time for which it is expected to act. Given that the components of morphogen signalling cascades are highly conserved in evolution, the mechanisms revealed in the *Drosophila* wing are probably applicable to the action of morphogens in vertebrates.

Morphogen gradients are regulated at several levels and are shaped, in part, by feedback loops. Many molecular events are involved in the gradient formation, so it would not be surprising if additional molecules, with novel functions, were found to have roles in each signalling pathway. It should be noted that the mechanisms described here are based on the assumption that cells closer to the source receive the morphogen and transmit it to the neighbouring downstream cells, regardless of the underlying mechanisms. However, some doubt has been cast over this premise by the discovery of cytonemes — actin-based long processes that imaginal cells extend towards the A/P border, where Dpp is expressed[98]. By means of cytonemes, even the cells far from the source of morphogen can make direct contact with cells that express the morphogen, thereby removing the need for an intercellular transport system to disperse it. Although we have not so far needed the function of cytonemes to explain the observations or the effects of Dynamin mutant clones on Dpp distribution, further analysis might discover the function of cytonemes and pave the way to understanding the novel mechanisms by which morphogens organize patterning.

Links

DATABASE LINKS

TGF-β | Hh | Wnt | Activin | BMP | Shh | FGF8 | Dpp | Wg | *en* | *sal* | *omb* | *fringe* | Notch | *apterous* | *Dll* | *vg* | Tkv | Sax | Put | Nrt | *dishevelled* | *armadillo* | Cd2 | Ptc | Sqt | *shi* | Rab5 | Rab7 | Smo | *sgl* | *sfl* | *dally* | *dally-like* | Disp | *ttv* | human multiple exostoses | Ihh | *Ext1* | Mad | Dad | *mtv* | Fz | Dfz2 | Dfz3 | Brk | *Ubx* | Sog | Med

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