Title: Non-canonical biogenesis of centrioles and basal bodies

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Abstract

Centrioles and basal bodies (CBBs) organize centrosomes and cilia within eukaryotic cells. These organelles are composed of microtubules and hundreds of proteins performing multiple functions such as signalling, cytoskeleton remodelling and cell motility. The CBB is present in all branches of the eukaryotic tree of life and, despite its ultrastructural and protein conservation, there is diversity in its function, occurrence (i.e. presence/absence) and modes of biogenesis across species.

In this review, we provide an overview of the multiple pathways through which CBBs are formed in nature, with a special focus on the less discussed, non-canonical ways. Despite the differences among each mechanism here presented, we highlighted some of their common principles. These principles, governing different steps of biogenesis, ensure that CBBs may perform their multitude of functions in a huge diversity of organisms but yet retained their robustness in structure throughout evolution.

Centrioles and Basal Bodies (CBBs) are microtubule-based structures that assemble centrosomes and cilia. The centrosome is the dominant microtubule organizing centre (MTOC) in most animal cells, thereby regulating intracellular transport and spindle pole formation, and establishing cellular polarity and migration. Each centrosome is composed of two cylindrical centrioles, often nine-fold symmetric, surrounded by dynamic pericentriolar material (PCM). The PCM is responsible for anchoring and nucleating microtubules. Centrioles, then called basal bodies, can also anchor to the cell membrane and template the growth of motile and immotile cilia. In animals, most cell-types form only one cilium (the primary cilium) but others can form hundreds (multiciliogenesis). These organelles are required for both cell and flow motility and sensing environmental cues.

It is essential that a cell regulates CBBs biogenesis to ensure they assemble at the right place, time and number. Failure in regulating this process can lead to cellular defects and diseases. If cells possess more than two centrosomes at mitotic onset, they may assemble multipolar spindles and segregate the genome unevenly. This leads to aneuploidy, genomic instability and cancer (Peel et al. 2007; Ganem et al. 2009; Silkworth et al. 2009; Godinho and Pellman 2014; Levine et al. 2017). Similarly, problems in cilia assembly cause a plethora of ciliopathies (Badano et al. 2006). In some cases, those may arise directly from basal body defects, for e.g., some mutations causing Bardet-Biedl Syndrome (Ansley et al. 2003).

CBBs are well conserved structures present across the eukaryotic tree of life and probably derived from a basal body-like organelle already present in the last eukaryotic ancestor (LECA) (Cavalier-Smith 2002; Hodges et al. 2010). They have been lost within plant, fungi and amoebae lineages or reduced to some particular tissues or life-cycle stages in other groups, acquiring new morphologies and modes of biogenesis.

CCBs can assemble by several pathways; the best characterized one is centriole duplication (Loncarek and Bettencourt-Dias 2018). This, hereafter called canonical pathway,

occurs through the formation of two daughter centrioles close to pre-existing ones. In mitosis, one centrosome is segregated to each daughter cell, ensuring that cells maintain a correct centriole number when they proliferate. Canonical biogenesis is always coupled to the cell cycle, ensuring that CBBs only form once. On the other hand, centriole biogenesis can occur through non-canonical pathways. Less is known in terms of their regulation and origin, though they are widespread in nature.

In this Review we describe the diverse pathways through which CBBs are formed. We focus mostly on the non-canonical strategies, which have been less explored in the literature, and that we differentiated into two categories: deuterosome-mediated biogenesis, when centrioles form in bulk in the presence of pre-existing centrioles, and *de novo*, strictly referring to biogenesis without any previously existing centrioles in the cell/organism. We highlight the similarities and differences between these pathways and discuss both their evolution and underlying molecular and cellular mechanisms.

Pathways of biogenesis

1. The canonical pathway (Centriole duplication)

In cycling cells, centrioles assemble in G1 to S transition, forming one daughter centriole orthogonally to each mother. The daughter centrioles elongate and, in late G2, undergo centriole-to-centrosome conversion losing the cartwheel (in some species) and recruiting PCM (Fu et al. 2016). Then, the two centrosomes migrate towards opposite poles of the cell organizing the mitotic spindle. After mitosis, each daughter cell inherits exactly one pair of centrioles (Fig. 1).

Although we are not yet aware of all the details governing this process and preventing re-duplication, the molecular pathways involved in triggering and coupling centriole duplication to the cell cycle have been extensively studied in recent years (Matsumoto et al. 1999; Meraldi et al. 1999; Harrison et al. 2011; Zitouni et al. 2016). Such mechanisms are not detailed here but they have been covered by numerous reviews (Loncarek and Bettencourt-Dias 2018; Nigg and Holland 2018).

2. Deuterosome-mediated Biogenesis

Post-mitotic cells containing two resident centrioles can differentiate into multicilated cells (MCCs), assembling CBBs in large-scale through the deuterosome-mediated pathway (Meunier and Azimzadeh 2016) (Fig. 2). Many multiciliated vertebrate tissues - the respiratory tract, the oviduct, skin, efferent ducts and the brain ependymal – are composed of MCCs. These cells produce fluid flow and particle movement, through the coordinated beating of their motile cilia. We hereby describe multiciliogenesis in vertebrate MCCs, whose molecular aspects have been characterized in recent years, showing that deuterosome-mediated and canonical biogenesis share part of their molecular cascade (Vladar and Stearns 2007; Azimzadeh et al.

2012; Klos Dehring et al. 2013; Zhao et al. 2013; Mori et al. 2017). We also speculate that a similar mechanism might contribute to the formation of multiciliated sperm in some invertebrates, such as in molluscs (*C. malleata* [Gall 1961] and *P. ebeninus* [Healy and Jamieson 1981]) and the insect *M. termites* (Baccetyi and Dallai 1978; Riparbelli et al. 2009).

In primary ciliogenesis, a single cilium derives directly from a CBB formed canonically whereas in multiciliogenesis, hundreds of basal bodies are generated which nucleate hundreds of cilia. Centriole biogenesis in MCCs does not rely only on the association with pre-existing centrioles but instead, depends of additional specialized structures (deuterosomes) to efficiently assemble a large number of CBBs. Electron microscopy (EM) studies described the formation of electron-dense granules (‘fibrogranular material’) in the cytosol; usually in the apical cell region and in the vicinity of resident centrioles, as the first morphological evidence of ciliogenesis (Fig. 2A and E)(Sorokin 1968; Steinman 1968; Kalnins and Porter 1969; Dirksen 1971; Hagiwara et al. 2004; Vladar and Stearns 2007) . Progressively, these granules increase in size and condense into large spherical bodies, the deuterosomes, which show no discernible structure and are extremely electron-dense (Fig. 2B, C and G); suggesting they consist of concentrated proteins. Frequently, numerous Golgi cisternae, small vesicles and microtubules were seen in the vicinity of deuterosomes (Fig. 2A and E)(Sorokin 1968; Kalnins and Porter 1969; Dirksen 1971; Vladar and Stearns 2007), suggesting these organelles might contribute to deuterosome formation and pro-centriole biogenesis. While Golgi and vesicles, together with microtubule activity can supply the deuterosome with precursors, pre-existing centrioles might contribute with activating enzymes catalysing biogenesis from the centriolar precursors. One such case, can be mediated by the activity of the Polo-like kinase 4 (Plk4), a master regulator and upstream player in centriole assembly (Bettencourt-Dias et al. 2005; Habedanck et al. 2005).

Several evenly spaced pro-centrioles assemble simultaneously from each deuterosome (Fig. 2B, C and G). In most tissues, pro-centrioles form both around the amorphous deuterosome

(acentriolar-mediated) (Fig. 2G) and the pre-existing centrioles (centriolar-mediated) (Fig. 2F) (Sorokin 1968; Anderson and Brenner 1971; Hagiwara et al. 2004; Al Jord et al. 2014). During ependymal MCC differentiation, deuterosomes arise from the wall of the (pre-existing) daughter centriole (Al Jord et al. 2014). Nonetheless, in all tissues, most of the centrioles (70-90%) are generated via deuterosomes rather than directly from centrosomal centrioles. The specific centriole amplification mechanism used by different MCCs, might then depend on the number of cilia they produce (Meunier and Azimzadeh 2016). Pro-centrioles separate from the clusters, mature and become typical basal bodies nucleating motile cilia.

Only recently, the molecular mechanisms driving deuterosome formation started to be understood. The multiciliogenesis program starts with downregulation of the Notch signaling pathway in MCCs precursors. Then, MCCs activate a cascade, mediated by the GemC1-Multicilin-E2f4/5 complex, triggering cell-cycle exit, cytoskeleton remodeling and upregulation of several centriole biogenesis components, including Cep152/Asl, Plk4, Cpap/Sas4, Sas6, Stil/Sas5 and centrin (Vladar and Stearns 2007; Hoh et al. 2012; Zhao et al. 2013; Mori et al. 2017; Arbi et al. 2017). These proteins are usually at very low abundance in cycling cells, hence limiting the number of centrioles that are formed. MCCs also express deuterosome-specific components; Deup1 (a paralog of Cep63) and Ccdc78, which localize to the centre of the deuterosome (Klos Dehring et al. 2013; Zhao et al. 2013). Deup1 binds Cep152/Asl, which then recruits Plk4, kick-starting the centriole biogenesis molecular cascade (Zhao et al. 2013; Al Jord et al. 2014; Mori et al. 2017). As MCCs start differentiating, E2f4 moves from the nucleus to the cytosol, where it interacts with Deup1 (Mori et al. 2017). Cep152/Asl, Plk4 and Centrin are subsequently enriched at the deuterosome and at the pre-existing centrioles, seeding the biogenesis of multiple CBBs. E2f4 has a dual role in the cell; first driving the transcription of centrosomal components and later participating in their assembly in the cytoplasm.

Knowing how the seeding of new procentrioles starts, remains unanswered how centriole amplification stops. Is there a feedback mechanism that terminates centriole amplification? Or does it simply result from exhaustion of centrosomal components?

3. *De novo*

Centrioles can assemble *de novo*, i.e. without centriolar structures present in the cell, in several species. However, in most naturally occurring cases (Fig. 6, Table S1), the mechanisms remain poorly understood. Centrioles may arise as single units (Fig. 3), as two centrioles coaxially oriented (Bicentriole, Fig. 4) or in electron-dense spheres (Blepharoplasts, Fig. 5) where the number of CBBs assembled varies (Miki-Noumura 1977; Riparbelli et al. 1998; Renzaglia and Garbary 2001).

Amoebae to flagellate transition in *Naegleria gruberi* is accompanied by the biogenesis of two centrioles. Since amoebae lack centrioles and microtubules, and so far no basal body precursor was found, it was proposed that centrioles assemble *de novo* (Dingle and Fulton 1966; Fulton and Dingle 1971). By studying the localization of centrin and γ-tubulin during the transition, Fritz-Laylin and colleagues (2016) have shown that only the first centriole assembles *de novo* while the second one appears to duplicate from the first. There is no EM support for the underlying pathway and despite some molecular insights from recent studies (Suh et al. 2002; Kim et al. 2005; Fritz-Laylin et al. 2010; Lee et al. 2015; Fritz-Laylin and Fulton 2016) the exact cascade is still unknown.

Other examples of *de novo* biogenesis of single centrioles take place in parthenogenetic insect eggs (in *Muscidifurax uniraptor* [Riparbelli et al. 1998] [Fig. 3], and *Drosophila mercatorum* [Riparbelli and Callaini 2003]) and artificially activated eggs of sea urchin (Dirksen 1961; Miki-Noumura 1977) and *Spisula solidissima* (Kuriyama et al. 1986; Palazzo et al. 1992) (Fig. 6; Table S1). As in most animals, centrioles are lost during oogenesis (Fig. 3A) and are delivered to the

egg by the sperm upon fertilization. In insect eggs, *w*hen development is triggered without fertilization, single centrioles are formed *de novo* and nucleate tubulin monoasters (Fig. 3B) (Miki-Noumura 1977; Palazzo et al. 1992; Riparbelli et al. 1998; Riparbelli and Callaini 2003). In activated hemynopteran eggs, multiple microtubule asters containing single centrioles are formed along the cortex (Fig. 3B). These migrate towards the centre of egg. Parthenogenetic development is initiated when two asters are captured by the female pronuclei forming the first mitotic spindle (Fig. 3C) (Riparbelli et al. 1998; Tram and Sullivan 2000).

The centriole in the mouse sperm is unable to nucleate microtubules after fertilization (Schatten et al. 1985; Gueth-Hallonet et al. 1993), so the first embryonic divisions are acentrosomal (Gueth-Hallonet et al. 1993; Courtois et al. 2012) and centrioles are only detected by EM from 64-cell stage onwards (Gueth-Hallonet et al. 1993). Throughout the first mitotic divisions, the spindles become progressively more focused and are enriched with PCM and centriolar components, such as centrin, pericentrin and CP110. Nevertheless, the trigger underlying centriole assembly is still unclear. A gradual concentration of PCM and centriolar components throughout the mitotic cycles, could allow crossing a molecular threshold that enables the formation of centrioles (Courtois et al. 2012).

Oocytes represent a very particular cell-type that is loaded with several centriolar components therefore, mechanisms blocking spontaneous centriole assembly could be present. Thought in most eggs centrioles do not assemble spontaneously, overexpression of Plk4 is enough to drive *de novo* formation of multiple centrioles (Peel et al. 2007; Rodrigues-Martins et al. 2007).

In most cases, centrioles assembled *de novo* seem to be able to replicate through the canonical pathway (Palazzo et al. 1992; Rodrigues-Martins et al. 2007; Fritz-Laylin et al. 2016). Therefore, in cases where several centrioles are observed, we cannot exclude that, some could result from duplication following *de novo* biogenesis. Moreover, in *Naegleria*, both CBBs form

cilia, highlighting that centrioles formed *de novo* and canonically are equally capable of nucleating cilia without the need of a full cell cycle to mature.

3.1 Bicentriole

De novo centriole biogenesis through bicentrioles is known to occur in plants with biflagellated sperm, such as bryophytes, as well as in the protist *Labyrinthula spp.* (Perkins 1970) (Fig. 6, Table S1). A bicentriole is composed of two centrioles oriented end-to-end, aligned along the same axis and connected by a continuous cartwheel hub, while the triplet microtubules between centrioles are discontinuous (Fig. 4C and F) (Moser and Kreitner 1970; Robbins 1984).

In land plants, two bicentrioles appear simultaneously in the sperm mother-cell. First, an electron-dense body without any recognizable structure is detected in the outer surface of the nucleus. Microtubules emanate from this structure, suggesting that it has MTOC activity (Fig. 4A). Next, it separates into two different lobes (pro-bicentrioles) with a lighter stained central core surrounded by a darker matrix (Fig. 4B) (Robbins 1984). Before mitosis, the two pro-bicentrioles separate, migrate towards the poles of the cell and mature into bicentrioles, assembling MT-triplets (Robbins 1984; Renzaglia and Duckett 1987). Each bicentriole at the spindle pole contains two coaxial centrioles (Fig. 4C and F) (Moser and Kreitner 1970; Robbins 1984).

Each spermatid inherits one bicentriole. The central hub breaks at its midpoint and the two resulting centrioles undergo planar rotation becoming almost parallel to each other, with their proximal ends facing the same direction (Fig. 4D) (Moser and Kreitner 1970; Kreitner and Carothers 1976; Robbins 1984). Centriole reorientation is accompanied by the development of the multi-layered structure (MLS), immediately bellow the centrioles (Fig. 4E and G). The MLS is composed of a bundle of parallel microtubule singlets – the spline (Fig. 4G - asterisk) - and by the lamellar strip (layers of electron-dense material) (Fig. 4G – arrowhead). The centrioles

anchor to the MLS and become basal bodies for ciliogenesis (Moser et al. 1977; Renzaglia and Duckett 1987).

There is no available molecular data on centriole assembly through bicentrioles, except that these structures appear to contain γ-tubulin (Shimamura et al. 2004)**.** The only study reporting the early stages (before bicentriole assembly) of *de novo* bicentriole assembly is from Robbins (1984) on spermatogenesis in the bryophyte *Riella americana*. Early land plants, such as *Marchantia polymorpha, Physcomitrella patens* and *Selaginella moellendorffii* are model organisms that assemble CBBs through the bicentriole pathway and therefore, could be used to better describe this pathway and understand its regulatory mechanisms.

3.2 Blepharoplast

In land plants with multiciliated sperm such as ferns, cycads and *Ginkgo* (Fig. 6, Table S1), CBBs are formed through blepharoplasts. The blepharoplast arises *de novo* as a spherical electron-dense organelle which is initially amorphous (Fig. 5A), and during maturation it becomes intercalated by lighter cylinders embedded in an electron-opaque matrix. These cylinders mature into centrioles that later give rise to the basal bodies of multiple cilia (Fig. 5) (Hepler 1976; Gifford and Larson 1980).

Blepharoplast biogenesis starts with the appearance of two hemispherical densely stained structures near the cell nucleus (Fig. 5B and F). Then, cylinders organize within the electron-dense matrix (Fig. 5G – arrowheads), with microtubules emanating from the blepharoplast. These structures grow and become spherical, giving rise to two blepharoplasts (Mizukami and Gall 1966; Hepler 1976; Hoffman and Vaughn 1995). The two blepharoplasts separate (Fig. 5G) and migrate to the spindle poles of the mitotic cell, where they appear to act as MTOC (Fig. 5C) (Hepler 1976; Gifford and Larson 1980; Doonan et al. 1986). In the metaphase-anaphase transition of the last mitosis, the blepharoplast becomes more diffuse and loses its MT-

nucleating ability. The cylinders acquire a nine-fold symmetry and a hub-and-spokes configuration, therefore resembling pro-centrioles. Each daughter cell inherits one blepharoplast (Norstog 1967; Gifford and Lin 1975; Hepler 1976). Sperm development proceeds as centrioles are formed (Fig. 5D and H) (Hepler 1976; Renzaglia and Maden 2000). The blepharoplast eventually collapses, resulting in individualized centrioles. The centrioles dock into the MLS and function as basal bodies nucleating axonemes (Fig. 5E) (Mizukami and Gall 1966; Doonan et al. 1986; Norstog 1986).

Molecular characterization of blepharoplast assembly is still scarce. However, a few studies have reported the localization of centrin, acetylated, tyrosinated and β-tubulins at the blepharoplast (Doonan et al. 1986; Klink and Wolniak 2001; Vaughn and Renzaglia 2006). Centrin's function was studied in *M. vestita*, where RNAi experiments highlighted its requirement for proper blepharoplast and centriole biogenesis (Klink and Wolniak 2001).

To this date, there is no evidence for centriole duplication in multiciliated plant cells. It appears that each CBB formed *de novo* only gives rise to one cilium (Mizukami and Gall 1966; Norstog 1967; Gifford and Lin 1975; Norstog 1986).

Mechanisms underlying CBBs assembly

Regulation of centriole number is still not fully understood. While in the canonical pathway regulation is partially achieved by coupling of the centriole and cell cycles, this cannot be the case in non-canonical pathways. It is possible that centriole number only depends on the amount of its building blocks and as centrioles are assembled, these are depleted. In this case regulation takes place at the levels of transcription and translation. Another strategy would be the activation of a negative feedback mechanism wherein, once the right amount of centrioles are assembled, any further biogenesis is inhibited. In that regard, even non-canonical pathways

show some centriole number regulation since several cell-types that form centrioles *de novo* consistently form a defined and similar amount.

In spite of the diversity of pathways, their outcome is the same: the generation of CBBs with a conserved ultrastructure and function. The mechanism used by each cell-type and organism to build it seems highly dependent on the number of CBBs they have to begin with and how many will be generated. While in the canonical pathway, a single daughter is generated per mother per cycle, in most non-canonical pathways that number regulation is seemingly loss, allowing a variable number of CBBs to assemble.

Nevertheless, canonical and non-canonical pathways share many striking similarities. Two centriolar proteins - Sas6 and centrin - and pericentriolar components γ-tubulin and pericentrin have been shown to be present in both canonical and non-canonical pathways in multiple species (Table 1). Sas6 is the most conserved centriolar protein and the major molecular component of the cartwheel, forming nine-fold symmetrical stacks at the core of the centriolar barrel, and being required for centriole and basal body assembly (Nakazawa et al. 2007; van Breugel et al. 2011; Kitagawa et al. 2011). In plants, centrin and γ-tubulin are enriched in the blepharoplast of *Ceratopteris richardii* (Hoffman et al. 1994) and functional studies demonstrated that centrin is needed to form the blepharoplast and therefore, the cilliary apparatus in *Marsilea vestita* sperm (Klink and Wolniak 2001). *De novo* CBB formation in *Naegleria gruberi* is preceded by the formation of a γ-tubulin, pericentrin and myosin II complex, at the site where Sas6 and centrin-positive centrioles assemble (Fritz-Laylin et al. 2010; Lee et al. 2015; Fritz-Laylin and Fulton 2016). In vertebrates, all of these previously mentioned components along with others, localize to centrioles generated *de novo* in mammalian culture cells (Khodjakov et al. 2002; La Terra et al. 2005; Uetake et al. 2007) and are upregulated in multiciliogenesis (Vladar and Stearns 2007; Klos Dehring et al. 2013; Zhao et al. 2013; Mori et al.

2017). Though the molecules are the same, differential regulation of their levels allows overcoming the canonical biogenesis regulation and assembling multiple CBBs.

The location where pro-centrioles assemble is determined by the site where its precursors concentrate, here called "concentrator". Even though the "concentrator" might be morphologically distinct in each centriolar or acentriolar pathways, components must first accumulate in a defined location in the cytosol to then seed the growth of CBBs. In the canonical pathway the mother centriole acts as a concentrator, whereas in the non-canonical pathways organisms evolved multiple structures where centriolar components are specifically enriched – the blepharoplast, the deuterosome and other electron-dense structures. This way, the concentrator regulates the location and number of CBBs assembled (Table 1).

The microtubule cytoskeleton helps transporting components to the concentrator (Table 1). CHO cells, upon centriolar removal and if treated with nocodazole, no longer form centrioles *de novo* (Khodjakov et al. 2002). Multiciliogenesis is accompanied by cytoskeleton remodelling that promotes assembly of stable cytoplasmic microtubules (more resistant to depolymerisation) (Vladar and Stearns 2007). Microtubule enrichment is also detected close to the fibrogranular material preceding deuterosome formation (Steinman 1968; Dirksen 1971) and microtubules grow from the blepharoplast, after depolymerization (Vaughn and Bowling 2008). Overall, multiple observations hint that microtubules are important for CBBs assembly, however it is still left to determine when exactly they are critical. Are they needed in the very early stages of precursor concentration? Or do they only facilitate recruitment once there is already a MTOC primordium? Some components might have evolved affinity for the MTs, naturally concentrating at the MTOCs and facilitating the process. Amongst those components, PCM proteins are known to be required to stabilize centrioles and allow efficient centriole duplication (Dammermann et al. 2004; Pimenta-Marques et al. 2016). Proteins like chTOG/XMAP215, members of the Tacc family, Cpap/Sas4 and γ-tubulin are important for PCM

assembly and microtubule organization and are widely present in eukaryotes (Dammermann et al. 2004; Peset and Vernos 2008; Hodges et al. 2010). PCM might help concentrating centriolar proteins, hence wherever PCM stably aggregates in the cytosol it can create a suitable environment for CBBs biogenesis (Table 1) (Varmark et al. 2007; Dzhindzhev et al. 2010).

Finally, self-assembly and catalytic activity of centrosomal components are important in driving CBBs biogenesis. In several animals, Plk4 is the main kinase triggering centriole biogenesis. PLK4 controls its own activation by *trans*-autophosphorylation, which results in a positive feedback loop dependent on Plk4 accumulation (Lopes et al. 2015). Self-assembling properties facilitate Sas6 oligomerization *in vitro* (Kitagawa et al. 2011). Together with Cep135/Bld10, these two *Chlamydomonas* proteins are able to assemble a cartwheel, the first step in building the centriolar core (Guichard et al. 2017). Recent studies have also shown that some centrosomal components spontaneously form condensates *in vitro*. Above a critical concentration, *C. elegans* Spd5 (a master PCM recruiter), forms a supramolecular scaffold where other PCM proteins can bind (Woodruff et al. 2017). Spd5 condensates enriched with chTOG and TPX2, are capable of concentrating α- and β-tubulin and organizing microtubule asters. Future work should dissect the role of self-assembling *in vivo.*

Evolutionary history of CBBs and their pathways

Numerous evidences support that CBBs are the same identity which was co-opted throughout evolution to perform different functions within the eukaryotic cell. Not only CBBs are ultrastructurally similar and co-occur across distinct *taxa*, but the same gene network, the core centriolar assembly, is conserved in the genome of ciliated species (Woodland and Fry 2008; Carvalho-Santos et al. 2010; Hodges et al. 2010). Indeed, CBBs are found in all 7 major eukaryotic lineages (Fig. 6, Table S1), suggesting they were already present in the LECA but apparently not before (Carvalho-Santos et al. 2010). The ancestral CBB was most likely a basal

body-like organelle composed of nine microtubule triplets arranged in a radially symmetrical cylinder (Beisson and Wright 2003), involved in the nucleation of motile cilia (Carvalho-Santos et al. 2011; Azimzadeh 2014). CBBs (and their gene repertoire) have been independently lost in several lineages and are frequently absent in: some plants (Archaeplastida), fungi (Opisthokonts) and amoebae (Amoebozoa) (Fig. 6) (Renzaglia and Garbary 2001; Woodland and Fry 2008; Carvalho-Santos et al. 2011; Judelson et al. 2012; Yubuki and Leander 2013).

Throughout evolution, the requirement for ciliary motility imposed a functional constrain on basal body architecture, since absence of cilia allowed for complete centriole loss and the generation of MTOCs with very distinct morphology like the Spindle Pole Body (SPB) of fungi and the Nuclear-Associated Body (NAB) of amoebae (Table S1) (Hodges et al. 2010; Azimzadeh 2014).

Though cilia are seemingly ancestral structures, centrosomes most probably are not. A good example is the animal centrosome, which is it is mostly composed by Holozan-specific components (Holozoa is an Opisthokont sub-division including animals and closely related organisms except fungi) (Hodges et al. 2010). Recently, Gouw et al. (M. Gouw, unpubl) employed maximum parsimony landscapes to assess the probability of the cilium and the centriole-based centrosome being ancestral in specific eukaryotic lineages. This analysis favoured a convergent evolution hypothesis for the origin of centriole-based centrosomes, suggesting that centrioles were co-opted as part of the centrosomes independently in different eukaryotic lineages. The acquisition of centrosomal functions might have occurred in a stepwise manner. First, by becoming part of the spindle poles, CBBs could segregate equally to daughter cells upon cell division. This could favour an enrichment in PCM, potentiating MTOC activity. Finally, the acquisition of cell cycle components (Lange 2002) would link centrosome biogenesis and segregation to cell cycle progression, allowing a much tighter regulation of its activity and copy-number in cells (Nigg and Holland 2018).

All pathways share components; a specific set of centriolar proteins - Sas6, Cpap/Sas4, Cep135/Bld10, Poc1, centrin – as well as α-, β- and γ-tubulin, is found in the genome of most eukaryotic species that assemble CBBs (Table 1) (Carvalho-Santos et al. 2010; Hodges et al. 2010). Functional studies and expression data are still scarce outside Opisthokonts, but are needed to validate the function of these components in each pathway.

Canonical duplication is the most prevalent pathway and probably, the ancestral one. It is present in every main branch of the eukaryotic tree, though the mechanism is somewhat different in specific taxa. In some oomcytes such as *S. ferax* and *P. infestans* (Stramenopiles) and in *Plasmodiophora* spp. (Rhizaria) (Fig. 6, Table S1), daughter centrioles assemble in a 180-degree angle from their mother (coaxial orientation), rather than the usual 90 degrees, forming a Bicentriole, similar to the one found in some plants (Heath and Greenwood 1970; Heath 1974a, 1974b; Garber and Aist 1979).

Similarly, to the centriole-based centrosomes, the deuterosomes, the bicentriole and blepharoplast are all evolutionary innovations, arising relatively recently in eukaryotic history (Fig. 6). A recent study argued that the deuterosome-mediated pathway is vertebrate-specific, arising just before tetrapode divergence. That is because Deup1, a specific component of the deuterosome and resulting from Cep63 duplication, is only found in the genomes of lobe-finned fish and tetrapods (Zhao et al. 2013). Some gastropodes (*C. malleatus* and *P. ebeninus*); annellides (*Tubifex spp.*) and the termite *M. darwiniensis* produce multicilliated sperm (Fig. 6, Table S1) (Gall 1961; Baccetyi and Dallai 1978; Healy and Jamieson 1981; Ferraguti et al. 2002; Riparbelli et al. 2009). In these naturally occurring cases, the sperm basal bodies might derive from a mechanism similar to the deuterosome. In all these studies, no typical deuterosomes were detected, only occasional clouds of electron-dense material containing microtubules.

Archaeplastida, the group including plants and some algae, suffered multiple events of centriole loss; both in basal groups (in some green algae and in red algae altogether), but also in

gymnosperms after the split of conifers and gnetales from cycads and ginkgophytes and once again before angiosperm evolution (Bremer et al. 1987; Finet et al. 2010). Within this vast group, *de novo* mechanisms are the most prevalent, based either on the bicentriole or the blepharoplast, since most plants lack CBBs throughout their life cycle except in sperm. The bicentriole appeared in land plants, it is present in most Marchantiophyta and Bryophyta, and in some species of Anthocerotophyta and Lycopodiophyta, but not in the basal species of Archaeplastida (reviewed in Renzaglia and Garbary 2001) (Fig. 6, Table S1). Interestingly, a bicentriole is also formed *de novo* in *Labyrinthula spp.*, a Stramenopila (Fig. 6, Table S1). It is possible that the blepharoplast from the Pteridophyta and some gimnosperms derived from the bicentriole. The blepharoplast is mechanistically very similar to the deuterosome, suggesting a scenario of convergent evolution. CBBs are required for species that form motile cilia and, somehow depend on a moist environment for fertilization. Gimnosperms (Pinaceae and Gnetales) and all angiosperms (Magnoliophyta) no longer use motile cilia, since fertilization takes place by means of a pollen tube with immotile sperm cells.

It also remains to be understood if, in all the species of Amoebozoa assembling CBBs *de novo* upon ameboid to flagellate transition (for e.g. *Physarum spp*.) the mechanisms resemble those found in animals (for e.g. in female eggs) or if these have evolved their own specific precursor and uncharacterized pathway. Fungi with CBBs seem to conserve the ancestral canonical pathway of biogenesis, but likely suffered more than one event of centriole loss (Fig 6).

Throughout the eukaryotic tree, there are several examples of convergent evolution where unrelated groups appear to share similar strategies to assemble CBBs. This suggests that the possibilities for how to make CBBs are somewhat limited, indicating some sort of morphological (perhaps even molecular) constraint inherent to the process.

Conclusions

In this review, we have discussed that non-canonical modes of CBBs assembly are widespread in the eukaryotic tree. Thought the pathways display some lineage-specificity there are several examples of convergent evolution, suggesting that when it comes to making centrioles, the options are limited and mostly governed by numbers.

Most descriptions of non-canonical assembly were done by EM in chemically fixed samples. However, new techniques are now available, such as High-Pressure Freezing followed by Automated Freeze Substitution (HPF+AFS) and Cryo-EM, which can improve the quality of the data and help to unravel the true representation of each step of these processes. Super resolution microscopy, in particular 3D-structured illumination microscopy, allows correlating different proteins within the organelles at much better resolution and, potentially, following *live* these biogenesis processes.

Molecular studies on non-canonical centriole biogenesis are scarce and focused on a few species (such as *Naegleria gruberi* and *Drosophila spp.*) and biased towards the deuterosome-mediated pathway in vertebrate multiciliated cells. One reason is the absence of tools to study other systems, which can now be overcome with CRISPR/Cas9 technology and the increasing availability of genomic data. More gene expression data and functional studies should expand our molecular knowledge outside the Opisthokonts, in order to understand what are the universal principles underlying centriole assembly as well as the specific properties inherent to each pathway.

Many of the core centriolar components and some regulators (Polo-like kinases, PCM components and MT regulators) appear to be conserved across evolution (Hodges et al. 2010; Carvalho-Santos et al. 2010, 2011), suggesting a common molecular cascade across all centriole assembly pathways. However, non-canonical centriole biogenesis appears more restrictive; in specific cell-types during differentiation (multiciliated cells in vertebrates – deuterosome-

mediated pathway) or life-cycle stages (*N. gruberi* and spermatogenesis in plants – *de novo* pathways), suggesting that centriole assembly must be under developmental regulation. In the future, it will be important to unravel how the multiple pathways operate in different organisms; how the PCM components, together with the MT network, create a suitable environment where unassembled centriolar precursors concentrate, forming a scaffold for centriole assembly. Only then we will fully understand CBBs function and its upstream and downstream molecular machinery.

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Figure Legends

Figure 1. Canonical Biogenesis in cycling cells. In early G1, cells have one centrosome with two centrioles (mother and daughter) orthogonally oriented. Before duplication, the two centrioles disengage (G1), losing their orthogonal configuration and both become mother centrioles (Robbins et al. 1968). From G1 to S transition, one pro-centriole forms orthogonally to each mother. The pro-centrioles elongate during the G2 phase and each centrosome starts recruiting PCM components (Robbins et al. 1968; Kuriyama and Borisy 1981). From G2 to mitosis, the two centrosomes separate and migrate towards opposite poles of the cell. Mitotic centrosomes

recruit more PCM allowing them to help to organize the mitotic spindle. Upon mitotic completion, each daughter inherits exactly one pair of centrioles. At the beginning of each G1 phase, the oldest centriole acquires both distal and sub-distal appendages (Kong et al. 2014). Pro-centriole assembly relies on the stepwise incorporation of conserved molecules (depicted in the inset). Cep152/Asl recruits Plk4, which phosphorylates downstream substrates, allowing the formation of the Sas6 and Cep135/Bld10 cartwheel, thus building the centriole core. Cep152/Asl also interacts with Cpap/Sas4, promoting incorporation of PCM components (Cizmecioglu et al. 2010; Dzhindzhev et al. 2010; Gopalakrishnan et al. 2011; Sonnen et al. 2013).

Figure 2. Deuterosome-mediated biogenesis in vertebrate multicilated cells (MCCs). Multiciliogenesis starts with the formation of electron-dense 'fibrogranular material' ((A) and depicted within the white square in the EM micrograph (E)) in the cytosol, close to pre-existing centrioles. This dense material is usually enriched with microtubules (MTs), Golgi cisternae and vesicles (A, E - arrowheads). The 'fibrogranular material' condenses and deuterosomes – electron-dense hollow spheres– are formed (B, G – arrows). A recent study in ependymal cells demonstrated that the resident daughter centriole is capable of generating multiple deuterosomes, which detach from its wall and give rise to many pro-centrioles (B, C and G) (Al Jord et al. 2014). Additionally, pro-centrioles assemble directly around the resident centrioles (C), as shown in the EM micrograph (F). Hundreds of CBBs are formed in the cytosol, which then migrate and dock to the cell membrane assembling hundreds of cilia (D). (E [x37000] and F [x50000]: Adapted, with permission, from Sorokin 1968, Journal of Cell Science, 3: 207-230; G [x96000]: Adapted, with permission, from Dirksen 1971, Journal of Cell Biology, J51(1):286-302 DOI: 10.1083/jcb.51.1.286).

Figure 3. *De novo* centriole biogenesis in parthenogenic insect eggs. Unfertilized eggs do not have centrioles but contain high levels of centriolar precursors (A). Upon egg activation and

meiotic resumption, centrioles are formed *de novo* along the cell cortex (B). These single centrioles nucleate MT asters. Meiosis is completed and the free centrosomes migrate towards the egg centre (C). Two asters interact with the female pro-nucleus, assembling the first mitotic division and triggering embryonic development (C – black rectangle). The remaining centrosomes degenerate (Riparbelli et al. 1998).

Figure 4. Bicentriole-mediated biogenesis in land plants with biciliated sperm. During spermatogenesis, electron-dense material enriched in microtubules (MTs) is found near the nuclear envelope (A). This material assembles into two light lobes, surrounded by a darker matrix (B). As mitosis begins, the two lobes separate and migrate towards the poles of the spindle and mature into bicentrioles (C). Bicentrioles are composed of two coaxial centrioles connected by their central hub and with discontinuous MT triplets (F – white arrow). Each daughter cell (spermatid) inherits one bicentriole that breaks in half and separates into? two centrioles (D) that will migrate to the edge of the cell and anchor to the multi-layered structure (MLS), serving as basal bodies during ciliogenesis (E and G). The MLS is composed of a bundle of parallel MTs – the spline (G – asterisk) – and layers of electron-dense material – the lamellar strip (G – arrowhead). (F [x50000] and G [x50000]: Adapted, with permission, from Moser and Kreitner 1970, Journal of Cell Biology, 44 (2): 454-458 DOI: 10.1083/jcb.44.2.454).

Figure 5. Blepharoplast-mediated biogenesis in land plants with multiciliated sperm. In plants with multiciliated sperm, an electron-dense agglomerate of material and microtubules (MTs) is first detected near the nuclear envelope of the sperm mother-cell (A). This material develops into two darker hemispherical lobes, intercalated by lighter cylinders (B, F and G - arrowheads). As the cell approaches mitosis, the lobes keep developing and separate (G). Each lobe migrates to a pole of the mitotic spindle and assembles a blepharoplast (C). Each spermatid inherits one blepharoplast, where many centrioles are assembled. The blepharoplast eventually collapses

releasing the individual centrioles (D and H) that will migrate and anchor into the MLS, giving rise to the basal bodies of the several cilia (E). (F [x37000] and G [x37000]: Adapted, with permission, from Hepler 1976, Journal of Cell Science, 21: 361-390; H [x21000]: Adapted, with permission, from Mizukami and Gall 1966, Journal of Cell Biology, 29 (1): 97-111 DOI:10.1083/jcb.29.1.97).

Figure 6. Consensus eukaryotic tree of life (selected groups; following Burki 2014 and Worden et al. 2015). The distinct centriole biogenesis pathways are represented in different colours. Canonical biogenesis (in black) is the most prevalent pathway and probably, the ancestral one. Deuterosomes (blue), the bicentriole (green) and blepharoplast (purple) are all evolutionary innovations, arising relatively recently in the eukaryotic history. Some pathways are more restricted to some groups, for e.g. the canonical and deuterosome pathways are predominant in vertebrates, while most plants assemble CBBs through a bicentriole or a blepharoplast. There are some striking *exceptions*, like the presence of a deuterosome-like mechanism in the sperm of some invertebrates. While in gastropods (Mollusca) the non-canonical pathway seems to be centriolar, where up to 20 CBBs assemble only around the existing centrioles, the sperm from annelids and *M. darwiniensis* (Arthropoda) possesses a very high number of CBBs, likely formed via both centriolar and acentriolar ways. Similarly, within the Class Parabasalia (Excavata) some protists undergo massive centriole amplification. It is proposed that biogenesis is driven by resident centrioles along a “ladder”-like configuration (Tamm and Tamm 1980). In all these studies, no typical deuterosomes were detected, only occasional clouds of electron-dense material containing microtubules. There are other examples of convergent evolution among pathways, such as the presence of a bicentriole in Labyrinthulae (Stramenopila). Future studies should be expanded to more species in less known groups to clarify the mechanism involved in *de novo* biogenesis (orange) and understanding if they are all a result of lineage-specific

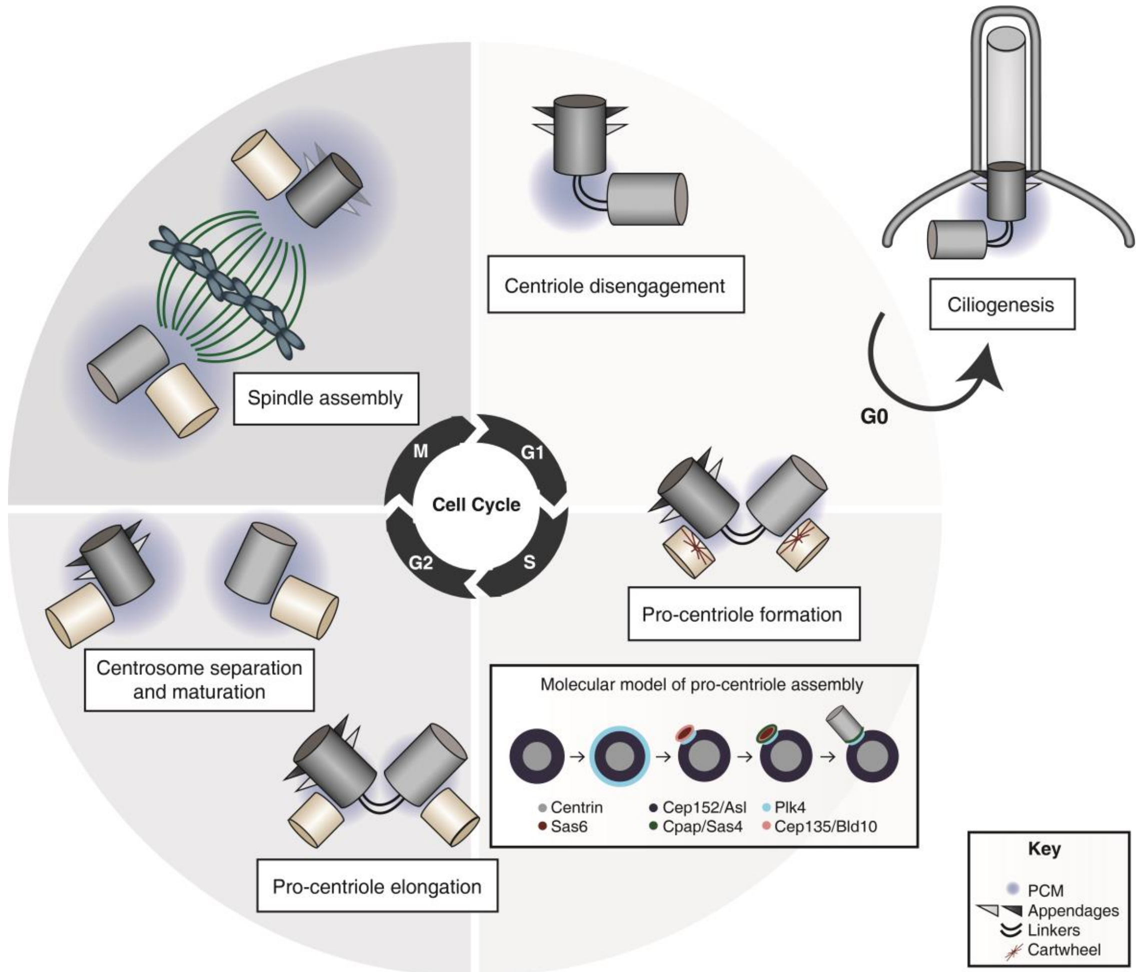
evolution (convergent evolution). CBBs were lost in multiple lineages (red lines and crosses: absent in all species within the groups; red crosses – lost in only some species within the lineage).

Tables

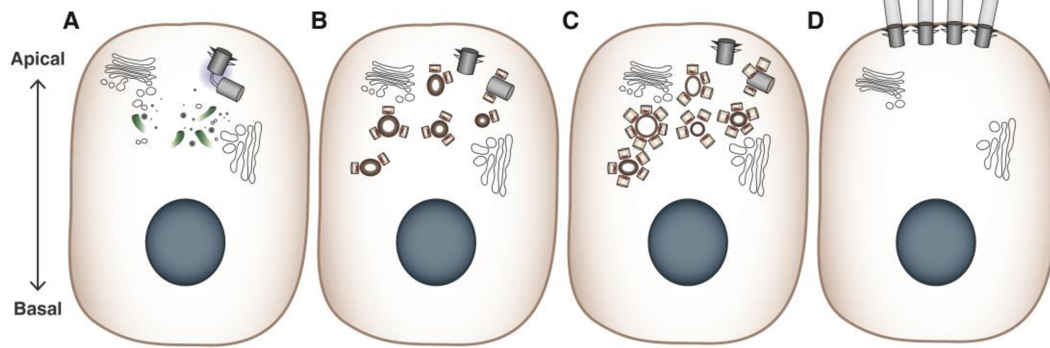
Table 1. Common principles underlying centriole biogenesis among known pathways.

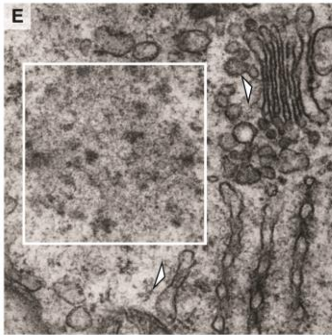
Supplementary Figures

Suppl. Table 1. References consulted to build the main Figure 6. Here, we dissected the information on CBBs presence/absence, their biogenesis pathway in specific cell-types or tissues, across the eukaryotic tree of life. ***No bona-fide deuterosome were detected but pro-centrioles always form in the vicinity of resident centrioles suggesting a similar centriolar-mediated process. ****Coaxial centriole duplication.

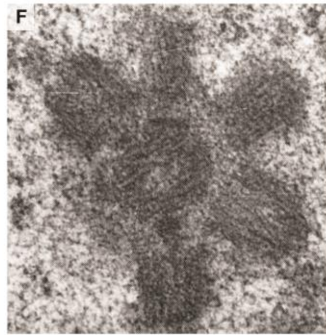

Canonical biogenesis
Centriole disengagement
Ciliogenesis
G0
Spindle assembly
M
G1
Cell Cycle
G2
S
Pro-centriole formation
Centrosome separation and maturation
Molecular model of pro-centriole assembly
Centrin
Sas6
Cep152/Asl
Cpap/Sas4
Plk4
Cep135/Bld10
Pro-centriole elongation
Key
PCM
Appendages
Linkers
Cartwheel

Deuterosome-mediate biogenesis

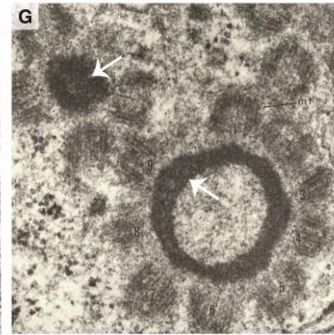




Rat - Lung epithelia
(Sorokin 1968)

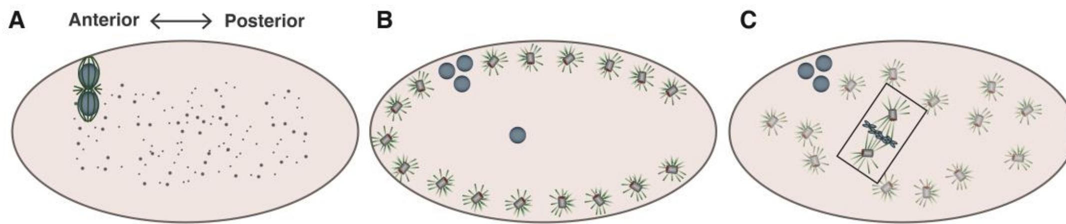


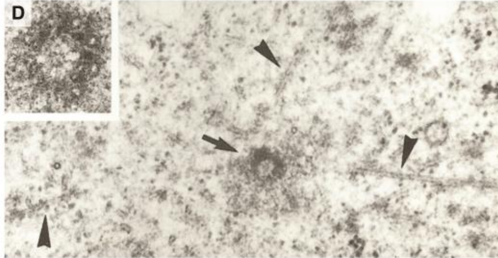
Rat - Lung epithelia
(Sorokin 1968)



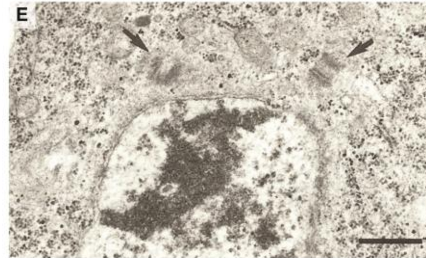
Mouse - Oviduct epithelia
(Dirksen 1971)

***de novo* Biogenesis**





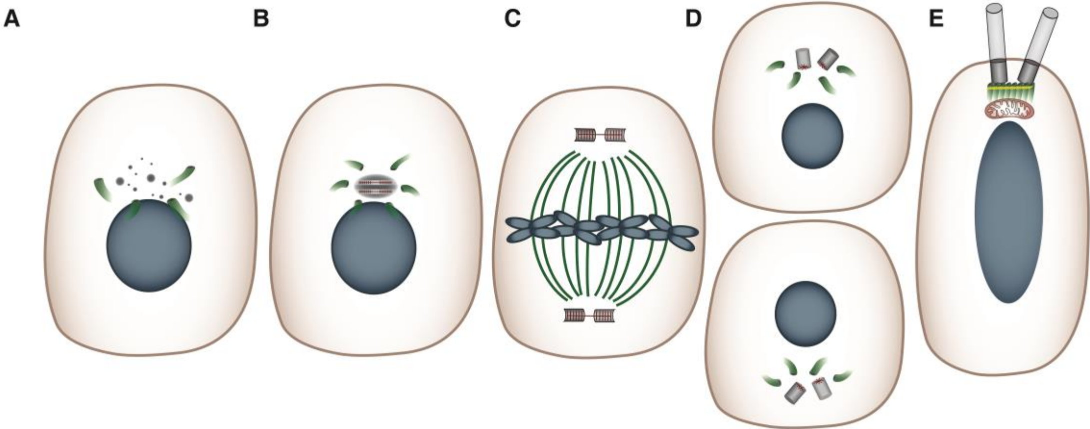
Wasp (*M. uniraptor*) - Parthenogenic egg (Riparbelli et al. 1998)

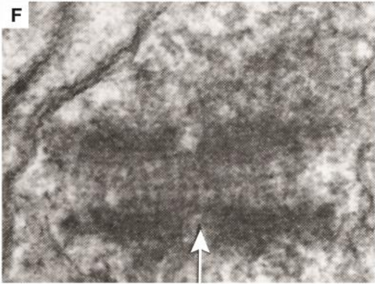


Wasp (*M. uniraptor*) - Parthenogenic egg (Riparbelli et al. 1998)

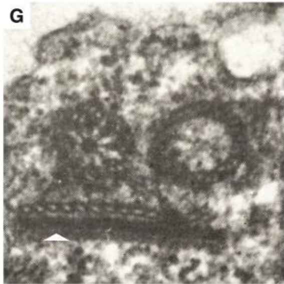
Key
Centriolar precursors
Nucleus
Microtubules
Centriole

Bicentriole-mediated biogenesis





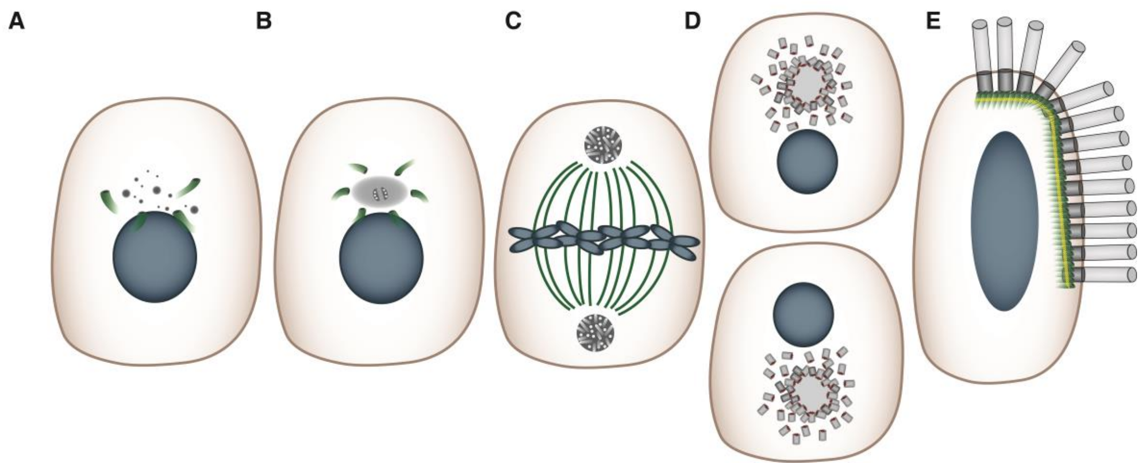
Liverwort (*M. polymorpha*) - Spermatid
(Moser and Kreitner 1970)

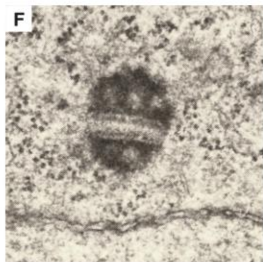


Liverwort (*M. polymorpha*) - Spermatid
(Moser and Kreitner 1970)

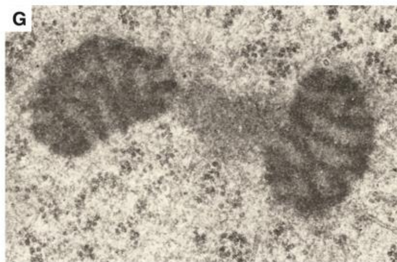
Key
Centriolar precursors
Nucleus
Microtubules
Centriole
Mitochondria
Bicentriole

Blepharoplast-mediated biogenesis

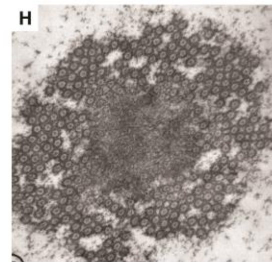




Fern (*M. vestita*) - Spermatid (Hepler 1976)



Fern (*M. vestita*) - Spermatid (Hepler 1976)



Cyca (*Z. pumila*) - Spermatid (Mizukami and Gall 1966)

Key

Centriolar precursors
Nucleus
Microtubules
Centriole
Blepharoplast
Bicentriole

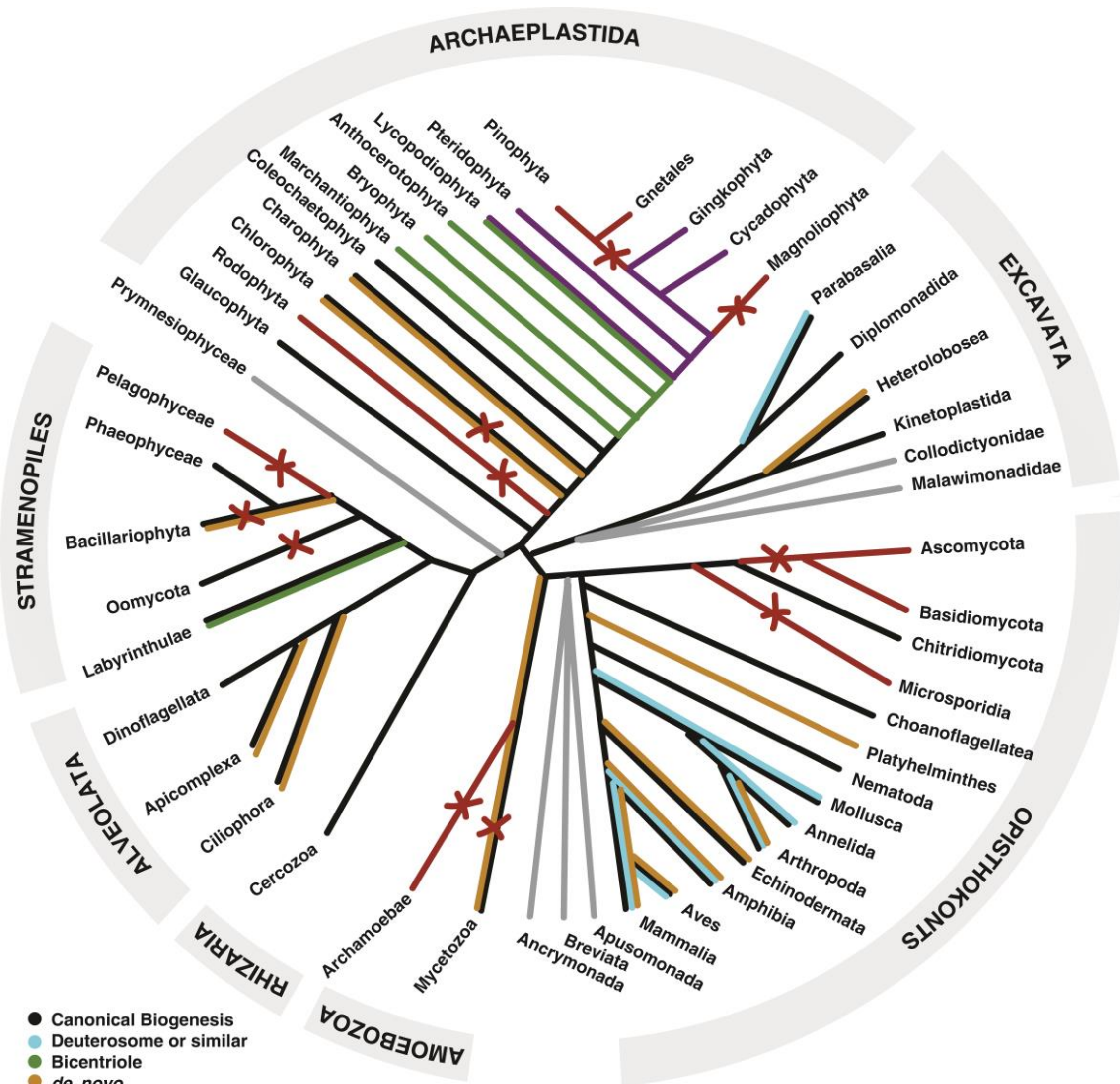

ARCHAEPLASTIDA
EXCAVATA
OPISTHOKONTS
AMOEBOZOA
RHIZARIA
ALVEOLATA
STRAMENOPILES
Pinophyta
Gnetales
Gingkophyta
Cycadophyta
Magnoliophyta
Pteridophyta
Lycopodiophyta
Anthocerotophyta
Bryophyta
Marchantiophyta
Coleochaetophyta
Charophyta
Chlorophyta
Rodophyta
Glaucophyta
Prymnesiophyceae
Parabasalia
Diplomonadida
Heterolobosea
Kinetoplastida
Collodictyonidae
Malawimonadidae
Ascomycota
Basidiomycota
Chitridiomycota
Microsporidia
Choanoflagellatea
Platyhelminthes
Nematoda
Mollusca
Annelida
Arthropoda
Echinodermata
Amphibia
Aves
Mammalia
Apusomonada
Breviata
Ancrymonada
Mycetozoa
Archamoebae
Cercozoa
Ciliophora
Apicomplexa
Dinoflagellata
Labyrinthulae
Oomycota
Bacillariophyta
Phaeophyceae
Pelagophyceae
Canonical Biogenesis
Deuterosome or similar
Bicentriole
de novo
Blepharoplast
CBBs lost in all/some species
Unconsensual taxonomy

Table 1. Common principles underlying centriole biogenesis among known pathways

PATHWAYS	Electron-dense precursors (PCM?)	Microtubule enrichment	"Concentrator"	PCM enrichment	Pro-CBB assembly	CBB
Canonical	1 per Mother Centriole	Yes	Mother Centriole	Yes	Yes	Yes
Deuterosome	Many	Yes	Deuterosomes and Resident Centrioles	Yes	Yes	Yes
de novo	Not clear	Yes	Not clear	Yes	Yes	Yes
Bicentriole	1	Not clear	2 Pro-bicentrioles	Yes	Yes	Yes
Blepharoplast	1	Yes	2 Blepharoplasts	Yes	Yes	Yes

Key Microtubules (MTs); PCM (γ-tubulin); MTs-wall (MTs triplet); Cartwheel (Sas6 + Cep135/Bld10); Sas4/Cpap + Centrin + ?