The Decapentaplegic morphogen gradient: from pattern formation to growth regulation

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Abstract | Morphogens have been linked to numerous developmental processes, including organ patterning and the control of organ size. Here we review how different experimental approaches have led to an unprecedented level of molecular knowledge about the patterning role of the *Drosophila melanogaster* morphogen Decapentaplegic (DPP, the homologue of vertebrate bone morphogenetic protein, or BMP), the first validated secreted morphogen. In addition, we discuss how little is known about the role of the DPP morphogen in the control of organ growth and organ size. Continued efforts to elucidate the role of DPP in *D. melanogaster* is likely to shed light on this fundamental question in the near future.

To understand how shape and size of animal organs are regulated during embryogenesis lies at the heart of developmental biology. The concept of gradient morphogens to explain body patterning was put forward more than a century ago. Concentration gradients of 'form-generating' substances were proposed to arise through signalling molecules that emanate from a localized source eliciting distinct responses at different distances (reviewed in REFS 1,2). Over the past 20 years, secreted proteins of the WNT, Hedgehog (HH), epidermal growth factor (EGF), fibroblast growth factor (FGF) and transforming growth factor-β (TGFB) families have been recognized as candidate substances to specify positional information by such mechanisms (reviewed in REF. 3). An unambiguous demonstration of the existence of extracellular morphogens was obtained some 10 years ago in the *Drosophila melanogaster* wing system, where Decapentaplegic (DPP), the fly homologue of vertebrate bone morphogenetic proteins BMP2 and BMP4, functions directly at a distance to specify gene expression patterns in a concentration-dependent manner. Here we review how progress over the past decade has provided us with an unprecedented level of molecular knowledge about a morphogen system that regulates organ pattern. At the same time, we discuss our poor understanding of DPP's function in the regulation of organ size, a property that continues to elicit fascination and spur research.

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doi:10.1038/nrg2166

Is DPP a morphogen?

Classically, a morphogen is defined as a substance that spreads from a localized source such that its concentration declines in a continuous and predictable fashion, providing a series of concentration thresholds that control the behaviour of surrounding cells as a function of their distance from the source. The advent of modern molecular genetics saw the identification of many genes that encode proteins with apparent long-range organizing activities, but the way in which these molecules achieve their long-range organizing influence was not clear. Although they were suspected to function as gradient morphogens, another plausible possibility was that they act as short-range inducers that initiate a sequential chain of secondary signals, which ultimately elicit distinct responses at different positions.

In situ hybridization experiments and the analysis of reporter genes have shown that DPP is expressed in a small set of cells that form a stripe along the anteroposterior compartment boundary of the larval wing imaginal disc (BOX 1). Ectopic expression of DPP in anterior or posterior cell clones caused reorganizations of the wing pattern that indicated a long-range activity[4,5]. To analyse whether DPP functions as a bona fide morphogen, it was necessary to ask whether DPP exerts its organizing influence on responding cells directly and in a concentration-dependent manner, or whether its activity is mediated indirectly by a chain of secondary signals (FIG. 1). Four tools were required to address this

Box 1 | ***Drosophila* imaginal discs**

Drosophila imaginal discs are epithelial structures that give rise to the adult body structures. The wing disc contains about 30 cells at the beginning of the first larval instar and, at metamorphosis almost 4 days later, the number of cells reaches about 50,000 (REF. 78). The adult wing is produced by the eversion of the wing disc, and the cells of the wing neither divide nor grow. The size of the adult wing is therefore predetermined by the final size of the wing imaginal disc[62]. Wing disc size seems to a large extent to be regulated disc-autonomously, because transplantation of early discs into the abdomen of adult flies results in discs of normal size[84,85]. The effects of disc-extrinsic inputs, such as nutrient availability, which affects organismal size, are discussed elsewhere[86].

experimentally: a crucial component for the transduction of the DPP signal in receiving cells; an altered form of that component that activates the pathway constitutively; a method to eliminate the wild-type component or express its activated form in marked cell clones; and molecular readouts for the apparent long-range activity of DPP.

The first condition was fulfilled with the genetic and molecular identification of the DPP type I and type II receptors, Thickveins (TKV) and Punt, respectively[6–10] (FIG. 2). Their structure as membrane-spanning serine–threonine kinases and their functional similarities with mammalian TGFB receptors also allowed the construction of a constitutively active form of TKV, TKV^{QD}. This variant type I receptor carries a mutation in the vicinity of its GS domain and structurally mimics the phosphorylated form, so that it potently activates the DPP pathway in a ligand-independent manner[11,12]. The third tool — methods to create marked cell clones — was traditionally well developed in *D. melanogaster*. In particular, loss-of-function mutations could be rendered homozygous in single cells of an otherwise heterozygous animal by somatic recombination, a procedure that became highly efficient with the advent of the Flp recombinase system[13,14]. Conversely, transgenes could be clonally activated by the newly developed Flp-out method, and the resulting clones identified by the concomitant loss of a marker gene[15]. Finally, a molecular surrogate was used to forecast the formation of morphological pattern. The *lacZ* reporter genes for *optomoter-blind* (*omb*, also known as *bifid*) and *spalt* (also known as *salm*) were ideally suited for this fourth requirement, as they not only provided markers for the potential long-range action of DPP, but their nested expression patterns, which were centered on the DPP source (FIG. 3a), also allowed researchers to address the existence of concentration-dependent outputs of DPP signalling.

Key to scrutinizing the morphogen hypothesis was to compare the consequences of ectopic expression of the secreted ligand DPP with those of ectopic activation of its receptor system (in rare clones of only a few cells) (FIG. 1). If DPP operates indirectly through the induction of secondary signals, then the local ectopic activity of the receptor system alone in cells that are positioned at a distance from the source should be as effective as ectopic expression of the ligand itself in exerting a long-range influence on the surrounding tissue. By contrast, if DPP operates as a gradient morphogen, only ectopic activity of the secreted ligand, but not that of its receptor system, should have this property. The outcome was crystal clear: cells that ectopically secreted DPP expressed *omb* and *salm*, and also induced the expression of these genes in overlapping but distinct populations of surrounding cells (*omb* was induced in a broader region than *salm*); conversely, cells that expressed the constitutively active DPP receptor TKV^{QD} expressed both *omb* and *salm*, but did not induce ectopic expression of these reporter genes in neighbouring cells. These and further experiments led to the conclusion that DPP exerts its long-range influence on wing patterning by acting directly at a distance as a gradient morphogen, and not indirectly as a short-range inducer of other signals. Later, a fluorescent form of DPP was found to be distributed at a significant distance from its source (see below), an observation that provides descriptive support, but not functional evidence, for long-range morphogen activity.

The availability of loss-of-function mutations in the *tkv* gene (tool number one) was not strictly required for the strategy described above. Although adding support for the argument that TKV is essential for transducing all known responses to DPP in *D. melanogaster*, mutant *tkv* alleles were essential for challenging the 'cellular memory' or 'ratchet' mechanism that was also put forward to account for the long-range action of DPP[11]. According to this model, DPP would act directly, but only at short range, to alter surrounding cells so that these and their descendents would heritably express genes like *salm* and *omb*, even if they moved out of contact with DPP-secreting cells as a consequence of wing growth[11]. However, the loss of *omb* expression in late-induced *tkv* mutant cell clones, located at a significant distance from the DPP source, argued that all cells in the *omb* expression domain continuously require, and hence receive, DPP input throughout wing disc development[12].

More recent studies of a second *D. melanogaster* BMP-family member, Glass bottom boat (GBB), suggest that DPP might not act alone in specifying BMP-signalling-dependent positional information along the anterior–posterior axis[16]. GBB seems to contribute to the BMP morphogen gradient, most strongly in the posterior compartment. Either alone or in heterodimers with DPP, GBB uses the same signal transduction system as DPP, and might influence some of the properties of morphogen gradients that are discussed below.

The demonstration of the action of DPP as a morphogen in wing development called immediate attention to three biological problems. How does DPP spread from its source to form an activity gradient in the disc epithelium? How do wing cells read and interpret the graded DPP signal to respond in a manner that is appropriate for their position? And finally, as altered DPP signalling strongly affects wing size, how does DPP affect growth? The desire to understand how DPP organizes wing development can be largely ascribed to the elusive combination of DPP's ability to regulate cell fates as well as cell proliferation.

How does DPP spread to form a gradient?

The local synthesis of the ligand and its action at a distance indicated that DPP forms a protein gradient

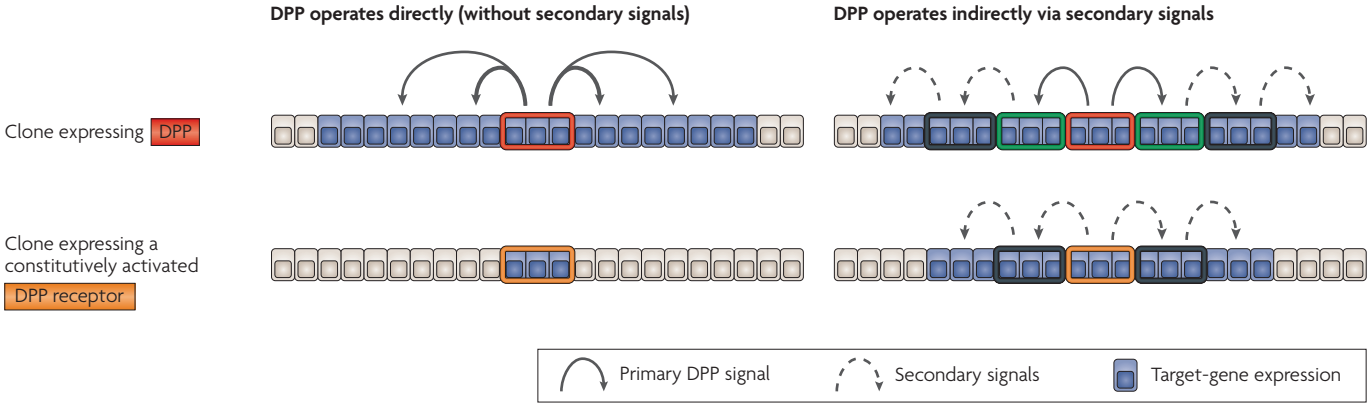

Figure 1 | **An experimental test for Decapentaplegic (DPP) morphogen function.** The long-range activity of a secreted signalling molecule can either be direct or indirect. If DPP operates indirectly, through the induction of secondary signals, then the local ectopic activity of the receptor system alone in cells that lie at a distance from the source should be as effective as ectopic expression of the ligand itself in exerting a long-range influence on surrounding tissue (right side). By contrast, if DPP operates as a gradient morphogen, only ectopic expression of the ligand, but not that of its receptor system, has this long-range effect (left side).

with its highest level in the centre of the wing along the anteroposterior compartment boundary, with levels declining as the distance from this boundary increases. Different scenarios have been proposed to participate in or regulate gradient formation, the two major ones being facilitated diffusion and planar transcytosis.

Several studies point to a crucial involvement of extracellular matrix components, in particular members of the heparan sulphate proteoglycans (HSPGs)[17,18], in the formation and stabilization of the DPP gradient. The proteoglycans of the glypican subgroup, Dally and Dally-like protein (DLP) seem to enhance the spreading of DPP on the cell surface. Extracellular DPP fails to move across cell clones that lack both Dally and DLP, a phenomenon which is also seen in clones that are mutant for *sulphateless*, which is essential for the biogenesis of glypicans[19]. In addition to affecting morphogen spreading, glypicans seem to be important for effective signalling in a cell-autonomous manner, possibly by influencing the presentation of DPP to its receptors[19–21].

Movement of a biologically active GFP–DPP fusion protein was impaired in discs with large patches of cells lacking Dynamin, a protein that is essential for endocytosis[22]. This result was taken as evidence of a role for 'planar transcytosis' in morphogen transport: upon internalization by receptor-mediated endocytosis at the cell surface, DPP is re-secreted and taken up by adjacent cells[22,23]. This model of morphogen distribution has been challenged by theoretical and experimental studies[24,19]. Further analysis is required to determine the exact role of endocytosis and facilitated extracellular diffusion in the generation of a stable DPP gradient. In principle, both mechanisms could work hand-in-hand to stabilize and fine-tune the gradient[25]. A recent kinetic study using fluorescence recovery after photobleaching (FRAP) showed that GFP–DPP indeed requires Dynamin to redistribute into a region that has been photobleached, and diffuses more slowly upon partial inactivation of Dynamin[26]. However, because Dynamin is not only required for endocytosis, but also for exocytosis[27,28], it is possible that its effect on GFP–DPP distribution reflects altered cell-surface or extracellular matrix properties.

The controversy about the distribution of the relevant portion of DPP and the mechanisms of gradient formation reminds us that the mere whereabouts of a signalling protein in a tissue cannot tell us whether a morphogen mechanism is at work, because the fraction of the observed protein that is responsible for the patterning output is not known. Without analysing both ligand distribution and signalling activity concomitantly in different experimental situations, a clear statement about the contribution of either mechanism will be difficult to argue in the case of DPP.

How is the DPP signal transduced?

To understand how cells respond to the DPP morphogen gradient in molecular terms, two important issues had to be addressed. On the one hand, the components involved in signal transduction from the cell surface to the nucleus needed to be identified, characterized and ordered by genetic and molecular epistasis analysis. On the other hand, assuming that the prime response to DPP with regard to pattern formation was a change in nuclear transcription, direct target genes had to be isolated and their control regions had to be carefully dissected.

Because signalling mediators downstream of mammalian TGFB receptors were unknown at the time, the study of functional homologues in *D. melanogaster* was not possible, and genetic approaches were used to identify pathway components. Soon after the identification of the DPP receptors, mutations in two genes, *Mothers against dpp* (*Mad*) and *Medea*, were reported to enhance a hypomorphic *dpp* mutant phenotype, suggesting that these genes have a positive role in DPP signalling[29,30]. The putative protein encoded by *Mad* showed similarity to *Caenorhabditis elegans* and vertebrate proteins, and within a short time biochemical analysis and cell-culture

Facilitated diffusion
The extracellular diffusion of molecules mediated by cell-surface or extracellular matrix proteins.

Planar transcytosis
Active transport of a molecule via endocytosis and re-secretion, so that it moves from one cell to a neighbouring cell in a planar fashion within an epithelial sheet.

Glypican
Membrane-associated proteoglycan with a GPI (glycosylphosphatidylinositol) anchor at the C terminus.

Endocytosis
The capture of extracellular molecules via the uptake of membrane vesicles or vacuoles derived from the plasma membrane.

Photobleach
To bleach or destroy the fluorescence of a molecule by intense illumination in a given area of a biological sample. Fluorescence recovery can then be studied as a re-equilibration of the fluorescent signal by molecules from the non-bleached environment.

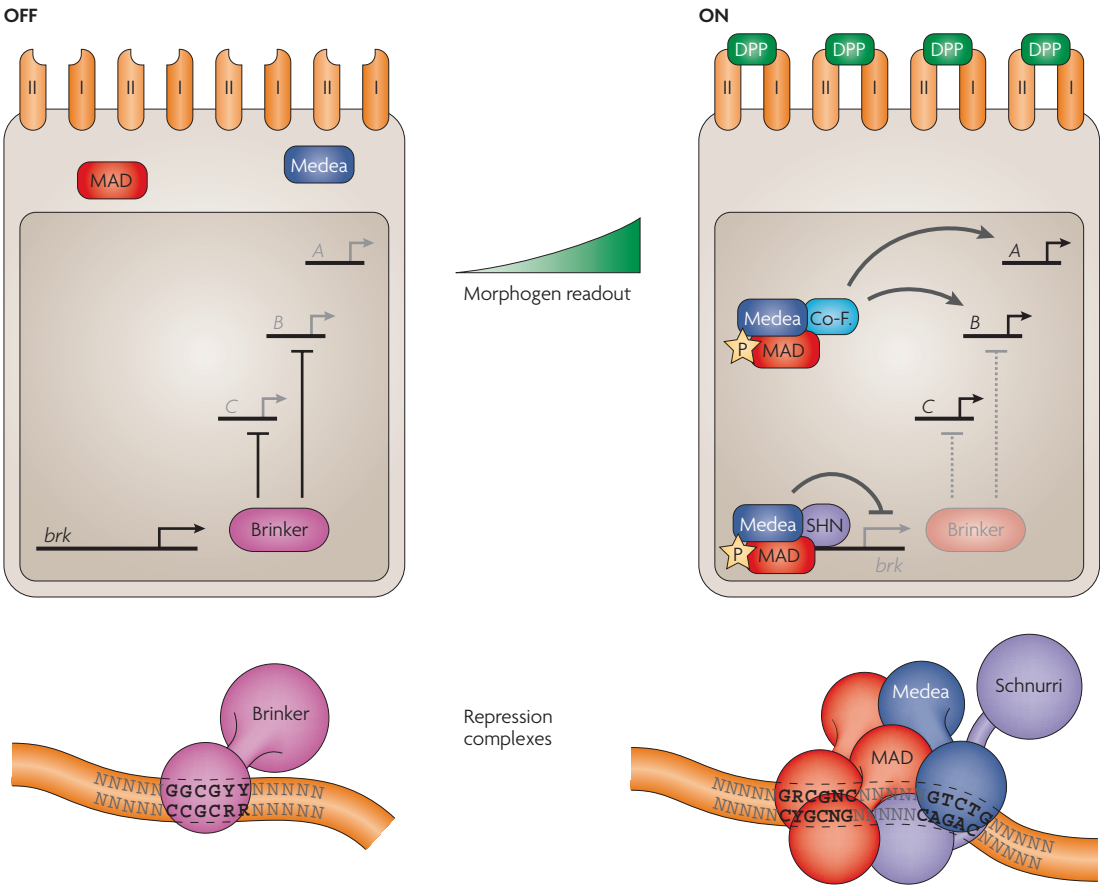

Figure 2 | **Decapentaplegic (DPP) signal transduction components.** In the absence of DPP signalling, the levels of the Brinker (BRK) transcriptional repressor are high. BRK represses most DPP target genes via the sequence GGCGYY in their regulatory region. When DPP reaches a cell, it interacts with its two receptors, the type II receptor Punt and the type I receptor Thickveins (TKV). Within this ligand–receptor complex, the constitutively active receptor Punt phosphorylates TKV, which in turn phosphorylates the receptor SMAD (R-SMAD) Mothers against *dpp* (MAD). Phosphorylated MAD (pMad) subsequently interacts with the common-mediator-SMAD (co-SMAD) Medea, and this complex is translocated into the nucleus. Binding of the pMAD–Medea complex to the silencer elements at the *brk* locus results in the recruitment of Schnurri (SHN), which represses the transcription of *brk*. The removal of BRK by DPP signalling results in the 'derepression' of several genes, such as *optomoter-blind* (*omb*). These genes are expressed under the control of uncharacterized activators. The removal of BRK and the binding of pMAD–Medea to binding sites in certain genes (such as *spalt major* (*salm*)) result in derepression and concomitant activation. Certain genes might not be repressed by BRK in the absence of DPP signalling, but still interact with pMAD–Medea and become activated upon signalling. Additional genes are repressed via silencer elements in certain tissues; other genes might be repressed upon DPP signalling by different molecular mechanisms that are better characterized in mammalian cells[31,32].

experiments showed unequivocally that vertebrate SMAD proteins were prime mediators of responses elicited by TGFB receptors (see REFS 31,32 for comprehensive reviews on SMAD proteins). SMAD proteins have since been subdivided into three classes. Receptor-SMADs (R-SMADs) act as substrates of the type I receptor kinase and become phosphorylated on formation of the ligand–receptor complex. The common-mediator-SMADs (co-SMADs; only a single member, encoded by the *Medea* gene in *D. melanogaster*, has been identified in all animal systems analysed so far) interact with phosphorylated R-SMADs (pMAD in *D. melanogaster*) leading to the accumulation of the heteromeric complex in the nucleus. Inhibitory SMADs (I-SMADs) can be induced at the transcriptional level following SMAD signalling to act as competitive feedback inhibitors at various levels in the signalling cascade.

At first, the link between SMAD proteins and signal-regulated transcriptional control remained obscure. Experiments with *D. melanogaster* MAD, the R-SMAD acting in the DPP signalling pathway, were the first to show that one of the two protein domains of the SMAD proteins, the so-called SMAD-homology domain 1, behaves as a DNA-binding domain that recognizes a CG-rich sequence motif[33]. Such sequence elements were identified in DPP-responsive genes, strongly suggesting that the heteromeric SMAD complexes participate directly in gene regulation, a proposal that was subsequently confirmed for mammalian SMAD proteins by experiments in cultured cells[31,32].

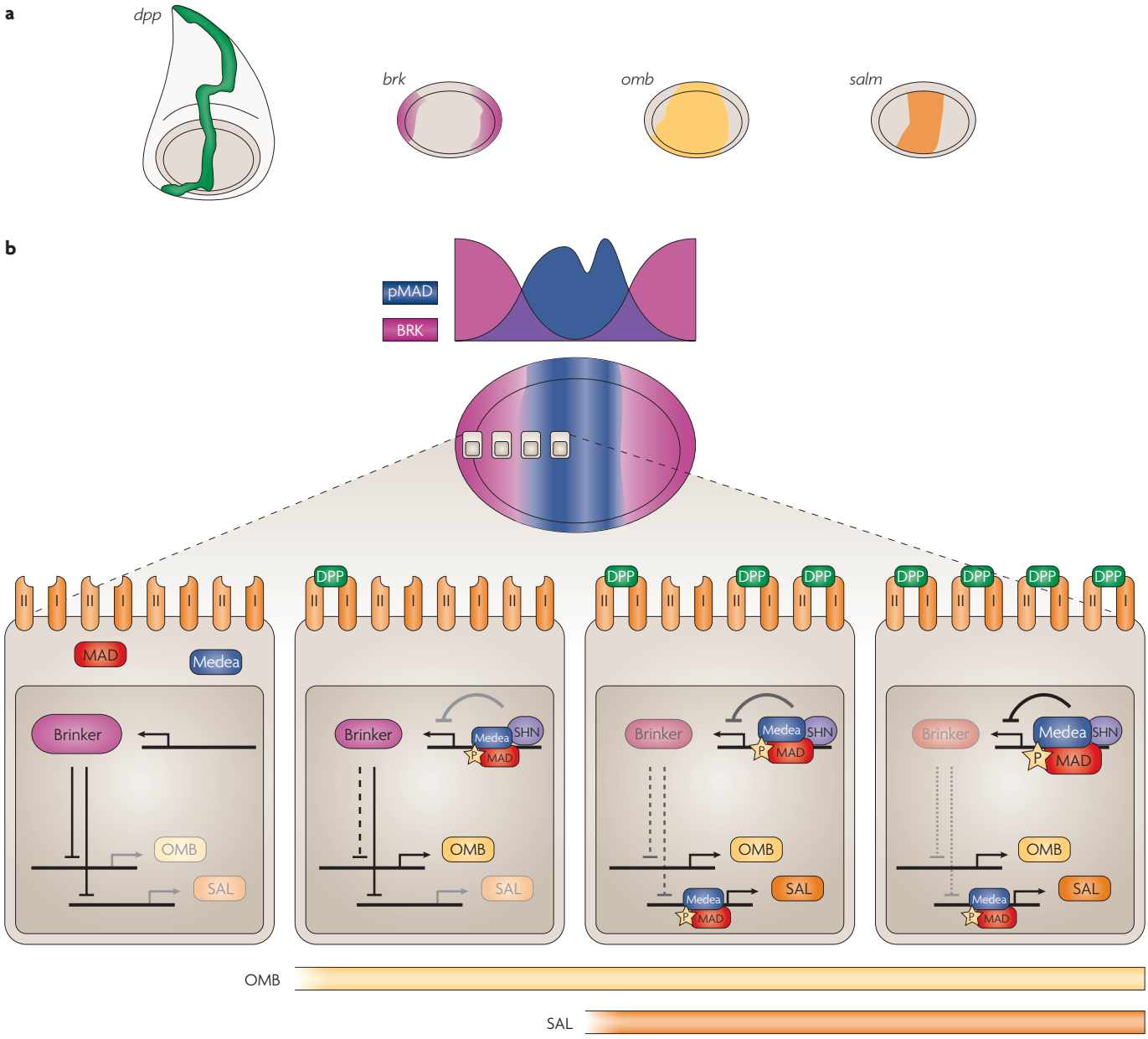

Figure 3 | **Decapentaplegic (DPP) morphogen readout: gene expression patterns in the developing wing imaginal discs. a** | Expression domains of *dpp*, *brinker* (*brk*), *optomoter-blind* (*omb*) and *spalt major* (*salm*) in third instar wing imaginal discs. DPP is secreted from the site of production in the centre of the disc and spreads into the anterior and posterior compartment, establishing a concentration gradient with the highest values in the centre and lower values towards the periphery. The expression domain of *omb*, centred around the source of DPP, is broader than that of *salm*; *brk* is only expressed at the periphery of the disc. Expression data are based on REFS 12,37. **b** | The graded distribution of the DPP ligand leads to differences in the activation levels of the Punt–Thickveins (TKV) receptor complex, and ultimately to decreasing levels of phosphorylated Mothers against *dpp* (pMAD)–Medea with increasing distance from the DPP source (the indentation of the pMAD profile in the centre of the disc is caused by a local transcriptional downregulation of the *tkv* gene[90] and will not be considered further here). The amount of pMAD–Medea is sensed at the silencer elements in the *brk* locus and, through the recruitment of Schnurri (SHN), *brk* transcription is gradually repressed by an increasing concentration of pMAD–Medea. This mechanism leads to the formation of a gradient of BRK in which the levels of BRK are inversely correlated to the amount of pMAD–Medea. Genetically, this graded expression seems to require *brk*, but the molecular bases for this requirement have not been elucidated[49,52]. SHN is present in all cells of the imaginal disc, but is functional only in DPP-induced repression when recruited to the silencer element via pMAD–Medea. BRK levels are important in setting the expression boundaries of *omb* and *salm*; *omb* is repressed by high levels of BRK (and is therefore broadly expressed in the wing imaginal disc) whereas *salm* is repressed by lower levels of BRK (and is thus expressed in a more narrow domain). Therefore, nested transcription domains are generated as a readout of the levels of the BRK repressor. Transcription of both *omb* and *salm* is activated by unknown factors that are present in the wing disc; in addition, *salm* transcription is further increased by direct binding of pMAD–Medea to its regulatory region.

What is the role of Schnurri and Brinker?

Although the isolation of MAD and Medea were key to the molecular understanding of the DPP signalling pathway, two more proteins turned out to be instrumental with regard to the readout of the DPP morphogen gradient in the wing imaginal disc: Schnurri (SHN) and Brinker (BRK). In *shn* mutant animals, most cells, including those of the imaginal discs, fail to respond to DPP signalling, even following introduction of a transgene expressing the constitutively active version of the TKV receptor[34–36]. These observations, and the fact that *shn* encodes a large nuclear protein with eight zinc fingers, led to the proposal that *shn* might act as a nuclear cofactor that contributes to gene regulation by DPP. BRK turned out to be a general repressor of DPP target genes[37–39], and functions as a nuclear protein with a sequence-specific DNA-binding domain that folds into a homeodomain-like structure[40]. BRK also has several protein motifs that are capable of interacting with the transcriptional co-repressors Groucho and C-terminal-binding protein (CtBP)[41–46]. BRK binds to many DPP target genes via the sequence GGCGYY and helps to repress them in the absence of DPP signalling, thereby acting as a default repressor. When *brk* is expressed ectopically, cells that normally respond to DPP become refractory to it. Therefore, in order to activate target genes, the DPP signalling pathway must remove BRK. This downregulation of *brk* occurs at the transcriptional level, requires DPP signalling through MAD and Medea and, strikingly, functions only in the presence of SHN[47,48].

The genetic characterization of these two genes led to the proposal that an important role of *shn* is to assist DPP signalling in repressing *brk* transcription, and that the molecular role of SHN in DPP signalling had to be unravelled in the context of *brk* repression (FIG. 2). Dissection of the *brk* *cis*-regulatory region led to the identification of two separate elements with opposing properties, a constitutive enhancer and a DPP-regulated silencer element[49]. The short silencer element serves as a direct target for a protein complex that consists of pMAD and Medea as well as the SHN protein[49,50]. pMAD and Medea bind to specific sites in the silencer elements (GRCGNC binds two molecules of MAD[51] and GTCTG binds one molecule of Medea); when these sites are properly spaced and oriented, — that is, with five nucleotides between GRCGNC and GTCTG — the pMAD–Medea complex recruits SHN and represses transcription (FIG. 2).

How are the borders of DPP target genes set?

What about *omb* and *salm*, the target genes that were initially used as molecular surrogates to document the patterning readout of the DPP morphogen gradient? Are these genes directly activated in different domains by pMAD–Medea complexes, or are they indirectly regulated by signalling, possibly through BRK?

Owing to the inverse correlation between the levels of DPP signalling and the levels of BRK repressor, which are generated via pMAD, Medea and SHN, the extracellular DPP morphogen gradient leads to the formation of an inverse nuclear gradient of BRK repressor in the developing wing imaginal disc (FIG. 2,3). The level of BRK repressor is in turn instrumental in controlling the activity states of DPP-induced genes in the morphogen field[44,49,52] (FIG. 3b). Although *brk* is important in setting the expression boundaries of *salm*, the rate of *salm* transcription within the expression domain depends in part on DPP signalling and in part on unknown activators[37,38,47]. These conclusions were nicely corroborated by the detailed analysis of a *cis*-regulatory element of the *salm* gene, which recapitulates *salm* expression in the wing disc[53]. The removal of BRK-binding sites broadened the expression domain, whereas the inactivation of SMAD-binding sites reduced but did not abolish expression levels within the expression domain, and did not change the expression boundary. Similar to *salm*, the expression boundaries of *omb* are set by BRK; *omb* is less sensitive to BRK than *salm*, so its expression domain is broader than that of *salm*.

However, and in sharp contrast to *salm*, *omb* transcription *per se* does not require DPP signalling or SMAD proteins, and its transcriptional activity is brought about by unknown factors that are present in the wing imaginal disc. The question of how BRK represses *omb* and *salm* at different concentrations has not been addressed in detail. Evidence from transgene expression studies suggests that distinct repression domains of BRK are sufficient to repress *omb* but not *salm*[44]. Thus, *omb* and *salm* seem to show not only quantitative but also qualitative differences in their response to BRK, and it will be important to elucidate in more detail the mechanisms involved. It is not known how many threshold responses are set by the DPP gradient, but at present one would argue that there are only a few across the anteroposterior axis of the imaginal wing disc.

Gene regulation and morphological patterning

In the mature *D. melanogaster* wing, DPP patterning outputs are manifested by the positioning of wing veins along the anteroposterior axis. How do the early expression patterns of *brk*, *omb* and *salm* determine the patterning events that are instrumental for subsequent vein positioning? Like *brk*, *omb* and *salm* encode transcriptional regulators, and seem to be directly involved in controlling the venation pattern. Extensive genetic analyses have shown that veins are positioned by both cell-autonomous and non-cell-autonomous events at 'boundaries', and that this positional information indeed depends on the function of the three DPP target genes. The positioning of vein 2 in the anterior compartment depends on the expression domains of *brk* and *salm*, whereas that of vein 5 in the posterior compartment depends on *brk* and *omb* (for a more detailed description of these regulatory events, see REFS 54–57). Thus, despite the need to elucidate many details, a rather complete picture emerges: DPP is secreted locally and the extracellular DPP protein gradient is established; the DPP gradient is converted into a nuclear pMAD–Medea gradient with both positive and negative transcriptional outputs; the inverse BRK gradient is generated, which regulates the 'on' and 'off' states of target genes at

Homeodomain-like structure
Homeodomains are DNA-binding domains encoded by homeobox proteins. The 60 amino-acid homeodomain folds into a globular domain containing a helix-turn-helix motif, which interacts with residues in the major groove of the target DNA. Additional binding affinity of homeodomains is provided by a flexible N-terminal arm that interacts with the minor groove.

Box 2 | **What can we learn from DPP for other patterning systems?**

Soon after the demonstration that Decapentaplegic (DPP) acts as a true morphogen in the patterning of the wing imaginal disc, the methods described in the main text were used to demonstrate that other signalling molecules, such as Wingless (WG) and Hedgehog (HH), also act as morphogens at certain times during *Drosophila melanogaster* development. Because recombinase-based methods to generate mutant patches of cells in a heterozygous environment were not available in vertebrate systems, the identification of the first validated morphogen in such animals relied on cell-transplantation experiments. Using such assays, Chen and Schier[87] demonstrated that Squint (a signalling molecule of the transforming growth factor-β (TGFB) superfamily) acts as a secreted morphogen that does not require a relay mechanism during mesoderm formation and patterning.

An interesting feature of the DPP morphogen gradient is the establishment of an instructive inverse gradient of a transcriptional repressor protein. Could such a scenario with opposing gradients contribute to the precision of reading out the morphogen gradient? This is not the case on a cellular level, because the subordinate Brinker (BRK) gradient does not provide DPP-independent positional information. But on the level of gene expression, the employment of two inverse gradients can be advantageous. Nuclei at increasing distances from the DPP source not only experience decreasing levels of phosphorylated Mothers against *dpp* (pMAD)–Medea, but concomitantly increasing levels of BRK. Because these two activities have opposing transcriptional effects, expression boundaries of target genes that are directly regulated by both inputs gain in sharpness. At present, there is no direct experimental evidence to support the idea that the borders of *optomoter-blind* (*omb*) or *spalt major* (*salm*) are set directly by binding sites for both factors. However, other target genes such as *Daughters against dpp* (*Dad*) are likely to read out both activities. Future studies will have to address this issue in more detail.

Inverse gradients of opposing activities are also generated by other morphogens. In the case of HH and WNT signalling, the primary transcriptional effectors of these pathways mediate transcriptional repression in the absence of signalling, but are then converted to transcriptional activators upon signalling[88]. The net effect of signalling at different strengths thus produces the formation of inversely proportional levels of transcriptional activators and repressors. These two molecular species presumably act in collaboration on the same target genes, with important contributions to gene regulation by both repressor and activator. Detailed *cis*-regulatory analyses are needed to test this proposal and gain further insight into the precise mechanisms of target gene regulation in the HH and WNT pathways. Intuitively, however, the utilization of inverse gradients of nuclear regulators with opposite effects seems to be a fundamental means to increase the precision of target-gene expression in response to morphogen signalling.

Organizer
A piece of tissue that can induce appropriately organized structures in neighbouring cells.

Intercalary growth
Regeneration that occurs at a tissue boundary between parts that are not normally neighbours.

Columnar epithelium
A single-cell layered sheet of elongated epithelial cells arranged on a basement membrane. Cells are joined to their neighbours by specialized junctions such as septate junctions and adherens junctions in flies.

different activity thresholds such that, finally, the patterning landmarks are positioned correctly. Whether these features of DPP morphogen readout show similarities to transcriptional readouts of other secreted morphogens can be determined only with more detailed studies of the cellular responses to WNT, HH and other morphogen signals (BOX 2).

DPP and growth

The morphogen concept in its original form did not have a bearing on the regulation of growth. It was the discovery of organizers and intercalary growth, through grafting and regeneration experiments in insects and amphibians, that led to the idea that morphogens might also be linked to cell proliferation[1,58–61]. In particular, it was proposed that the slope of a morphogen gradient is used by cells as a determinant for proliferation[62] (see below). Several of the secreted signalling molecules known to date have growth-factor-like activities *in vivo*. However, only a few of them have such impressive effects on organ shape and size as DPP exerts when expressed ectopically *in vivo*[4]. It is of no surprise, therefore, that the role of DPP in wing development has been viewed as a twofold task, regulating growth in addition to pattern. The DPP system has often been used to make the point that patterning by morphogens is in general intimately linked to the regulation of cell proliferation[64,65].

The case for DPP

What exactly is the evidence that DPP signalling is linked to wing growth? Two principally different situations should be distinguished: loss or gain of DPP signalling. Loss or severe reduction of *dpp* expression in the wing primordium by means of hypomorphic alleles reduces the wing to a little stump[4,66]; hence, DPP is required for the wing to assume its normal size. Cell clones mutant for *tkv*, *shn* or *Mad* fail to survive[67–71]. These two observations led to the suggestions that DPP functions as a survival factor for wing cells. By sharp contrast, gain of DPP signalling causes overgrowth[4,5,11,12,72]. Particularly impressive are the effects of clones that are genetically programmed to secrete DPP ectopically: if such clones cross the dorsoventral compartment boundary and comprise both dorsal and ventral cells, they organize the formation of extra tissue, which can assume the shape of winglets[4]. The ectopic DPP-producing clones form only a small central patch at the tip of such winglets, the rest being made by wild-type cells under the control of the secreted DPP. Undoubtedly, imaginal discs containing such ectopic DPP sources are composed of a greater number of cells than their untreated counterparts. Moreover, ubiquitous expression of DPP or its constitutively active receptor TKV^{QD} causes massively enlarged imaginal discs, which are expanded to both sides along the anteroposterior axis[5,12,72]. Such experimental findings have been taken as evidence for a role of DPP as a potent growth promoter.

Loss or gain of DPP: two sides of the same coin?

The apparent dichotomy of effects of too little versus too much DPP signalling has reinforced the concept of DPP signalling as a growth-regulating process, and championed DPP as a prime example of a controller of cell proliferation and organ size (that is, growth) (BOX 3). However, recent analyses of the fate of cells that transduce too little DPP have undermined the role of DPP as a cell-survival promoter, and suggest that, instead, the primary role of DPP is to ensure the correct architecture of epithelial cells[74,75]. Cell-biological analysis of *tkv* mutant cells revealed that they undergo cytoskeletal changes and extrude from the columnar epithelium of the wing disc. In most cases, this behaviour is associated with apoptosis. If the role of DPP is to maintain an essential morphological property of disc cells, should it still be called a survival factor? Arguments for either view are valid. It is also possible that DPP signalling is required for epithelial integrity as well as for cell proliferation, and that both aspects are independent outcomes of this pathway. It emerges, however, that the dependence of imaginal disc cells on DPP input and the overgrowth that is caused by extra DPP might not necessarily be opposite sides of the same coin. Moreover, it is so far not understood whether changes in epithelial architecture occur only when small

cell clones that lack a DPP-reception component are generated, or whether they also occur in cells of discs that are entirely mutant for *dpp*. Hence, it is possible that at least the deficit in growth observed upon global loss of DPP signalling is indeed a phenomenon directly related to the overgrowth that occurs upon global gain of DPP signalling.

How does a gradient direct uniform growth?

A particularly demanding challenge for any mechanistic model of DPP's role in growth is the finding that cell divisions occur all over the wing disc, with a pattern and rate that are approximately uniform over the entire area of the disc epithelium[76–78]. This uniformity seems to be at odds with the graded distribution and activity of DPP along the anteroposterior axis that is described above, which might suggest that growth should occur preferentially in the centre, where DPP activity is highest.

Several models have tried to accommodate this discrepancy, only five of which will be discussed here (FIG. 4). In the first of these, Day and Lawrence[62] argued that local growth could depend on local reading of the steepness of the DPP concentration gradient (FIG. 4a). Thus, instead of the absolute levels, it would be the differences in DPP levels perceived by adjacent cells that stimulates proliferation. This model is attractive because it also provides a mechanism for the determination of organ size. In its simplest form, the model proposes that the high DPP level in the centre of the disc and the low DPP levels at its periphery are fixed (that is, the gradient is scaled and somehow adjusts to changes in wing disc size during growth). Growth anywhere in the disc extends the gradient and thus reduces its rake. Cells grow only when the local DPP gradient is sufficiently steep, and therefore cell proliferation ceases when the local steepness falls below a threshold[62]. This model predicts that growth does not occur in an experimental disc with homogeneous DPP signalling, as the DPP slope is near zero in such discs. However, considerable growth occurs in wing discs with homogeneous DPP signalling[12,70,72], contradictory to this most simple form of the gradient model in the case of the wing disc. Moreover, the model suffers from the problem that a twofold increase in DPP signalling should result in a twofold increase in wing disc size, which is not observed.

The second model discussed here (FIG. 4b) has been put forward by Rogulja and Irvine[72] as a refinement of the above gradient model. In support of the idea that a steep DPP signalling gradient triggers growth, these researchers found that activation of the DPP pathway in clones expressing the constitutively active TKV^{QD} seems to transiently stimulate proliferation of cells in the vicinity of the clone boundaries; similar effects were seen when the pathway was locally inhibited[72]. These observations were attributed to the juxtaposition of cells with different levels of DPP pathway activity. Uniform expression of DPP or TKV^{QD} inhibited proliferation in the centre of the disc and caused overproliferation primarily in lateral disc regions. Hence, Rogulja and Irvine proposed that the disc is subdivided into two classes of cells that respond differently to DPP: medial cells proliferate only in response to differences in DPP pathway activity, whereas lateral cells proliferate in response to even low absolute levels of DPP, and also when these are uniform. Although this model answers the problem of homogeneous DPP pathway activity discussed above, it creates a new conundrum: how are the two cell populations specified along the anteroposterior axis? In the absence of a plausible alternative, this would have to be accomplished by prior DPP signalling (or some unidentified lateral influence, see below). So, this model is basically a cellular context model, relying on a mechanism that ensures that lateral cells are programmed during early disc development to be more sensitive to DPP than medial cells. Moreover, neither of these first two models can explain growth at the actual source of DPP, where cells of a considerable region are exposed to saturating and equal signal levels.

The third model (FIG. 4c) provides an alternative view to explain uniform growth in response to graded DPP distribution, and assumes that an inhibitor of cell proliferation is secreted by DPP-expressing cells to form a gradient in parallel with that of DPP, such that all cells along the anteroposterior axis are subjected to the same net growth stimulus[79]. The expression of such an inhibitor could either be controlled by DPP or independent of DPP. In the first case, homogeneous expression of DPP should lead to homogeneous growth, as both DPP and the inhibitor would be uniformly distributed; however, this is not observed (see above[72]). In the second case, homogeneous expression of DPP should lead to an increase of proliferation laterally and possibly also in the centre of the disc, but it should not cause a decrease of proliferation in the centre, as is known to occur[72]. Furthermore, if the source of the inhibitor were not specified by DPP signalling,

Box 3 | Relating growth to cell size and cell number and time

What is meant by 'growth' in the context of imaginal discs? A convenient simplification is the two-dimensional nature of these epithelia. A second simplification, which we base on the lack of counter-evidence, is the assumption that DPP signalling does not affect the final size of a cell. Finally, we note that the period of imaginal disc development, and hence the time window during which disc cells can proliferate, is finite and essentially defined by the initiation of puparium formation, a time point determined by disc-extrinsic factors that are presumed to be independent of DPP. However, this window does not directly define the wing disc size, because transplantation of early discs into the abdomen of adult flies results in discs with normal size[84,85].

Under these premises and using the definition 'growth = (cell size x cell number)/time', we can directly relate growth to the net number of cells at the end of larval development, which in turn should be reflected in the size of the disc that is being analysed. The net number of cells is the sum of the cells generated by division minus those lost by apoptosis. In the absence of exogenous insult, the number of cells undergoing programmed cell death is small in wild-type discs, and can therefore be ignored[78], leading to the assumption that the size of a disc reflects the amount of cell proliferation.

It should be added for completeness, however, that experimentally induced alterations in cell proliferation are often compensated for by changes in cell size[89]. The size of experimentally modified wing discs is not a direct function of cell number, but an integration of the number and size of cells. Moreover, it is possible that the size of the adult wing is further adjusted by an undocumented phase of cell death at early pupal stages (G. Schubiger, personal communication). In this Review, the discussion about organ size is therefore limited to larval wing imaginal discs composed of normally sized cells.

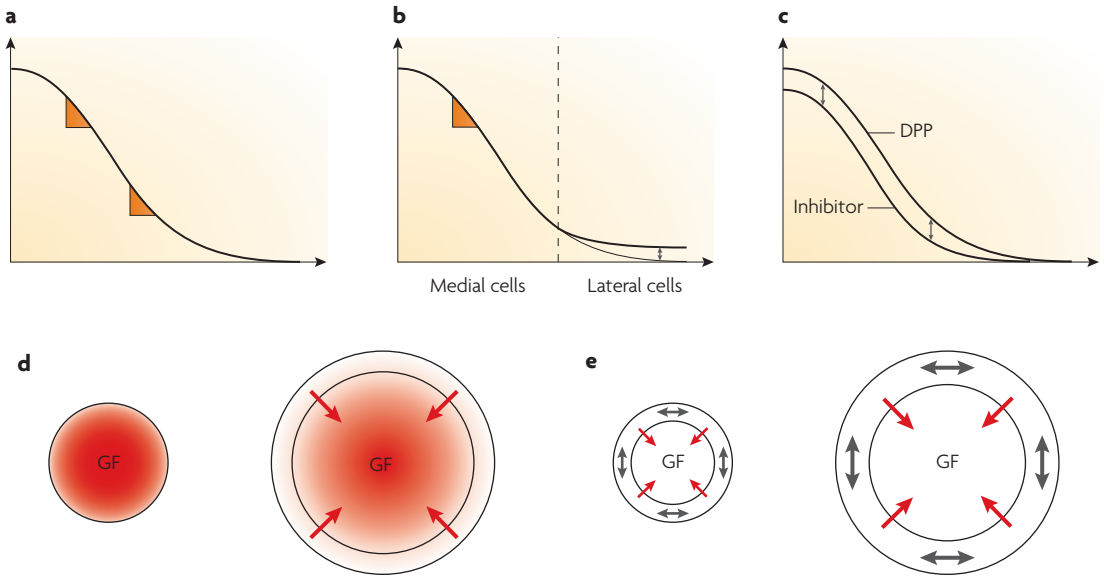

Figure 4 | **Models addressing the function of Decapentaplegic (DPP) in growth control.** Different models have been proposed that could explain how graded expression of a morphogen could lead to uniform cell-division patterns in a field of cells. **a** | Model 1. The slope of the DPP gradient, and not absolute values, determines the proliferation behaviour of cells in different parts of the disc. **b** | Model 2. A modification of model **a** in which the disc is subdivided into two regions: for medial cells, it is the slope of the DPP gradient that drives proliferation, whereas cells in lateral regions also divide in response to increases in absolute DPP levels. **c** | Model 3. A parallel gradient of a growth inhibitor evens out the vast differences in DPP signalling across the disc. **d** |Model 4. The DPP morphogen distribution does not adapt to disc size. Once the disc has grown (shown on the right), peripheral cells receive too little DPP, stop proliferating, and thus exert a compressing force onto medial cells, with the effect that these cells also stop proliferating. **e** | Model 5. Growth is determined by morphogens emanating from the centre of the disc and an interplay of stretching and compression. Owing to growth-factor signalling in the middle, the disc centre grows (left panel). This growth leads to stretching in the periphery (indicated by black arrows). The stretching then causes growth in the periphery, with some stretching still remaining. Residual stretching in the periphery and further growth in the centre cause compression (indicated by red arrows), which inhibits growth. Over time, the stretched region gets wider and thus the compression on the centre becomes stronger, until an equilibrium is reached with the disc achieving its final size. GF, growth factors.

it would almost certainly have to arise in response to HH. However, uniform expression of HH throughout the disc causes overproliferation of anterior compartment cells[63], a response that should then be prevented by the concomitant induction of inhibitor expression. Moreover, no such inhibitor has been identified. It seems unlikely therefore that parallel gradients of positive and negative proliferation signals can account for the DPP-mediated growth control in the wing imaginal disc.

A completely different explanation for uniform growth was given by Shraiman, who proposed an effect of mechanical forces on growth[80]. He argued that local differences in growth rates lead to mechanical stresses, which could in turn affect cell divisions, and hence provide the basis for an 'integral-feedback' mechanism, stabilizing uniform growth. Shraiman, Cohen and colleagues recently extended this idea in order to account for the termination of growth[81] (model four here (FIG. 4d)), whereby the eventual size of the wing disc is set by the DPP gradient. Contrary to their previous work[82], they argue that the DPP morphogen distribution in the wing imaginal disc does not adapt to disc size; therefore, as the disc grows to a certain size, the morphogen concentration to which cells at the boundary are exposed falls below a threshold, and these peripheral regions then stop growing. The more central regions of the disc continue to grow until they become compressed by the outer boundary. This compression is assumed to inhibit growth, such that eventually growth stops in the whole disc. A theoretical disadvantage of this model is the dependence of wing disc size on a morphogen threshold level in a region where the morphogen gradient is shallow and the absolute levels are low, making it sensitive to small absolute variations in morphogen activity levels. However, it is possible that there are additional mechanisms present in the disc to ensure sufficient robustness.

A similar model (number five here (FIG. 4e)) that yields uniform growth and autonomous termination was developed independently by Aegerter-Wilmsen *et al.*[83] As in model four, growth is induced by morphogens and terminated by compression in the centre; in contrast to model four, however, the final size of the wing disc does not depend on a morphogen threshold. Instead, model five crucially depends on the assumption that mechanical stretching stimulates growth above a certain threshold. This stretching occurs in the peripheral regions because of the growth-factor-induced growth in the centre. Because the stretching is not completely compensated

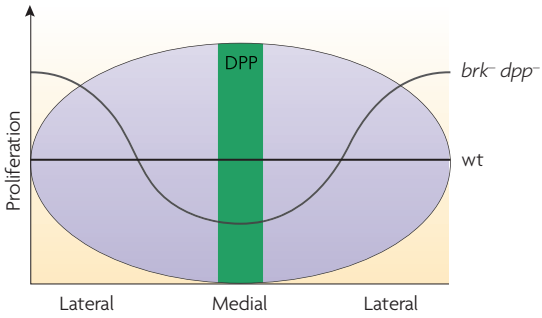

Figure 5 | **An uneven ground state of growth in the absence of the Decapentaplegic (DPP)–Brinker (BRK) system?** The main function of DPP signalling is the repression of *brk*. Global removal of BRK function seems to have the same consequences for cell proliferation as simultaneous removal of BRK and DPP, or uniform expression of Thickveins (by the constitutively active form TKV^{QD}): overproliferation laterally and underproliferation medially[72]. Thus, the DPP–BRK system seems to correct an inherently uneven proliferation pattern into a uniform pattern along the anteroposterior axis. wt, wild type.

for by the induced growth, the peripheral regions will compress the centre of the disc. The larger the disc, the stronger this compression automatically becomes. Growth ceases when the growth factors can no longer overcome the inhibiting effect of the compression.

Both models four and five are purely hypothetical, and at this stage are difficult to distinguish between experimentally. Until we know more about the effects of mechanical forces on the proliferation behaviour of epithelial cells, and until we can quantitatively measure and experimentally manipulate intercellular forces *in vivo*, the plausibility of both mechanical stress models cannot be assessed.

The purpose of BRK in the regulation of growth

One way to deal with the difficulties of explaining how DPP controls growth and organ size is to acknowledge that we still know too little about the growth-related target genes of the DPP signalling system to make mechanistic predictions about how DPP affects growth. Below we attempt to increase our understanding of DPP's role in growth by applying some of the insights obtained from DPP's patterning function.

One of the key discoveries following the identification of DPP as a morphogen was the unexpected twist that the DPP signalling gradient is first converted into an inverse BRK repression gradient[37–39]; BRK levels then determine the expression boundaries of the genes that are instrumental in the patterning function of DPP. What is the relationship between DPP and BRK with regard to growth? Could DPP also control growth indirectly by repressing *brk* transcription? Surprisingly, this is probably the case, although the actual evidence is fragmentary. The effects of uniform TKV^{QD} expression are reminiscent of those that occur when *brk* is removed genetically: *brk* mutant discs overproliferate and are similar in appearance to discs in which the DPP pathway has been activated ubiquitously[12,37]. Cell clones that are mutant for *Mad* fail to survive, but can be rescued by simultaneously eliminating *brk* function[47,73]. Indeed, *brk*, *Mad* double-mutant clones behave like *brk* single-mutant clones. So, it seems that the growth-promoting function of DPP consists primarily, if not exclusively, in repressing *brk* expression.

If one fully equates the ubiquitous activation of the DPP pathway and the complete removal of BRK function, one would predict that discs of both the *brk* mutant and the *brk*, *dpp* double mutant would show elevated proliferation rates in lateral regions and reduced rates in central regions of the disc (as observed in discs that express TKV^{QD} ubiquitously[72]) (FIG. 5). So, in the absence of the DPP–BRK system, growth rates would not be uniform in the disc epithelium. This prediction would lead to two further inferences. The growth differences posited to exist in the absence of the DPP–BRK system would have to stem from other inputs affecting growth. It is possible, for example, that cells in lateral regions have the propensity to proliferate more quickly owing to their proximity to the peripodial membrane, the squamous epithelium that connects the lateral regions and covers the disc. Whether this surrounding tissue might exert an influence by affecting mechanical parameters of disc cells, or by serving as a source for a growth factor, is at best speculation. However, whatever the cause for the above inferred unevenness of growth traits might be, it would have to be the function of the BRK gradient to correct it. Hence, the main purpose of the DPP–BRK system in growth might be to even out regional differences in proliferation rates to achieve homogeneity. A better understanding of this complex system would require identifying, on the one hand, the origin of such differences and, on the other hand, the target gene(s) of BRK by which these presumed differences are evened out.

Where do we go from here?

It becomes evident from the conceptual differences of the models described above that there is still a long way to go to reach a satisfactory level of understanding of DPP's role in growth. There are many questions and few answers in sight. Are mechanical constraints important? Is the naive wing disc a uniform structure, or is it prepatterned? What are the targets for growth control? Is the control exerted at the transcriptional level?

Much has been learned about the patterning function of DPP, and some surprises have surfaced (such as the key role of the transcriptional repressor BRK, signal-induced repression, and others). These discoveries were only possible with comprehensive genetic and molecular analysis. To unravel the mysteries of growth control by morphogens, we must embark on a long journey, involving target identification, functional studies, the development of new methods to assess and alter mechanical forces, and much more. As in any scientific endeavour, it will be important to develop carefully devised questions, precise enough to be answerable and unbiased enough so that researchers in the field agree on them.

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Acknowledgements
We would like to thank A. Weiss for help with the figures and G. Pyrowolakis, A. Weiss, G. Schwank, T. Aegerter-Wilmsen and P. Gallant for discussions and comments and on the manuscript.

Competing interests statement
The authors declare no competing financial interests.

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