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Fascin, may the Forked be with you

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The FGFR pathway triggers a wide range of key biological responses. Among others, the Breathless (Btl, *Drosophila* FGFR1) receptor cascade promotes cell migration during embryonic tracheal system development. However, how the actin cytoskeleton responds to Btl pathway activation to induce cell migration has remained largely unclear. Our recent results shed light into this issue by unveiling a link between the actin-bundling protein Singed (Sn) and the Btl pathway. We showed that the Btl pathway regulates *sn*, which leads to the stabilization of the actin bundles required for filopodia formation and actin cytoskeleton rearrangement. This regulation contributes to tracheal migration, tracheal branch fusion and tracheal cell elongation. Parallel actin bundles (PABs) are usually cross-linked by more than one actin-bundling protein. Accordingly, we have also shown that *sn* synergistically interacts with *forked* (*f*), another actin crosslinker. In this Extra View we extend *f* analysis and hypothesize how both actin-bundling proteins may act together to regulate the PABs during tracheal embryonic development. Although both proteins are

required for similar tracheal events, we suggest that Sn is essential for actin bundle initiation and stiffening, while F is required for the lengthening and further stabilization of the PABs.

Parallel actin bundles organize cortical cell protrusions

Parallel actin bundles (PABs) are present in a wide variety of cortical cell protrusions, such as the microvilli, microspikes, filopodia, invadopodia or podosomes. These cortical protrusions are stabilized by tightly packed filaments of actin that possess sufficient rigidity, but yet are flexible enough to be deformed.[1] PABs are formed by the addition of actin monomers to the barbed-plus ends of the filaments, which are bundled by the action of cell type specific crosslinkers. The nature and concentration of the actin-bundling proteins determine the degree of packing of the filament bundles,[1] thereby determining the specific type of protrusion in each cell type context.

For instance, brush border microvilli are finger-like projections emanating from the apical surfaces of certain absorptive epithelia. They increase the apical membrane extension and the epithelial absorption,[2] and they defend the surface area against pathogens.[3,4] Each microvillus is supported by a central core bundle of ~19 actin filaments that are cross-linked by the two actin bundling proteins Fimbrin and Villin.[5,6] The core bundle contains also a small amount of a third actin-crosslinker, the Small Espin isoform,[7] which appears to accumulate only later during brush border assembly.[8]

In contrast to the persistent microvilli, the non-protruding microspikes and protruding filopodia, podosomes or invadopodia are transient cell protrusions usually related to cell adhesion, migration or cell-cell interactions. Such protrusive structures, typically at the leading edge of motile cells, are also formed by parallel, unipolar bundles of actin that require the activity of several actin-crosslinkers. Besides motility and adhesion roles, invadopodia (present in many carcinoma cell types) and podosomes

also possess the ability to degrade the extracellular matrix (ECM), providing the cell with capacity to extravasate and invade other tissues.[9-11] In contrast, filopodia are non-invasive thin finger-like structures with multiple roles in cell motility. Classically, filopodia have been proposed to act as guiding devices that explore the environment ahead of the lamellipodium, serving as sensors that can determine the direction of migration. Additionally, they have more recently been proposed to operate as mechanical devices at sites of signaling and adhesion with the surrounding ECM, generating the traction force required to move the cell body forward,[1,12] thus modifying cell morphology and cell function in response to extracellular stimuli.[13]

The actin-bundling protein Fascin tightly packs actin bundles

Fascin is the major actin bundling protein present in filopodia. This globular 55KDa protein contains two actin-binding sites that have been highly conserved throughout evolution.[14-18] Fascin-family proteins are present in a wide range of cell types, where they tightly pack PABs, providing sufficient strength to overcome the membrane resistance and protrude several microns from the leading edge of the cell.

In humans there are three Fascin isoforms. Fascin-2 and Fascin-3 are retina and testis specific.[19,20] In contrast, Fascin-1 is more widely expressed, but importantly it is only present at low levels in specific tissues of healthy adults (i.e., nervous system and highly migratory mesenchymal cells[21,22]). Fascin-1 appears clearly upregulated in several carcinoma cell lines, and this upregulation correlates with poor prognosis in cancer patients. It is proposed that Fascin-1 promotes motility and invasiveness in carcinoma cells, as Fascin-1 appears at the front of the tumors,[12] stabilizes the F-actin bundles of invadopodia, and potentiates protrusive invasion.[9,10] Therefore, Fascin-1 is currently considered a marker for some of the most aggressive cancers[23] and has

become a potential therapeutic target. The Fascin specific inhibitor Migrastatin (and synthesized analogs) is assayed as a possible oncogenic drug.[24,25]

Singed is the only *Drosophila* Fascin homolog

The *Drosophila* genome encodes one single protein of the Fascin family, named Singed (sn). *sn* is expressed in several tissues and structures throughout fly development. During embryonic development *sn* is expressed and required in blood cells for the organization of microspikes during their migration[26] and in tracheal cells for filopodia organization (see following section). During larval development *sn* is required in a specific subset of sensory neurons to establish the pattern of the actin-rich terminal branchlets of their dendrites.[27] In addition, *sn* is required during oogenesis and for adult fly bristle formation .

Cooperation between different actin-bundling proteins during PAB formation has been reported in *Drosophila*. During oogenesis *sn* plays a role in the process of dumping, by which the nurse cells empty their content into the oocyte. Dumping requires that filopodia-like structures hold the nuclei of the nurse cells in place during the process. In the absence of *sn* these actin-rich structures are not properly organized and as a result the nuclei block and collapse the ring canals, preventing proper dumping.[28] A similar phenotype is observed in the absence of a variety of actin-associated elements, including another actin-crosslinker, named Quail (Qua, Villin-like protein). It was found that *sn* and *qua* act in concert in the formation of the actin cables in nurse cells.[27] Similarly, during the formation of the neurosensory adult bristles, *sn* acts in concert with another actin-bundling protein, named Forked (F).[29] Bristles are curved and long protrusions that contain multiple parallel actin bundles positioned beneath the plasma membrane.[30] During bristle formation *sn* and *f* play critical roles in proper organization of this actin-based cytoskeleton, which consists of tightly packed

actin bundle modules that are connected end-to-end.[31] Whereas in the absence of *f* the number of actin filaments per bundle decreases, in the absence of *sn* there are many actin filaments per bundle, but these are not hexagonally packed. Lack of both proteins leads to almost no actin filaments arranged into bundles,[30,31] giving rise to very short, thick and twisted bristles.

The Breathless/FGFR pathway positively regulates *singed* tracheal expression

The tracheal system is the respiratory organ of the fly and an excellent model system for the analysis of cell migration. During tracheal embryonic development, the Branchless (Bnl, a *Drosophila* FGF homolog)/Breathless (Btl, *Drosophila* FGFR1) pathway plays a prominent role[32]. Bnl was shown to act as a chemoattractant for the tracheal cells,[33] which express the FGF Receptor Btl. The cells at the tip of the branches receive the highest levels of Bnl, and thanks to a positive feed-back loop mediated by Ras-MAPK, maximally activate the pathway.[34] These tip cells which lead the collective migration of the branches toward the source of Bnl,[35] display a high migratory behavior and particular morphological properties. For instance, leading cells extend numerous filopodia which have been proposed to contribute to migration. However, the molecular mechanisms underlying this process are not well understood.[36]

We have recently found that *sn* is specifically expressed in the leading cells of the tracheal branches and that this expression is positively regulated by the Btl/FGFR pathway.[37] To better understand how *sn* contributes to cell migration in vivo we have analyzed the requirements for *sn* in tracheal cells.

Functional analysis of *singed* during tracheal development

We focused our analysis on a particular type of tracheal branches, the so-called dorsal branches (DBs).[37] Our functional study indicated that *sn* is required in leading

cells, which accumulate high levels of Sn in their cytoplasm and in cellular protrusions. We found that *sn* is required for the guided and timely migration of DBs, since in *sn* mutants the DBs are often missguided and their extension is delayed. The two tip cells of the DBs are, under normal conditions, highly specialized and accomplish specific activities. One of them (the fusion cell) mediates the fusion of tracheal branches and the other one (the terminal cell) extends a terminal branch.[32] We found that *sn* is also required for the correct activity of these tip cells, since in *sn* mutants both branch fusion and terminal branching are compromised (**Fig. 1**. A-D, ~~G~~F).

Singed is required for the organization of the Actin cytoskeleton in the leading cells

To better understand these effects we analyzed in detail *sn* requirements at single cell resolution. We found clear and reproducible defects in filopodia and morphology of *sn* mutants tip cells. While in control embryos DB tip cell filopodia were long, straight and stiff and the terminal cells extended organized cell fronts, in *sn* mutants filopodia were curved and bent with an apparent flaccid aspect and the cell fronts were typically irregular (**Fig. 2A,B**). To better characterize these defects we quantified filopodia number and length in fixed tissue and compared control and *sn* mutants. We detected around 17 filopodia per cell, typically long (~4,4 μm) and conspicuously straight in appearance in control embryos. *sn* mutants displayed a slight but stastistically significant lower number of filopodia but with a normal length (~13,5 filopodia/terminal cell) (**Fig. 2E-F**).

To better characterize *sn* requirements we analyzed actin organization in tracheal leading cells. A first analysis of wild type embryos revealed the technical difficulties to visualize actin rearrangements due to the small size of leading cells and to the fast development of the tracheal system. Nevertheless, an analysis with high

temporal and spatial resolution allowed us to visualize the organized movement of tracks of actin toward the cell edge during migration, forming the lamellipodia. We also detected non-protruding actin bundles and actin-pools, which accumulated toward the direction of migration, fusion or terminal cell elongation during stages 14 and 15. In addition, we also detected bundles of actin perpendicular to the membrane and protruding beyond the cell edge, forming the filopodia. In contrast, lack of *sn* generated a disorganized movement of actin pools that barely formed a continuous lamellipodia in the deformed cell edge, neither long and thick bundles of intracellular actin. Therefore we concluded that *sn* acts in tracheal leading cells as a link between the Bnl/FGF signal and the actin cytoskeleton.[37]

Forked is required for tracheal formation

As already mentioned, most cortical cell protrusions based on actin-bundles require at least two actin-crosslinkers to maximise the bundle packing.[38] This is true for the neurosensory bristles (with *f* and *sn*) and filopodia-like cables of nurse cells (with *qua* and *sn*) in *Drosophila,* for microvilli (with Fimbrin and Villin), and also for hair cell stereocilia (with Fimbrin, Espin and Fascin[38,39]) (for a review see ref.[40]). Hence, we investigated the possible requirements of other actin cross-linkers in tracheal development.

qua (villin-like) single mutants did not show reproducible tracheal phenotypes. When tested in the absence of *sn*, we did not detect any clear genetic interaction.

In contrast, *f* mutant embryos displayed tracheal defects that were similar, and even more penetrant, than the ones found in *sn* mutants. In particular, we detected a high-percentage of DB missguidances (in 71% of *f* mutants, compared with 36% of *sn* mutant embryos or 9% in the wild type) (**Fig. 1A**). The fusion of the contralateral DBs at the end of embryogenesis was also compromised in all *f* embryos analyzed (in 100%

of *f* embryos, vs. 58% of control embryos, and 95% of *sn* mutants)~~.~~ (**Fig. 1B** and white arrow in **Figure ~~1H~~1G**). Lumen guidance inside the terminal cells was also strongly affected in *f* mutants (100% of *f* mutants displayed missguided terminal lumina, compared with 16% in the control, and 80% in *sn* mutants) (**Fig. 1C** and blue arrows in **Figure ~~1H~~1H**). Occasionally, this terminal lumen was also shorter (**Fig. 1H**), in spite of an apparent normal extension of the terminal cell. Finally, we also found an excess of terminal-DSRF positive cells (in 97% of *f* mutants compared with 53% of control embryos, and 89% of *sn mutants*) (**Fig. 1D** and purple arrow in **Figure ~~1H~~1G**).

Given the similarities between *sn* and *f* tracheal requirements, and the fact that both are actin-bundling proteins, we speculated that F would also contribute to organize the actin cytoskeleton. To evaluate this possibility we analyzed tip cell morphology and filopodia and compared the results to *sn* mutants. In vivo analysis of *f* mutants using the Src membrane marker confirmed that the cell fronts of the leading cells were as defective and irregular as in *sn* mutants (**Fig. 2B,C**) and that when filopodia were long enough, these also seemed weakly bent and curved. However, we also observed that *f* filopodia were shorter than those of control and *sn* mutants (**Fig. 2C**). These differences in filopodia defects between *sn* and *f* mutants, suggested differences in the molecular and cellular mechanisms of Sn and F. To confirm this observation we analyzed and quantified filopodia parameters in fixed tissue. Interestingly, we found that *f* mutants have a normal number of filopodia but shorter than in the control (~3,6 µm vs. ~4,4 µm of control terminal filopodia) or *sn* mutants, while *sn* mutants have less filopodia but with a normal length (**Fig. 2E-G**). These results show that although these two genes are required for filopodia formation, they act differently.

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Singed and Forked interact during filopodia formation

To better characterize the contribution of *sn* and *f* to filopodia formation, we also analized tracheal filopodia in *sn f* double mutants. *In-vivo* analysis showed extremely irregular cell shapes with apparently inefficient cell fronts from where only a few and short filopodia protrude (**Fig. 2D**). To have a more accurate measure of these defects we quantified filopodia parameters in fixed embryos and compared them to those of *sn* or *f* single mutants. This analysis corroborated that the number and length of filopodia was differently affected in the three mutant conditions: Sn affects the number of filopodia, *f* mutants display shorter filopodia, and *sn f* mutants extend shorter and fewer filopodia (**Fig. 2E-G**).

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We also analyzed the consequences of *sn f* absence at the tracheal tissue level in double mutants. We detected a synergisitic genetic interaction between the two genes as new tracheal phenotypes were observed (**Fig. 1~~J-M~~I-L**). *sn f* double mutants lacked some branches, in particular some DBs (**Fig. ~~1J~~1I**), and also lacked DSRF-expressing terminal cells in the formed DBs (**Fig. 1~~K-M~~I-L**). This lack of terminal cells is particularly intriguing since each mutant on its own produced an increase in the number of terminal cells (**Fig. 1D**). We can propose different explanations for such observations.[37] On the one hand, we speculate that terminal cells cannot receive the Bnl signal and correctly respond to it by expressing DSRF due to delayed migration (caused by inefficient filopodia) or to an impaired reception of the signal (in case the tracheal filopodia act as chemosensitive organelles by carrying Btl receptors[41]). Alternatively, the abnormal specification of the terminal cells could be due to the defective actin-rearrangements, as it has been described that actin can modulate the expression of the terminal determinant DSRF.[42,43] In this case, the disorganized actin-cytoskeleton in terminal cells could also explain the short intracellular lumen inside the correctly specified terminal cells found in *sn f* mutants (blue arrows in **Figure ~~1K-~~1J and ~~M~~L**).

From our analysis of single and double mutants we draw several conclusions. First, since we find that in the absence of both *sn* and *f* filopodia can still form, we suggest that another actin-bundling protein may also participate in the process. Second, we find that while the morphogenetic defects found in *sn* and *f* mutants are very similar, and though both proteins are proposed to act as actin cross-linkers, our analysis at the cellular level reveals that they affect filopodia formation differently, suggesting that they play different molecular roles. In the following section we elaborate on this aspect and propose a model of how each protein can contribute to filopodia organization.

Model for singed and forked function in filopodia formation

Tilney, Guild and colleagues put forward a model for Sn and F during actin organization in *Drosophila* bristle formation. They proposed that in a first step a yet unidentified actin cross-linker forms small and disordered actin bundlets. Subsequently, F aggregates them into loosely ordered modules. In a third step F also facilitates Sn entry into the bundles, and Sn in turn displaces F and tightly organizes the bundles into hexagonally packed structures that become straight and stiff. Finally, the different actin modules that form each bundle are glued by actin filaments that are held by F (for a review see Ref.[44]). However, filopodia are not formed by actin modules. This opens the question of what is the exact role of each actin cross-linker in the organization of PAB during filopodia formation.

Here, we propose a model for the role of each protein during filopodia formation based on our results. We propose that Sn acts in the first step of filopodia formation while F acts later facilitating filopodia elongation. In addition, the binding of each protein along the PAB stabilizes the bundle. Together both proteins control the correct number, shape, stiffness and length of the filopodia.

The fact that in *sn* mutants the number of filopodia decreases may indicate that *sn* is required at the initial steps of filopodia formation, in the region of the lamellipodia or the lamellae. This is in agreement with several observations we made, including the cytoplasmic localization of both the active and inactive bundling forms of Sn, and the disorganization of the cytoplasmic actin-cytoskeleton in the mutants (including the partial loss of cytoplasmic microspikes or actin-bundles).[37] Previously published data also supports this hypothesis: 1) the recruitment or activation of fascin to the VASP clustered barbed ends of the filament precursors (called Λ-precursors) initiate filament bundling and allow the growth of the nascent filopodium[45,46]; 2) *sn* is required for the formation of cytoplasmic actin cables such as those of the nurse cells[26,28]; 3) *sn* is also found in branched filaments in the lamellipodia, not only in parallel bundles.[47]

In contrast to *sn*, *f* mutant filopodia are shorter. This would fit with a model in which F facilitates actin monomer addition, to compensate for actin monomer elimination at the minus end during treadmilling, either by acting directly on actin polymerization or by stabilizing the bundles thereby preventing depolymerization. In agreement with this hypothesis it is interesting to point out the similarity of F to the Espin family of actin-bundling proteins. In particular, there is a 39% homology between F and a 66aa peptide at the Espin C-terminus, which corresponds to the actin-bundling module (ABM) and to the ankyrin-repeat motive.[48] The ABM is necessary and sufficient for the lengthening of the parallel actin-bundles in microvilli. Mechanistically, it is suggested that Espin provides to the initial short microvillar PABs a net barbed-end elongation of treadmilling actin filaments, giving rise to longer bundles without joining shorter modules.[7] Additionally it has been reported that the magnitude of the PAB lengthening is dependent on the level of espin expression.[49] It is also possible that F has an uncapping function in filopodia, in such a way that in its

absence the filaments are capped at their barbed ends and then the constant actin depolymerisation leads to filopodia dismantling.

Taken together we propose that during tracheal filopodia formation Sn acts first (before F, in contrast to bristle formation) probably just after Ena/VASP initiate the filopodia precursors[46] to initiate the first bundling of the actin filaments and to generate the first packing of the PABs. Thus, in the absence of *sn* less filopodia form, and as the bundles are not tightly organized, these filopodia are wavy and bent. F acts later on the initial and short filaments of actin, providing a maintained barbed-end elongation of actin, allowing for filopodia length increase. Hence, *f* mutant filopodia are shorter, in agreement with shorter microvilli, stereocillia and bristles in *espin* and *f* mutants.[7,49,50] These reports also showed that *f* and *espin* mutants have a decreased number of filaments giving rise to thinner protrusions. Our imaging resolution level does not allow us to address this aspect in tracheal filopodia; however, fewer actin filaments could contribute to the weak bending of filopodia observed. In *sn f* double mutants the first actin-bundling step would be compromised. Later, those PABs that successfully form would soon have an actin treadmilling displaced toward filament de-polymerisation and final reabsorption. Thus, only very few and short filaments would form under these conditions.

In summary, we propose that Sn and F together are necessary to bundle and elongate several filaments of actin and give them enough strength to protrude and elongate several microns from the cell edge generating straight and long filopodia. Stiff filopodia provide the cell with the trailing force to move forward and to change shape in response to the signals at play.

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Figure 1. The actin-bundling protein Forked is required during tracheal system development. (**A-D**) Percentage of embryos with the indicated number (in different colors) of dorsal branches (DBs) misguided (**A**), unfused DBs (**B**), terminal intracellular lumen misguided or bifurcated (**C**), or with extra DSRF-terminal cells (**D**). n is the total number of fixed embryos analyzed. Note that *f* mutant embryos (f^{36} FBal0003950) display stronger defects than *sn* mutants (sn^{P1}. FBal0035641) in all aspects analyzed. *** $P < 0{,}001$ analyzed by Fisher's exact test (http://www.langsrud.com/fisher.htm#INTRO). *(***E-G**) Confocal projections of fixed control (*yw*) (**E**), sn^{P1} (**F**) and f^{36a} (**G**) embryos at the end of embryogenesis stained with the luminal marker 2A12 (red) that labels the tracheal tree and the terminal cell marker *D*SRF (white). **(H**) Close-up of two DBs of a f^{36a}*; btlGal4 UAS-srcGFP* mutant labeled with 2A12, *D*SRF and GFP (which highlights cell morphology due to the membrane marker Src fused to GFP under the control of the breathless (btl)-Gal4 tracheal driver). **(I-L)** Confocal projections of fixed sn^3f^{36a} embryos (FBst0306268) stained with 2A12 (red) and *D*SRF (white). (K-L) are close-ups of two DBs of different embryos. **White arrows** point to the presumptive point of fusion between contralateral DBs at the dorsal midline. **Blue arrows** point to the intracellular terminal lumen of terminal branches. Note that terminal lumina correctly extends ventrally in control embryos (E), but often turn dorsally (F and H) or hardly extend (J and L) in mutant conditions. **Purple arrows** point to the individual (E), extra (F-H) or missing (I-L) *D*SRF-expressing cells of DB in different conditions. **Yellow**

asterisks (I) indicate missing DBs. In all cases *yw* was used as the control. Scale Bar: 15 μm.

Figure 2. Singed and Forked control the number, length and stiffness of tracheal filopodia. (**A-D**) Stills from *in-vivo* movies taken approximately every 10 s of embryos carrying *btlGal4 > SrcGFP* (which allows to clearly visualize the morphology and dynamics of filopodia) in an otherwise wild-type (**A**), sn^{P1} (**B**), f^{36a} (**C**) or $sn^{3}f^{36a}$ (**D**) backgrounds. The images show the tip of DBs of stage 14–15 embryos. **Arrows** point to straight (in control, A) or curved filopodia (in *sn*, *f* or *snf* mutants). Note also the presence of conspicuously shorter filopodia in *f* or *snf* mutants. Scale Bar: 5 μm. (**E**) Quantification of the number of filopodia in the terminal cells of DBs of fixed embryos at stage 16 carrying *btlGal4 > SrcGFP* in a control or, sn^{P1}, f^{36a} and $sn^{3}f^{36a}$ mutant background. Note the decrease in the number of filopodia in *sn* mutants, as well as *sn f* double mutants. * $P < 0,05$; ** $P < 0,01$; *** $P < 0,001$ were obtained using two-tail *t* test of R-Commander software. n = number of terminal cells analyzed. (**F**) Quantification of the length (in microns) of filopodia in the terminal cells of DBs of fixed embryos at stage 16 carrying *btlGal4 > SrcGFP* in a control or, sn^{P1}, f^{36a} and $sn^{3}f^{36a}$ mutant background. *f* terminal cell filopodia are significantly shorter than control and *sn* mutant ones. *sn f* terminal cell filopodia are much shorter than the previous cases. * $P < 0,05$; ** $P < 0,01$; *** $P < 0,001$ were obtained using two-tail *t* test of R-Commander software. n = number of terminal cells analyzed. (**G**) Distribution of the length of filopodia of terminal cells (represented in different color/micron) in *control*, sn^{P1}, f^{36a} and $sn^{3}f^{36a}$ fixed embryos at stage 16. Note that in *sn f* mutants almost 50% of filopodia are shorter than 2 μm and hardly any filopodia extend longer than 10 μm. Typically, long filopodia (> 10 μm) are associated

504 with the ventral cytoplasmic extension that generates the terminal branch. n =
505 number of terminal cells analyzed. In all cases *btlGal4 > SrcGFP* was used as
506 control.
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