The transcription factors KNIRPS and KNIRPS RELATED control cell migration and branch morphogenesis during *Drosophila* tracheal development

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SUMMARY

Cell migration during embryonic tracheal system development in *Drosophila* requires DPP and EGF signaling to generate the archetypal branching pattern. We show that two genes encoding the transcription factors KNIRPS and KNIRPS RELATED possess multiple and redundant functions during tracheal development. *knirps/knirps related* activity is necessary to mediate DPP signaling which is required for tracheal cell migration and formation of the dorsal and ventral branches. Ectopic *knirps or knirps related* expression in lateral tracheal cells respecifies their anteroposterior to a dorsoventral migration behavior, similar to that observed in the case of ectopic DPP expression. In dorsal tracheal cells *knirps/knirps related* activity represses the transcription factor SPALT; this repression is essential for secondary and terminal branch formation. However, in cells of the dorsal trunk, *spalt* expression is required for normal anteroposterior cell migration and morphogenesis. *spalt* expression is maintained by the EGF receptor pathway and, hence, some of the opposing activities of the EGF and DPP signaling pathways are mediated by *spalt and knirps/knirps related*. Furthermore, we provide evidence that the border between cells acquiring dorsal branch and dorsal trunk identity is established by the direct interaction of KNIRPS with a *spalt* cis-regulatory element.

Key words: *knirps, spalt, Drosophila melanogaster*, Tracheal system, Cell migration, Morphogenesis

INTRODUCTION

Directed cell migration is an essential process during the development of multicellular organisms. This process involves an integrated interplay between locally distributed signaling molecules, their receptors and intracellular signal transduction pathways (Hynes and Lander, 1992; Montell, 1994; Lauffenburger and Horwitz, 1996). A model for study of the molecular mechanisms underlying directional cell migration and branch formation is the developing *Drosophila* tracheal system. It originates from ten metamerically ordered placodes on each side of the embryo. These placodes, which consist of about 90 ectodermal cells each, invaginate in a coordinated manner into the underlying mesoderm and form a stereotyped pattern of tubular structures. Cell migration is the predominant mechanism during branch formation, since tracheal development is a cell-proliferation-independent process. Some of the primary branches grow along the dorsoventral body axis to establish the dorsal, the lateral and the ganglionic branches. An additional set of primary branches extends along the anteroposterior axis to form the dorsal trunk and the visceral branches. The individual tracheal metameres on each side of the embryo eventually connect by fusion of the dorsal trunk and the lateral trunk branches. Unicellular secondary branches are subsequently formed and the two halves of the system become interconnected into a three-dimensional network by anastomosis formation. Finally, additional fusion and terminal branching lead to the highly branched tracheal network, which starts its function, the transport of gases, during the first larval instar stage (for detailed descriptions see Manning and Krasnow, 1993; Samakovlis et al., 1996).

During primary branch outgrowth, three different signaling pathways play a prominent role in directed tracheal cell migration. *branchless* (*bnl*), which codes for an FGF-like secreted molecule (BNL; Sutherland et al., 1996), is expressed in cell clusters surrounding the tracheal placodes. Its activity promotes guided tracheal cell migration, a process mediated by the FGF receptor tyrosine kinase BREATHLESS (*BTL*; Klämbt et al., 1992; Lee et al., 1996). In contrast to BNL signaling, which prefigures tracheal cell migration of all primary tracheal branches, the signaling molecules SPI (SPITZ) and DPP are required for anteroposterior and
dorsoventral tracheal cell migration, respectively. SPI is ubiquitously expressed and processed into a potent ligand by the transmembrane proteins RHOMBOID (RHO) and STAR (Schweitzer and Shilo, 1997). RHO is tightly regulated and its expression is confined to the central part of the tracheal placode (Wappner et al., 1997). Secreted SPI activates the EGF receptor (EGFR) signaling pathway in the tracheal placodes, which results in anteroposterior tracheal cell migration (Llimargas and Casanova, 1997; Wappner et al., 1997). DPP is expressed dorsally and ventrally of the developing placodes. The dorsal- and ventralmost tracheal cells respond to the DPP signal via the receptor serine-threonine kinases THICK VEINS (TKV) and PUNT (PUT), which ultimately lead to cell migration along the dorsoventral body axis (Affolter et al., 1994; Ruberte et al., 1995; Vincent et al., 1997). Hence, the EGF and DPP pathways are likely to specify directional tracheal cell migration by antagonistic effects and they determine localized gene expression patterns as monitored by spalt (sal) and knirps (kni) marker gene expression (Vincent et al., 1997; Wappner et al., 1997). sal encodes a zinc-finger-type transcription factor (SAL), which is expressed in the dorsal parts of all tracheal placodes (Kühnlein et al., 1994). During branch outgrowth, SAL expression persists in the cells of the dorsal trunk anterior and dorsal trunk posterior but decreases in the dorsal branch cells. SAL is essential for dorsal trunk cell migration in the anteroposterior direction, which ultimately leads to dorsal trunk formation (Kühnlein and Schuh, 1996).

Here we show that knirps (kni) and knirps related (knrl), which encode sequence-related zinc finger transcription factors (Rothe et al., 1989), are expressed in overlapping patterns and share redundant functions during tracheal development. Region-specific tracheal kni/knrl expression mediates DPP signaling required for cell migration along the dorsoventral body axis. Ectopic kni/knrl expression in lateral tracheal tracheal cells reprograms tracheal cell migration from anteroposterior to dorsoventral direction. Additionally, kni/knrl represses sal function and can thereby prevent dorsal trunk formation. In wild-type embryos, kni/knrl-dependent sal expression occurs in dorsal tracheal cells and is a prerequisite for normal dorsal branch morphogenesis. kni/knrl-dependent sal expression most likely involves a direct interaction of KNI with sal upstream sequences and results in the establishment of a distinct border between cells that gives rise to dorsal trunk and dorsal branches, respectively.

### MATERIALS AND METHODS

#### DNA analysis

Methods of DNA manipulation were performed according to standard protocols (Sambrook et al., 1989). To generate UAS-kni, the kni coding region of cDNA-cJ15 (Rothe et al., 1989) was cloned in the P-element vector pUAST (Brand and Perrimon, 1993). The resulting pUAST-kni plasmid was used for P-element mediated transformation of flies to generate UAS-kni effector lines. Electrophoretic mobility-shift assays were carried out as described (Kühnlein et al., 1997) and purified KNI (80 ng/µl) from bacterial extracts was provided by R. Rivera-Pomar.

#### Fly stocks

The following mutant fly lines were used in this study: kniFC13, rhoTM7, spiA14, flh2W74, fhp2C82, sal445, Df(3L)irX11 (Lindsley and Zimm, 1992).

#### Immunostainings

Whole-mount immunostainings were performed as described (Goldstein and Fryberg, 1994). The secondary antibody was revealed either by the horseradish peroxidase Elite ABC kit (Vector Laboratories) or by staining for alkaline phosphatase activity. To stain tracheal lumen the monoclonal antibody 2A12 was used (DSHB, Iowa). To visualize β-galactosidase a monoclonal antibody (Promega) or a polyclonal antibody (Cappel) was applied. The anti-KNRL rabbit antiserum was provided by M. Hoch and the anti-SAL antibody was described previously (Kühnlein et al., 1994). Immunostained embryos were viewed with a Zeiss Axiopt microscope. Embryos stained with fluorescent antibodies were analyzed by laser scanning microscopy as described (Kühnlein et al., 1996).

#### In situ hybridizations

RNA in situ hybridizations to whole-mount embryos were performed as described (Goldstein and Fryberg, 1994). The RNA probes used in our experiments derived from bnl (Sutherland et al., 1996), bil (Klambt et al., 1992), kni (Rothe et al., 1989), lacZ and sal (Kühnlein et al., 1994).

#### Reporter gene constructs and generation of transgenic fly lines

sal upstream DNA fragments (see Fig. 8C) were cloned into the vector pCaSpeRhs43lacZ (Thummel and Pirrotta, 1992). Transgenic fly lines of the recombinant DNA constructs were generated by P-element-mediated germline transformation and for each construct several independent fly lines were analyzed.

#### RESULTS

**kni and knrl are expressed in overlapping patterns during tracheal branch formation**

The kni and knrl genes encode sequence-related transcription factors, which are expressed in overlapping patterns during embryogenesis, including a posterior expression domain during blastoderm stage (Rothe et al., 1989). Whereas kni activity in this domain is required for abdominal segmentation, knrl lacks segmentation function due to a 23 kb primary transcript, which is aborted during the short mitotic cycles at blastoderm stage. However, a knrl minigene rescues the segmentation defects of kni mutant embryos (Rothe et al., 1992). Furthermore, knrl can compensate for the lack of kni activity required for normal stomatogastric nervous system (SNS) formation. Thus, KNI and KNRL carry redundant functions during SNS development (González-Gaitán et al., 1994).

**kni expression has also been described during tracheal development** (Wimmer, 1995). To examine and eventually distinguish tracheal expression of kni and knrl, we performed...
in situ embryo staining using a kni-specific hybridization probe and KNRL-specific antibodies. The expression of kni and KNRL appears during stage 10 in the tracheal placodes (Fig. 1A,D). During primary branch formation expression of kni and KNRL decreases and restricts to the dorsal- and ventralmost cells as well as visceral branch cells (Fig. 1B,E). kni and KNRL expression persists in the cells of dorsal, visceral, lateral trunk and ganglionic branches (Fig. 1C,F,G). Thus, kni and KNRL are expressed in the same spatio-temporal patterns, suggesting that kni and knrl may also share redundant functions during tracheal development.

**Tracheal branch outgrowth is affected in embryos lacking kni/knrl activity**

DPP signaling is required for the directed migration of dorsal and ventral tracheal cells. It activates kni expression and has been proposed to control target gene expression via KNI (Vincent et al., 1997). To elucidate kni function in dorsal and ventral tracheal cells we examined tracheal formation in kni mutant embryos and in embryos mutant for the deficiency Df(3L)riXXI, which uncovers both kni and knrl (Rothe et al., 1989).

In contrast to wild-type embryos, which develop ten tracheal metanemes (Fig. 2A), kni and Df(3L)riXXI mutant embryos develop only five (Fig. 2B,C). This result reflects the lack of five abdominal segments in both kni and Df(3L)riXXI mutant embryos (Rothe et al., 1989). However, while the remaining tracheal metanemes in kni mutant embryos develop many aspects of a wild-type branching pattern (Fig. 2B), Df(3L)riXXI mutant embryos develop only rudimentary tracheal metanemes, which invaginate but lack primary branching and interconnections (Fig. 2C). This suggests a functional back-up by knrl activity in kni mutant embryos. Furthermore, the lack of primary branching in Df(3L)riXXI mutant embryos suggests that kni/knrl activity participates in early primary branch outgrowth and hence hampers the analysis of a potential kni/knrl function during later stages of branch formation.

To overcome both segmentation and primary tracheal branch defects, we rescued kni and Df(3L)riXXI embryos by a kni transgene that provides both kni segmentation gene function and kni placode expression (Rothe et al., 1992; Fig. 2D). kni mutant embryos bearing the kni transgene develop a normal number of tracheal metanemes as well as a wild-type-like tracheal branching (not shown). In Df(3L)riXXI embryos bearing the kni transgene (DfriXXI/kni-transgene), dorsal trunk formation is similar to wild-type (Fig. 2E), whereas dorsal branch outgrowth is lacking and lateral trunk fusion occurs only partially. In addition, visceral and ganglionic branches fail to contact the gut and the central nervous system, respectively (Fig. 2F,G). Thus, the region-specific kni/knrl tracheal expression in the dorsal, ventral and visceral branches is required for their formation. The wild-type-like branch outgrowth in the remaining tracheal anlagen of kni mutant embryos suggests that knrl can substitute for kni activity in such embryos.

To unambiguously demonstrate that kni/knrl activity rather than the activity of another gene uncovered by the Df(3L)riXXI is necessary for branch formation, we performed tissue-specific rescue experiments. UAS-kni or UAS-knrl effector constructs were expressed under the control of a tracheal-specific GAL4 driver (btl-Gal4; see Materials and methods) in DfriXXI/kni-transgene embryos. Such embryos exhibit wild-type-like dorsal, visceral and gangleonic branch outgrowth as well as lateral trunk formation (Fig. 2H,J and not shown), although the ectopic kni or knrl expression causes a reduced diameter and occasional interruption of the dorsal trunk (Fig. 2H,I and not shown; see also below). This indicates that persisting kni or knrl expression during tracheal development is sufficient to rescue the lack of branch outgrowth of DfriXXI/kni-transgene embryos. Thus, kni and knrl share redundant functions during tracheal development and the region-specific tracheal kni/knrl expression is indeed a prerequisite for wild-type tracheal branch formation.

**kni/knrl activity is necessary for directed outgrowth of specific tracheal branches**

The embryonic tracheal development exclusively depends on Fig. 1. kni and KNRL are expressed in overlapping tracheal patterns. Whole-mount in situ hybridization with a kni antisense RNA probe of wild-type embryos at stages 10 (A), 13 (B) and 14 (C). Whole-mount antibody staining with anti-KNRL antibodies of wild-type embryos at stages 10 (D), 13 (E) and 14 (F). The expression of kni (A) and KNRL (D) in the first and tenth tracheal placode is indicated by arrows. (G) Schematic drawing of a tracheal metamere during stage 13; black regions indicate region-specific kni and KNRL expression. Note that KNRL is detected in the cell nuclei.
cell migration and cell extension processes (Manning and Krasnow, 1993). Therefore, the lack of specific tracheal structures caused by mutations is either due to cell death or to stalled cell outgrowth. To further characterize the role of kni/knrl during tracheal outgrowth we monitored the fate of tracheal cells marked by reporter gene expression driven by the region-specific kni enhancer (Wimmer, 1995) in both wild-type and Df(3L)rici XT1 kni-transgene embryos. Reporter gene expression is observed in a kni-like pattern in wild-type tracheae (Fig. 3A). In Df(3L)rici XT1 kni-transgene embryos the reporter gene product is expressed in the stalled ventral and visceral branches and in cell clusters associated with the dorsal trunk (Fig. 3B). These cell clusters are arranged in a segmentally repeated pattern at dorsal positions of the dorsal trunk where dorsal branches form in wild-type embryos. Thus, the lack of tracheal branch formation in Df(3L)rici XT1 kni-transgene embryos is caused by the failure of proper cell migration and not by tracheal cell death. The close association of β-galactosidase expressing cells with the dorsal trunk suggests that these cells may participate in dorsal trunk formation in the absence of kni/knrl activity. To confirm this, we examined reporter gene expression in combination with SAL expression, which serves as a marker for dorsal trunk cells (Kühnlein and Schuh, 1996). Interestingly, the dorsal trunk cells coexpress SAL and β-galactosidase (Fig. 3C), showing that dorsal branch progenitor cells are integrated into the dorsal trunk and express SAL in response to the lack of kni/knrl activity. Thus, the kni/knrl activity is necessary for the directed outgrowth of dorsal, ventral and visceral tracheal cells and it determines dorsal branch cell fate.

kni/knrl acts independently of FGF and EGF signaling key components

FGF signaling controls tracheal branching by guiding tracheal cell migration (Lee et al., 1996; Sutherland et al., 1996). The key component in this process is the chemoattractant BNL, which directs tracheal branches towards their targets.
(Sutherland et al., 1996). The BNL signals are received by the FGF receptor BTL (Klämbt et al., 1992).

We examined whether stalled branch outgrowth in \textit{DfriXT1} knt-transgene embryos is caused by altered \textit{bnl} and/or \textit{btl} expression. In such embryos, \textit{bnl} expression is the same as in wild-type embryos, including the dorsalmost \textit{bnl} patches which, however, fail to direct dorsal branch outgrowth in \textit{DfriXT1} knt-transgene embryos (Fig. 4A,B). Furthermore, \textit{DfriXT1} knt-transgene embryos show normal \textit{btl} expression in their rudimentary tracheal system (Fig. 4C) and region-specific \textit{knt} expression is not affected in \textit{btl} mutant embryos (not shown). Thus, the region-specific \textit{kni}/\textit{knrl} activity does not interfere with FGF signaling, although the results do not exclude the possibility that \textit{kni}/\textit{knrl} may affect downstream components of the FGF pathway.

The EGF pathway is activated by the generation of activated SPITZ (SPI) in the tracheal placode and is necessary for dorsal trunk and visceral branch formation (Wappner et al., 1997). As this visceral branch phenotype is similar to the one of \textit{DfriXT1} knt-transgene embryos, we monitored whether \textit{knt} expression is still occurring in \textit{spi} mutant embryos. As shown in Fig. 4D \textit{knt} expression is normal in \textit{spi} mutant embryos. Taken together, these results indicate that region-specific \textit{kni}/\textit{knrl} expression is not controlled by FGF or EGF signaling and that \textit{kni}/\textit{knrl} activity does not affect key components of these pathways.

\textbf{\textit{kni}/\textit{knrl} activity is necessary but not sufficient to mediate DPP-dependent tracheal patterning}

DPP signaling is required for dorsal and ventral tracheal branch formation and for \textit{knt} expression (Vincent et al., 1997). The tracheal mutant phenotypes of embryos lacking the DPP receptors TKV and PUT are reminiscent of the \textit{knt} tracheal phenotype, suggesting that \textit{knt} is necessary to mediate functional aspects of DPP signaling (this work; Vincent et al., 1997). To link \textit{knt} activity and DPP signaling more directly, we expressed \textit{knt} in \textit{tkv} mutant background by using the tracheal-specific \textit{btl}-Gal4 driver and the UAS-\textit{knt} effector. Since \textit{tkv} mutant embryos lack the dorsalmost patches of \textit{bnl} expression that are necessary for dorsal branch outgrowth (Vincent et al., 1997), we focused our analysis on ventral branch formation in the presence of \textit{knt} expression. These embryos develop a rudimentary ventral tracheal system that is indistinguishable from the branching of \textit{tkv} mutants (not shown). Thus, the activation of \textit{knt} expression by DPP is necessary but not sufficient for ventral branch formation. This result also suggests that DPP signaling controls additional genes different from \textit{kni}/\textit{knrl} that are necessary for branch outgrowth (see Discussion).

Ectopic DPP expression in all tracheal cells leads to dorsoventral cell migration, which causes the lack of dorsal trunk and visceral branches that are normally formed by anteroposterior migration (Llimargas and Casanova, 1997; Vincent et al., 1997; Wappner et al., 1997). It also leads to ectopic \textit{knt} expression in all tracheal cells (Vincent et al., 1997). Our finding that ectopic \textit{knt} expression also interferes with dorsal trunk formation (Fig. 2H) suggests a role of \textit{knt} activity in mediating ectopic DPP signaling. Thus, we examined the tracheal phenotypes generated by either ectopic \textit{dpp} or ectopic \textit{knt} expression. Ubiquitous tracheal \textit{dpp} expression by \textit{btl}-Gal4 driver and UAS-\textit{dpp} effector causes the lack of anterioposterior branch formation and the migration of supernumerary cells towards dorsal (Vincent et al., 1997; compare Fig. 5A,B with 5C,D). Ubiquitous tracheal UAS-\textit{knt} expression in response to one copy of \textit{btl}-Gal4 leads to a reduced dorsal trunk and an increased number of cells migrating dorsally (compare Fig. 5A,B with E,F), whereas UAS-\textit{knt} driven by two copies of \textit{btl}-Gal4 results in the absence of the dorsal trunk and the migration of supernumery cells towards dorsal positions (compare Fig. 5C,D with G,H). Thus, \textit{knt} activity leads to a dorsal tracheal cell migration, as observed for DPP.

In summary, our results indicate that the role of DPP in directing tracheal cells to adopt a dorsoventral migration behaviour is mediated by \textit{kni}/\textit{knrl} activity, but \textit{kni}/\textit{knrl} is not sufficient to mediate DPP-dependent branch formation.

\textbf{Ectopic \textit{kni}/\textit{knrl} expression interferes with anteroposterior cell migration by repressing \textit{sal}}

To gain insight into the mechanisms of \textit{kni}/\textit{knrl}-dependent reprogramming of tracheal cells from anteroposterior to dorsoventral migration, we first examined the activities of genes known to be required for dorsal trunk formation. In embryos mutant for genes of the EGFR pathway the dorsal trunk is strongly affected and the visceral branches are reduced, suggesting a role of this pathway in specifying anteroposterior tracheal cell migration (Wappner et al., 1997). In \textit{sal} mutant embryos dorsal trunk progenitor cells grow out in an erratic manner resulting in a tracheal phenotype reminiscent of EGFR signaling mutant phenotypes, with the difference that \textit{sal} mutant embryos exhibit normal visceral branch formation (Kühnlein and Schuh, 1996). The observation that \textit{sal} expression is reduced in the rudimentary tracheal system of \textit{rho}
mutant embryos (Wappner et al., 1997) suggests that sal expression may be controlled by EGFR signaling.

To investigate this possibility we monitored sal expression at different stages of tracheal development in embryos that lack components of EGFR signaling. In embryos mutant for rho, spi or faint little ball (flb), sal expression is not affected during early processes of primary branch formation (stage 11; Fig. 6A,B; not shown). However, during stage 12 sal tracheal expression is strongly reduced in embryos lacking EGFR signaling (compare Fig. 6C with D; not shown). These results suggest that the EGFR pathway is not necessary for the initiation but for the maintenance of tracheal sal expression.

Ectopic kni expression in dorsal trunk progenitor cells causes a phenotype that is reminiscent of the tracheal phenotypes caused by the lack of EGFR signaling or by the lack of sal, respectively. Hence we tested whether ectopic kni activity interferes with the EGFR pathway or the activation of its transcriptional target sal by examining both the activated state of the EGFR pathway (Wappner et al., 1997) and the SAL expression pattern. The double-phosphorylated form of mitogen-activated protein kinase (dp-ERK) is detectable in similar amounts in wild-type embryos and in embryos that express ectopic kni in response to the btl-Gal4 driver (not shown). Thus, kni does not interfere with the activation of the EGFR pathway in tracheal cells. In contrast, SAL expression is repressed by ectopic kni expression (Fig. 6G-J). UAS-kni expression driven by one copy of btl-Gal4 reduces dorsal trunk SAL expression and dorsal trunk diameter (compare Fig. 6E,F with G,H), whereas UAS-kni expression driven by two copies of btl-Gal4 causes the lack of SAL expression and dorsal trunk formation (Fig. 6IJ). Thus, kni activity results in repression of sal in dorsal trunk cells and consequently in a lack of dorsal trunk formation. Furthermore, these results also indicate that the lack of anteroposterior dorsal trunk cell migration due to ectopic DPP is mediated by the transcriptional activation of kni/knrl, which in turn represses SAL.

Dorsal branch development requires SAL repression by kni/knrl activity

The observation that ectopic kni/knrl activity in the tracheal system leads to the repression of SAL prompted us to ask whether such an interaction is also necessary for normal tracheal development. In wild-type embryos SAL is expressed in the dorsal parts of all tracheal metameres. However, prior to dorsal trunk formation, SAL fades away in the dorsal branch cells and persists in the dorsal trunk cells only (Kühnlein and Schuh, 1996). Double staining experiments show that the decreasing SAL expression correlates with the activation of β-galactosidase expression driven by the region-specific tracheal kni enhancer in dorsal branch cells (Fig. 7A,B). This suggests that kni/knrl is involved in the repression of sal in dorsal branch cells. Furthermore, the lack of kni expression in dorsal branch progenitor cells of DfriXT1/knrl-transgene embryos results in persistent SAL expression in these cells and their integration into the dorsal trunk (see above). While these observations correlate well with the proposal that kni/knrl activity represses sal in wild-type dorsal branch cells, they do not provide evidence that repression of sal is necessary for normal dorsal branch development. Therefore, we examined dorsal branch

Fig. 5. kni/knrl activity leads to dorsoventral tracheal cell migration. Whole-mount antibody staining with anti-β-galactosidase antibodies of stage 16 embryos bearing the btl-Gal4 driver (A,B), the btl-Gal4 driver and the UAS-dpp effector (C,D), the btl-Gal4 driver and the UAS-kni effector (E,F), two copies of the btl-Gal4 driver and the UAS-kni effector (G,H) and the UAS-lacZ effector construct, which drives nuclear-localized β-galactosidase. Embryos are shown in a lateral (A,C,E,G) and dorsal (B,D,F,H) view. Note: UAS-knrl results in equivalent tracheal cell migration as found for UAS-kni (not shown).
KNI binds in vitro to sal regulatory DNA sequences that direct tracheal expression

The expression patterns of sal and kni as well as their genetic interaction suggest that sal tracheal expression is directly repressed by KNI in dorsal branch cells. To identify cis-regulatory sequences of sal that respond to KNI, we monitored β-galactosidase reporter gene expression driven by the sal tracheal enhancer (salTSE1000; Fig. 8A,E; Kühnlein and Schuh, 1996; Kühnlein et al., 1997). In embryos that express KNI ubiquitously in the tracheal system, β-galactosidase expression of salTSE1000 is abolished (not shown) as found for wild-type sal expression. This indicates that the salTSE1000 contains binding sites for KNI-mediated repression. To examine whether KNI directly interacts with the salTSE1000, we performed electrophoretic mobility-shift assays using KNI protein and different DNA-subfragments of the 1 kb salTSE1000 enhancer region (Fig. 8B). In addition, we delimited the sal tracheal cis-control region by generating transgenic embryos expressing reporter genes under the control of various subfragments of salTSE1000 (Fig. 8C). From five subfragments analyzed, only a 230 bp DNA fragment (salTSE230) binds to KNI (Fig. 8B,D; not shown) and conducts a sal-like β-galactosidase tracheal expression pattern (Fig. 8C,F). Furthermore, salTSE230-dependent reporter gene expression is repressed by ectopic KNI or DPP (Fig. 8G; not shown). These results indicate that the activation of sal expression in tracheal cells and its KNI-dependent repression in dorsal branch cells are mediated by a 230-bp cis-regulatory element containing sites for a direct interaction with KNI.

DISCUSSION

kni and knrl share redundant functions during tracheal development

The transcription factors KNI and KNRL are expressed in overlapping patterns during tracheal development. Tissue-specific rescue experiments of kni and knrl deficient embryos by kni- or knrl-expressing transgenes show that the genes share redundant tracheal functions, as has been described for SNS development (González-Gaitán et al., 1994). kni/knrl activity is involved in distinct cellular processes of tracheal cell differentiation. During placode formation, kni/knrl is expressed in all tracheal cells. Embryos lacking kni/knrl expression develop rudimentary tracheal branches, a phenotype reminiscent of branchless (Sutherland et al., 1996), breathless (Klämbt et al., 1992) or tracheae defective (Eulenberg and Schuh, 1997) mutants. This suggests that the early tracheal kni/knrl activity is required for tracheal development prior to primary branch formation.

In contrast to early kni/knrl expression in all tracheal cells, kni/knrl is expressed in a region-specific manner during primary branch formation in a subset of the major branches. The absence of kni/knrl expression in visceral branch primordial cells results in rudimentary visceral branches, as has been observed in mutants lacking components of the EGF pathway (Llimargas and Casanova, 1997; Wappner et al., 1997). However, this aspect of kni/knrl expression is not dependent on EGF signaling (unpublished results and Wappner et al., 1997), suggesting that EGF signaling and kni/knrl activity function in parallel. Embryos that lack the dorsal and ventral kni/knrl expression lack branches which grow out dorsally (dorsal branch), whereas branches that extend ventrally (ganglionic branch, lateral trunk) are rudimentary. This phenotype is reminiscent of mutants affecting DPP signaling and supports previous observations suggesting that dorsal and ventral kni expression are activated by DPP (Vincent et al., 1997). Moreover, the finding that ectopic kni/knrl expression is able to reprogram tracheal cell migration along the dorsoventral body axis and causes cells to migrate in a dorsal direction, is similar to the altered migration in response to ectopic DPP (Vincent et al., 1997). Thus, the proposed role of DPP to regionalize the tracheal placode by selecting groups of cells that adopt specific migration behaviors appears to be mediated by kni/knrl activity. However, rescue experiments by artificial kni expression in homozygous tkv mutant embryos does not overcome the lack of DPP signaling in the tracheal system. Therefore, kni/knrl activity is necessary but not sufficient to mediate DPP signaling in the tracheal system and indicates that additional DPP-dependent genes are required for dorsal and/or ventral branch outgrowth. For ventral branch formation, unplugged (unp) represents such a candidate gene. unp is necessary for ganglionic branch morphogenesis, but its DPP dependence has not yet been investigated (Chiang et al., 1995).

Repression of sal by kni/knrl controls dorsal branch morphogenesis

kni/knrl activity is necessary for the initiation of dorsal branch formation, i.e. the migration of dorsal branch cells towards the dorsal side of the embryo (see above). In addition, kni plays a role in subsequent dorsal branch development by repression of sal activity in these cells. sal repression is essential for normal dorsal branch formation, as shown by ectopic sal expression experiments. Whereas the migration of dorsal branch cells is not affected, proper dorsal branch morphogenesis does not occur in the presence of SAL. In wild-type embryos the six (approx.) dorsally migrating cells are of cuboidal shape and arranged side-by-side at embryonic stage 12. Up to stage 15, the branches continue to extend to the dorsal side and the cells elongate and intercalate to an end-to-end organization. The two leading dorsal branch cells, DB1 and DB2, develop long...
cytoplasmic extensions, termed secondary branches. The extension of the DB1 cell approaches the dorsal trunk, whereas the DB2 branch grows out toward the dorsal midline and fuses with its contralateral partner, thus forming the dorsal anastomosis (Samakovlis et al., 1996). Sal activity interferes with several developmental steps of dorsal branch formation. In the presence of sal, cell elongation and intercalation is delayed. Furthermore, dorsal anastomosis formation and outgrowth of the DB1 cell back to the dorsal trunk fails and normal terminal branching of DB1 cells during stage 16 was not detectable in response to sal. Thus, ectopic sal interferes with dorsal branch extension and it prevents secondary and terminal branch formation. Since tracheal cell intercalation, secondary and terminal branch formation are not found during dorsal trunk morphogenesis (Samakovlis et al., 1996) it is likely that a function of sal is to prevent these processes during normal dorsal trunk development. This assumption is supported by mutant analysis and sal overexpression studies, which indicate a
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role of sal in the repression of molecular processes that lead to secondary and terminal branch formation (C.-K. Chen and R. Schuh, unpublished). Taken together, these findings establish that repression of sal in dorsal branch cells is required for normal dorsal branch morphogenesis.

Several lines of evidence indicate that kni/knrl activity causes the repression of sal. (1) sal expression is detectable during the placode stage in the dorsal half of the tracheal metamers. After kni expression is turned on in dorsal branch primordial cells sal expression gradually decreases during dorsal branch formation. This indicates a strict and inverse spatial and temporal correlation between sal and kni/knrl expression. (2) Dorsal branch primordial cells lacking kni/knrl activity continue sal expression and these cells become integrated into the dorsal trunk. (3) Gain-of-function experiments indicate that ectopic kni/knrl expression represses sal in a concentration-dependent manner in dorsal trunk cells. (4) KNI binds in vitro to sal cis-regulatory sequences that mediate tracheal expression. These findings indicate that kni/knrl-dependent sal repression in dorsal branch cells involves the direct binding of the transcription factors KNI/KNRL.

EGFR signaling maintains sal expression

sal expression in dorsal tracheal cells is necessary for the migration of dorsal trunk progenitor cells along the anteroposterior axis and for dorsal trunk morphogenesis (Kühnlein and Schuh, 1996). The sal mutant phenotype in the dorsal trunk is similar to the phenotype observed in embryos lacking components of the EGFR pathway (Kühnlein and Schuh, 1996; Llimargas and Casanova, 1997; Wappner et al., 1997). Thus, EGFR and sal may act in the same genetic circuitry. This inference is confirmed by the finding that EGFR signaling is necessary to maintain sal expression in dorsal trunk cells prior to fusion and subsequent morphogenesis, whereas EGFR signaling is not required for the initiation of sal expression. The mutant EGFR pathway causes a lack of tracheal branches to migrate in anteroposterior direction and thus affects the morphogenesis of the dorsal trunk and the visceral branches (Llimargas and Casanova, 1997; Wappner et al., 1997). In sal mutant embryos the dorsal trunk is absent, whereas visceral branches develop normally (Kühnlein and Schuh, 1996). Therefore, sal may function in a patterning system that has been proposed to represent the basis for the distinction between anteroposterior migrating dorsal trunk and visceral branch cells within the domain controlled by EGFR signaling (Wappner et al., 1997).

The DPP and EGF pathways interact via their target genes kni and sal

It has been proposed that the DPP and EGF signaling generates three different cell fates in the developing placode that confer the capacity to migrate in distinct directions (Wappner et al., 1997). We have shown that kni/knrl activity is necessary to mediate DPP signaling for dorsal and ventral cell migration. In addition, repression of the EGFR signaling target sal by kni/knrl establishes a border between the dorsally and anteroposteriorly migrating dorsal branch and dorsal trunk cells, respectively. However, the repression of sal is not necessary for normal dorsoventral tracheal cell migration but rather for morphogenetic processes that occur independently of cell migration. Thus, tracheal cells that express sal and kni/knrl still adopt a dorsoventral migration behavior. Ectopic expression of kni/knrl in dorsal trunk cells has two
consequences. Firstly, it represses SAL, which results in the lack of anteroposterior migration of dorsal trunk cells. Secondly, ectopic kni/knrl leads primordial dorsal trunk cells to adopt a dorsoventral migration behavior. Thus, the observation that ectopic DPP causes altered tracheal cell migration and lack of dorsal trunk formation (Llimargas and Casanova, 1997; Vincent et al., 1997; Wappler et al., 1997) is consistent with the proposal that these processes are mediated in part via kni/knrl. However, we noted that in contrast to ectopic DPP, which inhibits visceral branch formation, ectopic kni/knrl tracheal expression does not affect anterior outgrowth of visceral branches. This observation is not unexpected since kni/knrl is expressed in visceral branch cells and is necessary for normal visceral branch morphogenesis. Thus, kni/knrl acts within the genetic circuitry of visceral branch cell fate determination in a different way from during dorsal branch development and it does not involve mediation of dorsoventral determination in a different way from during dorsal branch development.

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