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Cross regulation of intercellular gap junction communication and paracrine signaling pathways during organogenesis in *Drosophila*

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Abstract

The spatial and temporal coordination of patterning and morphogenesis is often achieved by paracrine morphogen signals or by the direct coupling of cells via gap junctions. How paracrine signals and gap junction communication cooperate to control the coordinated behavior of cells and tissues is mostly unknown. We found that hedgehog signaling is required for the expression of *wingless* and of *Delta/Notch* target genes in a single row of boundary cells in the foregut-associated proventriculus organ of the *Drosophila* embryo. These cells coordinate the movement and folding of proventricular cells to generate a multilayered organ. hedgehog and wingless regulate gap junction communication by transcriptionally activating the *innexin2* gene, which encodes a member of the innexin family of gap junction proteins. In *innexin2* mutants, gap junction-mediated cell-to-cell communication is strongly reduced and the proventricular cell layers fail to fold and invaginate, similarly as in *hedgehog* or *wingless* mutants. We further found that *innexin2* is required in a feedback loop for the transcriptional activation of the hedgehog and wingless morphogens and of Delta in the proventriculus primordium. We propose that the transcriptional cross regulation of paracrine and gap junction-mediated signaling is essential for organogenesis in *Drosophila*.
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Keywords: *Drosophila*; Gap junctions; Morphogens; Hedgehog; Wingless; Innexin2; Delta; Foregut; Organogenesis

Introduction

Patterning and morphogenesis of the gut share numerous features between *Drosophila* and vertebrates. The underlying signaling events have been studied in some detail for the development of the foregut (see Pankratz and Hoch, 1995; Fuss et al., 2004; Grapin-Botton and Melton, 2000, for review). In the chick and in the mouse, Sonic hedgehog (Shh), mesodermally expressed BMP4 and members of the *Hox* and *ParaHox* gene families are required for the generation of boundaries, which determine the development of foregut-associated organs such as the thyroid, lungs, liver, and pancreas (see Hebrok et al., 1998; 2000; Pepicelli et al., 1998; Roberts et al., 1998; Ramalho-Santos et al., 2000; Kim et al., 1998; Grapin-Botton and Melton, 2000; Kiefer, 2003; Stainier, 2005, for review). Aberrant *hedgehog* signaling is involved in small cell lung cancer (SCLC), as well as in upper gastrointestinal malignancies arising from the pancreas, esophagus and stomach (see Watkins and Peacock, 2004; Radke et al., 2006, for review). In the *Drosophila* embryo, conserved signaling cascades including hedgehog, wingless, Delta/Notch and JAK/STAT pathways were shown to be required for patterning, invagination and folding of the posterior foregut epithelium to generate the proventriculus, a gut-associated organ mediating food passage in the larva (Pankratz and Hoch, 1995; Hoch and Pankratz, 1996; Fuss and Hoch, 1998; Fuss et al., 2004; Josten et al., 2004). How paracrine signaling pathways control patterning and morphogenesis of gut development is not well understood in both *Drosophila* and vertebrates.

An integration of metabolic and signaling activities in cells and tissue layers is also achieved by direct cell-to-cell communication via gap junctions. Gap junctions allow the direct exchange of ions and small molecules (<1 kDa) among neighboring cells (Goodenough et al., 1996). Three gene families have evolved to construct gap junctions, the *connexins* and *pannexins* in deuterostomes including mammals and the *innexins* in protostomes including *Drosophila* and *Caenorhabditis elegans* (Panchin et al., 2000; Bauer et al., 2005; Phelan, 2005; Barbe et al., 2006). Members of all three gene families encode structurally very similar four-pass trans-

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membrane proteins with two extracellular loops, cytoplasmic N- and C-termini and a cytoplasmic loop domain. Connexin proteins were shown to oligomerize into hexameric complexes called hemichannels or connexons. End-to-end docking of two connexons, with each connexon provided by one of two neighboring cells, allows the formation of gap junction channels; channel assembly along the lateral membrane domain of epithelial cells results in the formation of gap junction plaques (Martin and Evans, 2004; Segretain and Falk, 2004). The *Drosophila* innexin family of gap junction proteins consists of 8 members (Phelan, 2005; Bauer et al., 2005). For some of them, functions have been assigned using mutant and expression analysis and heterologous expression in the *Xenopus* oocyte system (see Bauer et al., 2005; Phelan, 2005, for reviews). Innexin2 was shown to be required for cell polarity and epithelial tissue organization in the *Drosophila* embryo (Bauer et al., 2002; 2004; Lehmann et al., 2006). In maternal and zygotic mutants of *innexin2* (named *kropf* mutants), epithelia fail to develop (Bauer et al., 2004), whereas zygotic *kropf* mutants show severe organogenesis defects, including a failure of proventriculus folding and invagination, which is also observed in *hedgehog* and *wingless* mutants (Pankratz and Hoch, 1995; Bauer et al., 2002). How paracrine signaling and intercellular gap junction communication cooperate to allow the coordinated behavior of cell and cell layers during foregut morphogenesis is unknown.

We have studied the development of the foregut-associated proventriculus organ of the *Drosophila* embryo. We show that *hedgehog* activates *wingless* and *Delta/Notch* target genes in a single row of cells in the proventriculus primordium, the anterior boundary cells, which are crucial for cell movement and folding of the proventriculus epithelium during organogenesis. *wingless* in turn activates the transcription of *innexin2*, a core component of gap junction channels in the invaginating cells, thus connecting paracrine hedgehog and wingless signaling with the regulation of gap junction communication. We further show that in a feedback loop, *innexin2* is essential for the transcriptional activation of *hedgehog*, *wingless* and *Delta* during foregut morphogenesis. Our results provide first evidence that the mutual transcriptional cross regulation of paracrine and gap junction-mediated signaling is essential for organogenesis in *Drosophila*.

Results

hedgehog controls the invagination of the ectodermal proventriculus precursor cells

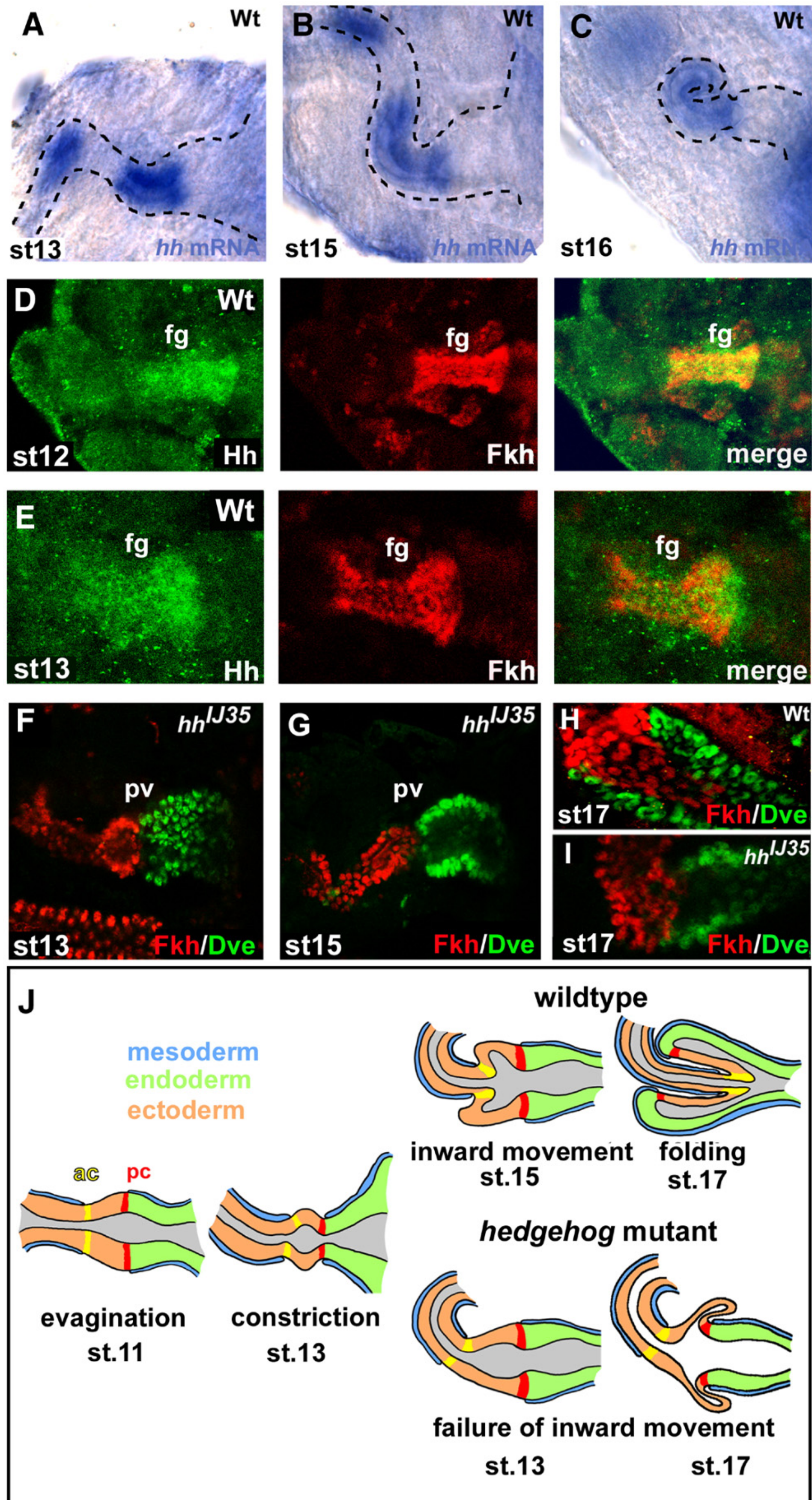
The proventriculus is a multiply folded, cardia-shaped organ, which functions as a valve to regulate food passage from the foregut into the midgut in *Drosophila* larvae (Campos Ortega and Hartenstein, 1997). Two rows of cells, the anterior and the posterior boundary cells, play special roles during proventriculus morphogenesis (Fuss et al., 2004). The posterior boundary cells are positioned at the ectoderm/endoderm boundary of the foregut and the midgut; they provide "stiffness" function at the rim of the endodermal layer, which forms a pocket-like endoderm opening. The anterior boundary cells form the tip of an ectodermal cell population invaginating into the endodermal pouch. The specification and morphogenetic function of both the anterior and posterior boundary cells is dependent on local activation of the Notch signaling pathway in these cell rows (Fuss et al., 2004). In the posterior boundary cells, the JAK/STAT signaling cascade cooperates with Notch signaling to control the expression of *short stop*, which encodes a cytoskeletal crosslinker protein of the spectraplakin superfamily (Gregory and Brown, 1998; Strumpf and Volk, 1998; Röper et al., 2002); *short stop* stabilizes the cytoskeleton in the posterior boundary cells to provide a stiffness function essential for the invagination of the anterior cells to invaginate (Fuss et al., 2004; Josten et al., 2004). How the anterior boundary cells forming the tip of the invaginating ectodermal cells are allocated is not known.

In a search for regulators controlling the activities of the anterior boundary cells, we identified *hedgehog* as a key regulator. *hedgehog* mRNA (Figs. 1A–C) and protein (Figs. 1D, E) are expressed from early to late embryonic stages in a broad expression domain in the posterior foregut covering cells of the developing esophagus and all the ectodermal cells of the proventriculus primordium, which invaginate during the formation of the multilayered organ. In amorphic *hedgehog* mutants, evagination and the constriction of the proventriculus primordium are not affected (Fig. 1F). However, inward movement and folding of the ectodermal proventricular cells fail to occur (Fig. 1G). As a consequence, the ectodermal cells stay behind on top of the endodermal layer resulting in a cardia arrest phenotype in late stage 17 *hedgehog* embryos (Fig. 1I, compare to wild type in H; Pankratz and Hoch, 1995). In summary, these results suggest that hedgehog signaling is essential for the invagination of the ectodermal proventricular cells (Fig. 1J).

Hedgehog is required for the specification of the anterior boundary cells

We have previously shown that the inward movement of the ectodermal proventricular cells is controlled by the anterior boundary cells, in which the Notch signaling pathway is activated (Fuss et al., 2004; Josten et al., 2004). To test whether the anterior boundary cells are compromised in *hedgehog* mutants, we first used the Grainyhead (Grh)/Suppressor of

Fig. 1. *hh* is required for proventriculus development. (A–C) *Hh* mRNA expression in the proventriculus of stages 13 (A), 15 (B), and 16 (C) wild-type embryos. The epithelial gut tube is depicted by dashed lines; *hh* expression is detected in the ectodermal part of the proventriculus. (D, E) Co-immunostaining of Hh (green) and Fkh (red) in the foregut (fg) of stage 12 (D) and stage 13 (E) wild-type embryos. (F–I) Anti-Fkh (red)/anti-Dve (green) immunostaining of the proventriculus (pv) of stage 13 (F), stage 15 (G), and stage 17 (I) *hh* loss of function mutants, and of a stage 17 (H) wild-type embryo. The evagination and constriction stages during proventriculus development are not affected; however, the inward movement of the ectodermal cells from stage 15 onwards fails to occur. The wild-type proventriculus shows the invaginating ectodermal part (H, red). (J) Schematic representation of the proventriculus development in wild-type and *hh* mutants. Anterior boundary cells (ac) in yellow and posterior boundary cells (pc) in red.


A
Wt
st13
hh mRNA
B
Wt
st15
hh mRNA
C
Wt
st16
D
Wt
fg
st12
Hh
fg
Fkh
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merge
E
Wt
fg
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F
hhIJ35
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J
mesoderm
endoderm
ectoderm
ac
pc
evagination
st.11
constriction
st.13
wildtype
inward movement
st.15
folding
st.17
hedgehog mutant
failure of inward movement
st.13
st.17

Hairless (Su(H)-lacZ) reporter construct (Gbe-*Su(H)m8-lacZ*; Furriols and Bray, 2001; Bray and Furriols, 2001), that we have applied previously to monitor the activity of the Notch signaling pathway (Fuss and Hoch, 2002; Fuss et al., 2004). This construct carries multiple Su(H)-binding sites from the *E(spl) m8* gene combined with binding sites for the transcription factor Grainy-head (Grh) (Furriols and Bray, 2001; Bray and Furriols, 2001). In cells, in which Notch signaling is active and Grh is expressed, Su(H) cooperates with Grh to yield high levels of reporter gene expression whereas reporter gene expression is repressed in cells in which Notch is inactive (Furriols and Bray, 2001). Reporter gene expression in corresponding transgenic embryos reflects the range of Notch signaling.

In *hedgehog* mutants, we find that reporter gene expression is specifically lost in the anterior boundary cells, whereas it is not altered in the posterior boundary cells (Fig. 2B, compare to wild type in A). This is consistent with the invagination defect observed in *hedgehog* mutants. Cell counts indicate that the number of cells in the proventriculus primordium of *hedgehog* mutants is not altered as compared to wild type in which the cell number is 35±2. To further examine whether *hedgehog* has a specific role in the allocation of the anterior boundary cells, we monitored the expression of *wingless*, which is also expressed in the anterior and the posterior boundary cells in the proventriculus primordium (Fig. 2C; Josten et al., 2004). In *hedgehog* mutants, we find a specific loss of *wingless* expression in the anterior boundary cells (Fig. 2D, asterisk; compare to wild type in C), whereas the posterior *wingless* domain is not affected. This is consistent with previous findings that temperature-sensitive *wingless* mutants show an invagination defect of the ectodermal proventricular cells (Pankratz and Hoch, 1995), which is very similar to the phenotype observed in *hedgehog* mutants. In summary, these data provide evidence that hedgehog is required for the expression of Notch target genes and of *wingless* in the anterior boundary cells, whereas JAK/STAT signaling induces the expression of the Notch target genes such as *short stop* in the posterior boundary cells (Fig. 2E; Fuss et al., 2004; Josten et al., 2004).

To further test this, we analyzed the expression of a wingless target gene in the proventriculus primordium, the *innexin2* gene (Bauer et al., 2002). Innexin2 encodes a member of the innexin multiprotein family of gap junction protein proteins (see Bauer et al., 2005; Phelan, 2005, for reviews) and zygotic *innexin2* (*kropf*) mutants show a feeding defect, which is associated with a failure of proventriculus formation (Bauer et al., 2002). *innexin2* mRNA is initially expressed in the early evagination stage in a broad domain covering both the ectodermal and endodermal precursor cells of the proventriculus primordium (Figs. 3A–C). In the subsequent keyhole stage, when the ectodermal cells start to invaginate into the endodermal layer, high levels of *innexin2* mRNA expression are found in the ectodermal cell population including the anterior boundary cells that move inward and to endodermal cells later forming the rim of the proventriculus chamber, as shown in Figs. 3B and C. In the proventricular cells, we find an accumulation of innexin2 protein in the membranes, as shown by anti-innexin2/anti-FASIII double

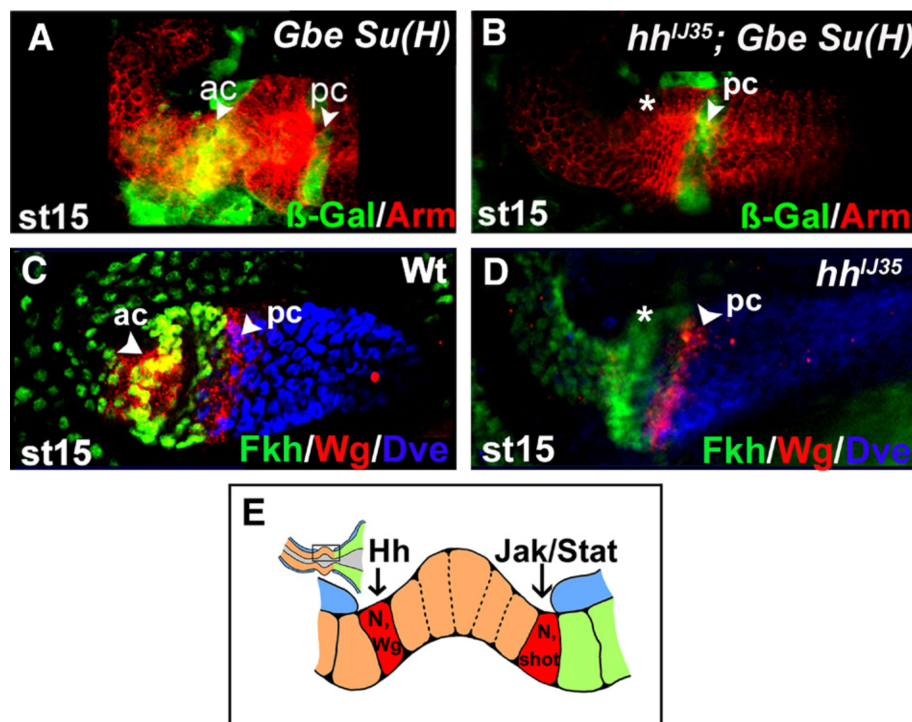


Fig. 2. *Notch* and *wingless* activities in the anterior boundary cells are dependent on *hedgehog*. (A, B) Notch signaling activity monitored by Gbe-*Su(H)m8-lacZ* expression (green) in the anterior (ac) and posterior boundary cells (pc) of stage 15 wild-type (A) and *hh* mutant (B) embryos. Arm (red) is used as a membrane marker. Note the loss of Notch signaling activity in the ac cells in the mutants (asterisk). (C, D) Anti-Fkh (ectoderm; green)/anti-Wg (red)/anti-Dve (endoderm; blue) antibody stainings of stage 15 wild-type (C) and *hh* mutant embryos (D). Whereas *wg* is expressed in the ac and pc in wild-type embryos, the ac domain is missing in *hh* mutants (asterisk). (E) Schematic representations of the Hh and JAK/STAT pathways controlling ac and pc allocation and proventriculus morphogenesis. For further details, see text.

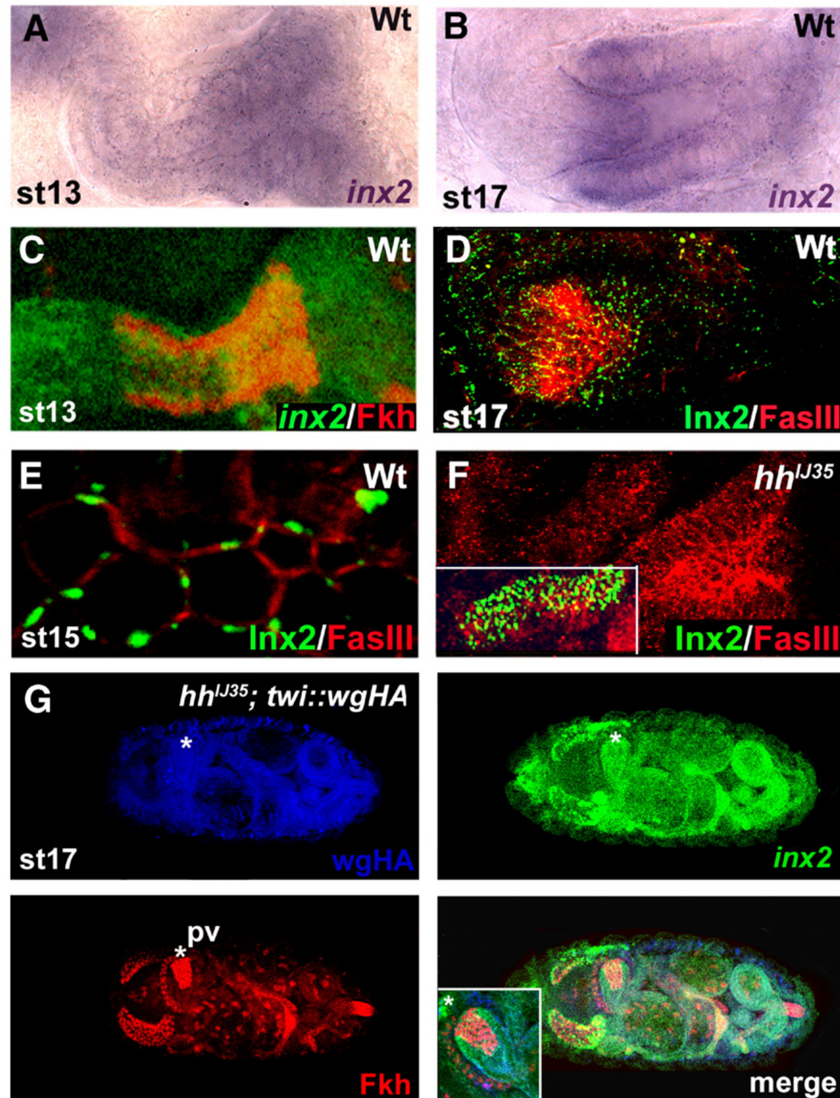


Fig. 3. *hedgehog* regulates innexin2 expression in the proventriculus. (A, B) *Inx2* mRNA expression in the proventriculus of stages 13 (A) and 17 (B) wild-type embryos. *inx2* expression is found in the ectodermal and endodermal cells of the proventriculus. (C) Fluorescence *in situ* hybridization of *inx2* mRNA (green) costained with the ectodermal foregut marker Fkh (red). (D) Anti-Inx2 (green)/anti-FasIII (red) antibody staining of a stage 17 wild-type proventriculus. (E) Wild-type stage 15 proventriculus. Inx2 protein is localized within gap junctions in the lateral membranes of proventricular cells. (F) Anti-Inx2 (green)/anti-Arm (red) antibody stainings of a stage 13 *hh* mutant embryo. Inx2 protein expression is absent in the proventriculus, however, inx2 expression is still visible in the salivary glands (F, inset). (G) Rescue of *inx2* expression in *hh* mutants by overexpression of *wg*. Fluorescence *in situ* hybridization of *inx2* mRNA (green) combined with antibody stainings against wgHA (blue) and Fkh (red). Inset in merged picture shows magnification of the invaginated proventriculus. The asterisk (*) marks the proventriculus. For further details, see text.

staining (Figs. 3D, E; Bauer et al., 2001), consistent with its function as a gap junction protein. In *hedgehog* mutants, *innexin2* expression is specifically lost in the proventriculus (Fig. 3F) whereas it is still present in the salivary glands of the embryo (Fig. 3F, inset). When we re-supplied wingless in the genetic background of *hedgehog* mutants by using the *twi*-Gal4 driver and UAS-*wgHA* effector lines (Materials and methods), *innexin2* expression was rescued (Fig. 3G), providing further evidence that *innexin2* is a target gene of wingless in the proventriculus primordium. Furthermore, the cardia arrest phenotype of *hedgehog* mutants was rescued and the ectodermal cells properly invaginated into the endodermal portion of the proventriculus primordium (Fig. 3G, inset in merge).

To investigate whether innexin2-dependent gap junction communication is affected, we performed dye tracer injection experiments with isolated proventriculi of early stage 13 embryos (Fig. 4; Materials and methods). We used Lucifer yellow, which was previously shown to be passed via gap junctions from cell to cell (Warner and Lawrence, 1982) and injected the dye into the proventricular endoderm of wild type (Figs. 4A, C, E), and *kropf* (Figs. 4B, D, F) mutant embryos. Whereas after 2 h, we find that Lucifer yellow has diffused in average 6- to 8-cell diameters in wild-type embryos, the passage of the dye is strongly reduced in *kropf* mutants. After 16 h, the dye diffused approximately into 20 cells in wild type, as compared to three cells in a *kropf* mutant proventriculus. Since cell

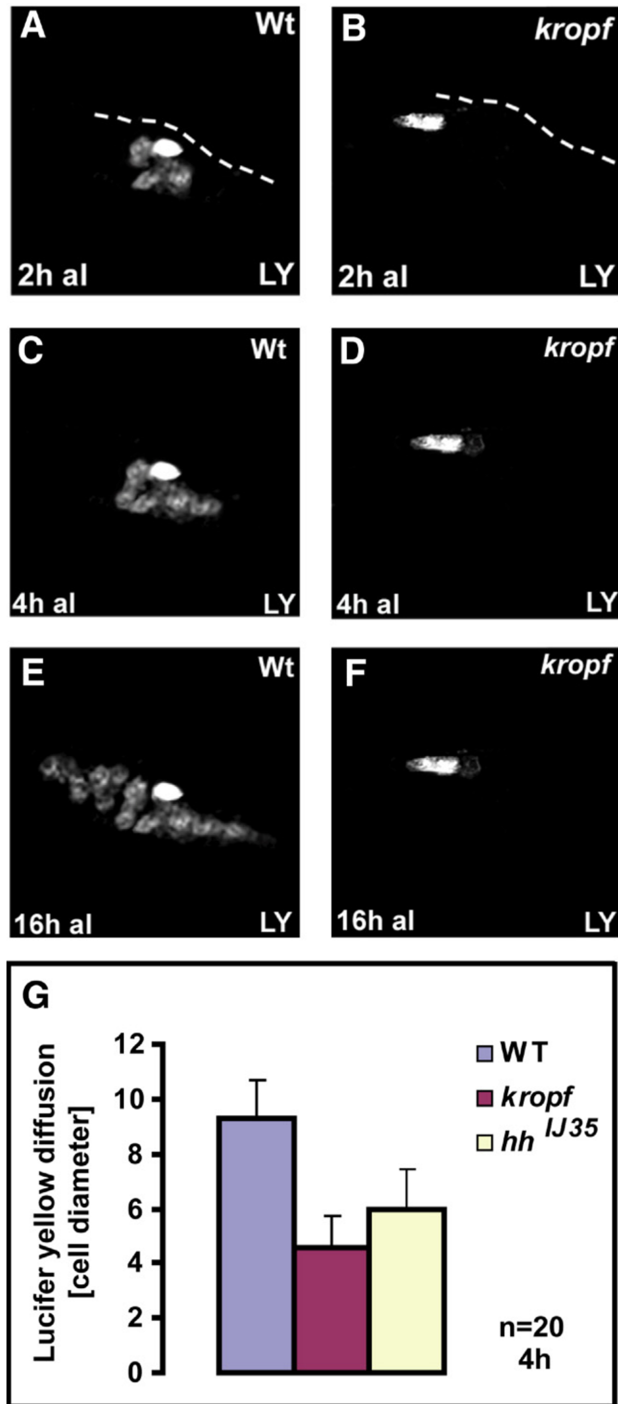


Fig. 4. Reduced gap junction-mediated dye transfer in *kropf* and *hh* mutants. (A–F) Lucifer yellow injection into proventriculi of wild-type (A, C, E), *kropf* (B, D, F) stage 15 mutants; dye transfer was monitored 2 h (A, B), 4 h (C, D), and 16 h (E, F) after injection. After 16 h, the injection dye spread into approximately 20 cells in wild-type, as compared to 3–4 cells in *kropf* mutants. (G) Lucifer yellow diffusion through wild-type proventricular cells compared to diffusion through proventricular cells of *kropf* and *hh* mutants 4 h after injection. Dye diffusion is strongly decreased in both, *kropf* and *hh* mutant proventriculi.

death did not occur in the proventricular endoderm until 24 h after injection, as shown by TUNEL experiments (data not shown), we conclude that *innexin2* mutants have defects in gap junction-mediated cell-to-cell coupling. A significant reduction of dye coupling was also observed upon injection of Lucifer yellow into the endoderm of the proventriculi of *hedgehog* mutants (Fig. 4G), consistent with *innexin2* being genetically downstream of hedgehog in the proventriculus.

Innexin2-dependent activation of hedgehog, wingless, and Delta transcription in the proventriculus primordium

The above data in combination with the similarity of the proventriculus invagination defects observed in *hedgehog*, *wingless* and *kropf* mutants are consistent with a model in which *hedgehog* acts on *wingless* thereby regulating *innexin2*-dependent gap junction activity required for the invagination of the ectodermal cells of the proventriculus primordium.

To test whether gap junction communication is, in turn, required for paracrine signaling during proventriculus development, we analyzed the expression of *hedgehog* and *wingless* in *kropf* mutants and in innexin2 overexpression experiments. In the proventriculus primordium of stage 13 *kropf* mutants, we find a loss of *hedgehog* (Fig. 5B, compare to A) and a strong reduction of *wingless* expression (Fig. 5E, compare to D), as compared to wild-type embryos. Overexpression of *innexin2* in the proventricular cells by using the *14-3fkh*-Gal4 driver in combination with the UAS-*innexin2* flies results in a dramatic expansion of the *hedgehog* (Fig. 5C, compare to A and Figs. 1A–C) and *wingless* expression domains (Fig. 5F, compare to D and Fig. 2C). A similar ectopic expression of wingless in the proventricular endoderm is obtained upon ectopic *hedgehog* expression (Figs. 5G, H, compare to F). The expression domain of Delta encoding a ligand of the Notch receptor also expands into the proventricular endoderm when *innexin2* is overexpressed (Fig. 5J, compare to I). These data indicate that innexin2 is essential for the transcriptional activation of *hedgehog*, *wingless* and *Delta*.

To further test this, we performed a series of quantitative real-time (RT) PCR experiments on mRNA isolated from *kropf* mutant embryos and from embryos in which *innexin2* was either knocked down by RNAi or overexpressed by using UAS-*innexin*2 (Fig. 6). The expression levels of *actin 5C* (*Act5C*, *act*) and *Ribosomal protein L32* (*RpL32*, *rp49*) were used as reference genes for normalization (Materials and methods). For the knockdown of *innexin2* expression in embryos, we generated a transgenic line carrying a RNAi knockdown construct for *innexin*2 (UASwiz*inx2*) in which part of its coding regions was cloned into a "tail to tail" orientation (Materials and methods). In zygotic *kropf* mutants, which still show considerable maternal *innexin2* mRNA expression (Bauer et al., 2004), we found significant reductions of *hedgehog*, *wingless*, and *Delta* mRNA levels, as compared to wild type (Fig. 6A). When we used the *hs*-Gal4 driver in combination with UASwiz*inx2* and heat shocked the transgenic embryos for 30 min followed by a 2.5 h incubation time to achieve RNAi knockdown of *innexin2*, we obtained a 5-, 4-, and 1.25-fold reduction of the *hedgehog*, *wingless*, and *Delta* mRNA levels, respectively, as compared to wild type (Fig. 6A). This effect was even more dramatic when using the 14-3 *fkh*-Gal4 driver which constitutively drives in most of the tissues of the embryo from early

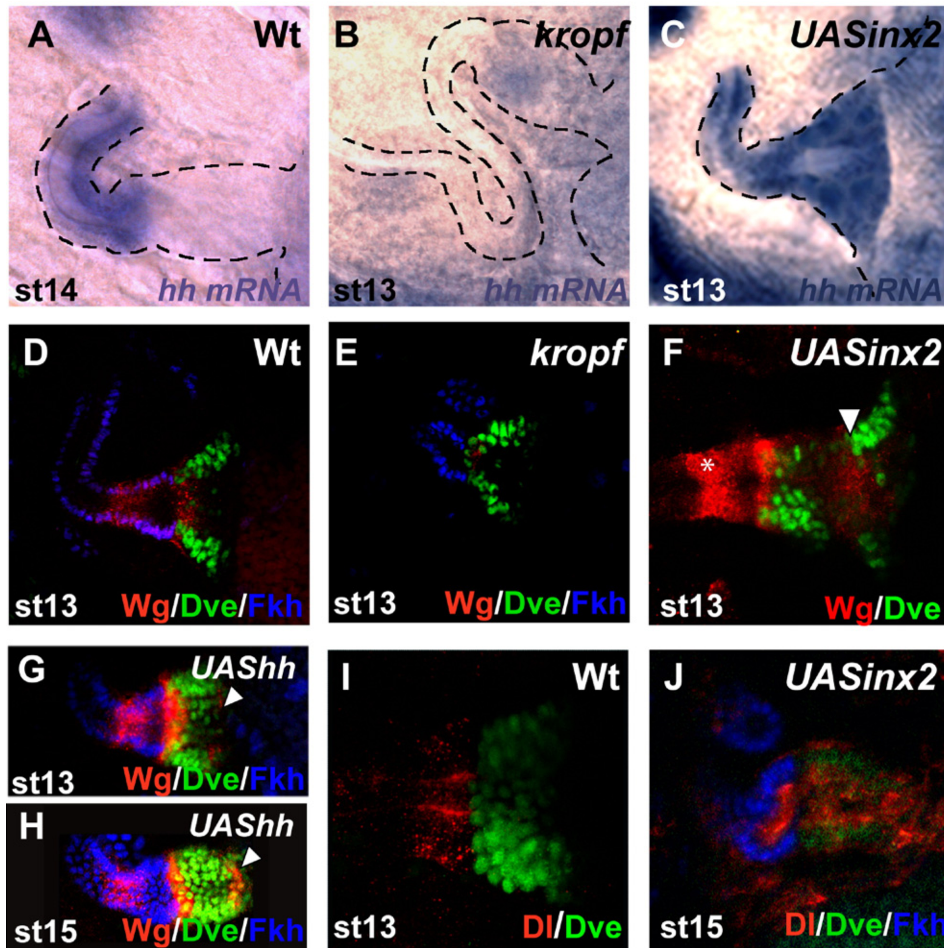


Fig. 5. Innexin2 controls the transcription of *hh*, *wg* and *Delta*. (A–C) *hh* mRNA expression in the proventriculus of a stage 14 wild-type embryo (A), a late stage 13 *kropf* mutant (B) and a late stage 13 embryo overexpressing *inx2* (C). Whereas *hh* is localized in the ectodermal part of the proventriculus (A), expression is strongly reduced in *kropf* mutants (B) and strongly expanded into the endodermal part of the proventriculus upon overexpression of *inx2* (C). (D–F) Anti-Fkh (blue)/anti-Wg (red)/anti-Dve (green) antibody stainings of a stage 13 wild type (D), a *kropf* mutant (E), and an embryo in which inx2 was overexpressed (F). Note the loss of Wg expression in the *kropf* mutant, the expanded expression of *Wg* (asterisk) and the ectopic Wg expression domain (white arrowhead) upon overexpression of *inx2*. (G, H) Anti-Fkh (blue)/anti-Wg (red)/anti-Dve (green) antibody staining of the proventriculus of a stage 13 (G) and a stage 15 (H) embryo, overexpressing *hh*. Note the ectopic wg expression domain in the dve-positive endoderm (white arrowhead; compare to panel F). (I, J) Anti-Dl (red)/anti-Dve (green) antibody stainings of a stage 13 wild-type embryo (I), and an embryo in which *inx2* was overexpressed (J). Dl, which is restricted to the ectodermal cells in wild type, expands into the proventricular endoderm upon *inx2* overexpression.

stages onwards (Supplementary Figure 1), in combination with UASwiz*inx2*. In these embryos, *hedgehog* mRNA is reduced by 8.3-fold, *wingless* mRNA by 33-fold, and *Delta* mRNA by 8.3-fold (Fig. 6A), as compared to the wild-type control. Consistent with these results, we find a downregulation of the mRNA expression of *hedgehog*, *wingless* and *Delta* during embryogenesis in *kropf* mutants, as shown by situ hybridization experiments (Figs. 6D, E; not shown). For the *innexin2* overexpression experiments, we used exclusively stage 13 embryos in which the ectodermal proventricular cells start to invaginate. After a heat shock for 1 h followed by 2.5 h incubation time the mRNA was prepared and used for quantitative RT PCR analysis. We find that the levels of *hedgehog* mRNA are increased in these embryos by 2.5- fold whereas *wingless* and *Delta* mRNA expression increases by 4-fold and 10-fold, respectively, as compared to the controls (Fig. 6B). In contrast, when we used a transgenic line carrying an innexin2 protein variant with a deletion of the cytoplasmic C-terminus, *inx2ΔCT*, for the ectopic expression experiments (*hs*-Gal4::UAS-*inx2ΔCT*), we found a similar downregulation of the *hedgehog*, *wingless*, and *Delta* mRNA levels, as obtained in *kropf* mutants or in *innexin2* RNAi knockdown embryos (Fig. 6C, compare to A). These data provide evidence for the direct or indirect involvement of the innexin2 C-terminus in the transcriptional control of the signaling molecules.

Discussion

Cell movement and folding of epithelial cell layers during foregut organogenesis require hedgehog and innexin2-mediated gap junction communication

In both vertebrates and invertebrates, the posterior foregut constitutes a center of organogenesis from which gut-associated

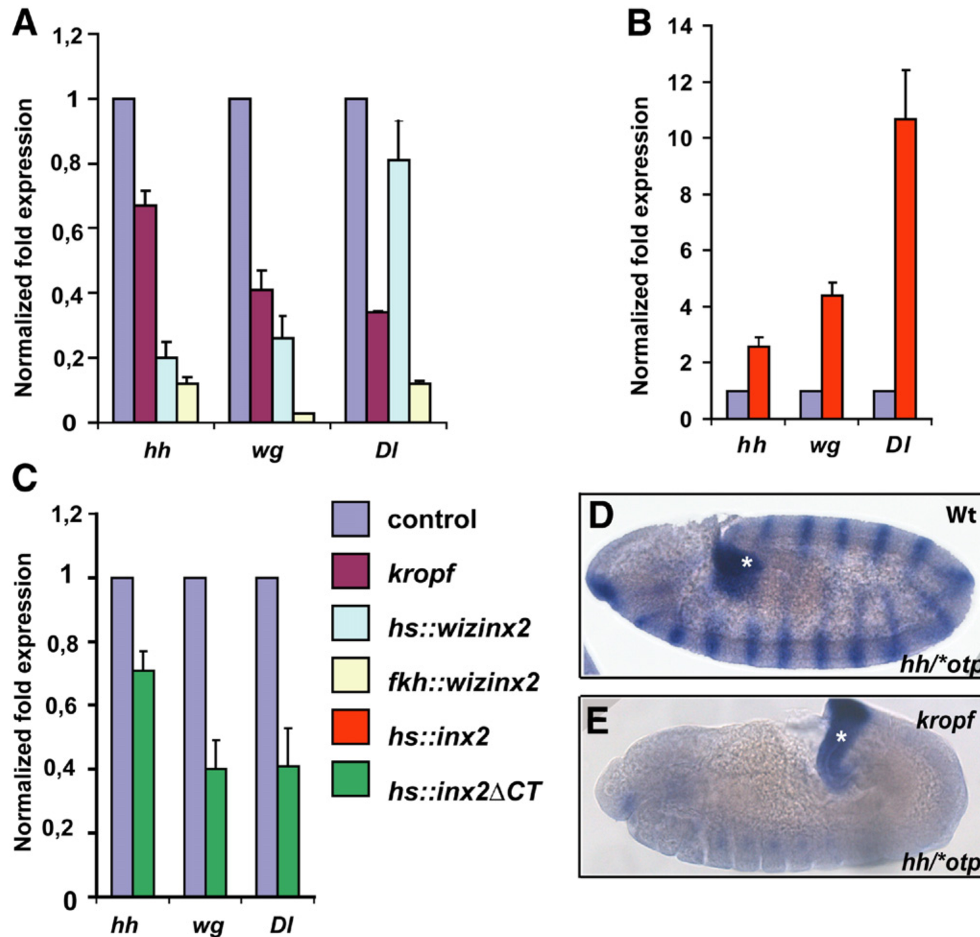


Fig. 6. Quantitative real-time PCR demonstrating the transcriptional regulation of *hh*, *wg* and *Delta* in response to innexin2 activity. (A) Decreased expression of *hh*, *wg* and *Dl* mRNA levels in *kropf* mutants as well as in *inx2* RNAi knockdowns using the UAS-wiz*inx2* in combination with *hs*-Gal4 or 14-3 *fkh*-Gal4 driver lines (Materials and methods; see text). (B) In contrast, overexpression of *inx*2 by using UAS-*inx*2 in combination with *hs*-Gal4 results in enhanced *Dl*, *wg* and *hh* mRNA levels, as compared to the control. (C) Overexpression of *inx2ΔCT* containing a C-terminal deletion (Inx2 aa 301–367) with *hs*-Gal4 results in a similar reduction of *hh*, *wg* and *Dl* mRNA, as observed in *kropf* mutants (compare to panel A). (D, E) In situ hybridization of wt (D) and *kropf* (E) mutant embryos using antisense probes against hh and the hindgut marker otp which was used as a staining control. Note the significant reduction of *hh* expression in *kropf* mutants.

organs such as the lung in vertebrates or the proventriculus in *Drosophila* develop (Grapin-Botton and Melton, 2000; Fuss and Hoch, 1998; Fuss et al., 2004). Proventriculus development involves the folding and invagination of epithelial cell layers to generate a multiply-folded organ. Two cell populations, the anterior and the posterior boundary cells, were shown previously to control cell movement and the folding of the proventriculus organ (Fuss et al., 2004; Josten et al., 2004). In the posterior boundary cells, which organize the endoderm rim of the proventriculus, the JAK/STAT signaling cascade cooperates with Notch signaling to control the expression of the gene *short stop* encoding a cytoskeletal crosslinker protein of the spectraplakin superfamily (Gregory and Brown, 1998; Strumpf and Volk, 1998; Röper et al., 2002). Thereby the Notch signaling pathway is connected to cytoskeletal organization in the posterior boundary cells, which have to provide a stiffness function to enable the invagination of the ectodermal foregut cells (Fuss et al., 2004; Josten et al., 2004). The findings in this paper provide evidence that *hedgehog* is essential for the Notch signaling-dependent allocation of the anterior boundary cells. In amorphic *hedgehog* mutants, evagination and the formation of the constriction at the ectoderm/endoderm boundary are not affected, however, the inward movement of the anterior boundary cells is not initiated at the keyhole stage (Fig. 1G). The lack of cell movement of the ectodermal proventricular cells is consistent with our finding that *hedgehog* specifically controls Notch target gene activity in the anterior boundary cells (Fig. 2E). Our genetic experiments further identify *wingless* as a target gene of *hedgehog* in the anterior boundary cells. *wingless*, in turn, controls the transcription of the *innexin2* gene, which is expressed in the invaginating proventricular cells. When wingless is re-supplied in the genetic background of *hedgehog* mutants, *innexin2* expression is rescued (Fig. 3G), providing further evidence that *innexin2* is a target gene of wingless in the proventriculus primordium. Innexin2 encodes a member of the innexin family of gap junction proteins and is essential for the development of epithelial tissues (Bauer et al., 2004). In the proventriculus, *innexin2* mRNA is initially expressed in the early evagination stage in a broad domain covering both the ectodermal and endodermal precursor cells of

the proventriculus primordium (Figs. 3A–C). When the ectodermal cells start to invaginate into the proventricular endoderm, *innexin2* expression is upregulated in the ectodermal cell layer. Invagination of the ectodermal cells fails in *hedgehog*, *wingless* and *kropf* mutant proventriculi and our dye tracer injection experiments demonstrate that *hedgehog* and *kropf* mutants show a strong reduction of gap junction communication (Fig. 4G). These data suggest that the direct coupling of cells via innexin2-containing gap junctions, which are induced in response to *hedgehog* and *wingless* activities, is important for the coordinated movement of the ectodermal cell layer. It is known from extensive studies in mammals that the coupling of cells and tissues via gap junctions enables the diffusion of second messengers, such as Ca^{2+}, inositol-trisphosphate (IP3) or cyclic nucleotides to allow the rapid coordination of cellular behavior during morphogenetic processes such as cell migration and growth control (Chen et al., 1995; Wei et al., 2004). Cell movement and folding involves a modulation of cell adhesion and of cytoskeletal architecture of the proventricular cells. A functional interaction of innexin2 with the cell adhesion regulator *D*E-cadherin, which is a core component of adherens junctions has been shown recently by co-immunoprecipitation, yeast two-hybrid studies, and genetic analysis (Bauer et al., 2004). In mutants of *D*E-cadherin, innexin2 is mislocalized and vice versa suggesting that the regulation of cell adhesion and gap junction-mediated communication may be linked (Bauer et al., 2004; 2006). Similar evidence for a coordinated regulation of connexin activity and N-cadherin has been obtained in mammals during migration of neural crest cells (Wei et al., 2005).

Gap junctions are crucial for the transcriptional activation of morphogen-encoding signals

In *kropf* mutants or innexin2 knockdown animals, *hedgehog*, *wingless* and *Delta* transcription is strongly reduced as shown by *in situ* hybridization and by quantitative RT PCR experiments using mRNAs isolated from staged embryos. Furthermore, hedgehog, wingless and Delta are ectopically expressed and their mRNA is upregulated in embryos in which innexin2 is overexpressed. In summary, these experiments provide strong support that the gap junction protein innexin2 plays an essential role enabling or promoting transcriptional activation of *hedgehog*, *wingless* and *Delta*. These data point towards an essential requirement of gap junction communication for the transcriptional activation of morphogen-encoding genes activating evolutionary conserved signaling cascades essential for patterning in animals (Serrano and O'Farrell, 1997; for review, see Crozatier et al., 2004). It is of note that gap junctions are established at very early stages of embryonic development, correlating with a maternal and zygotic expression of innexin2 and other innexin family members (Bauer et al., 2002, 2004). *kropf* mutant animals, which are devoid of maternal and zygotic innexin2 expression are early embryonic lethal and develop no epithelia (Bauer et al., 2004), consistent with a fundamental role of gap junctions in development, on top of which pattern formation of tissues and organs may occur. It has been shown previously that gap junctions are essential for *C. elegans*, *Drosophila*, and vertebrate embryogenesis from early stages onwards (Bauer et al., 2005; Wei et al., 2004). In the nematode *C. elegans*, a transient network formed by the innexin gap junction protein NSY-5 was recently shown to coordinate left–right asymmetry in the developing nervous system (Chuang et al., 2007). Previous findings in chick and *Xenopus laevis* embryos have suggested an essential role of connexin43-mediated gap junction for the determination of the left–right asymmetry of the embryos (Levin and Mercola, 1999). Treatment of cultured chick embryos with lindane, which results in a decreased gap junctional communication, frequently unbiased normal left–right asymmetry of *Sonic hedgehog* and *Nodal* gene expression, causing the normally left-sided program to be recapitulated. An important role of connexin43 (Cx43)-dependent gap junction communication for *sonic hedgehog* expression was also observed in limb patterning of the chick wing (Law et al., 2002). Additionally, modulation of gap junctions in *Xenopus* embryos by pharmacological agents specifically induced heterotaxia involving mirror-image reversals of the heart, gut, and gall bladder (Levin and Mercola, 1998, 1999). These data in combination with our findings indicate that the transcriptional regulation of hedgehog and other morphogen-encoding genes by gap junction proteins may be evolutionary conserved between deuterostomes (vertebrates) and protostomes (*Drosophila*), although the *Drosophila innexin* gap junction genes share very little sequence homology with the *connexin* genes (Bauer et al., 2005; Phelan and Starich, 2001). The molecular mechanism underlying *innexin2*-mediated transcriptional regulation of *hedgehog*, *wingless* and *Delta* is not clear. It has been proposed that the nuclear localization of the carboxy-tail of connexin43 may exert effects on gene expression and growth in cardiomyocytes and HeLa cells (Dang et al., 2003). This would infer a cleavage of connexin43 to release the C-terminus, however, *in vivo* evidence for this event is still lacking. Sequence analysis reveals a nuclear receptor recognition motif (LXXLL motif; Savkur and Burris, 2004) within the C-terminus of innexin2 (Bauer et al., 2005). It has been demonstrated that this recognition motif mediates the interaction of coactivators with nuclear receptors. However, we have no immunohistochemical evidence for a nuclear localization of innexin2 or the innexin2 C-terminus in *Drosophila* embryonic cells (H. Lechner and M. Hoch, unpublished) indicating that a direct involvement of innexin2 in regulating transcription of target genes may not occur. The direct association of a transcription factor with gap junctions has been recently proposed for the mouse homolog of ZO-1-associated nucleic acid-binding protein (ZONAB). This transcription factor binds to ZO-1, which is associated with oligodendrocyte, astrocyte and retina gap junctions (Penes et al., 2005; Ciolofan et al., 2006). It is possible that innexin2-dependent transcriptional regulation may involve a similar type of mechanism: a still unknown transcriptional regulator associated with the C-terminus of innexin2-containing gap junctions could be released upon modulation of gap junction composition thereby modulating the transcription of innexin2-dependent target genes.

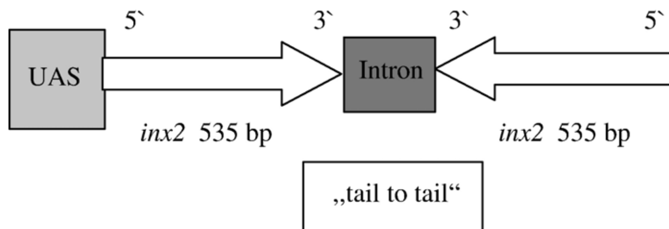
Materials and methods

Drosophila stocks and analysis of mutants

The Oregon R strain was used as wild type, for mutant analysis we used hh^{IJ35} (Pankratz and Hoch, 1995) and *kropf* mutant embryos (Bauer et al., 2002). For phenotypic analysis all *kropf* alleles were balanced over *FM7c*, P{*kr*-Gal4}, P{UAS-*GFP*} and mutant larvae were scored based on loss of fluorescence detection. Notch signaling activity was detected by the Gbe-*Su(H)m8-lacZ* reporter construct (Furriols and Bray, 2001). Ectopic expression studies were performed using the Gal4 driver lines *hs*-GAL4 (Bloomington stock center) and 14-3 forkhead (*fkh*-Gal4 (Fuss and Hoch, 1998)). 14-3 *fkh*-Gal4 drives expression from stage 10 onwards in the esophagus and in the endodermal part of the proventriculus primordium. As UAS effector strains, we used UAS-*inx2*, UAS-wiz*inx2*, and *UAS-wgHA*. For rescue of *innexin2* expression in hh^{IJ35} mutants by re-supplying *wg*, flies of the genotype *twi*-Gal4/+; +/+; hh^{IJ35}/+ were crossed to +/+; UAS-*wgHA*/+; hh^{IJ35}/+. Homozygous hh^{IJ35} mutants were easily identified based on their reduced size (Tabata et al., 1992).

Overexpression, RNAi constructs and stable transgenic fly lines

Cloning of recombinant plasmids for the creation of stable RNAi inducible fly strains was performed as described by Lee and Carthew (2003). A 535-bp DNA fragment of *innexin2* (bases No. 323-857 of the *inx2* cDNA) was amplified by PCR with PCR primers containing at their 5′ ends an *Xba*I restriction site, which is compatible with *Avr*II and *Nhe*I. The RNAi construct was made by two sequential insertions of the same PCR product into the *Avr*II and *Nhe*I sites of pWIZ (gift from R.W. Carthew), which was dephosphorylated with alkaline shrimp phosphatase (Roche) prior to ligation. For transformation we used SURE competent cells (Stratagene). Recombinants with the insert in opposite orientation to the first were screened and selected.



UAS-*inx2ΔCT*, coding an innexin2 variant with a deletion of the cytoplasmic C-terminus (amino acid (aa) 301–367) was generated by PCR reaction from pIB*inx2* containing the entire *innexin2* cDNA. *inx2ΔCT* was inserted into pP{UAST} using *Eco*RI/*Xba*I to produce pP{UAST-*inx2ΔCT*}. Transgenic fly lines were generated by standard P element transformation procedures (Rubin and Spradling, 1982; Brand and Perrimon, 1993).

Immunostainings and in situ hybridizations

Embryos were staged and stained as described previously (Fuss et al., 2004). The following antibodies were used: anti-Arm (1:50), anti-FasIII (1:5), and anti-Wg (1:20) from Developmental Studies Hybridoma Bank. Additionally, we used anti-Dve (1:1000, Fuss et al., 2004), anti-Fkh (1:100, P. Carrera), anti-βGal (1:100, Promega), anti-Hh (1:1000, Tabata and Kornberg, 1994) anti-HA antibody (1:200, St. Cruz, CA), and anti-Inx2 (Bauer et al., 2002). As secondary antibodies, we used $Alexa^{488}$ (1:400), $Alexa^{555}$ (1:400), Cy-3 (1:200) and $Alexa^{633}$ (1:200), all from Molecular Probes. Fluorescent images were recorded using a Leica TSP2 confocal microscope (Leica, Wetzlar, Germany) and a Zeiss Axiovert 200 with ApoTome. Images of multi-labeled samples were acquired sequentially on separate channels. For *in situ* hybridization, full-length *hh* and *inx2* digoxygenin-labeled RNA antisense probes were generated by *in vitro* transcription according a protocol described previously (Josten et al., 2004). Fluorescent detection of *innexin 2* transcripts was performed as described previously (Kosman et al., 2004) in combination with the Tyramide Signal amplification (TSA) kit (Perkin Elmer) and by antibody staining. Briefly, the digoxygenin-labeled *innexin2* probe was detected with an anti-Dig antibody (sheep, 1:500, Dianova), followed by the incubation with a biotinylated anti-sheep–biotin antibody (donkey, 1:300, Dianova). The embryos were washed thoroughly with PBT in between and then incubated with Vectastain ABC reagent (Vector Laboratories, Ohio) for 45 min, followed by extensive washes with PBT. After incubation with TSA-Cy3 (20 min) and extensive washing the embryos were costained with different antibodies.

RNA isolation and real-time PCR

Drosophila embryos (stage 13) were washed thoroughly with water, transferred to lysis buffer (supplied with RNA isolation kit) and homogenized (Ultra-Turrax T25basic) at full speed for 1 min. Total RNA was isolated by using NucleoSpin RNA II kit (Macherey and Nagel, includes on-column DNaseI treatment).

First strand cDNA reaction was carried out with 1 μg total RNA using the iScript Transcription Kit (BioRad) including DNaseI treatment following the supplier's protocol. For real-time PCR the reaction consisted of cDNA template (1 μl of 1:100 diluted cDNA first strand reaction), forward and reverse primers (200 nM final concentrations, primer sequences see below) and iQ SYBR Green Supermix (BIO-RAD) in a total volume of 25 μl. Per template 3 reactions were done in parallel. These were repeated with independently isolated RNA samples from different egg collections. The experiments were performed with iQ5 Real-Time PCR Detection System from BIO-RAD. Expressions of *actin 5C* (*Act5C*, *act*) and *Ribosomal protein L32* (*RpL32*, *rp49*) were used as reference genes for normalization (Fuss et al., 2006). Standard control PCR reactions were carried out to test for contaminations. Real-time PCR was analyzed using BIO-RAD iQ5 Optical System software (version 1.1.1442.OCR), following the instruction provided by the supplier, and Microsoft Excel. The following oligonucleotides were used for real-time PCR analysis:

Act5C: GTGCACCGCAAGTGCTTCTAA (act-Sy-F1)
TGCTGCACTCCAAACTTCCAC (act-Sy-R1)
RpL32: GCTAAGCTGTCGCACAAATG (rp49-Real-F1)
GTTCGATCCGTAACCGATGT (rp49-Real-R1)
Hedgehog: AATCGGCAAACTCCCGGAG (hh-Sy-F1)
CAAAGGGCTTGAACTCGCC (hh-Sy-R1)
Wingless: GTGCAAGCTGTGTCGGACCA (wg-Sy-F1)
AGAACGAAGAGGGCGGCTTC (wg-Sy-R1)
Delta: GCTGTTTTCTCCGTTGCGAT (Dl-Sy-F1)
GCGTCGTCCTTTTCCTGAGC (Dl-Sy-R1)
Innexin2: CCTACTCCGAGCCCGTTCC (inx2-Sy-F1).
TGCCCAGCTGATAGAGCAGG (inx2-Sy-R1);

Dye tracer injection assays

Flies were allowed to lay eggs for 1 h on apple juice agar plates followed by an incubation of these plates at 25 °C for 16 h. Finally, 20 proventriculi of manually dechorionated embryos of each genotype were prepared under *Drosophila* ringer solution. Following the preparation, proventriculi were pinned with wolfram pins on Soelgard media.

For microinjection an ultrathin glass capillary was loaded with Lucifer Yellow, which was previously shown to be passed via gap junctions from cell to cell (Warner and Lawrence, 1982). The injection procedure was performed with the aid of an Eppendorf micromanipulator (injector Eppendorf TransferMan NK2) connected to a FemtoJet microinjector (Eppendorf FemtoJet) under an Olympus CKX31 (Olympus) inverse microscope. Images were recorded using a Leica TSP2 confocal microscope (Leica, Wetzlar, Germany).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2007.07.008.

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