

Different strategies for midline formation in bilaterians

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The evolutionary emergence of the bilateral body plan and the central nervous system required the establishment of a midline organizer. The formation of a solitary, elongated but narrow organizing region for the dorsoventral (or mediolateral) axis requires rather complex molecular interactions. Different modes of midline formation evolved in vertebrates, insects and planarians, indicating that midline formation had a crucial role in the diversification of higher organisms.

Radially symmetrical animals — such as the phylum *Cnidaria*, which includes the freshwater polyp *Hydra* — have a diffuse nerve net. Although relatively simple, it allows the animal to perform complex tasks: for example, catching prey, changing position by loosening and re-attaching the foot, and spilling out the indigestible remains of a meal. A crucial step towards the evolution of higher organisms was the emergence of the bilateral body plan. With the newly formed mediolateral axis, it was possible to bundle the nervous system and to localize muscle cells. Together, these properties allowed manoeuvres such as controlled undulation of the long-stretched body, which resulted in much more efficient locomotion.

A bilateral body plan requires the establishment of a midline organizer in a former radially symmetrical animal. This organizer can be a source or sink region of a morphogenetic substance that provides positional information for the dorsoventral (DV) or mediolateral axis. Neurons for the CNS are allocated in regions close to the midline organizer. In *Drosophila melanogaster*, for example, specified nerve cell progenitors arise at particular distances from the midline¹. If progenitors are transplanted closer to the midline, their commitment changes according to the new position, but their commitment remains unchanged on transplantation further away from the midline. This type of behaviour is typical for systems that interpret positional information².

The midline organizer has a different geometry to the usual Spemann-type organizer. It extends along the whole antero-posterior (AP) axis, but has only a small

extension mediolaterally. Although it seems simple just to 'draw a line' along one side of the long axis of the body, the generation of such an organizer is actually a subtle process. How is it that cells lateral to the midline are excluded from the organizer, whereas the cells along the midline maintain this property? If some sort of lateral inhibition is involved, why does the midline not break up into separate patches? How is its correct orientation along the AP axis assured, and why does it not curl up in a corkscrew-like manner around the body? All developmental processes are coordinated by the interaction of molecules, so what types of molecular interactions could generate a midline organizer with the required properties?

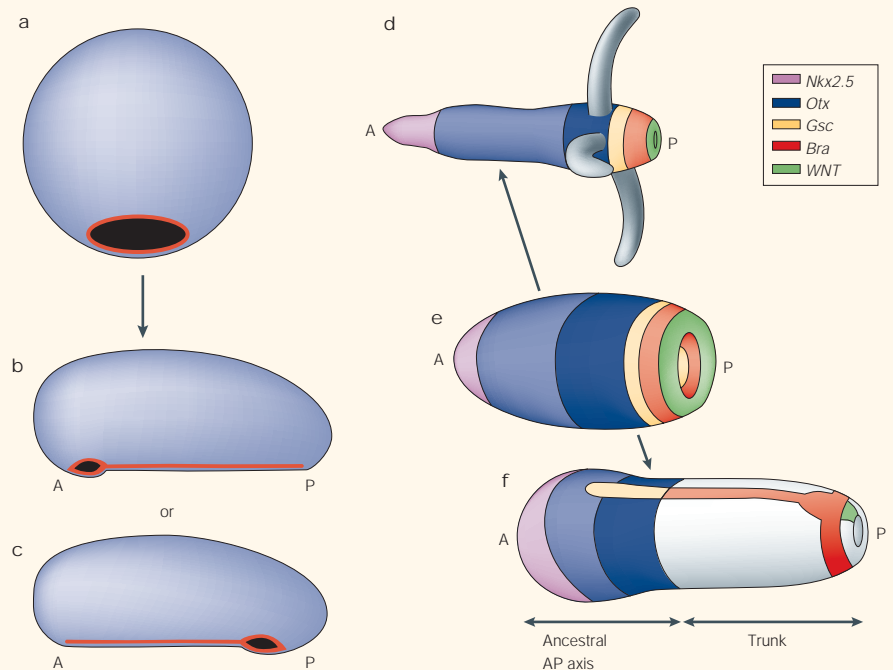


Figure 1 | Hypotheses for the transition from radially symmetrical to bilaterally symmetrical animals. According to the protostomia-deuterostomia concept (a–c), the blastopore (red/black) of a gastrula-shaped ancestor is transformed into a slit (a process known as amphistomy), which forms the midline and consequently the future neural side^{9–11}. Either the anterior (A, part b) or the posterior end (P, part c) of this slit remained as an opening of the gastric cavity, forming the mouth in protostomia (b) or the anus in deuterostomia (c). d–f | Alternative hypothesis as implied by the expression patterns of several homologous genes in *Hydra* (d) and higher organisms (f). The blastopore remains the most posterior structure, and it is orientated perpendicular to the midline. The body of a hypothetical radially symmetrical ancestor (e) evolved into the brain region of higher organisms²⁸ (f). The closed side antipodal to the blastopore is the anterior region that gave rise to the forebrain and midbrain (blue) and heart. A separate signalling system is required to generate the midline (yellow/red). This enables a mediolateral organization, and consequently the formation of a CNS. The midline system and the trunk are later evolutionary additions.

In this article, I combine well-known data from the literature with insights obtained from a theoretical analysis of pattern-forming reactions to argue that such a pattern can be generated only by coupling several pattern-forming reactions. Comparative analysis of vertebrates, insects and planarians reveals that different strategies for midline formation emerged during evolution. In vertebrates, a dorsal organizer causes initiation and elongation of the midline in anterior and posterior directions, and the midline — and consequently the nerve cord — develops dorsally. By contrast, in insects, a dorsal organizer represses midline formation, so the midline occurs at the ventral side. This might provide a new explanation for the well-known fact that under the influence of the same signalling system, the nerve cord develops dorsally in vertebrates but ventrally in insects^{3,4}. In vertebrates, the midline is sequentially elongated along the AP axis, whereas it refines along the DV axis in insects. In planarians (flatworms), there is evidence that the DV axis is hierarchically the primary pattern. The border between cells with dorsal and ventral specifications, which encircles

the whole organism, acquires organizing properties secondarily, and it localizes the dorsal midline by repulsion.

At present, it is commonly thought that bilaterally symmetrical organisms evolved from ancestral radially symmetrical cup- or gastrula-shaped organisms^{5,6}, that this transition occurred only once in evolution, and that this event gave rise to an urbilaterian^{7,8}. The diversity of present-day higher organisms is assumed to be the result of a subsequent diversification. The different strategies for midline formation described here raise questions about the concept of a unique urbilaterian ancestor and indicate scenarios for essential steps in the evolution of bilateral organisms.

One hypothesis on midline formation considers the midline as a remnant of the blastopore — the gastric opening of the cup-shaped gastrula^{9–11}. In this scheme, it is assumed that the initially ring-shaped blastopore became stretched in one direction and squeezed in the other to form a long-extended slit-like structure — the future midline and neuronal side. (FIG. 1a–c). A variation is the 'radial head hypothesis'¹², according to which the midline results from a posterior elongation of the originally circular blastopore. However, recent gene expression studies provide support for the classical view that the blastopore forms the most posterior pole^{13,14} and not the midline (FIG. 1d–f). For instance, *Brachyury* is generally assumed to be a marker of the blastopore. If the blastopore/midline model is correct, we would expect *brachyury* to be expressed along the insect midline. However, its expression is actually restricted to the posterior pole¹⁵. This raises a crucial question: if there is no elongated blastopore that precedes the midline, how can a signalling system emerge that can generate a narrow but long extended source or sink region?

The problem of forming a stripe
Several observations indicate that midline formation is based on a genuine pattern-forming reaction. Markers that correspond to the midline can regenerate after its removal^{16–18}, indicating that an existing midline organizer exerts some sort of lateral inhibition. In general, pattern formation is possible if a substance that self-enhances its own production interacts with a long-range antagonist^{2,19,20}. The *nodal-lefty2* interaction has the predicted properties: *nodal* activation is self-enhancing and regulates the production of its antagonist *lefty2*, which in turn blocks *nodal* activation^{21–23}. However, mathematical modelling of pattern-forming processes has revealed

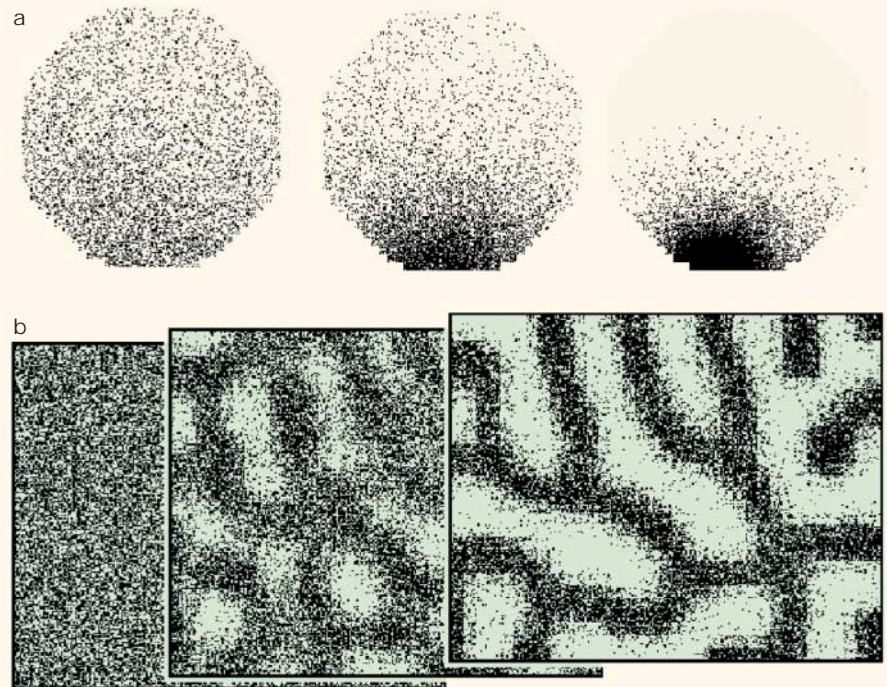


Figure 2 | The problem of generating a stripe-shaped organizing region. a | High concentrations with a patch-like geometry can be generated by an interaction between a short-ranging autocatalytic activator and a long-ranging inhibitor^{2,19,20}. The *nodal-lefty2* interaction^{21–23} is of this type. In this simulation, the pixel density indicates the activator concentration. The initial distribution was uniform, except for small random fluctuations. Early, intermediate and final stable patterns are shown. If the range of the inhibitor covers the size of the field, a single peak with a patch-like geometry emerges at a marginal position (a). The activator or inhibitor distribution (not shown) could provide positional information for a cell concerning its distance to the peak — the 'organizing region'. **b** | If the autocatalysis saturates at high activator concentrations owing to a rate-limiting step, stripe-like patterns emerge when triggered by random fluctuations^{24,25}. These stripes bend and bifurcate; the stripe and the inter-stripe regions are approximately of the same width (see online [supplementary information S1](#) (simulation)). A single straight stripe cannot be generated by strengthening of the lateral inhibition, as this would lead to a decay of the stripes into patches. So, accessory reactions are required to enable the formation of the single straight stripe-shaped organizer that is required to pattern the dorsoventral axis (see FIGS 3,5–7).

that a straight solitary stripe-shaped organizing region cannot be generated by a single pattern-forming reaction. In the absence of other constraints, activated regions formed in this way have a spot- or patch-like geometry, because the gradation around the maximum is the same in all directions (FIG. 2a). However, activated regions with a stripe-like geometry can emerge if the self-enhancing reaction saturates owing to rate-limiting steps^{24,25} (FIG. 2b). The restricted maximum peak height causes an enlargement of the activated regions until an equilibrium between the self-enhancing and the antagonistic reaction is achieved. However, for a stable activation, lateral inhibition requires the proximity of non-activated cells into which the antagonist can be dumped, or from which a necessary co-factor can be obtained. These seemingly contradictory requirements — large activated regions and non-activated cells in the direct neighbourhood — are satisfied in stripe-like patterns.

In this situation, each activated cell has activated neighbours along the stripe and non-activated neighbours perpendicular to it.

On its own, however, such a stripe-forming system is still insufficient to generate a solitary straight stripe. After initiation by random fluctuations, meandering stripes are formed that might bifurcate (FIG. 2b). The width of the stripes and the inter-stripe regions are of the same order, reminiscent of the ocular dominance columns in the visual cortex²⁶, or patterns on zebras or tropical fish²⁷. The problem of multiple stripes cannot be circumvented by strengthening the lateral inhibition, because the stripe would decay into individual patches. Therefore, accessory pattern-forming reactions are required that allow the formation of just one stripe — the future midline organizer. If these accessory patterns are disabled by mutations, the default periodic character of the midline system can become manifest.

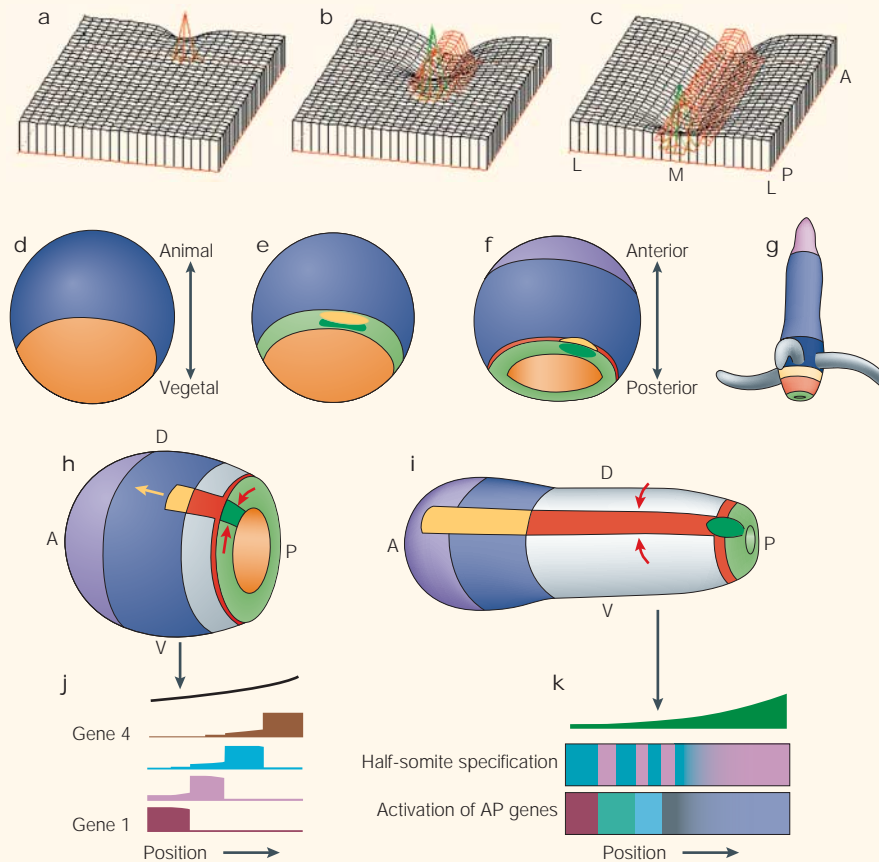


Figure 3 | Vertebrate solution: midline formation by local elongation owing to a moving organizer. **a–c** | Simplified model: a spot-shaped organizing system (green) triggers a stripe-forming system (red), which, in turn, causes a shift of the organizing region in front of the tip — a process that leads to the emergence of a single straight line (see online [supplementary information S2](#) (simulation)). A, anterior; L, lateral; M, medial; P, posterior. **d–i** | Schematic drawing showing the generation of a near-Cartesian coordinate system starting from a sphere of cells (as in the frog). **d** | Separation of the ectoderm and endoderm occurs along the animal–vegetal axis. **e** | Mesoderm (green) becomes induced at the border. Both endoderm and mesoderm invaginate; the site of invagination — the blastopore — has the geometry of a large ring. **f** | The gastrula after invagination of the endoderm and mesoderm. The blastopore (marginal zone) becomes the organizing region for the anteroposterior (AP) axis and is posterior. This early gastrula forms essentially the brain and the heart, and is proposed to be homologous to the body of the *Hydra* (**g**). **h, i** | The spot-like Spemann-type organizer (dark green/yellow), which is localized on the blastopore, gives rise to the two parts of the midline that govern dorsoventral (or mediolateral) patterning. Those mesodermal cells that move anteriorly, and that were close to the organizer, form the prechordal plate (yellow, *gooseoid* expression), the anterior part of the midline^{60,62}. The trunk arises by cell proliferation around the blastopore, causing the formation of a cylinder (light grey). The blastopore and the organizer (dark green) riding on them remain the most posterior structure (D, dorsal; V, ventral). The organizer leaves behind the posterior midline (red). By the convergence–extension movement towards the midline (red arrows), the short thick cylinder becomes narrower and more extended¹⁰⁵. **j, k** | The AP patterning of the head and trunk relies on different mechanisms. The head region is specified by a *Wnt* gradient that has its origin at the blastopore^{51–53}, causing an anterior-to-posterior transformation. In the trunk, an oscillation takes place at the posterior end^{66,67} that causes sequential activation of the Hox genes^{67,68} in the typical 3′–5′ order. Models have predicted both mechanisms².

An ancestral body became the brain. Cnidarians are assumed to closely resemble the ancestral metazoan organisms before bilaterality occurred in evolution. Therefore, animals such as *Hydra* can be regarded as living fossils that provide information about axis formation before bilaterality evolved. A comparison of the expression patterns of some crucial genes indicates that almost the

entire body of a radially symmetrical *Hydra*-like ancestor evolved into the brain and the heart of higher organisms²⁸ (FIG. 1d–f). *Otx*, which is indicative of the forebrain and midbrain, is expressed throughout the body of *Hydra*, except for the most terminal regions²⁹. The homologous gene in the short germ insect *Tribolium castaneum* is expressed in the anterior half of the embryo and is

indispensable for head formation³⁰. The *Nkx2.5* expression pattern³¹ implies that the foot of the present-day *Hydra* has the same positional address as the forebrain and heart of higher organisms, and that it represents the most anterior neuronal and endodermal structures^{32–34}. The *Hydra* peduncle has pumping properties, and the frequency of the constrictions can be influenced by peptides that also alter the beating frequency of the mammalian heart^{35,36}.

By contrast, as indicated by *brachyury* and *Wnt* expression^{37,38}, the so-called *Hydra* head, which includes the hypostome and gastric opening (anus), represents the most posterior structure, namely the blastopore. This indicates that the gastric opening was formed at a position that in present-day higher organisms lies just posterior to the midbrain (posterior *Otx* border). In *Hydra*, the *gooseoid*³⁹ and *brachyury* homologues³⁷ are expressed in adjacent rings close to the gastric opening. In vertebrates, *gooseoid* is involved in head patterning⁴⁰ and *brachyury* is involved in tail formation⁴¹, so this implies that the trunk (the largest portion of present-day higher animals) evolved and differentiated next to the posterior end of the ancestral organism. This view provides a rationale for the otherwise puzzling observation that genes that are typically expressed in the brain (for instance, homologues of *aristless*⁴² and *ems*⁴³) and the tail (*brachyury*⁴¹) are expressed at the same end of the body column. On the other hand, the typical Hox genes with their 3′–5′ collinear arrangement that are characteristic for trunk formation in higher organisms have not been found in *Hydra*^{44,45}. This supports the view that *Hydra* preserves an ancestral axial pattern from before trunk and midline formation emerged during evolution.

So, comparisons of molecular expression patterns in a coelenterate and higher organisms reveal that the AP axis of higher organisms consists of two parts. The ancestral anterior part was used to pattern the body of radially symmetrical ancestors, and in higher organisms it extends from the most anterior position to the posterior border of the midbrain. It includes the signalling systems for heart and germ-cell formation. This scheme provides a rationale for why the heart and the germs cells are laid down much more anteriorly in comparison with their final position in the adult body. The highly conserved pattern of the brain of higher organisms^{46–48} is proposed to have its origin in the preserved body pattern of radially symmetrical common ancestors. The newly evolved part — the trunk — resulted from sequential addition of more posterior structures, starting posterior to the

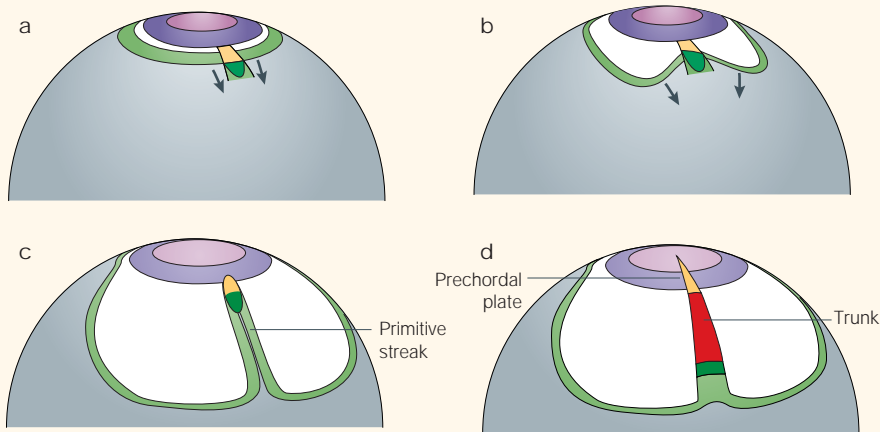


Figure 4 | Formation of a near-Cartesian coordinate system in the chick. **a** | The proliferating cells on the yolk form the *area pellucida*. The outer border of this region (light green) corresponds to the blastopore. The pattern of the bag-like ancestral organism (FIG. 1e) has become flattened into a circle. The organizer (posterior marginal zone or Koller's sickle (dark green)) forms on this ring. **b** | In a process that can be best described as partial epiboly, cells on both sides of the organizer move over the yolk (arrows). This is in sharp contrast to the complete and radially symmetrical epiboly in the fish. The organizing region remains in place, giving rise to Hensen's node. Again this is in contrast with the fish, where the shield moves with the germ ring. **c** | The deformed blastopore forms a hairpin-like loop — the primitive streak. Although the orientation of the streak is the same as the future anteroposterior (AP) axis, it is equivalent to the marginal zone in amphibians or to the germ ring in the fish; that is, it is a pattern that is conventionally seen as a dorsoventral pattern. **d** | With the regression of Hensen's node, the proper posterior part of the AP axis, the trunk (red), is generated. The brain is laid down in the ancestral, original central part of the *area pellucida*. The anterior midline is generated by the prechordal plate (yellow), which moves, as in the frog and fish, away from the blastopore underneath the brain ectoderm (purple).

brain (FIG. 1f). This sequence of events is recapitulated in the ontogenetic development of many animals, including vertebrates, short germ insects and annelids.

The vertebrate solution

The AP organizer became a large ring. As outlined above, the patterning of the body of ancestral radially symmetrical organisms and the elementary AP patterning of the brain in higher organisms are considered to be homologous processes. Classical experiments have revealed that in *Hydra*, the hypostome acts as an organizing region for the single axes. β -Catenin, Tcf, Wnt and *brachyury* are expressed in this organizing region, Wnt expression being restricted to a small spot-like region surrounding the gastric opening³⁸. By contrast, the equivalent zones in amphibians and fish — the marginal zone and germ ring, respectively — are rings with a diameter nearly as large as the diameter of the embryo. There is good evidence that the ancestral portion of the AP axis, that is, the region that stretches from the anterior end of the body to the posterior border of the brain, is still controlled by the blastopore, despite its enlargement to a ring. This AP organization does not require a Spemann-type organizer, as has been most clearly demonstrated for the zebrafish. The formation of any dorsal

organizer was suppressed by removal of cytoplasmic components from the fertilized egg⁴⁹. Nevertheless, *Otx* and *Krox20*, which are markers for the anterior part of the AP axis, are expressed in the normal anterior-to-posterior order in a radially symmetrical fashion (in this experiment the expression of neuronal markers was enabled despite the loss of a dorsal signal through the suppression of the *swirl/Bmp2* gene). So, the blastoporal ring (the region of *Wnt/brachyury* expression) rather than the Spemann-type dorsal organizer is equivalent to the organizer of the radially symmetrical ancestor. Sequential activation of more posterior cells takes place in the marginal zone⁵⁰, except for the Spemann organizer. There is direct evidence that a *Wnt* acts as a morphogen for the AP patterning of the head^{51–53}, but other molecules might also be involved¹³.

The large blastoporal ring is necessarily orientated perpendicular to the AP axis. A secondary spot-like organizer that emerges on this ring is, therefore, an appropriate candidate to organize a further axis perpendicular to the AP axis. The widening of the small patch to a large ring was a precondition to generate an off-axis position to organize this second axis. Unlike the small patch-like organizer region in *Hydra*, the large ring-shaped blastopore in vertebrates cannot be generated in a spontaneous way, and it must be specified by a signalling

system. In *Xenopus laevis*, it depends on the formation of a large coherent endodermal region. The boundary between the endoderm and ectoderm has the geometry of a large ring, and it forms the mesodermal marginal zone and the blastopore (FIG. 3d–f). By contrast, the endodermal cells in the early *Hydra* embryo appear first in a scattered arrangement⁵⁴. So, during evolution, important changes in early development were required to proceed from the small patch-shaped to the large ring-shaped blastopore. Strong asymmetries, such as the Nieuwkoop centre in amphibians⁵⁵, are required to ensure that only a single organizer is formed on the large ring. This is especially crucial for large embryos such as the frog, because an inhibitory interaction around the large marginal zone would require too much time (see REF. 56 for modelling). By contrast, such an asymmetry is less important for small embryos such as the mouse, where each of the blastomeres of a four-cell embryo is able to form the complete organism.

In *Hydra*, re-aggregated cells can form normal animals⁵⁷, whereas a similar manipulation in vertebrates gives only partial structures⁵⁸. In terms of the model proposed, it is straightforward to regenerate a single organizing region (FIG. 2a). However, the blastoporal ring cannot be reconstituted in a spontaneous self-regulating way, making a spontaneous reconstitution of the whole coordinate system impossible.

Midline formation by elongation. The mechanism that is envisioned for midline formation in vertebrates can be illustrated by the following analogy. Clouds of many shapes can be formed spontaneously. However, a single straight cloud on a blue sky results only as a trace behind a high-flying aeroplane: a moving spot-like device leaves behind a narrow but elongated stripe-like structure. Analogously, the Spemann-type organizer or node is proposed to act as a moving spot-like structure that leaves behind the midline. The DV (or mediolateral) axis is organized by the midline, not by the organizer itself. A strong and wide-range lateral inhibition of the organizer ensures that a single midline is formed. The midline itself can consist of a chain of stably differentiated cells, or a stripe-forming system that needs only moderate lateral inhibition. In both cases, the stripe would be stable against decay into separate patches. As the lateral inhibition of the midline is weak, two midlines can coexist in proximity under experimental conditions. By contrast, a new organizing region can only be triggered at a greater distance⁵⁹. FIGURE 3 shows a simulation in which a spot-like pattern-forming system triggers a stripe-forming

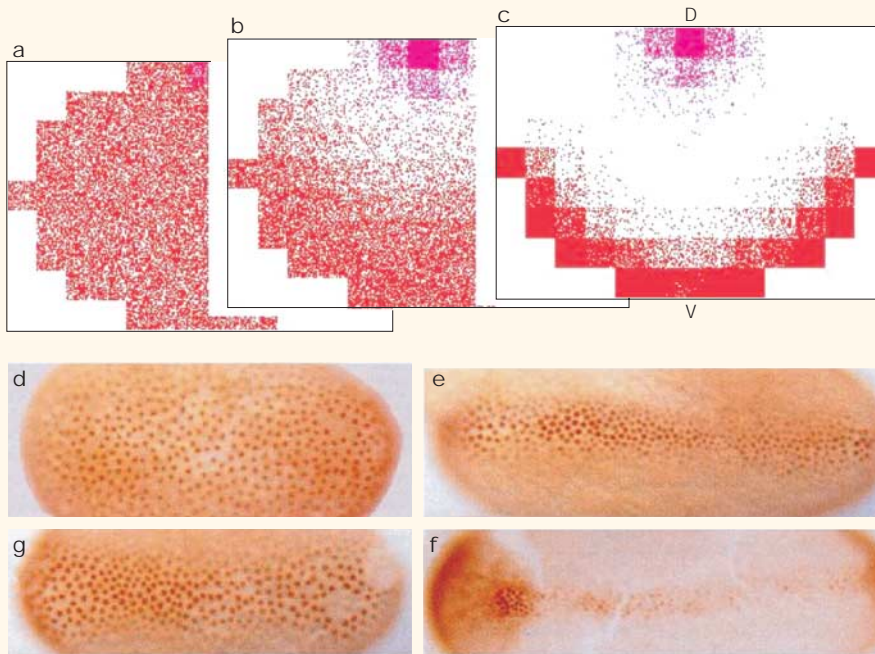


Figure 5 | The insect solution: ventral midline formation by repression from a dorsal organizer. **a–c** | Simplified model: it is assumed that there are two pattern-forming systems, one with patch-forming (pink) and one with stripe-forming (red) characteristics. The first has an inhibitory influence on the second. High-level activation of the stripe-forming system is possible only at a distance from the patch-shaped organizer. The midline appears simultaneously along the whole anteroposterior (AP) axis, but sharpens over time along the dorsoventral (DV) axis. Initial, intermediate and final stable patterns are shown (see online [supplementary information S3](#) (simulation)). **d–g** | Experimental indication for the sharpening of the midline along the DV axis in the short germ insect *Tribolium castaneum*; the activation of the *Dorsal* gene on the ventral side⁷³. Starting from an initially uniform activation, the activation becomes restricted over time to a fine stripe at the ventral side. Molecular details of this process are not yet known. As more segments are added during further development in *T. castaneum*, this initial midline will be also elongated towards the posterior. Figure kindly supplied by Siegfried Roth, University of Cologne.

system, which in turn displaces the spot-forming system, causing an elongation of the stripe. Over time, a single straight stripe is generated.

The position at which the organizer first appears is usually regarded as anterior-dorsal, but this is not entirely correct. Its early position is anterior only in relation to the not-yet formed trunk, but it is posterior to the brain; that is, it is posterior in relation to the ancestral AP axis. This is most obvious in the chick, where the head and heart form anterior to the early node position (FIG. 4). Therefore, the midline in vertebrates is formed in two parts. The anterior part extends anteriorly from the early node, and it is required for the DV patterning of the brain. This is achieved by mesodermal cells, which move from the blastopore at an early stage and migrate underneath the future head ectoderm⁶⁰. Organizer-derived cells that express particular genes, such as *chordin* and *gooseoid*, obtain a stripe-like geometry within this sheet of mesodermal cells, forming the prechordal plate. Through induction,

the prechordal plate allows the formation of neurons in the overlying ectoderm.

The posterior elongation of the midline is based on several processes. Cells from more lateral positions move towards the organizer and form the midline, together with cells derived from a stem-cell pool of the organizer. This process causes the ring-shaped arrangement of mesoderm that is perpendicular to the AP axis to be converted into a rod-shaped arrangement that is parallel to the AP-axis (FIG. 3), as shown by *brachyury* expression during gastrulation^{61,62}. In the amphibian organizer, head and trunk organizing regions can be distinguished at early stages⁶³. The notochord is generated during posterior elongation and trunk formation.

Cells antipodal to the dorsal organizer are usually regarded as ventral. However, recent fate mapping studies have shown that these 'ventral' cells form essentially posterior structures^{64,65}. This conflict disappears if it is kept in mind that it takes a long time for antipodal cells to get close to the organizer. During this time, the midline attains a substantial AP

extension. Therefore, cells antipodal to the organizer become integrated into the axial pattern at a relatively late stage, and they form posterior structures. According to the proposed mechanism, dorsal or ventral fate is decided not by the distance to the organizer, but by the final distance to the midline that is left behind the moving organizer.

The midline is not the only structure that forms in two parts: different mechanisms are used to pattern the anterior and posterior portions of the embryo. In the brain region, *Wnt* molecules are used in a concentration-dependent manner^{51–53}. By contrast, in the trunk, an oscillation takes place in the posterior-most region that leads to periodic somite formation^{66,67}. The same oscillation is also involved in the sequential activation of Hox genes^{68,69}. The coupling of a temporal periodic pattern and sequential gene activation was predicted on theoretical grounds² to account for the generation of a spatial periodic pattern — the somites — in which the individual units differ from each other in a systematic and reproducible way. This allows the periodic pattern (the somites) and the pattern of AP-specific gene activation to be formed precisely in register.

The midbrain–hindbrain border is an important organizing region of the brain⁷⁰, and the posterior border of *Otx* has an important role in its establishment. The corresponding posterior border of *Otx* expression in *Hydra* is next to the hypostomal organizing region²⁹ (FIG. 1). This implies that the posterior end of the ancestral organism corresponds to the posterior end of the midbrain in vertebrates. Indeed, the AP organization of the hindbrain and the first 6–8 somites seems to resemble a third AP system, in which a gradient of retinoic acid functions as a morphogen^{71,72}. This implies that AP patterning is a three-step process: first, the most anterior ancestral parts are patterned by a *Wnt* gradient; second, the initial stages of posterior elongation are controlled by a second gradient system, which uses retinoic acid to form the hindbrain and the first somites; and last, even more posterior structures are generated by oscillation-driven gene activation. Therefore, the midbrain–hindbrain organizing region might be a descendant of the primary organizing region that once ruled in radially symmetrical ancestors. *Wnt* has a crucial role in both of these organizing regions, and both are posterior to a region of *Otx* expression.

The insect solution
The strategy for midline formation in insects is fundamentally different to the vertebrate strategy. In *D. melanogaster*, there is a dorsal

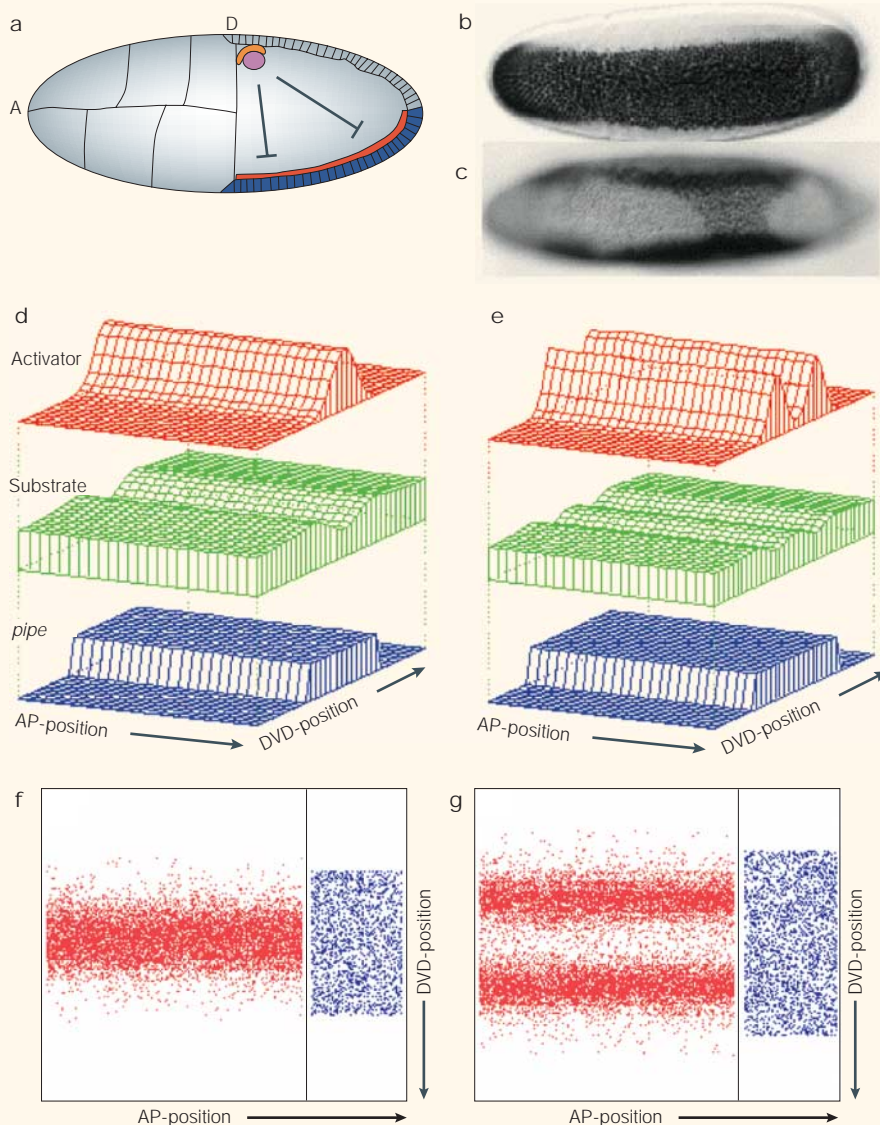


Figure 6 | Midline formation in *Drosophila melanogaster*. **a** | Schematic drawing showing the geometry of the embryo (A, anterior; D, dorsal). The nucleus (pink circle) triggers a dorsal organizing centre, causing the local expression of *gurken* (orange). Its inhibiting influence restricts *pipe* expression (blue) to a broad ventral band in follicle cells. By a complex cascade, a high nuclear concentration of the dorsal protein (red) accumulates only on the ventral side of the embryo^{74,75}. **b** | The downstream gene *twist* forms a single long-extended stripe. **c** | Reduced *gurken* activity leads to two midlines at a more lateral position⁸³. **d, e** | Simulation on an unrolled cylinder. Confined by the repressing influence from the dorsal organizer, *pipe* expression (blue) occurs in a plateau-like fashion over a large area at the ventral (V) side. It is assumed that a diffusible precursor (green) is modified and/or released by *pipe*-expressing cells. This precursor is processed by a cascade, in which at least one component (red) provides positive feedback. The depletion of the precursor molecules in a larger surrounding causes lateral inhibition^{2,19}, and therefore sharpening. A straight stripe-like activation results that is much narrower than the region of *pipe* expression. P, posterior. **e** | If the expression domain of *pipe* is broader owing to reduced *gurken* signalling, two peaks are formed at more lateral positions, whereas the central region is deactivated. **f, g** | The same simulations (ventral view), showing the relationship between *pipe* expression (blue) and the final signal (red) for the normal and the duplicated midline (**b** and **c** kindly supplied by Siegfried Roth, University of Cologne; see online [supplementary information S3](#) (simulation)).

organizing region, but it is not located on the blastopore and it does not elongate the midline. No ring-to-rod conversion occurs, and the midline has its full AP extension from the outset. It was proposed that repression emanates from a dorsal spot-shaped organizer, and this

restricts the stripe-forming midline system to the antipodal ventral side. The refinement of the ventral midline to a narrow, long-extended stripe is achieved by a patterning mechanism that is based on self-enhancement and long-range inhibition²⁴ (FIG. 5). The two main

implications of this proposal — simultaneous midline formation along the AP axis and its refinement along the DV axis — have been most clearly demonstrated in the short germ insect *T. castaneum*⁷³ (FIG. 5d–g).

In *D. melanogaster*, midline formation depends on communication between the oocyte and the surrounding follicle cells^{74,75}. At the crucial stage, the oocyte has the shape of a cone. The nucleus moves from the posterior tip to a marginal position on the circular anterior side⁷⁶, a process that is necessarily connected with a break of symmetry⁷⁷. Communication between the nucleus and the nearby follicle cells by means of the epidermal growth factor (EGF) signalling pathway leads to the formation of a dorsal organizing centre⁷⁸, a process that involves autoregulatory and inhibitory components⁷⁹, in accordance with our general theory^{2,19,20}. At this stage, the follicle cells on the ventral side are all about the same distance from the dorsal organizer (FIG. 6a). A repressing influence of the *Gurken* protein, which is produced at the dorsal organizer, restricts the expression of the *pipe* gene to the ventral follicle cells^{80–82}. The resulting *pipe* expression is much broader than the final midline. Through a complex cascade, the plateau-like *pipe* activation in the follicle cells gives rise to the proper ventral midline in the oocyte^{74,75}. The observation that a broader *pipe* stripe (owing to reduced *gurken* activity) leads not to a broader midline, but to a split of the midline into two parallel midlines⁸³ (FIG. 6c), is a clear-cut indication that a genuine pattern-forming process is involved. A mechanism that accounts for this observation is illustrated in FIG. 6. So, in *D. melanogaster*, the problem of multiple stripes that is inherent in the mechanism of stripe formation is solved by a repression that emanates from a dorsal spot-like organizer. If this repressive influence is not fully functional, the stripe-forming system does what is theoretically expected, resulting in multiple, bending and bifurcating stripes⁸³ (see FIG. 2).

Flatworms: a circumventing DV border
A closer look at planarians shows that the list of possible solutions of the stripe problem is not exhausted by the examples given above. Planarians are well known for their almost unlimited capability for regeneration^{84–86}, and the overall hierarchy of the AP and DV axes seems to be completely different from that in insects and vertebrates. In planarians, a confrontation between dorsal and ventral cells is a precondition for the regeneration of the anterior- or posterior-most terminal regions^{87,88}, whereas in vertebrates, the ancestral AP pattern can be formed without any DV

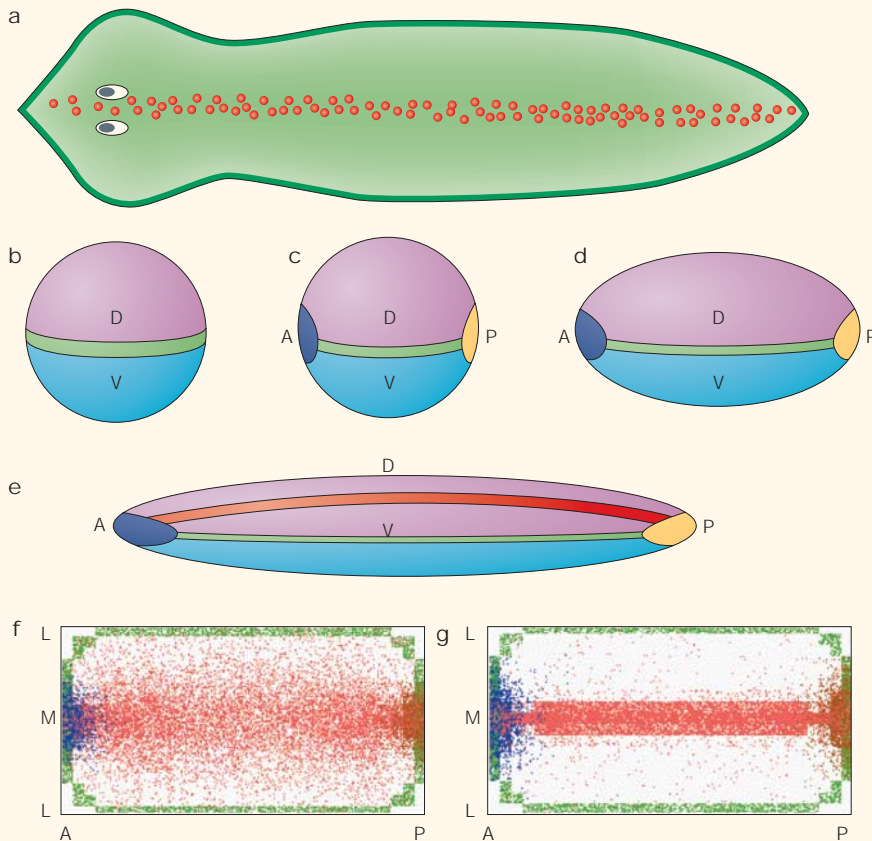


Figure 7 | Midline formation by repulsion from a circumventing border: the flatworm solution.
a | Schematic drawing of *Bmp* expression (red) in *Dugesia japonica* (drawn after Orii *et al.*⁹⁰). **b–e** | Proposed mechanism for midline formation. A primary dorsoventral (DV) subdivision (pink/blue) is assumed. The resulting DV border (green) acquires organizing properties. It is the precondition to initiate the most anterior (A, blue) and most posterior (P, yellow) structures. Intercalation of the pattern along the AP axis leads to the long extension of the animal. The dorsal midline (red) is localized by an inhibitory influence from the circumventing organizing border (green). **f, g** | Simulations. A stripe-forming system (red) is assumed. The stripe emerges at the largest possible distance from the circumventing DV border. It sharpens in the course of time (**g**). If the most anterior (blue) and the most posterior structures (brown) weaken the repelling influence, the midline can reach the poles (see online [Supplementary information S4](#) (simulation)). L, lateral; M, medial.

pattern⁴⁹. As shown below in more detail, the available data indicate that in planarians the lateral positions are determined first, and midline formation is a secondary event.

Not much information is available on midline formation in planarians, although a bone morphogenetic protein (BMP) homologue has been cloned in *Dugesia japonica* (FIG. 7a) that forms a sharp midline on the dorsal side along most of the AP axis. Together with the observations listed above, the following mechanism is suggested (FIG. 7b–d). Very early in development, a DV pattern is formed, which specifies the dorsal and ventral parts of the organism⁸⁹. This determination remains unchanged during all subsequent pattern regulations. The border between the dorsal and the ventral part, which surrounds the organism, acquires organizing properties. Anterior or posterior terminal structures depend on this organizing border,

and can only be generated at or near to it. As long as the distances are small and no other constraints are superimposed, the most anterior and posterior structures emerge at the largest possible distance from each other. Intercalation of structures between the poles leads to the long extension of the organism. The DV border that circumvents the elongated organism acts as the origin for the mediolateral organization by repelling midline formation. In this way, the two main body axes are kept perpendicular to each other. For instance, the formation of the stripe of BMP expression mentioned above can be explained by assuming that BMP is part of a pattern-forming system, and that an inhibitory influence spreads from the circumventing DV border (FIG. 7e, f). In keeping with this model, Orii *et al.*⁹⁰ found that in long fragments from either side that do not contain the midline,

BMP expression reappears first with a broad distribution, which sharpens over time.

These dynamics are consistent with our standard model of pattern formation, in which regeneration of the midline occurs in the following steps. Removal of the BMP-expressing midline relieves the inhibition against *BMP* activation. Initially, a more or less uniform activation occurs, but through local self-enhancement and long-range inhibition, a competition for *BMP* activation sets in, which will be won by those cells that have the greatest distance from the DV border. Although no data are available for planarians, autoregulation of *BMP* expression and a diffusible *BMP* antagonist has been described for other systems^{91,92}. In the regeneration experiment mentioned above, the first *BMP* localization appears closer to the wounded side⁹⁰. Presumably, it takes some time during regeneration until the DV border becomes fully functional and acquires its repelling influence. Such a mechanism would imply that in planarians, the most lateral position is specified first, and the midline is laid down secondarily at the largest possible distance.

The separation into sharply confined upper and lower parts in flatworms is analogous to other flat systems in biology. The sheet-like shape of a leaf or of a *D. melanogaster* wing depends likewise on a confrontation of two layers with different determinations: dorsal and ventral in the imaginal disk⁹³ and abaxial and adaxial in the leaf^{94,95}. In both cases, a primary border between two early determinations acquires organizing functions, a feature that was predicted for both systems on theoretical grounds^{96,97}.

Conclusions

In higher organisms, the establishment of the DV axis is a precondition for generating a CNS. The formation of a corresponding organizing region, with its long extension along the AP axis and its short extension perpendicular to it, requires several coupled reactions. The examples discussed in this article demonstrate that a surprisingly diverse range of midline-generating mechanisms have evolved, yet many of the same molecular ingredients are used for their realization.

In vertebrates, midline formation occurs by sequential elongation under the driving force of the organizer. The midline appears on the same side of the embryo as the organizer (FIG. 3). In insects, a dorsal organizer repels the midline. The midline, and consequently the CNS, appears on the ventral side. The full AP extension is present from the outset (FIG. 5), and no moving node or prechordal plate movement is required. In planarians, it is pro-

posed that the opposite sequence of events occurs: the most lateral positions are specified first and the midline is secondary (FIG. 7). We have probably not yet exhausted the list of possible mechanisms.

This diversity raises the question of whether the transition from a radially symmetrical to a bilaterally symmetrical body plan occurred only once, as postulated by the urbilaterian concept. An alternative possibility, and perhaps the most probable one, is that the patterning systems that are required for the AP and DV axes had already evolved in the radially symmetrical ancestors, and that both are involved in organizing the AP axis (the only axis that has a systematic pattern in these organisms). Indeed, the *chordin/Bmp* signalling pathway, which is responsible for DV patterning in all higher organisms⁹⁸, is present in the radially symmetrical coelenterates^{44,99–101} (T. Holstein, F. Rentzsch and B. Hobmayer, personal communication). Both the *chordin-Bmp* and the β -*catenin-Wnt-Nkx2.5* patterns are orientated essentially parallel to the AP axis. Therefore, the formation of the second axis in bilateria was presumably based on a mutual reorientation of two pre-existing, originally near-parallel patterning systems and not on the invention of a new system. Different phyla found different mechanisms for the separation of the two patterning systems and for the transformation from a spot- to a stripe-shaped organizer.

The midline and nerve cord appear dorsally in vertebrates, but ventrally in insects^{3,4} (FIGS 3h,i and 6a). Possible origins of this remarkable DV–VD inversion have been widely discussed^{102–104}. The schemes that I propose in the present paper suggest an alternative possibility: the deviation from radial symmetry was achieved in both phyla by the establishment of an off-axis organizer that marks the dorsal side. In insects, this dorsal organizer repels the midline, which therefore appears ventrally together with the nerve cord. By contrast, in vertebrates the organizer elongates the midline, causing it to be localized dorsally. Therefore, the DV–VD conversion could have its origin in the fundamentally different mechanisms of midline formation.

It is conceivable that the innovation of different modes of midline formation was the point of no return in the separation of different phyla. Alternatively, if an urbilaterian once existed, the different modes of midline formation described above would indicate a surprising flexibility to modify the original mode of midline formation. In any case, a linkage of several pattern forming reactions that are based on local self-enhancement and long-range antagonistic effects provide a

powerful toolkit to generate complex patterns in a reliable and self-regulating way. The midline is only one of them.

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doi:1038/nrn1410

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Competing interests statement

The author declares that he has no competing financial interests.

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