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The *pruned* gene encodes the *Drosophila* serum response factor and regulates cytoplasmic outgrowth during terminal branching of the tracheal system

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SUMMARY

We identified a *Drosophila* gene, *pruned*, that regulates formation of the terminal branches of the tracheal (respiratory) system. These branches arise by extension of long cytoplasmic processes from terminal tracheal cells towards oxygen-starved tissues, followed by formation of a lumen within the processes. The *pruned* gene is expressed in terminal cells throughout the period of terminal branching. *pruned* encodes the *Drosophila* homologue of serum response factor (SRF), which functions with an ETS domain ternary complex factor as a growth-factor-activated transcription complex in mammalian cells. In *pruned* loss of function mutants, terminal cells fail to extend cytoplasmic projections. A constitutively activated SRF drives formation of extra projections that grow out in an unregulated fashion. An activated ternary complex factor has a similar effect. We propose that the *Drosophila* SRF functions like mammalian SRF in an inducible transcription complex, and that activation of this complex by signals from target tissues induces expression of genes involved in cytoplasmic outgrowth.

Key words: branching morphogenesis, tracheal development, cytoplasmic outgrowth, serum response factor, *Drosophila*, *pruned*

INTRODUCTION

A general problem for all animals is the supply of oxygen to internal tissues. The common structural solution is formation of a branched tubular network that transports air or oxygenated blood to the sites of utilization. In both the mammalian circulatory system and the insect tracheal (respiratory) system, fine branches ramify on or near the oxygen-requiring tissues, with the extent of branching matched to the oxygen needs of the tissue. Angiogenesis has been intensively studied, and a number of angiogenic factors have been identified in cell culture assays, such as acidic and basic fibroblast growth factors and vascular endothelial growth factor (Folkman and Shing, 1992). It is not yet known how these signaling molecules are involved in angiogenesis in vivo or how such signals are transduced into capillary sprouting and growth toward target tissues.

We have initiated a study of tracheal terminal branch (tracheole) formation in *Drosophila melanogaster*, where the branching process can be analyzed at cellular resolution and dissected genetically. Tracheal tubes are a monolayer epithelium that forms an elaborate tubular network with branches to all body tissues (Rühle, 1932; Manning and Krasnow, 1993). Terminal branches contact the tissues, with most cells lying close to or directly contacted by fine branches (Samakovlis et al., 1996). Oxygen enters the tracheal network through the spiracular openings and diffuses along the primary and secondary branches and finally into the blind-ended terminal branches, where it passes across the tracheal epithelium into surrounding tissues.

Terminal branch formation is distinguished from other tracheal branching events by its cellular mechanism and patterns of outgrowth. Terminal branches arise from individual tracheal cells (called terminal cells) as long cytoplasmic extensions that resemble neuronal processes (Keister, 1948; Wigglesworth, 1972; Samakovlis et al., 1996). The extensions grow towards tracheal target tissues, often reaching 100 μm or more in length. The extensions form a lumen in their cytoplasm, thus becoming fine tubules capable of oxygen transport. The extent and pattern of terminal branching, unlike the earlier stages of branching, is not stereotyped (Rühle, 1932). It is regulated during development by tissue oxygen need, so that tissues with greater need receive a denser supply of branches.

In this paper we report the identification of a gene, *pruned*, that is required for terminal branch formation. Molecular char-

acterization shows that it encodes the *Drosophila* homologue of mammalian serum response factor (SRF) and that it is specifically expressed in terminal cells during terminal branching. SRF has been intensively studied in cultured mammalian cells where it functions with ternary complex factors in growth-factor-activated transcription complexes that regulate expression of c-*fos* and other cellular immediate early genes, and also is involved in muscle-specific gene expression (reviewed by Treisman, 1994, 1995). Although SRF is involved in transduction of a variety of extracellular signals in cultured cells, its function in animals is unknown. We show that the critical role of SRF in early *Drosophila* development is to regulate cytoplasmic outgrowth during terminal branching. The results suggest that the *Drosophila* and mammalian proteins are functional homologues, and that the *Drosophila* protein functions like mammalian SRF in an inducible transcription complex. We propose that activation of this complex by signals from tracheal target tissues stimulates expression of genes involved in cytoplasmic outgrowth.

MATERIALS AND METHODS

Fly strains and genetics

The *pruned*^{1} allele was isolated in a screen for tracheal enhancer trap lines (Terminal-1; Samakovlis et al., 1996). It carries a P[*lacZ, rosy*$^{+}$] element and corresponds to strain l(2)3267 of the Spradling collection (Spradling et al., 1995). Additional alleles and revertants were created by excision of the P element, after crossing in a third chromosome carrying Δ2-3 to supply transposase. Eighty-five *rosy*$^{-}$ males from independent excision events were isolated and used to establish stocks. Excision alleles used here were: *pruned*^{ex84} (early pupal lethal, severe tracheal phenotype); *pruned*^{ex53} and *pruned*^{ex87} (lethal as pharate adults, intermediate tracheal phenotype); *pruned*^{ex3}, *pruned*^{ex8},and *pruned*^{ex40} (viable revertants, no tracheal phenotype). CyO balancer chromosomes carrying P[*hunchback-lacZ, rosy*$^{+}$] or P[*fushi tarazu-lacZ, rosy*$^{+}$], and chromosome translocation TSTL14 carrying the *Tubby* mutation were used to distinguish heterozygous *pruned* embryos and larvae from homozygotes. Chromosome deficiencies Px4, Px and Px2 have been described (Kimble et al., 1990). (The tracheal phenotypes previously reported for these deficiencies (Kimble et al., 1990; Affolter et al., 1994), in which primary tracheal branches are disrupted and discontinuous, are due to the deletion of another gene or more than one gene in the region.) Canton-S was the wild-type strain. Other *lacZ* enhancer trap lines were the general tracheal markers *1-eve-1* (Perrimon et al., 1991) and 6-81a (Bier et al., 1989), and terminal cell markers Terminal-2 to 5 (Samakovlis et al., 1996).

Histology and immunohistochemistry

Embryos were fixed and immunostained as described (Samakovlis et al., 1996). Rat polyclonal serum against the *Drosophila* SRF (Affolter et al., 1994) was used at 1:300 dilution. mAb FMM5 against muscle myosin (from D. Kiehart and C. Goodman) was used at a 1:5 dilution. Other tracheal antibodies are described elsewhere (Samakovlis et al., 1996). Embryo staging was according to Campos-Ortega and Hartenstein (1985).

To visualize tracheal cells in sectioned embryos, embryos heterozygous for the *1-eve-1* enhancer trap marker were fixed in 4% formaldehyde and 0.5% glutaraldehyde. After immunostaining for β-galactosidase, individual embryos, staged under a dissecting microscope by their tracheal and gut morphology, were dehydrated through an ethanol series, infiltrated overnight with Epon-Araldite, and embedded in fresh resin in flat embedding molds. Blocks were hardened for 48 hours at 70°C. Sections (5 μm thick) were cut on a Reichard ultramicrotome, counterstained with 1% methylene blue, and mounted on microscope slides with Permount.

Larvae were dissected in phosphate-buffered saline and fixed in either 1% glutaraldehyde or 2% formaldehyde for 4′,6′diamidino-2-phenylindole (DAPI) staining or horseradish peroxidase immunocytochemistry, respectively, as described by Talbot et al. (1993). Trachea of live larvae were examined under phase contrast optics after anesthetization with sodium azide and mounting in halocarbon oil.

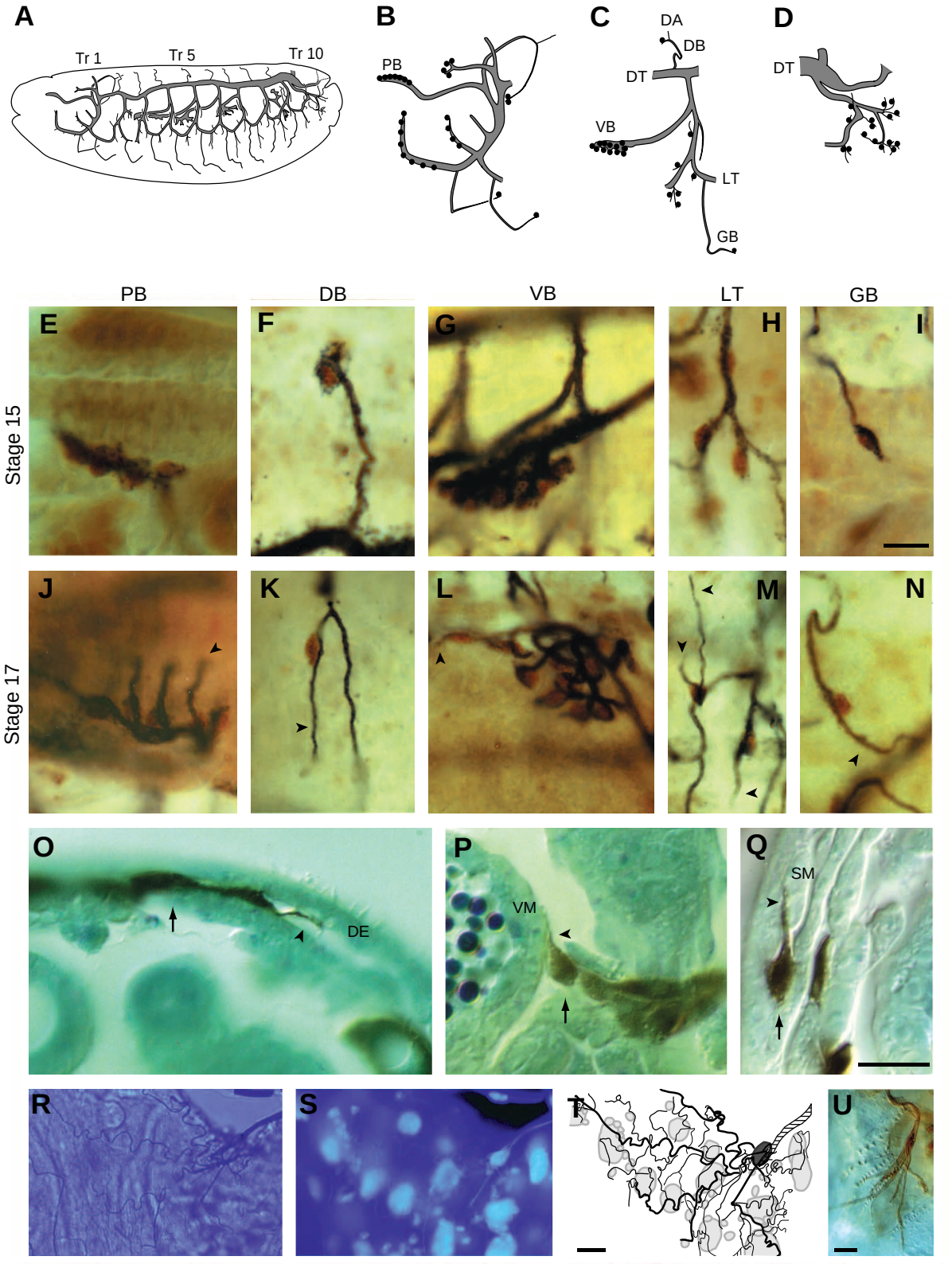
Expression of SRF and Elk-1 derivatives

Flies carrying the activated SRF or Elk-1 constructs described below were crossed to flies carrying a tracheal Gal4p expression construct, and the resulting embryos were aged at 29˚C for maximal Gal4p activity (Brand et al., 1994). Late stage embryos were fixed and stained for tracheal antigens. The C38 (Lin and Goodman, 1994) and Tracheal GAL4 (A. Brand, personal communication) lines both

Fig. 1. The *pruned lacZ* enhancer trap marker is expressed in all tracheal terminal cells. (A) Diagram of left side of tracheal system of a stage 15 embryo stained with a lumen-specific antibody (mAb 2A12). The first, fifth and tenth tracheal hemisegments (Tr1, Tr5, Tr10) are indicated. Dorsal is top and anterior is left in this and all other figures. (B-D**)** Typical nuclear positions (filled circles) of *lacZ* (β-galactosidase)-expressing cells in Tr1 (B), Tr5 (C), and Tr10 (D) of stage 15 embryos carrying the *pruned*^{1} marker. The expressing cells are called terminal cells and give rise to terminal branches. The major tracheal branches from which the terminal branches shown in E- N arise are labeled (PB, pharyngeal branch; DB, dorsal branch; VB, visceral branch; LT, lateral trunk; and GB, ganglionic branch), along with the dorsal trunk (DT) and the dorsal anastomosis (DA) which connects to the contralateral hemisegment. (E-I) Light micrographs of selected β-galactosidase-expressing cells at stage 15, with terminal cell nuclei stained brown (β-galactosidase) and the tracheal lumen stained black (mAb 2A12). The lumen does not extend beyond the terminal cell nuclei at this stage. Only two lateral trunk terminal cells are clearly visible in H. (J-N) β-galactosidase-expressing cells as in E-I but at stage 17. Note lumenal extensions (arrowheads) distal to the nuclei, which are the first terminal branches to form in each cell. Occasionally, a cell has formed more than one branch at this stage (top two arrowheads in M). The terminal visceral branches are still very short at stage 17 (L, arrowhead), but like other terminal branches they ramify extensively during the larval period (see R-T). (O-Q) Transverse sections of a stage 17 embryo showing intimate contact between terminal branches and target tissues. Tracheal cells are stained brown (*1-eve-1* marker). Terminal cell nuclei are indicated by arrows and cytoplasmic extensions by arrowheads. A dorsal branch terminal cell (DB1) extends a terminal branch along the dorsal epidermis (DE) in O. A visceral branch terminal cell extends a terminal branch along the visceral mesoderm (VM) in P. A terminal cell from the lateral trunk extends a terminal branch along a somatic muscle (SM) in Q. (R-T) Ramification of a visceral branch terminal cell on the gut in a third instar larva. R is a phase contrast image in which the air-filled terminal branches appear as thin dark curves. S is a fluorescence micrograph of the same field showing DAPI-stained nuclei. T is a composite tracing of R and S. The secondary tracheal branch is cross-hatched and its terminal branches are shown as thin solid lines; the terminal cell nucleus (dark grey fill) is located near the point where the branch begins to ramify. The nuclei of the larval and imaginal gut cells are shown as large and small circles (in light grey fill), respectively. (U) Light micrograph of a visceral tracheal branch from a heterozygous *pruned*^{1} third instar larva showing continued expression of β-galactosidase in the terminal cell nucleus. Fine branches were lost during fixation and staining. Bars in I (for E-N), Q (for O-Q), R (for R-T) and U, 10 μm.

express Gal4p in all tracheal cells beginning at the end of stage 12, as assayed with a UAS:lacZ construct, and both gave very similar results with all UAS constructs. Experiments with dominant negative Elk-1 were carried out in a similar fashion except that embryos were reared at 18˚C. This reduces Gal4p activity (Brand et al., 1994) and it reduced the penetrance of early tracheal defects that were also seen with dominant negative Elk-1. Apparently, ternary complex factor plays a role earlier in tracheal development, but the function in terminal branching is more sensitive to dominant negative Elk-1.

The structure of the constitutively active SRF-VP16 fusion gene (Dalton and Treisman, 1992), the constitutively active Elk-1-VP16 gene (Hill et al., 1994), and the dominant negative Elk-1 gene (Hill



et al., 1993) have been described. Each was subcloned into the P element vector pUAST (Brand and Perrimon, 1993) downstream of the Gal4p UAS, and germ-line transformants were generated (Spradling and Rubin, 1982). At least two independent transformants of each construct were tested and gave similar results.

Molecular biology

Genomic DNA flanking the *pruned*^{1} P element was isolated by plasmid rescue in *E. coli* (Pirotta, 1986). Flanking genomic DNA (approx. 15 kb) was recovered from an *Spe*I digest of genomic DNA, and 1.4 kb extending upstream of the *pruned* transcription unit were recovered from an *Xba*I digest. For Southern blots (Fig. 5), a λDashII (Stratagene) phage clone (DSRFØ2) containing 15 kb of genomic DNA and a 2.2 kb DSRF cDNA (Affolter et al., 1994) were digested separately with *Eco*RI and *Hin*dIII and hybridized with *Xba*I and *Spe*I rescue plasmid probes. The sequence of the junctions between P element and genomic DNA in the rescue plasmids was determined by dideoxy sequencing, as was the corresponding region of the wild-type chromosome from DSRFØ2.

RESULTS

The *pruned* marker is expressed in all terminal tracheal cells throughout the period of terminal branching

The *pruned*^{1} mutation was isolated in a screen of *D. melanogaster* enhancer trap strains carrying a *lacZ* (β-galactosidase) P element transposon at random genomic positions (Samakovlis et al., 1996). The strain was identified by its highly specific pattern of β-galactosidase expression in the developing tracheal system. Tracheal hemisegments schematized in Fig. 1B-D show the nuclear positions of β-galactosidase-expressing cells in a *pruned*^{1} heterozygote at stage 15, when primary and secondary branches have formed. Each expressing cell gives rise to terminal branches, and all are affected in *pruned* mutants (see below). Our description will concentrate on the tracheal cells and branches shown at stage 15 in Fig. 1E-I. Fig. 1J-N shows the cells approx. 5 hours later when each has begun to form its first terminal branch. These terminal extensions contact different target tissues (Fig. 1O-Q). In the larval period terminal cells continue to grow and ramify on the target tissues (Samakovlis et al., 1996; Fig. 1R-T). The *pruned*^{1} marker continues to be expressed in terminal cells during this time (Fig. 1U).

Terminal branches are missing in *pruned* mutants

In homozygous *pruned*^{1} embryos, major tracheal branches and connections between branches were almost always normal but terminal branches were truncated or absent, giving the tracheal tree a 'pruned' appearance. *pruned*^{1} is a recessive mutation and the phenotype is fully penetrant. The embryonic phenotype was most easily appreciated in the dorsal and ganglionic branches (Figs 2A,B and 4B,C below), where terminal extensions are normally very long at this stage, but other terminal branches were similarly affected (Fig. 2C,D). Essentially every terminal branch was severely truncated or absent in mutant embryos.

When *pruned*^{1} was tested over Df(2R)Px4, which removes a large chromosomal region including the *pruned*^{1} locus, the hemizygous embryos exhibited the same tracheal phenotype with the same penetrance and expressivity as *pruned*^{1} homozygotes. The same phenotype was also observed for strong excision alleles of the *pruned*^{1} P element, for two strong EMS alleles, and for small chromosomal deficiencies that remove the *pruned* gene (see below; J. Montagne, J. G and M. A., unpublished data). Thus *pruned*^{1} is a loss of function allele, and it represents the null condition for this function.

pruned^{1} is homozygous lethal with poor larval viability and no survivors beyond the early pupal stage. Examination of surviving larvae revealed a striking phenotype. Instead of the extensive networks of terminal branches seen in wild type (see Figs 1R-T and 2E), the parental branches of homozygous *pruned*^{1} larvae ended abruptly without ramification (Fig. 2F). The parental branches and branches that interconnect the tracheal network were generally intact (DB and DA in Fig. 2F). Thus the *pruned*^{1} mutation prevents formation of terminal branches during both embryonic and larval periods.

Despite the absence of terminal branches, the surviving mutant larvae were still able to move about, although they were more sluggish than their heterozygous siblings. Many mutant larvae contained dark tissue masses typically associated with the fat body. These might be necrotic fat body tissue resulting from inadequate oxygen supply (see Abrahamson and Fanale, 1959).

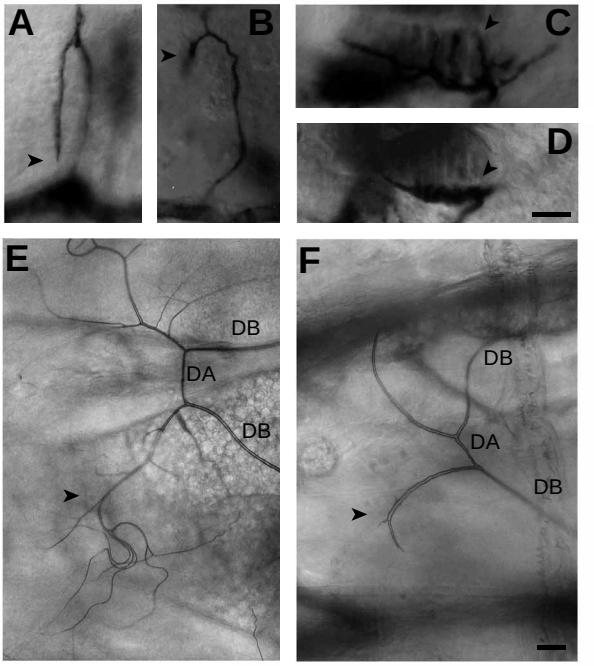


Fig. 2. Terminal branches are truncated in *pruned* embryos and larvae. The dorsal branch (A,B) and end of a pharyngeal branch (C,D) in stage 17 heterozygous *pruned*^{1} embryos (A,C) and homozygous *pruned*^{1} embryos of the same age (B,D), stained for tracheal lumen (mAb 2A12). Arrowheads show the ends of normal and truncated terminal branches. (E,F) Left and right dorsal branches of wild-type (E) and *pruned*^{1} homozygous (F) third instar larva in dorsal view. In the mutant, the dorsal branches end prematurely (arrowhead in F) and without ramification, near the point where the dorsal branch ramifies extensively in wild type (arrowhead in E). The proximal portion of the dorsal branches (DB) and the dorsal anastomosis (DA) connecting the right and left dorsal branches are unaffected in the mutant. Bars in D (for A-D) and E (for E-F), 10 μm.

Absence of terminal branches results from failure of tracheal cells to extend cytoplasmic processes

The cellular events leading to formation of a terminal branch in wild type are shown in Fig. 3A-D. At early stage 15, the terminal cell begins to extend a cytoplasmic process (Fig. 3B, arrowhead). A lumen is present in the terminal cell but only up to the level of the nucleus (Fig. 3B,C); this is a secondary tracheal branch. The cytoplasmic projection continues to grow, becoming very thin as it extends along the surface of the target (Fig. 3C,D). A lumen begins to form in the projection. Lumenal staining appears in short patches at variable positions, initially discontinuous with the lumen of the secondary branch (arrowhead in Fig. 3C). Patches expand and coalesce, forming a continuous lumen extending out to the tip of the projection (Fig. 3D). During the larval period many more cytoplasmic processes grow out and develop lumens, generating multiply-branched cellular structures like the one shown in Fig. 1R-T.

The terminal cells of *pruned*^{1} embryos did not have long cytoplasmic projections beyond the cell body (compare Fig. 3E and G with D and F). In some cases rudimentary projections were seen, but these were always short and broad, resembling those in the wild type at early stage 15, and they never supported a lumen. Thus, the absence of terminal branches in *pruned*^{1} embryos is a consequence of the failure of terminal cells to extend long cytoplasmic projections to their targets.

Defects in cytoplasmic outgrowth in *pruned*^{1} mutants were detectable several hours after initiation of *pruned* gene expression and only after terminal cells had migrated close to their targets. Using tracheal cytoplasmic (*1-eve-1*), nuclear (6-81a), and lumenal markers (mAb 2A12), we showed that tracheal cell migration and lumen formation during earlier branching events were little affected by the *pruned*^{1} mutation. We also tested whether *pruned* was required for expression of four terminal-cell-specific enhancer trap markers (Terminal-2 to 5), all of which are first expressed at or shortly after initiation of *pruned* expression but before outgrowth of cytoplasmic processes (Samakovlis et al., 1996). None of these markers exhibited altered expression patterns in embryos homozygous for *pruned*^{ex84}, a null allele of *pruned* that does not express *lacZ* (data not shown). Thus, the earliest cellular or molecular defect manifest in *pruned* embryos is the inability of terminal cells to extend cytoplasmic processes to target tissues.

The *pruned* gene encodes the *Drosophila* serum response factor

To begin molecular characterization of the *pruned* locus, we first established that the tracheal phenotype and lethality of the *pruned*^{1} mutation were due to the P element. Excision of the P element, which carries a *rosy*$^{+}$ marker, was induced by introduction of a source of transposase. Twenty-one per cent (18 of 85) of the independent *rosy*$^{-}$ strains generated by excision were fully viable, and the tracheal phenotype was reverted in all three strains examined. Thus, the *pruned*^{1} lethality and tracheal phenotype are caused by the P element. Among the lethal *rosy*$^{-}$ strains obtained, several had tracheal phenotypes identical to *pruned*^{1}, including *pruned*^{ex84} which lacked all β-galactosidase expression. Other excision alleles (see Materials and Methods) had less expressive phenotypes, with less severe truncations of terminal branches.

The P element in *pruned*^{1} mapped to cytological position 60C. This is the same chromosomal region as the recently isolated homologue of mammalian serum response factor (Affolter et al., 1994). The *Drosophila* serum response factor (DSRF) is strongly expressed in a subset of embryonic tracheal cells, and more weakly in somatic muscles, so it was a good candidate for the gene that is affected in *pruned* mutants. We established that *pruned*^{1} is a loss of function mutation in the DSRF gene by the following tests.

First, tracheal expression of DSRF protein was indistinguishable from the expression pattern of the β-galactosidase marker in embryos and third instar larvae carrying a single copy of the *pruned*^{1} marker (compare Fig. 4A and C, and data not shown). Expression of both DSRF and β-galactosidase was turned on at stage 13 in all terminal cells, and expression persisted in these cells throughout embryonic and larval life.

Second, the *pruned*^{1} mutation mapped to the DSRF locus. The *pruned*^{1} lethality was mapped genetically to the region of overlap (60C5-6 to 60D1-2) between chromosomal deficiencies Px and Px2, the same interval as DSRF (Affolter et al., 1994). Genomic DNA flanking the *pruned*^{1} P element was then isolated by plasmid rescue and used for molecular mapping. The P element was inserted in the first exon of DSRF, 350 bp upstream of the assigned translation start site (Fig. 5).

Finally, a DSRF antiserum was used to examine DSRF protein levels in *pruned*^{1} mutants. There was no detectable DSRF expression in the tracheal cells of mutant embryos or larvae (Fig. 4B and data not shown). This is consistent with the genetic data indicating that *pruned*^{1} is a null allele for the tracheal function of the gene. DSRF protein expression in somatic muscles of mutants was weak and difficult to detect above background. Two of the strongest *pruned* alleles (*pruned*^{1}, *pruned*^{ex84}) were examined for muscle defects, using an antibody to *Drosophila* muscle myosin. No abnormalities were detected in the number or general organization of the somatic musculature in homozygous or hemizygous embryos (data not shown).

An activated SRF causes extra cytoplasmic processes and terminal branches

Mammalian SRF is a MADS box transcription factor that forms a complex with a subset of ETS-domain transcription factors, called ternary complex factors, at the promoters of immediate early genes such as c-*fos*. The complex is normally inactive until the cells are exposed to various growth factors, which trigger signaling cacades that culminate in modification of one or more components of the complex. SRF can be made into a constitutive activator of transcription independent of the signaling cascades by fusion of the Herpes simplex virus VP16 transcriptional activation domain to the carboxyl terminus of the protein (Dalton and Treisman, 1992; Fig. 6A). We tested the effect of constitutively activated SRF on tracheal development by expressing an SRF-VP16 chimera in developing tracheal cells using the GAL4 indirect expression system (Brand and Perrimon, 1993).

Activated SRF drove formation of extra cytoplasmic processes and terminal branches that grew out earlier than usual and in an unregulated fashion (Fig. 6). Normally, terminal cells like DB1 form just a single cytoplasmic projection with a single lumen by stage 17 (Fig. 3D). Embryos expressing the activated SRF formed more and longer cytoplasmic extensions than normal (Fig. 6B,C). Many of these

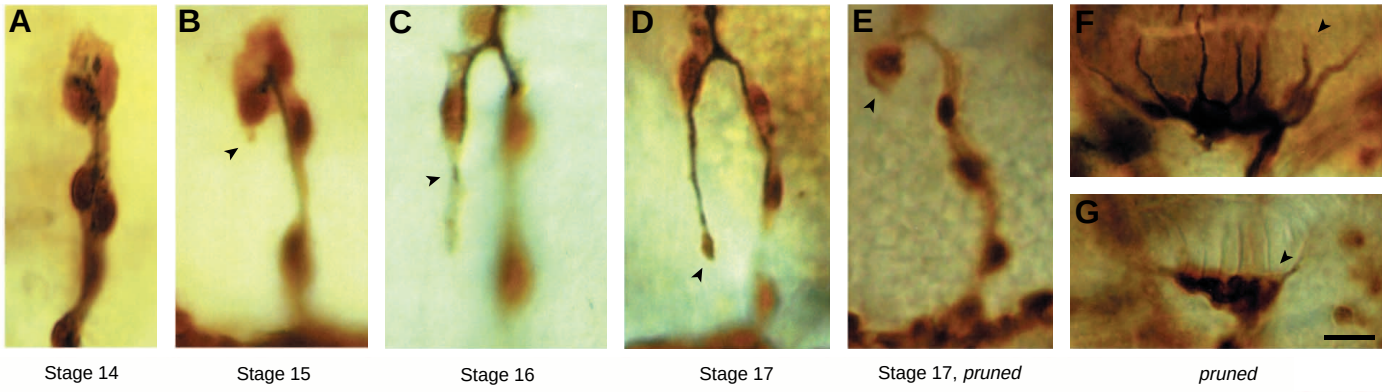


Fig. 3. Terminal cells in *pruned* mutants do not extend long cytoplasmic processes. Embryos were stained for the *1-eve-1* marker (cytoplasm in light brown and nuclei in dark brown) and the mAb 2A12 lumenal antigen (in black). (A-D) Dorsal branch in wild-type embryos at the stages indicated, focused in the plane of the terminal cell, DB-1. (A) Stage14, approx. 11 hours after egg lay (AEL). (B) Early stage 15, approx. 11.5 hours AEL. A short cytoplasmic projection emanates from the terminal cell (arrowhead). (C) Early stage 16, approx. 13.5 hours AEL. A short segment of lumenal staining (arrowhead) is visible within the long thin cytoplasmic projection. (D) Stage 17 embryo, approx. 16 hours AEL. Note bulbous ending of cytoplasmic projection (arrowhead). (E) *pruned*^{1} homozygote, stage 17. The terminal cell is present (arrowhead) but it lacks the long cytoplasmic process seen in wild type (compare to D). (F,G) Distal portion of the pharyngeal branch of a stage 17 wild-type embryo (F) and *pruned*^{1} homozygote (G). Cytoplasmic processes (arrowhead) are missing in the mutant. Bar, 10 μm.

went on to develop a lumen, as indicated by staining with mAb 2A12 (Fig. 6D,E). Most of the extra terminal branches that formed arose from the normal terminal cells, but other tracheal cells sometimes formed ectopic terminal branches. These were almost always cells adjacent to terminal cells, which sometimes express terminal markers and form terminal branches in wild type (Fig. 6H).

The cytoplasmic processes that formed in cells expressing SRF-VP16 often did not grow toward their usual targets but instead grew out in random directions, invading territories normally supplied by other tracheal branches (Fig. 6G,H). Outgrowing branches sometimes reversed direction abruptly (Fig. 6D, arrowhead) and traversed other tracheal branches (Fig. 6E,G arrowheads), events that rarely if ever occur in wild type. These observations demonstrate that extra cytoplasmic outgrowth and branching can be driven by activated SRF, and that it is independent of the normal guidance and growth controls.

An activated ternary complex factor has similar effects as activated SRF

In mammalian cells, SRF typically functions in conjunction with a ternary complex factor such as Elk-1. The domain of SRF that interacts with ternary complex factors is conserved in the DSRF protein (Affolter et al., 1994), suggesting that such a factor may be present in *Drosophila*, although none has been identified. To test for involvement of a ternary complex factor in terminal branching, we used constitutively activated and dominant negative forms of mammalian Elk-1 protein. The dominant negative form is a C-terminal truncation of Elk-1 that removes its regulated transcriptional activation domain, so that the protein can still interact with SRF and bind DNA but fails to activate transcription (Hill et al., 1993; Fig. 7A). The constitutively activated form of Elk-1 has the regulated transcriptional activation domain replaced with the VP16 activation domain (Hill et al., 1994; Fig. 7A). Expression of the dominant negative Elk-1 during the period of terminal branching resulted

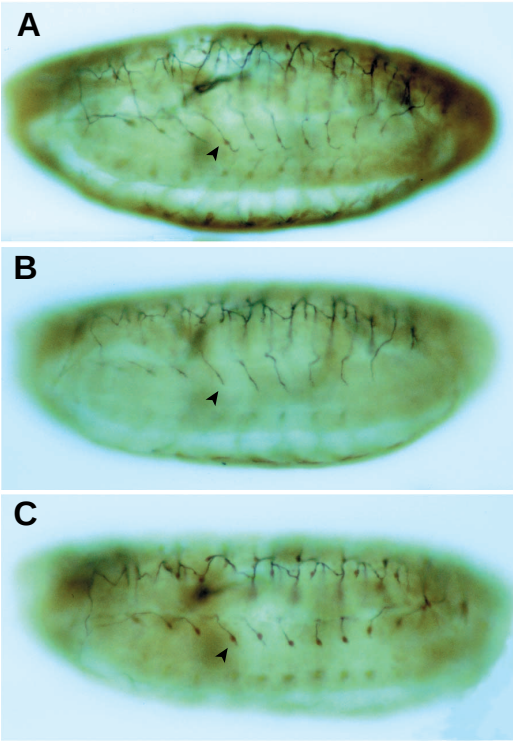


Fig. 4. *Drosophila* serum response factor (DSRF) is expressed in the same pattern as the *pruned*^{1} marker and expression is absent in *pruned* mutants. (A,B) Ventrolateral views showing the tracheal ganglionic branches of stage 17 heterozygous (A) and homozygous (B) *pruned*^{1} embryos double stained together for DSRF (brown) and mAb 2A12 lumenal antigen (black). DSRF staining is visible in the nucleus (arrowhead) of terminal cells in A but not the *pruned*^{1} homozygote in B. The *pruned*^{1} homozygote has truncated terminal branches. **(**C**)** Similar view of a *pruned*^{1} homozygote double stained for β-galactosidase and mAb 2A12, showing the same expression of β-galactosidase as DSRF expression in A.

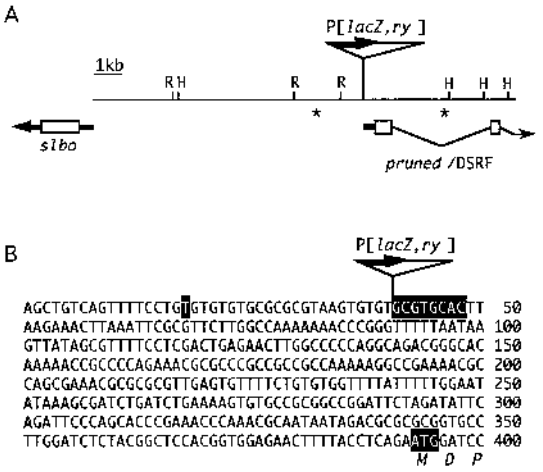


Fig. 5. Molecular mapping of the *pruned* mutation. (A) Restriction map of a 15 kb genomic clone containing the 5′ portion of the *pruned*/DSRF transcription unit. Positions of *pruned* and the neighboring *slbo* (Montell et al., 1992) transcription units are shown. Open boxes, coding sequences; filled bars, 5′ and 3′ untranslated regions; diagonal lines, introns. The position of the *pruned*^{1} P element and orientation of the P element promoter are indicated. The 1.8 kb and 6.2 kb *Eco*RI genomic fragments which hybridized on a Southern blot to both the *Xba*I and *Spe*I P element rescue plasmids are indicated by asterisks. R, *Eco*RI site; H, *Hin*dIII site. (B) Nucleotide sequence of the 5′ portion of the *pruned*/DSRF transcription unit. The P element insertion site in *pruned*^{1} is shown by the vertical line next to the eight nucleotides of genomic DNA that were duplicated at the insertion site (boxed, nucleotides 41-48). The first nucleotide of the DSRF cDNA sequence (nucleotide 18) and the predicted ATG translational start (nucleotides 393-396) are also boxed (Affolter et al., 1994). The first nucleotide shown is a putative transcription initiation site identified by primer extension analysis that is consistent with the length of the major *pruned* transcript (2.2 kb) detected on northern blots.

in truncated terminal branches very similar to those observed in the *pruned* loss of function mutants (Fig. 7C). Expression of the constitutively activated Elk-1 protein resulted in extra terminal branches that grew out in an unregulated fashion, as with the activated SRF protein (Fig. 7D). Although results with Elk-1 were essentially the same as those obtained with SRF in most respects, the extra, unregulated branching observed with activated Elk-1 was completely dependent on the function of the endogenous *pruned* gene. While *pruned*^{1} mutants expressing activated SRF formed rudimentary terminal branches (data not shown), *pruned*^{1} mutants expressing activated Elk-1 displayed the *pruned* loss of function phenotype and did not form terminal branches (Fig. 7E).

DISCUSSION

The *Drosophila* serum response factor pruned is a key regulator of terminal branching of the tracheal system. We have shown both that it is required for terminal branch formation, and that an activated form of SRF is sufficient to drive excessive terminal branching. The results further suggest that DSRF functions like the mammalian protein, together with a ternary complex factor. While mammalian cell culture experiments and in vitro studies have elucidated the mechanism of SRF action, they cannot provide information about the process SRF regulates in vivo. Our data show that DSRF controls cytoplasmic outgrowth during terminal branching, a novel function not anticipated from previous work.

A model for DSRF function in cytoplasmic outgrowth during terminal branching

DSRF is a structural and functional homologue of mammalian SRF. It displays greater than 90% sequence identity with the functional core of the mammalian protein (Affolter et al., 1994), which includes its DNA binding, homodimerization, and accessory protein interaction domains. The residues that dictate binding specificity and complex formation with ternary complex factor are completely conserved. The *Drosophila* protein also has the same DNA binding specificity as the mammalian protein (see Treisman, 1987). More compelling is the fact that mammalian SRF derivatives can provide *pruned* function in *Drosophila*, and a mammalian ternary complex factor can function with DSRF.

The close relationship between DSRF and mammalian SRF suggests a simple molecular model for how DSRF controls cytoplasmic outgrowth during terminal branching. Like mammalian SRF, DSRF presumably functions as a DNA binding transcription factor. Mammalian SRF binds and regulates the c-*fos* proto-oncogene and other ‘immediate-early’ genes (Treisman, 1990). In our model, DSRF controls expression of one or more genes that are necessary for extension of long cytoplasmic processes from terminal cells. Its targets might encode components necessary to form or stabilize cytoplasmic processes, or adhesion molecules that encourage their outgrowth.

Mammalian SRF forms a ternary complex with Elk-1 or related ternary complex factors at the c-*fos* serum response element (Treisman, 1994). Expression of dominant negative and constitutively activated forms of Elk-1 mimicked the *pruned* loss-of-function phenotype and the extra branching phenotype of activated SRF, respectively. The effect of activated Elk-1 required *pruned*$^{+}$ function, as expected if activated Elk-1 requires an SRF for binding (Shaw et al., 1989). These results suggest that DSRF functions in the developing tracheal system with an as yet unidentified ternary complex factor, and forms analogous ternary complexes at its targets.

Tracheogenic signals and the activation of the DSRF transcription complex

The SRF transcription complex is activated by extracellular signals. Although the signal transduction pathways that activate the transcription complex are still being elucidated, at least in some instances they appear to involve Ras, Raf, and MAP kinases, or the small Ras-like GTPases Rac, Rho, and Cdc42, and result in the phosphorylation of one or more proteins in the complex (reviewed by Treisman, 1995). In our model, DSRF transcription complexes are similarly quiescent until terminal cells receive an extracellular signal. In mammalian cells, the signal requirement can be bypassed by fusion of the VP16 transcriptional activation domain to either protein of the complex, or by expression of activated forms of Ras or other upstream signaling components, resulting in constitutive expression of target genes. Expression of activated

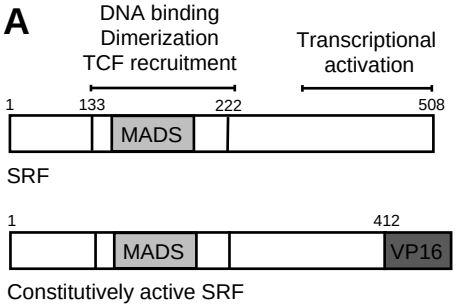
forms of SRF or Elk-1 in tracheal cells, or activated forms of upstream signaling components (K. G. and M. K., unpublished data), leads to formation of extra terminal branches that grow out in an unregulated pattern, presumably because DSRF target genes are constitutively expressed.

A likely source of the activating signal is the tracheal target tissues. Experiments in other insects suggest that tracheal targets produce a signal that induces formation of new branches and attracts them to the target (reviewed by Manning and Krasnow, 1993). When a target tissue is ablated during larval development the terminal branches that normally supply the tissue fail to form (Wigglesworth, 1954), the same consequence as inactivating the *pruned* gene. According to our model, both manipulations result in missing terminal branches because in one case (target ablation) the tracheogenic signal is removed, and in the other case (*pruned* mutant) the signal is present but cannot be transduced into an effect on cytoplasmic outgrowth. Conversely, transplantation of a metabolically active tissue causes nearby terminal branches to proliferate and cover the tissue (Wigglesworth, 1954). Proliferation of terminal branches is also observed in animals reared at low oxygen pressures or other conditions that induce tissue hypoxia (Wigglesworth, 1954; Locke, 1958). In these experiments the oxygen-starved tissues are thought to produce increased levels of a tracheogenic signal that causes the observed proliferation of terminal branches, an effect that is mimicked by activated SRF or Elk-1.

An important difference between the extra terminal branches induced by tissue anoxia and by expression of activated SRF or Elk-1 is seen in the outgrowth and spatial patterning of branches. In wild type, terminal branch patterns are not stereotyped, but they are nevertheless highly regulated. Branches usually remain within specific spatial domains, and they rarely if ever cross over other tracheal branches even though they densely cover the target. With activated SRF or Elk-1, terminal branches grew out in various directions, invading new territories and growing over other branches. The normal mechanisms that control the direction of cytoplasmic outgrowth and the distribution of terminal branches are apparently saturated or bypassed by activated SRF or Elk-1. The data suggest that the DSRF transcription complex regulates the decision to form a new cytoplasmic extension, but not necessarily where it will grow. There must be another mechanism, functioning in parallel to the DSRF transcription complex, that controls where on the cell surface the outgrowth will form and the direction it grows.

A general role for SRF in regulation of the cytoskeleton and cytoplasmic outgrowth?

The role of DSRF in regulating cytoplasmic outgrowth of terminal tracheal cells is the first demonstrated function for an SRF in an animal. Studies of mammalian SRF in cultured cells



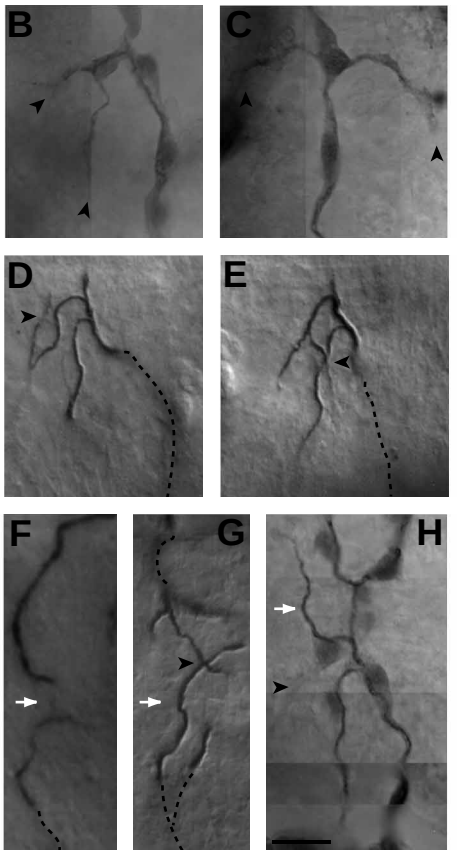


Fig. 6. Expression of an activated SRF in the tracheal system causes extra cytoplasmic extensions and terminal branches. (A) Primary structure of human SRF and a constitutively active derivative containing the VP16 transcriptional activation domain. MADS box homology and functional domains of SRF are shown. (B,C) Dorsal branches of late stage embryos expressing SRF-VP16, double-stained for the *1-eve-1* marker and tracheal lumen (mAb 2A12). The DB-1 terminal cell in B and C have each formed two cytoplasmic processes with lumen (arrowheads). The two long cytoplasmic processes in C extend in atypical directions (compare with Fig. 3D), as does the top process in B. (D,E) Dorsal branch of late stage embryos expressing SRF-VP16, stained for tracheal lumen (mAb 2A12). In D, two terminal branches have formed, one of which sharply reverses direction (arrowhead). In E, three terminal branches have formed and one traverses another (arrowhead). (F,G) Ventral view of right and left ganglionic branches of a control wild-type embryo (F) and an embryo expressing SRF-VP16 (G). In G the right (upper) ganglionic branch has formed three terminal branches and the left (lower) ganglionic branch has formed two terminal branches, one of which has grown across the midline over another terminal branch (arrowhead). White arrow, ventral midline. (H) Dorsal view of a right and left dorsal branch of an embryo expressing SRF-VP16, stained as in B. The normal terminal cell (DB1) and an additional cell (DB3) of the left (lower) dorsal branch have both formed long terminal branches; the terminal branch emanating from DB1 has inappropriately crossed the midline (white arrow). Each of these cells has also formed a second cytoplasmic projection that traverse one another (arrowhead). Bar (B-H), 10 μm.

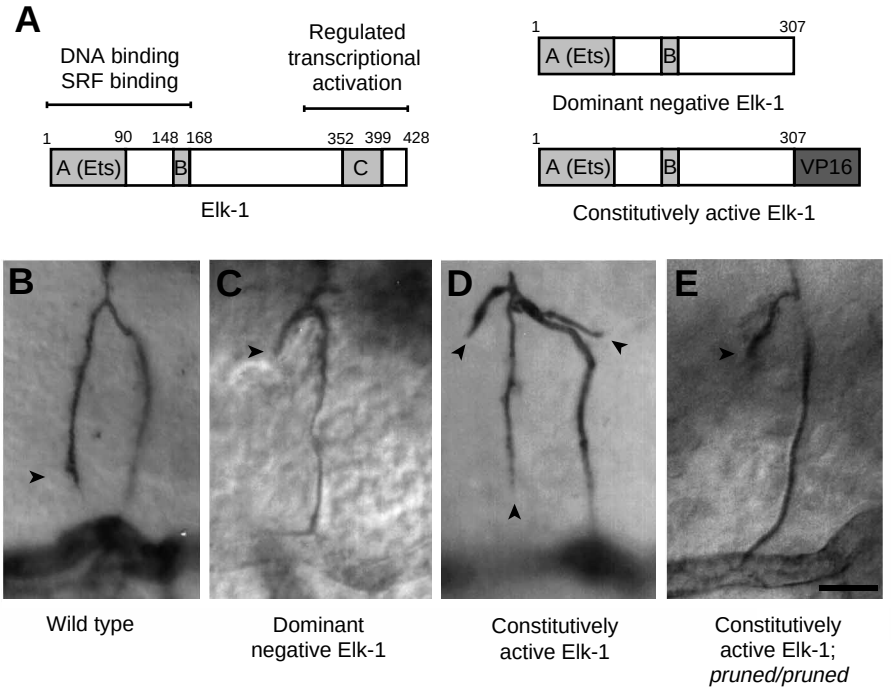


Fig. 7. Effect of dominant negative and constitutively active forms of Elk-1 on terminal branching. (A) Primary structure of ternary complex factor Elk-1 and two derivatives, showing the positions of functional domains and three signature motifs (A, B, C) of ternary complex factors. (B) Dorsal branch lumen of a wild-type stage 17 embryo. Arrowhead points to the tip of the terminal branch. (C) Stage 17 embryo expressing dominant negative Elk-1. The terminal branch is truncated (arrowhead). (D) Stage 17 embryo expressing Elk-1-VP16. Three terminal branches have formed and grown out in different directions (arrowheads). (E) Dorsal branch lumen of a stage 17 *pruned*^{1} homozygote expressing Elk-1-VP16. The terminal cell expresses the *pruned*^{1} marker but does not form a terminal branch (arrowhead). The phenotype is indistinguishable from *pruned*^{1} homozygotes that do not express Elk-1-VP16. Bar (B-E), 10 μm.

have focused on possible roles in mitogenesis, but additional evidence implicates SRF in regulation of the cytoskeleton. SRF targets include a number of cytoskeletal genes (Treisman, 1995), and treatment of cells with agents that disrupt the cytoskeleton, such as cytochalasin D, activates SRF-mediated transcription (Zambetti et al., 1991; Hill et al., 1995). Recent evidence has implicated SRF in signaling pathways involving the small GTPases Rho, Rac and Cdc42 (Hill et al., 1995) that regulate cytoskeletal rearrangements (reviewed by Chant and Stowers, 1995; Ridley, 1995). Perhaps Rho, Rac, and Cdc42 activation of SRF stimulates expression of genes that reinforces the more direct effects of these small GTPases on the cytoskeleton.

Not only does *pruned* regulate cytoplasmic outgrowth during terminal branching, but it has also been found to function during morphogenesis of the wing in a process that also involves polarized cell shape changes (Montagne et al., unpublished results). One of the roles of MCM1 protein, a related protein in yeast, is in the pheromone response pathway in which the cell undergoes polarized cell growth (shmoo formation) toward an extracellular signal (reviewed by Dolan and Fields, 1991). Common to all these examples may be an SRF homologue regulating changes in cytoskeletal components that lead to stable alterations in cell shape.

The isolation of mutations in the *pruned* gene opens the way to a detailed analysis of DSRF functions and the proposed signaling pathway in vivo. The implication of a ternary complex factor suggests the involvement of Ras and MAP kinase upstream in signaling. The *breathless* fibroblast growth factor receptor may also function in this pathway. Although *breathless* plays crucial roles in tracheal development long before terminal branching begins, it was recently shown that a dominant negative form of the protein expressed late in embryogenesis can give rise to truncated terminal branches resembling those of the *pruned* loss-of-function mutants (Reichman-Fried and Shilo, 1995).

Genetic control of branching morphogenesis: *pruned* defines a regulated late program

Although tracheal branches arise sequentially from a single homogenous cluster of tracheal precursor cells in each hemisegment, there are morphological, molecular and functional differences between early and late branching events (Samakovlis et al., 1996). The *pruned* phenotype demonstrates that the early and late branching processes are separable genetically and it defines the late phase. Tracheal development thus appears to be under the dual control of a hard-wired early genetic program that controls the outgrowth of the stereotyped major branches and positions them close to their targets, and a regulated late program mediated by *pruned* that governs the outgrowth of fine branches to tissues according to tissue oxygen need. The early program also controls the highly specific expression pattern of *pruned*, ensuring that only terminal cells express *pruned* and are competent to respond to signals and form terminal branches (Samakovlis et al., 1996). There are striking morphological and functional parallels between terminal branching and mammalian angiogenesis. It seems likely that the system for sensing and responding to tissue oxygen need is ancient, and it will be interesting to see if SRF is involved in angiogenesis and if any of these parallels extend to the molecular level.

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