A role for fascin in preventing filopodia breakage in *Drosophila* tracheal cells

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Filopodia are long and thin finger-like protrusions essential for cell migration. They are formed by parallel actin bundles tightly packed by cell type and context dependent actin-bundling proteins. Our recent work analyzing the role of Fascin during tracheal development in *Drosophila* has shown that Singed (the *Drosophila* Fascin homolog) acts as a molecular link between the Branchless (FGF)/Breathless (FGFR) pathway and the actin cytoskeleton. We have reported that the lack of Singed (Sn) leads to wavy and flaccid filopodia due to the disorganization of the tracheal actin cytoskeleton. Here we describe for the first time filopodia breakage in *Drosophila*, and show that Fascin plays a role in this event. We propose that actin filaments in sn mutant filopodia buckle under membrane pressure due to lower bending stiffness, eventually undergoing breakage. Both Filopodia buckling and breakage would impair correct cell navigation and migration.

Filopodia are thin and long finger-like protrusions that contain unipolar parallel filaments of actin, with their barbed-polymerization ends located at the distal tip of the protrusion. These filaments are packed by actin-bundling proteins, which confer stiffness to the actin-bundles. Filopodia typically protrude from the cellular leading edge and adhere to the substratum or to other cells.

Filopodial protrusions are essential for navigation and migration in several types of motile cells, both in health and disease conditions. They have been described to play 2 main roles. On the one hand they serve as guiding devices that sense the environment ahead of the lamellipodium, thus determining the correct direction of the migration. On the other hand they are able to act as mechanical devices by adhering to the substratum and generating and distributing the tension required to pull the cell forward.[1,2] These functions confer to the filopodia the ability to control the activity and morphology of the cell depending on the extracellular stimuli.[3]

The exact mechanism of filopodia initiation is still under discussion. The convergent nucleation model proposes that filopodia are formed from the so-called Λ-precursors at the leading edge of the Arp2/3 branched actin-network of the lamellipodia.[4] Once initially formed, actin-bundling proteins crosslink the uncapped parallel filaments, and the bundle elongates by polymerization at its tip, pushing the membrane forward.[5] It has been estimated that to overcome the resistance imposed by the membrane and protrude some microns from the cell edge, the bundles need to be formed by at least 10 actin-filaments.[2] Filopodia actin-bundles act as effective elastic rods that hold the constant membrane resistance. From different studies it emerged that the kinetics of filopodia growth depends on the resistance of the cell membrane to be deformed, the transport of G-actin monomers to the growing filopodia tip, the actin polymerization rate, and the mechanical buckling of the bundle[6] **Figure 1.**

We have been recently analyzing the role of the major actin-bundling protein in filopodia, the glomerular Fascin,[5] during *Drosophila* tracheal (respiratory) system development. The formation of the tracheal system relies on collective cell migration, cell shape changes, and reorganization of the cells within the organ. Collective cell migration requires the activity of the FGF/FGFR pathway (Branchless, Bnl/Breathless, Btl pathway in the case of Drosophila tracheal formation): the ligand Bnl acts as chemoattractant and directs the migration of the tracheal cells that express the receptor Btl.[7-9] It was proposed that the high activation of the pathway at the tip of the branches (where the ligand signal is received) regulates the migratory behavior of the leading cells, which in turn pull the stalk cells. However, the mechanism

by which the activation of the Btl pathway triggers cell migration was not elucidated.[9] We have recently shed light into this aspect by showing that *Drosophila* Fascin (named Singed (Sn)), acts as a molecular link between the Btl pathway and the actin cytoskeleton in the trachea.[10]

We have described that Sn accumulates at high levels at the tip of the tracheal branches in response to activation by the Btl pathway and there it regulates branch navigation, speed of migration, actin-cytoskeleton organization and cell shape changes. We also found that *sn* mutant filopodia were
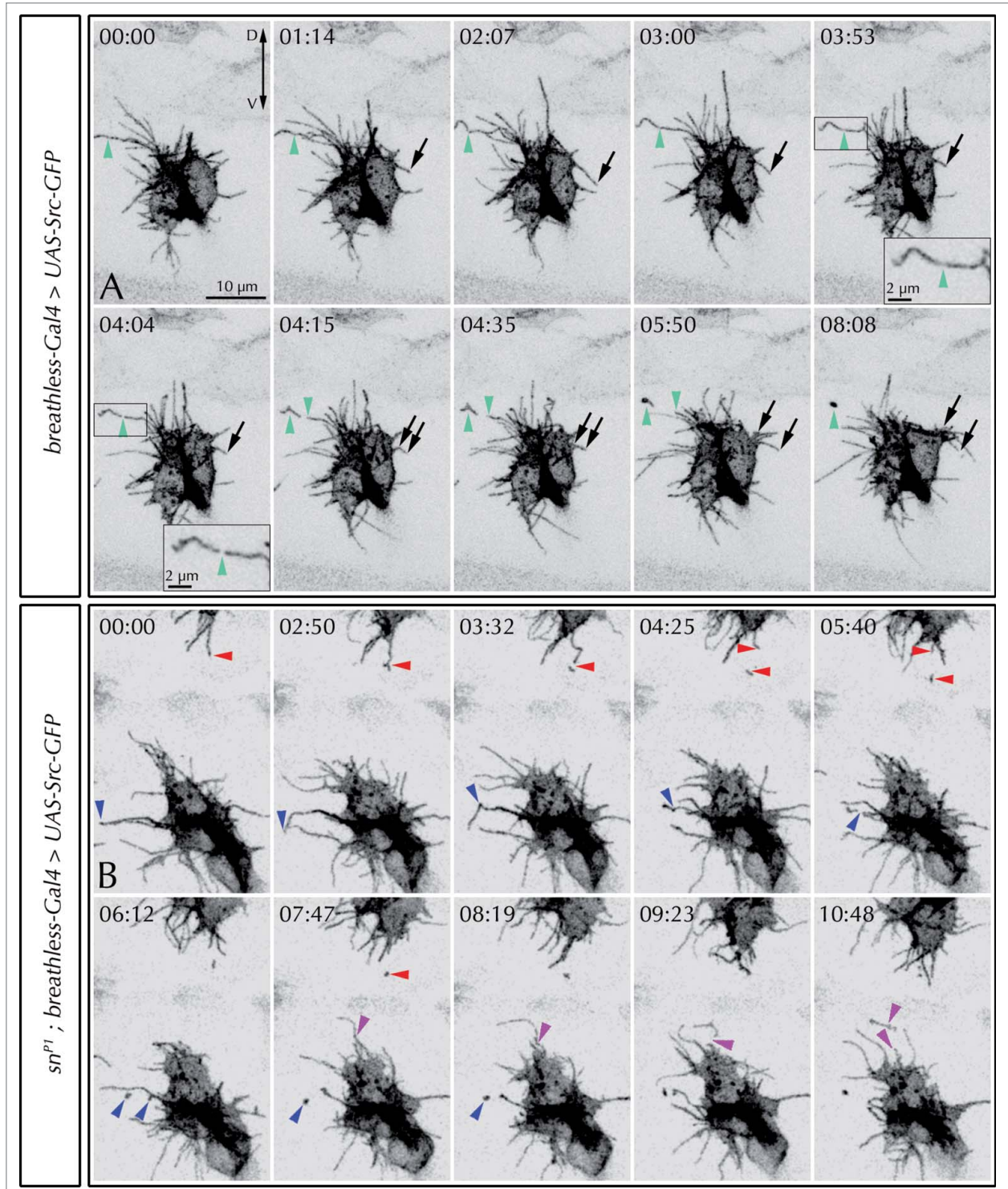
Figure 1. For figure legend, see page 3.

extremely bent or wavy, with a flaccid appearance, as seen in other cell type protrusions,[11-15] and that at late stages of development the number of filopodia in the tip-terminal cells was decreased. We proposed that Sn tightly packs actin bundles in tracheal filopodia, conferring enough stiffness and stability to the bundles to push the membrane edge and correctly migrate forward.[10]

During the course of our in vivo analysis of filopodial dynamics we observed a striking phenomenon: several filopodia break, usually during retraction and after undergoing a remarkable bend. Visual inspection suggested that this phenomenon was more frequent in *sn* mutant embryos than in control ones, but the type of breakage was comparable in both conditions. Quantifications corroborated that the frequency of breakage of filopodia is significantly much higher in *sn* mutant embryos than in control embryos. Typically, we found around 10,65 breaks/hour in *sn* mutant dorsal branch tip cells as compared to 1,55 breaks/hour in the control. After filopodium breakage, the free filopodia remnants end up shrinking into a "ball" of membrane, although this process takes some time. This shrinking could either reflect the internal tension under which the filopodium was or, alternatively, it could reflect an actin treadmilling displaced toward depolymerization. Interestingly the free filopodium, while shrinking, continues moving (see below).

While there are other possible explanations, we propose that this filopodial breakage is due, at least in part, to mechanical buckling. Buckling occurs when an applied load, in this case the resistance of the membrane, becomes large enough to unstabilize the actin-filaments, which will experience a lateral deformation. When the load exceeds the internal resistance of the rod the breakage or catastrophe of the structure occurs. The critical force at which the internal resistance is not enough and buckles the rod depends on the length and stiffness of the bundle.[2] The stiffness is controlled by the number of filaments and by the nature and concentration of the actin-bundling proteins.[16] Therefore, the type of actin bundling protein in the actin-bundle will determine the type and characteristics of the resulting protrusion.[17] For instance, larger and flexible cross-linking proteins (such as α-actinin) or higher distances between crosslinkers along the filament generate weakly bundled or loose bundles that buckle at short distances. In our experimental conditions, the lack of Sn in filopodia would decrease the stiffness of the filopodia bundles, which would not sustain the resistance of the cell membrane, inducing filopodial buckle. This would result in the curved and wavy aspect of filopodia that we observe. In addition, a minimum concentration of the actin-bundling protein Sn could lead to a decrease of the number of actin filaments per bundle, which would also favor breakage, in addition to an excessive membrane resistance in a particular filopodia.

We noticed that the filopodia fracture is more frequent in the distal part in control embryos (67% distal. n = 9 breaks) but more frequent in the proximal part in *sn* mutants (48% distal. n = 25). This observation could reflect an increased requirement of *sn* activity at the base of the filopodia during retraction, where likely there are more actin filaments and more tightly bundled than at the tip. This is consistent with observations indicating a retrograde flow of phospho-/dephosphorylated fascin after filopodia extension.[18]

To our knowledge this is the first time filopodia breakage is described in an *in vivo* system. A recent *in vitro* analysis on bipolar shaped endothelial human cells migrating over gelatin fibrils described the rare phenomenon of the fracture of filopodia during cell migration.[19] They observed that the tip of the filopodia that will break first tend to cluster, then there is a "necking" process and finally the scission occurs, leading to some retraction of the cell body. In our case, and in agreement with this report, occasionally we also observe a small "necking" prior to breakage, which could represent a region in the filopodia with decreased number of filaments, which would eventually break.

During our in vivo analysis we also noticed cycles of re-elongation of tracheal filopodia; i.e., after extension, filopodia start retraction but they are not completely reabsorbed, and instead they use the same path to elongate again. Previous models for filopodia dynamics had proposed consecutive steps of initiation, elongation, stasis and retraction. However, recent studies in human keratinocytes showed that most of their filopodia undergo at least one re-elongation cycle,[18] in which a part of an old filopodia is re-used to elongate a new filopodia. This process is energetically favorable since it allows recycling of actin filaments and other signaling and scaffolding proteins from a previously existing filopodia. Thus, our analysis offers an in vivo example of filopodia re-elongation, suggesting that this can be a general mechanism.

Figure 1 (See previous page). Filopodial breakage and re-elongation occur during tracheal tissue morphogenesis. Time-lapse images of *in vivo* movies of tip cells from tracheal dorsal branches (DBs) during their approach toward the dorsal midline. (**A**) Tip cells of a DB of a stage 15 control embryo projecting stiff and straight filopodia. We show the rare case of a filopodium breaking (**green arrowheads**). During the retraction phase, this long filopodium first bends almost 90° (at 02:07 min) and later breaks in 2 pieces (04:04, magnified in the inset). The freed piece eventually shrinks, the cell body-attached piece retracts and is reabsorbed (04:15-08:08). Before breakage it is possible to observe the "necking" of the filopodium (03:53, magnified in the inset) just where the filopodium will bend and break. **Black arrows** show the process of re-elongation. The arrows point to one filopodium that elongates (01:14 to 02:07), retracts (02:07 to 03:53) and shows a period of stasis (03:53-04:35). Subsequently, instead of being reabsorbed, the filopodium elongates again (04:35-05:50) to finally retract and reabsorb. Note the extension of the lamellipodium toward this re-elongating filopodium (04:15-04:35). Also note that this lamellipodium extension continues after the retraction of the marked filopodium, likely helped by the formation formation of more filopodia in the same direction (04:35-08:08). Images were taken every 10s624ms. (**B**) Tip cells of a DB of a stage 15 sn mutant embryo (sn^P1^. FBal0035641) show bent filopodia and very irregular cell edges and cell shapes. We show 3 cases of filopodia breakage (**red, blue and purple arrowheads**). All filopodia break during the retraction phase in regions of pronounced bending. The free filopodia remnant shrinks and the attached filopodia retracts toward the cell. Images were taken every 10s627ms. In order to visualize tracheal cells and filopodia, the Src membrane protein tagged with GFP is expressed in the tracheal tissue driven by the specific tracheal driver *breathless-Gal4* in both control and sn mutant embryos. Live-time imaging was performed in a TCS-SP5 Leica confocal microscope HCX PL APO lambda blue 63.0×1.40 Oil UV. Images were imported into Fiji and Photoshop software. **Scale Bar** 10 μm, and 2 μm in magnifications. **Time** is shown in min:sec.

Analysis in keratinocytes also revealed the formation of filopodial focal complexes (FX) along the filopodia, which are small but fully organized focal adhesions that adhere to the substrate. When these FXs are reached by the lamellipodium they enlarge and give rise to stable focal adhesions (FAs), subsequently driving force transmission.[20] These filopodial adhesions may favor the re-elongation cycles of filopodia, since after FA formation, the filopodium is able to re-elongate again, which may in turn promote faster and more efficient movement of the lamellipodia.[18,20] As it is the case in keratinocytes, it remains possible that tracheal filopodia also contain focal complexes. The presence of these adhesions could determine the movement of broken filopodia over the substrate, because, as mentioned before, the filopodia free remnants, while shrinking, continue moving. The presence of adhesions in tracheal filopodia is also consistent with our observation of re-elongation cycles, which would promote fast migration of tracheal cells. In *sn* mutant conditions, the increase in filopodial breakage would lead to a decrease of force cell transmission, providing an additional explanation for the observed slower speed of migration in *sn* mutant tracheal extension.[10]

Further work to determine the presence of adhesion complexes in tracheal filopodia would open new horizons on the in vivo roles and dynamics of filopodia in cell migration.

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