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Segmenting the fly embryo: logical analysis of the role of the Segment Polarity cross-regulatory module

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ABSTRACT **Initially activated by the *pair-rule* genes, the expression patterns of the segment polarity genes *engrailed* and *wingless* become consolidated through inter-cellular interactions between juxtaposed cells. We delineate a logical model focusing on a dozen molecular components at the core of the regulatory network controlling this process. Our model leads to the following conclusions: (1) the pair-rule signals, which activate *engrailed* and *wingless* genes independently of each other, need to be operative until the inter-cellular circuit involving these two genes is functional. This implies that the pair-rule pattern is instrumental both in determining the activation of the genes *engrailed* and *wingless* in rows of adjacent cells, and in consolidating these expression patterns; (2) the consolidation of *engrailed* and *wingless* expression patterns requires the simultaneous activation of both autocrine and paracrine Wingless-pathways, and the Hedgehog pathway; (3) protein kinase A plays at least two roles through the phosphorylation of *Cubitus interruptus*, the effector molecule of the Hedgehog signalling pathway and (4) the roles of Sloppy-paired and Naked in the delineation of the *engrailed* and *wingless* expression domains are emphasized as being important for segmental boundary formation. Moreover, the application of an original computational method leads to the delineation of a subset of crucial regulatory circuits enabling the coexistence of specific expression states at the cellular level, as well as specific combination of cellular states inter-connected through Wingless and Hedgehog signalling. Finally, the simulation of altered expressions of segment polarity genes leads to results consistent with the published data.**

KEY WORDS: *Drosophila, systems biology, pattern formation, segmentation, regulatory circuit*

Introduction

The *Drosophila* embryo encompasses a series of repeated units (segments). The first signs of segmentation are transient grooves (visible on the surface of the embryo after gastrulation) (Martínez-Arias and Lawrence, 1985). This initial (para)segmental organisation of the embryo gives rise to the overt segments shown by the larva and later on by the adult. The posterior portion of one segment plus the anterior portion of the next posterior segment originate from one individual embryonic parasegment. Parasegments thus span the same length as segments, but their borders lie between the segment boundaries.

Segmentation originates from the action of maternal organisers on zygotic genes (reviewed in Ingham and Martínez-Arias, 1992; Pankratz and Jäckle, 1993; Sprenger and Nüsslein-Volhard,

1993; Rivera-Pomar and Jäckle, 1996). These zygotic segmentation genes can be classified into three categories depending on the number of segments affected by their mutations. Mutations in *gap* genes affect several contiguous segments, while the *pair-rule* gene mutations delete complete alternate segments. Finally, the *segment polarity* genes affect each segment. Each of these three sets of genes forms a cross-regulatory module wherein all genes are involved in intertwined feedback circuits (for a discussion on genetic cross-regulatory modules, see Thieffry and Sánchez, 2004). These modules form a hierarchical system in which interactions take place in a temporal order. The maternal products involved in segmentation act upon the zygotic genome, resulting

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Abbreviations used in this paper: Abr, Abbreviation; Abrs, Abbreviations.

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in the activation of *gap* genes. Maternal and gap factors control the activation of *pair-rule* genes. Finally, *pair-rule* genes control the expression of *segment polarity* genes, ultimately determining which cells will express *engrailed (en)* in juxtaposition to cells that express *wingless (wg)* along the anterior-posterior axis of the embryo trunk. The juxtaposition of these two types of cells determines the formation of the parasegmental boundaries: cells expressing *en* mark the anterior (posterior) region of each parasegment (segment) and cells expressing *wg* mark the posterior (anterior) region of each parasegment (segment).

The formation of parasegments occurs at the blastoderm stage. When the embryo cellularises, the blastomeres become able to express either *en* or *wg* in response to the *pair-rule* signal, with the main role played by *even-skipped* and *fushi-tarazu* (reviewed in Ingham, 1991; Ingham and Martínez-Arias, 1992; for a model analysis, see Sánchez and Thieffry, 2003). The *pair-rule* products activate the genes *en* and *wg* in 14 adjacent narrow stripes along the anterior-posterior region of the embryo trunk. The initial activation of *en* and *wg* is independent of each other, but the consolidation of their expressions depends upon inter-cellular regulatory interactions: the expression of *en* requires the expression of *wg* in the anterior adjacent cells and the expression of *wg* requires the expression of *en* in the posterior adjacent cells. Thus, the originally independent expression patterns become dependent upon each other. Later in development, when the segments are already formed, the expressions of *en* and *wg* become again independent of each other.

Two other genes, *sloppy-paired* and *naked*, may be critical in limiting the competence of these cells to receive the cell-specifying signals. The *sloppy paired (slp)* gene shows a *pair-rule* as well as a *segment polarity* expression pattern overlapping *wg* but not *en* expressing cells. Considering its ability to activate *wg* and repress *en*, Cadigan *et al.* (1994) proposed that *slp* could be involved in the delineation of *en* and *wg* competence domains. *Naked (nkd)* is a segment polarity gene whose lack of function produces multiple segmentation defects (see *e.g.* Jürgens *et al.*, 1984). It shows a very complex pattern of expression. At the blastoderm stage, *nkd* is almost ubiquitously expressed. High levels of Nkd are found in the 2-3 cells anterior to the En-stripe and low levels are found in cells just posterior to this stripe, whereas the cells expressing *en* show very scant Nkd levels. In loss-of-function *wg* mutants, the expression of *nkd* is initiated normally but is markedly reduced later. On the other hand, ectopic expression of *nkd* mimics the phenotype of loss-of-function *wg* mutants. Thus, *wg* induces the expression of *nkd*, which itself acts as a repressor of *wg* (Zeng *et al.*, 2000).

Detailed genetic and molecular studies allowed the identification of *segment polarity* genes and regulatory interactions underlying the inter-cellular dependence of *en* and *wg* expressions. The gene *en* encodes a transcription factor that promotes the transcription of gene *hedgehog (hh)*, whose product undergoes several post-translational modifications before being secreted. Hh secreted from the En-cell binds to its receptor Patched (Ptc) at the surface of neighbouring cells. The transduction of Hh signal ultimately results into the conversion of the Cubitus interruptus (Ci) protein into a transcriptional activator of gene *wg* (this situation corresponds to the *activation* of Hh pathway). *Ci* gene encodes a transcription factor that enables the expression of Wg, a secreted glycoprotein, which binds to its receptor Frizzled (Fz)

in the adjacent (En) cell. Consequently, the degradation of Armadillo (Arm) is prevented, leading to the activation of *en* (this situation corresponds to the *activation* of Wg pathway) (reviewed in Ingham and McMahon, 2001; Nybakken and Perrimon, 2002; Kalderon, 2002; Jiang, 2002).

Among the proteins that have been identified as components of the Hh-signalling pathway, Ci is the only transcription factor. The native Ci is a large protein that contains three domains: a N-terminal domain characteristic of transcriptional repressors, a zinc finger domain, and a C-domain typical of transcription activators (Alexandre *et al.*, 1996). Ci exists in two distinct functional forms (depending on phosphorylation and on interactions with other factors): a full-length Ci-155 form and a truncated form Ci-75, which derives from Ci-155 by proteolysis of its transcriptional activation domain. Ci-75 acts as a transcriptional repressor, Ci[rep], whereas uncleaved Ci-155 acts as a transcriptional activator, Ci[act], of Hh-responsive genes (Aza-Blanc *et al.*, 1997). Although *ci* is transcribed uniformly in all cells that are responsive to Hh producing the Ci[rep], the Ci[act] form accumulates specifically in cells adjacent to those secreting Hh (Aza-Blanc *et al.*, 1997; Motzny and Holmgren, 1995; Wang and Holmgren, 1999), suggesting that Hh acts by inhibiting the proteolytic cleavage of Ci-155.

The Hh-signalling pathway comprises other components regulating the conversion of Ci into one of its two forms. These proteins include the membrane protein Smoothened (Smo), as well as the cytoplasmic proteins Protein kinase A (Pka), Costal-2 (Cos2), Suppressor-of-Fused (Su(fu)) and Fused (Fu). Smo is required to transduce the Hh signal. In the absence of Ptc, Smo activity is independent of the Hh ligand and constitutively activates the Hh signalling pathway. Thus, the binding of Hh to Ptc is thought to liberate Smo from repression by Ptc (Alcedo *et al.*, 1996; Chen and Struhl, 1996; van den Heuvel and Ingham, 1996; Denef *et al.*, 2000; Strutt *et al.*, 2001; Martin *et al.*, 2001). Pka inhibits Hh-signalling by phosphorylating Ci-155 (Lepage *et al.*, 1995; Pan and Rubin, 1995; Jiang and Struhl, 1995; Li *et al.*, 1999, Wang *et al.*, 1999). Cos2 is a kinesin-related protein (Sisson *et al.*, 1997), whose capacity to bind microtubules is reversed by Hh-signalling (Robbins *et al.*, 1997). It is required for proteolytic processing of Ci-155 (Wang and Holmgren, 2000). Su(fu) is not involved in the conversion of Ci-155, but it appears to block the transport of Ci[act] into the nucleus (Méthot and Basler, 2000; Wang *et al.*, 2000). Fu is a serine-threonine protein kinase whose activity is necessary for some but not all levels of response to Hh (Alves *et al.*, 1998; Ohlmeyer and Kalderon, 1998; Thérond *et al.*, 1999). Fu is itself phosphorylated in an Hh-dependent manner (Thérond *et al.*, 1993; Thérond *et al.*, 1996) and phosphorylates Costal-2 (Nybakken *et al.*, 2002). Apparently, Fu kinase opposes cytoplasmic retention of Ci[act] by Su(Fu) (Méthot and Basler, 2000; Wang *et al.*, 2000).

More precisely, the processing of Ci-155 to generate Ci[rep] requires the sequential phosphorylation of Ci by three kinases. Pka phosphorylates multiple sites in the C-terminal region of Ci and promotes further phosphorylation by Glycogen synthase kinase 3/Zeste white 3 (GSK3/Zw3) and caseine kinase I (CKI) at adjacent sites (Wang *et al.*, 1999; Chen and Struhl, 1999; Price and Kalderon, 1999; Jia *et al.*, 2002; Price and Kalderon, 2002). Pka plays a key role in this process because it targets full-length Ci-155 for proteolysis into Ci[rep] and also inhibits the conversion

of Ci-155 into Ci[act] (Wang and Holmgren, 1999; Wang *et al.,* 1999).

Several proteins have been identified as components of the Wg-signalling pathway (reviewed in Kalderon, 2002). One of these is Armadillo/beta-catenin, which is the effector of the Wg-signalling pathway. Other components of this pathway are Dishevelled (Dsh) and Zw3 (GSK3). Dsh is a positive regulator of Wg-signalling pathway activity (Li *et al.,* 1999; Salic *et al.,* 2000) and is required by the receptor complex to initiate the intra-cellular Wg-signalling cascade (Yanagawa *et al.,* 1995; Axelrod *et al.,* 1998; Boutros and Mlodzik, 1999; Rothbacher *et al.,* 2000). Zw3 phosphorylates Arm, targeting it for degradation and thereby keeping the cellular pool of Arm low (Yost *et al.,* 1996; Pai *et al.,* 1997). In the absence of Wg-signal, Arm is continuously degraded (Cliffe *et al.,* 2003). The binding of Wg-signal to its receptor Fz prevents the degradation of Arm, which is then stabilised and enters into the nucleus, where it binds the (constitutively expressed) transcription factor TCF and co-activates the transcription of target genes, including *nkd*, *slp* and *en* (reviewed in Bienz and Clevers, 2003).

An important feature of the mutual inter-cellular regulatory interactions between *en* and *wg* is the fact that these two genes remain active in adjacent rows of cells. This is so because the diffusions of secreted Hh and Wg are both limited. In the case of Hh, one of the consequences of the Hh-signalling is to increase the transcription of *ptc* in the adjacent *wg* expressing cell (*Wg-cell*, for short). At high levels, Ptc can then sequester all the secreted Hh, thereby impeding its diffusion towards more anterior cells (Chen and Struhl, 1996). With respect to Wg diffusion, it has been shown that its transport or stability is reduced in *en*-expressing cells (*En-cell*, for short) (Sanson *et al.,* 1999).

In *Drosophila* embryos, secreted Wg can act upon the cell that secretes it (*autocrine* pathway) as well as upon neighbouring cells (*paracrine* pathway). These two pathways use the same receptor, Fz, as well as the component Dsh of the Wg-signalling cascade. However, the paracrine pathway passes through Zw3 and then Arm, whereas the autocrine pathway seems to involve Ci (Hooper, 1994).

The *Drosophila* segmentation process has stimulated a wealth of theoretical studies (Meinhardt, 1982, 1986; Bodnar, 1997; Reinitz *et al.,* 1998; von Dassow *et al.,* 2000; von Dassow and Odell, 2002; Houchmandzadeh *et al.,* 2002; Albert and Othmer, 2003; Ingolia, 2004; Jaeger *et al.,* 2004a,b; Alves and Dilão, 2006). Using a logical approach (cf. Materials and Methods section), we have previously developed qualitative models for the *gap* (Sánchez and Thieffry, 2001) and *pair-rule* (Sánchez and Thieffry, 2003) cross-regulatory modules. The present manuscript focuses on the analysis of the network of cross-regulations among the *segment polarity* genes. More specifically, it aims at better understanding how the expressions of *en* and *wg* in juxtaposed cells, once activated by the *pair-rule* network, become consolidated through inter-cellular interactions and various kinds of intra-cellular regulatory interactions. These events take place in a short period spanning embryonic stages 9-10 of Campos-Ortega and Hartenstein (1985), approximately 3:40 – 5:20 h after egg laying.

The *segment-polarity* gene network has already been modelled in terms of ordinary differential (von Dassow *et al.,* 2000; von Dassow and Odell, 2002; Ingolia, 2004) or Boolean (Albert and

Othmer, 2003) equations. These models present fundamental differences, regarding the regulatory elements and the regulatory interactions considered (see the supplementary figures, Figs. S1 to S3). As the data sets enlarge and more interactions become known, there have been refinements made to these models in the hopes of explaining the complete set of phenomena and delineating new experiments. The model of von Dassow and co-workers includes inhibitions of *ptc* and *en* by Ci[rep]. However, the background level of Ptc neither rises in the absence of Ci protein (Dominguez *et al.,* 1996; Hepker *et al.,* 1997; Méthot and Basler, 1999), nor disappears when Ci[rep] is over-expressed (Muller and Basler, 2000). Furthermore, ectopic expression of *ci* does not repress the expression of *en* (Hepker *et al.,* 1997). Von Dassow's model further encompasses an inhibition of *wg* by Ci[rep], which is known to play a role later on during the specification of the cuticle formation, but not in the formation of the parasegment boundaries (Méthot and Basler, 1999; Gallet *et al.,* 2000; Méthot and Basler, 2001). Von Dassow *et al.* consider that the Ci-155 protein obtained after translation of its mRNA is already the activatory form (i.e. Ci[act]), and they thus derive the repressor form (Ci[rep]) directly from Ci[act]. In contrast, in our model, we explicitly consider the three reported *Ci* products, Ci-155, Ci[act] and Ci[rep], together with their differential regulation by the Hh pathway. This is important because it has been shown that Wg expressing cells respond to particular thresholds of Hh signal (Wang and Holmgren, 1999). Finally, the model of von Dassow *et al.* does not include *slp* and *nkd*, which both contribute to restrict *en* and *wg* expressions (Cadigan *et al.,* 1994; Zeng *et al.,* 2000). Leaning on the preceding model, Ingolia (2004) also assumes that *wg* expression is dependent on Hh signalling. However, Ingolia removed the repression of *en* by Ci[rep] (denoted CN), and introduced a mutual inhibition between *slp* and *en*, with *slp* mediating the positive effect of *en* upon *hh*. Next, Hh directly inhibits the conversion of Ci[act] (denoted CI) into Ci[rep], thus making Ptc implicit. Finally, cell division is incorporated into the model. Albert and Othmer (2003) considered the repression of *ptc* and *wg* by Ci[rep], but neglected the requirement of *wg* function for the maintenance of *slp* expression, as well as the repression of *slp* and *wg* by *en*. Similarly, the role of *nkd* is not taken into account. Furthermore, their model does not recognize the autocrine and paracrine Wg-signalling pathways, thus impeding a proper analysis of the link between the Wg- and the Hh-signalling pathways. Finally, the use of a pure Boolean approach impeded the modelling of some subtle aspects of the behaviour of the *segment polarity* network, such as the regulation of *ptc*. Since *ptc* is basally expressed in the Wg-cell, there is certain low amount of Hh receptor present. After reception of Hh signal, the transcription of *ptc* increases significantly, thereby allowing the sequestration of all secreted Hh and preventing further anterior diffusion. Consequently, the gene *wg* becomes transcribed only in the cell adjacent to the *engrailed*-expressing cell that secretes Hh.

In this paper, we use the logical formalism developed by René Thomas and collaborators (Thomas, 1991; Thomas *et al.,* 1995) to model the segment polarity module, focusing on the roles of the signalling pathways enabling the consolidation of En and Wg expression patterns beyond the syncytial stage. This logical approach (cf. Materials and Methods section) relies on the delineation of a regulatory graph, which represents the epistatic relationships inferred from genetic experiments. Subtler (e.g.

synergic) relationships are encoded in terms of logical rules. The developmental properties derived from the resulting model are then systematically analysed for a strip of cells, representing a whole parasegment, and on different genetic backgrounds (*e.g.* gene knockouts, ectopic gene expressions, or combinations thereof).

Results

Delineation of the segment polarity regulatory graph

Our logical model of the segment polarity cross-regulatory module includes a series of simplifications, enabling the skeleton of the module to be more readily seen. In this simplified scheme, the Hh-signalling pathway is initiated by the binding of Hh to its receptor Ptc (Fig. 1A). As Smo merely connects the receptor and cytoplasmic components of the Hh-pathway, it is implicitly considered behind the arrow connecting Ptc and Pka. The cytoplasmic components are represented by Pka, which plays a key role in the conversion of Ci, the target of the Hh-signalling pathway. We further distinguish the native Ci-155 form (Ci in Fig. 1A), the functional activator form (Ci[act]), and the repressor form (Ci[rep]). Ci[rep] operates later in embryogenesis and during the development of imaginal discs, but it

does not play important roles in the formation of the parasegment boundaries (Méthot and Basler, 1999). Although apparently not essential for wild type development, the repression of *hh* by Ci[rep] has been incorporated in the *segment polarity* regulatory graph because it might be detrimental when Hh-signalling is weakened (Gallet *et al.,* 2000; Méthot and Basler, 2001). For sake of simplicity, the genes *fu* and *Su(fu)* are omitted because *fu* kinase activity is partially dispensable in wild type animals and entirely dispensable in animals lacking *Su(fu)* (Préat; 1992; Préat *et al.,* 1993). The model thus assumes that Ci[act] translocates into the nucleus once it is formed. Finally, the role of Cos2 in the proteolytic processing of Ci-155 is implicitly considered behind Ci-155 phosphorylation by Pka.

In our model, Fz, the receptor of the Wg-signal, together with Dsh represent the Wg signalling pathway. Although there are two Fz proteins (Fz and Fz2) acting as Wg receptors, a single receptor of the Wg ligand is considered since the presence of either protein appears to be sufficient to transduce the Wg signal (Chen and Struhl, 1999). Dsh is assumed to play a role in both autocrine and paracrine Wg pathways. For simplification, Arm is omitted, as it is either destroyed or stably maintained depending on the activity of the Wg-signalling pathway, so that its activity follows that of Fz and Dsh. This is different from the situation found for Ci-155, the effector of the Hh-signalling pathway, which does not directly lead to functional Ci[act], but depends on phosphorylation by Pka, which in turn depends on Hh signal. Zw3 function is implicitly represented by two different arcs: an activatory arc from Dsh upon En and a negative arc from Dsh upon Ci[rep].

The positive arc from Dsh towards

A

B

Wg

Hh

Para-segmental border
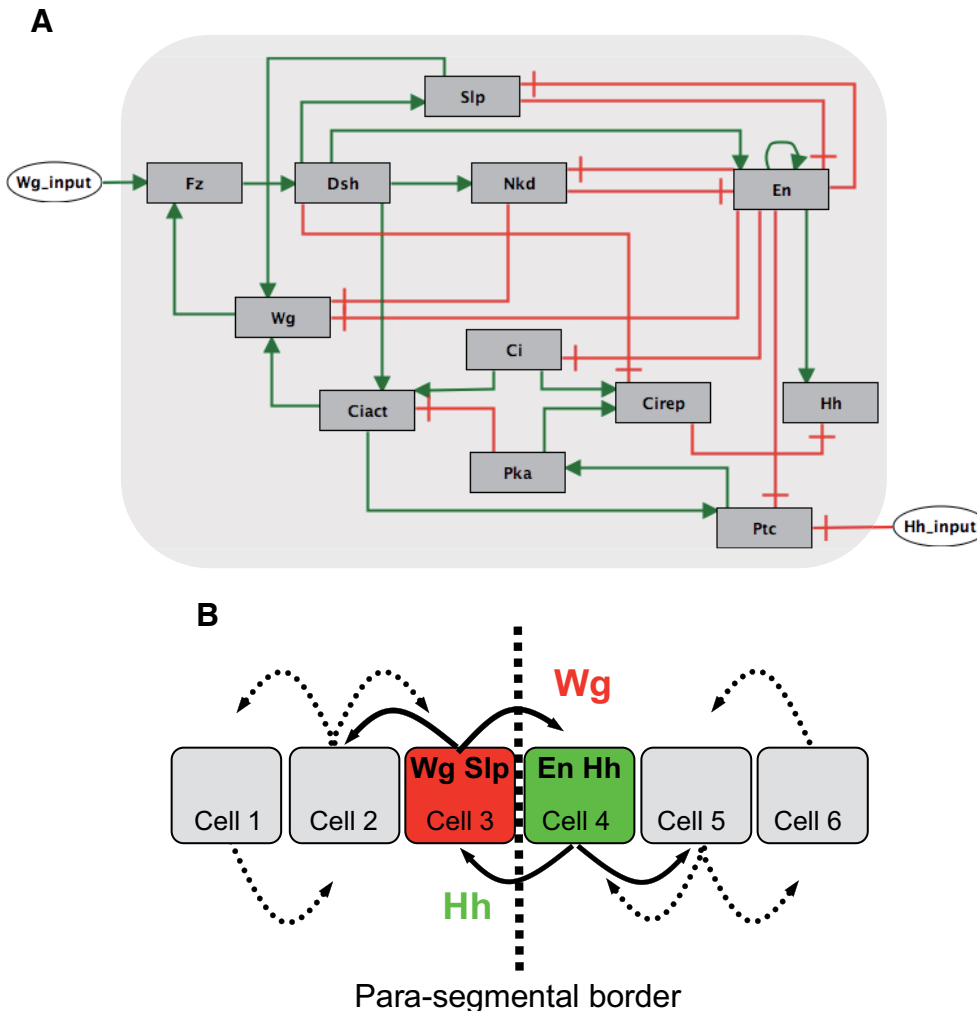
Fig. 1. The segment polarity network. (A) *Cross-regulations between* segment polarity *regulatory factors. This regulatory graph explicitly encompasses the regulatory components Wg, En, Hh, Slp, Nkd, Fz, Dsh, Pka, Ptc, as well as Ci-155 and its two functional forms Ci[act] and Ci[rep]. Normal and blunt arrows represent positive and negative interactions between these regulatory elements, respectively. Once Hh and Wg are excreted, they diffuse and interact with their respective receptors Ptc and Fz, which are present at the surface of the neighbouring cells. During normal development, this occurs between the cells flanking the para-segmental border (see text for further explanation).* **(B)** *Stripe encompassing six cells with inter-cellular connections through Wg and Hh signalling. These cells are numbered from 1 to 6, with cell 3 denoting the cell just anterior to the border, cell 4 the cell just posterior to the border, whereas the other cells are linearly arranged along the anterior-posterior axis, according to their numbering. For more details see text.*

TABLE 1

INTERACTIONS BETWEEN THE *SEGMENT POLARITY* REGULATORY PRODUCTS

Interactions	Main experimental observations	Bibliographical references
En→Hh	In loss-of-function *en* mutants, *hh* is not expressed	1, 2, 3, 4
En⊣Wg	Ectopic *en* expression suppresses *wg* expression	1, 5
En⊣Ci	Expression of *en* represses *ci* transcription	1, 6, 7, 8
En⊣Ptc	Ptc protein is not detected in cells expressing *en*	9
En⊣Slp	Ectopic *en* expression suppresses *slp* expression	1, 10
En⊣Nkd	*nkd* expression is strongly reduced in *en*-expressing cells	Novel interaction derived from published data (see text for details)
Wg→Fz	The binding of secreted Wg to its receptor Fz causes its activation	11, 12
Slp→Wg	All the cells expressing *wg* in loss-of-function *ptc* mutants require functional *slp* for *wg* expression	13
Slp⊣En	In loss-of-function *slp* mutants, *en* is ectopically expressed: ectopic *slp* expression represses *en*	13
Nkd→Wg	Ectopic *nkd* expression results in decreasing *wg* expression	14
Nkd⊣En	In *nkd*⁻ embyos, *en* is de-repressed	5, 15, 16, 17
Hh⊣Ptc	The binding of secreted Hh to its receptor Ptc causes its inactivation	9, 15
Ptc→Pka	Ptc inhibits Smo that, in turn, inhibits Pka	Cf. Introduction
Pka⊣Ci[act]	Low levels of phosphorylation of Ci-155 by Pka prevents the formation of Ci[act]	18
Pka→Ci[rep]	Conversion of Ci into Ci[rep] form requires Pka activity	18
Ci[act]→Wg	In loss-of-function *ci* mutants, *wg* expression fades, whereas ectopic *ci* activates *wg*	16, 19
Ci[act]→Ptc	In loss-of-function *ci* mutants, *ptc* expression fades, whereas ectopic *ci* activates *ptc*	16, 19
Ci[rep]⊣Hh	In *ci* mutant clones, there is activation of *hh*	6, 7
Fz→Dsh	Dsh serves as a sensor of Fz activity and is required for Wg-signalling	20, 21, 22, 23, 24
Dsh→En	Dsh inhibits Zw3, the inhibitor of Arm that activates gene *en*	Cf. Introduction
Dsh→Nkd	In *wg*⁻ embryos, *nkd* expression decays, and ectopic *wg* expression enhances *nkd* expression	14
Dsh→Ci[act]	Dsh participates in the autocrine Wg-signalling pathway via Ci	25
Dsh⊣Ci[rep]	Dsh inhibits Zw3, which participates in the proteolysis of Ci-155 into Ci-75	Cf. Introduction

Interactions between the *segment polarity* regulatory factors considered in our model. In the first column, normal and blunt arrows represent positive and negative interactions, respectively. Experimental evidence supporting each of these interactions are summarised in the middle column, and documented by bibliographical references in the last column. (1) Alexandre and Vincent, 2003; (2) Lee *et al.,* 1992; (3) Tabata *et al.,* 1992; (4) Tabata *et al.,* 1995; (5) Heemskerk *et al.,* 1991; (6) Dominguez *et al.,* 1996; (7) Méthot and Basler, 1999; (8) Eaton and Kornberg, 1990; (9) Ingham *et al.,* 1991; (10) Kobayashi *et al.,* 2003; (11) Chen and Struhl G, 1999; (12) Wodarz and Nusse, 1998; (13) Cadigan *et al.,* 1994; (14) Zeng *et al.,* 2000; (15) Martínez-Arias *et al.,* 1988; (16) van den Heuvel *et al.,* 1993; (17) Mullen and DiNardo, 1995; (18) Jiang, 2002; (19) Hepker *et al.,* 1997; (20) Yanagawa *et al.,* 1995; (21) Axelrod *et al.,* 1998; (22) Boutros and Mlodzik, 1999; (23) Rothbacher *et al.,* 2000; (24) Noordermeer *et al.,* 1994; (25) Hooper, 1994.

Nkd implements the activatory effect of Wg upon *nkd* expression (Zeng *et al.,* 2000). We also consider direct repression of *wg* (Zeng *et al.,* 2000) and *en* by Nkd (Martínez-Arias *et al.,* 1988; Heemskerk *et al.,* 1991; van den Heuvel *et al.,* 1993; Mullen and DiNardo, 1995). In fact, the gene *nkd* is initially expressed in the cells where *en* expression is also initiated, yet the En-cell shows later scant Nkd levels (Zeng *et al.,* 2000). We thus assume that *en* acts as a repressor of *nkd*. Consequently, in our model, we consider mutual negative interactions between *en* and *slp,* as well as between *en* and *nkd*.

During the consolidation of the mutual dependence of *en* and *wg* expressions, *en* does not appear to be able to maintain its own expression alone. In contrast, the action of Wg (from the Wg-cell) upon the En-cell is necessary and sufficient to maintain *en* expression (Heemskerk *et al.,* 1991). Yet, we consider *en* auto-regulation in our model to encompass the transition between the formation of the parasegmental boundaries and the formation of the final segmental boundaries, which depends on *en*-autoregulation (when *en* expression becomes independent of Wg-signalling). Once the parasegment boundary is formed, *en*, initially expressed in one single cell (cell 4 in our model), becomes expressed into 2-3 cells following cell rearrangement and division, so that the daughter cells inherit the auto-regulatory state of *en*.

In brief, although simplified, the resulting regulatory graph preserves the main regulatory relationships between selected components of the *segment polarity* network (see Fig. 1A).

These regulatory relationships have been derived from the published phenotypes of *segment polarity* mutants. A listing of experimental results backing these interactions is provided in Table 1, together with bibliographical references.

Parameterisation of the* segment polarity *regulatory graph

For parsimony, we have assumed that most *segment polarity* genes (and then their products) have a single functional threshold. However, Wg, Ptc, Ci[act], Pka, and Nkd, are each assumed to have two significantly distinct functional levels (*i.e.* the corresponding logical variables can take three different values: 0, 1 and 2).

In *ptc* mutants, the Wg-domain broadens (Ingham *et al.,* 1991). Wg itself modulates this expansion since in double *ptc* and *wg* loss-of-function mutants, *wg* expression disappears, remaining only in occasional cells (Hooper, 1994; Ingham and Hidalgo, 1993; Bejsovec and Wieschaus, 1993; Yoffe *et al.,* 1995). Thus, *wg* up-regulates its own expression by an Hh-independent mechanism (autocrine *wg* function). In *ptc wg^{ts}* double mutants with low levels of functional Wg, large clusters of *wg* expressing cells replace the small clusters seen in *ptc wg* double mutants with no functional Wg protein. Importantly, these Wg-cell clusters are not adjacent to cells expressing *en* (Yoffe *et al.,* 1995). Thus, the cells expressing *wg* produce levels of Wg sufficient to activate the autocrine *wg* function, but insufficient to induce expression of *en* in adjacent cells, *i.e.* to activate the paracrine *wg* function. Consequently, we consider that the amount of secreted Wg

protein needed to induce the autocrine Wg-pathway in the Wg-cell is lower than that needed to activate the paracrine Wg-pathway in the En-cell (Wg ≥ 1 for autocrine function; Wg = 2 for paracrine function).

The receptor Fz is considered to be active (Fz = 1) when it is bound to its ligand, the secreted Wg protein. Dsh level follows that of Fz, since Dsh transduces Wg signal towards cytoplasmic components. Indeed, loss-of-function mutations of *fz* or *dsh* show similar altered segment polarity phenotypes.

ptc is expressed at a basal level in the cells that do not express *en*. We consider that the receptor Ptc is active when it is available in its free form, *i.e.* not bound to Hh. Ptc is assumed to be inactive (Ptc = 0) when it is bound to Hh. The transcription of *ptc* is increased when the Wg-cell responds to the Hh signal. Two functional levels are thus introduced for free Ptc, which correspond to basal expression (Ptc = 1) and to transcriptional activation (Ptc = 2).

Pka is considered to be active (Pka ≠ 0) when it is able to phosphorylate Ci-155. As Pka activation depends on free Ptc level, we also consider two different functional levels for Pka, correlated with those of Ptc.

We further assume that the presence of the Ci-155 protein is totally dependent on whether En is present. The levels of Ci[act] and Ci[rep] depend on the levels of the factors controlling the conversion of Ci-155. In this respect, we assume that Ci[act] has two functional levels, matched by Wg: when Ci[act] reaches the value 1, the gene *wg* is expressed at an intermediate level (Wg=1), whereas, when Ci[act] reaches the value 2, the gene *wg* is expressed at a higher rate (Wg = 2). Furthermore, we assume

TABLE 2

LOGICAL PARAMETERS DEFINING THE EFFECT OF REGULATORY ELEMENTS IN AND BETWEEN THE ANTERIOR AND POSTERIOR CELLS FLANKING THE PARA-SEGMENTAL BOUNDARY

Network element	Maximal variable value	Logical parameter values
Wg	2	$K_{Wg}(\{Slp, 1\},\{Ci[act], 1\}) = 1$ $K_{Wg}(\{Slp, 1\},\{Ci[act], 2\}) = 2$ $K_{Wg}(\{Slp, 1\},\{Ci[act], 2\},\{Nkd, 2\}) = 2$
Fz	1	$K_{Fz}(\{Wg, [1,2]\}) = 1$
Dsh	1	$K_{Dsh}(\{Fz, 1\}) = 1$
Slp	1	$K_{Slp}(\{Dsh, 1\}) = 1$
Nkd	2	$K_{Nkd}(\varnothing) = 1$ $K_{Nkd}(\{Dsh, 1\}) = 2$
En	1	$K_{En}(\{Dsh, 1\}) = 1$ $K_{En}(\{Dsh, 1\},\{En, 1\}) = 1$ $K_{En}(\{Dsh, 1\},\{Nkd, [1,2]\}) = 1$ $K_{En}(\{Dsh, 1\},\{En, 1\},\{Nkd, [1,2]\}) = 1$
Hh	1	$K_{Hh}(\{En, 1\}) = 1$
Ptc	2	$K_{Ptc}(\varnothing) = 1$ $K_{pPtc}(\{Ci[act], [1,2]\}) = 2$
Pka	2	$K_{Pka}(\{Ptc, 1\}) = 2$
Ci	1	$K_{Ci}(\varnothing) = 1$
Ci[act]	2	$K_{Ci[act]}(\{Ci, 1\}) = 1$ $K_{Ci[act]}(\{Ci, 1\},\{Dsh, 1\},\{Pka, [1,2]\}) = 1$ $K_{Ci[act]}(\{Ci, 1\},\{Dsh, 1\}) = 2$
Ci[rep]	1	$K_{Ci[act]}(\{Ci, 1\},\{Pka, 2\}) = 1$

Interactions are denoted by the name of their source node, together with the level(s) for which they are operative. The value of a parameter defines the effect of a given combination of interactions (*set* in parentheses) operating on a given regulatory element (sub-index of the parameter). An empty set (φ) denotes a basal functional level. $K_{Wg}(\{Slp, 1\},\{Ci[act], 1\})$ denotes the target value of Wg in the presence of Slp and Ci[act] (both at level 1) but in the absence of Nkd. Note that only the non-zero parameters are listed (i.e. all other combinations of inputs on a given gene drive it to the level 0 per default).

that both the autocrine and paracrine Wg-pathways are required to reach the highest level of Ci[act] (Ci[act]=2). Note that, in *cos2* mutants, the ectopic activation of *wg* is significantly delayed with respect to the increased activation of *ptc* in the Wg-cell (Forbes *et al.,* 1993), suggesting that these genes may require a different amount of Ci[act] for their activation.

The phosphorylation of Ci-155 by Pka plays a dual role. It targets the proteolysis of Ci-155 to form Ci[rep], and it inhibits the conversion of Ci-155 into Ci[act]. The pathway chosen by Ci-155 depends on its degree of phosphorylation: a higher level prompts the conversion of Ci-155 into Ci[rep], whereas the lack (or lower level) of phosphorylation causes its transformation into Ci[act] (Wang *et al.,* 1999; Price and Kalderon, 1999; Jia *et al.,* 2002; Price and Kalderon, 2002; Chen *et al.,* 1999). Alteration of individual Pka-phosporylation sites on Ci-155 abolishes its conversion into Ci[rep] but does not transform it into Ci[act]. Alteration at multiple sites not only inhibits conversion of Ci155 into Ci[rep] but also promotes its transformation into Ci[act] (Wang *et al.,* 1999). This further justifies the distinction between two significant functional levels for Pka, with high levels (Pka = 2) stimulating the formation of Ci[rep] (phosphorylation), whereas medium or high levels (Pka ≥ 1) prevent the conversion of Ci-155 into Ci[act] (residual phosphorylation of Ci-155).

Finally, we also consider two functional thresholds for Nkd. Medium or high levels (Nkd ≥ 1) enable the repression of *en*, whereas highest levels (Nkd = 2) are needed to further repress *wg*. Indeed, high levels of Nkd are found anteriorly to the En-stripe (in cells 2 and 3 of our model, see Fig. 1B), whereas low levels are detected in the cells posterior to that stripe (cells 5 and 6 of our model). Although high levels of Nkd are expressed in cells 2 and 3, repression of *wg* by Nkd only occurs in cell 2. Consequently, we assume that the activation of Hh-pathway in cell 3 overcomes the repression effect of Nkd upon *wg*, leading to high levels of Ci[act] (Ci[act] = 2). In contrast, in cell 2, the Hh-pathway is not activated due to the lack of Hh input. Consequently, cell 2 cannot produce much Ci[act], and Nkd can repress Wg. In cell 4, both *en* and *nkd* are initially activated. By assuming that the pair-rule signal can overcome the repression of *en* by Nkd, En can repress *nkd* in this cell, ultimately leading to the fading out of Nkd (the detected scant levels of Nkd are considered as negligible).

On the basis of the regulatory interactions and activity levels just delineated, we can now define the logical rules (or *logical parameters*) enabling each regulatory component to reach significant expression levels. Table 2 lists all the non-zero parameter values used in our simulations. This set of values has been progressively specified by comparing simulation results with published experimental data, describing wild-type as well as mutant phenotypes, and applying simplicity criteria (*i.e.* selecting the lowest parameter value) whenever different values are compatible with available data.

To delineate the essential (wild type and mutant) properties of the *segment polarity* network in the formation of the parasegmental boundaries, it suffices to consider six cells representing the three classes of cells (or regions, since one cell of the model might represent several cells at some point during the differentiation process) within the putative parasegment, which are determined by the expression pattern of the *pair-rule* genes (represented in the model by Slp, together with proper initial patterns for Wg, En, and Slp). These classes of cells are defined by their capacity to

express either *en* or *wg* under the action of the pair-rule signal. Cells 1 and 6 represent cells lacking both *en* and *wg* expressions. Cells 2 and 3 represent cells initially expressing *wg*. Cells 4 and 5 represent cells initially expressing *en*. However, only the juxtaposed cells 3 and 4 (where the parasegmental boundary will be formed) maintain *wg* and *en* expression. Therefore, the intracellular network just described has been iterated six times to represent the different gene expression domains flanking each parasegmental border. The cells are numbered from 1 to 6, with cell 3 denoting the cell just anterior to the border, cell 4 the cell just posterior to the border, whereas the other cells are linearly arranged along the anterior-posterior axis, according to their numbering (Fig. 1B). Altogether, these six cells represent a full parasegment, traversed by the future border at its middle. Communications between these cells occur through Hh and Wg signals, and in the wild-type case, we assume that these intercellular interactions are restricted to neighbouring cells. (However, this restriction can be released to account for particular situations, as, for example, to simulate specific perturbations, cf. Discussion). As we shall see, the logical parameters allow us to qualitatively recover all documented mutant phenotypes, including expanded *wg* or *en* expression domains combined with ectopic *en* or *wg* expression stripes.

Dynamics of the segment polarity *network*

On the basis of the regulatory graph delineated in Fig. 1A and implemented with logical parameter values (cf. Table 2), we can show that a given cell can reach five different stable states depending on inputs or initial conditions (cf. Table A1 in Appendix). One of these stable states accounts for the normal Wg expressing region, with Wg, Fz, Dsh, Slp, Nkd, Ci and Ci[act] all expressed at their maximal levels, whereas the remaining factors are OFF. Another stable state represents the normal En expressing region, with En and Hh ON, but also high Fz and Dsh expressed, due to Wg input from the anterior cell (all the other components being OFF). The three other asymptotical states are devoid of Wg, En and Hh expressions, but vary in terms of the other component expressions, depending on input signals (cf. supplementary material for more details).

Considering now a strip of six cells connected through Wg and Hh signalling between neighbouring cells (Fig. 1B), we obtain 65 stable configurations of the five states just mentioned. Some of these stable states correspond to symmetrical situations. Counting pairs of symmetrical states only once, we still obtain 29 different stable configurations for the six connected cells (cf. supplementary material). However, most of these configurations may not be reachable from realistic initial conditions.

To delineate which stable configurations are indeed reachable from plausible initial conditions, we have translated our logical models into discrete Petri nets and applied the reachability analysis tool of INA (cf. Materials and Methods section). In the wild-type situation, the initial conditions in each cell result from the activity of the *pair-rule* module. We have considered that Wg and Slp could be initially expressed in a few cells just anteriorly to the forming parasegmental border (cells 2 and 3 in our model encompassing 6 cells). Similarly, we have considered that En and Hh could be initially expressed in both cells 4 and 5. As a consequence, Pka, Ptc, Nkd and Ci are initially expressed in all cells, excepting cell 4 and 5, due to the presence of En. Initially

expressed in a ubiquitous manner (see Introduction), Nkd is considered to be active in all cells at the initial state, although it becomes quickly repressed in cell 4 by En.

To further simplify the dynamical analysis, we have taken into account generic differences in the speed of logical transitions, assigning a low priority to all transitions corresponding to *de novo* protein syntheses (cf. Materials and Methods). Fig. 2 shows the initial state used for our strip of six cells, together with the two significant stable configurations reachable from these initial conditions.

The first configuration corresponds to the wild type situation, wherein each of the cells flanking the forming segmental border expresses a specific combination of functional regulatory products. The cell located just anteriorly to the forming segmental border (cell 3) is characterised by the expression of *wg, slp, nkd* and *ci*, the presence of Ci[act], as well as of Fz and Dsh, both participating in the autocrine Wg pathway. The remaining *segment polarity* genes/products are inactive. Note that after the reception of the Hh signal by the Wg-cell, there is no active Ptc at the stable state because all the Ptc product (including that from newly transcribed *ptc*) is bound to the secreted Hh, thereby preventing Hh diffusion towards more anterior cells. In contrast, the cell located just posteriorly to the forming border (cell 4) expresses genes *en* and *hh*, secretes Hh, with both Fz (the Wg-receptor) and Dsh activated by Wg diffusing from the neighbouring anterior cell (paracrine Wg-pathway), whereas the remaining *segment polarity* genes/products are inactive. The four other cells considered (cells 1, 2, 5 and 6) reach a state devoid of En and Wg expression, even when we considered the possibility of a significant initial expression of En (in cell 2) or Wg (in cell 5).

However, from realistic initial conditions, the system can also reach another stable state, which is characterised by the loss of Wg and En expression in the whole segment. All cells are then trapped in the same stable state, with Nkd moderately expressed (at its intermediary level 1), and Ci, Ci[rep], Pka and Ptc all highly expressed.

On the basis of these results, we can now delineate the conditions required for the *segment polarity* network to reach the wild type stable state. Firstly, both autocrine *and* paracrine Wg-pathways need to be functional. This means that the maximal functional level of *wg* expression in the Wg-cell can only be attained when this cell receives auto-secreted Wg and simultaneously receives Hh secreted by the adjacent En-cell, as a consequence of paracrine Wg signalling on this cell, which requires a high Wg level (Wg = 2) (cf. the section devoted to the Analysis of genetic perturbation, in particular the analysis of *wg* partial loss-of-function). Secondly, as schematised in Fig. 2, the pair-rule inputs upon *en* and *wg* need to last long enough to enable full activation of both Wg- and Hh-signalling pathways. If the pair-rule signal fades away before the two signalling pathways are fully functional, the *segment polarity* system reaches a final state characterised by the absence of expression of *en* and *wg*, and consequently the parasegment boundary cannot be formed.

Feedback circuit analysis

The network described into Fig. 1A encompasses 20 intracellular regulatory circuits, involving from one to eight regulatory components. Using a novel algorithm to compute the corresponding functionality domains (cf. Materials and Methods and Naldi *et*
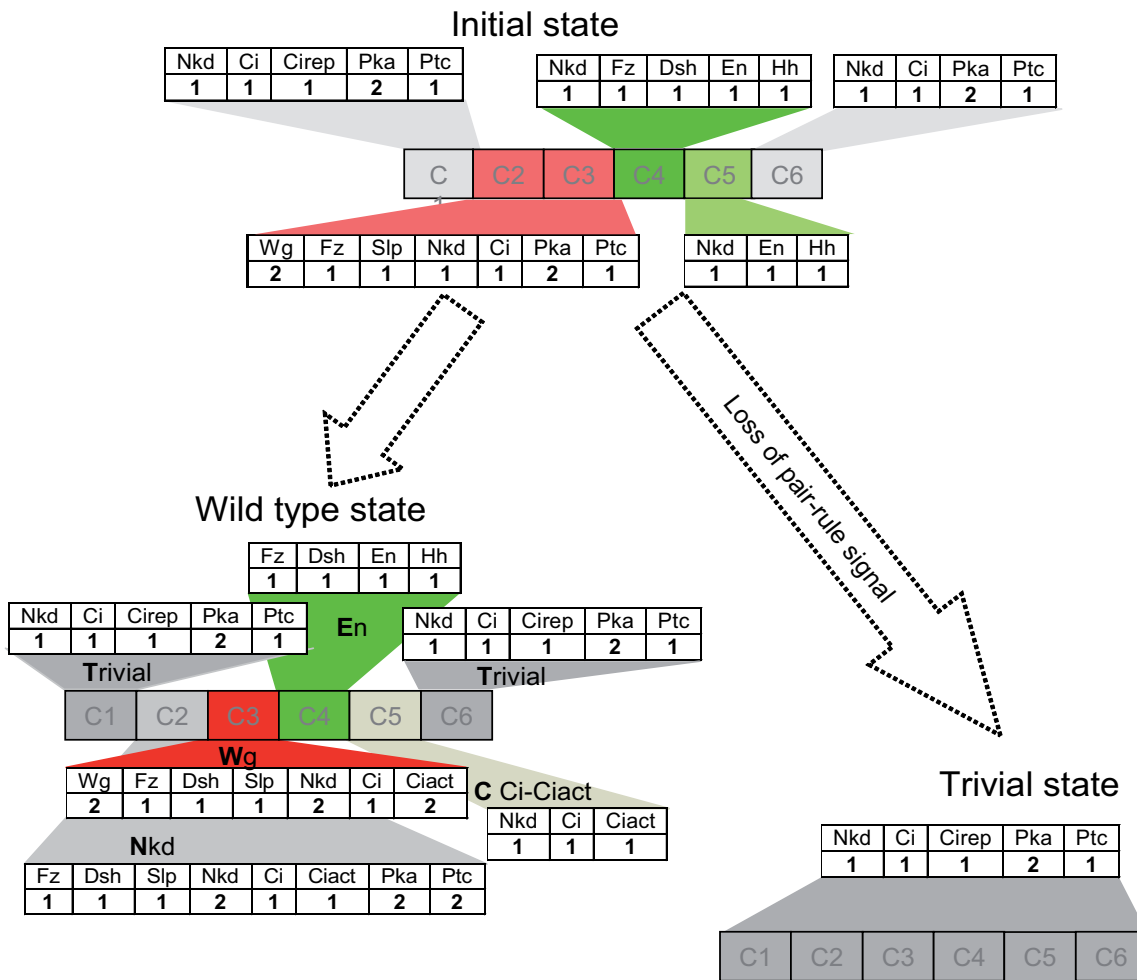
Initial state

Nkd	Ci	Cirep	Pka	Ptc
1	1	1	2	1

Nkd	Fz	Dsh	En	Hh
1	1	1	1	1

Nkd	Ci	Pka	Ptc
1	1	2	1

C	C2	C3	C4	C5	C6

Wg	Fz	Slp	Nkd	Ci	Pka	Ptc
2	1	1	1	1	2	1

Nkd	En	Hh
1	1	1

Wild type state

Fz	Dsh	En	Hh
1	1	1	1

En

Nkd	Ci	Cirep	Pka	Ptc
1	1	1	2	1

Nkd	Ci	Cirep	Pka	Ptc
1	1	1	2	1

Trivial   **T**rivial

C1	C2	C3	C4	C5	C6

Wg

Wg	Fz	Dsh	Slp	Nkd	Ci	Ciact
2	1	1	1	2	1	2

C Ci-Ciact

Nkd	Ci	Ciact
1	1	1

Nkd

Fz	Dsh	Slp	Nkd	Ci	Ciact	Pka	Ptc
1	1	1	2	1	1	2	2

Loss of pair-rule signal

Trivial state

Nkd	Ci	Cirep	Pka	Ptc
1	1	1	2	1

C1	C2	C3	C4	C5	C6

Fig. 2. Simulation of the wild-type dynamics of the segment polarity network. *C1 to C6 represent the six cells of Fig. 1B. The vectors with gene names and numbers describe the levels of activated regulatory factors in the different cells, the rest of the genes being OFF. The initial state is provided by the expression of the pair-rule module, in terms of En, Wg and Slp levels, plus ubiquitous expression of* nkd. *Fz is further activated in the three cells (C2-4) receiving Wg signal, whereas Ptc, Pka and Ci are activated in all cells devoid of En. Here, we consider fuzzy initial expression patterns for En and Wg, each spanning over two cells, a situation which nevertheless normally leads to crisper (one cell-wide) expression domains. For these initial conditions, the system can follow multiple trajectories, leading to two significant stable states: a stable state qualitatively matching the wild-type pattern, as well as a* trivial *state lacking both Wg and En across the whole segment. This trivial state is reached when the initial En and Wg pattern (set by the pair-rule module) fades before the locking of the inter-cellular circuit involving Hh and Wg signalling pathways (cf. Fig. 3).*

al., 2007), we found that only four of these circuits are functional for the parameter values selected and for specific constraints on the values of input components:

- The positive (cross-inhibitory) circuit En-Slp is functional in the presence of Dsh. This circuit ensures mutual exclusion between En and Slp expressions and enables the generation of two stable states in the presence of Wg signalling, with En ON and Slp OFF, or the opposite configuration. In absence of Dsh, both En and Slp are switched OFF.

- The positive circuit Dsh-Ci[act]-Wg-Fz (involving two different thresholds of Ci[act]) is functional in the absence of En, and consequently when the Hh-pathway is not induced, and in the presence of Slp and Ci. This circuit corresponds to the action of Wg autocrine loop.

- The positive circuit Dsh-Slp-Wg-Fz is also functional in the absence of En (in the same cell) and consequently when the Hh-pathway is not induced, and in the presence of Ci[act]. This circuit implies a role of Slp in the maintenance of Wg. Together with the preceding positive circuit (also involving Dsh, Wg and Fz, but Ci[act] instead of Slp), this circuit enables the generation of three alternative stable states when En is absent, characterised with low, moderate or high levels of Wg activity, respectively.

- Finally, the negative circuit Wg-Fz-Dsh-Nkd is functional in the absence of En (in the same cell), but functions only in the presence of Slp and of moderate levels of Ci[act] (Ci[act]=1), as well as for low or moderate Wg input (Wg ≤ 1). However, at the cellular level, these conditions are simultaneously met only in cell 2 in our six-cell model, and this cell is normally flanked by a cell (cell 3) expressing a high level of Wg. Consequently, this circuit cannot generate oscillatory gene expression in the wild-type situation. However, *wg*-repression by Nkd plays a crucial role in cell 2 by antagonising the autocrine-Wg pathway.

Once we connect neighbouring cells through Wg and Hh signalling pathways, we obtain 265 additional inter-cellular circuits (counting symmetrical circuits only once). However, only one of these circuits is functional, involving Hh, Fz, Dsh and En in one cell, and Ptc, Pka, Ci[act] and Wg in the neighbouring cell. This positive inter-cellular circuit thus involves both Wg and Hh signalling pathways (see Fig. 3). In the wild-type case, the functionality constraints (cf. supplementary material) are met only in the pair of cells flanking the forming segmental border (cells 3 and 4 in our model), leading to maintenance of Wg *versus* En

expressing states in these neighbouring cells. The role of this circuit consists thus in forcing the right combination of stable states at the right location to specify the position of the forming segmental border.

Analysis of genetic perturbations

To test the consistency and the qualitative robustness of our model, we have simulated a series of perturbations of the *segment polarity* module. Single and double loss-of-function mutations were simulated, as well as various ectopic expression experiments, and combination thereof (cf. the Materials and Methods section and supplementary material). Selected examples are presented in what follows and listed in Table 3, together with corroborating references.

In loss-of-function mutants of genes *wg*, *en*, *hh* and *ci*, a unique stable state is attained, lacking both *wg* and *en* expressions (in the case of Ci, there is neither Ci[rep]), in agreement with published experimental data (cf. references in Table 3).

In loss-of-function *ptc* mutants, the system encompasses up to 64 stable states, down to 36 after discarding the symmetrical ones. Using two priority classes and INA (cf. Materials and Methods section), only two stable states are reached. In both states, we have an anterior expansion of Wg expression, together with a posterior expansion of Ci[act]. In one of these states, we further observe an ectopic expression of En in cell 1, in agreement with published results (Ingham *et al.,* 1991).
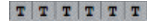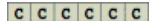
In loss-of-function *nkd* mutants, the system has up to 106 non-symmetrical stable patterns, combining four among the five stable cellular states (that characterised by Nkd expression is lacking, see Table A1 in Appendix), and a novel cellular state characterised by medium levels of Wg and Ci[Act], together with active Wg and Hh signalling components: Fz, Dsh, Slp, Ci, Pka and Ptc (cf. Table 3). This large set of stable states includes a state corresponding to the observed experimental situation. The Wg-stripe expands anteriorly, whereas the En-stripe expands posteriorly and induces *wg* in the adjacent posterior cell (Martínez-Arias *et al.,* 1988; van den Heuvel *et al.,* 1993). However, this state is difficult (if not impossible) to reach from realistic initial conditions, even using the priorities mentioned above. Tentatively, this discrepancy results from an increased diffusion of Wg, which appears to be restricted by Nkd *in vivo* (Zeng *et al.,* 2000; Moline *et al.,* 1999). To test this hypothesis, we have analysed a variant of our model allowing diffusion of Wg towards posterior cells. In this situation, the system can indeed easily reach a state corresponding to the expected pattern (cf. Table 3 and supplementary material). Notice that the complete simulation of these mutants could not be performed in a model based on four instead of six cells, since the ectopic expression of Wg that characterises these mutants occurs in cell 6, which is adjacent and posterior to the cell 5, which is ectopically expressing En.

The double loss-of-function *ptc; hh* mutant shows the same phenotype as the loss-of-function *ptc* mutant, where *wg* and *en* remain active, in contrast with the single loss-of-function *hh* situation, where *wg* and *en* are both inactivated. This matches the

TABLE 3

MOST SIGNIFICANT STABLE STATES FOR THE WILD-TYPE AND FOR KNOWN MUTANTS

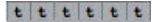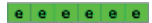| Genetic background		Main outcome(s)		Bibliographical references
Wild-type	T N W E C T / T T T T T T	**T** Trivial cell **N** Nkd cell **W** Wg cell **E** En cell **C** Ci, Ci-act cell		
Loss-of-function of *wg* Loss-of-function of *en* Loss-of-function of *hh* Loss-of-function of *ci*	T T T T T T			5, 15, 26, 27 15, ,26 9, 16 16, 25, 28, 29, 30
Loss-of-function of *ptc* (and loss-of-function of *ptc* and *hh*)	E W W E C C			9, 15, 16, 26, 31
Loss-of-function of *nkd*	T A W E E W	**A** Cell with medium levels of Wg and Ci[act], highest levels of Fz, Dsh, Slp, Ci, Pka and Ptc		15, 16
Loss-of-function of *ptc* and *wg*	C C C C C C			25, 26
Loss-of-function of *ptc* and partial loss-of-function of *wg*	C W W C C C	**W** Wg cell with a medium level of Wg		25
Ectopic expression of *en*	e e e e e e	**e** Cell expressing only En and Hh		5, 31, 32
Ectopic expression of *wg*	n n W e e e	**n** Nkd cell expressing *wg* transgene **e** En cell expressing *wg* transgene		24, 33, 34
Ectopic expression of *nkd*	t t t t t t	**t** Trivial cell with high level of Nkd		14
Ectopic expression of *wg* and loss-of-function of *slp*	e e e e e e	**e** En cell expressing *wg* transgene		34

Segment polarity expression patterns obtained for wild-type, loss-of-function or ectopic gene expression backgrounds. The first column lists selected genetic background, whereas the second column displays the significant reachable stable state(s), using a colour and letter code (see also Fig. 2 and Table A1 in the Appendix). Note that the consideration of six different cells enables the recovery of subtle phenotypes such as the combination of Wg or En domain expansion combined with ectopic En or Wg stripes (cf. the rows describing *ptc* and *nkd* loss-of-functions, respectively). (26) DiNardo *et al.,* 1988; (27) Bejsovec and Martínez-Arias, 1991; (28) Forbes *et al.,* 1993; (29) Slusarski *et al.,* 1995; (30) Hidalgo, 1991; (31) Bejsovec and Wieschaus, 1993; (32) Yoffe *et al.,* 1995; (33) Sampedro *et al.,* 1993; (34) Noordermeer *et al.,* 1995; for the rest of references see footnote to Table 1. (See Supplementary material for a complete description of simulation results).

Fig. 3. Functional feedback circuits. *The functional intra-cellular circuits are shown at the top, while the unique functional inter-cellular circuit is depicted in the bottom panel (highlighted with thicker lines).*

finding that Hh signal is dispensable in the absence of Ptc, indicating that the role of Hh is to inhibit Ptc, in order to allow the activation of the Hh-pathway (cf. Introduction).

Double loss-of-function *wg; ptc* mutants have a unique stable state lacking both *wg* and *en* expressions. The lack of Wg impedes the activation of Dsh in the En-cell, further leading to the loss of *en* expression, in agreement with experimental results (Hooper, 1994; DiNardo *et al.,* 1988). However, for a loss-of-function *ptc* mutation combined with a partial loss-of-function *wg* mutation, *wg* expression remains possible at a low level in some cells, while *en* expression is completely lost, in agreement with published results (Hooper, 1994). Indeed, as *wg* is expressed at its medium level (i.e., Wg = 1), the autocrine but not the paracrine-Wg pathway is functioning. Consequently, the expression of *en* is lost.

On the basis of our logical *segment-polarity* model, we can also easily simulate selected ectopic gene expressions. For example, when *en* is ectopically expressed, a unique stable state is obtained, lacking *wg* expression, in agreement with experimental results (Heemskerk *et al.,* 1991; Bejsovec and Wieschaus, 1993; Yoffe *et al.,* 1995). This naturally results from the repression of *wg* by En (Heemskerk *et al.,* 1991; Alexandre and Vincent, 2003).

When *wg* is ectopically expressed, the system has up to 41 non-symmetrical stable states. High Wg activity activates Dsh in all cells and thus enables the functionality of the positive Slp-En circuit. As a consequence, we obtain different patterns combining cells expressing En and cells expressing Slp (together with high levels of Nkd, Ci and Ci[act]). Using the same priority classes as above, the system primarily reaches states characterised by a posterior expansion of En stripe, conserving the wild-type anterior border, as observed experimentally (Sampedro *et al.,* 1993; Noordermeer *et al.,* 1994; Noordermeer *et al.,* 1995). Thus, in

these embryos, two cell types can be distinguished: cells expressing genes *wg* and *slp* but not *en*, on the one hand, and cells expressing genes *wg* and *en* but not *slp* on the other hand. (Notice that, here, En and Wg can be both expressed in the same cell because En cannot repress the *wg* transgene).

Although the ectopic *wg* expression does not normally induce *en* expression throughout the whole parasegment, it does so in embryos lacking *slp* function, thereby indicating that *slp* prevents the induction of En throughout the parasegment despite the ubiquitous *wg* expression (Noordermeer *et al.,* 1995). Indeed, the simulation of embryos ectopically expressing *wg* and deficient for *slp* leads to a unique cell type, with expression of both *wg* and *en*.

Discussion

In *Drosophila melanogaster*, segmentation originates from the action of maternal organisers upon the zygotic segmentation gene system. This hierarchical system is composed of three cross-regulatory modules (sub-systems): the *gap,* the *pair-rule* and the *segment polarity* modules. Whereas the interactions among the *gap* and among the *pair-rule* genes consist essentially in intra-cellular transcriptional regulations, the interactions among the *segment polarity* genes occur at the intra- and inter-cellular levels. This manuscript proposes a consistent formal qualitative model of the role of the *segment polarity* cross-regulatory module in the segmentation process in *Drosophila* embryo. Specifically, it accounts for the consolidation of the *en* and *wg* expression pattern, initially set under the control of the *pair-rule* module, through specific inter-cellular interactions and signal transduction pathways.

In the introduction, three other *segment polarity* model analyses have been mentioned (von Dassow *et al.,* 2000; von Dassow and Odell, 2002; Albert and Othmer, 2003; Ingolia, 2004). Each of these analyses aims at integrating diverse genetic and molecular data and at delineating the most crucial components and constraints involved in the formation of correct segmental borders. All these models (including ours) assume that the dynamics of the *segment polarity* gene network is largely defined by its topology (i.e. the interactions between the genes, the sign of each of these interactions and their different weights), rather than by fine-tuning of kinetic parameters. To some extend, this idea was already present in the model proposed by Meinhardt and Gierer (1980), well before the identification of the segmentation genes and the molecular nature of their interactions.

The delineation of the definite topology of the segment-polarity network from the analysis of published experimental data is nevertheless far from trivial. Indeed, the different studies mentioned above significantly differ with respect to the regulatory interactions considered (cf. Introduction), and consequently lead to different conclusions.

On the basis of their ODE model analysis, von Dassow *et al.* (2000) concluded that the segment polarity system is robust and

that only loose constraints on the initial conditions have to be met to obtain the wild-type biological pattern. This pattern can even be attained when the initial conditions do not include an initial pulse of En and Wg. Ingolia's model proposes that the robustness of the *segment polarity* network is due specifically to the positive feedback of gene products on their own expression, and in the cross-inhibitory circuit between *slp* and *en* which plays an important role in the establishment of the final expression pattern of *en* and *wg*. On the basis of their Boolean model analysis, Albert and Othmer (2003) found that the deletion of *ci* does not affect the pattern of the genes *en, wg* and *hh*. To explain this result, Albert and Othmer proposed that *slp* maintains the activation of *wg*, which in turn maintains the expression of *en* and *hh*. However, it has been shown that *slp* cannot be continuously expressed in the absence of *wg* function (Cadigan *et al.,* 1994). Furthermore, the maintenance of *wg* expression in the Wg-cell requires the Hh input from the adjacent En -cell and the Hh-signalling pathway in the Wg-cell, which passes necessarily through the formation of Ci[act] (Méthot and Basler, 2001). Albert and Othmer further concluded that *slp* plays a crucial role in the delineation of the *segment polarity* expression pattern, through its control on *wg*. Finally, they proposed that a proper expression of *wg* and *ptc* suffices to initiate a proper wild-type *segment polarity* expression pattern.

The revised model for the *segment polarity* module presented here (regulatory graph of Fig. 1A, together with the logical parameter values of Table 2) has been iterated and properly interconnected through Wg and Hh signallings to model a row of six cells (or regions, as each of them might encompass more than just one cell), thus covering a whole segment of the embryo (Fig. 1B).

This model accounts for the mutual activation of *en* and *wg* in two juxtaposed cells, flanking the forming segmental borders. En-cell and Wg-cell are characterised by a particular combination of active *segment polarity* genes (or gene products), corresponding to the autocrine and paracrine Wg-signalling pathways (operative upon the Wg-cell and the En-cell, respectively) together with the Hh-signalling pathway (operative in the Wg-cell). Acting upon the En-cell, the paracrine Wg-pathway induces the formation and secretion of Hh, ultimately leading to the activation of the Hh-signalling pathway in the adjacent Wg-cell, thereby forming an inter-cellular positive circuit.

Although most *segment polarity* factors could be considered as binary devices, we were led to distinguish two functional levels for Wg, Nkd, Ci[act], Pka, and Ptc. We further assumed that the autocrine Wg-signalling pathway requires lower Wg levels than the paracrine pathway. This allowed us to uncover subtle behaviours such as the maintenance of *wg*, in the Wg-cell, without *en* induction in the adjacent cells, in loss-of function *ptc* and partial loss-of-function *wg* mutants. Fully functional Wg (autocrine and paracrine) and Hh pathways are needed to reproduce all experimental observations (in particular the effect of' *wg* partial loss-function). More precisely, high levels of *wg* expression require high levels of Ci[act], which in turn require the combination of autocrine and paracrine Wg-signalling, together with Hh-signalling pathways.

Our model analysis implies that the consolidation of *en* and *wg* expression patterns requires the autocrine function of *wg*, *i.e.*, the direct action of the secreted Wg product upon the cell that secretes it. Could Hh, secreted from the En-cell, also perform an autocrine function? It has been reported that Hh can be internalised

into posterior compartment cells (Callejo *et al.,* 2006), in spite of the fact that these lack Ptc receptor because the gene *en* expressed in these cells represses *ptc* expression (Ingham *et al.,* 1991). How such a process takes place and whether such internalisation of Hh performs any biological function remain open questions. If it has a function, it must be implemented through a different Hh pathway because the cells expressing *en* and secreting Hh lack Ci, the effector of the canonical pathway studied here (Dominguez *et al.,* 1996; Méthot and Basler, 1999; Alexandre and Vincent, 2003; Eaton and Kornberg, 1990)

The analysis of the wild-type model behaviour suggests that the pair-rule pattern is instrumental not only in determining the activation of genes *engrailed* and *wingless* in rows of adjacent cells, but also for the consolidation of their expressions. This explains the observed temporal overlapping of *pair-rule* and *segment polarity* gene expressions. In this respect, according to our analysis, Slp and Nkd play a crucial role in the delineation of *engrailed* and *wingless* expression domains beyond their specification by pair-rules factors (such as Eve and Ftz), to enable the full activation of Wg and Hh signalling pathways. We further assumed that the highest level of Ci[act] overcomes *wg* repression by Nkd, thereby explaining why, in the wild-type, Nkd represses *wg* only in cell 2 (and not in cell 3, where Nkd is also initially expressed) (Zeng *et al.,* 2000). Furthermore, we considered that En, either directly or indirectly, represses *nkd*. Thus, we obtain similar cross-inhibitory relationships between *en* and *nkd* and between *en* and *slp*. Consequently, *en* can be expressed in the presence of Nkd, provided the absence of Slp. These assumptions have been introduced to account for the initial expression of *nkd* in cell 4, where *en* is also expressed, ultimately leading to *nkd* repression (Zeng *et al.,* 2000). When we selected parameters allowing the diffusion of Wg towards the posterior cells, the model predicted a state corresponding to the expected pattern that has been observed in the loss-of-function *nkd* mutants. This suggests a series of investigations designed to see if Nkd retards Wg diffusion.

Finally, our model analysis emphasises the dual role played by Protein kinase A through the phosphorylation of Cubitus interruptus, the effector molecule of the Hedgehog signalling pathway.

The consistency of our model with available experimental data has been evaluated through systematic simulations of loss-of-functions mutations or ectopic expressions of *segment-polarity* genes, which all led to results in qualitative agreement with published data. In particular, we could recover the peculiar behaviour of loss of function *ptc* mutants, with an anterior ectopic expression of *en* associated with an anterior expansion of the *wg* expression domain. Similarly, we could successfully simulate the striking phenotype of loss-of-function *nkd* mutants, where the posterior expansion of En-stripe induces *wg* in the adjacent posterior cell. In this respect, as we had to allow an increased diffusion of Wg to reach this result, our analysis supports a possible role of Nkd in the restriction of Wg diffusion (Zeng *et al.,* 2000; Moline *et al.,* 1999).

Prospects

Primarily based on genetic data, the *segment polarity* model presented here aims at better understanding how the expression of *en* and *wg* in juxtaposed cells, once activated by the *pair-rule*

network, becomes consolidated through inter- and intra-cellular interactions between *the segment polarity* genes. This corresponds to the last step of segmentation in *Drosophila* embryos, *i.e.* the establishment of the (para)segmental boundaries, which occur at the juxtaposition of cells expressing *en* and cells expressing *wg*. Certainly, we have overlooked the roles of additional genes here, as we have focused on representative genes of the Hh- and Wg-signalling pathways, which implement the interactions between the En *versus* Wg expressing cells. Furthermore, some of the considered genes are also involved in other biological processes, such as, for example, the internal patterning and cell specification of the segments, once these became formed, or yet the growth and organisation of adult appendages, such as the wing and the leg. We believe that the core of our model can be used to study different differentiation involving the canonical Hh- and Wg-signalling pathways. Notwithstanding, we are aware that specific Hh- and/or Wg-target genes should be added to develop more realistic biological analyses of these processes.

The model presented here can also be applied to the development of the wing and leg discs, with the addition of another gene, *decapentaplegic (dpp)*, whose function is under the control of the Hh signalling pathway. For example, the wing disc is composed of two distinct populations of cells, which form the anterior and the posterior compartments. Posterior compartment cells express continuously *en* and secrete Hh, which induces *dpp* expression in the anterior compartment cells adjacent to the anterior-posterior (AP) compartment border. This activation involves the production of Ci[act]. However, in the anterior compartment cells away from the AP boundary, the default form of Ci, Ci[rep], represses *dpp* (reviewed in Ingham, 1998; McMahon, 2000; Ingham and McMahon, 2001). Similarly to the situation of the formation of parasegment boundaries, simulation of loss-of-function and dominant-negative mutations in gene *ci* can be simulated through the selection of appropriate values for the variables corresponding to Ci, Ci[act], Ci[rep] and Dpp.

Materials and Methods

Logical modelling of genetic networks

In the context of this qualitative approach, genetic regulatory interactions are represented in terms of a directed graph (Chaouiya *et al.,* 2003). In a *logical regulatory graph*, each node (gene or regulatory product) is assigned a *maximal level,* which defines the highest qualitative functional level taken by the regulatory node (this maximal level equals 1 in the simplest, *Boolean* case). Whenever distinct functional concentrations of the same regulatory product need to be considered, multi-level variables are used. Each arc embodies a regulatory interaction and is assigned a threshold. This threshold defines the smallest functional level of the interaction source for which the interaction is operative. *Logical parameters* qualitatively describe the effects of each interaction or combination of interactions controlling the functional state of a given element of the network. Thus, for a node, there are as many parameters as the number of possible combinations of interactions acting on this node. Per default, these parameters are set to zero. Defining non zero parameters thus amounts to single out the combinations of interactions which enable the activation of the different regulatory components and the qualitative recovery of all documented wild type and mutant gene expression patterns.

A state of the network is represented by a vector, which encompasses the current (discrete) level of each node. Given a state, one can determine which interactions are operative on each gene. Using the values of the

logical parameters, one can then identify the nodes called to change their levels. In general, for a given state, we consider all possible elementary transitions (*i.e.* switching of only one regulatory component, to a neighbouring integer level), thus leading to as many outgoing arrows as updating calls (asynchronous updating). Depending on the structure of the regulatory graph and on the values of the logical parameters, the network encompasses a large but finite number of dynamical pathways.

Computational tools for the analysis of logical model properties

The *GINsim* software supports the definition of logical regulatory graphs, from the network structure to the logical parameters, through a user-friendly interface (Gonzalez *et al.,* 2006). It further allows the simulation and the analysis of logical models. A simulation is performed on the basis of selected initial state(s) and results in a *state transition graph,* which represents all possible trajectories from the specified initial conditions. *GINsim* further allows the identification of stable states, as well as the computation of a shortest path between two given states. *GINsim* can be freely downloaded from a dedicated web site, which also provides extensive documentation and a model repository (http://gin.univ-mrs.fr/GINsim).

When facing large regulatory networks (encompassing dozens of components), it becomes impossible to perform exhaustive simulations. As we are particularly interested in the delineation of differentiated expression states, usually represented by stable states, we have used the following strategy.

First, we have developed an efficient algorithm to determine all the stable states of a logical model, without simulating the system. Our approach leans on a compact computational structure (Ordered Multivalued Decision Diagrams) for the representation of the logical functions associated to the regulatory components (Naldi *et al.,* 2007).

Next, it is necessary to verify which of the identified stable states can be actually reached from proper initial conditions. In the discrete modelling context, this problem has already been extensively addressed by computer scientists, leading to the development of various computational tools. Here, we have leaned on *INA* (for *Integrated Net Analyzer*), a software package supporting the analysis of discrete *Petri nets* (http://www2.informatik.hu-berlin.de/~starke/ina.html). Petri nets constitute a convenient mathematical and graphical framework to represent discrete models with concurrency (for an introduction to Petri nets, see Murata, 1989). Logical models can easily be translated into standard Petri nets (Chaouiya *et al.,* 2006), and a specific module has been added to *GINsim* (version in development) to automatically export logical models into INA storage format.

Given a Petri net and an initial state, *INA* builds the state transition graph and determines the stable states (terminal nodes). Unfortunately, for large regulatory systems, the size of the state transition graph can be huge and thus impossible to build entirely. However, in practice, it is sufficient to determine the closest stable states, or the stable states lying within some distance interval, which can be easily defined with *INA*. We have further used an option allowing a reduction of the state transition graph by ignoring the paths, which lead to the same states (*stubborn* reduction).

When asynchronous updating is used for really complex cases, the definition of simple *transition priority classes* becomes valuable (Fauré *et al.,* 2006). Indeed, the consideration of priorities amounts to discard relatively slow transitions, which are associated with a lower priority. In the present case, two priority classes have been defined. The high priority class includes all transitions corresponding to protein modification events (receptor in/activation, proteolysis, de/phosphorylation). This class includes Ci[act], Ci[rep], Fz, Dsh and Pka. The low priority class encompasses all *de novo* gene expression processes. This class comprises Ci, Wg, Nkd, En, Slp, Hh and Ptc.

The logical framework also provides original tools to delineate the dynamical roles of specific *regulatory circuits* embedded within complex regulatory networks (for an introduction to feedback circuits, and their

roles see Thomas *et al.,* 1995; Thieffry, 2007). In the discrete (graph-theoretic) framework, a regulatory circuit can be defined as a simple circular chain of oriented interactions. When interaction signs are fixed (positive for activations, negative for inhibitions), the sign of a circuit amounts to the product of the signs of its constitutive interactions. This leads to the definition of two classes of circuits, *positive* versus *negative circuits*, which can generate strikingly different biological and dynamical properties: multiple stable states (or, more generally, multiple attractors) and differentiation *versus* homeostasis and sustained oscillatory behaviour. However, the sole presence of a circuit in a regulatory graph does not suffice to ensure such properties. When a circuit does generate the associated behaviour, we say that it is *functional*. In the logical framework, the functionality of a circuit depends on the values of the logical parameters associated to each regulatory component involved in the circuit, and thus on the values of the external inputs controlling these elements. We have recently implemented a novel algorithm into *GINsim* to efficiently compute circuit functionality domains (in terms of the values of the input components) (Naldi *et al.,* 2007). Applied to our model of the segment polarity network, this approach has led to the identification of a very limited number of specific intra- and inter-cellular circuits, each playing a crucial role in the dynamical behaviour of the system. These results have been further validated through the simulation of genetic perturbations specifically affecting each of these circuits.

The simulation of genetic perturbations is straightforward in the logical framework. A *loss-of-function mutation* at a given gene implies that this gene produces a non-functional product (or no product at all), which amounts to assign the value zero to the corresponding logical variable (regulatory product concentration) and parameters. In contrast, the *ectopic expression* of a gene implies that this gene is expressed in an unregulated manner beyond its normal spatial-temporal expression domain. This can be accomplished by forcing the corresponding element to take higher values (for a more detailed description of this formal treatment of mutations see Thieffry and Sánchez, 2002). In this respect, *GINsim* provides an intuitive interface to trap single or multiple components into selected intervals of discrete values, in combination with the specification of proper initial conditions.

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Appendix

Simulations and stable state analyses

Considering the different situations (wild-type, and genetic perturbations), we have answered two questions. First, what are the potential stable states, independently of any initial condition? Second, given an initial state (compatible with the wild-type initial state shown in Fig. 2), what are the reachable stable states ?

Wild-type stable states for the isolated cell submitted to input Hh and Wg signals

A preliminary analysis of the model amounts to consider a single isolated cell submitted to two input signals (Wg and Hh).

In the case of the wild-type situation, Table A.1 lists all possible stable states depending on external inputs.

Wild-type analysis

We consider the complete model of 6 connected cells (Fig. 1). Note that with the 5 possible stable states of the above table for the isolated cells, there are 15625 possible combinations (5^6) for the 6 cells interconnected, that is 15625 potential stable states. The complete State Transition Graph (STG), without specifying any initial condition, contains a huge number of states: $(3^5.2^7)^6 > 10^{26}$ (this is the number of all possible states of the system).

But the inter-cellular interactions severely restrict the number of possible stable states, as there are exactly 65 stable states (among which 29 are symmetrical, *eg* TTTCEW and WECTTT are symmetrical). Hence the model gives rise to 36 different patterns, in the wild type case.

It was not possible to construct the whole STG. But, we have verified that the two following states are reachable, the wild type state (1rst one below) and the trivial state.

T	N	W	E	C	T
T	T	T	T	T	T

Single loss-of-function wg, en, hh and ci mutants

From initial conditions similar to those taken in the wild type case, but compatible with the considered loss-of-function, we have obtained the following unique stable state in the four situations:

T	T	T	T	T	T

In the four cases, this state is unique and reachable. In the case of the loss-of-function *ci* mutant, the stable state is rather similar to the trivial state, with Ci=0.

Loss-of-function ptc *mutant*

There are exactly 64 stable states (among which 28 are symmetrical). Hence the model gives rise to 36 different patterns. The reachability analysis showed a number of reachable stable states, but using the two priority classes (as described in the text), we were able to prove that only the two following states are reachable.

W	W	W	E	C	C
E	W	W	E	C	C

Loss-of-function nkd *mutant*

In this mutant situation, there are exactly 196 stable states (72 are symmetrical), hence 124 possible patterns. We reproduce in the table hereafter the 5 cellular states (a new one appears with a medium level of Wg, it has been called A):

Wg	Fz	Dsh	Slp	Nkd	En	Hh	Ci	Ciact	Cirep	Pka	Ptc	
0	0	0	0	1	0	0	1	0	1	2	1	T (trivial)
0	0	0	0	1	0	0	1	1	0	0	0	C (CiCiact)
2	1	1	1	2	0	0	1	2	0	0	0	W (Wg)
0	1	1	0	0	1	1	0	0	0	0	0	E (En)
1	1	1	1	0	0	0	1	1	0	2	2	A (Ciact)

We have not been able to observe the reachability of the expected stable state, generating a large STG, limiting the depth of the exploration, or yet considering priority classes. The stable states reached were not compatible with the experimental observations.

Consequently, we have modified the wiring, allowing Wg to

TABLE A1

STABLE STATES FOR AN ISOLATED CELL SUBMITTED TO FIXED WG AND HH INPUTS

External inputs		Wg	Fz	Dsh	Slp	Nkd	En	Hh	Ci	Ciact	Cirep	Pka	Ptc	Letter code
Wg	Hh													
0	0	0	0	0	0	1	0	0	1	0	1	2	1	T (trivial)
0	1	0	0	0	0	1	0	0	1	1	0	0	0	C (CiCiact)
0	1	2	1	1	1	2	0	0	1	2	0	0	0	W (Wg)
1	0	0	1	1	0	0	1	1	0	0	0	0	0	E (En)
1	0	0	1	1	1	2	0	0	1	1	0	2	2	N (Nkd)
1	1	2	1	1	1	2	0	0	1	2	0	0	0	W
1	1	0	1	1	0	0	1	1	0	0	0	0	0	E

The first two columns give the input values (4 possible combinations of external signals Wg and Hh), the 12 following columns give the level of each component in the cell. The last column gives the name of the cellular state, together with a letter code. Finally, a color has been associated to each cellular state.

diffuse towards more posterior cells. This new wiring leads to a slight restriction of the number of possible stable states (132 rather than 196 previously). Then, we were able to verify the presence in the reachability graph of the following expected stable pattern:

T	A	W	E	E	W

Double loss-of-function ptc, hh *mutant*

There are exactly 64 stable states (among which 28 are symmetrical). Hence the model gives rise to 36 different patterns which are the same as for the single loss-of-function *ptc* mutant (but the En cell lacks Hh). Moreover, we got the same reachability result, with the same 2 stable patterns.

Double loss-of-function ptc, wg *mutant*

We found a unique stable state, which is reachable from initial conditions similar to those of the wild type situations (with Ptc and Wg to zero):

C	C	C	C	C	C

Loss-of-function ptc *and partial loss-of-function* wg *mutant*

There are exactly 64 stable states (among which 28 are symmetrical). Hence the model gives rise to 36 different patterns. A partial construction of the STG showed the reachability of the following patterns (the Wg cells have a medium level of Wg):

C	w	w	C	C	C
C	w	C	C	C	C
C	C	w	C	C	C
C	C	C	C	C	C

Ectopic en *expression*

One single stable pattern with the six cells in the same state where only En and Hh are expressed. This state is reachable from initial conditions similar to those of the wild type situation (with En=1 for all cells).

e	e	e	e	e	e

Ectopic wg *expression*

There are exactly 64 stable states (among which 28 are symmetrical). Hence the model gives rise to 36 different patterns. The simulation, using the 2 priority classes and considering the same initial condition as for the wild type case (but with Wg=2 for all cells), the partial STG shows the following 4 reachable stable patterns (note that En and Nkd cells have an ectopic expression of Wg):

n	n	W	e	e	W
n	n	W	e	e	e
e	W	W	e	e	W
e	W	W	e	e	e

Ectopic wg *expression and loss-of-function* slp *mutant*

There exists a unique stable state, which is reached from the initial conditions:

e	e	e	e	e	e

Ectopic nkd *expression*

There are 65 stable patterns (29 are symmetrical), hence 36 patterns are possible.

Among these, the trivial stable state is shown to be reachable (partial construction of the STG, limitation of the depth to 50, meaning that the trivial state is closer from the initial state than other possible pattern):

t	t	t	t	t	t

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