

## Dual function of the region-specific homeotic gene *spalt* during *Drosophila* tracheal system development

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### SUMMARY

We report that the region-specific homeotic gene *spalt* affects the *Drosophila* tracheal system at two different stages of embryonic development. Both lack-of-function and gain-of-function experiments show that blastodermal *spalt* activity restricts tracheal development to 10 bilaterally positioned pairs of tracheal placodes in the trunk region by repressing placode formation in parasegments 2, 3 and 14. The results suggest that the activity of the zinc-finger type transcription factor encoded by *spalt* suppresses the molecular pathway that establishes tracheal develop-

ment. *spalt* function is also necessary for the directed migration of the dorsal trunk cells, a distinct subset of tracheal cells. This process is a prerequisite for the formation of the dorsal trunk generated by fusion of adjacent tracheal metameres into a common tubular structure. The directed cell migration, in which *spalt* gene function participates, seems to be independent of branch fusion and general tracheal cell migration processes.

Key words: *Drosophila*, embryogenesis, development, trachea, *spalt*

### INTRODUCTION

The *Drosophila* tracheal system is an excellent system to reveal the molecular mechanisms underlying the formation of a three-dimensional interconnected network of tubular structures during embryogenesis (for review see Manning and Krasnow, 1993). It involves a number of cellular mechanisms such as directed cell migration, target tissue recognition, pathfinding, branch fusion and branch point control. The primary function of the tracheal system is the transport of gases by passive diffusion to target tissues during larval and adult stages. It may also provide endoskeleton-like structural support and guidance function in neuronal pathfinding (Chapman, 1982; Giniger et al., 1993; Manning and Krasnow, 1993).

Tracheal system development begins about 4 hours after egg deposition during stage 10 (stages according to Campos-Ortega and Hartenstein, 1985) and is initiated by the appearance of ten tracheal placodes from lateral clusters of dorsoectodermal cells on either side of the embryo. About 1.5 hours later at embryonic stage 11, cells of the tracheal placodes invaginate into the underlying mesoderm to form the tracheal pits along each side of the embryo. At this stage, each tracheal metamere consists of about 90 cells. The metameres do not undergo any further cell division up to the end of larval development and, thus, the assembly of the tracheal system is exclusively reduced to proliferation-independent cell migration processes.

Tracheal cell migration is initiated shortly after the invagination of the tracheal placodes and follows a stereotype branching pattern. Some of the outgrowing branches extend towards defined target tissues while other branches meet each

other and fuse to interconnect the isolated metameres to a three-dimensional network. The fusion of tracheal branches is initiated by specialized tracheal cells, the homotip cells. After about 10 hours of embryonic development, this process leads to the main anterior-posterior connection, the dorsal trunk. Subsequently, a second longitudinal as well as dorsal and ventral connections are linked together. Additional tracheal cell outgrowth and branching leads finally to the highly branched tracheal network which starts its physiological function during the first instar larval stage (for detailed description see Manning and Krasnow, 1993; Samakovlis et al., 1996).

Only a few genes have been identified that are required for formation of the tracheal system including *breathless* (*btl*) (Klämbt et al., 1992; Reichman-Fried et al., 1994), *pointed* (*pnt*) (Klämbt, 1993) and *drifter* (*dfr*) (Anderson et al., 1995). In the absence of *btl* gene activity the tracheal placodes form normally but the tracheal cells fail to migrate. In the absence of *dfr* or *pnt* activities, the tracheal cells only partially migrate. The *btl* gene encodes a receptor tyrosine kinase, a *Drosophila* homolog of the fibroblast growth factor receptor (DFGF-R1). This suggests that tracheal cell migration depends on the activity of a signal transduction pathway (Reichman-Fried et al., 1994; Reichman-Fried and Shilo, 1995). In contrast, the genes *pnt* and *dfr* encode potential transcription factors containing an ETS domain (Klämbt, 1993) and a POU domain (Anderson et al., 1995), respectively. These three genes are expressed in all tracheal cells during placode formation, while the expression of the gene *pruned*, a *Drosophila* homolog of the vertebrate serum response factor, turns on during late tracheal development, at a stage when the main branching pattern is already established. Its mutant phenotype suggests

that *pruned* activity may serve as a key regulator of terminal tracheal branch outgrowth (Affolter et al., 1994a; Guillemain et al., 1996). Furthermore, *decapentaplegic* (*dpp*)-dependent signaling, mediated by the receptor serine/threonine kinases *punt* and *thick veins* (*tkv*), is necessary for the development of dorsal and ventral tracheal structures (Affolter et al., 1994b; Ruberte et al., 1995).

The region-specific homeotic gene *spalt* (*sal*) has also been reported to be expressed in the tracheal system although its function in tracheal development is yet unknown (Kühnlein et al., 1994). The *sal* gene was initially identified by mutations leading to an incomplete homeotic transformation of cuticle pattern elements of the posterior head to adjacent thoracic structures and of the anterior tail to adjacent abdominal structures, i. e. it promotes the specification of terminal pattern elements as opposed to trunk segments (Jürgens, 1988). The gene product of *sal* is a zinc finger protein likely to function as a transcription factor (Kühnlein et al., 1994). *sal* participates in the regulatory network of homeotic genes, since *Ultra-bithorax* (*Ubx*) (Casanova, 1989) and *teashirt* (*tsh*) (Röder et al., 1992) are ectopically expressed in *sal* mutants, and *Antennapedia* (*Antp*) represses *sal* activity in the mesothoracic leg disc (Wagner-Bernholz et al., 1991).

Here we show that *sal* is required to confine the tracheal cell anlagen to 10 tracheal placodes on each side of the blastoderm embryo. Its activity suppresses the genetic pathway which directs the development of the tracheal cell fate from ectodermal cells. In addition, *sal* function is required at a later stage in specific tracheal cells. During tracheal system branch outgrowth, *sal* activity is necessary for the directed migration of the dorsal branch cells to generate the dorsal trunk along the anterior-posterior axis of the embryo.

## MATERIALS AND METHODS

### Immunostainings

Whole-mount embryo immunostaining was performed according to standard protocols (Ashburner, 1989). Secondary antibodies were either conjugated with biotin or alkaline phosphatase. Biotinylated secondary antibodies were revealed using the VECTASTAIN Elite ABC-peroxidase system (Vector Laboratories) with DAB as substrate. In double-staining experiments, alkaline phosphatase stainings were performed after the horseradish peroxidase stainings. To detect  $\beta$ -galactosidase expression, a monoclonal antibody (Promega) was used. Spalt was visualized using the rabbit anti-Spalt antiserum described by Kühnlein et al. (1994). The anti-Engrailed antibody was kindly provided by Nipam Patel. Immunostained embryos were viewed with a Zeiss Axiophot microscope. Embryos stained with fluorescent antibodies were analyzed by laser scanning microscopy (LSM) using a Zeiss LSM 10 under the following conditions: FITC; excitation wavelength 488 nm, bandpass filter 530 nm; CY3; excitation wavelength 514, longpass filter 575 nm. Acridine orange stainings was performed as described by Abrams et al. (1993).

### Image reconstruction and stereoimages

Greyscale images of fluorescence double-labeled embryos were transformed into coloured images and superimposed using Adobe Photoshop Version 2.5.1. For stereoimages, 25 LSM confocal sections of 3  $\mu$ m intersection distance were recovered and superimposed in stacks. Left and right projections (total projection angle 16°) were calculated using the NIH program Version 1.5.4.

### Scanning electron microscopy

Scanning electron microscopy on dechorionated and devitelinated embryos was performed as described (Turner and Mahowald, 1977). Photographs were taken with a Cambridge Stereoscan 150 electron microscope.

### Fly strains

The *spalt* null alleles that were used include *sal*<sup>l16</sup> and *sal*<sup>l445</sup> (Jürgens, 1988; Kühnlein et al., 1994). The *lacZ* enhancer trap line *l-eve-1* was used to mark tracheal cells with cytoplasmic  $\beta$ -galactosidase expression. The P-element is inserted on the third chromosome close to the *trachealess* gene (Perrimon et al., 1991; Wilk et al., 1996).

The *lacZ* enhancer trap line G6 was used to mark dorsal trunk homotip cells by  $\beta$ -galactosidase expression. The P-element is inserted on the second chromosome close to the *escargot* gene (Whiteley et al., 1992). The 40o homozygous viable fly line contains a P-vector construct on the second chromosome which drives Gal4 expression in the central *Kr* expression domain (*Kr*-Gal4 driver) (Castelli-Gair et al., 1994).

### Cloning of *sal* heat-shock and *sal* GAL4 system vectors hs-*sal*

The coding region for Spalt was excised from Bluescript vector (Stratagene) as a *Sma*I restriction fragment. This fragment extends from nucleotide position 370 to 5555 of the composite *sal* cDNA (Kühnlein et al., 1994). After addition of *Not*I linkers, the DNA fragment was cloned in the *Not*I site of pCaSpeRhsp*Not*I (Rothe et al., 1992) resulting in *sal* gene expression which is under the control of the *Drosophila* heat-inducible hsp70 promoter. The pCaSpeRhsp-*Not*I-*sal* heat-shock construct was injected into homozygous *ry*<sup>506</sup> embryos as described (Rubin and Spradling, 1983) and three transgenic lines designated as hs-*sal* were obtained.

### UAS-*sal*

The *sal* coding region was excised from Bluescript vector as a *Sma*I restriction fragment and subcloned in the *Xho*I site, which was made blunt by filling in with the Klenow fragment of DNA polymerase I, of the P-element vector pUAST (Brand and Perrimon, 1993). The resulting UAS-*sal* construct was used for P-element-mediated transformation of flies. Several independent fly strains bearing the UAS-*sal* construct on different chromosomes were generated (UAS-*sal* effector strains).

### *sal*-TSE-*lacZ*

A 1 kb *Eco*RI-*Bam*HI restriction fragment comprising the *cis*-regulatory sequences of the *sal* tracheal system enhancer element (*sal*-TSE; R. P. Kühnlein and R. Schuh, unpublished data) was cloned in pCaSpeRhs43 $\beta$ -gal (Thummel and Pirrotta, 1992), and several independent transgenic fly strains were generated after injection of the construct into homozygous *white*<sup>-</sup> embryos.

### *sal*-TSE-Gal4

The *Eco*RI-*Bam*HI restriction fragment of the *sal*-TSE element was made blunt by filling in with the Klenow fragment of DNA polymerase I and, after addition of *Pst*I linker and *Pst*I restriction digest, it was cloned in the *Pst*I site of T2.2 vector (G. Micklem and M. Leptin, personal communication). The T2.2 vector is a pCaSpeR construct containing eight UAS repeats and a truncated Gal4 fragment. Transgenic fly strains were generated as described above.

### *sal* heat-shock experiments

To analyze the phenotypic consequences of ectopic Spalt expression on tracheal development, we crossed homozygous viable males of the second chromosome hs-*sal* integration (hs-*sal*2) to females of the *l-eve-1* tracheal marker line. To mark dorsal trunk cells, homozygous hs-*sal*2 males were crossed to homozygous females of a third chro-

mosomal *sal-TSE-lacZ* integration. Embryos of this cross were staged under the microscope and collections of blastoderm embryos were subjected to one 15 minute heat shock at 37°C air temperature (early heat shock), and postblastoderm embryos were subjected to two 15 minutes heat shocks at 37°C, which were separated by a 30 minutes recovery at 25°C (late heat shock). After heat shock, the embryos were developed at 25°C until analysis at an appropriate stage.

**Crosses and genetic analysis**

To analyze ectopic *Spalt* expression in the central *Kr* expression domain, we used the yeast GAL4-directed transcription system (Brand and Perrimon, 1993). For this, females homozygous for a *UAS-sal* integration on the X chromosome (*UAS-salX*) and bearing the *1-eve-1* chromosome were crossed to homozygous males of the *Kr-Gal4* driver inserted on the second chromosome of line 40o. The progeny were analyzed by immunostaining as described above.

To rescue the *sal* tracheal system phenotype, *UAS-salX*; *sal<sup>16</sup>/CyO* females were crossed to *sal<sup>445</sup>/CyO* males containing the *sal-TSE-Gal4* integration on the third chromosome. Homozygous *sal* mutant embryos were identified by *Spalt* expression exclusively in the tracheal system.

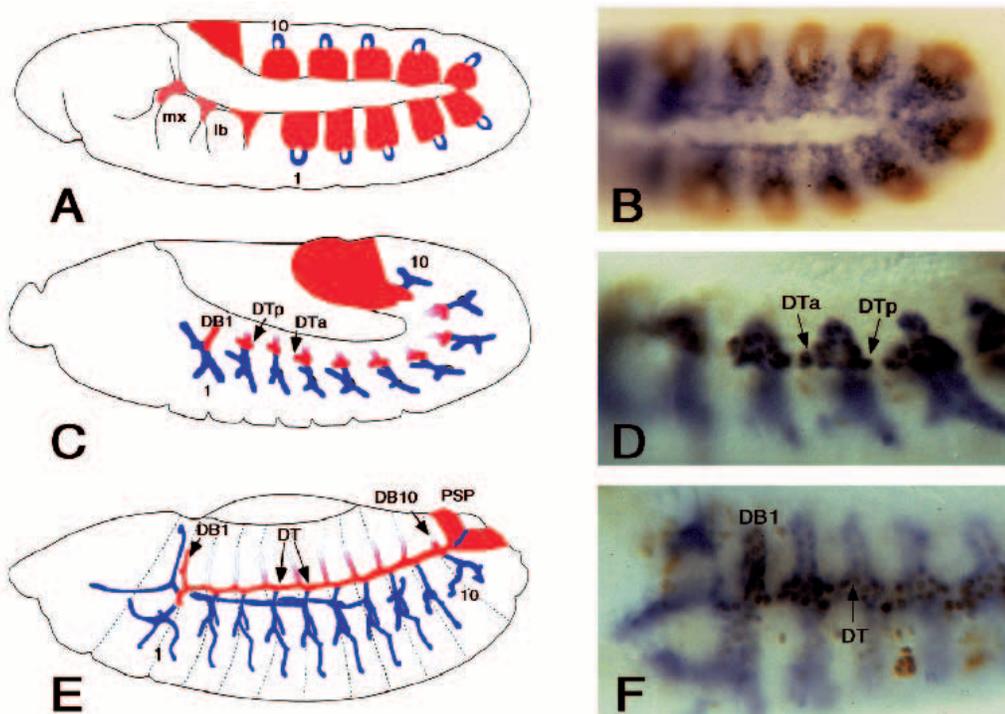
To detect dorsal trunk cell identity in a *sal* mutant background, *sal<sup>445</sup>/CyO* males containing the *sal-TSE-lacZ* integration on the third chromosome were crossed to *sal<sup>16</sup>/CyO* females. Homozygous *sal* mutant embryos were identified by their β-galactosidase expression in the ectopic tracheal placodes TP0 and TP11 of *sal* mutants.

**RESULTS**

***Spalt* expression during tracheal system development**

After its initial, region-specific blastoderm expression in parasegments (PS) 1-3 as well as 14 and 15, *sal* protein (*Spalt*)

is observed in the developing central nervous system and the tracheal system (Kühnlein et al., 1994). In order to examine tracheal expression of *Spalt* in more detail, we performed anti-*Spalt* antibody staining of embryos bearing a *1-eve-1* chromosome (Perrimon et al., 1991). The *1-eve-1* enhancer trap line shows *lacZ* expression in all tracheal cells from stage 10 onwards and thus serves as a specific cell marker for tracheal identity (see Materials and Methods). At late stage 10, the tracheal primordia are first recognized as 10 metameric placodes along each side of the embryo. They are located in the dorsolateral ectoderm between the second thoracic (T2) and the eighth abdominal segment (A8). During this stage broad dorso-ectodermal fields of *Spalt* expression are detectable (Kühnlein et al., 1994). At stage 11 when the tracheal cells invaginate into the underlying mesodermal tissue, tracheal pits are transiently formed. The *Spalt* field domains overlap with the dorsal parts of all tracheal placodes, and *Spalt* expression decreases in the non-tracheal cells and becomes restricted to tracheal cells (Fig. 1A,B). At stage 12, the basic branching pattern of the tracheal metameres is generated by directed migration of tracheal cells forming finger-like extensions. *Spalt* is found in the dorsal parts of all tracheal metameres, in the outgrowing dorsal trunk anterior and dorsal trunk posterior as well as in the dorsal branch, while no *Spalt* expression is detectable in central and ventral tracheal structures (Fig. 1C,D). In stage 14, *Spalt* expression persists in the cells of the dorsal trunk anterior and dorsal trunk posterior when these structures of adjacent metameres fuse to generate the dorsal trunk (Fig. 1E,F). During stage 14 and 15, when branch outgrowth continues and lateral trunk fusion occurs, *Spalt* expression becomes weaker in the dorsal branches but persists in the dorsal trunk cells. Between stages



**Fig. 1.** *Spalt* expression during the embryonic tracheal system development.

(A,C,E) Schematic representation of the developing tracheal system of a stage 11 embryo (A), a stage 12 embryo (C) and a stage 14 embryo (E). *Spalt* expression associated with the tracheal system is shown in red, while all other tracheal structures are shown in blue.

(B,D,F) Whole-mount antibody double staining of *1-eve-1* embryos at stage 11 (B), stage 12 (D) and stage 14 (F) using anti-β-galactosidase (brown in B and blue in D,F) and anti-*Spalt* (blue in B and brown in D,F) antibodies.

Anterior is left and dorsal up. The β-galactosidase expression of the *1-eve-1* enhancer trap line serves as a tracheal-specific cell marker.

Abbreviations: 1, tracheal

metamere 1; 10, tracheal metamere 10; DB1, dorsal branch 1; DB10, dorsal branch 10; DT, dorsal trunk; DTa, dorsal trunk anterior; DTp, dorsal trunk posterior; lb, labial bud; mx, maxillary bud; PSP, posterior spiracles primordia.

15 and 17 when the tracheal system terminally differentiates, Spalt expression continues in all dorsal trunk cells and acquires a posterior-anterior graded appearance (not shown).

### Tracheal system development is affected in *sal* mutant embryos

In order to elucidate the *sal* function for tracheal development, we examined tracheal development in *sal* mutant embryos using  $\beta$ -galactosidase expression of the *1-eve-1* enhancer trap line as the tracheal cell marker. Lateral and ventral structures of the tracheal system are not affected by the absence of *sal* activity. However, the dorsal trunk fails to be properly assembled in *sal* mutant embryos, i.e. the tracheal metameric units fail to connect the dorsal trunk anterior and dorsal trunk posterior branches (Fig. 2A,B). As compared to wild-type, the dorsal trunk cells of *sal* mutant embryos do not migrate in strict anterior or posterior directions but, instead, migrate predominantly in dorsal directions (Fig. 2A,B). Sporadic horizontal branch connections are formed; however, they occur at ectopic dorsal positions of tracheal metameres (Fig. 3). This mutant phenotype indicates that the misrouted branches maintain their potential to connect in *sal* mutant embryos. Indeed, *sal* mutant embryos specify the dorsal trunk homotip cells, which are cells at the distalmost position of each outgrowing dorsal branch and are thought to be essential for dorsal branch fusion (Manning and Krasnow, 1993). In wild-type embryos, these homotip cells express Spalt and  $\beta$ -galactosidase and are controlled by the *escargot* (*esg*) gene (arrows in Fig. 2C), which specifically mark the homotip cells (Whiteley et al., 1992). In *sal* mutant embryos, the homotip cells are still formed, although dorsal to their normal position and in an erratic manner (Fig. 2D). This suggests that *sal* is needed for the oriented dorsal trunk cell migration so that the homotip cells of adjacent tracheal metameres can meet and fuse to form the dorsal trunk. General cell migration and branch fusion processes seem not to be affected in *sal* mutant embryos.

The analysis of dorsal trunk development revealed an additional tracheal phenotype of *sal* mutant embryos, the extension of the tracheal system toward the termini of the embryo. *sal* mutants develop three ectopic tracheal placodes Tr-1, Tr0 and Tr11 (Fig. 2F) in addition to the ten wild-type placodes (Tr1 to Tr10), as visualized by  $\beta$ -galactosidase expression provided by the *1-eve-1* marker (Fig. 2E,F). The ectopic placodes are localized in the anterior compartments of the labial (Tr-1; PS 2), the prothoracic (Tr0; PS 3), and the ninth abdominal segment (Tr11; PS 14), respectively. Their relative positions correspond to their wild-type counterparts between PS 4 and PS 13 as revealed by *engrailed* (*en*) marker gene expression (Fig. 2F). Whereas the size of Tr0 resembles that of an average placode, the sizes of the ectopic placodes Tr-1 and Tr11 are variable and reduced (Fig. 2F). The ectopic placodes participate in the coordinated invagination of tracheal cells and possess a subsequent branching competence. (Fig. 2B). Scanning electron microscopy of stage 11 embryos reveal that the ectopic placodes undergo transient formation of tracheal pits corresponding to Tr0 and Tr11 (compare Fig. 2G and H). A presumptive ectopic tracheal pit corresponding to Tr-1 might be obscured by the furrow between the maxillary and labial bud of stage 11 *sal* mutant embryos (Fig. 2H). Note, that with the exception of the ectopic pits no apparent epidermal alterations were caused

by the lack of *sal* gene function during germ band extension (Fig. 2G,H).

Taken together the lack of *sal* activity during tracheal cell determination results in ectopic tracheal placodes and, during branch outgrowth, results in the lack of dorsal trunk formation (Fig. 3). These two apparently unrelated mutant phenotypes suggest dual roles of *sal*, which function at different stages of tracheal development.

### Early *sal* activity confines tracheal placode formation to the trunk region

The ectopic tracheal placodes in *sal* mutant embryos suggest that the region-specific *sal* expression during blastoderm suppresses tracheal development in PS 2, 3 and 14. In order to demonstrate this function more directly, we ectopically expressed Spalt in a central region of the blastoderm embryo by use of the yeast GAL4-directed transcription system (Brand and Perrimon, 1993), i.e. Spalt expression was provided through a UAS-*sal* DNA construct activated by GAL4 expression mediated by the *Kr* central domain enhancer element (Castelli-Gair et al., 1994; for details see Materials and Methods). In embryos containing the two transgenes, ectopic Spalt is detectable in a patchy expression pattern in the central region of embryos during blastoderm (Fig. 4A) through germ band extension stages (Fig. 4B). In such embryos, both the number of tracheal primordial cells in tracheal metameres 2 to 6 and the differentiated tracheal system in the corresponding region, are strongly reduced (Fig. 4C). Since no associated defects were observed in the cuticle of such larvae (data not shown) and the basic segmental organisation of the embryonic peripheral nervous system is unaffected (Fig. 4D), *sal* may suppress the developmental fate of tracheal versus epidermal development.

In order to assess the phenocritical period during which Spalt may interfere with early tracheal development, we induced *sal* expression under the control of the *hsp70* heat-shock promoter (*hs-sal*; Materials and Methods). Stage 5 embryos bearing the *hs-sal* expression construct were subjected to a single heat-shock pulse of 20 minutes at 37°C. Such embryos were found to ubiquitously express Spalt 30 minutes after the heat-shock pulse (data not shown). They show a normal *engrailed* (*en*) expression pattern, indicating that the metameres are not affected by the heat shock (Fig. 4E,F). However, in such embryos, reduced numbers of tracheal cells per metamere and the absence of tracheal metameres were observed (Fig. 4F). The embryos nevertheless develop into normally segmented larvae, which hatch from their egg shells but lack an interconnected tracheal system (Fig. 4G,H). The larvae move slowly and die shortly after hatching. These results indicate that ectopic Spalt expression during stage 5 specifically interferes with tracheal system development.

The phenocritical period during which ectopic Spalt expression can interfere with tracheal cell fate determination is restricted to stage 5. Embryos subjected to Spalt heat-shock treatment later than stage 5 develop normal tracheal placodes (data not shown). Thus, the region-specific Spalt expression at blastoderm stage is likely to restrict tracheal development to the trunk region by suppression of a genetic pathway that leads to tracheal instead of epidermal cell fate in distinct regions of the stage 5 ectoderm.

### Late *sal* activity is necessary for dorsal trunk cell migration

*sal* mutant analysis suggests that *sal* activity is necessary for the directed outgrowth of dorsal branch cells leading to the formation of the dorsal trunk. In order to assess whether the dorsal trunk defect is caused by the lack of *sal* activity in dorsal trunk cells, we performed a tracheal-specific rescue experiment with *sal* mutant embryos. For this, we fused the genuine *sal* tracheal system enhancer (*sal*-TSE; Kühnlein and Schuh, unpublished data) excluding the *sal* field domain enhancer to a yeast Gal4 driver construct (see Materials and Methods). *sal* mutant embryos bearing both the *sal*-TSE-Gal4 driver construct and the UAS-*sal* cDNA effector construct (see above) express Spalt exclusively in dorsal trunk cells of the tracheal system from stage 12 onwards. Upon Gal4-driven Spalt expression, the dorsal trunk branches fuse at high frequency, leading to dorsal trunk fragments which span several tracheal metameric units (Fig. 5A). The partial, instead of a complete rescue, can be attributed to the late accumulation of Spalt in the dorsal trunk cells from stage 12 onwards. Spalt is normally expressed in the corresponding tracheal cells of stage 10 wild-type embryos (see above). These results suggest that the *sal* activity in dorsal trunk progenitor cells is necessary for dorsal trunk formation.

Tracheal development is largely independent of cell proliferation but rather depends on the migration of cells that are already present in the placodes (Manning and Krasnow, 1993). Yet, the late tracheal defects, observed in the absence of Spalt expression, could be attributed to cell death of dorsal trunk cells. In order to exclude cell death as the cause of defective dorsal trunk formation in *sal* mutant embryos, we generated a *sal*-TSE element-based *lacZ* reporter gene. The  $\beta$ -galactosidase expression of this reporter gene serves as a specific tracheal cell marker to monitor the developmental fate of *sal* gene expression in dorsal trunk cells (Fig. 5B). In *sal* mutant embryos the  $\beta$ -galactosidase-expressing cells were not found to be altered throughout tracheal development when compared to wild-type (Fig. 5C). In addition, no enhanced cell death is detectable in the lateral trunk region of stage 10 to 14 *sal* mutant embryos by acridine orange staining when compared to wild type (data not shown). Thus, this suggests that the defects observed in the tracheal system of *sal* mutant embryos are not caused by cell death but due to the abnormal cell migration of the dorsal trunk anterior and posterior cells.

### Ectopic Spalt expression interferes with dorsal trunk cell migration

Ectopic expression of Spalt between stage 6 and stage 8 had no scoreable effects on tracheal development (see above). However, ectopic Spalt expression during stage 9 (see Materials and Methods) affects the migration of dorsal trunk cells and causes severe defects in the dorsal trunk of such embryos. The dorsal tracheal system structures develop normally up to stage 14. During stage 15, however, when dorsal trunk fusion is completed, tracheal cells continue to migrate from the dorsal trunk to the ventral side of the embryo and form ectopic tracheal branches (Fig. 6A). The cells of these branches retain their normal dorsal trunk cell identity as shown by *sal*-TSE element-based *lacZ* reporter gene expression and, hence, the ectopic branches involve only cells that normally express Spalt (Fig. 6B). This finding suggests that the ectopic

branches are caused by hypermigration of dorsal trunk cells, which could be a consequence of the increased level of heat-shock-dependent Spalt in these cells. This also suggests that the hypermigration of dorsal trunk cells is caused by the increased levels of *sal* activity in a cell autonomous way in dorsal trunk cells rather than due to *sal* expression in the surrounding cells.

## DISCUSSION

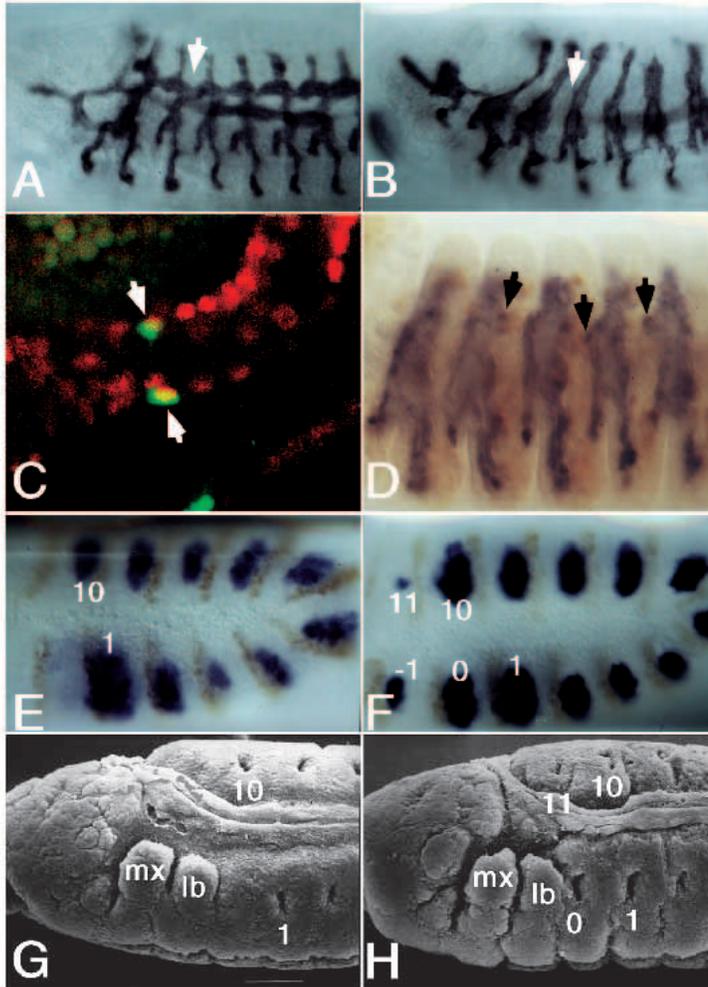
We present evidence that the *sal* gene functions in two distinct developmental pathways required for *Drosophila* tracheal system development. During blastoderm stage, *sal* activity prevents tracheal cell fate determination. It thereby restricts tracheal development to trunk segments exclusively, by acting as a suppressor of tracheal cell fate in ectodermal cells. Once the placodes are formed and tracheal metamers are established, *sal* activity is necessary for the directed outgrowth of specific tracheal cells, the dorsal trunk anterior and posterior cells. This process is essential for dorsal trunk formation, the main anterior-posterior connection of the *Drosophila* tracheal system.

### Early *sal* gene activity suppresses tracheal cell fate

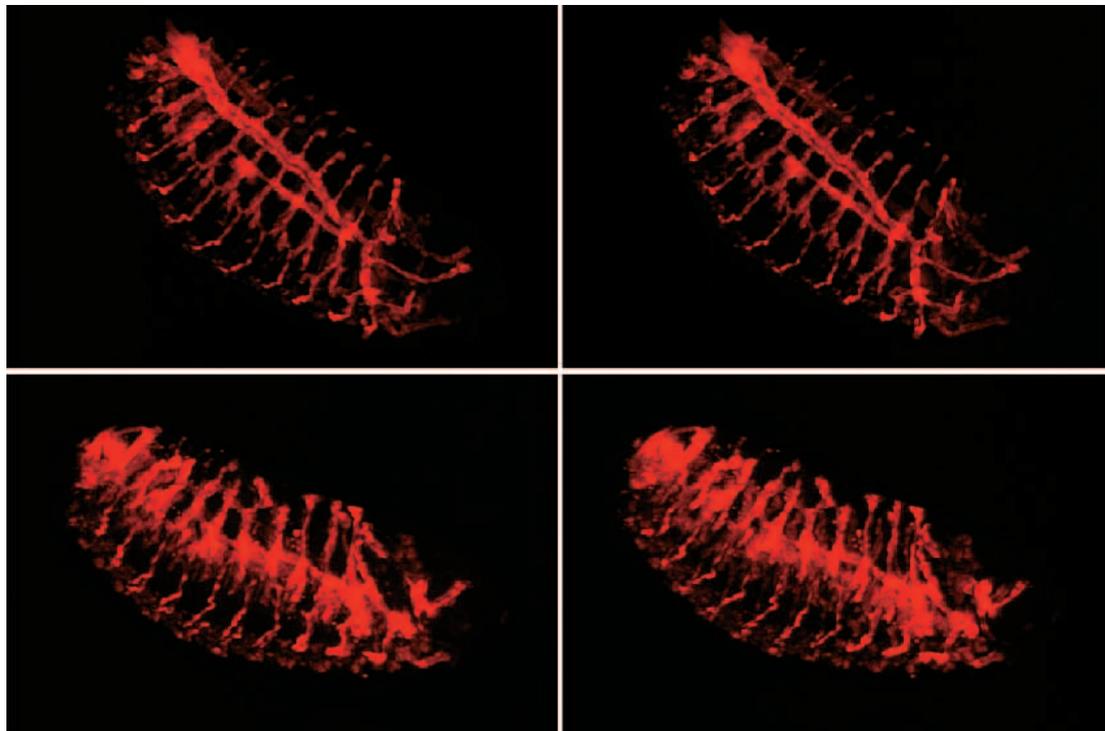
Tracheal system development is initiated by the differentiation of ectodermal precursors into tracheal cells. This process results in ten lateral groups of cells, the tracheal metamers, along each side of the embryo. *sal* mutant embryos develop three additional tracheal metamers in parasegments 2, 3 and 14, respectively. In the *sal* mutants, cuticle structures derived from these parasegments are partially transformed into the adjacent trunk structures, leading to the region-specific homeotic function of *sal* as described earlier (Jürgens, 1988).

This observation suggests that the ectopic tracheal metamers of *sal* mutant embryos may result from homeotic transformation, implying that the absence of tracheal development in wild-type parasegments 2, 3 and 14 is linked to segmental identity generated through *sal* activity. However, both ectopic *sal* gene expression in the central region of the blastoderm embryo, as well as ubiquitous *sal* expression induced by heat-shock-treated stage 5 embryos, suppress tracheal development without otherwise apparently altering segment identity. This finding indicates that repression of tracheal development by *sal* activity is rather specific, and it suggests that Spalt does not act during blastoderm outside its normal domains of expression by interfering with the activity of other homeotic genes. However, Spalt specifically interferes with tracheal development in all positions of the embryo. The simplest explanation for this finding is that *sal* activity acts upon or interferes with the activity of a control gene that determines tracheal cell fate versus epidermal development in the ectoderm.

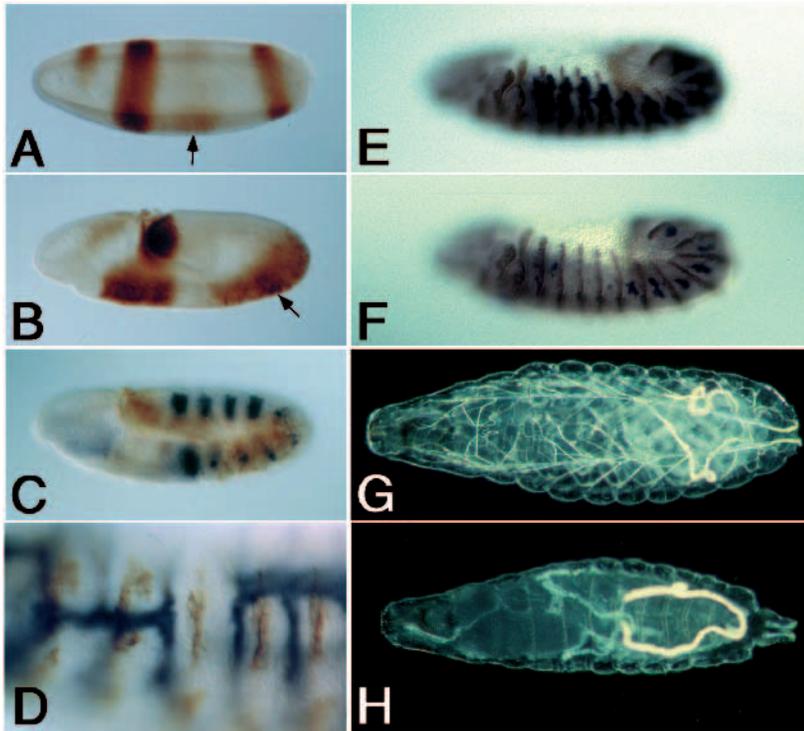
The *trachealess* (*trh*) gene has recently been described as a control gene for tracheal cell fate determination and tubulogenesis (Wilk et al., 1996; Isaac and Andrew, 1996). Ectopic expression of *trh* induces tracheal cell fates and tracheal pits similar to the ectopic placode and pit formation as observed with *sal* mutant embryos (Wilk et al., 1996). The fact that Spalt regulates *lacZ* expression of the *1-eve-1* enhancer trap line, which represents a P-element insertion in the *trh* gene, indicates a genetic dependence of *trh* expression by Spalt. This



**Fig. 2.** *sal* gene expression is required for tracheal system development. (A,B) Whole-mount anti-β-galactosidase staining of a stage 14 wild-type (A) and *sal* mutant (B) embryo bearing the *1-eve-1* chromosome. The arrow points to the dorsal trunk in A and to the lacking dorsal trunk in B. (C) Whole-mount double staining of a stage 15 embryo bearing the G6 chromosome using anti-Spalt antiserum (red, revealed with CY3-coupled secondary antibodies) and anti-β-galactosidase antibodies (green, revealed with fluorescein-coupled secondary antibodies). Superimposition of the CY3 and fluorescein pattern reveals coexpression of Spalt and β-galactosidase (arrows) indicating Spalt expression in the homotip cells of the dorsal trunk. (D) Whole-mount double staining of a stage 15 *sal* mutant embryo bearing the *1-eve-1* chromosome using anti-Escargot (Fuse et al., 1994) and anti-β-galactosidase antibodies. The coexpression of Escargot (black, nuclear) and β-galactosidase (brown, cytoplasmic) in dorsal trunk anterior and posterior cells of the *sal* mutant embryo indicates the presence of dislocated homotip cells (arrows). (E,F) Whole-mount double staining of a stage 11 wild-type (E) and *sal* mutant (F) embryo bearing the *1-eve-1* chromosome using anti-β-galactosidase (blue) and anti-*en* (brown) antibodies. Numbers refer to positions of wild-type (1 and 10) and ectopic (0, -1 and 11) tracheal placodes. (G,H) Scanning electron microscopy (SEM) of a stage 11 wild-type (G) and a *sal* mutant (H) embryo. Numbers refer to positions of wild-type (1 and 10) and ectopic (0 and 11) tracheal pits. mx, maxillary bud; lb, labial bud.

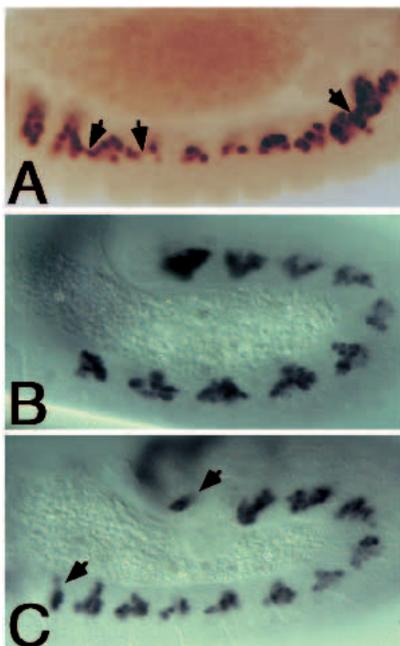


**Fig. 3.** Stereomicroscopy representation of a wild-type and *sal* mutant tracheal system. Stereomicroscopy representation of the tracheal system of a stage 15 wild-type (upper panels; dorsolateral view) and *sal* mutant (lower panels; ventrolateral view) embryo. 3D image reconstructions were based on the image processing of antibody stainings against β-galactosidase expression of the *1-eve-1* tracheal cell marker. Note, there is a sporadic horizontal branch connection between tracheal metamere 5 and 6 in this *sal* mutant embryo.



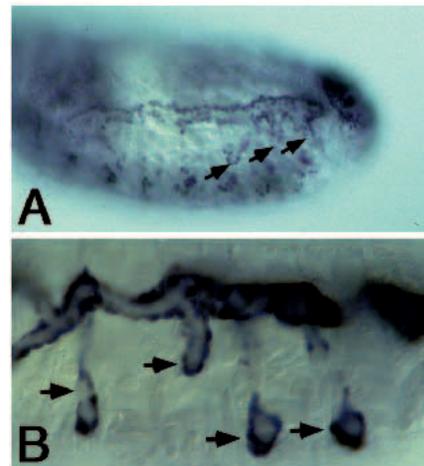
**Fig. 4.** Repression of tracheal development by Spalt. Whole-mount anti-Spalt antibody staining of embryos (A,B) and double staining of *I-eve-I* embryos (C,D) bearing *Kr-Gal4* driver and *UAS-sal* effector constructs (see Materials and Methods). (A) Late stage 5 and (B) stage 9. The ectopic Spalt expression in a region corresponding to the *Kr* central domain expression is first detectable during late stage 5 (arrow in A), accumulating to higher levels in a patchy pattern towards the end of germband extension (arrow in B). (C) Stage 11 staining using anti- $\beta$ -galactosidase (blue) and anti-Spalt (brown) antibodies. Tracheal placode formation is drastically reduced in the region which corresponds to ectopic Spalt expression (TP2-TP6). (D) Stage 16 staining using anti- $\beta$ -galactosidase (blue) and 22C10 (brown) antibodies. Tracheal system formation is drastically affected most obvious by the partial lack of dorsal trunk formation. In contrast, no gross morphological defects of the peripheral nervous system are detectable by ectopic Spalt expression, using monoclonal antibody 22C10 (Zirpursky et al., 1984) as a marker. (E,F) *I-eve-I* embryos at stage 11 lacking the *hs-sal* (E) or bearing the *hs-sal* (F) construct were double stained with anti- $\beta$ -galactosidase (blue) and anti-*engrailed* (brown) antibodies after early heat-shock treatment. In contrast to the highly affected tracheal system (blue), the metamerization process as visualized by *engrailed* expression (brown) is not affected after early ectopic

Spalt expression. (G,H) Dark-field images of living wild-type first instar larvae that lack the *hs-sal* (G) or contain the *hs-sal* (H) construct after early heat-shock treatment. In contrast to the highly branched wild-type tracheal system of first instar larvae shown in G, *hs-sal*-induced ectopic Spalt during blastoderm generates first instar larvae that lack a tracheal system shown in H.



**Fig. 5.** Spalt expression is necessary for dorsal trunk cell migration but not for cell viability. Whole-mount anti-Spalt antibody staining of a stage 14 *sal* mutant embryo (A) bearing the *UAS-sal* and *sal-TSE-Gal4* construct. The Spalt expression in the dorsal trunk of the *sal* mutant embryo causes a partial rescue of the *sal* tracheal phenotype. Several metameres are fused via a dorsal trunk (arrows). Whole-mount anti- $\beta$ -galactosidase antibody staining of a

stage 12 wild-type (B) and a stage 12 *sal* mutant (C) embryo bearing the *sal-TSE-lacZ* construct. In wild-type and *sal* mutant embryos, a similar number of cells express  $\beta$ -galactosidase. Note the  $\beta$ -galactosidase expression in tracheal cells of the ectopic tracheal metameres TP 0 and TP11 of *sal* mutant embryos (arrows).



**Fig. 6.** Ectopic branch formation caused by late heat-shock-induced Spalt expression. Whole-mount anti-Spalt antibody staining of a stage 15 embryo bearing the *hs-sal* construct after late heat-shock (see Materials and Methods) treatment (A). The ectopic outgrowth of branches (arrows) is predominantly detectable from the

posterior region of the dorsal trunk. Whole-mount anti- $\beta$ -galactosidase antibody staining of a stage 15 embryo bearing the *sal-TSE-lacZ* construct and the *hs-sal* construct after late heat-shock treatment (B). The  $\beta$ -galactosidase expression of the *sal-TSE-lacZ* construct serves as a marker for dorsal trunk cell identity and indicates that the ectopic branches (arrows) retain dorsal trunk cell identity. Note: The lumina of the transverse connectives are in a different focal plane.

suggests that the amount of ectopically provided Trachealess is sufficient to overcome the repression of tracheal cell fate by *sal* activity in parasegments 2, 3 and 14. The question whether Spalt interacts directly with *trh* expression or with other components of the genetic circuitry underlying the distinction between epidermal and tracheal cell fate will be addressed in the future.

### Late *sal* gene activity is necessary for directed dorsal trunk cell migration

Once tracheal placodes are formed, *sal* is expressed in the dorsal cells. In these cells *sal* is coexpressed with genes that function at the early stages of tracheal development including *breathless* (*btl*), *pointed* (*pnt*) and *drifter* (*dfr*) (Anderson et al., 1995; Klämbt, 1993; Reichman-Fried et al., 1994). The activities of these genes were shown to be essential for general migration of tracheal cells. In the absence of *dfr* or *pnt* activity, the tracheal cells stop migration halfway through the formation of the tracheal system while, in the absence of *btl* activity, a *Drosophila* homolog of the fibroblast growth factor receptor (DFGF-R1), tracheal cells fail to initiate migration and remain in the epidermis. Since the absence of *sal* activity does not affect general migration of tracheal cells at any stage of embryonic development, we conclude that *sal* does not interfere with the activities of *btl*, *pnt* and *dfr*. In addition, Spalt is expressed normally in tracheal cells lacking *btl* and *pnt* gene activity (data not shown). These observations suggest that *sal* acts independent of these genes.

In contrast to the genes *btl*, *pnt* and *dfr*, which are necessary for general tracheal cell migration during early tracheal system development, the *sal* gene is required for a specific and late aspect of tracheal development; i.e., the directed outgrowth of dorsal trunk anterior and dorsal trunk posterior cells to link the individual tracheal metamers throughout the embryo. In order to fulfill this function, *sal* needs to be expressed from stage 10 onwards, since delayed *sal* expression, as provided by the Gal4-directed transcription system (Brand and Perrimon, 1993), only partially rescues dorsal trunk fusion in *sal* mutant embryos. Thus, the process of dorsal trunk fusion is possibly not only dependent on *sal* activity per se but may also be sensitive to its level of activity. Indeed, the level of *sal* activity seems to be crucial for the directed outgrowth of dorsal trunk cells as increased level of Spalt provided by the heat-shock-dependent Spalt expression cause hypermigration of dorsal trunk cells.

In the absence of *sal*, the outgrowth of dorsal trunk anterior and posterior occurs in an erratic manner. However, each one is led by a homotip cell as observed in wild type, and those cells have not lost their potential to fuse with other homotip cells as observed by occasional ectopic fusion of dorsal trunk branches in *sal* mutant embryos. This implies that *sal* activity is not required for the general migration of dorsal trunk cells or homotip cell formation but rather to provide dorsal trunk cells with the ability of directed outgrowth in an anterior or posterior direction. By this process, the corresponding homotip cells of the adjacent metamers can meet and the branches fuse to establish the normal structure of the dorsal trunk. In order to perform this function, *sal* gene activity may be restricted to dorsal trunk homotip cells by providing these cells with the ability of a directional outgrowth. Homotip cells would then lead and guide dorsal trunk cells by cell adhesion and by phys-

ically pulling tracheal cells behind them. We do not favor this model as it would imply a non-functional Spalt expression in all dorsal trunk cells except homotip cells. Therefore, we favor a model in which all dorsal trunk cells respond to Spalt activity by their directed outgrowth. However, on the basis of our data, we cannot distinguish between either possibility.

### How does *sal* control directed tracheal cell migration?

The *sal* gene encodes a potential transcription regulator, a zinc finger protein (Kühnlein et al., 1994). This may suggest that the *sal* activity regulates the expression of general cell adhesion molecules in dorsal trunk cells that are also expressed on cells surrounding tracheal cells. Cell adhesion mediated by a homophilic interaction may promote the tracheal cells extension but it does not necessarily impart the directional information. Such interactions have been shown to be provided through surface molecules such as N-CAM and N-cadherin to mediate adhesion between axons and neural epithelial cells. On the basis of their uniform expression in most tissues of the developing nervous system, it has been suggested, however, that they do not play a specific role in promoting directional outgrowth (Keynes and Cook, 1995). In addition, *sal* gene activity may control the expression of gene products which play primary roles as directional guidance cues. Such functions have been described for homeodomain proteins encoded by the *Caenorhabditis elegans* genes *mab-5* and *unc-4* (Miller et al., 1992; Salser and Kenyon, 1992). Mutations in the *unc-4* gene cause the connection of specific motor neurons with inappropriate interneurons while mutations in the *mab-5* gene cause a reversal in the direction of posteriorly directed cell migration of migratory neuroblasts to adopted anterior migration. Therefore the *mab-5* and *unc-4* genes appear to be involved in the control of reading guidance cues. Furthermore *sal* target genes may affect the architecture of the cytoskeleton and thereby cause alterations in directed tracheal cell motility (Gumbiner, 1992; Kemler, 1993). Based on the data presented here, we cannot distinguish between these possibilities.

We thank M. Leptin and G. Micklem for providing the T2.2 construct and the 40o Gal4 expressing fly line, S. Hayashi for anti-Escargot antibodies, N. Patel for anti-Engrailed antibodies, P. Buchenau for help with the LSM. We are grateful to B.-Z. Shilo for sharing unpublished results. We thank G. Dowe, M. Hoch, M. Pankratz, B. Purnell and particularly H. Jäckle for helpful comments on the manuscript and for discussions. We are thankful to H. Jäckle for providing a stimulating environment. The work was supported by the Deutsche Forschungsgemeinschaft (SFB 271)

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