Centrosomes in branching morphogenesis

Sofia J. Araújo

Dept. of Genetics, Microbiology and Statistics, School of Biology, University of Barcelona, Spain

Abstract

The centrosome, a major microtubule organizer, has important functions in regulating the cytoskeleton as well as the position of cellular structures and orientation of cells within tissues. The centrosome serves as the main cytoskeleton-organizing centre in the cell and is the classical site of microtubule nucleation and anchoring. For these reasons, centrosomes play a very important role in morphogenesis, not just in the early stages of cell divisions but also in the later stages of organogenesis. Many organs, such as lung, kidney and blood vessels develop from epithelial tubes that branch into complex networks. Cells in the nervous system also form highly branched structures in order to build complex neuronal networks. During branching morphogenesis, cells have to rearrange within tissues though multicellular branching or through subcellular branching, also known as single-cell branching. For highly branched structures to be formed during embryonic development, the cytoskeleton needs to be extensively remodelled. The centrosome has been shown to play an important role during these events.

Keywords

Centrosome, branching, single-cell, tubulogenesis, neuron, axon, dendrite, trachea, endothelium, microtubule, vasculogenesis

1. Centrosomes in branching morphogenesis

The general role of centrosomes during embryonic development has mainly been studied in regard to cell division. However, it is becoming clear that subcellular events that rely on centrosome positioning, movement and activity are key to many

developmental processes (Tang and Marshall, 2012). From cell migration to force generation and both multicellular and subcellular branching, the role of the centrosome at the subcellular level can influence many morphogenetic processes. Branching morphogenesis has been extensively studied in many model organisms and distinct organs and despite our knowledge on many of the molecular players involved in branching, the centrosome's role in branching morphogenesis has only been reported clearly in a few of these systems.

2.The active role of the centrosome in tubulogenesis

2.1. The tracheal system

Tracheal system development has been broadly studied in the fruitfly *Drosophila melanogaster* and is an important model for the analysis of branching morphogenesis the developmental process that gives rise to many vertebrate organs including the lung, vascular system, kidney, and pancreas (Affolter and Caussinus, 2008, Manning and Krasnow, 1993, Ghabrial et al., 2003, Hayashi and Kondo, 2018).

During *Drosophila* embryogenesis, the tracheal primordial cells appear as 10 pairs of tracheal placodes, of about 80 cells each, that invaginate into the body cavity while maintaining epithelial integrity, and undergo stereotyped branching and fusion processes to form a continuous network of tubular epithelium providing all tissues with the necessary amount of oxygen (Fig. 1 A) (reviewed in (Hayashi and Kondo, 2018)). Interestingly, embryonic tracheal development proceeds without the need for cell division, which makes it an excellent system to study branching morphogenesis in post-mitotic cells.

A key process in branching tubulogenesis is the invagination of the epithelial placodes, which converts flat cellular sheets into three-dimensional structures and positions some of the cells of these sheets below the surface on which they were originally positioned. Tracheal invagination starts by the apical constriction in a small group of cells at the centre of the placode. These cells begin the internalization events which are then

followed by distinct rearrangements of the adjacent cells in the dorsal and ventral part of this placode. This process is regulated by the activity of the Trachealess (Trh) transcription factor and Epidermal Growth Factor Receptor (EGFR) signalling (Nishimura et al., 2007, Ogura et al., 2018). In invaginating placodes at stage 11 of *Drosophila* embryogenesis, the nucleus is basally located, the apical domain faces the future lumen, and centrosomes localize within a subapical domain (Brodu et al., 2010). Here, an apical array of short microtubules (MTs), organized into a meshwork, forms a cap-like structure at the apical domain whereas long MT fibres are distributed along the basolateral cell domain. As Microtubule Organizing Centres (MTOCs), these centrosomes colocalize with gamma-tubulin at this stage. However, this colocalization changes as invagination progresses and by embryonic stage 13, MTOCs relocate, from the centrosome to the apical membrane, by a two-step process controlled by the transcription factor Trh. The first step involves a Spastin-mediated microtubule release from the centrosome, followed by Pio-pio (Pio) facilitated anchoring of microtubules to the apical plasma membrane (Brodu et al., 2010). This dynamic centrosomal two-step mechanism, controls MT-network reorganization during *in vivo* development and these changes are essential for tracheal branching morphogenesis (Brodu et al., 2010). However, despite these studies of the centrosomal involvement during invagination and preliminary lumen formation stages, centrosome localization and dynamics has not yet been studied during cell migration stages that occur after placode invagination. During these stages, the main chemoattractant responsible for cell migration is the FGF homologue Branchless (Bnl) (Sutherland et al., 1996). Bnl activates the FGF receptor (FGFR) Breathless (Btl) on tracheal tip-cells, which leads to the concerted cell migration towards the Bnl source (Klämbt et al., 1992, Lee et al., 1996). These tip-cells actively migrate, extending filopodia towards the chemoattractant until they reach their targets.

Centrosome position has been shown to be important for many cell migratory events (Barker et al., 2016). In mammalian cell *in vitro* systems, the involvement of the centrosome in cell movement has been analysed and in some cases it has been shown

that centrosome amplification is able to increase the activity of Rho GTPase Rac1 in the cell and promote migratory invasive events (Godinho et al., 2014). In other cases, excess centrosomes perturb the cytoskeleton resulting in reduced migration (Kushner et al., 2016). Thus, it will be interesting to know if centrosomes are also involved in the tracheal migratory steps that lead to both multicellular and single-cell branching events.

In *Drosophila*, after most of the tracheal migration is accomplished, specific cells at strategic positions within the tracheal network differentiate into different cell types (Fig. 1 B). Of these, two very special cell-types in the *Drosophila* tracheal system are able to form lumina within the cytoplasm of one cell. These are the tip or terminal-cells (TCs) that form a luminal space inside their cytoplasm as they elongate (Fig. 1 B); and the fusion cells (FCs) that mediate the fusion between anastomosing branches of this complex system in order to assure network continuity (Sigurbjörnsdóttir et al., 2014). These two cell types form a type a lumen called subcellular due to its “intracellular” characteristics, which arises by *de novo* growth of an apical membrane toward the inside of the cell (Sigurbjörnsdóttir et al., 2014). Tracheal TC lumen formation depends on cytoplasmic extension, asymmetric actin accumulation, and vesicle trafficking guided by the cytoskeleton (Gervais and Casanova, 2010, Schottenfeld-Roames and Ghabrial, 2012, Schottenfeld-Roames et al., 2014). The beginning of TC subcellular lumen formation relies on centrosomes that localize to the apical junction between the terminal and the stalk-cell. From this centrosome pair, microtubules are organized in the direction of cell elongation and form two tracks that are thought to facilitate membrane delivery and concomitant lumen formation (Fig. 1 C) (Ricolo et al., 2016). Subsequent lumen extension requires microtubule polymerization and asymmetric actin accumulation at the basolateral tip of the terminal cell (Gervais and Casanova, 2010). An increase in centrosome number is able to induce the growth of additional lumina inside the terminal cell and centrosome loss impairs terminal tubulogenesis (Ricolo et al., 2016). So, at embryonic stages, the centrosome plays an active role in TC subcellular lumen formation, the first branching step of the TC. In fusion cell anastomosis and subcellular

lumen formation the participation of the centrosome has not yet been analysed. It is interesting to note that tracheal TCs at subcellular lumen initiation stages display a pair of centrosomes per TC as visualized by distinct centrosomal markers (Ricolo et al., 2016).

At larval stages, tracheal TCs ramify extensively to form many new terminal branches, long cytoplasmic extensions that grow toward oxygen-starved cells and then form a cytoplasmic, membrane-bound lumen, creating tiny tubes inside one cell in an intricate single-cell branching process. The localisation of cytoskeletal markers in larval TCs suggests that the overall principles of growth that start at embryonic stages are maintained at larval stages (Sigurbjörnsdóttir et al., 2014). Acetylated MTs line the gas-filled tube and extend beyond it or branch off it into filopodia (Jayanandanan et al., 2014). The gamma-tubulin remains restricted to the apical membrane after lumen formation, suggesting that MT orientation remains the same as during lumen elongation (Gervais and Casanova, 2010). And End-binding protein 1 (EB1), a marker for polymerising MT tips, shows growth of MTs throughout branches (Schottenfeld-Roames and Ghabrial, 2012). However, the debate is still open in regard to both the positioning and possible participation of centrosomes at this stage.

2.2. Vertebrate vasculature

Like the fruitfly tracheal system, the vertebrate vasculature is a highly ramified branched organ (Fig. 2) (Kotini et al., 2018). The vasculature, ensures the proper transport and distribution of relevant molecules to every organ. The initial vessels are generated during embryonic development by a process called “vasculogenesis” and these serve as the substrate for the formation of most of the fine, branched vascular networks that are formed later. Like the tracheal system, growth of new vessels requires directional migration, cell rearrangements, and cell shape changes, and it is mostly stereotyped in the early developing embryo. Unlike the tracheal system, vascular system branching

requires endothelial cell (EC) division. After vasculogenesis, new branches arise through a process called “angiogenesis”. Angiogenesis is responsible for the addition of new vessels to the already formed vascular system in response to the physiological needs of growing tissues and organs. There are two modes of angiogenesis called “sprouting angiogenesis” (SA) and “intussusceptive angiogenesis” (IA) (Makanya et al., 2009). SA involves the formation of tip cells that form numerous filopodial extensions and explore the environment to react to several positive and negative guidance cues; IA implicates the insertion of new branching points within the tubular branches of the network or the splitting of existing branches into finer ones. Both processes require considerable changes in cell shape and fast cytoskeletal reorganization (Ochoa-Espinosa and Affolter, 2012).

There are many differences and as many similarities between cells of the vertebrate vascular system and the invertebrate tracheal system. Animal blood circulation does not contain tree-like blunt-ended branches such as those present in insect tracheal systems, because branching capillaries need to anastomose with each other in order to ensure full blood circulation. However, parallels can be drawn regarding branching mechanisms due to equal needs for extensive cytoskeletal remodelling during branching events.

As in tracheal cells, endothelial branching requires cell movement. In many cases, centrosomes orient and sustain migratory polarity via nucleation of MTs and centrosome reorientation relative to the nucleus is believed to be required for proper migration (Tang and Marshall, 2012). In cell culture, ECs establish a centrosome-forward orientation, with the centrosome in front of the nucleus relative to migration direction (Luxton and Gundersen, 2011).

Furthermore, 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). However, non-centrosomal MTs have also been reported during sprouting angiogenesis (Martin et al., 2018).

Another vascular branching mechanism where centrosomes have been reported to play an active role is in oriented cell divisions (Tang and Marshall, 2012). As such, orientation of spindles along the long axis of developing tubes is seen in blood vessel development (Zeng et al., 2007) and EC cell divisions occur in coordination with tubular architecture avoiding disruptions in the tubular network (Aydogan et al., 2015). Hence, centrosome positioning during cell division is important for vascular architecture.

Notably, as in the tracheal system, the centrosome is important for EC lumen formation as shown by 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 and lumen initiation between adjacent cells (Rodríguez-Fraticelli et al., 2012). However, it is as yet unclear what happens to the EC lumen in conditions where supernumerary centrosomes are present in the EC cytoplasm as may happen during cancer progression.

3. Centrosomes in axonal growth specification

The extraordinary morphological transformations neurons undergo as they migrate, extend axons and dendrites and establish synaptic connections, imply a precisely regulated process of structural reorganization and dynamic remodelling of the cytoskeleton (Fig. 3 A). Mutations in genes encoding centrosomal proteins cause severe neurodevelopmental disorders (NDDs) (Bonini et al., 2017) leading to several diseases, such as lissencephaly, microcephaly and schizophrenia and centrosomal proteins have been shown to be involved in neurodegeneration (Diaz-Corrales et al., 2005, Madero-Perez et al., 2018). For this reason, unravelling the importance of centrosomal participation in axonal growth and branching is of foremost importance.

Many studies on the neuronal centrosome have focused on neuronal migration, a developmental phase many neurons undertake prior to axonal and dendritic development (Kuijpers and Hoogenraad, 2011). In migrating neurons, most microtubules

are attached to the centrosome, an interaction necessary for pulling along the cell body and nucleus as the neuron moves to its final destination (Higginbotham and Gleeson, 2007). However, what happens to this centrosome during branching, once the neuron finishes migration and starts growing an axon and dendrites?

Neurons are highly polarized cells that generally extend a single thin, long axon which transmits signals, and multiple shorter dendrites, which are specialized in receiving signals. Both axon and dendrites are rich in microtubules (Baas et al., 2016). The microtubule arrays within these processes are essential for providing architectural support, for enabling axons and dendrites to take on different shapes and branching patterns, and allowing for organelle distribution within the whole differentiated cell (Conde and Cáceres, 2009). They are indispensable for the complex neuronal architecture. However, most of the highly organized microtubules in axons and dendrites are not attached to the centrosome or any recognizable organizing structure. Instead, the microtubules are free at both ends, and take on various lengths within the axon and dendrites (Baas et al., 2016). In both vertebrate and invertebrate neurons, axons have a uniform arrangement of microtubules with plus ends distal to the cell body (plus-end-out), and dendrites have equal numbers of plus- and minus-end-out microtubules (Conde and Cáceres, 2009, Kapitein and Hoogenraad, 2015).

Initial research on centrosomes and their role as MTOCs in neurons resulted in the proposal that the neuronal centrosome acts as the initiator of microtubules for both the axon and dendrites (Ahmad and Baas, 1995, Ahmad et al., 1998). After this pioneer role of the centrosome, a consequence of earlier developmental stages, MTs are released and transported into the axon and dendrites by molecular motor proteins. Several observations have, since then, associated the centrosome with the primary site of axonal extension.

Cultured cerebellar granule neurons, after they stop migrating, grow a single axon, followed by a second axon to attain a bipolar morphology. Afterwards, they extend several short dendrites from the cell body. In these neurons, the centrosome is first

positioned near where the initial axon develops and then moves to where the secondary axon develops, suggesting that the position of the centrosome is related to the development of each of the two axonal extensions (Zmuda and Rivas, 1998).

It was reported that in cultured hippocampal neurons, the axon consistently arose from the first immature neurite forming after the final mitotic division of the neuroblast, and that the Golgi and endosomes (which generally cluster together with the centrosome) clustered in the location where the first neurite formed (de Anda et al., 2005). These observations are consistent with a previous report on cerebellar granule neurons described by Zmuda and Rivas (Zmuda and Rivas, 1998). Interestingly, these authors also found that light inactivation of the centrosome prevented normal polarization of *Drosophila* neurons (de Anda et al., 2005). Moreover, they observed that a small number of hippocampal neurons had two centrosomes and such neurons consistently formed two axons. It is interesting that most neurons normally have a single centrosome and according to the “one-neurite-only” growth signal, the centrosome may be the originator of this signal (Craig and Banker, 1994, de Anda et al., 2005). Thus, it has been proposed that the singularity of the axon and the singularity of the centrosome are somehow related, with cells with one centrosome extending one axon and cells with 2 centrosomes extending two. Nonetheless, in neurons such as cerebellar granule neurons, one centrosome is also capable of inducing the sprouting of 2 axons by changing its position (Zmuda and Rivas, 1998). Centrosomal movement was also shown to be important for neurite formation *in vivo*. Using live imaging of zebrafish Rohon-Beard (RB) sensory neurons, a spatiotemporal relationship between centrosome position and the formation of RB axons was established (Fig. 3 B) (Andersen and Halloran, 2012).

However, conflicting evidence correlates the role of the centrosome as an active MTOC with inhibition of neurite outgrowth. Centlein, a protein required for centrosome cohesion, is also a microtubule associated protein (MAP) exerting its function by stabilizing microtubules. It was found that overexpression of centlein inhibited neurite outgrowth, associating centlein and the centrosome as negative regulators of neurite formation (Jing

et al., 2016). Furthermore, many non-centrosomal MT networks have been associated with axonal extension (Stiess et al., 2010, Conde and Cáceres, 2009, Sanchez-Huertas et al., 2016, Nguyen et al., 2011).

Taken together, these many observations indicate that there is no ‘‘one size fits all’’ scenario for the location of the neuronal centrosome or its function as the main MTOC during axonal specification. Even so, it would certainly appear that the centrosome is an important structure in the neuron, for axonal formation, at least during the early stages of development.

4 Centrosomes and dendritic arborisation

Many of the most fundamental differences between axons and dendrites directly or indirectly result from distinct patterns of microtubule orientation in each type of process. In the axon, nearly all of the microtubules are oriented with their plus ends distal to the cell body, whereas in the dendrite, the microtubules have a mixed pattern of orientation (Delandre et al., 2016). In addition, unlike the case with the axon, dendrites are almost always multiple in numbers (Fig. 3 A), and it would be hard to visualize a centrosome that could be so mobile in the cell body as to move from dendrite to dendrite and then back to the axon to serve each neurite one at a time. Interestingly, it was reported several years ago what appears to be streams of microtubules flowing from the centrosome into developing dendrites of cultured hippocampal neurons, with a location roughly centralized among the dendrites (Sharp et al., 1995). So, we could envisage that the centrosome may act as the main MTOC and then MT fragmentation could lead to the varied pattern of MT polarity within dendrites. Microtubule fragmentation occurs during dendritic specification and growth as was shown from the genetic disruption of MT severing enzymes such as Spastin or Katanin, all necessary for correct dendritic patterning (Yu et al., 2008). Nonetheless, localization of gamma-tubulin to axons and dendrites, as well as the close tie between microtubule polarity and gamma-tubulin

activity, argues that many microtubules in mature neurons are likely to be generated locally at the dendrite rather than transported from the cell body (Nguyen et al., 2014).

The centrosome importance in dendritic arborization has also been shown 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).

Other structures in addition to the centrosome can function as MTOCs. Membranous organelles such as the nuclear envelope, endosomes, mitochondria and Golgi can also act as nucleation sites as has been shown for many cell types (Petry and Vale, 2015). In addition, the Golgi apparatus tends to cluster at the centrosome, because membranous elements that comprise the Golgi are transported by cytoplasmic dynein toward minus ends of microtubules (Corthesy-Theulaz et al., 1992). So, many of the molecular components used by centrosomes to organize MT nucleation can also be found at the Golgi.

In *Drosophila* sensory neurons, MT nucleation was observed at Golgi fragments situated within dendrites and called Golgi outposts that also share composition with centrosomes (Ori-Mckenney et al., 2012). In *Drosophila*, Centrosomin (Cnn) and Pericentrin-like protein (Plp) link MT nucleation to dendritic Golgi outposts (Yalgin et al., 2015, Ori-Mckenney et al., 2012), and both can also be found at the Golgi (Sanders and Kaverina, 2015). However, removing Golgi outposts from dendrites did not prevent gamma-tubulin MT nucleation at these dendrites arguing that gamma-tubulin in dendrites is associated with some other internal structure (Nguyen et al., 2014). Furthermore, MTs can nucleate from other MTs, an event mediated by the Augmin complex and this has been shown to control neuronal microtubule polarity (Sanchez-Huertas and Luders, 2015, Sanchez-Huertas et al., 2016).

Again, as it seems, a “one-model-fits-all” appears not to exist regarding MT nucleation in dendrites. Dendritic arbours are very diverse and this diversity may be controlled by distinct ways of MT polymerization and branching.

4. Other branching organs

Centrosomes are also important for the development of branching structures in other organs. In kidney tubules, similarly to the vascular system, cells primarily divide along the proximal–distal (longitudinal) axis of the epithelium, leading to lengthening of the tubule, while maintaining a constant diameter. In mouse and rat models for polycystic kidney disease, a ciliopathy, the orientation of tubule epithelial cell divisions is randomized, leading to increased tubular diameter and subsequent cysts showing that the control of the orientation of cell division is crucial (Fischer et al., 2006). In mouse knockout models and in human renal tissue from polycystic kidney disease patients *in vivo*, supernumerary centrosomes were found in cells (Battini et al., 2008). The presence of supernumerary centrosomes was detected in normal tubular cells in these mutant conditions, suggesting that centrosome amplification is an early event that precedes cyst formation (Battini et al., 2008). In addition, centrosome amplification was sufficient to induce rapid cystogenesis both during development and after ischemic renal injury (Dionne et al., 2018). Thus, despite centrosome amplification not having been shown as the direct cause of these renal pathologies, it is likely that changes in centrosome number affect the orientation of tubular cell divisions and induce defects in cilia, leading to the appearance of cysts.

Longitudinally oriented cell divisions also occur in the developing lung, and using a mathematical model, it was recently demonstrated that a change in airway shape can be explained entirely on the basis of the distribution of spindle angles, without requiring oriented changes in other cellular processes, such as proliferation or cell shape (Tang et al., 2011). There are, of course, other cases, such as in mouse lung development, where centrosome dynamics directly affects branching by affecting cell proliferation

(Schnatwinkel and Niswander, 2012).

5. Conclusions

It has been shown in various types of cells that centrosomal localization is important for branching morphogenesis. The location of the centrosome may be functionally important not just as a MTOC but also as a provider of factors necessary for cytoskeletal rearrangements. Furthermore, the centrosome may be responsible for the localization of Golgi outposts or the transport of Golgi-derived vesicles. Another possibility is that the centrosome is important to gather together various proteins that form the pericentriolar material (PCM). For example, the PCM is rich in kinases (Hames et al., 2005), and hence the centrosome could act as a processing centre to phosphorylate functionally important proteins. Alternatively, the PCM might act as a sink for various proteins that would otherwise be widely distributed in the cytoplasm or as a hub for different protein machinery such as the proteasome (Avidor-Reiss and Gopalakrishnan, 2013, Puram et al., 2013).

Thus, in branching morphogenesis, the centrosome is important developmentally, and may also be required in more mature differentiated cells in which the centrosome appears to have become vestigial. Perhaps under certain circumstances, the centrosome is reactivated to enable the cell to meet a particular challenge, such as breaking its symmetry or changing its branching pattern in response to disease or during regeneration.

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Figure legends

Fig. 1. *Drosophila* tracheal system. A) The tracheal system of an embryonic stage 16 Drosophila embryo, with all tracheal cells labelled in green (UASGFP expression under the control of btlGAL4) and tracheal lumen in red by Whest Germ Aglutinin (WGA). B) Detail of the ganglionic branch terminal cells (TCs) marked with an antibody against Drosophila Serum Response Factor (DSRF) (blue), tracheal cells in green and lumen in

red as in A. C) Schematic representation of the involvement of centrosomes (green dots) in subcellular lumen (red) formation: two centrosomes localize apically to the junction of the TC with the stalk cell. There, they are active MTOCs and provide the cytoskeletal structure necessary to start forming the ingrowing subcellular lumen. The MTs that emanate from this centrosome-pair grow towards the basolateral membrane (tip) of the TC, forming two tracks through which membrane can be delivered and the new lumen built.

Fig. 2. **Vertebrate vasculature**. The vascular system of a four-day-old zebrafish embryo vascular system expressing EGFP under the control of the endothelial specific promoter of the *flk1* gene (TG:flk1:EGFP).

Fig. 3. Neuronal branching. A) *Drosophila* third instar larval c4da neuron with long, branched dendrites; this branching pattern has not been shown to depend on centrosomes, at least in some of these neurons. B) Schematic representation of a zebrafish Rohon-Beard (RB) sensory neuron, showing centrosome localization during axonal extension (adapted from (Andersen and Halloran, 2012)).