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The *Drosophila* homologue of SRF acts as a boosting mechanism to sustain FGF-induced terminal branching in the tracheal system

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SUMMARY

Recent data have demonstrated a crucial role for the transcription factor SRF (serum response factor) downstream of VEGF and FGF signalling during branching morphogenesis. This is the case for sprouting angiogenesis in vertebrates, axonal branching in mammals and terminal branching of the *Drosophila* tracheal system. However, the specific functions of SRF in these processes remain unclear. Here, we establish the relative contributions of the *Drosophila* homologues of FGF [Branchless (BNL)] and SRF [Blistered (BS)] in terminal tracheal branching. Conversely to an extended view, we show that BNL triggers terminal branching initiation in a DSRF-independent mechanism and that *DSRF* transcription induced by BNL signalling is required to maintain terminal branch elongation. Moreover, we report that increased and continuous FGF signalling can trigger tracheal cells to develop full-length terminal branches in the absence of *DSRF* transcription. Our results indicate that DSRF acts as an amplifying step to sustain the progression of terminal branch elongation even in the wild-type conditions of FGF signalling.

KEY WORDS: *Drosophila*, FGF, SRF (BS), Morphogenesis, Trachea, Tube formation

INTRODUCTION

Branching morphogenesis is a widespread process in development that requires important cell shape changes, such as elongation and lumen formation. Vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) signalling play a conserved role during the branching of ramified cellular systems upstream of the MADS-box transcription factor SRF (serum response factor), which controls the expression of cytoskeletal proteins involved in branching. This is the case for axonal branching in mammals (Alberti et al., 2005; Knoll et al., 2006; Wickramasinghe et al., 2008) and tubulogenesis during sprouting angiogenesis in vertebrates (Franco et al., 2008) and during terminal branching of the *Drosophila* tracheal system (Affolter et al., 1994; Guillemin et al., 1996; Sutherland et al., 1996).

The *Drosophila* tracheal system is a well-established model for the formation of complex branched tubular structures, which displays strong similarities with blood vessel sprouting in mammals (Franco et al., 2008). The development of tubes is crucial to higher multicellular organisms as these organisms rely on those structures to supply oxygen and nutrients to their cells. *Drosophila* embryonic tracheal cells invaginate and migrate in a stereotypical pattern in response to a set of positional cues in order to form each of the primary tracheal branches. All tracheal cells express Breathless (BTL), an FGF receptor (Klambt et al., 1992), allowing them to respond to the FGF homologue BNL, which is expressed by nearby cells at each position where a new branch will form and subsequently extend (Sutherland et al., 1996). These epithelial tubes can form through multiple mechanisms (for reviews, see Ghabrial et al., 2003; Lubarsky and Krasnow, 2003). The main branches have extracellular lumens, as their cells fold to form a tube. Subsequently, terminal cells, a set of specialised cells in some of the main branches, form fine tubes through the generation of an intracellular lumen. Terminal branch formation in the embryo is controlled by DSRF (Blistered, BS – FlyBase) expression, which is triggered by BNL signalling (Affolter et al., 1994; Guillemin et al., 1996; Sutherland et al., 1996) and is required for proper actin cytoskeleton reorganisation (Gervais and Casanova, 2010). Here, we address the question of the respective contribution of FGF signalling and SRF and establish a reinforcement mechanism for these factors in promoting fine branch development in a two-step process. On the one hand, FGF triggers initial terminal cell development, whereas on the other hand, FGF induces *DSRF* transcription, which in turn ensures the progression of terminal cell elongation. However, the DSRF-mediated step is dispensable when high levels of FGF are continuously supplied.

MATERIALS AND METHODS

Drosophila stocks and genetics

The following stocks are described in FlyBase (http://flybase.org/): *dof1* (*stumps*^{09904b}), *btlLG19*, UAS-srcGFP, UAS-act42A-GFP [UASp-GFP-Act42A (Bloomington 9251)], 69BGal4 [P(GawB)69B (Bloomington 1774)], P(PZ)ena^{02029} (ena^{02029}). *pruned1* (*blistered*^{03267}) is a DSRF mutant that has been described previously (Guillemin et al., 1996). We used the GAL4 system (Brand et al., 1993) for over- or ectopic expression experiments. We used btlGal4 as a pan-tracheal driver and Term-Gal4 (Guillemin et al., 2001) and a Gal4 insertion in DSRF (from M. Calleja and G. Morata, Madrid, Spain) as specific terminal cell drivers. We checked that btlGal4 is functional in *btl* mutants. To ectopically express BNL, we used 69BGal4. We also used UAS-PHGFP (Pinal et al., 2006), UAS-bnl (Sutherland et al., 1996), UAS-DSRF, UAS-DMRTF (Han et al., 2004) and UAS-hSRFVP16 (Guillemin et al., 1996).

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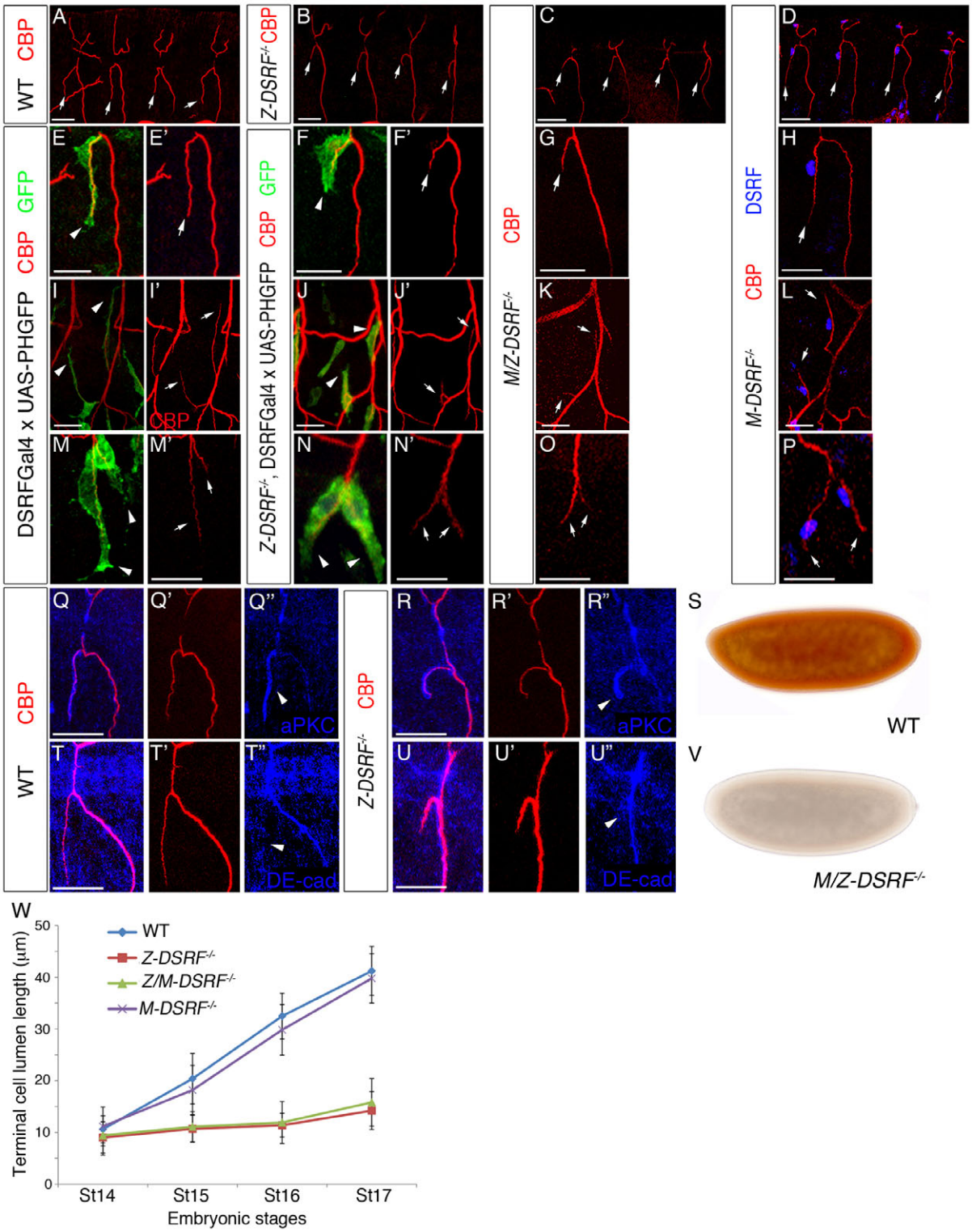

Fig. 1. DSRF loss-of-function blocks terminal branch progression but not the establishment of terminal cells. (**A-R″**,**T-U″**) Dorsal terminal branches (A-H) and lateral terminal branches (I-P) of stage 16 wild-type (A,E,E′,I,I′,M,M′,Q-Q″,T-T″), Z-DSRF (B,F,F′,J,J′,N,N′,R-R″,U-U″), M/Z-DSRF (C,G,K,O) and M-DSRF (D,H,L,P) mutant embryos. Terminal cells are outlined by PH-GFP (E,F,I,J,M,N), aPKC (arrowheads in Q″,R″) or DE-cad (arrowheads in T″,U″) distribution. Z-DSRF and Z/M-DSRF mutant terminal cells prematurely stop cytoplasm (arrowheads in E versus F, I versus J, M versus N) and lumen elongation (arrows in A versus B; C,E′ versus F′; G,I′ versus J′; K,M′ versus N′,O). M-DSRF mutant terminal cells display no defects (compare with wild type; arrows in A versus D; E′ versus H; I′ versus L; M′ versus P). Terminal cell fate is properly established in DSRF loss-of-function as these cells still form a seamless tube (no cadherin) (arrowhead in T″ versus U″) in the proper direction by inwardly elongating an apical membrane (Q″ versus R″). The lumen is detected by Chitin-binding protein (CBP; red). Nuclei of terminal cells (D,H,L,P) is detected by DSRF expression in blue. (**S**,**V**) No DSRF protein is detected in M/Z-DSRF mutant embryos (V) compared with wild type (S). (**W**) Quantification of lumen length for each genotype in stages 14 to 17. Error bars represent standard deviation from the mean. Scale bars: 20 μm.

Immunohistochemistry and image acquisition

Embryos were staged as described by Campos-Ortega and Hartenstein (Campos-Ortega and Hartenstein, 1985) and stained following standard protocols. For immunostaining, embryos were fixed in 4% formaldehyde for 20-30 minutes or 10 minutes for DE-Cadherin antibody. To visualise microtubules, embryos were fixed as described by Lee et al. (Lee et al., 2003). We used antibodies that recognise DE-Cad (Developmental Studies Hybridoma Bank), DSRF (BS) (Active Motif), GFP (Molecular Probes and Roche), βGal (Cappel and Promega), acetylated tubulin (Sigma), dpERK (Sigma), actin (MP Biomedicals), aPKC (Santa Cruz Biotechnology) and biotinylated, Cy2-, Cy3- and Cy5-conjugated secondary antibodies (Jackson ImmunoResearch). Chitin was visualised with Chitin-binding probe (CBP; New England Biolabs). Fluorescent images were obtained by confocal microscopy (Leica TCS-SP5-AOBS system, Leica DMI6000B microscope). Images are maximum projections of confocal sections.

RESULTS AND DISCUSSION

DSRF is required for progression of the terminal branch but not for terminal cell specification or for the initial elongation of cell and lumen

Terminal branch formation is controlled by the transcription factor DSRF, whose expression in terminal cells is triggered by BNL signalling (Affolter et al., 1994; Guillemin et al., 1996; Sutherland et al., 1996). To gain a better insight into the role of DSRF, we examined the morphology of terminal cells in *DSRF* zygotic mutants (Z-DSRF) using the null allele *pruned1*. In these mutants, terminal cells began to differentiate and started elongation in the normal direction. The dorsal terminal branches made the characteristic ventral turn as in wild-type embryos (Guillemin et al., 1996) (Fig. 1A,B,E,F). In some cases, filopodia pointed in the proper direction of elongation, although they were smaller than in the wild type (Fig. 1E,F,J,I). Furthermore, lumen elongation started properly (Fig. 1B,F). As in the wild type, in the mutant the tubes grew inwards into the cytoplasm (Fig. 1F) by elongation of an apical membrane (Fig. 1R) and formed seamless tubes (Fig. 1U). However, both elongation and lumen formation stopped prematurely, which led to a terminal cell with short cytoplasm and lumen extensions (in 85% of the cases; *n*=67) (Fig. 1B,F,W). The same defects were observed in the terminal cells of all the embryonic branches (Fig. 1F,J,N; data not shown).

There is also a maternal contribution of DSRF. Thus, in these and previous experiments (Guillemin et al., 1996) it could be argued that maternally provided DSRF could account for the initial development of terminal branches in zygotic mutants for

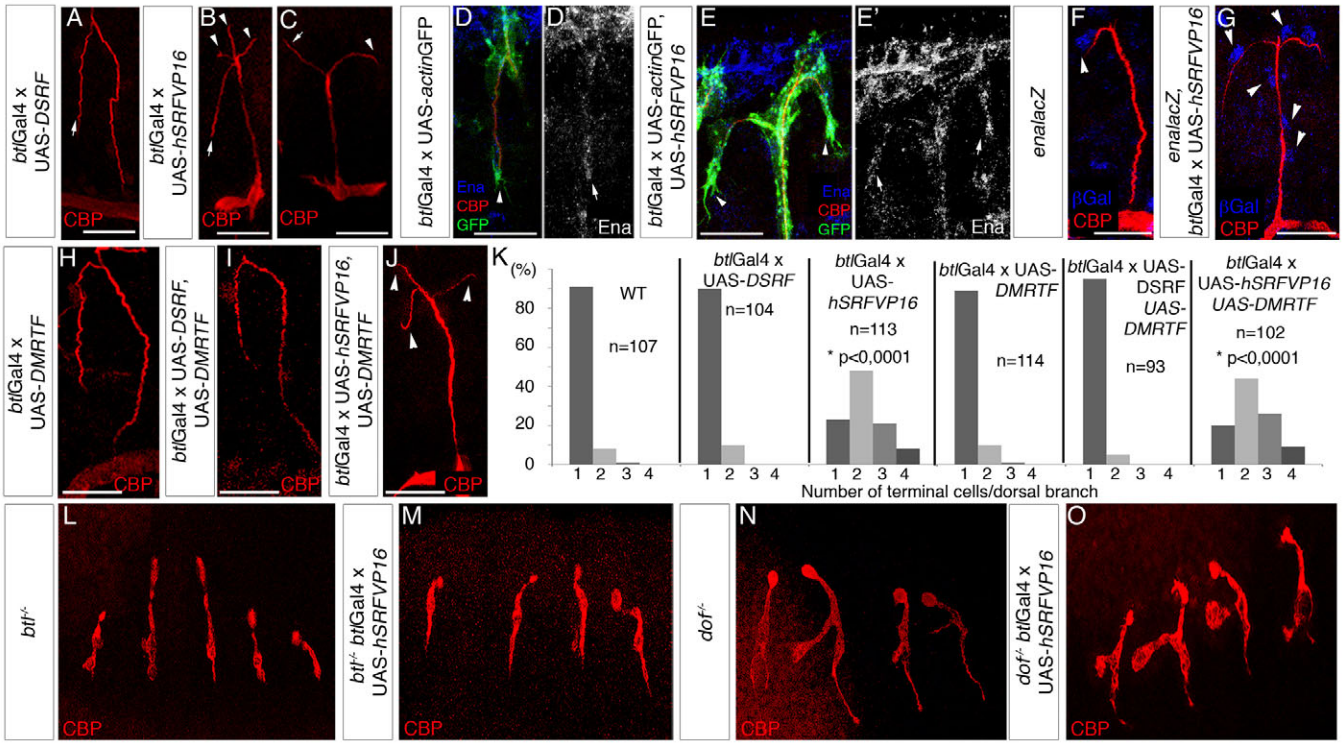

Fig. 2. Spatial restriction of extra terminal branch formation upon expression of an activated form of SRF. (**A-C**) Dorsal branches of stage 16 embryos expressing DSRF (A) or hSRFVP16 (B,C) in all the tracheal cells. Only the activated form of SRF has the capacity to induce extra terminal branches (arrowheads in B,C). Arrows indicate normal terminal cells. (**D**,**D′**,**E**,**E′**) Normal asymmetric actin (GFP; green) and ENA accumulation (Ena; blue in D,E white in D′,E′) at the tip of the elongating terminal cells of stage 16 embryos expressing hSRFVP16 (arrowheads in E, arrows in E′) compared with the wild type (arrowhead in D, arrow in D′). (**F**,**G**) When expressed in all the tracheal cells, hSRFVP16 induces pan-tracheal enhancement of *ena* expression [indicated by β-galactosidase (β-Gal) expression; G] compared with the restricted strong *ena* expression in the terminal cell in wild type (F). Arrowheads indicate tracheal cell nuclei. (**H-J**) DMRTF expression in all tracheal cells alone (H), with DSRF (I) or with hSRFVP16 (J) has no impact on formation of extra terminal cells. (**K**) Analysis of the number of terminal cells per dorsal branch in stage 16 embryos, either wild type or expressing DSRF, hSRFVP16 and/or DMRTF in all the tracheal cells. The frequency of extra terminal branches is significantly increased upon activation of DSRF overexpression, whereas non-activated DSRF or DMRTF have no effect (two-tailed Fisher's exact test). (**L**,**M**) A stage 16 *btl* mutant embryo (L) and a *btl* mutant embryo expressing hSRF-VP16 in the entire tracheal system (M) stained for the lumen. In both cases, there is no formation of terminal branches. (**N**,**O**) A stage 16 *dof* mutant embryo (N) and a *dof* mutant embryo expressing hSRF-VP16 in the entire tracheal system (O) stained for the lumen. In both cases, there is no formation of terminal branches. The lumen is detected by Chitin-binding probe (CBP; red). Scale bars: 20 μm.

DSRF. However, RNA and protein fade away during germ band extension (Affolter et al., 1994). In addition, the only DSRF protein detected at the stage of terminal cell specification derives from zygotic transcription (L.G. and J.C., unpublished). Nevertheless, in order to test whether a maternal contribution is required for the initial elongation of terminal cells and lumens, we generated females with *pruned1* mutant germlines and crossed them with *pruned1* heterozygous males. Half of the embryos were maternal and zygotic mutants (M/Z-DSRF) and no DSRF protein was detected (Fig. 1V). The M/Z-DSRF mutant phenotypes were identical to those of zygotic mutants (Fig. 1C,G,K,O), both in strength (Fig. 1W) and penetrance (78% of the cases; *n*=114). This observation indicates that maternal DSRF does not contribute to terminal branch formation. Thus, in the absence of DSRF protein, terminal branches initiate their development, thereby indicating that terminal cell specification is independent of DSRF. Instead, DSRF was required to sustain the progression of terminal branch morphogenesis rather than to trigger the process.

An activated form of SRF has the capacity to induce terminal cell formation only at specific positions in the tracheal system

DSRF expression is regulated by BNL signalling (Sutherland et al., 1996). More precisely, it has been proposed that an initial round of BNL signalling drives *DSRF* transcription and later, in a second round, this signalling in the same cells triggers the activation of the DSRF protein (Guillemin et al., 1996; Ghabrial et al., 2003). Accordingly, whereas expression of DSRF in all tracheal cells did not induce the formation of additional terminal branches (*n*=104) (Fig. 2A), expression of an activated form of human SRF promoted the sprouting of supplementary terminal branches (*n*=113) (Fig. 2B,C) (Guillemin et al., 1996). However, these supplementary branches arose only from cells in proximity to the normally occurring terminal branches (one to three extra terminal cells for each dorsal branch) (Fig. 2B,C,E,G,K) (Guillemin et al., 1996), where BNL signalling is thought to be higher (Ghabrial and Krasnow, 2006). These additional terminal cells displayed a normal morphology and, like wild-type terminal cells, distinctly accumulated actin and the actin binding protein Enabled (ENA) (Fig. 2E). The new terminal branches arose from other cells in the dorsal branch, among which was the fusion cell as judged by molecular markers (see Fig. S1 in the supplementary material) and the absence of the fusion branch (data not shown).

Myocardin and myocardin-related transcription factors (MRTFs) are co-activators of SRF in mammalian cells (Wang et al., 2001; Wang et al., 2002) and in the *Drosophila* tracheal system (Han et al., 2004). We verified that restricted induction of extra terminal branches is not due to the absence of *Drosophila* MRTF in the other tracheal cells. Through overexpressing MRTF alone or with DSRF in all the tracheal cells, we did not observe the formation of additional terminal branches (*n*=37) (Fig. 2H,I,K). Similarly, co-expression of MRTF and the activated form of SRF did not induce more terminal branches than those induced upon expression of an activated form of SRF alone (*n*=102) (Fig. 2J,K).

Additional information supports the notion that DSRF activity alone is not sufficient to drive terminal branching. Upon expression of an activated form of SRF, *ena*, which encodes a downstream effector of DSRF (Gervais and Casanova, 2010), was uniformly transcribed in the tracheal cells (Fig. 2G). This finding indicates that under these conditions SRF is indeed active in all the tracheal cells even when supplementary branches arise only from cells in proximity to the normally occurring terminal branches.

These results suggest that factors other than DSRF or its co-factors, which are probably also dependent on BNL signalling, are required to promote terminal branch formation among tracheal cells. Consistent with this hypothesis, we did not observe any terminal branch formation upon tracheal expression of the same activated form of SRF in an otherwise *btl* mutant background (*n*=14) (Fig. 2M). Similarly, we found no terminal branch formation upon tracheal expression of activated SRF in mutant backgrounds for *dof* (*downstream of FGFR*; *stumps* – FlyBase), a component of FGF signalling (Vincent et al., 1998) (Fig. 2O).

High levels of BNL can bypass the requirement of DSRF for terminal branch formation

The results above indicate that terminal branch morphogenesis initiates normally in *DSRF* mutants. To examine this issue further, we took advantage of a BNL overexpression assay used to illustrate the capacity of BNL signalling to induce terminal branches

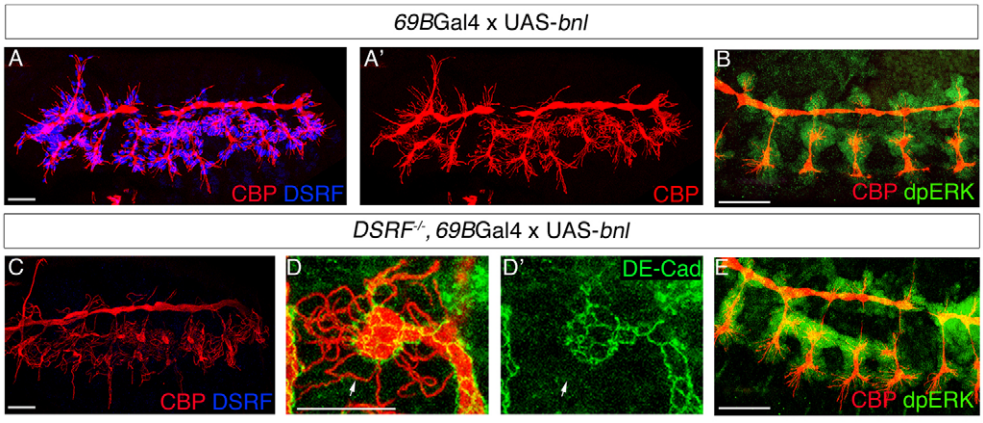

Fig. 3. High and persistent levels of BNL induce terminal cell formation even in the absence of DSRF. Stage 16 embryos broadly and persistently expressing *bnl* in the epidermis in an otherwise wild-type (**A**,**A′**,**B**) or *DSRF* mutant (**C-E**) background. Extra terminal branches are formed ectopically either in presence or absence of DSRF. Induced branches are seamless tubes, without adherens junctions, as shown by the absence of DE-Cadherin (green) along the lumen (arrows in D and D′). (B,E) General dpERK distribution (green) in the tracheal cells upon ectopic activation of the BNL-BTL pathway. The lumen is detected by Chitin-binding probe (CBP; red). Scale bars: 40 μm.

(Sutherland et al., 1996). This induction has been attributed to the concomitant general triggering of DSRF expression in tracheal cells (Fig. 3A), which is accompanied by BNL signalling (as assessed by dpERK staining) (Fig. 3B). However, in a *DSRF* mutant background BNL overexpression also induced terminal branch formation (100%, *n*=19) (Fig. 3C). These ectopic branches are true seamless branches with an intracellular lumen, as shown by the absence of DE-Cadherin (DE-Cad) autocellular junctions (Fig. 3D). Therefore, high levels of BNL signalling initiated terminal branch induction in the absence of *SRF* transcription. Furthermore, on the basis of the length of the intracellular tubes (Fig. 3C,D), continuous BNL activity also supported the progression of terminal branch elongation even in the absence of DSRF.

Terminal branch formation is dependent on the reorganisation of actin and microtubule (MT) networks (Oshima et al., 2006; Gervais and Casanova, 2010). To study further how BNL overexpression induces terminal branch development in a DSRF mutant background, we examined actin and MT network reorganisation in these conditions. At the initial steps of ectopic terminal cell formation, actin was detected at the cell periphery (Fig. 4A,B) and then after, as tubes elongated, it accumulated at the tip of the cells as in the wild-type terminal cells (Fig. 4C). We also detected increased levels of ENA in all the newly elongating terminal cells (100%, *n*=8) (Fig. 4E), thus indicating that specific ENA expression in terminal cells is also triggered by a DSRF-independent mechanism. Also, like wild-type terminal cells, in all the newly elongating terminal cells the MTs reorganised along the lumen and ahead of it, pointing toward the tip of the cell, reaching the area of high ENA and actin accumulation (Fig. 4G,H). These observations indicate that BNL signalling, in the absence of DSRF, triggers the reorganisation of the terminal cell cytoskeleton in a pattern similar to that which occurs in the wild-type terminal cells with DSRF expression.

Concluding remarks

Our results contribute to clarification of the roles of BNL and DSRF in terminal branch formation. First, *DSRF* transcription is dispensable for terminal branch initiation but is a crucial requirement for the progression of this process in wild-type

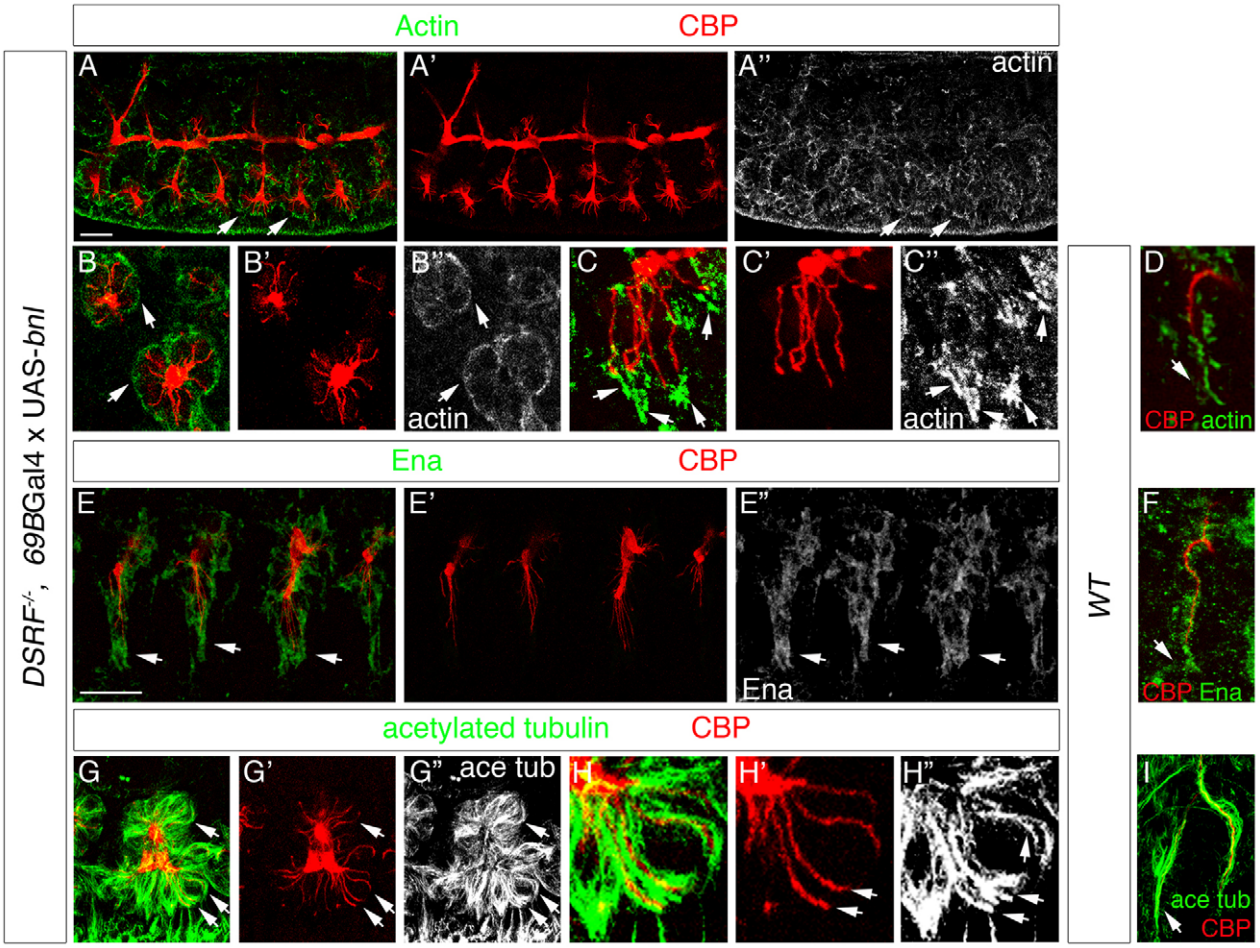

Fig. 4. High and persistent levels of BNL trigger cytoskeleton reorganisation in the terminal cell in the absence of DSRF. (**A-C″**,**E-E″**,**G-H″**) Images of embryos broadly and persistently expressing *bnl* in the epidermis in *DSRF* mutants. (**D**,**F**,**I**) Dorsal terminal branches of wild-type embryos. As in wild type, actin (green in A,B,C,D; white in A″,B″,C″) accumulates asymmetrically at the tip of the elongating extra terminal cells upon overexpression of *bnl* in *DSRF* mutants (arrows; A-C″ compared with D). *bnl* overexpression in *DSRF* mutants induces ENA ectopic expression (Ena; green in E, grey in E″) in all the tracheal cells, similar to that of wild-type terminal cells (F). Upon *bnl* overexpression in DSRF mutants, acetylated microtubules (acetylated tubulin, ace tub; green in G,H,I, grey in G″,H″) are normally distributed along the intracellular tubes of the terminal cells (arrows) compared with wild type (I). H is a magnification of G. The tracheal lumen is detected by Chitin-binding probe (CBP; red). Scale bars: 40 μm.

embryos. Second, a constitutively activated form of DSRF still requires BNL signalling to achieve terminal branch formation, thereby indicating that an additional outcome from BNL signalling is required for terminal branch development. Third, high levels of BNL signalling give rise to terminal branches independently of DSRF transcription. All these observations indicate that branches with an intracellular lumen can initiate their development in the absence of DSRF activity. As these are the specific features of terminal cells, we can conclude that DSRF is not a general determinant of terminal cell fate.

On the basis of these results, we propose the following model for terminal branch development. On the one hand, BNL signalling triggers the initial phases of cell elongation and intracellular lumen formation. This step is independent of *DSRF* transcription, probably because BNL levels at this stage are high enough to promote terminal branch initiation. On the other hand, BNL signalling activates DSRF, which in turn allows the progression of cell elongation and intracellular lumen formation [see Fig. S2 in the supplementary material for the timing of BNL signalling as visualised by activated MAPK (dpERK) and DSRF transcription]. Indeed, as mechanical tension has been proposed as a means to active DSRF expression (Somogyi and Rorth, 2004), it could well be that the same elements involved in the triggering of cell elongation by BNL signalling might also mediate activation of DSRF expression in the terminal cell. Irrespective of the mechanism promoting its expression, DSRF activity can be considered to be a boosting mechanism that, together with other outputs from BNL signalling, ensures that the cellular modifications required for elongation and intracellular lumen formation are kept active in the wild-type conditions of BNL signalling. Consequently, this process appears to spatially restrict induction of terminal branching to places of high BNL signalling, which are often found at the tip of the branches. Finally, although high levels of BNL signalling do not reproduce physiological wild-type conditions, the observation that such high levels bypass the requirement of *DSRF* transcription for the growth of terminal branches can be of relevance in stress conditions, such as in hypoxia. Likewise, a similar mechanism could be of significance for the induction of angiogenesis in disease.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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