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Cytoskeletal players in single-cell branching morphogenesis

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ABSTRACT

Branching networks are a very common feature of multicellular animals and underlie the formation and function of numerous organs including the nervous system, the respiratory system, the vasculature and many internal glands. These networks range from subcellular structures such as dendritic trees to large multicellular tissues such as the lungs. The production of branched structures by single cells, so called subcellular branching, which has been better described in neurons and in cells of the respiratory and vascular systems, involves complex cytoskeletal remodelling events. In *Drosophila*, tracheal system terminal cells (TCs) and nervous system dendritic arborisation (da) neurons are good model systems for these subcellular branching processes. During development, the generation of subcellular branches by single-cells is characterized by extensive remodelling of the microtubule (MT) network and actin cytoskeleton, followed by vesicular transport and membrane dynamics. In this review, we describe the current knowledge on cytoskeletal regulation of subcellular branching, based on the terminal cells of the *Drosophila* tracheal system, but drawing parallels with dendritic branching and vertebrate vascular subcellular branching.

1. Tracheal terminal cells: a model for subcellular branching

The main tubes in the *Drosophila* tracheal system are multicellular and generate an extracellular lumen during developmental stages. Multicellular tubes can be further divided into tubes formed by cells with intercellular junctions or autocellular junctions (reviewed in (Hayashi and Kondo, 2018)). However, in some branches, there are cells able to form subcellular lumina within their cytoplasm. In this case, these lumina are called subcellular or seamless, due to the lack of intracellular or autocellular junctions. Cells able to form a subcellular seamless lumina are of two types: fusion cells (FCs) and terminal cells (TCs). FCs are cells that elongate shortly and undergo anastomosis to connect different branches in the network (Gervais et al., 2011; Samakovlis et al., 1996). TCs are the tip-cells of the main branches, which elongate extensively during embryonic development and, from hatching, are able to deliver oxygen to target tissues (Burguete et al., 2019; Guillemin et al., 1996; Jarecki et al., 1999). At larval stages, these TCs, branch extensively, greatly increasing their surface area and, consequently, their oxygen delivering capacity (Fig. 1). Most studies on TCs have been performed mainly in Dorsal Branch (DB) TCs, due to easier imaging during development and larval stages. However, subcellular branching mechanisms can be generalized to ganglionic branch (GB) TCs (Ricolo and Araújo, 2020; Ricolo et al., 2016) as well as other TCs. TC growth is primarily controlled by the Fibroblast Growth Factor (FGF) Branchless (Bnl), which TCs sense using the corresponding receptor tyrosine kinase (FGFR) Breathless (Btl) (Klämbt et al., 1992; Sutherland et al., 1996). TC fate is determined by Drosophila Serum Response Factor (DSRF) expression, which is triggered by Btl/Bnl signalling (Affolter et al., 1994; Guillemin et al., 1996; Sutherland et al., 1996). Depending on the branch where TCs locate, branch tip topology can be different. For example, in GBs the TC has a junction with only one stalk-cell (SC), whereas in DBs the TC has junctions with both the SC and the DB fusion cell (FC). However, in both cases, the TC lumen arises from the junction with the SC (Gervais and Casanova, 2010; Gervais et al., 2011) (Fig. 2).

TCs create a lumen by invaginating membrane from the intercellular junction with the stalk cell and extending it towards the inside of the cell. This will form a seamless tube without any junctional structures (Sundaram and Cohen, 2017). Topologically, the 'inside' of the cell is the

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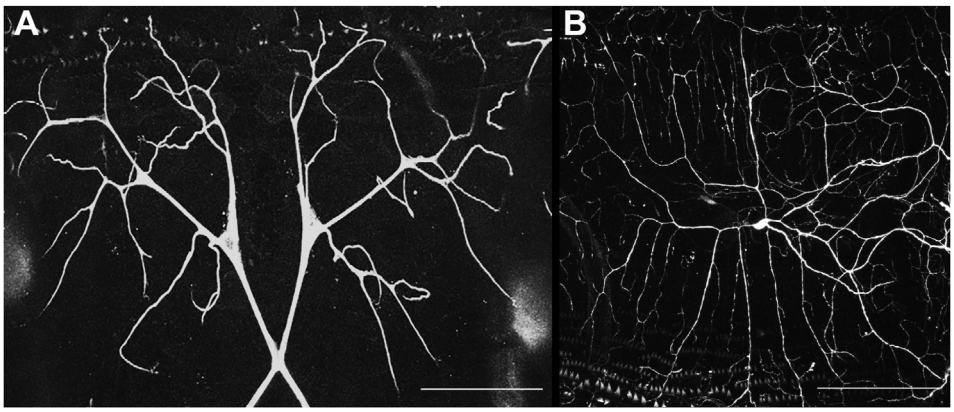

Fig. 1. Single-cell branching models - The highly branched structures formed by (A) tracheal terminal cells and (B) dendritic arborisation neurons in *Drosophila* larvae. (A) Two L3 TCs expressing GFP, fused at the dorsal midline; (B) An L3 ddaC neuron, expressing GFP. Anterior is up. Scale bars 50 μm.

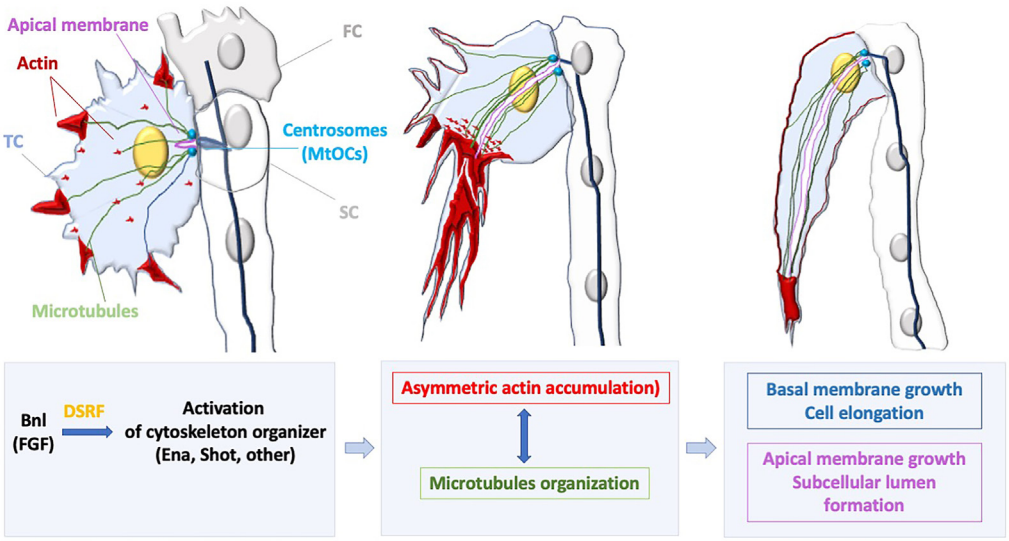

Fig. 2. Subcellular lumen formation is associated with cell elongation. Representation of dorsal TCs from stage 14 to st. 16 in light blue. DB stalk cells (SCs) in white with nuclei in grey, FC in light grey with nucleus in grey, TC in light blue with nucleus in yellow and lumen in blue. (A) At stage 14, the TC, (basolateral membrane in dark grey, apical membrane in pink, actin network in red and microtubules in green) next to FC in grey and SC in white, emits short filopodia in all directions in response to Btl/Bnl signalling. (B) At stage 15, long filopodia extend in the direction of cell elongation and apical membrane grows in the same direction, giving rise to the subcellular lumen. Consequently, the subcellular lumen is filled with chitin (in white). (C) At stage 16 the TC is elongated, and the subcellular lumen is stabilized within the cytoplasm of the cell.

cytoplasm between the apical tube membrane and the outer basolateral cell membrane, therefore the term 'subcellular' to describe these tubes (Sigurbjörnsdóttir et al., 2014). *De novo* subcellular lumen formation at embryonic stages starts the single-cell branching morphogenesis process in tracheal terminal TCs. During larval stages, each TC branches out, through subcellular branch sprouting and elongation, increasing its tissular contacts and becoming a highly branched structure resembling a neuron with its branched-out dendrites (Fig. 1) (Affolter and Caussinus, 2008). Single-cell branching morphogenesis can be studied in TCs at embryonic stages, where a lumen is formed *de novo*, or during larval stages, where most luminal and cellular branching occur.

2. Terminal cell differentiation

TCs have high Btl/Bnl signalling activity triggering the expression of several factors implicated in cell elongation and subcellular lumen formation. Terminal branch formation in the embryo is controlled, during the initial phases, directly by Bnl signalling and later on, during the progression of cell elongation and subcellular lumen formation, by DSRF(encoded by the *pruned/blistered* gene), whose expression is also triggered by Bnl (Affolter et al., 1994) (Fig. 2). Mutant embryos for the null allele *pruned* display TCs where subcellular lumen initiation begins, but cell elongation and subcellular lumen formation stop prematurely (Guillemin et al., 1996). However, high levels of Bnl can bypass the requirement of DSRF for the initiation of terminal branch formation (Gervais and Casanova, 2011). So, DSRF is dispensable for terminal branch initiation, probably because Bnl levels at this stage are high enough to promote directly the first steps of terminal branch formation. Nonetheless, DSRF is a crucial requirement for the progression of cell elongation and subcellular lumen formation (Gervais and Casanova, 2011).

Through DSRF, Btl/Bnl signalling regulates the expression of genes involved in cytoskeleton regulation, including the gene encoding for Enabled (Ena), a VASP protein substrate for the nonreceptor tyrosine kinase Abelson (Abl), which accumulates in areas of actin remodelling and promotes filopodia formation (Gervais and Casanova, 2010), Singed (Sn), encoding *Drosophila* Fascin (Okenve-Ramos and Llimargas, 2014), or Short-stop (Shot), the only spectraplakin in *Drosophila*, involved in the crosstalk between MTs and actin (Ricolo and Araújo, 2020). This cytoskeletal reorganization is instrumental in the communication between apical and basal membranes, probably by directing intracellular trafficking, thus coupling cell elongation and subcellular lumen formation.

3. Subcellular lumen *de novo* formation

Subcellular lumen formation in *Drosophila* TCs requires the cell to break its symmetry and establish a specific pattern of apical-basal polarity. TC elongation begins with the formation of filopodia from the

basolateral membrane as the TC leads the migration of the branch. This transient and dynamic single-cell branching activity begins with the formation of filopodia from the basolateral membrane in random directions and continues with more directed filopodial extension towards the direction of the chemoattractant Bnl (Okenve-Ramos and Llimargas, 2014). At initial stages, the TC has a ring of apical membrane at the junction with its adjacent stalk cell (SC) (Fig. 2). The peripheral membrane of the TC, including the membrane at the cell tip, has basolateral properties, making the epithelial TC resemble more of a mesenchymal cell. The subcellular lumen develops by invagination of the apical membrane inside the cell, and this defines the first step in subcellular lumen formation. The ingrowing apical membrane accumulates specific apical markers like the PAR-polarity complex components aPKC/Par6/-Baz, and Crumbs (Crb) (Best, 2019; Jones and Metzstein, 2011; Mathew et al., 2020). Following membrane formation, as for all tracheal tubes, chitin is accumulated inside the lumen creating the apical ECM (aECM) (Gervais and Casanova, 2010; Öztürk-Çolak et al., 2016).

Cell elongation and subcellular lumen formation are intimately associated (Fig. 2). During the first steps of cell elongation, at stage 14, the TC emits short filopodia in all directions and immediately after, larger cellular protrusions start to form, indicating the way of future lumen elongation (Fig. 2A). Subcellular lumen is already apparent at early stage 15, as a new apical membrane growing towards the tip of the cell (Fig. 2B). *In vivo* analysis demonstrated that the incorporation of new apical material is not restricted to specific sites in this new apical membrane, such as the tip or the base, but accumulation of vesicles carrying luminal components and recycling endosomes are localized all along all the length of the lumen. The subcellular tube grows in unison with the basolateral domain of the cell, and as the cell and its tube extend, trafficking markers are seen throughout the cell, often associated with the tube itself (Gervais and Casanova, 2010; Jayanandanan et al., 2014; Mathew et al., 2020; Schottenfeld-Roames et al., 2014). Rab11 and dRip11, proteins involved in the trafficking of recycled and new synthesized proteins to the apical plasma membrane, are localized near and ahead the forming lumen. Other components involved in membrane protein transport, like the exocyst complex proteins Sec6 and Sec8 localize to the tip of the cell during elongation and subcellular lumen formation and this localization is lost upon MT depolymerization (Gervais and Casanova, 2010). Likewise, TC endosomes have been shown to act as a hub in coordinating plasma membrane redistribution. They are enriched at the tip of TCs, between the growing lumen and the extending filopodia. In addition, an organelle carrying markers of late endosomes and multivesicular bodies (MVBs) has been suggested to act as a transit station for membrane destined to be redistributed both apically and basally in TCs (Mathew et al., 2020) (Fig. 3).

Thus, subcellular lumen formation involves the structured expansion of the apical plasma membrane through the dynamic modulation of vesicle transport, actin and microtubule cytoskeleton. These mechanisms are also used in other systems, such as endothelial cells (ECs), for *de novo* lumen formation, and in neurons during dendritic branching (Dong et al., 2015; Sigurbjörnsdóttir et al., 2014).

4. Actin organization in embryonic TCs

TCs require proper actin organization for their polarized elongation and subcellular lumen formation. From the beginning of TC specification, actin is organized in different structures. Initially, a multitude of basal filopodia extends in all directions and actin dots are diffuse in the cytoplasm. Later on, filopodia grow towards the direction of cell elongation and F-actin matures in a central core at the distal tip of the cell, which resembles the growth cone of an axon (Oshima et al., 2006) (Fig. 4). During their development, TCs generate at least three distinct populations of actin that have different functions. First, the "growth-cone--like" F-actin accumulation is involved in the correct cell elongation and subcellular lumen formation. Ena mediates the remodelling of actin filaments and promotes filopodial growth. During cell elongation, Ena localizes at the cell tip, where it co-localizes with F-actin and Moesin (Moe), Moe is the only member, in *Drosophila,* of the Ezrin-Radixin-Moesin (ERM) family, involved in connecting the cytoskeleton with the plasma membrane (McCartney and Fehon, 1996; Polesello et al., 2002). Accordingly, *ena* mutants display defective terminal branch formation characterized by short and disorganized TC filopodia. Ena might also function in guiding luminal growth, as in *ena* mutants the lumen elongates in an incorrect direction (Gervais and Casanova, 2010; Sigurbjörnsdóttir et al., 2014). Second, at the basolateral cell membrane, cortical actin is essential for the stability of the TC branches. Integrin molecules bind to the extracellular matrix through their extracellular domains and bind to adaptor molecules such as Talin

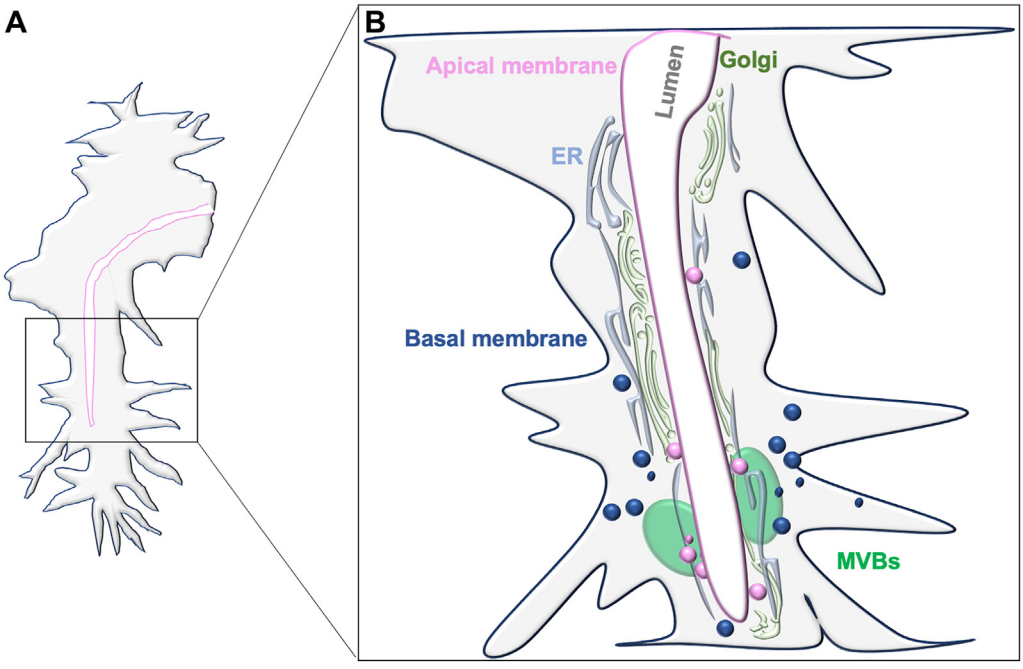

Fig. 3. Organelles and TC membrane delivery. (A) Representation of a TC and its magnification (B) at stage16. (A) Cytoplasm is in grey, apical membrane in pink. ER (in light blue) and Golgi apparatus (in light green) are all around the luminal space, next to the apical membrane. Multivesicular bodies (MVBs) (in dark green) are involved as a transit station for membrane destined to be redistributed both apically and basally, vesicles addressed to the basal membrane (in blue) and vesicles directed to apical membrane (in pink). (Figure adapted from (Mathew et al., 2020)).

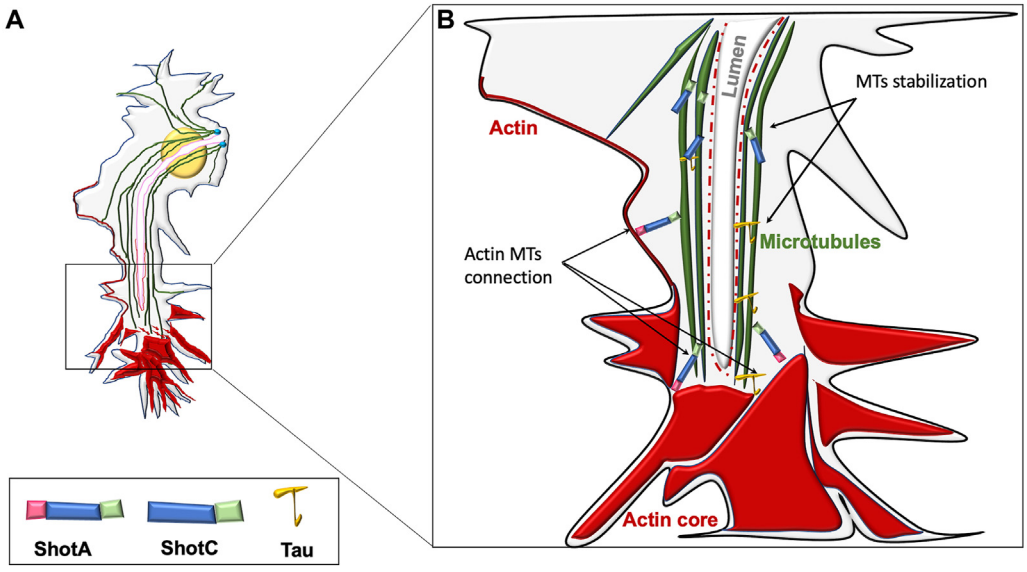

Fig. 4. Shot and Tau dynamically modulate the cytoskeleton during subcellular lumen formation. (A) Schematic representation of stage16 embryonic TCs and its magnification (B); cytoplasm in grey, nucleus in yellow, baso-lateral membrane in blue, apical membrane in pink and luminal space in white. Cytoskeletal components in a *wt* embryo are represented with the actin-network (red) and MTs (green). Shot and Tau are able to organize the cytoskeleton: i) by crosslinking MTs and different actin network populations. The longer isoform of Shot (ShotA, represented with the actin domain in red and the MT binding domain in green) as well as Tau (represented in golden) mediate the crosstalk between actin and MTs. ii) by stabilizing MTs. The shorter isoform of Shot (ShotC, represented with only the MT binding domain in green) lacks part of the actin binding domain (ABD) and, as Tau, is able to stabilize MTs around the growing subcellular lumen.

which organize the basal cortical actin (Levi et al., 2006). Third, a dense actin network surrounds the luminal tube as it grows. This pool of actin is required for the delivery of apically targeted material and possibly also for structuring the tube. The interaction between this actin network and luminal membrane is mediated by a complex comprising Moe and Bitesize (Btsz), which aids the incorporation of apical membrane material into the growing luminal tube (Jayanandanan et al., 2014; Oshima et al., 2006) (Table 1).

Shot downregulation leads to defects in the actin network, suggesting a possible direct role of this spectraplakin in organizing TC actin (Ricolo and Araújo, 2020). According to this hypothesis, Shot/ACF7 can promote filopodia formation in axons (Lee et al., 2007; Sanchez-Soriano et al., 2009). In particular, during axonal extension in the Central Nervous System (CNS) midline, this function of Shot depends on the interaction of the two EF-hand motifs and the translational regulator of filopodia assembly Krasavietz (Kra), which is necessary for filopodial formation (Lee et al., 2007; Sanchez-Soriano et al., 2009). However, a direct role of Shot, possibly through this same EF-hand motif, in regulating the dynamic actin organization in the TC has not yet been studied.

A role for the late endocytic pathway in organizing actin for proper cell morphogenesis has also been proposed, in addition to its known role in membrane and protein trafficking. In this model, late endosomes act as centres for actin nucleation ahead of the growing tube, a process that allows the crosstalk and coordination between distinct cytoskeletal pools within the cell (Rios-Barrera and Leptin, 2021).

5. TC microtubule network

The MT network has been well characterized in tracheal TCs. At early stages of subcellular lumen formation, MT bundles emanate from the junction between the TCs and the stalk cells, with their plus end towards the tip of the cell and running in parallel to the subcellular lumen (Fig. 2). As the subcellular tube begins to extend, MTs are organized along the apical membrane and ahead of it, interacting with the rich actin area at the tip of the cell. Bundles of stable MTs are detected along the entire cell, strongly accumulating next to the apical surface. Conversely, tyrosinated tubulin, found in newly assembled MTs, specifically accumulates at the tip and ahead of the lumen, suggesting it may have a luminal guiding role (Gervais and Casanova, 2010).

TC elongation and lumen formation correlate with correct MT network stabilization and MT transport. MT depolymerization, by overexpression of Spastin in TCs, induces an arrest in cell elongation and subcellular lumen formation. Also, mutants in *Lissencephaly-1* (*Lis-1*), a dynein motor co-factor, which binds MTs and is involved in cellular transport, display subcellular lumen formation defects and TCs that fail to elongate. Interestingly, in such mutant conditions TCs still emanate filopodia in the correct direction and the actin network is not apparently affected (Gervais and Casanova, 2010). Conversely, in mutants like *ena*, which disturb TC actin, the MT-network is affected suggesting that actin is involved in MT organization at the cell tip, as postulated for the growth cone in axons (Lowery and Van Vactor, 2009).

6. Centrosomes are microtubule-organizing-centres (MTOCs) in TC

Despite being differentiated cells, TCs have one centrosome pair. This is unusual since generally 2 centrosomes characterize dividing cells and in most differentiated cells only one (or none) centrosome is present (Nigg and Stearns, 2011).

The mechanism by which TCs maintain two centrosomes after cell division is an open question; we may speculate that TCs have started endo-replication as reported in larval TCs (Burguete et al., 2019) or, perhaps, that TCs inherit one centrosome after cell division and later on, during differentiation, this centrosome is duplicated, allowing subcellular lumen formation. From our data, we have proposed that differentiated TCs maintain/generate a pair of centrosomes, thus allowing for the particular MT organization needed to organize the first steps of subcellular lumen formation.

At stages before subcellular lumen *de novo* formation, the TC centrosome-pair localizes apically, at the junction of the TC with the stalk cell. Here, centrosomes are active MTOCs allowing the MT organization necessary to start forming the extending subcellular lumen. In particular, two main MT-bundles emanate from each centrosome and grow towards the tip of the cell, alongside and ahead of the ingrowing subcellular lumen. Through these MTs tracks, vesicles can be directed to deliver membrane and/or chitin, and the new lumen can be built (Gervais and Casanova, 2010). Moreover, the two MT tracks may have also a structural function, in maintaining the subcellular lumen framework and stabilizing the extending TC.

Centrosomes are defined as the primary MTOC during the first steps of subcellular lumen formation in TCs because not only the MT nucleator factor γ-tubulin localizes at centrosomes at early stages of TC

Table 1
– Different *Drosophila* cytoskeletal-related proteins involved in TC lumen formation and branching and their parallel functions in endothelial cells and dendritic arborisation neurons. Function in TCs in either embryos or larvae; broad function of orthologs in endothelial cell lumen formation; function in da neurons. n/d no data available. For references see Table 2 and main text.

Drosophila gene	Terminal cells (TCs)	Endothelial cells (EC)	da neurons
blistered/pruned DSRF (bs)	TC fate induction	Tip-cell induction Cytoskeletal organization (SRF)	n/d
enabled (ena)	Subcellular lumen formation	n/d	Dendritic branching
actin	Cellular extensions Lumen directionality	Cellular extensions/ protrusions Lumen formation	Cellular extensions Dendritic outgrowth and branching
moesin (moe)	Subcellular lumen formation and branching	Cellular extensions/ protrusions Lumen formation	Dendritic branching
Lissencephaly-1 (Lis-1)	Subcellular lumen formation	Tube formation and sprouting (PAFAH1B1/Lis1)	Dendritic branching
bitesize (btsz)	Subcellular lumen formation and branching	Component of the Weibel-Palade Body (WPB) (Slp4-a/Sytl4)	n/d
Syntaxin7 (Syx7)	Cellular branching	n/d	n/d
short-stop (shot) Spectraplakin	MT-actin crosstalk Subcellular lumen formation and branching	n/d (DYS and MACF1)	Dendritic outgrowth and branching
whacked (wkd) RabGAP	Subcellular lumen formation and branching	Modulators of angiogenesis (TBC1D10A-C)	n/d
twinstar (tsr) Cofilin	Lumen formation	n/d	Dendritic branching
singed (sn)	Cellular extensions Lumen directionality	n/d	Dendritic outgrowth (filagree phenotype)
forked (f)	Cellular extensions Lumen directionality	n/d	n/d
washout (wash)	Subcellular lumen formation Lumen directionality	n/d	n/d
spaghetti squash Myosin regulatory light chain *(sqh)*	No effect	EC migration and lamellipodia formation	n/d
PAR complex *(par-6, baz, aPKC)*	Subcellular lumen formation and cellular branching	Endothelial polarity and lumen formation	No effect
Exocyst complex (*Sec5, 6, 10, 15*)	Subcellular lumen formation and cellular branching	n/d	Dendritic outgrowth and branching
Rab GTPases	Subcellular lumen formation and cellular branching	Tube formation and sprouting	Dendritic outgrowth and branching
Shibire/dynamin (shi)	Subcellular lumen formation and branching	Angiogenesis/ branching morphogenesis (Dnm2)	Dendritic outgrowth and branching
shrub/Snf7 ESCRT-III component *(shrb)*	Subcellular lumen formation	n/d	Dendritic outgrowth and branching
Transport and Golgi Organization 1 (Tango1)	Subcellular lumen formation and branching	n/d	n/d
Organelles			
centrosome			n/d
	Subcellular lumen formation and branching	Endothelial polarity and lumen formation	
Golgi outposts (GOs)	n/d	n/d	Dendritic outgrowth and branching

development, but also that the centrosomes are active MTOCs (Ricolo et al., 2016). However, it was previously shown that, when the lumen is already formed, γ-tubulin also localizes around the subcellular lumen and, as consequence, it has been proposed that the minus end of MTs face the apical membrane and are probably used for the secretion of molecules into the lumen (Gervais and Casanova, 2010; Sigurbjörnsdóttir et al., 2014). So, it is also possible that during development, secondary acentrosomal points of MT nucleation are formed on the apical membrane as observed in other tracheal cells (Brodu et al., 2010). In addition, there is still the possibility that this γ-tubulin population around the apical membrane is not involved in nucleating MTs but is involved in other aspects of MT network, such as the organization of plus-end dynamics (Oakley et al., 2015).

7. Crosstalk between microtubules and actin during lumen formation and branching

The proper cytoskeletal configuration allowing TC *de novo* lumen formation and branching depends on the coordination between actin and MTs during TC extension and guidance. Recently, we have studied the role of Shot during TC extension and subcellular lumen formation (Ricolo and Araújo, 2020). Spectraplakins are conserved giant proteins, characterized by an ability to bind and coordinate different cytoskeletal elements through its various domains (Dogterom and Koenderink, 2019; Röper et al., 2002). In *shot* mutant TCs the actin network and MT bundles are highly disorganized, which leads to defects in lumen formation and branching. Rescue experiments showed that both the microtubule (C-tail) and actin binding domain (ABD) of Shot are required, in the same molecule, to restore the cytoskeletal configuration and the correct cellular branching and lumen formation (Ricolo and Araújo, 2020). Hence, we have proposed that in TCs the spectraplakin Shot, behaves like in neuronal cells, crosslinking actin and MTs (Lee et al., 2007; Lee and Kolodziej, 2002b; Ricolo and Araújo, 2020; Zhang et al., 2017) (Table 1).

Shot with its complete N- and C-terminal domains (ShotA) is involved in actin-MT crosstalk, a process necessary for the correct cell elongation and subcellular lumen formation. Suitably, Shot co-localizes with the area where MTs and actin overlap between stage 14 and stage 16, when most cell elongation and subcellular lumen formation take place. In neurons, the ABD present in Shot-A is necessary for the proper linkage between actin and the MT-network and for the correct neuronal morphogenesis (Bottenberg et al., 2009; Sanchez-Soriano et al., 2009). In contrast, it has been suggested that in tracheal FCs, the binding sites for actin and MTs appear to be functionally redundant; the actin binding domain is essential when the microtubule binding site is absent, and the microtubule binding site is essential when the F-actin binding site is absent (Lee and Kolodziej, 2002a). So, according to our observations the role of Shot in TCs resembles more its function in neuronal cells than its function in FCs. Moreover, Shot maintains its accumulation on stable MTs around the subcellular lumen and here the C-tail is involved in sustaining MT stabilization and in turn maintaining the integrity of the subcellular lumen (Ricolo and Araújo, 2020).

We also found that the MT-associated protein Tau, previously described for its function in actin and microtubule crosstalk (Cabrales Fontela et al., 2017; Elie et al., 2015), is involved in the maintenance of cytoskeletal organization and branching during TC maturation, functionally overlapping Shot (Ricolo and Araújo, 2020).

One human orthologue of Shot, the spectraplakin Dystonin (DST), when mutated gives rise to Hereditary Sensory and Autonomic Neuropathy (HSAN) Type VI and recent observations point to an emerging role of cytoskeleton organization and function in this disease (Fortugno et al., 2019). Mouse models lacking *dystonin* display nervous system defects together with muscle cell cytoskeletal abnormalities. The other orthologue, Microtubule-Actin Crosslinking Factor 1 (MACF1; aka Actin Crosslinking Family 7/ACF7), when mutated gives rise to Lissencephaly 9 (LIS9) and mouse models show early neurodevelopmental abnormalities together with impaired heart and gut physiology (Voelzmann et al., 2017). Nonetheless, none of these vertebrate homologues of Shot, have been implicated in EC lumen formation to date.

8. Extra lumen and extra branching of embryonic TCs

Regulator of cyclin A1 (*Rca1)* is the *Drosophila* orthologue of vertebrate Emi1 and a regulator of APC/C activity at various stages of the cell cycle (Grosskortenhaus and Sprenger, 2002). *Rca1* mutants have supernumerary centrosomes in TCs and these develop extra subcellular lumina at embryonic stages, in association with higher levels of stable MTs and higher levels of apical complex proteins (Ricolo et al., 2016). According to our model, in supernumerary centrosome conditions, more centrosomes can be active as MTOCs and the following steps are triggered: 1) more centrosome/apical complex proteins interactions are established; 2) more MT tracks are generated and 3) more MT bundles are available to direct membrane delivery. These events cause the immediate expansion of the apical membrane and hence the extra branching phenotype. Accordingly, in *Rca1* and other mutants affected in centrosome number, a bifurcated subcellular lumen arises at the beginning of TC maturation, mostly from the apical junction (Ricolo et al., 2016). In association with the bifurcated lumen, the expansion of the cytoplasmatic protrusion at the tip of the cell and its own asymmetric actin accumulation at the tip are observed. Probably, the initial surplus of MT-bundles and the establishment of a double intracellular polarized symmetry push the cell to split and branch prematurely. These observations reinforce the notion that subcellular lumen formation is intimately associated with cell elongation (Gervais and Casanova, 2010) also in cases of supernumerary lumina (Ricolo et al., 2016).

The correlation between centrosome amplification and TC subcellular architecture is in agreement with phenotypes observed in Spindle Assembly Abnormal 4 (*Sas-4*) mutants. In *Sas*-4 TCs, without centrosomes, subcellular lumen does not extend within the TC and the cell is able to elongate only partially, probably due to the scarcity of MTs directing vesicle trafficking and the failed interaction between apical complex proteins and centrosomes. In *Rca1;Sas-4* double mutant conditions, TCs lose the extra subcellular lumen branching capacity, because a balance between the effects of the two mutations restores a *wt*-like condition. Interestingly, also in cases in which only one centrosome localizes to the apical region of the TCs, the subcellular lumen is not able to grow properly, reinforcing the notion that two centrosomes are necessary for luminal development and extension (Ricolo et al., 2016).

Extra subcellular branching can also be induced by changing other cytoskeletal players. The overexpression of *shot* in the tracheal system induces extra TC luminal branching and this effect does not depend on centrosome amplification (Ricolo and Araújo, 2020). Our data indicate that the C-tail domain of Shot is involved in inducing the extra branching phenotype because tracheal overexpression of this domain alone is able to induce extra-subcellular luminal branching and a construct in which the C-tail has been removed fails to induce extra subcellular lumina (Ricolo and Araújo, 2020). Studies using different models have shown that spectraplakins interact with MTs using two conserved C-terminal domains, the Gas2-related domain (GRD) and the adjacent C-tail domain (C-tail). Such domains associate with MT bundles and protect them against the MT destabilizing drug nocodazole (Alves-Silva et al., 2012; Lee and Kolodziej, 2002b; Sun et al., 2001). In different tissues, the C-tail has been reported to associate with either MT bundles (Alves-Silva et al., 2012) or EB1 at polymerizing MT plus ends (Applewhite et al., 2013). So, spectraplakins work as MT-stabilizing factors similar to classical MAPs and they also have roles as regulators of MT plus ends similar to + TIPs. For this reason, the cause of the generation of extra subcellular lumen in conditions of Shot overexpression is likely to be the over stabilization/polymerisation of MTs, a process in which the C-tail domain is involved. Accordingly, by the analysis of endogenous Shot or by the overexpression of isoforms containing the C-tail domain, we have detected the localization of this spectraplakin on stable MT bundles surrounding the growing subcellular lumen. In most cases, the extra subcellular lumen in Shot overexpression arises from the main lumen where an excess of this spectraplakin could generate the formation of new MT bundles (Fig. 5).

Thus, the current model takes into account two concurrent mechanisms in TCs during *de novo* formation of extra subcellular branches in embryos: one dependent on centrosome numbers and the other acentrosomal (Figs. 2 and 5) (Ricolo and Araújo, 2020; Ricolo et al., 2016). According to the models proposed, centrosomes are necessary for the first steps of *de novo* lumen formation and supernumerary centrosomes induce the formation of extra subcellular lumina from the junction of the TC with the SC (Ricolo et al., 2016). Overexpression of MT binding proteins such as Tau or Shot (or only the MT binding domain of Shot, ShotCtail) is capable of inducing extra subcellular lumina, acentrosomally, from various points distributed along the length of the preexisting luminal membrane (Ricolo and Araújo, 2020). Interestingly, overexpression of Bnl ahead of the TC migration path, does not induce detectable changes in centrosomal number, but is able to induce subcellular luminal branching acentrosomally from the luminal membrane (Ricolo et al., 2016). Likewise, constitutively activation of Btl in TCs can also induce extra subcellular lumina (Rios-Barrera and Leptin, 2021), possibly due to higher DSRF and Shot expression (Ricolo and Araújo, 2020) (Figs. 4 and 5).

9. Larval subcellular lumen branching

De novo subcellular lumen formation takes place in TCs at embryonic stages, but the gross luminal growth and branching occurs during larval stages. Most studies on larval branching have been performed in wandering third instar (L3) larvae with an overall view of the final branching morphology, but not the progression from embryonic stages, through L1 and L2, to L3. In wild-type L3 larvae, all cellular extensions contain one single lumen per branch, which is airfilled, but there are different mutant conditions that can affect this balance and create branches without a lumen or branches with extra lumina (Table 2) (Ricolo and Araújo, 2020). Therefore, during larval stages we can observe two different and separable aspects of subcellular branching: cellular branching and luminal branching. Nonetheless, many studies focus and report either one or the other phenotype, making it difficult to ascertain which factors affect both luminal branching and TC cellular branching.

Regarding the cytoskeleton, what is known so far indicates that most of the players observed in embryonic TCs are maintained in the larva (Best, 2019). Cellular branching and extension can also be regulated by Btl/Bnl signalling, although Bnl expression stops being stereotypically determined, as in embryonic stages, to become expressed according to the oxygen needs of the environment (Jarecki et al., 1999; Lee et al., 1996). Current models relate localization of Bnl expression with the spatial induction of new TC branches (Best, 2019).

Inside the larval TC, the tube maintains its apical identity and its actin cortex as well as MT bundles parallel to the subcellular lumen (Fig. 6). F-actin is enriched in three distinct larval TC subcellular domains: surrounding subcellular tubes, decorating filopodia and outlining short stretches of basolateral membrane (Schottenfeld-Roames and Ghabrial, 2012). The connection between the basal actin network and the outside is made through Talin, which links the cytoskeletal network to the extracellular matrix (ECM) via the integrin complex (Levi et al., 2006). Regulation of the luminal actin is done by Bitesize (Btsz), a Moe

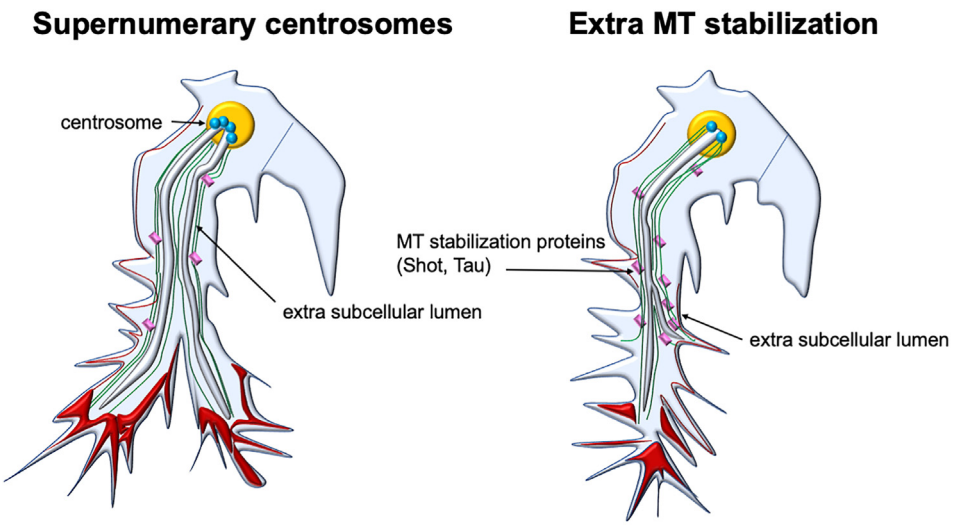

Fig. 5. Extra subcellular lumen formation mechanisms In *Drosophila* tracheal TCs extra subcellular lumina can arise dependent on centrosome numbers and/or by acentrosomal MTOCs. Centrosomes are necessary for the first steps of *de novo* lumen formation and supernumerary centrosomes induce the formation of extra subcellular lumina from the junction of the TC with the stalk cell. Extra activity of MT binding proteins such as Tau or Shot are able to induce extra-subcellular lumina, acentrosomally, from various points distributed along the length of the pre-existing luminal membrane.

Table 2
– Effect of different mutations affecting the cytoskeleton, LOF or downregulation conditions, on TC and da branching in *Drosophila*. Embryonic TC branching: (−) impaired subcellular lumen; (+) extra subcellular lumen. Larval TC luminal branching at L3: (−) lower numbers of lumina per branch/cell; (≈) 1 lumen per branch, as in wild-type; (+) extra subcellular lumina per branch/cell. Larval TC branching at L3: (−) TC with less branches; (+) TC with more branches. da neurons: (−) less branches/endpoints; (+) more branches/endpoints. * lumen overgrowth; ** mushroom body neurons; *** *lava lamp* (*lva*) mutations. n/d no data available.

Drosophila gene (downregulation)	Embryonic TC luminal branching	Larval TC luminal branching	Larval TC cell branching	da neuron branching	References
enabled (ena)	–	n/d	n/d	–	(Gervais and Casanova, 2010; Li et al., 2005)
twinstar (tsr)	n/d	n/d	n/d	–	(Nithianandam and Chien, 2018; Skouloudaki et al., 2019)
moesin (moe)	n/d	+	–	–	(Freymuth and Fitzsimons, 2017; Jayanandanan et al., 2014; Schottenfeld-Roames et al., 2014)
short-stop (shot)	–	–	–	–	(Ricolo and Araújo, 2020) (Nithianandam and Chien, 2018)
Syntaxin7 (Syx7)	n/d	≈	–	n/d	Schottenfeld-Roames et al. (2014)
whacked (wkd)	n/d	+*	n/d	n/d	Schottenfeld-Roames and Ghabrial (2012)
PAR complex (par-6, baz, aPKC)	n/d	n/d	–	No effect **	(Jones and Metzstein, 2011; Jones et al., 2014; Rolls and Doe, 2004)
Lissencephaly-1 (Lis-1)	–	–	–	- **	(Gervais and Casanova, 2010; Liu et al., 2000; Satoh et al., 2008; Schottenfeld-Roames and Ghabrial, 2012)
bitesize (btsz)	+	+	–	n/d	Jayanandanan et al. (2014)
Rab GTPases	n/d	n/d	Rab5 – Rab6 – Rab10 -Rab11 -Rab18 + Rab35 +	Rab1 – Rab5 – Rab11 +	(Best and Leptin, 2020; Schottenfeld-Roames et al., 2014)}(Copf, 2014; Harish et al., 2019; Jones et al., 2014; Satoh et al., 2008; Ye et al., 2007)
shibire (shi)	+	–	–	–	(Mathew et al., 2020; Schottenfeld-Roames et al., 2014; Yang et al., 2011)
Exocyst complex (*Sec5, 6, 10, 15, 23*)	n/d	n/d	–	–	Ye et al. (2007)
Transport and Golgi Organization 1 (Tango1)	n/d	–	–	n/d	Ríos-Barrera et al. (2017)
shrub (shrb)	–	n/d	n/d	+	Sweeney et al. (2006)
Organelles					
Supernumerary centrosomes	+	n/d	n/d	n/d	Ricolo et al. (2016)
Golgi outposts (GOs)***	n/d	n/d	n/d	–	Ori-Mckenney et al. (2012)

interacting protein, present at the apical membrane (Jayanandanan et al., 2014). Membrane interactions with actin are required for proper TC morphology, and mutations in either the *Drosophila* Talin gene *rhea* or in *btsz* induce multiple convoluted lumina per TC (Jayanandanan et al., 2014; Levi et al., 2006).

In larval as in embryonic TCs, acetylated MTs line the subcellular tube membrane and extend beyond it or branch off it into filopodia (Jayanandanan et al., 2014). γ-tubulin remains restricted to the apical membrane and EB1 is localized close to the tube and parallel to it, indicating that MT orientation is maintained (Schottenfeld-Roames and Ghabrial, 2012). Tubulin immunostaining ahead of the tube sometimes colocalises with small patches of apical markers (Schottenfeld-Roames and Ghabrial, 2012), which could be the same as the reported embryonic MVBs and late endosomes (Mathew et al., 2020) (Rios-Barrera and Leptin, 2021).

Unlike embryonic TCs, branching larval TCs have to deal with the added complexity of having to deliver membrane material to subcellular locations at a large distance from the cell body. Rab35 has been implicated in the directed transport of apical membrane vesicles to the growing tips of the branches. Constitutive activation of Rab35 (by loss of *whacked*, *wkd*) results in tube overgrowth at TC tips, whereas defective activation causes formation of ectopic tubes surrounding the TC nucleus (Schottenfeld-Roames and Ghabrial, 2012). Likewise, the exocyst complex is required for subcellular lumen formation and branching, under the control of the PAR complex (Jones and Metzstein, 2011; Jones et al.,

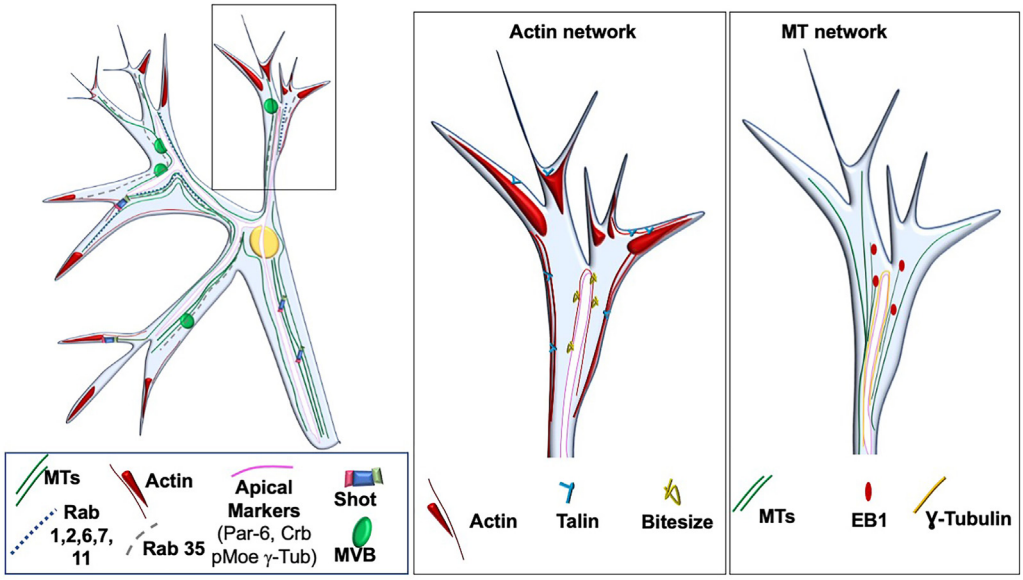

Fig. 6. Larval TC cytoskeletal players Representation of a larval TC during larval development. Cytoplasm is in light blue and nucleus in yellow. MTs, in green, are all around the subcellular lumen and invade all the cytoplasmatic protrusions. The actin network, in red, is at the tip of the branches and distributed throughout the branches as cortical actin and apical actin. MTs are shown in green. Apical components (Par6/Baz/Crb) and pMoe and γtub), in pink, are organized around the subcellular lumen. Rab proteins presence in vesicles are represent in blue spots (Rab 1,2,6,7 and 11) or grey dashes (Rab35); MVB are in light green. Actin network is shown in a schematic magnification with Talin in blue and Bitesize in yellow; and the MT network is shown in a magnification with EB1 in red and apical γtubulin in orange.

2014). Furthermore, within immature branches, late endosomes and MVBs that seem to contain material destined for the subcellular lumen have been identified using electron microscopy and correlative light electron microscopy (CLEM) (Mathew et al., 2020; Nikolova and Metzstein, 2015), reiterating the importance of vesicular trafficking in TC larval branching.

In larval TCs, extra subcellular branching can be achieved by cell-autonomous activation of the Btl/Bnl pathway and by mutations in the Hippo pathway member *warts/lats1* (aka *miracle-gro*), and the TOR pathway inhibitor, *Tsc1* (aka *jolly green giant*) (Ghabrial et al., 2011), as well as by mutations in *btsz* and *wkd* as previously mentioned. More lumina per TC branch can also be induced by overexpression of proteins such as Shot and Tau (Ricolo and Araújo, 2020). Interestingly, higher numbers of lumina per TC do not always correlate with more TC branches (Jayanandanan et al., 2014; Ricolo and Araújo, 2020). The effect of centrosome amplification at larval stages has not yet been determined. In contrast, downregulation of proteins affecting actin such as moesin, and endocytic pathway components like Syntaxin 7, Rab5 or Shibire (Dynamin) cause drastic reductions in TC branching (Schottenfeld-Roames et al., 2014). The same effect is achieved by downregulating cytoskeletal modulators such as Shot and Tau (Ricolo and Araújo, 2020) and intracellular transport factors such as Lis-1 (Schottenfeld-Roames and Ghabrial, 2012), Transport and Golgi organization 1 (Tango1) (Ríos-Barrera et al., 2017) and exocyst components (Jones et al., 2014). Interestingly, most loss of function conditions, induce reduced TC branching capacity (Table 2), though exceptions have been reported for Rab18 and Rab35 mutant TCs (Best and Leptin, 2020; Schottenfeld-Roames et al., 2014). Reported discrepancies in TC phenotypes may be due to differences in experimental set-up. In many cases the phenotypes were analysed using the overexpression of Rab mutant variants, which may interfere with other Rabs in the cell or not allow a full downregulation of protein levels. In contrast, other setups used RNAi or degradation of tagged proteins, which might not deplete Rabs sufficiently (Best and Leptin, 2020; Schottenfeld-Roames et al., 2014).

10. Parallels between tracheal and endothelial subcellular branching

The mechanisms that TCs use to build a subcellular tube resemble those in other lumen-building structures (Sigurbjörnsdóttir et al., 2014). The smaller capillaries of the vertebrate vascular system, are formed by a single cell (seamless tubes) or chains of several cells connected between them by interendothelial cell-contacts (seamed tubes) forming a chain that is only one cell thick (Lubarsky and Krasnow, 2003; Wolff and Bär, 1972). Capillaries in the vertebrate vascular system that are seamless tubes, like tracheal TCs, can adopt branched structures like neurons (Sundaram and Cohen, 2017). Endothelial cell (EC) capillary architecture differs significantly from one organ to another. For example, in mammals, single-cell seamless tubes make up to 50% of the endothelial cells in the renal glomerolum and duedoenal villi and 30% in cerebral cortex (Bär et al., 1984). In contrast, in other systems like the mesentery or in the urinary bladder, seamless capillaries are a minority group. In other organs, both types of capillaries are found in the same ratio. Generally, seamless tubes localize in anastomoses and branch points and intercalate most frequently in those capillaries that develop last in the terminal vascular bed (Bär et al., 1984). Abnormalities in capillary development and maintenance are involved in many diseases. For example, some Mendelian syndromes like Cerebral Cavernous Malformation (CCM), Cerebral Autosomal Recessive Arteriopathy with subcortical infarcts and leukoencephalopathy (CARASIL) and Osler–Weber–Rendu disease display phenotypes related with defects in smaller blood vessels (Sundaram and Cohen, 2017). Genes involved in these hereditary diseases are associated with dysfunctional behaviours in growth factor signalling, vesicle trafficking and cytoskeleton organization (Sundaram and Cohen, 2017). Homologues of these human vascular disease genes also cause tubulogenesis defects in *Drosophila melanogaster* and *C. elegans,* and, conversely, many genes involved in epithelial subcellular lumen formation and branching are also implicated in endothelial lumen formation (Table 1) (Song et al., 2013) (Lant et al., 2015; Xu and Cleaver, 2011).

Seamless tubes are also implicated in angiogenesis, a “common denominator” underlying many deadly and debilitating conditions, including cancer, skin diseases, age-related blindness, diabetic ulcers, cardiovascular disease, stroke, and many others (Muñoz-Chápuli, 2011). Angiogenesis can be induced by hypoxia, a condition in which tissues undergo a reduction of oxygen. Hypoxia induces the sprouting of vascular tip cells, many of which later form seamless tubes, which lead the outgrowth of new vessels into under-vascularized tissue (Semenza, 2012). In parallel, in *Drosophila* tissues, hypoxia induces TCs to respond by extending more branches (Jarecki et al., 1999). Angiogenenesis depends on the coordination between chemoattractant sensing, polarization and motility of endothelial cells (ECs). The concurrent change of cell shape and migration requires cytoskeletal proteins that play a central role in angiogenesis. Nonetheless, not much is known about the intracellular signalling that orchestrates the rearrangement in cell shape and motility

required for a complex process such as capillary sprouting. Rapid changes in cytoskeletal dynamics occur in response to extracellular stimuli, through the activation of specific signalling leading to changes in morphology and/or cell behaviour (Franco et al., 2013).

Just like for the correct branching of tracheal system of *Drosophila*, SRF is required for correct sprouting angiogenesis and the maintenance of vascular integrity in vertebrates.

SRF ablation in ECs leads to embryonic death at around 14 days (E14) of mouse embryonic development with mutant embryos showing a severe decrease in vascular density, oedema, aneurismal structures and haemorrhages. These vascular defects were associated with compromised tip cell morphology a decreased EC migratory capacity, and altered cytoskeletal organization (Franco et al., 2008).

Another important functional similarity in the development of the vertebrate vascular and invertebrate tracheal systems is the establishment of the apical–basal polarity, as this is a crucial event for the organization of the subcellular lumen (Iruela-Arispe and Davis, 2009). In ECs, disrupting the Par polarity complex apical distribution and downstream signalling, results in altered EC polarity and impairs vascular lumen formation (Giampietro et al., 2015).

The arrangement of actin and MT networks in endothelial cells invading 3-D collagen matrices, which mimics angiogenic sprouting initiation, resembles the cytoskeletal architecture observed in TC cytoplasmatic protrusions. Large, highly aligned tubulin bundles are observed and maintained continuously throughout the extensions of peripheral cellular processes and actin foci are observed at critical subcellular locations, at the tip of these peripheral processes (Fig. 7) (Bayless and Johnson, 2011). Moreover, both moesin and actin have been reported to play a role during tubulogenesis, specifically during lumen formation (Xu and Cleaver, 2011). Interestingly, in ECs, in contrast to TC migration, Myosin-IIA is involved in migration and lamellipodia formation (Figueiredo et al., 2020; Ochoa-Espinosa et al., 2017).

Comparably to TC lumen development, EC lumen formation requires vesicle transport. Dynamin is essential for angiogenesis *in vitro* and *in vivo* as shown by the impairment of cell migration and branching morphogenesis upon Dynamin2 (Dnm2) downregulation (Lee et al., 2014). Likewise, the dynein transport machinery is important, as revealed by knockdown of Platelet-activating factor acetyl hydrolase 1B1 (PAFAH1B1, also known as Lis1) leading to impaired tube formation and decreased sprouting of HUVEC (Josipovic et al., 2016). Other cytoskeletal players have also been shown to play a role in EC lumen formation. Rab GTPases and Rab GTPase-activating proteins (RabGAPs), as regulators of vesicle-trafficking, are also involved in EC subcellular lumen formation (Norden et al., 2020; Xie et al., 2019).

As in TCs, the centrosome is important for EC lumen formation. In ECs centrosomes reorient within cells as they begin sprouting angiogenesis during tube formation and centrosome number regulation is necessary for appropriate blood vessel sprouting (Gierke and Wittmann, 2012; Kushner et al., 2014). The localization of centrosomes adjacent to the vacuoles needed to build the lumen (Davis et al., 2007) and by the centrosomal localization at junctional membranes (Rodríguez-Fraticelli et al., 2012) equally depict the importance of this organelle in EC lumen formation. Furthermore, EC centrosome number regulates proper lumenization downstream of effects on apical–basal polarity and cell–cell junctions (Buglak et al., 2020).

In conclusion, in what relates to cytoskeletal regulation, EC sprouting angiogenesis and endothelial lumen formation have strong parallels with *Drosophila* tracheal cell specification and subcellular lumen formation.

11. Parallels between tracheal and neuronal subcellular branching

At a glance, neurons and tracheal cells are diverse and appear to develop differently. However, despite many differences in cell architecture and in the development of the organs they integrate, branching tracheal TCs and neurons share many common morphological features (Affolter and Caussinus, 2008). After embryonic development, neurons and TCs branch extensively, forming highly arborised structures at the end of larval development and growth (Fig. 1). In *Drosophila,* dendritic arborisation (da) neurons, a subset of multi-dendritic (md) neurons from the Peripheral Nervous System (PNS), are the most common model cells to study dendritic development and neuronal single-cell branching morphogenesis (Jan and Jan 2010; Lefebvre et al., 2015). The pattern of single-cell branching morphogenesis of tracheal TCs and da neurons shares common mechanisms in the formation of single-cell branched structures. Both cells form branched structures involving extensive cytoskeletal reorganization and directed membrane growth, increasing their cell surface area immensely. Nonetheless, the two systems differ in the way branches are stabilized from within: by a subcellular lumen and the cytoskeleton in TCs and by only the cytoskeleton in da neurons.

As in tracheal TCs, dynamic cytoskeletal regulation is involved in dendrite growth and branching and many of the involved players have been studied in recent years (Dong et al., 2015; Lüders, 2020) (Fig. 8). Actin filaments and MTs are the main components of the developing neuronal cytoskeleton during single-cell branching (Medina et al., 2006). In neurons, actin and MTs are involved in cell polarization and cell-shape determination and affect axon and dendrite growth and branching (Kapitein and Hoogenraad, 2015; Lüders, 2020). Neuronal branching starts when membrane protrusions that will give rise to neurites start to appear in the neuronal soma, breaking its round shape. These extending

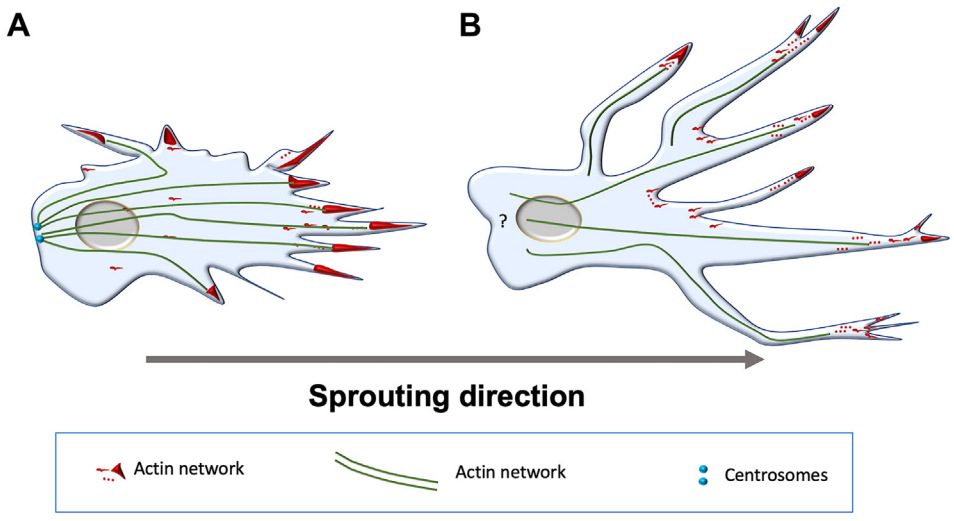

Fig. 7. Comparison of cytoskeleton organization of *Drosophila* TCs and activated HUVECs in a 3D matrix. Representation of TCs at early developmental stages (A) and early activated HUVECs in 3D matrix (B). Cytoplasm (light blue), actin network (red), MTs bundles (green). In both cases filopodia emanate indicating the direction of sprouting and MT bundles invade the cytoplasmatic protrusions. In TCs the MTOCs are composed of a pair of centrosomes (dark blue) in HUVECs the MTOCs are unknown.

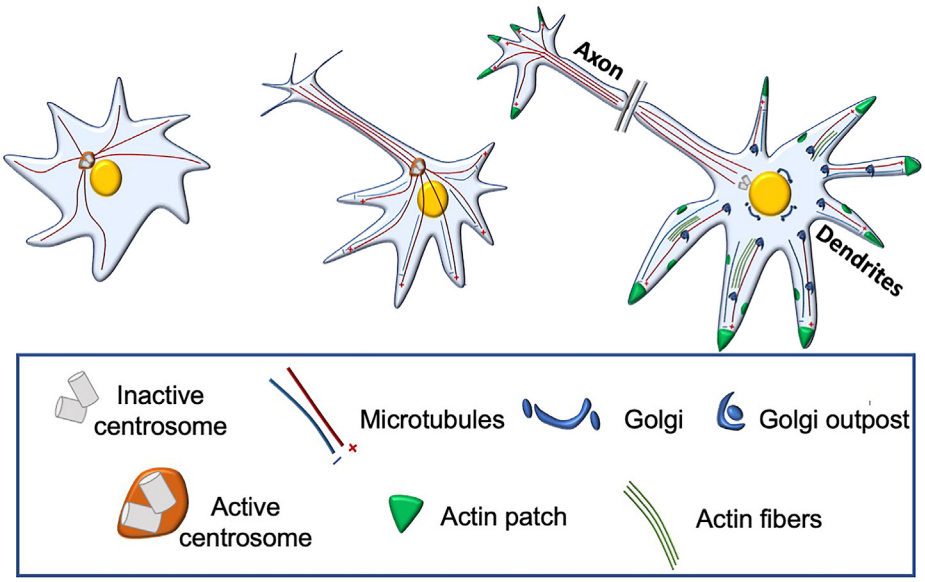

Fig. 8. Cytoskeletal organization during neurite morphogenesis. Schematic representation of neuron development and its cytoskeleton elements, the cytoplasm is in light blue, the nucleus is in yellow. Inactive centrosome is in grey, active centrosome is represented whit the pericentriolar material in orange, positive ended MTs are in red and negative ended MTs are in blue. Golgi outposts (dark blue) and actin patch and actin fibres in green. (A) At the beginning of neuronal morphogenesis, before polarization, positive ended MTs emanate through the soma, from the active centrosome. (B) At early stages of neuronal polarization, the axon is specified from the branch closest to the active centrosome. Dendrites develop with a mix MTs polarization. (C) At later stages centrosome is inactive and positive ended MTs emanates from Golgi outposts. The actin network is represented only in C.

neurites contain bundled MTs and an actin-rich growth cone. Nascent branches start with protrusions like filopodia and lamellipodia. This initial structure is stabilized through MT invasion and polymerisation, which leads to branch extension and maturation (Dogterom and Koenderink, 2019). MTs are uniformly plus-end out in axons, whereas dendrites possess minus-end-out MTs (Mattie et al., 2010; Stone et al., 2008). MT growth is directed at dendrite branch points through a mechanism dependent on an EB1-kinesin complex (Chen et al., 2014; Mattie et al., 2010).

In da neurons like in TCs, actin plays a central role in the formation and branching of dendrites. F-actin-rich patches accumulate in dendrites and premark the directed site of future dendritic filopodia emergence (Andersen et al., 2005) (Fig. 8). There are many different proteins involved in dendritic branching, which modulate actin nucleation and polymerisation, such as the polymerizing factor Ena and Twinstar (Tsr), the *Drosophila* homologue of cofilin. Ena, promotes the formation of both dendritic branches and actin-rich spine-like protrusions of da neurons, an effect opposite to that of Abl (Li et al., 2005). In neurites, Tsr/Cofilin binds to actin to induce its cleavage and, depending on its activity levels, induces increased actin polymerisation or depolymerization (Nithianandam and Chien, 2018). In *Drosophila* da neurons, downregulation of either Ena or Tsr results in lower levels of dendritic branching (Table 2) (Li et al., 2005; Nithianandam and Chien, 2018). Similarly, in tracheal TCs, Ena downregulation negatively affects TC extension and subcellular lumen formation (Gervais and Casanova, 2010) but *tsr* downregulation was not analysed in these cells (Skouloudaki et al., 2019).

The actin nucleator Actin-related protein (Arp2/3) accumulates in sites before dendrite branch formation was proposed to be the major actin nucleator, under the control of the activator WAVE and the small GTPase Rac1 (Stürner et al., 2019). As seen in TCs, F-actin distribution within the neuron is highly dynamic and heterogeneously distributed in dendritic shafts with enrichment at terminal dendrites (Nithianandam and Chien, 2018). These data suggest that actin localization and remodelling is important to guide subcellular branch formation in both systems. Accordingly, Moesin overexpression and knockdown leads to defects in dendritic arborisation and has reported to be involved in long-term memory formation (Freymuth and Fitzsimons, 2017).

As in TCs, neuronal actin and MTs need to interact to coordinate cellular movements and the structural support provided by these cytoskeletal components relies on the crosstalk between them. In *Drosophila* neurons, as in TCs, this is achieved by Shot, whose role has been widely investigated in neurons (Prokop, 2020; Voelzmann et al., 2017); *shot* mutations affect terminal branch formation of embryonic motoneurons and local sprouting of their dendrites in the central nervous system. Also, dendrites of sensory neurons in *shot* mutants are usually shorter, failing in their branching and arborisation (Bottenberg et al., 2009; Gao et al., 1999; Prokop et al., 1998).

Centrosome position in the neuron varies during development. During proliferation it is attached next to the nucleus through perinuclear MTs. In newly polarized neurons the centrosome is an active MTOC with a dynamic position in the cell, but over time, it gradually loses its activity. In mature neurons the MT cytoskeleton is not linked with the centrosome, showing both ends free (Fig. 8) (reviewed in (Lüders, 2020)). Not much is known about the influence of higher or lower numbers of centrosomes on dendritic branching. During dendritic arborisation, the centrosome has been shown to be important as a nucleator of other factors/complexes necessary for dendrite formation. One of them is the Cell division cycle 20-Anaphase Promoting Complex/Cyclosome (Cdc20-APC/C) complex, whose subcellular location at the centrosome was shown to be critical for its ability to drive dendrite development (Kim et al., 2009). In dendrites, other structures in addition to the centrosome can function as MTOCs. In *Drosophila* da neurons, acentrosomal MT nucleation was observed at Golgi fragments situated within dendrites and named “Golgi outposts” (Ori-Mckenney et al., 2012). Golgi outposts are specialized Golgi organelles first described in mammalian neurons, which are preferentially associated with branching points in dendrites, sharing their composition with centrosomes (Horton and Ehlers, 2003; Ori-Mckenney et al., 2012). However, removing Golgi outposts from dendrites did not prevent γ-tubulin MT nucleation at these dendrites arguing that γ-tubulin in dendrites is associated with some other internal structure (Nguyen et al., 2014). In a more recent study, an association of the γ-tubulin complex with somatic Golgi in da neurons has been shown, leading to the proposition that the somatic Golgi can also work as an MTOC in these neurons (Mukherjee et al., 2020).

Drosophila Lis-1 is highly enriched in the nervous system, where it cell-autonomously regulates the cytoskeleton and is required for dendritic growth, branching and maturation as seen for other dynein motor-protein genes (Liu et al., 2000; Satoh et al., 2008; Zheng et al., 2008). Together with similar phenotypes in TCs, these data highlight the importance of intracellular transport during single-cell branching.

Like in TCs, plasmatic membrane extension is required in developing neurons for neurite elongation and branching. This membrane delivery is achieved via the secretory/endocytic pathway, and is mediated through different mechanisms that occur in the neuron (Pfenninger, 2009). A

genetic screen in *Drosophila* found a group of genes entitled dendritic arborisation reduction (dar) genes, which affect dendritic morphogenesis, leading to a decrease in dendritic arborisation. Proteins encoded by *dar2* (*Sec23*), *dar3* (*Sar1*) and *dar6* (*Rab1*) regulate the endoplasmic reticulum (ER) to Golgi transport, and affect dendritic but not axonal growth (Ye et al., 2007). Early endosomes localize to dendrites and have been shown to control dendritic branching. Rab5 mutant neurons extend axons and major dendrites but they display a significant decrease in the number of dendritic terminals (Satoh et al., 2008). In addition, disruption of clathrin-mediated endocytosis reduces the number and length of dendrites (Yang et al., 2011) and *shrub* mutations, affecting the formation of MVBs, increase dendritic branching (Sweeney et al., 2006). Furthermore, vesicle tethering elements from the exocyst octameric complex are involved in neurite growth, as was shown by a decrease in dendritic branching in *Sec5* and *Sec6* mutant da neurons (Peng et al., 2015). Last but not least, the existence of Golgi outposts and their association with dendrite branching points, reinforces the importance of the secretory pathway in dendritic branching.

Overall, dynamic cytoskeletal regulation and consequent intracellular vesicle movement is essential for single-cell branching morphogenesis both in neurons and in TCs.

12. Concluding remarks

Single-cell branching morphogenesis is of foremost importance for the development and physiological homeostasis of organisms. Depending on the cell and tissue type, higher and lower levels of single cell branching are involved in different pathologies. Here, we presented three main models used to study single-cell branching, based on the parallels with the *Drosophila* tracheal TC single-cell branching morphogenesis. As analysed, there are unifying principles in the way single cells branch, from tracheal TCs to endothelial cells and neurons. There is a great deal we can learn by studying these mechanisms in parallel and many clues can be unravelled by analysing these systems on the light of these common principles.

So, while a lot is already known regarding cytoskeletal regulation during single-cell branching morphogenesis, many questions still remain open. We are yet not able to give a detailed answer to the important question of how a single cell can form long and stable branches from its cytoplasm or how it triggers branching from a specific point upon extracellular signalling. In addition, we are still in the dark on how, in lumen forming cells, the newly formed membrane is assembled in a tubular shape.

Most importantly, changes in single-cell branching have been observed during the aging process, but we still do not know how much of this is cause or consequence, or what are the cellular mechanisms involved. What are the consequences of changes in single-cell branching for the whole living organism? Some studies are beginning to arise, where consequences of changes in dendritic branching are studied in organismal behaviour (Freymuth and Fitzsimons, 2017). It will be important to know more about what changes in organismal behaviour can be triggered upon changes in neuronal, TC or EC branching.

Studies of neurons, TCs and endothelial cells can gain from comparison of single-cell branching mechanisms in each of them and from filling-in the gaps in one system with knowledge from the other. For example, it is known that single-cell branching in neurons and TCs may be driven by cell-autonomous and non-autonomous programmes (reviewed in (Dong et al., 2015)). Position-specific extracellular branching signals have been identified in *C. elegans* (Dong et al., 2013; Salzberg et al., 2013) and in tracheal TCs (Centanin et al., 2008; Jarecki et al., 1999), but evidence is not conclusive regarding da neurons or ECs (Hoyer et al., 2018; Palavalli et al., 2020).

Regarding the cytoskeleton, Cofilin has been shown to promote branching in neurons by facilitating MT protrusion, but not much is known about its role in TC or EC branching mechanisms. Alternatively, the spectraplakin Shot in *Drosophila* has been shown to be a key regulator in TC and neuronal branching, but it is still to be tested if MACF1 or DYS (the vertebrate homologues of Shot) can play the same role in ECs. Centrosomes have been shown to be involved in TC and EC branching, but evidence is not conclusive regarding da neurons in *Drosophila* (de Anda et al., 2005; Nguyen et al., 2011; Taylor et al., 2010). These and other factors could be easily tested and additional knowledge gathered in one system, could be used to gain novel insight in the other single-cell branching systems.

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