The *Drosophila* gap junction channel gene *innexin 2* controls foregut development in response to Wingless signalling

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Summary

In invertebrates, the direct communication of neighbouring cells is mediated by gap junctions, which are composed of oligomers of the innexin family of transmembrane proteins. Studies of the few known *innexin* mutants in *Drosophila* and *C. elegans* have shown that innexin proteins, which are structurally analogous to the connexins in vertebrates, play a major structural role as gap junctional core components in electric signal transmission. We show that *Drosophila innexin 2* mutants display a feeding defect that originates from a failure of epithelial cells to migrate and invaginate during proventriculus organogenesis. The proventriculus is a valve-like organ that regulates food passage from the foregut into the midgut. Immunhistological studies indicate that *innexin 2* is functionally required to establish a primordial structure of the proventriculus, the keyhole, during the regionalisation of the embryonic foregut tube, which is under the control of Wingless and Hedgehog signalling. Our genetic lack- and gain-of-function studies, and experiments in *Drosophila* tissue culture cells provide strong evidence that *innexin 2* is a target gene of Wingless signalling in the proventricular cells. This is the first evidence, to our knowledge, that an invertebrate gap junction gene controls epithelial tissue and organ morphogenesis in response to the conserved WNT signalling cascade.

Key words: Gap junctions, Wingless signalling, *innexin 2*, Proventriculus, *Drosophila*

Introduction

Cell-to-cell communication is essential for the generation of the diverse tissues and organs of the body in multicellular organisms (Gurdon, 1992). One class of molecules important for such interactions are secreted signalling molecules that are involved in intercellular signalling processes. Several conserved families of secreted growth and differentiation factors have been identified and it has been shown that assorted combinations of these factors control cell differentiation and morphogenesis in a variety of organisms (for reviews, see Massague, 2000; Taipale and Beachy, 2001; Wodarz and Nusse, 1998; Grapin-Botton and Melton, 2000). The *Drosophila* gene *hedgehog*, which encodes a secreted protein, and the genes *wingless* and *decapentaplegic*, which encode members of the WNT and TGFβ families of growth factors, respectively, have been studied extensively during early embryogenesis; limb and eye patterning, and gut development (Affolter and Mann, 2001; Bienz, 1997; Dahmann and Basler, 1999; Lawrence and Struhl, 1996; Stringini and Cohen, 1999). In the ectodermal fore- and hindgut of *Drosophila*, Wingless, Decapentaplegic and Hedgehog have been shown to coordinate morphogenesis in signalling centres that become established at the junctions of the ectodermal and endodermal tissue layers (Hoch and Pankratz, 1996; Pankratz and Hoch, 1995). At the junction of the foregut and midgut, they direct the formation of the proventriculus, a gut-associated organ that mediates food passage in the larva (Campos-Ortega and Hartenstein, 1997; King, 1988; Snodgrass, 1935; Strasburger, 1992; Skaer, 1993). It has remained largely elusive, however, how the signals are transformed into local activation of target genes that induce the morphogenetic movements.

Another class of molecules that is involved in the direct communication between neighbouring cells are transmembrane channel proteins that assemble into gap junctions (for reviews, see Kumar and Gilula, 1996; Goodenough et al., 1996). Gap junctions are composed of two hexameric hemichannel subunits that are transported to the plasma membrane, following the secretory pathway for transmembrane proteins (for reviews, see Falk and Gilula, 1994; Zhang et al., 1996; Yeager et al., 1998). If hemichannels of adjacent cells interact, they form a functional dodecameric gap junction channel directly linking cytoplasm of neighbouring cells (Unger et al., 1997). This allows cells to exchange ions and small molecules that can participate in signalling events. Gap junctions are clusters of intercellular channels and may consist of tens or thousands of channels forming so-called plaques in the membranes of cells. It is believed that the permeability of the intercellular channels for ions and small molecules depends on their molecular composition and on the size and charge of the permeant molecules (for reviews, see Kumar and Gilula, 1996; Goodenough et al., 1996). How plaque formation and localisation is controlled during developmental processes is largely unknown. In vertebrates, gap junction channel proteins

are coded by the connexin multigene family (Willecke, 1991). Many tissues express several different connexin isoforms with complex and overlapping temporal and spatial profiles. It has been shown that heteromeric channels can occur in vivo and that heterotypic ones can be formed in vitro, suggesting that the normal physiological functioning of cells may require expression of multiple connexins (for a review, see Yeager et al., 1998). In invertebrates, a separate gene family encoding gapjunction channel proteins has been identified, the *innexin* multigene family (Phelan et al., 1998a) (for review, see Phelan and Starich, 2001). Innexins are structurally and functionally analogous to connexins; however, they share no sequence homology (for a review, see Phelan and Starich, 2001). Electrophysiological evidence obtained in paired *Xenopus* oocytes suggests that innexins, like connexins, form heteromeric channels (Landesman et al., 1999; Phelan et al., 1998b; Stebbings et al., 2000). The *innexin* mutants that have been identified in *C. elegans* and *Drosophila* display defects in electrical signal transmission. Mutations in the *C. elegans unc-7* and *unc-9* genes cause uncoordinated phenotypes (Starich et al., 1996; Barnes and Hekimi, 1997), and *eat-5* mutants display an impairment of electrical coupling between pharynx muscles (Starich et al., 1996). In *Drosophila*, *shaking-B* mutants are characterised by a loss of electrical signalling at synapses of the giant fibre system (Phelan et al., 1996), and *ogre* mutants display a reduced optic lobe primordium (Watanabe et al., 1990). Most of the six other known *Drosophila innexin* genes (Adams et al., 2000) are expressed in a complex and overlapping temporal and spatial profile, with several members showing high levels of expression in the developing embryonic gut (Curtin et al., 1999; Stebbings et al., 2000).

In a screen of P-element insertion lines to identify novel regulators of gut development, we isolated four P alleles of a locus that we named *kropf*. The mutant phenotype of the different P alleles is highly similar. Most of the mutant animals are embryonic lethal; however, a few mutants reach the first instar larval stage and display a feeding defect. Molecular analysis has revealed that the *kropf* locus corresponds to the *innexin 2* transcription unit and that at least two P alleles are *innexin 2* transcript null alleles. Expression studies indicate that *innexin 2* is expressed maternally and zygotically. We now report that the feeding defect of *kropf* mutant larvae is due to a malformation of the proventriculus, an organ that controls food passage at the foregut/midgut boundary of the larval gastrointestinal tract. It originates during embryogenesis by the migration and subsequent folding of ectodermal and endodermal tissue layers. In *kropf* mutants, the formation of the keyhole, a primordial structure that was shown to be generated by the Wingless- and Hedgehog-dependent outfolding, and subsequent migration of proventriculus primordial cells, does not occur properly. Genetic lack- and gain-of-function studies and experiments in *Drosophila* tissue culture cells demonstrate that *innexin 2* transcription is induced by Wingless signalling in the foregut.

Materials and Methods

Stocks

Embryo hosts for injection were w^{118}. We used Oregon-R as wild-type flies. wg^{CX4}, dpp^{S4}, hh^{IJ35} and twi-Gal4 were obtained from the Bloomington stock centre. The 14-3fkh-Gal4 driver, which mediates Gal4 expression in the proventriculus and other regions of the gut, has been described previously (Fuß and Hoch, 1998). UAS-*wingless* and UAS-*decapentaplegic* were gifts from S. M. Cohen (EMBL, Heidelberg). We refer to the P lines *l(1)G0016*, *l(1)G0034*, *l(1)G0036* and *l(1)G0188* that were isolated by screening the Göttingen X-chromosome collection of P lines (Peter et al., 2002), as $kropf^{P16}$, $kropf^{P34}$, $kropf^{P36}$ and $kropf^{P188}$ alleles, respectively. We refer to the hemizygous *kropf* mutant male embryos in this manuscript as '*kropf* mutants'. Two-thirds of these mutants are embryonic lethal and one-third shows a lethality in the first instar larval stage (feeding defect). All four alleles showed similar mutant phenotypes in the embryo and all displayed a larval feeding defect. Using in situ hybridisation analysis, we determined that $kropf^{P16}$ and $kropf^{P36}$ were *innexin 2* transcript null alleles (Fig. 1). Expression studies (Fig. 4) are consistent with a maternal *innexin* 2 complement, which probably accounts for the variable phenotype of the mutants. To ascertain whether lethality was caused by the P-lacW insertion, the P element was remobilised to induce precise excision. The stocks were tested for loss of P-lacW insertion by sequencing of the genomic *innexin* 2 locus and for survival of hemizygous males. The embryonic defects and the larval feeding phenotype of the *kropf* mutants could be rescued by inducing low level of ubiquitous *innexin 2* expression in the background of homozygous $kropf^{P16}$ mutants using UAS-*innexin 2* (R. B. and M. H., unpublished) and *daughterless*-Gal4 or *armadillo*-Gal 4 driver lines. For mutant analysis, we used $kropf^{P16}$, if not stated otherwise.

Molecular characterisation of the kropf P alleles

Genomic DNA flanking the P-element insertions was recovered by plasmid rescue and inverse PCR. The precise location of the P-element insertion was determined by sequencing the rescued DNA and the amplified fragments. The P-element insertion sites are $kropf^{P16}$ +236, $kropf^{P34}$ –11, $kropf^{P36}$ +197 and $kropf^{P188}$ +188, with respect to the transcription start site at position +1 of the *innexin 2* gene (Stebbings et al., 2000).

Feeding assay

To monitor larval feeding behaviour, the animals were allowed to grow on grape agar plates and we fed them yeast that was dyed with Carmine Red (Sigma) (Pankratz and Hoch, 1995). Phenotypes were scored at various times under the dissecting microscope.

Antibody staining

The following antibodies were used: anti-Wingless (1:20 gift of S. Cohen), anti-Dve (1:1000; gift of F. Matsuzaki), anti-Fkh (1:100, gift of H. Jäckle) and mouse anti-β-Gal (1:1000; Developmental Studies Hybridoma Bank). For immunostaining, embryos were collected and processed following standard procedures (Fuß et al., 2001), incubated overnight at 4°C in primary antibody mix containing preabsorbed antibody. Biotinylated secondary antibodies, Vectastain and DAB staining reagents were used according to manufacturer's recommendations (Vector Laboratories). For fluorescent microscopy, secondary antibodies Alexa $Fluor^{488}$ and Alexa $Fluor^{546}$ from MoBiTec were used at dilutions of 1:200 and 1:400, respectively.

In situ hybridisation

For in situ hybridisation, digoxigenin- or fluorescein-labelled RNA antisense probes were generated by in vitro transcription of a C-terminal and full-length *innexin 2* fragment from the *innexin 2* cDNA clone LD clone 11658 (Berkeley Genome Project), according to the manufacturer's instructions (Roche, Mannheim). Fluorescent detection of *innexin 2* transcripts, co-immunostained with anti-Discs lost antibody, which marks the apical sides of epithelial cells (Bhat et

al., 1999), was performed as described previously (Hughes et al., 1996; Hughes and Krause, 1999). Rabbit anti-Discs lost and mouse anti-Fluorescein antibodies were used at dilutions of 1:750 and 1:2000, respectively. As secondary antibodies, we used Cy2-conjugated anti rabbit antibody (1: 200; Dianova) and Cy3-conjugated anti mouse antibody (1:400; Dianova). Images were obtained using a Leica DM IRB laser-scanning confocal microscope. Each fluorochrome was scanned individually to avoid crosstalk between channels. Images were subsequently combined using Adobe Photoshop 5.1.

Isolation and reverse transcription (RT)-PCR analysis in *Drosophila* S2 cells

Total RNA from *Drosophila* Schneider cells that were either transfected with the pIB expression vector alone or with pIBArm, which contains the Armadillo cDNA, was isolated using the Qiagen RNeasy Mini Kit, according the manufacturer's instructions (Qiagen). For RT-PCR analysis, 2 μg of DNase I-treated total RNA was reverse transcribed by using 25 U of avian murine virus reverse transcriptase (Roche, Mannheim) with an *innexin 2*-specific antisense primer. The reaction mixture contained total RNA, 5×reverse transcriptase buffer provided by the manufacturer, 35 U of RNase inhibitor (Pharmacia Biotech) and 0.2 mM of deoxynucleoside triphosphates (dNTP) in a final volume of 30 μl. The mixture was heated to 65°C for 5 minutes. AMV reverse transcriptase was added, and the mixture was incubated at 42°C for 60 minutes. After the completion of the first-strand cDNA synthesis, one-third of the reaction volume was amplified directly by using 5 U of Taq polymerase and 5 mM of *innexin 2*-specific antisense (5′-TTAGGCGTCGAAGGGCC-GCTT-3′) and sense primers (5′-CTGAGC-ATCATGTCGGGAATATCGC-3′). PCR was performed in a final volume of 50 μl by using the Roche 'High Fidelity PCR-kit'. The predicted PCR product was 273 bp. The predicted size for the actin amplification product (5′-AGCCAGCAGTCGTCTAAT-CCAG-3′ and 5′-CAGCAACTTTCTTCG-TCACACAT-3′), which was used as an internal control, is 210 bp.

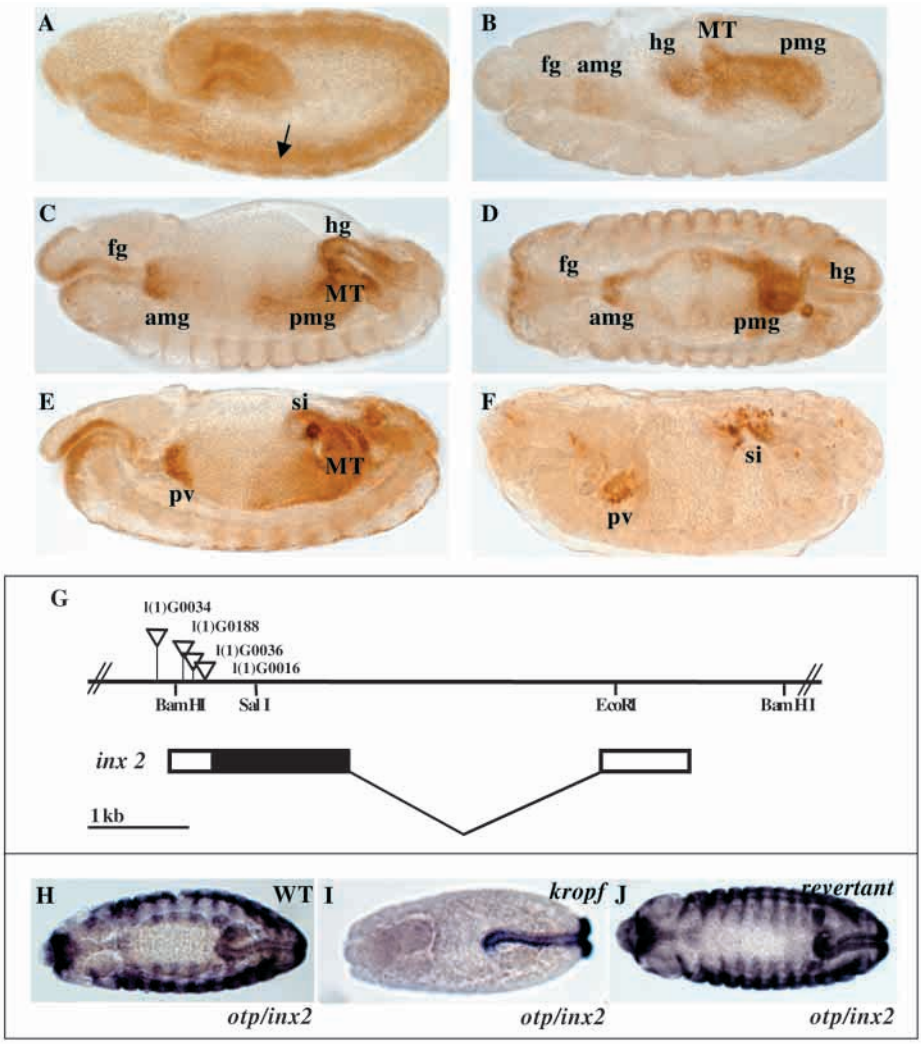

Fig. 1. Reporter gene expression pattern of the *kropf*^{P188} line and location of the P insertions in the *innexin* 2 transcription unit. (A-F) β-Gal expression monitored by anti-β-Gal antibody staining of whole-mount embryos. (A) Stage 10 embryo, showing uniform expression in the germ band (arrow). With the beginning of germ band retraction (B), the ubiquitous expression pattern resolves into localised expression in the ectodermal fore- and hindgut, the endodermal anterior and posterior midgut, and the excretory Malpighian tubule primordia. During germ band retraction, reporter gene expression is maintained in these tissues (C-E) and in stage 17 embryos (E,F), expression occurs predominantly in the endodermal parts of the proventriculus and in the small intestine in the hindgut. (G) The localisation of the P-element insertions is indicated by inverted triangles. The genomic organisation of the transcript is shown below (Stebbings et al., 2000). (H) In situ hybridisation analysis of wild type (H) and of a *kropf* mutant embryo (I) using antisense probes for innexin 2 (*inx2*) and *orthopedia* (*otp*; internal staining control). Note that the *inx2* pattern is abolished in *kropf* mutants, whereas otp is still expressed. amg, anterior midgut; fg, foregut; hg, hindgut; MT, Malpighian tubules; pmg, posterior midgut; pv, proventriculus; si, small intestine.

Results

kropf mutant larvae display a feeding defect

In a screen of the Göttingen P-element insertion lines (Peter et al., 2002) for reporter gene expression patterns in the developing gastrointestinal tract, we isolated four lines: *l(1)G0016*, *l(1)G0034*, *l(1)G0036* and *l(1)G0188*, in which the P element had inserted in a novel locus which we named *kropf*. The P lines showed a highly similar and dynamic reporter gene expression pattern, especially at the foregut/midgut and the midgut/hindgut junctions (Fig. 1). It is known that morphogenesis in both regions of the gut is under the control of similar regulatory networks involving *hedgehog*, *wingless* and *decapentaplegic* signalling activities (Pankratz and Hoch, 1995; Hoch and Pankratz, 1996). From early blastoderm stage onwards, until stage 10 (Campos-Ortega and Hartenstein, 1997), β-Gal expression occurs rather uniform in all embryonic tissues (Fig. 1A). The ubiquitous expression pattern resolves with the beginning of germband retraction into localised

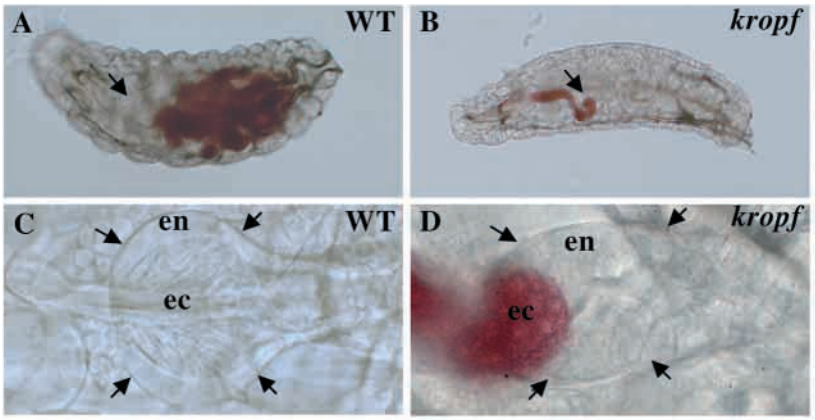

Fig. 2. *kropf* mutant larvae display a feeding defect. (A) Wild-type first instar larva fed with red dyed food (feeding assay). The food is predominantly found in the midgut. The multi-layered proventriculus is transparent (arrow). (B) In homozygous *kropf* mutant larvae, the food is stuck at the proventriculus (arrow). (C) Magnification of the proventriculus of the wild-type larva shown in A. Note that the ectodermal cells (ec) have migrated into the endodermal pouch (en, arrows). (D) Magnification of the proventriculus of the *kropf* mutant larva shown in B. Note that the ectodermal cells (ec) have failed to move into the endodermal pouch (en; arrows). As a result, the red food is stuck in the oesophagus causing a feeding defect.

expression in the ectodermal fore- and hindgut, the endodermal anterior and posterior midgut and the excretory Malpighian tubule primordia (Fig. 1B). High levels of reporter gene expression are maintained in these tissues (Fig. 1C,D), and expression occurs in late stages of embryogenesis predominantly in the proventriculus and in the small intestine of the hindgut, which abuts the endodermal posterior midgut (Fig. 1E,F). To clone the gene affected in the *kropf* locus, we isolated genomic DNA flanking the P insertion sites by plasmid rescue and used the sequencing information to perform a standard nucleotide blast search. We found that the P elements of the lines *l(1)G0016*, *l(1)G0036* and *l(1)G0188* were inserted in the untranslated leader region of the *innexin 2* gene (Fig. 1G), which encodes a member of the Innexin family of gap junction channel proteins (Curtin et al., 1999; Stebbings et al., 2000). In situ hybridisation analysis using riboprobes for *innexin 2* and *orthopedia*, a gene that is expressed in the hindgut and the anal pads (Simeone et al., 1994) and which serves as an internal staining control, was performed for the *kropf* P mutants *l(1)G0016* and *l(1)G0036*. It showed that these P alleles were *innexin 2* transcript null mutations (Fig. 1F). Mobilisation of the P element was performed in all four *kropf* P lines and resulted in independent excision lines that were homozygous viable and regained the *innexin* mRNA expression profile (Fig. 1J). And finally, experiments in which the *innexin 2* cDNA was cloned into an UAS vector and ubiquitously expressed in epithelial cells of *kropf* mutant embryos using *Daughterless* or *armadillo*-gal4 driver lines led to a rescue of the embryonic and the larval feeding phenotypes of the *kropf* mutants (see Materials and Methods). We therefore conclude that the transcription unit affected by the *kropf* P mutations corresponds to the *innexin 2* transcription unit.

The four *kropf*^{P} alleles all showed very similar mutant phenotypes. About two-thirds of the mutant animals were embryonic lethals; however, about one-third of the mutants died in the first instar larval stage (see Materials and Methods). As the P lines showed expression patterns in the developing proventriculus, we tested *kropf*^{P} larvae for their ability to feed dye-coloured yeast. We had shown previously that malformation of the proventriculus results in a feeding defect causing larval lethality (Hoch and Pankratz, 1995). Using this feeding assay, we found that in *kropf* mutant larvae, the food could not be efficiently transported into the midgut, resulting in an engorged oesophagus. Ectodermal cells of the foregut which in wild-type larvae migrate into the endodermal proventricular pouch tissue to form the multi-layered proventriculus organ (Fig. 2A, magnification in C), fail to move inwards in the mutant animals and are stuck on top of the endoderm cells (Fig. 2B, magnification in D). We observe lethality of the mutant larvae in the first instar stage, indicating that the feeding defect may result in death by starvation. For further mutant analysis, we used the *l(1)G0016* (*kropf* ^{P16}) allele.

Innexin 2 is required for keyhole formation during proventriculus development

The proventriculus is an organ that functions as a valve to regulate food passage from the foregut into the midgut in *Drosophila* larvae (Snodgrass, 1935; Strasburger, 1932). It develops during early embryogenesis at the junction of the ectodermal foregut and the endodermal anterior midgut primordia (Skaer, 1993; Fuß and Hoch, 1998; Nakagoshi et al., 1998). There is initially an outward buckling of the ectoderm, in a region that is referred to as the ‘keyhole’ structure (Pankratz and Hoch, 1995). This area will undergo further outward movement, then fold back on itself and move inwards into the endodermal proventricular pouch to form the mature, multi-layered proventriculus (King, 1988). Wingless is functionally required for cell migration of the proventricular cells and is initially expressed in wild-type embryos in a domain that includes the region from which the keyhole will form (Pankratz and Hoch, 1995; Fuß and Hoch, 1998) (Fig. 3A, magnification in C). With the onset of cell migration during keyhole formation, Wingless expression is either lost or repressed in the centre of its expression domain resulting into two domains that are split (Fig. 3B, magnification in D). One domain is at the anterior border of the keyhole in the ectodermal foregut cells and the other in endodermal cells posterior to the keyhole. In *kropf P* mutants, initial Wingless expression in the keyhole cells is normal (Fig. 3E, magnification in G). However, the Wingless expression in the central region persists (Fig. 3F, magnification in H). The cells that would normally form the keyhole stay fixed within the tightly organised epithelial foregut tube in the mutant until late stages of development, and the migration of the ectodermal cells into the endodermal pouch seems not to occur. Instead, these cells stay on top of the endoderm, as can be demonstrated using by anti-Fkh/anti-Dve double staining (Fig. 3I,J). We conclude from these

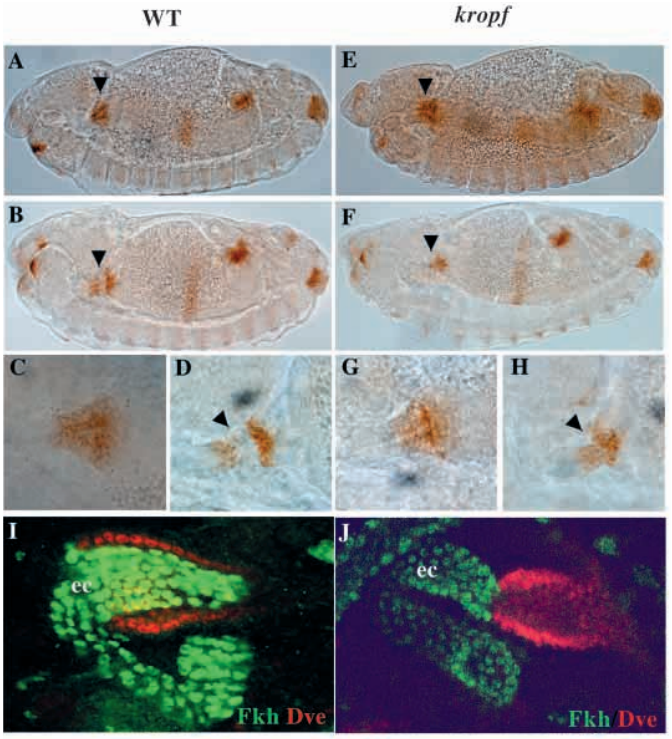

Fig. 3. Keyhole formation is disrupted in *kropf* P mutants. (A,B) Wild-type embryos stained with anti-Wingless antibody. (C,D) Magnifications of the keyhole region shown in A,B. (A) Stage 14 embryo. Note the striped Wingless expression domain prior to keyhole formation (arrow; magnification in C). (B) Stage 15 embryo. Note that the Wingless expression domain is split (arrow; magnification in D). (E,F) Anti-Wingless antibody staining of *kropf* mutant embryos. (E) Stage 14 embryo. Note that the striped Wingless expression domain is not affect in *kropf* mutants (magnification in G) when compared with wild type. (F) Stage 15 embryo. Note that the Wingless expression domain fails to split (arrow; magnification in H). (I,J) Confocal images of anti-Fkh (green)/anti-Dve (red) staining of stage 17 wild-type (I) and *kropf* mutant embryos (J). Although in wild type, the ectodermal cells (ec) have invaginated into the endodermal proventriculus pouch, the ectodermal cells are stuck on top of the endoderm in *kropf* P mutants (compare with the larval phenotype in Fig. 2D).

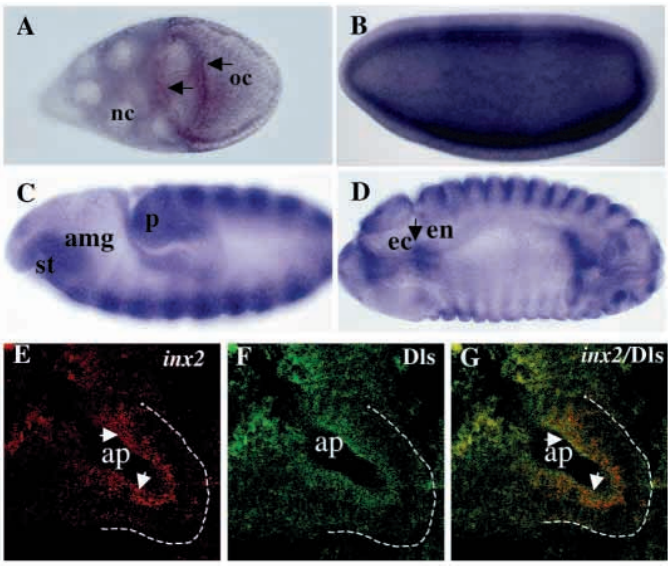

Fig. 4. Apical localisation of innexin 2 mRNA. In situ hybridisation using digoxigenin (A-D) or fluorescein-labelled RNA *innexin* 2 antisense riboprobes (E-G). (A) *innexin 2* transcripts are detected in the cytoplasm of the nurse cells (nc) and the oocyte (oc) during oogenesis. Note the localisation of the mRNA in the cortical region of the oocyte and the border cells (arrows). (B) Blastoderm embryo. Transcripts are ubiquitously distributed. (C) Germ band extension stage. mRNA expression occurs in the invaginating stomodeum (st), the anterior midgut primordium (amg) that abuts the involuted foregut tube, in the proctodeum (p) and in segment precursors (Stebbings et al., 2000). (D) During keyhole formation, *innexin 2* expression is found in the ectodermal (ec) and endodermal (en) tissue regions of the proventriculus primordium (arrow marks the boundary). Note that the *innexin* 2 mRNA expression pattern corresponds to the reporter gene expression pattern of the P lines (compare with Fig. 1). (E-G) Double immunostaining of a proventriculus of a stage 16 embryo (marked by the stippled line) using a fluorescein-labelled RNA *innexin 2* antisense riboprobe (E; red) and anti-Discs lost antibody (F; green), which marks the apical (ap, shown by arrows) sides of epithelial cells (Bhat et al., 1999). *innexin 2* mRNA is localised to the apical sides of the proventricular cells (merge in G).

results that *innexin 2* is required during early stages of proventriculus development for keyhole formation. In *kropf* mutants, the keyhole defect prevents the correct folding and inward movement of the proventriculus tissue layers most probably resulting in the feeding defect observed later in the mutant larvae.

Apical Innexin 2 mRNA localisation

In order to analyse the role of *innexin 2* during development further, we studied the mRNA expression pattern of the gene using an *innexin 2* antisense probe (see Materials and Methods). During oogenesis, *innexin 2* transcripts are found in the nurse cells, border cells and in a cortical localisation in the oocyte (Fig. 4A), consistent with a maternal complement that could explain the variability of the *kropf* mutant phenotype. *innexin 2* mRNA is ubiquitously distributed during the early embryonic blastoderm stage (Fig. 4B). A segmental expression pattern is found during germ band extension stage (Fig. 4C) (Stebbings et al., 2000) and we find expression in the stomodeal and proctodeal invaginations, which are the primordia of the foregut and the hindgut. During keyhole formation at the boundary of the foregut and the anterior midgut, *innexin 2* expression persists in the ectodermal and endodermal tissue regions of the proventriculus primordium (Fig. 4D). During the invagination of the ectodermal cells into the endodermal pouch, the expression pattern of *innexin 2* is restricted to the endodermal part of the proventriculus (not shown). Fluorescent detection of *innexin 2* transcripts (Materials and Methods) and co-immunostaining with anti-Discs lost antibody, which marks the apical sides of epithelial cells (Bhat et al., 1999) demonstrates that *innexin 2* mRNA is localised to the apical sides of the proventricular cells (Fig. 4E-G). In summary, our mRNA expression studies are compatible with a functional role of *innexin 2* during proventriculus development.

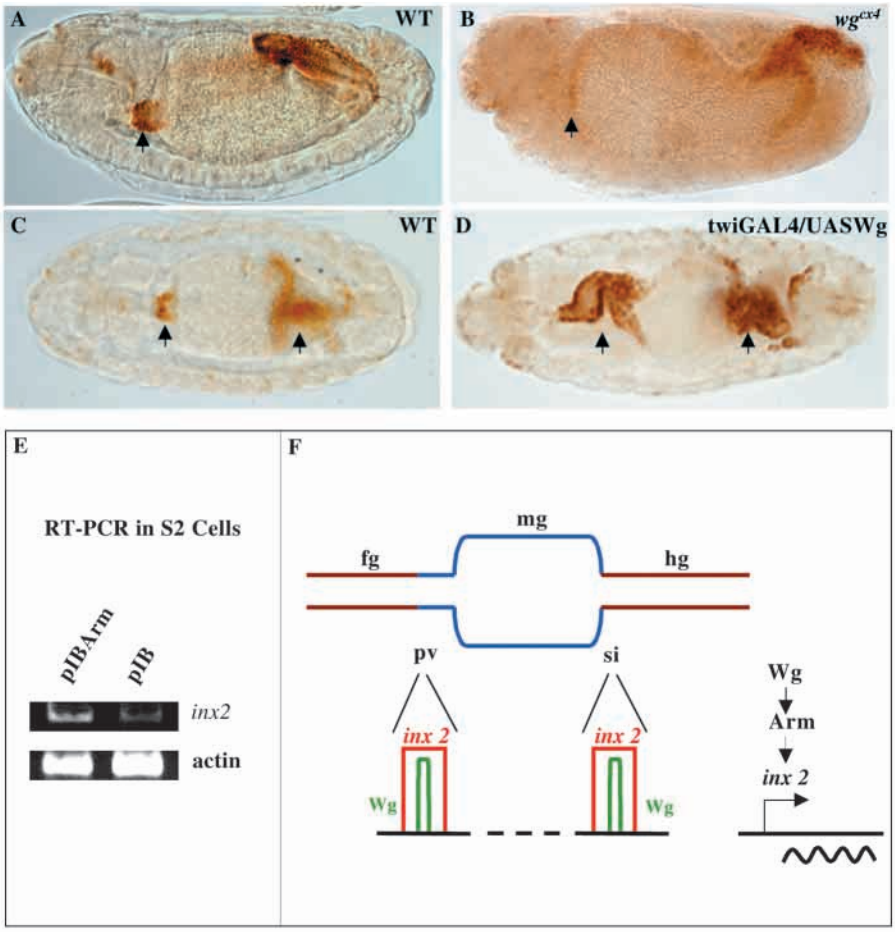

Fig. 5. *innexin 2* transcription is induced by Wingless signalling. The β-Gal expression of the *kropf*^{P16} line in stage 15 embryos of wild-type (A,C), homozygous *wingless* mutants (B) and in embryos in which β-Gal has been ectopically expressed by using the twi-Gal4 driver and the UAS-*wingless* effector lines (see text). Note that reporter gene expression is abolished in the proventriculus region in wingless mutants (compare regions marked by arrows in A,B), whereas it is dramatically expanded in the anterior and posterior region in the Wingless overexpression experiment (compare region marked by arrows in C,D). (E) RT-PCR experiments in *Drosophila* tissue culture S2 cells (Materials and Methods). Schneider S2 cells were either transfected with an expression vector for Armadillo, the β-catenin homologue that has been shown to serve as the transducer of the Wingless signalling cascade (pIBArm) or with the vector alone (pIB). *innnexin 2* mRNA levels were monitored by performing RT-PCR analysis (actin mRNA levels served as an internal control). Note that *innexin 2* mRNA levels are increased approximately five times in response to Armadillo when compared with the control reaction. (F) The regionalisation of developing gut tube. The foregut (fg) and the hindgut (hg) are of ectodermal (brown) and the midgut (mg) of endodermal origin (blue). At the ectoderm/endoderm boundaries of the gut, signalling centres arise that control the development of the proventriculus (pv) and the small intestine (si) in the anterior region and in the posterior regions, respectively. A working model suggests that Wingless (green) activates innexin 2 transcription (red) via the signal transducer Armadillo (right) in the boundary regions of the gut. Note, however, that the arrows do not imply direct molecular interactions (see Discussion). This induction may be required for enhanced gap junctional communication during the morphogenetic processes in both gut parts.

Innexin 2 is a target gene of the Wingless signalling pathway

Morphogenetic cell movements of the proventriculus epithelium and of the hindgut depend on the activities of the genes *hedgehog*, *wingless* and *decapentaplegic* (Pankratz and Hoch, 1995; Hoch and Pankratz, 1996). Through the restricted expression of these genes in local domains, they define signalling centres at the foregut/midgut and the midgut/hindgut boundaries. Wingless and Hedgehog were shown to control cell migration of epithelial gut cells whereas Decapentaplegic was shown to prevent morphogenesis in the gut (Pankratz and Hoch, 1995; Hoch and Pankratz, 1996). In order to analyse whether *innexin 2* transcription is dependent on these signalling cascades, we studied the transcriptional regulation of the gene by crossing the P lines into various mutant backgrounds or by determining the *innexin 2* mRNA profile (the nuclear ß-Gal expression pattern reflects the mRNA expression profile of *innexin 2* during proventriculus development). Whereas in *hedgehog* and *decapentaplegic* mutant embryos, *innexin 2* or reporter gene expression was not affected, they were absent in the proventriculus rudiment and reduced in the small intestine region of amorphic *wingless* mutants (Fig. 5A,B). Because the gut morphology in general is strongly affected in *wingless* mutants, we also performed a gain-of-function analysis using the UAS-Gal4 system (Brand and Perrimon, 1993). Ectopic expression of Wingless in the visceral mesoderm using the twi-Gal4 driver in combination with the UAS-*wingless* effector line or ectopic expression in the endoderm using the 14-3fkh-Gal4 driver in combination with the UAS-*wingless* effector, resulted in ectopic expression of *innexin 2* or β-Gal expression in the anterior and posterior regions of the gut (Fig. 5C,D). Both, the expression domains in the proventriculus and in the small intestine region that lies at the complementary endoderm/ectoderm boundary in the posterior region, dramatically expand (Fig. 5D). It is known that upon ectopic Wingless expression, endogenous *decapentaplegic* expression is ectopically activated and covers the region of parasegments 2-7 of the visceral mesoderm (Yu et al., 1996). However, upon ubiquitous expression of Decapentaplegic in the visceral mesoderm using UAS-*decapentaplegic* transgenes, in combination with twist-Gal4, we could not find a similar alteration of the *innexin 2* expression domains.

To further substantiate the conclusion drawn from our genetic experiments that *innexin 2* transcription is regulated in response to Wingless signalling, we performed transfection

experiments in *Drosophila* tissue culture S2 cells (see Materials and Methods). Total RNA was isolated from Schneider S2 cells that were either transfected with an expression vector alone or with the same vector containing the cDNA of Armadillo, the β-catenin homologue that has been shown to serve as the transducer of the Wingless signalling cascade (Peifer and Polakis, 2000). We monitored *innexin 2* mRNA levels by performing a RT-PCR analysis using actin mRNA levels as an internal control (see Materials and Methods). As shown in Fig. 5E, *innexin 2* mRNA levels are increased approximately five times in response to Armadillo when compared with the control reaction, whereas the actin mRNA levels remain constant in both situations. We conclude from our genetic lack- and gain-of-function studies, and from our tissue culture experiments that *innexin 2* is a target gene of the Wingless signalling cascade that is induced in wild-type embryos at ectoderm/endoderm boundaries during gut organogenesis (Fig. 5F).

Discussion

The regulatory networks controlling the assembly of gap junction channels to make cells competent for signalling during patterning and organogenesis are largely unknown in both vertebrates and invertebrates. We have investigated the role and the regulation of the *Drosophila innexin 2* gene, which encodes a core component of invertebrate gap junction proteins, during proventriculus development in the *Drosophila* embryo.

Innexin 2 is required for keyhole formation

Our results indicate that *kropf* mutant larvae which are devoid of *innexin 2* expression, display a feeding defect resulting from a failure of proventriculus fomation in the embryo: the ectodermal proventriculus cells fail to move into the endodermal pouch and are stuck instead on top of the endoderm tissue; a multi-layered organ does not form (Fig. 2). Our mutant analysis using Wingless and Fkh/Dve as markers to visualise the ectodermal/endodermal boundaries in the foregut strongly suggests that this late invagination defect originates from a failure of cells to form the keyhole structure at much earlier stages (Fig. 3). The keyhole is the first visible morphological structure that is formed by an outbuckling of primordial cells of the proventriculus, in a region of the caudal foregut primordium that is free of visceral mesoderm (Pankratz and Hoch, 1995). *innexin 2* mRNA is expressed in the primordial cells prior to the initiation of keyhole formation (Fig. 4). The analysis of Wingless expression in the proventriculus primordium of *kropf* mutants suggests that it is not the specification of the keyhole cells that is affected in the mutant. At the time when the keyhole forms, cell proliferation in the foregut has already been completed (Campos-Ortega and Hartenstein, 1997). Furthermore, we could not observe cell death of keyhole cells in *kropf* mutants using TUNEL analysis (data not shown). We therefore favour a model that suggests that gap junctional communication may be important for the cellular processes involved in the outbuckling, the folding and the subsequent migration of the keyhole cells, as has been observed previously for the migration of neural crest cells in vertebrates (Lo et al., 1999; Huang et al., 1998).

Innexin 2 is activated by Wingless signalling at the ectoderm/endoderm boundaries of the intestinal canal

Epistasis experiments indicate that *innexin* 2 transcription is abolished in the proventriculus rudiment that is left in *wingless* mutants and that expression of the gene is dramatically expanded upon ectopic expression of Wingless in the gain-of-function experiment (Fig. 5). Furthermore Armadillo, the signal transducer of the Wingless signalling pathway is able to induce *innexin 2* transcription in tissue culture cells, as show by RT-PCR experiments (Fig. 5E). In wild-type embryos, both *innexin 2* and *wingless* are co-expressed during early and late stages of proventriculus development (Fig. 3A,C, compare to Fig. 4D). In summary, these data provide strong evidence that the gap junction channel gene *innexin 2* is induced as a Wingless target gene during keyhole formation in the proventriculus primordium. Recently, *defective proventriculus* (*dve*), a novel homeobox gene, has been identified as another target gene of Wingless signalling in the proventriculus endoderm (Fuß and Hoch, 1998; Nakagoshi et al., 1998). *dve* mutants display, however, a late invagination defect caused by a malformation of the proventriculus endoderm (Fuß and Hoch, 1998; Nakagoshi et al., 1998). This suggests that Wingless has the potential to activate target genes that act during different phases of proventriculus development. *innexin 2* expression is, however, not only regulated by Wingless in the proventriculus primordium but also at the junction of the midgut and the hindgut. This region, which is called the small intestine (Hoch and Pankratz, 1996; Snodgras, 1935; Strasburger, 1932), is characterised by extensive morphogenetic cell movements and thus displays many similarities to the keyhole in the anterior region. Wingless-dependent gap junctional communication may also be required in this area of the gastrointestinal tract, although we have not observed gross morphological changes in this region in *kropf* mutants.

Whether the transcription factor complex that transduces the Wingless signal and which is composed of the β-catenin homologue Armadillo and Tcf/Lef DNA-binding proteins, directly interacts with the regulatory region of *innexin 2* to activate its transcription, is presently unknown. However it is noteworthy that a consensus Tcf/Lef binding site is found 5 kb upstream of the putative *innexin 2* promotor. Armadillo is distributed throughout the foregut, but is concentrated in specific areas of the developing proventriculus (Pankratz and Hoch, 1995). At early stages, it is strongly expressed near the keyhole region and at later stages it becomes highly concentrated in areas undergoing the most extensive cell movements. The proventriculus phenotype of *armadillo* mutant embryos is similar to that of *wingless* null embryos, strongly suggesting the requirement of *armadillo* during proventriculus morphogenesis. In addition, *innexin 2* transcription is induced by Armadillo in tissue culture cells. It is thus possible that Armadillo and Tcf/Lef directly regulate *innexin 2* transcription.

Apical localisation of *innexin* 2 gene products

Our expression studies show that *innexin 2* mRNA is localised to the apical side of cells. mRNA expression studies with other members of the *innexin* family indicate a similar apical localisation pattern (C. L. and M. H., unpublished). It is also

noteworthy that *wingless* mRNA is localised at the apical sides of epithelial cells (Baker, 1987). The apical localisation has been shown to depend on discrete elements within the *wingless* 3`UTR (Simmonds et al., 2001). It requires cytoplasmic dynein protein that assembles the apical RNAs selectively into particles that are transported apically along microtubules (Wilkie and Davis, 2001). Redistribution of the transcripts causes a dramatic loss of Wingless signalling activity. Furthermore, the subcellular localisation of WNT transcripts also occurs in other organisms, suggesting a conserved function (Simmonds et al., 2001). Various other components of the Wingless signalling pathway, such as the Wingless transmembrane receptor Frizzled, the β-catenin homologue Armadillo, Dishevelled and the Armadillo-modifying complex are also localised apically in the cells (for reviews, see Wodarz and Nusse, 1998; Peifer and Polakis, 2000). As we have shown that *innexin 2* is a target of Wingless signalling in the proventricular cells, it is likely that apical *innexin* transcript localisation is an important functional feature required for Wingless-dependent gap junctional communication in the keyhole cells. Furthermore, these data suggest the existence of yet unknown factors (most probably RNA-binding proteins) that are required for apical *innexin 2* mRNA localisation in epithelial cells. It remains to be shown whether connexin mRNAs are also localised and, if so, what functional role the localisation plays. Electrophysiological experiments in the heterologous *Xenopus* system have demonstrated that Innexin 2 forms heteromeric gap junctions with Innexin 3, another Innexin family member (Stebbings et al., 2000). Whether heteromerisation is also required for keyhole formation is not known because *innexin 3* mutants have not been identified, yet.

The WNT/Wingless pathway regulates the transcription of gap junction multigene families

Our results suggest that although invertebrate innexin genes share no sequence homology to the vertebrate connexin genes (Phelan et al., 1998a; Phelan and Starich, 2001), both multigene families might be regulated by an evolutionarily conserved signalling pathway, the WNT/Wingless signalling cascade. In the developing *Xenopus* embryo, it has been observed that Wnt1 expression leads to an enhancement of gap junctional communication in ventral cells (Olson et al., 1991; Olson and Moon, 1992; Krufka et al., 1998). Studies in the mouse have shown that ectopic expression of Wnt1 in the limb mesenchyme results in an increase of connexin 43 transcription (Meyer et al., 1997). Recent tissue culture studies using rat PC12 cell lines and cardiomyocytes have further provided molecular evidence that *connexin* 43 is a downstream target gene of WNT1 signalling (van der Heyden et al., 1998; Ai et al., 2000). Our studies about *innexin 2* regulation and the evidence from the *connexin* regulation in vertebrates suggest that WNT/Wingless signalling may be an evolutionarily conserved signalling pathway regulating the expression of gap junction-encoding multigene families.

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References

Adams, M. D., Celniker, S. E., Holt, R. A., Evans, C. A., Gocayne, J. D., Amanatides, P. G., Scherer, S. E., Li, P. W., Hoskins, R. A., Galle, R. F. et al. (2000). The genome sequence of *Drosophila melanogaster*. *Science* **287**, 2185-2195.

Affolter, M. and Mann, R. (2001). Development. Legs, eyes or wings – selectors and signals make the difference. *Science* **292**, 1080-1081.

Ai, Z., Fischer, A., Spray, D. C., Brown, A. M. C. and Fishman, G. I. (2000). Wnt-1 regulation of connexin 43 in cardiac myocytes. *J. Clin. Invest.* **105**, 161-171.

Baker, N. (1987). Molecular cloning of sequences from *wingless*, a segment polarity gene in *Drosophila*: the spatial distribution of a transcript in embryos. *EMBO J.* **6**, 1765-1773.

Barnes, T. M. and Hekimi, S. (1997). The *C. elegans* avermectin resistance and anesthetic respose gene *unc-9* encodes a member of a protein family implicated in electrical coupling of excitable cells. *J Neurochem.* **69**, 2251-2260.

Bhat, M. A., Izaddoost, S., Lu, Y., Cho, K. O., Choi, K. W. and Bellen, H. J. (1999). Discs Lost, a novel multi-PDZ domain protein, establishes and maintains epithelial polarity. *Cell* **96**, 833-845.

Bienz, M. (1997). Endoderm induction in *Drosophila*: the nuclear targets of the inducing signals. *Curr. Opin. Genet. Dev.* **7**, 683-688.

Brand, A. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401-415.

Campos-Ortega, J. A. and Hartenstein, V. (1997). The embryonic development of *Drosophila melangaster*. Berlin: Springer Verlag.

Curtin, K. D., Zhang, Z. and Wyman, R. J. (1999). *Drosophila* has several genes for gap junction proteins. *Gene* **232**, 191-201.

Dahmann, C. and Basler, K. (1999). Compartment boundaries: at the edge of development. *Trends Genet.* **15**, 320-326.

Falk, M. M. and Gilula, N. B. (1994). Membrane insertion of gap junction connexins: polytopic channel forming membrane proteins. *J. Cell Biol.* **127**, 343-355.

Fuß, B. and Hoch, M. (1998). *Drosophila* endoderm development requires a novel homeobox gene which is a target of Wingless and *Decapentaplegic* signalling. *Mech. Dev.* **79**, 83-87.

Fuß, B., Meißner, T., Bauer, R., Lehmann, C., Eckardt, F. and Hoch, M. (2001). Control of endoreduplication domains in the *Drosophila* gut by the *knirps* and *knirps-related* genes. *Mech. Dev.* **100**, 15-23.

Goodenough, D. A., Goliger, J. A. and Paul, D. L. (1996). Connexins, connexons and intercellular communcation. *Annu. Rev. Biochem.* **65**, 475-602.

Grapin-Botton, A. and Melton, D. A. (2000). Endoderm development: from patterning to organogenesis. *Trends Genet.* **16**, 124-130.

Gurdon, J. (1992). The generation of diversity and pattern in animal development. *Cell* **68**, 185-199.

Hoch, M. and Pankratz, M. J. (1996). Control of gut development by *fork head* and cell signalling molecules in *Drosophila*. *Mech. Dev.* **58**, 3-14.

Huang, G. Y., Cooper, E. S., Waldo, K., Kriby, M. L., Gilula, N. B. and Lo, C. W. (1998). Gap junction mediated cell-cell communication modulates mouse neural crest migration. *J. Cell Biol.* **143**, 1725-1734.

Hughes, S. C. and Krause, H. M. (1999). Single and double FISH protocols for *Drosophila*. *Methods Mol. Biol.* **122**, 93-101.

Hughes, S. C., Saulier Le Dean, B., Livne-Bar, I. and Krause, H. M. (1996). Fluorescence in situ hybridization in whole-mount *Drosophila* embryos. *Biotechniques* **20**, 748-750.

King, D. G. (1988). Cellular organization and peritrophic membrane formation in the cardia (proventriculus) of *Drosophila melanogaster*. *J. Morphol.* **196**, 253-282.

Krufka, A., Johnson, R. G., Wylie, C. C. and Hesman, J. (1998). Evidence that dorsal-ventral differences in gap junctional communication in the early *Xenopus* embryo are generated by β-catenin independent of cell adhesion effects. *Dev. Biol.* **200**, 92-110.

Kumar, N. M. and Gilula, N. B. (1996). The gap junction communication channel. *Cell* **84**, 381-388.

Landesman, Y., White, T. W., Starich, T. A., Shaw, J. E., Goodenough, D. A. and Paul, D. L. (1999). Innexin-3 forms connexin-like intercellular channels. *J. Cell Sci.* **112**, 2391-2396.

Lawrence, P. A. and Struhl, G. (1996). Morphogens, compartments, pattern: lessons from *Drosophila*? *Cell* **85**, 951-961.

Lo, C. W., Waldo, K. L. and Kriby, M. L. (1999). Gap junction communication and the modulation of cardiac neural crest cells. *Trends Cardiovasc. Med.* **9**, 63-69.

Massague, J. (2000). How cells read TGF-β signals. *Nat. Rev. Mol. Cell Biol.* **1**, 169-178.

Meyer, R. A., Cohen, M. F., Recalde, S., Zakany, J., Bell, S. M., Scott, W. J., Jr and Lo, C. W. (1997). Developmental regulation and asymmetric expression of the gene encoding Cx43 gap junctions in the mouse limb bud. *Dev. Genet.* **21**, 290-300.

Nakagoshi, H., Hoshi, M., Nabeshima, Y. and Matsuzaki, F. (1998). A novel homeobox gene mediates the *Decapentaplegic* signal to establish functional specificity within target cells. *Genes Dev.* **12**, 2724-2734.

Ohlson, D. J. and Moon, R. T. (1992) Distinct effects of ectopic expression of Wnt-1, activin B and bFGF on gap junctional permeability in 32-cell *Xenopus* embryos. *Dev. Biol.* **151**, 204-212.

Ohlson, D. J., Christian, J. I. and Moon, R. T. (1991). Effect of wnt-1 and related proteins on gap junctional communication in *Xenopus* embryos. *Science* **252**, 1173-1176.

Pankratz, M. J. and Hoch, M. (1995). Control of epithelial morphogenesis by cell signalling and integrin molecules in the *Drosophila* foregut. *Development* **121**, 1885-1898.

Peifer, M. and Polakis, P. (2000). Wnt Signalling in oncogenesis and embryogenesis – a look outside the nucleus. *Science* **287**, 1606-1609.

Peter, A., Schottler, P., Werner, M., Beinart, N., Dowe, G., Burkert, P., Mourkioti, F., Dentzer, L., He, Y., Deak, P. et al. (2002). Mapping and identification of essential gene functions on the X chromosome of *Drosophila*. *EMBO Rep.* **31**, 34-38.

Phelan, P. and Starich, T. A. (2001). Innexins get into the gap. *BioEssays* **23**, 388-396.

Phelan, P., Nakagawa, M., Wilkin, M. B., Moffat, K. G., O'Kane, C. J., Davies, J. A. and Bacon, J. P. (1996). Mutations in shaking-B prevent electrical synapse formation in the *Drosophila* giant fibre system. *J. Neurosci.* **16**, 1101-1113.

Phelan, P., Bacon, J. P., Davies, J. A., Stebbings, L. A., Todman, M. G., Avery, L., Baines, R. A., Barnes, T. M. and Ford, C. (1998a). Innexins: a family of invertebrate gap junction proteins. *Trends Genet.* **14**, 348-349.

Phelan, P., Stebbings, L. A., Baines, R. A., Bacon, J. P., Davies, J. A and Ford, C. (1998b). *Drosophila* Shaking-B protein forms gap junctions in paired *Xenopus* oocytes. *Nature* **391**, 181-184.

Simeone, A., D'Apice, M. R., Nigro, V., Casanaova, J., Graziani, F., Acampora, D. and Avantaggiato, V. (1994). *Orthopedia*, a novel homeobox-containing gene expressed in the developing CNS of both mouse and *Drosophila*. *Neuron* **13**, 83-101.

Simmonds, A. J., dosSantos, G., Livne-Bar, I and Krause, H. M. (2001). Apical localization of wingless transcripts is required for Wingless signalling. *Cell* **105**, 197-207.

Skaer, H. (1993). The alimentary canal. In *The Development of Drosophila melanogaster* (ed. A. Martinez Arias and M. Bate), pp. 941-1012. New York: Cold Spring Harbor Laboratory Press.

Snodgrass, R. E. (1935). *Principles of Insect Morphology*. New York: McGraw-Hill.

Starich, T. A., Lee, R. Y. N., Panzarella, C., Avery, L. and Shaw, J. E. (1996). *eat-5* and *unc-7* represent a multigene family in *C. elegans* involved in cell-cell coupling. *J. Cell Biol.* **134**, 537-548.

Stebbings, L. A., Todman, M. G., Phelan, P., Bacon, J. P. and Davies, J. A. (2000). The *Drosophila* Innexins are expressed in overlapping domains and cooperate to form gap junction channels. *Mol. Biol. Cell* **11**, 2459-2470.

Strasburger, M. (1932). Bau, Funktion und Variabilität des Darmtraktus von *Drosophila melanogaster* (Meigen). *Z. Wiss. Zool.* **140**, 536-649.

Stringini, M. and Cohen, S. M. (1999). Formation of morphogen gradients in the *Drosophila* wing. *Semin. Cell Dev. Biol.* **10**, 335-344.

Stringini, M. and Cohen, S. M. (2000). Wingless gradient formation in the *Drosophila* wing. *Curr. Biol.* **10**, 293-300.

Taipale, J. and Beachy, P. A. (2001). The Hedgehog and Wnt signalling pathways in cancer. *Nature* **411**, 349-354.

Teleman, A. A. and Cohen, S. M. (2000). Dpp gradient formation in the *Drosophila* wing imaginal disc. *Cell* **103**, 971-980.

Unger, V. M., Kumar, N. M., Gilula, N. B. and Yeager, M. (1997). Projection structure of a gap junction membrane channel at 7A resolution. *Nat. Struct. Biol.* **4**, 39-43.

van der Heyden, M. A., Rook, M. B., Hermans, M. M., Rijksen, G., Boonstra, J., Defize, L. H., and Destree, O. H. (1998). Identification of connexin 43 as a functional target for Wnt signalling. *J. Cell Sci.* **111**, 1741-1749.

Watanabe, T. and Krankel, D. R. (1990). Molecular cloning and analysis of *l(1) ogre*, a locus of *Drosophila melanogaster* with prominent effects on postembryonic development of the central nervous system. *Genetics* **126**, 1033-1044.

Wilkie, G. S. and Davis, I. (2001). *Drosophila wingless* and pair-rule transcripts localize apically by dynein-mediated transport of RNA particles. *Cell* **105**, 209-219.

Willecke, K., Hennemann, H., Dahl, E., Jungbluth, S. and Heynkes, R. (1991). The diversity of connexin genes encoding gap junction proteins. *Eur. J. Cell Biol.* **56**, 1-7.

Wodarz, A. and Nusse, R. (1998). Mechanisms of Wnt signalling in development. *Annu. Rev. Cell Dev. Biol.* **14**, 59-88.

Yeager, M., Unger, V. M. and Falk, M. M. (1998). Synthesis, assembly and structure of gap junction intercellular channels. *Curr. Opin. Struct. Biol.* **8**, 517-524.

Yu, X., Hoppler, S., Eresh, S. and Bienz, M. (1996). *decapentaplegic*, a target gene of the *wingless* signalling pathway in the *Drosophila* midgut. *Development* **122**, 865-874.

Zhang, J. T., Chen, M., Foote, C. I. and Nicholson, B. J. (1996). Membrane integration of in vitro-translated gap junctional proteins: co- and post-translated mechanisms. *Mol. Biol. Cell* **7**, 471-482.