Reviewed Preprint
v1 • May 13, 2024
Not revised

Reviewed Preprint
v2 • July 9, 2026
Revised by authors

The cell cycle variant in multiciliated cells incorporates 2 centriole biogenesis cycles

Amélie-Rose Boudjema[1,*], Rémi Balagué[1,*], Cayla E Jewett[2], Jacques Serizay[1,10], Gina M LoMastro[2,3], Olivier Mercey[4], Adel Al Jord[5,6], Adrien Candat[1], Marion Faucourt[1], Alexandre Schaeffer[7,8], Camille Noûs[9], Nathalie Delgehyr[1], Andrew J Holland[2], Nathalie Spassky[1], Alice Meunier[1]

[1]Institut de Biologie de l'École Normale Supérieure (IBENS), Paris Sciences et Lettres (PSL). Research University, CNRS, umR 8197, INSERM, U1024, Paris, France • [2]Department of Molecular Biology and Genetics, Johns Hopkins University School of Medicine, Baltimore, United States • [3]Seagen Inc., Bothell, United States • [4]Department of Molecular and Cellular Biology, University of Geneva, Geneva, Switzerland • [5]Centre for Genomic Regulation (CRG), The Barcelona Institute of Science and Technology, Barcelona, Spain • [6]Universitat Pompeu Fabra (UPF), Barcelona, Spain • [7]CytoMorpho Lab, Laboratoire de Physiologie Cellulaire & Végétale, Interdisciplinary Research Institute of Grenoble, University of Grenoble-Alpes, CEA, CNRS, INRA, Grenoble, France • [8]CytoMorpho Lab, umR8231, ESPCI, University PSL, CEA, Paris, France • [9]Cogitamus Laboratory, Paris, France • [10]Institut Pasteur, CNRS UMR3525, Université Paris Cité, Unité Régulation Spatiale des Génomes, Paris, France

For correspondence: alice.meunier@ens.fr

* These authors contributed equally to this work

Competing interests: No competing interests declared

Funding: See page 26

Reviewing editor: Jens Lüders, Institute for Research in Biomedicine, Spain

eLife Assessment

In this **important** study, Boudjema et al. use cell culture models and high quality advanced microscopic imaging to provide detailed analyses of the cellular processes underlying centriole amplification, apical migration, and assembly of hundreds of motile cilia in multi-ciliated cells. The authors present **convincing** evidence showing that in these cells all the molecular and cellular steps controlling centriole biogenesis that in cycling cells extend over almost two cell cycles, occur within a single cell cycle variant. This work provides a better understanding of the regulation and order of these processes and is of interest to all cell biologists and in particular researchers studying centrioles and cilia.

https://doi.org/10.7554/eLife.96584.2.sa3

Abstract

Multiciliated cell (MCC) differentiation is a calibrated version of the canonical cell cycle. The MCC cell cycle variant sustains amplification of centrioles for the nucleation of dozens of motile cilia, while avoiding cell division. In this study, we show that the MCC cell cycle variant is an accelerated version of the canonical cell cycle, which superposes two cycles of centriole biogenesis, in order to obtain multiple mature centrioles within a single -instead of double- cell cycle iteration. We further show that the precocious maturation of amplified procentrioles is even determinant for their spatial self-organization, disengagement and apical migration for cilia nucleation. Our findings collectively suggest that the decomposition of centriole biogenesis over two cycle iterations in dividing cells may have been adopted to ensure the growth of a solitary primary cilium, and exemplify how minimal deviations of the canonical cell cycle allow MCC progenitors to both amplify, and accelerate, centriole biogenesis for vital motile ciliogenesis.

Introduction

Centriole amplification is a characteristic of multiciliated cell (MCC) differentiation, necessary for the nucleation of dozens of motile cilia that propel physiological fluids in the brain, the respiratory and the reproductive tracts. However, it is a pathological mechanism in cancer cells, where it can

cause genomic instability that accelerates tumor formation (Nigg & Holland, 2018). It is interesting to note that centriole amplification dynamics in post-mitotic MCCs show similarities with the highly conserved process of centriole duplication that occurs in parallel with DNA during cell division (Al Jord et al., 2014; Herawati et al., 2016; Ma et al., 2014; Revinski et al., 2018; Stubbs et al., 2012; Tan et al., 2013; Vladar & Stearns, 2007; Zhao et al., 2013) . Consistently, MCC differentiation is driven by a true variant of the cell cycle, co-opting the mitotic oscillator and transcriptional topology of the cell cycle, but changing the cyclins, E2F transcription factor and some regulators of APC/C to hijack cell cycle activity and skip DNA duplication while (over-) replicating centrioles (Al Jord et al., 2017, 2019; Choksi et al., 2024; Khoury Damaa et al., 2025; Kim et al., 2022; Serizay et al., 2025; Vladar et al., 2018).

Three highly stereotyped sequential stages have been resolved by live imaging mouse brain MCCs: the amplification stage (called A-stage), during which dozens of latent procentrioles form sequentially around centrosomal centrioles and MCC specific deuterosome organelles; the growth stage (called G-stage), during which all procentrioles elongate synchronously; the disengagement stage (called D-stage), during which all centrioles detach from their growing platforms in a synchronized manner to finally migrate towards the apical membrane and nucleate motile cilia (Al Jord et al., 2014). Transitions between these stages, which share similarities with the S-, G2- and M-phase of the canonical cell cycle, are controlled by the mitotic oscillator. Specific inhibition or disinhibition of core components of this cell cycle clock-like regulatory circuit (CDK1, PLK1 and APC/C) results in altering centriole number, growth and disengagement, in a manner similar to centriole duplication (Al Jord et al., 2017; Kim et al., 2022; Revinski et al., 2018).

While the dynamics and molecular cascade are mostly comparable between centriole biogenesis in cycling and MCC differentiating cells, two major differences exist. The first one is the number of centrioles being produced : a dividing MCC progenitor produces one procentriole per parental centriole during the canonical cell cycle, but when it differentiates, the MCC progenitor cell enters a final modified iteration of the cell cycle that results in the production of several dozen centrioles. The second difference is the pace of centriole production : it takes almost two iterations of the canonical cell cycle to produce a mature « mother » centriole -or « basal body »- able to nucleate microtubules (MT) and grow a cilium, but a single iteration of the MCC cell cycle variant is needed to produce basal bodies, able to nucleate cytoplasmic MT and grow cilia.

Here, we used micro-patterned MCCs, new knock-in transgenic mice, ultrastructure expansion microscopy (U-ExM), correlative light and electron microscopy and MT/dynein pharmacological inhibitions, to explore these two specificities of the MCC cell cycle variant. In space, we found that centriole amplification emerges in a pericentrosomal « nest » concentrating core centriole/deuterosome elements, and that both the nest and the production of centrioles/deuterosomes are altered when MT or dynein are inhibited. In time, we found that the MCC cell cycle accelerates the centriole biogenesis program by superposing 2 canonical centriole cycles, leading to the concomitant elongation and maturation of procentrioles in a single cycle iteration. In this 2-in-1 cycle, the precocious maturation of procentrioles is moreover determinant for their subsequent spatial self-organization, disengagement and apical migration.

Results

MEF-MCCs grown on micropatterns localize the onset of centriole amplification at the pericentrosomal region

The origin of amplified centrioles in MCC remains controversial. Some live imaging experiments and electron microscopy suggest that the centrosome could constitute a nest for centriole and deuterosome biogenesis (Al Jord et al., 2014; Kalnins et al., 1972; Mori et al., 2017), but others have proposed that procentriole-loaded deuterosomes emerge independently from the centrosome location, all over the cytoplasm (Nanjundappa et al., 2019; Sorokin, 1968; Zhao et al., 2013, 2019). Interestingly, in the absence of deuterosome, procentrioles are massively amplified from and around centrosomal centrioles, yet, centrosomal centrioles are dispensable (Mercey et al., 2019a; Mercey et al., 2019b; Nanjundappa et al., 2019; Zhao et al., 2019).

Finally, in the absence of both centrosomes and deuterosomes, procentrioles are seen emerging from the middle of a self-organized microtubule organizing center (MTOC) suggesting that such MTOC could organize procentriole biogenesis (Mercey et al., 2019).

To further assess whether procentrioles are emerging from a particular location in MCCs, we took advantage of a cellular model based on the induced differentiation of MEFs into MCCs by ectopic adenoviral expression of MCIDAS, E2F4 and E2F4 transactivator Dp1 (Kim et al., 2018). This model of MCCs is advantageous because it is not tissue-specific and does not require epithelial cell-to-cell contact for differentiation. This allowed us to trigger MCC differentiatiation in single cells platted on micropatterns to impose a reproducible cytoplasmic organization facilitating spatial quantifications. We induced MCC differentiation in MEFs grown on crossbow-shaped micropatterns to analyze the global spatial dynamics of procentriole amplification (Fig. 1A). We first confirmed that, in MEF-MCCs, the same dynamics of amplification was observed as in brain MCC: an "amplification stage (A-stage)", marked by the progressive accumulation of immature non-polyglutamylated (GT335 "-") procentrioles is followed by a "growth stage (G-stage)" where procentrioles mature and become polyglutamylated (GT335 "+"). This maturation is followed by ciliation (MCC-stage) which is often partial, probably because of the absence of the typical epithelial apico-basal polarity (Fig. 1B). Consistently, in this cellular model, centrosomes can localize below the nucleus. Here, only cells with apically positioned centrosome were studied. By density mapping the location of centrosomes in multiple crossbow-shaped MEFs before MCC differentiation (precursor stage), we confirmed that centrosomes exhibit a reproducible position and are located at the center of convergence of microtubules (Fig. 1C, top row left) colocalized with PERICENTRIN (PCNT, Fig 1D, top row left). Then, by density mapping the position of SAS6+ procentrioles in differentiating MEF-MCCs containing less than 10 SAS6 foci (early A-stage), we observed that early procentriole emergence is localized in the centrosomal region, where PCNT also accumulates (Fig. 1C, 1D top row middle). Later during amplification (>10 SAS6+ foci, late A-stage and G-stage), procentrioles and PCNT occupy a larger portion of the cells centered around the centrosome location and surrounding the nuclear region (Fig. 1C-D top row right). These results suggest that, in this cellular model, procentrioles emerge in the centrosomal region.

We previously showed in brain MCC deprived of both centrosomes and deuterosomes, that centrioles emerged from the center of a self-organized microtubule network, within a PCNT cloud (Mercey et al., 2019b). To test whether microtubules drive the organization of a centrosomal nest from which procentrioles emerge, we treated MEF-MCCs with a dose of nocodazole affecting mildly the microtubule network (Fig. 1C, bottom row), before the onset of centriole amplification (see methods). In this condition, SAS6+ structure density mapping shows a clear dispersion of procentrioles compared to control cells (Fig. 1C bottom row). The centrosomal PCNT cloud also scatters in the cell cytoplasm (Fig. 1D bottom row). Since MT perturbation also induced centrosomal centriole splitting and decentralization of centrosomal centrioles (Fig. 1, bottom row leftC), we also measured the distance between emerging SAS6+ foci and the closest centrosomal centriole. This confirmed that procentriole-to-centrosome distance increases with nocodazole (Fig. 1 Supplementary 1A-B).

Altogether, these results suggest that, in this non tissue specific proxy of MCC progenitors, MT organize the onset of centriole amplification in the pericentrosomal region.

Correlative DEUP1 live-imaging and EM highlights the existence of a pericentrosomal "nest" in brain MCC

We then sought to study early onset of centriole biogenesis in physiological MCCs. Live monitoring centriole amplification in MCCs was only reported in brain cultured progenitors since respiratory and reproductive MCCs are integrated in stratified epithelia, hindering their live observation by inverted high-resolution live microscopy. It was done using GFP-tagged CENTRIN2 (CEN2-GFP) in control cells and in cells deprived of centrosome and/or deuterosomes through Centrinone treatment and/or DEUP1 gene invalidation (DEUP1 KO). These studies revealed that procentrioles, in presence (WT) or absence of deuterosomes (DEUP1 KO), where emerging from a pericentrosomal cloud of CEN2-GFP, suggesting that they were formed around the centrosome (Al

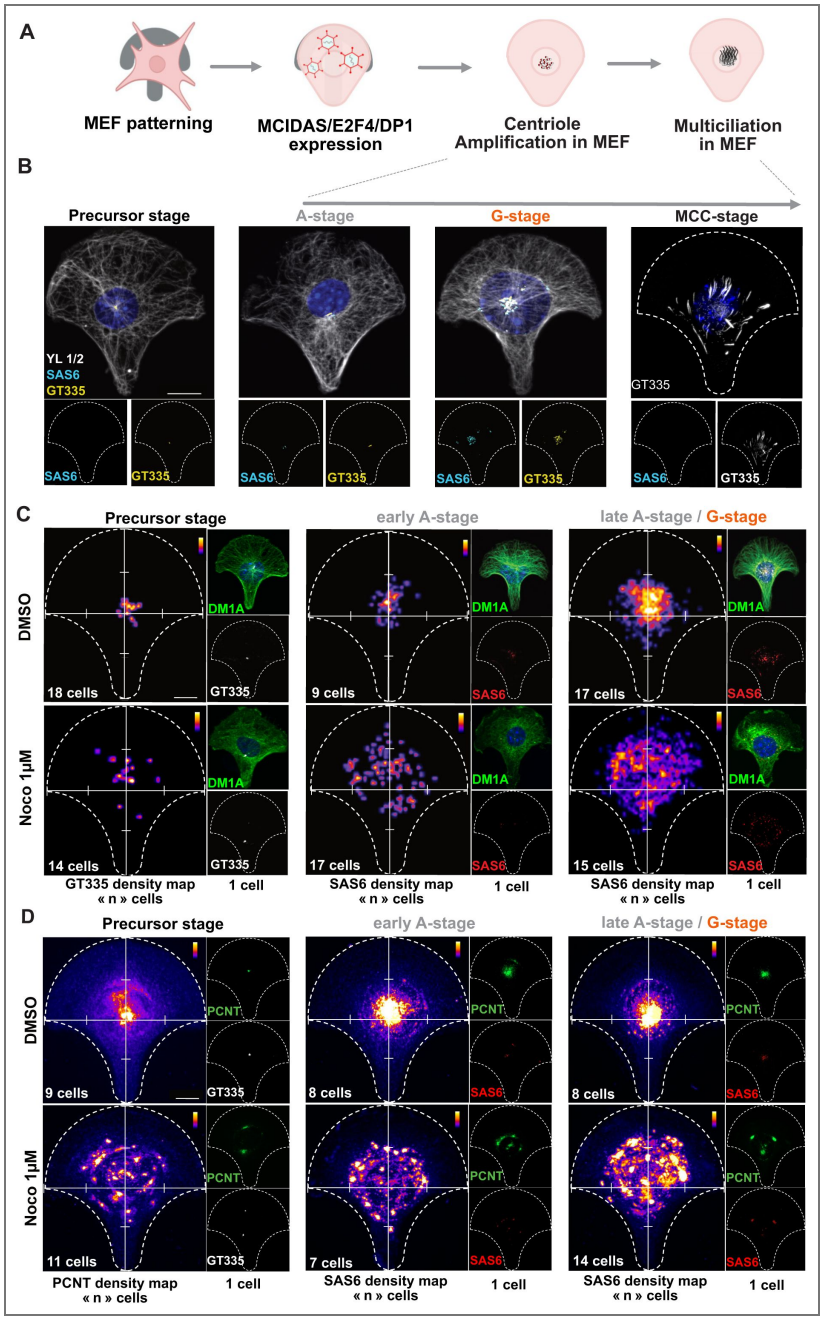

Figure 1. MEF-MCCs grown on micropatterns highlight the pericentrosomal focalization of procentriole production

(A) Scheme of the MEF-MCC differentiation process on crossbow micropatterns. **(B)** MCC-induced fibroblasts on micropatterns perform the typical step-wise dynamics of centriole amplification. Representative immunofluorescence images of MEF-MCC on crossbow micropatterns showing the 4 stages of differentiation. Scale bar, 10 µM. **(C)** Density mapping of centrosome in MEF-MCCs before amplification (precursor stage, left column), and of procentrioles during early amplification (cells with less than 10 SAS6 focis/cell, middle column) or late amplification (cells with more than 10 SAS6 focis/cell, right column) treated with DMSO (top row) or Nocodazole 1 µM (bottom row). Three independent experiments were quantified. Scale bar, 10 µM. **(D)** Density mapping of PCNT fluorescence signal during centrosome stage (left column) early A-stage (middle column) and late A-stage (right column) in MEF-MCCs under DMSO (top row) or Nocodazole 1 µM (bottom row). Two independent experiments were quantified. Scale bar, 10 µM. DM1A marks all MT, YL1/2 marks tyrosinated MT, SAS6 marks procentrioles, GT335 marks centrosome, mature procentrioles and cilia, PCNT marks PERICENTRIN. Dotted lines show the crossbow shape of the micropattern, white graduated lines show x and y axes. Insets on the right of each panel on (C) and (D) show one representative cell used for the density mapping.

Jord et al., 2014; Mercey et al., 2019a; Mercey et al., 2019b) . Further studies overexpressing a tagged version of DEUP1, contested these results by showing that DEUP1+ structures were appearing scattered in the cytoplasm (Zhao et al., 2019). However, these observations were done through DEUP1 overexpression, which induces the formation of non physiological DEUP1 aggregates, and in the presence of nocodazole, which can disturb the dynamics (Fig. 1C-D).

In order to catch the very fist events of centriole amplification, we developed a transgenic mouse line expressing CEN2-GFP, together with the core deuterosome component DEUP1, endogenously tagged with a mRuby fluorescent reporter (mRuby-DEUP1; see methods). Imaging brain MCC progenitors from these mice confirmed the absence of non physiological DEUP1 aggregates and the accurate monitoring of mRuby-DEUP1+ deuterosomes in addition to the CEN2-GFP+ procentrioles during the A-, G- and D-stages (Fig. 2A, Fig. 2 Supplementary 1A, Al Jord et al., 2014, 2017). To highlight the very early onset of amplification we live imaged early cultures and revealed that centriole amplification A-stage begins with the formation of a cloud of DEUP1, which we called “primordial” cloud (Fig. 2B, red arrowhead, 05:50 to 12:30; *Video 1*). This cloud forms and grows around the centrosome (Fig. 2B, white arrowheads, n>30 cells). Over time, DEUP1+ foci, which are also CEN2-GFP+, emerge from this cloud (Fig 2B, red and white arrowheads, 15:50-18:20). They represent procentriole-loaded deuterosomes characterizing the A-stage previously described using CEN2-GFP only (Al Jord et al., 2014).

To further assess the composition of the primordial cloud from which procentriole-loaded deuterosome emerge, we increased spatial resolution and identified the cloud as diffuse and/or composed of intermingled mRuby-DEUP1+ and CEN2-GFP+ foci of different sizes (Fig. 2 Supplementary 1B), which do not necessarily colocalize and can move independently from each others (Fig. 2 Supplementary 1B, *video 2*). We then used correlative light and electron microscopy (CLEM) to reveal the ultrastructure of the cloud and found that the mRuby-DEUP1+ diffuse signals outline regions rich in fibrogranular material, previously characterized as centriolar satellites (Zhao et al., 2021). This region was found to be either deprived of deuterosomes (Fig. 2C, Fig. 2 Supplementary 2) or embedding small nascent deuterosomes taking the form of amorphous electron dense structures, horseshoes or complete spheres (Fig. 2D, Fig. 2 Supplementary 3-6, *video 3*). As previously described, small deuterosomes can be seen connected to the centrosomal daughter centriole (“dc”, Fig. 2D, bottom right; Fig. 2 Supplementary 3, 4, *video 3*, (Al Jord et al., 2014; Khoury Damaa et al., 2025)). Nascent deuterosomes are either free (Fig. 2D, Fig. 2 Supplementary 3-4, blue stars), or loaded with small procentrioles (Fig. 2D, Fig. 2 Supplementary 3-5, black stars). Procentrioles seemingly not loaded on deuterosome can sometimes be detected (Fig. 2D, Fig. 2 Supplementary 4, red arrows; *video 3*). At this early stage, correlation between deuterosome size and the centriole number they are associated with is not evident: CLEM shows that even big deuterosomes are associated with only one or two procentrioles (Fig. 2D; Fig. 2 Supplementary 6). Live monitoring mRuby-DEUP1 further shows that the size of these young deuterosomes homogenizes with time. This is achieved through the splitting of big deuterosomes into smaller ones, when big deuterosomes are present (Fig. 2 Supplementary 1C), and through either fusion (never observed live) or growth, when amplification begins with small deuterosomes.

To better resolve how deuterosome and centriolar material concentrate around the centrosome, we then increased the temporal resolution (dt=5-15s for 1-4min) and revealed stochastic back and forth fluctuations to the centrosome for both early CEN2-GFP-/mRuby-DEUP1+ or CEN2-GFP+/mRuby DEUP1- structures (Fig. 2E, *Video 4-5*, 5/10 cells). These stochastic fluctuations are also visible later on CEN2-GFP+/mRuby-DEUP1+ deuterosomes (*Video 6*, Fig. 2 Supplementary 1D, 13/15 cells, white arrowheads). This movement to the centrosome is reminiscent to the dynamics of the centriolar satellite protein PCM1 in *Xenopus* and mice, and the PCM protein Centrosomin in *Drosophila* (Hall et al., 2023; Kubo et al., 1999; Megraw et al., 2002), suggesting that centriole and deuterosome components are connected to the centrosome, as other pericentrosomal components during A-stage. Interestingly, back and forth movements of mRuby-DEUP1+ structures to one centrosomal centriole are frequently observed in live (*Video 6*,

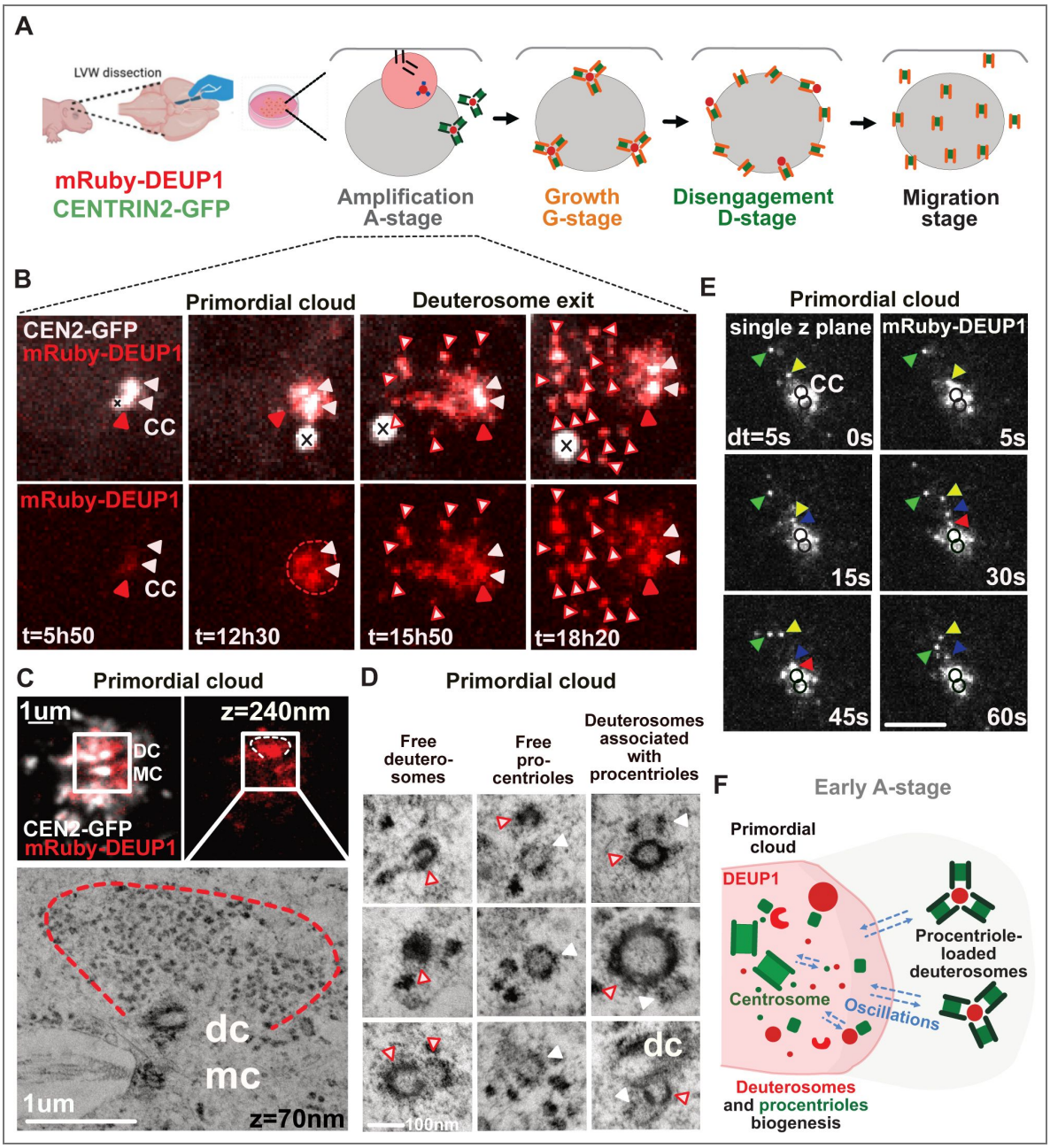

Figure 2. Live imaging endogenous DEUP1 highlights the first events of centriole amplification in brain MCC

(A) Scheme of the ependymal cell culture protocol. LVW: lateral ventricular wall. **(B)** Early A-stage dynamics in CEN2-GFP; mRuby-DEUP1+ cells. White arrowheads point the 2 centrosomal centrioles. The red dotted line corresponds to the cloud of DEUP1+ forming around the parental centrioles during early A-stage. The red arrowhead points to DEUP1 accumulation at one centrosomal centriole as compared to the other one. The red and white arrowheads point to CEN2-GFP+ structures corresponding to centriole loaded deuterosomes. The crosses rule out the CEN2-GFP+ aggregate not to be mistaken with a centrosome; CEN2-GFP in white, DEUP1 in red; scale bar, 5 µM. See associated *Video 1*. **(C)** Correlative light and electron microscopy resolves fibrogranular aggregate concentration in a mRuby-DEUP1+ primordial cloud. Light microscopy integrates the fluorescence signal of a z-stack of 240 nm. Electron microscopy z-sections are 70 nm. dc= centrosomal daughter centriole, mc= centrosomal mother centriole. **(D)** Representative ultrastructural elements present in the mRuby-DEUP1 primordial cloud resolved by correlative light and electron microscopy. Red and white arrowheads indicate deuterosome structures. White arrowheads indicate procentrioles. dc= centrosomal daughter centriole. See also *Video 3*. **(E)** Representative images of live imaged mRuby-DEUP1+ foci fluctuations in the primordial cloud (dt= 5s). A single z-slice of 0,7 µM is filmed. Black circles indicate the position of centrosomal centrioles located thanks to the CEN2-GFP signal, not shown here. mRuby-DEUP1+ foci are color-coded with arrowheads to appreciate their oscillatory behaviors towards the centrosomal mRuby-DEUP1+cloud. See associated *video 4*. Scale bar, 5 µM. **(F)** Scheme depicting the observations resulting from live-imaging CEN2-GFP; mRuby-DEUP1+ cells and correlative light and electron microscopy.

7, Fig. 2 Supplementary 1E) and may explain the observed connection of deuterosomes to the centrosomal daughter centriole on fixed samples during A-stage (this paper, (Boudjema et al., 2024; Khoury Damaa et al., 2025)),

Altogether live imaging mRuby-DEUP1/CEN2-GFP during early A-stage suggests that core deuterosome and centriole components are concentrated in a primordial cloud around the centrosome, which constitutes a nest where centrioles and deuterosomes concomitantly form before they move away from the centrosomal region (Fig. 2F). Back and forth fluctuations of CEN2-GFP+ and mRuby-DEUP1+ structures during A-stage suggest the existence of centrosome-related forces avoiding dispersion of primordial structures away from the centrosomal region.

S-phase like steps are recapitulated in the pericentrosomal nest

To gain molecular resolution on the early events occurring within the pericentrosomal nest, we performed ultrastructure expansion microscopy (U-ExM). U-ExM is an extension of expansion microscopy that allows the visualization of structures by optical microscopy with 4-fold increased resolution (Gambarotto et al., 2019).

U-ExM analysis confirmed the existence of a pericentrosomal cloud characterized by live imaging. In these fixed detergent-treated cells though, the cloud is around 10-fold dimmer than the centrosomal centrioles, and thus difficult to visualize without over-saturating the signal, explaining why it was not described before (Fig. 3 Supplementary 1A-E). In the primordial cloud, U-ExM identifies DEUP1 foci at and around the parent centrioles, with a light bias to the daughter centriole (Fig. 3A, H; Fig. 3 Supplementary 1C-E). This cloud of DEUP1 and CENTRIN also includes PCNT (Fig. 3 Supplementary 1F). Notably, CENTRIN and DEUP1 foci are often in close proximity but not necessarily colocalized, confirming live imaging, while PCNT puncta partially overlap with CENTRIN (Fig. 3 Supplementary 1F). At this stage, PLK4, the master regulatory kinase, and SAS6, one of the first centriolar components are either absent, or present as small foci within the cloud, often on the wall of the parent centrioles, also with a bias to the daughter centriole (Fig. 3A-B).

As DEUP1 accumulates, some DEUP1+ foci within the cloud become bigger, more regular in shape and colocalize with PLK4 and SAS6, which marks the association of nascent procentrioles with deuterosomes (Fig. 3C-D, yellow arrow). These procentrioles are however deprived of β-tubulin and therefore of a microtubule wall (Fig. 3C). Such “naked” cartwheel assembly was also recently revealed as the earliest step of centriole assembly in cycling cells (Laporte et al., 2024). As the number of nascent procentrioles increases, they migrate away from the centrosome and β-tubulin is recruited asynchronously on procentrioles, with procentrioles further away from the parents acquiring β-tubulin, and procentrioles closer to the parents often lacking β-tubulin (Fig. 3E-F, Fig. 3 Supplementary 1G). Consistent with a progression of procentriole assembly during radial migration away from the centrosome, the procentriole walls increase in length from 80 nm to 150 nm and in width from 120 nm to 220 nm as a function of distance from the parent centrioles (Fig. 3 Supplementary 1H-I). The range and correlation of procentriole length and width increases are consistent with the recent description of S-stage procentriole growth (Laporte et al., 2024). Around the same time as β-tubulin, CENTRIN becomes also visible on procentrioles (Fig. 3 Supplementary 1J). This stage is probably the first stage we previously detected by CEN2-GFP live imaging and correlative CEN2-GFP light and EM microscopy (Al Jord et al., 2014). The DEUP1 asymmetry to the daughter centriole previously described (Al Jord et al., 2014) becomes more visible than during earlier stage (Fig. 3 D-E, G-H). Centriolar microtubules are then acetylated on procentrioles migrating away from the parents (Fig. 3G, Fig. 3 Supplementary 1J). Procentrioles achieve their final width (Fig. 3 Supplementary 2 A-E) and will continue to elongate during G-stage – in the same range as described recently for G2 procentrioles – to finally reach the length of M-stage procentrioles during D-stage (Fig. 3 Supplementary 2 A-E, (Laporte et al., 2024)).

Altogether, these data reveal that (i) a pericentrosomal nest composed of CENTRIN, PCNT and DEUP1 is preassembled before the onset of centriole amplification, (ii) deuterosome and centriole biogenesis begin in this nest before what had been previously resolved using CEN2-GFP and EM,

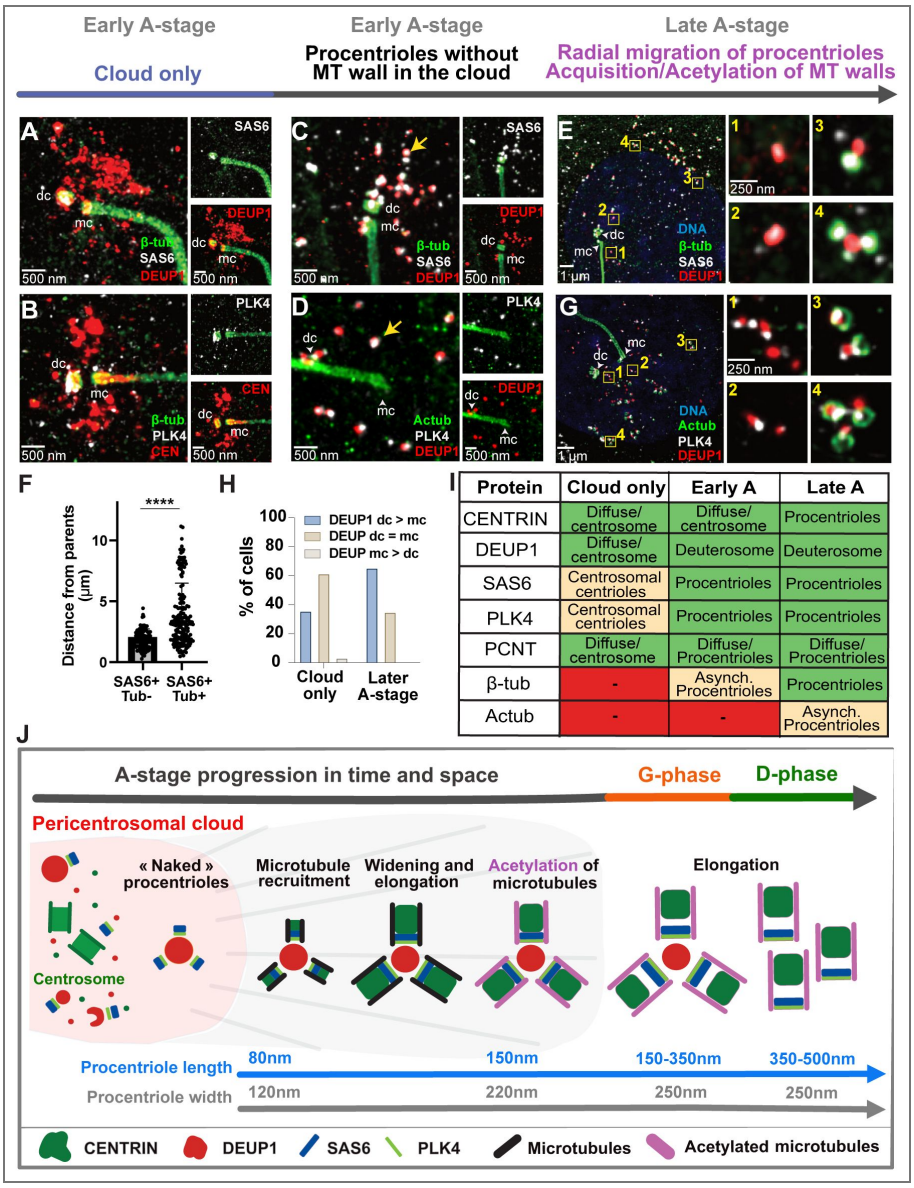

Figure 3. Procentriole assembly begins in a pericentrosomal nest and progresses during radial migration

(A-B) Representative U-ExM images of brain MCCs during early A-stage, when only a primordial cloud is visible but no procentriole-loaded deuterosomes can be identified. Cells were immunostained with antibodies to β-TUBULIN, SAS6, DEUP1 (A), β-TUBULIN, CENTRIN, PLK4 (B). mc: mother centriole, dc: daughter centriole. **(C-D)** Representative U-ExM images of brain MCCs during early A-stage, when procentriole-loaded deuterosomes are visible. Cells were immunostained with antibodies to β-TUBULIN, SAS6, DEUP1 (C) or Acetylated-TUBULIN, PLK4, DEUP1 (D). Arrows mark DEUP1 foci with either SAS6 (C) or PLK4 (D). mc: mother centriole, dc: daughter centriole. **(E)** Representative U-ExM images of brain MCCs during late A-stage. Boxes denote zoomed in regions on right, with 1-4 labels marking increasing distance from the parent centrioles. Cells were immunostained with antibodies to β-TUBULIN, SAS6, DEUP1 (E) **(F)** Quantitation showing the procentriole distance from parents is shorter in procentrioles without αβ-TUBULIN compared to procentrioles with αβ-TUBULIN. SAS6 is used as a marker for procentrioles associated with deuterosomes. Graphs show mean ± SD from n=7 cells across 3 different experiments. ****p<0,0001 using unpaired t-test. **(G)** Representative U-ExM images of brain MCCs during late A-stage. Boxes denote zoomed in regions on right, with 1-4 labels marking increasing distance from the parent centrioles. Cells were immunostained with antibodies to Acetylated-TUBULIN, PLK4, DEUP1. **(H)** Quantitation comparing percent of cells showing DEUP1 enrichment at daughter centriole over mother centriole (dc>mc), equal enrichment at both parent centrioles (dc = mc), or enrichment at mother centriole over daughter centriole (mc>dc) when cloud-stage and A-stage. A total of 54 cells were analyzed across 3 experiments. **(I)** Table summarizing protein localization as visualized by U-ExM along A-stage progression. "Cloud only" refers to the beginning of A-stage, when a cloud is visible but no procentriole-loaded deuterosomes can be identified. **(J)** Scheme representing A-stage progression in time and space.

(iii) procentrioles recapitulate the S-stage step-wise dynamics, recently revealed by U-ExM time-series reconstruction in cycling cells, during their radial migration from the pericentrosomal nest, and (iv) their subsequent G- and D-stage elongation follows the G2- and M-like elongation process of the canonical cell cycle (Laporte et al., 2024). In MCC as in cycling cells, centriole elongation therefore follows the same step-wise dynamics, correlated with cell cycle stages (Fig. 3I-J).

Dynein-dependent MT transport organizes the pericentrosomal nest and is required for deuterosome and centriole production

MEF-MCCs experiments suggest that the pericentrosomal cloud from which procentrioles emerge is organized by microtubules (Fig. 1 C-D). Consistently, in physiological brain MCC, dynamic tyrozinated microtubules are typically focalized to the centrosome before and during A-stage (Fig. 4A). This polarity is progressively lost during the following G- and D-stages to reappear at the basal body patch at the end of amplification, consistent with the known role of mature centriole appendages as MTOC in terminal MCC (Herawati et al., 2016; Kunimoto et al., 2012; Liu et al., 2020; Tateishi et al., 2017) (Fig. 4A).

Because the pericentrosomal nest builds at the convergence of MT minus ends, we tested the role of MT and the MT retrograde molecular motors dyneins in the formation of the nest, the deuterosomes and the centrioles. To proceed, we live imaged MCC progenitors under acute nocodazole (10 µM) or dynapyrazole (3 µM) (Steinman et al., 2017) treatments, added after the first time point (see methods and Fig. 4B, Fig. 4 Supplementary 1A-B). We found a reduction or a complete abolition of the propensity of MCC progenitor cells to enter A-stage during a 24h period (Fig. 4C). When added on cells that had already entered A-stage, drug treatments block DEUP1+ structure oscillations within minutes in both early or late A-stage (Fig. 4D, *video 8-11*) and dissolves the mRuby-DEUP1+/CEN2-GFP+ cloud in few hours (Fig. 4E, *Video 12-14*). Under dynapyrazole, no new mRuby-DEUP1 foci appeared and no new centrioles were produced after drug addition (Fig. 4F-G, *video 15*). By contrast, in nocodazole, the existing deuterosomes moved away from the centrosome and some new mRuby-DEUP1+/CEN2-GFP+ foci appeared scattered in the cytoplasm (Fig. 4 Supplementary 2A, *Video 12-14*). Although they can increase in size, they are less regular and the associated CEN2-GFP+ signal is fainter (*Video 12-13*) suggesting the A-to-G transition is perturbed, probably because of a block in MT wall polymerization. This precluded the counting of final centriole number.

To confirm and further document the role of MT and dyneins, we also performed milder pharmacological treatments, but over longer timescale ("chronic" nococazole 48h, 5 µM or dynapyrazole 16h, 3 µM), and counted the number of amplifying cells, deuterosomes and centrioles. Consistent with live imaging, nocodazole and dynapyrazole treatments lead to a sharp decrease in the number of amplifying cells (Fig. 4 Supplementary 2B). In those undergoing centriole amplification, deuterosomes are more numerous but smaller and loaded with fewer procentrioles than in the control (Fig. 4H, Fig. 4 Supplementary 2C-D). Eventually, the final number of procentrioles is decreased (Fig. 4I). Altogether, these results show that MT and dyneins are required for the formation and maintenance of the pericentrosomal nest, the production of deuterosomes and the biogenesis of centrioles.

To further highlight how deuterosome formation is affected by MT alteration, we performed super-resolution imaging and CLEM on early A-stage MCC treated with nocodazole (10 µM, 24h). Super resolution imaging revealed the presence of grapes of small deuterosomes seemingly loaded with only one centriole, instead of the usual flower-like arrangement of procentrioles around a spherical deuterosomes (Fig. 4J). An "inverted flower" phenotype, where mRuby-DEUP1+ subunits are organized around a CEN2-GFP+ core was also frequently observed (Fig. 4K). CLEM on these structures confirmed the presence of single centriole-loaded deuterosomes (Fig. 4 Supplementary 3) and show that the "inverted flower" phenotype corresponds to an inversion of deuterosome/centriole organization where small deuterosomes, growing a single procentriole each, face each-others, regrouping procentrioles at the center (Fig. 4K, Fig. 4 Supplementary 4). CLEM furher showed that mRuby-Deup1+ structures can also correspond to groups of fibrogranular aggregates, probably resulting from pericentrosomal cloud scattering (Fig. 4

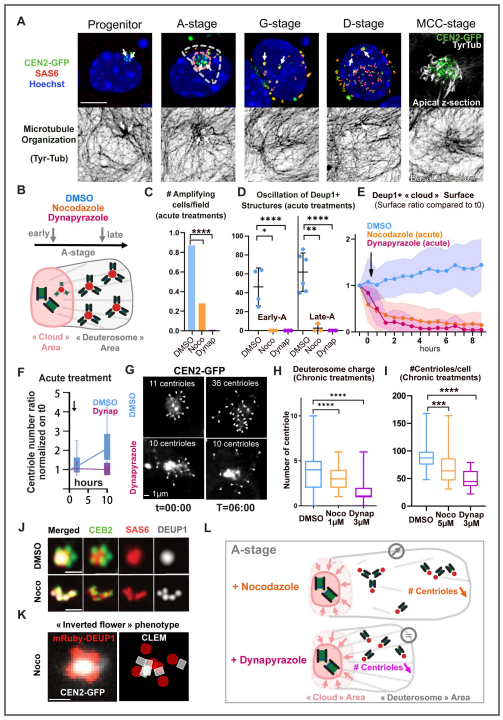

Figure 4. Dynein dependent MT transport organizes the pericentrosomal nest and is required for procentriole production

(A) Immunostaining profiles of procentrioles and microtubules during brain CEN2-GFP MCC differentiation. Procentrioles were counter-stained with anti-SAS6 antibodies (red), dynamic tyrosinated MTs were counter-stained with YL1-2 antibodies and the nucleus was counter-stained with Hoechst (blue). All micrographs are max projections of whole z-stacks except for the MCC-stage where max were done from apical or basal z-stacks selected from the same cell. Arrows show centrosomal centrioles, pink dotted line delineates the CEN2-GFP+ pericentrosomal cloud area, grey dotted line delineates the area occupied by deuterosomes. Scale bar, 5 µm. **(B)** Scheme depicting the experimental protocol. **(C)** Number of cells beginning centriole amplification (emergence of mRuby-DEUP1+/CEN2-GFP+ structures in the cell) in a microscope field during a 20h movie under acute DMSO (60 fields), Nocodazole (10 µM, 43 fields) or Dynapyrazole (3 µM, 30 fields) treatments. n>3 independent experiments. **(D)** Quantification of the percentage of early and late A-stage cells displaying at least one mRuby-DEUP1+ structure oscillation to and from the centrosome during 1-4min movies at dt5-15s, in DMSO, acute Nocodazole (10 µM) and acute Dynapyrazole (7.5 µM) treated cells. n>3 independent experiments were scored, n=81 DMSO cells, n=20 Nocodazole cells, n=56 Dynapyrazole cells analyzed. *p = 0.0108, **p=0.005, ****p<0.0001; Chi-square test with yates correction. See Videos 8-11. **(E)** Quantification of the area occupied by the mRuby-DEUP1+ cloud over time in control (DMSO), Nocodazole (10 µM) and Dynapyrazole (3 µM) treated cells. Black arrow indicates that DMSO and drugs were added right after the first acquisition. Three independent experiments were analyzed, n=12 DMSO cells, n=14 Noco 10 µM cells, n=14 Dynapyrazole 3 µM cells; ****p<0,0001; non-parametric Mann Whitney test; error bars represent mean ± SD. See Videos 12-14. **(F)** Centriole number ratio produced during A-stage under DMSO and Dynapyrazole (3 µM) treatment normalized on t0. DMSO and Dynapyrazole are added after the first time point. dt=40 min. **(G)** Representative images of CEN2-GFP movies used in (F). Arrowheads represent A-stage procentrioles. See also *video 15*. Scale bar, 1 µm. **(H)** Quantification of centriole number per deuterosome (using CEN2-GFP signal) in G-stage cells under DMSO, Nocodazole (1 µM) and Dynapyrazole (3 µM) chronic treatments (48h). Three independent experiments were scored, n_{deut}=459 in DMSO, n_{deut}= 215 in Nocodazole, n_{deut}=383 in Dynapyrazole. Error bars represent min to max ± median. **** P<0.0001; non-parametric Mann Whitney test. **(I)** Quantification of SAS6+ centriole number in G- and D-stage (pooled) cells under DMSO, Nocodazole (1 µM) and Dynapyrazole (3 µM) chronic treatments (48h). Three independent experiments were scored; error bars represent min to max ± median. n=42 DMSO cells, n=34 Noco 1 µM; n= 29 Noco 5 µM cells analyzed. Error bars represent min to max ± median. ***<0.001, **** P<0.0001; non-parametric Mann Whitney test. **(J)** Super resolution immunostaining profile of deuterosomes (DEUP1) and procentrioles (SAS6) under chronic Nocodazole (10 µM, 24h). Deuterosomes arrange in grapes and are reduced to small subunits scarcely loaded with procentrioles. Scale bar, 500 nm. **(K)** Super resolution fluorescence of the mRuby-DEUP1;CEN2-GFP "inverted flower" profile frequently observed under chronic Nocodazole (10 µM, 24h) along with the underlying deuterosome and procentriole arrangement revealed by correlative light and electron microscopy (CLEM). See Fig. 4 Supplementary 5 for serial CLEM sections. **(L)** Scheme representing the effect of Nocodazole and Dynapyrazole on A-stage. To be compared with the scheme of Fig. 4B.

Supplementary 3). These aberrant organizations of procentrioles and small deuterosomes in a 1:1 stoechiometry under nocodazole suggest that concentration of DEUP1 at the centrosome is required for DEUP1 subunits to merge into large spherical deuterosomes. Non exclusively, other MT-dependent forces may be involved in the gathering of centriole/deuterosome subunits into larger deuterosomes.

Altogether, these data show that MT and dyneins are required to concentrate centriolar and deuterosome core components within a pericentrosomal nest. MT depolymerisation and dynein inhibition dissolve this nest and hinder or even block, the formation of deuterosomes and centrioles (Fig. 4B, L).

Centriole maturation cycle superposes with centriole elongation cycle in the MCC cell cycle variant

We found that centriole elongation in MCC follows the same step-wise dynamics, correlated with cell cycle stages, as in the canonical cell cycle (Fig. 3J). A major difference however exists. After one canonical cell cycle the centriole cylinder is complete, has lost immaturity features such as cartwheel foundations, has been "modified" to gain MT nucleation capacity (Wang et al., 2011), but has not yet acquired full molecular maturity. This occurs during a second cell cycle and includes (i) the loss of daughter centriole proteins (e.g. centrobin, Cep97, Cep120) / acquisition of daughter centriole maturation proteins (e.g. C2CD3, ODF1, CEP350, CEP19, FOP) in S/G2 (Blanco-Ameijeiras et al., 2022), (ii) an enhanced capacity to nucleate MT, known as centrosome maturation at the G2/M transition (Joukov & De Nicolo, 2018), and (iii) the step-wise acquisition of distal (DA) and subdistal (SDA) appendages to dock at the membrane and anchor MT in early and late M stage respectively (Blanco-Ameijeiras et al., 2022). Called 'daughter centriole' at the end of the first cell cycle, the centriole is called "mother centriole" -or "basal body"- at the end of the second cell cycle (Fig. 5A) (Blanco-Ameijeiras et al., 2022; Nigg & Holland, 2018). By contrast, a single iteration of the MCC cell cycle variant allows mature basal bodies to form. We therefore sought to understand when and how centriole maturation occurs in this variant of the cell cycle.

Since (i) centriole elongation in MCC follows the dynamics of a canonical cell cycle (Fig. 3J), (ii) elongation and maturation cycles are driven by the same molecular cascade, (iii) which is also common to the MCC cell cycle variant (Al Jord et al., 2017), a parsimonious way of accelerating centriole biogenesis in the MCC variant would be to superpose the maturation "n+1" cycle, to the elongation "n" cycle. Consistent with this scenario, we found in our previous studies that the acquisition of centriole appendages is step-wise, and occurring in the second half of the MCC variant, like in the canonical cycle: distal appendages are acquired at the G-to-D transition (Al Jord et al., 2014) and subdistal appendages (basal feet) during early MCC (Fig. 5B, (Guirao et al., 2010)). Also, our single cell RNA sequencing data set comparing MCC and canonical cell cycle variants in brain progenitors (Serizay et al., 2025), shows that the expression of proteins involved in the "n" elongation cycle (Fig. 5 Supplementary 1A), superposes with the expression of proteins involved in the "n+1" maturation cycles (Fig. 5 Supplementary 1B) during the MCC variant. Additionnaly, at the end of MCC differentiation, procentrioles transiently display both "n" (SAS6+ foundations) and "n+1" (CEP164 appendages) characteristics (Al Jord et al., 2014), which is never observed in cycling cells.

To further test whether a true superposition exists, we tested whether and when the remaining maturity feature, i.e. the enhanced MT nucleation capacities, was happening during the MCC variant. In the canonical cell cycle, this is called centrosome maturation, occurs at the G2/M transition and is dependent on PCNT recruitment and the activation of an AurA-PLK1 kinase cascade(Joukov & De Nicolo, 2018)(Blanco-Ameijeiras et al., 2022). Staining for PLK1 and PCNT in MCC progenitors, we observed that PLK1 becomes visible at procentrioles during G-stage and that G-stage procentrioles recruit significantly more PCNT than A-stage procentrioles (Fig. 5C, Fig. 5 Supplementary 2A-C). This is true even in the absence of deuterosomes (DEUP1 knock out cells, Fig. 5 Supplementary 2D). To test whether procentrioles displayed enhanced MT nucleation capacities at the A-to-G transition, we performed MT regrowth experiments (see

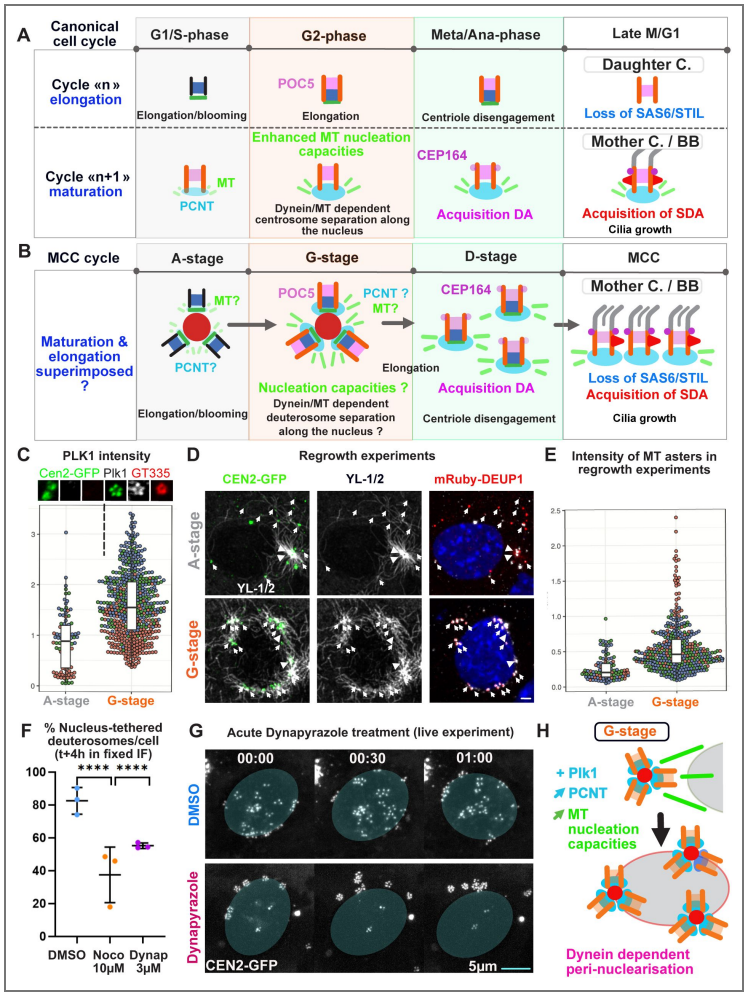

Figure 5. Centriole biogenesis is accelerated during a 2-in-1 MCC cell cycle variant

(A-B) Scheme comparing centriole biogenesis during centriole duplication in the canonical cell cycle and during centriole amplification in the MCC cell cycle variant. DA: distal appendages, SDA: sub-distal appendages, MT: microtubules. **(C)** Quantification of PLK1 intensity in A-stage and G-stage brain MCC samples using a linear regression model, with stages as the main effect and replicate as a fixed covariate, to account for batch variation. Statistical significance was assessed using Type II ANOVA. The lower and upper hinges correspond to the 25th and 75th percentiles. The upper (lower) whisker extends from the hinge to the largest (smallest) value no further than 1.5 * IQR from the hinge (where IQR is the inter-quartile range, or distance between the 25th and 75th percentiles); three independent experiments were quantified and colored differently; n= 32 cells; ****p<0,0001. Insets are from Figure 5 Supplementary 2A. **(D)** Immunostaining profile of YL1/2 in CEN2-GFP;mRuby-DEUP1 expressing brain MCCs in A- and G-stage during regrowth experiments (see methods). Arrowheads indicate centrosomal centrioles. Arrows indicate the position of A- or G-stage procentrioles. Scale bar, 1 µm. **(E)** Quantification of microtubule aster intensity in A-stage and G-stage brain MCC samples during regrowth experiments using a linear regression model, with stages as the main effect and replicate as a fixed covariate, to account for batch variation. Statistical significance was assessed using Type II ANOVA. The lower and upper hinges correspond to the 25th and 75th percentiles. The upper (lower) whisker extends from the hinge to the largest (smallest) value no further than 1.5 * IQR from the hinge (where IQR is the inter-quartile range, or distance between the 25th and 75th percentiles); three independent experiments were quantified and colored differently; n= 56 cells; ****p<0,0001. **(F)** Quantification of the percentage of tethered deuterosomes per G-stage cell in DMSO , Nocodazole (10 µM, 4h) and Dynapyrazole (3 µM, 4h). Deuterosomes above or below the nucleus are not quantified. Four independent experiments quantified; n=15 cells in DMSO, n=26 cells in Nocodazole, n=17 cells in Dynapyrazole. Error bars represent mean ± SD; ****p<0,0001; Chi-2 test with Yates' correction. **(G)** Still images from a movie of G-stage CEN2-GFP expressing cells treated with DMSO or Dynapyrazole 3 µM at t=0. While "centriole-loaded deuterosomes" keep a perinuclear distribution in DMSO treated cells, in Dynapyrazole treated cells, the "centriole-loaded deuterosomes" start untether from the nucleus at t+30 min. Shaded grey ovals outline the nucleus identified with contrasting CEN2-GFP. scale bar, 5 µM. See Video NEW17. **(H)** Scheme synthesizing the main findings of main and supplementary Fig. 5 on G-stage procentrioles maturation and nuclear tethering.

methods) in both MEF-induced and brain MCCs. We found that GT335+ G-stage procentrioles display a microtubule regrowth efficiency significantly greater than A-stage procentrioles which show a weak ability to nucleate MT (Fig. 5D-E, Fig. 5 Supplementary 2E-F). Consistent with a process comparable to the centrosome maturation occurring at the G2/M transition, scRNAseq analysis reveals that proteins involved in centrosome maturation (PLK1, AURKA, CEP192, PCNT) are expressed during the MCC variant (Fig. 5 Supplementary 1C),

In the canonical cell cycle, centrosome maturation is concomitant with the dynein dependent attraction of centrosomes to the nucleus and their consecutive migration on the nuclear membrane for bipolar spindle formation at mitosis onset (Fig. 5A). Interestingly, in MCC, the A-to-G transition is marked by a collective migration of centriole-loaded deuterosomes from the centrosome to the nucleus where can arrange as equally distant structures forming a belt around the nucleus (e.g in Fig. 6 Supplementary 1A, (Al Jord et al., 2017, 2019)). Live imaging mRuby-DEUP1/CEN2-GFP cells at this stage confirms this switch and further reveals that procentriole-loaded deuterosomes can migrate for several hours along the nuclear membrane, suggesting a strong and dynamic attachment (Fig. 5 Supplementary 2G-H, *video 16*). Nucleus migration, tethering and physical distancing suggest that the same mechanisms driving newly formed centrosomes to the opposite poles of the nucleus during early prophase, are also used by G-stage centrioles loaded on deuterosomes, to organize around the nucleus. Consistently, scRNAseq analysis reveals that most of the proteins involved in centrosome migration to the nucleus (CENPF and dynein subunits DYNLRB2, DYNLL1), centrosome dysjunction (TPX2, AURKA, NEK2) are expressed during the MCC variant and with a dynamics globally comparable to the canonical cell cycle (Fig. 5 Supplementary 1C). To further test this hypothesis, we treated MCC with nocodazole (10 µM) for 4h and found that this prevented the migration of G-stage procentrioles to the nuclear membrane, which was reversed in washout experiments (Fig. 5F, Fig. 5 Supplementary 2I-J). In addition, live treating cells in which centrioles have already migrated to the nucleus with nocodazole (10 µM) induce their detachment within minutes (Fig. 5 Supplementary 2K). A block in centriole tethering during G-stage was similarly observed in fixed and live conditions under dynapyrazole treatments (Fig. 5F-G) showing that centriole migration to the nuclear membrane is driven by a dynein and MT dependent mechanism, like during centriole duplication in the canonical cell cycle (Fig. 5H).

Collectively, these data show that in MCC, the elongation of procentrioles respects the step-wise dynamics observed during the canonical “n” cycle (Fig. 3J, 5A) and is superimposed by the acquisition of maturity features, which respects a canonical step-wise dynamics comparable to a canonical “n+1” cycle (Fig. 5B). By superimposing the two processes, the MCC variant skips the daughter centriole step and drives the production of mature basal bodies in a single cycle iteration (Fig. 5A-B). In addition, this “2-in-1” cycle confers procentrioles with high MT nucleation properties by G-stage, allowing their efficient self-organization around the nuclear membrane. Given the similarities in both centriole dynamics and expression of core molecular regulators, this perinuclearisation probably occurs through the same mechanisms as centrosome bipolarisation at mitotic entry (Fig. 5H) (Agircan et al., 2014).

Disengagement of centrioles in MCC involves deuterosome splitting and/or dissolution at the nuclear membrane

Once at the nuclear membrane, G-stage procentrioles disengage from deuterosomes or centrosomal centrioles, which constitute the so-called disengagement D-stage (Al Jord et al., 2017). Single deuterosome tracing through CEN2-GFP monitoring show that disengagement always occurs in contact with the nuclear membrane (Fig. 6A, Fig. 6 Supplementary 1A, *video 17*). Consistently, super-resolution imaging on CEN2-GFP+ G- and -D-stage cells immunostained with anti-nuclear pore complex proteins reveals procentrioles intermingled within nuclear pores (Fig. 6 Supplementary 1B, *video 18*). The global dynamics of disengagement has previously been characterized as the dismantling of empty and plain CEN2-GFP+ flower-like structures, representing CEN2-GFP+ procentrioles organized as flowers around CEN2-GFP negative deuterosomes or CEN2-GFP+ centrosomal centrioles respectively (“deuterosome” pathway and

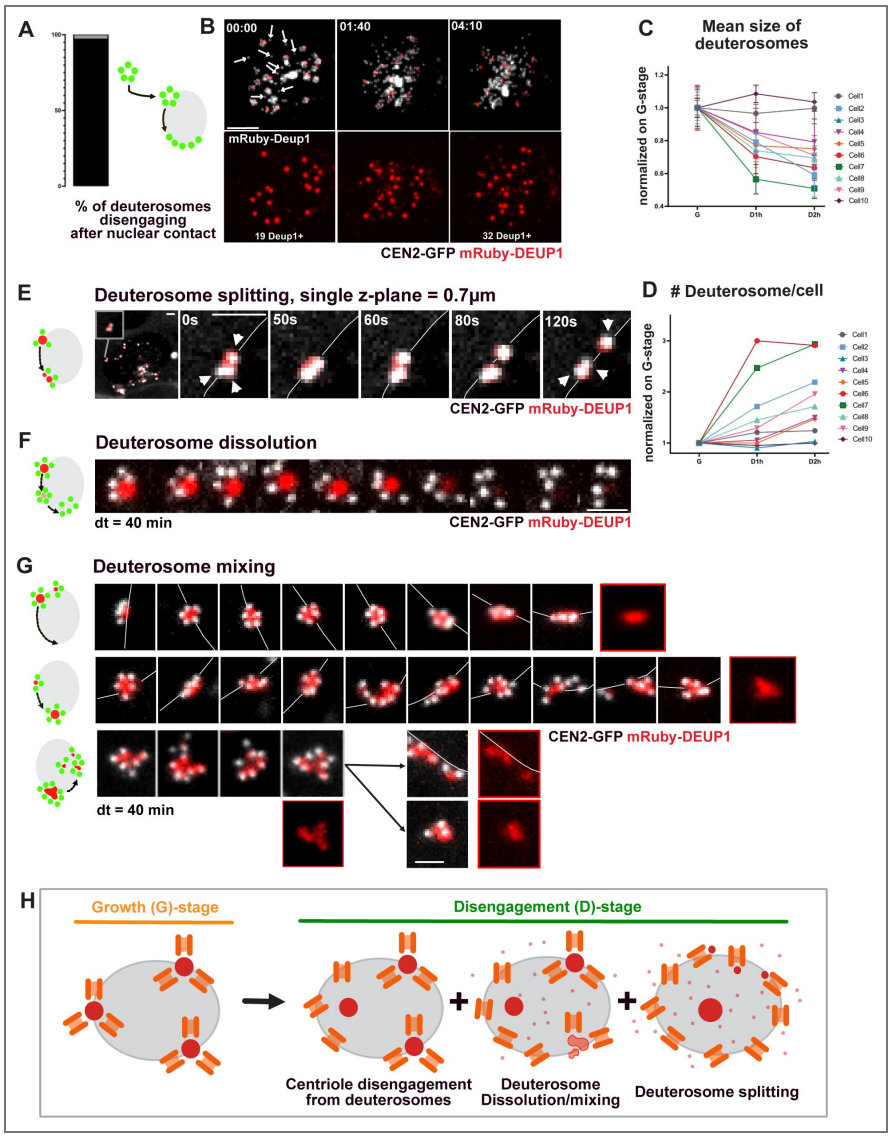

Figure 6. Disengagement of centrioles in MCC involves deuterosome splitting and dissolution

(A) Quantification of the proportion of procentrioles disengaging with a nuclear contact. Three independent experiments were quantified; n=98 deuterosomes, n=8 cells. See also *Video 17*. **(B)** CEN2-GFP; mRuby-DEUP1 dynamics in D-stage. Upper panels: in early D-stage (00:00), some procentrioles are already individualized, separated from DEUP1+ deuterosomes as shown by the white arrows. During D-stage, procentrioles disengaged from deuterosomes can keep a subunit of DEUP1 in their proximal portion, splitting them in smaller entities bearing fewer procentrioles not disengaged yet, as well as DEUP1 signal dissolution. Lower panels: grayscale showing the initial pool of 19 deuterosomes (00 :00) splitting to 32 DEUP1+ foci (4 :10). dt=50min; scale bar, 5 µM. See also *Video 19*. **(C-D)** Quantification of the size and number of deuterosomes per cell over time in D-stage relative to G-stage. n=10 cells analyzed were plotted individually to reveal the tendency to decrease in size, while increasing the number of DEUP1 foci. Each measurement in D-stage was normalized on G-stage. Three independent experiments were scored; error bars represent mean ± SD. **(E)** CEN2-GFP;mRuby-DEUP1 time lapse images of a single z-plane (0.7µm) of a D-stage cell showing a deuterosome which seemingly split into smaller deuterosomes migrating away from each others along the nuclear envelope. White dotted line outlines the nuclear membrane identified with contrasting CEN2-GFP signal. Left picture is the zoom-out picture of the D-stage cell, showing the position of the cropped still images. dt=5s, scale bar, 1 µm. See *Video 20* to see the entire movie. **(F)** Time lapse images of a D-stage cell showing the deuterosome mRuby-DEUP1+ signal dissolution correlated with CEN2-GFP rosette dismantlement. dt=40min, scale bar, 1 µm. See also *Video 21*. **(G)** CEN2-GFP;mRuby-DEUP1 time lapse images of a D-stage cell showing migration of a centriole-loaded deuterosome along the nuclear membrane (line 1), change of deuterosome shape and mixing with another deuterosome (line 2), followed by consecutive unmixing (line 3). dt=40min, scale bar, 1 µm. See *Video 22* to see the entire movie. **(H)** Scheme synthesizing the main findings of main and supplementary Fig. 6 on D-stage deuterosome dynamics and centriole disengagement.

"centriolar" pathways; Fig. 6 Supplementary 1A, (Al Jord et al., 2014)). To further highlight deuterosome behavior during disengagement, we monitored in live single cells expressing mRuby-DEUP1 in addition to CEN2-GFP.

At the global cell scale, the beginning of disengagement is marked by the apparition of single DEUP1 negative procentrioles suggesting that centrioles can detach from deuterosomes, like they disengage from parental centrioles in cycling cells (Fig. 6B, 00:00, white arrows). Then, quantifications reveal that the size of deuterosomes decreases while their number increases over time, in single cells, indicating that deuterosomes split during disengagement (Fig. 6B; 01:40; Fig. 6C-D, 10 cells; *Video 19*). In parallel, a DEUP1 diffuse signal, absent during G-stage, appears in the cytoplasm suggesting that splitting is accompanied by DEUP1 structure dissolution (*Video 19*). Sometimes, at the end of disengagement, mRuby-DEUP1+ structures aggregate into larger structures until ciliation, before dissolving (Fig. 6 Supplementary 1C).

We then increased spatial resolution and followed single deuterosomes. This allowed to observe deuterosomes that seemingly split around the nuclear membrane, giving rise to deuterosomes with single centrioles (Fig. 6E, *Video 20*). Also, we identified that dissolution of mRuby-DEUP1+ signal from the center of CEN2-GFP+ flowers was concomitant with centriole distancing (Fig. 6F, Fig. 6 Supplementary 1D, *Video 21*). Finally we found that deuterosomes can loose their spherical shapes and/or intermingle with others before the mRuby-DEUP1 signal split and finally disappears (Fig. 6G, *video 22*). All these events are observed along the nuclear membrane.

Altogether this suggests that disengagement is mediated by a change in the physical properties of DEUP1 aggregates allowing the splitting and/or dissolution of deuterosomes (Fig. 6H). Whether this results from post-translational modifications of DEUP1 by APC/C, PLK1 and/or separase, which are known to be required for centriole disengagement in both cycling cells and MCC, requires further examination (Al Jord et al., 2017; Prosser et al., 2012; Revinski et al., 2018; Tsou et al., 2009; Tsou & Stearns, 2006). In favor of a common molecular control, centriole disengagement from centrosomal centrioles and deuterosomes occurs concomitantly in differentiating MCC (Fig. 6 Supplementary 1A), and the dynamics of disengagement (duration and perinuclear location) of doublets or triplets of centrioles when deuterosomes are absent (DEUP1 knock-out cells), seems comparable to controls (Mercey et al., 2019b).

Disengagement of centrioles in MCC is assisted by MT and dyneins

Since centrioles disengage along the nuclear membrane which they reach through a dynein dependent process, we then sought to test if mechanical forces are also involved in centriole disengagement. In fact, if perinuclearisation mechanisms are the same in MCC progenitors and cycling cells, it means that dyneins bound to nuclar pores are running on MT nucleated by individual G-stage procentrioles. We reasoned that this would lead to procentrioles being pulled apart from each other's, which could contribute mechanically to deuterosome splitting and centriole disengagement. In favor of such a scenario, D-stage centrioles are on the same z-section (250 nm) as nuclear pores (Fig. 6 Supplementary 1B, *Video 18*), they continue to migrate along the nuclear membrane after deuterosome splitting (Fig. 7A, *Video 23*), and nocodazole (10 µM) induces their immediate detachment from the nucleus (Fig. 7 Supplementary 1A). Also, MT regrowth experiments show that single D-stage centrioles are nucleating MT (Fig. 7B) and super-resolution confocal imaging show that disengaging centrioles are co-localizing with MT (Fig. 7C, *video 24*). Finally, we identified a transient stage during which disengaging procentrioles redistribute in the 3 dimensions along the nuclear membrane, which is coherent with the existence of isotropically distributed traction forces on the nuclear surface (Fig. 6 Supplementary 1B, 4:30, *video 18*).

To functionally test whether MT attachment of the centrioles to the nuclear membrane assists mechanically their disengagement, we chronically treated brain MCCs with increasing doses of nocodazole (1-5 µM, 48h) and analyzed whether procentrioles were able to individualize. We observed that at low doses, the proportion of cells in "D-stage" remained unchanged, but significantly more "D"-stage cells showed incomplete disengagement compared to controls (Fig.

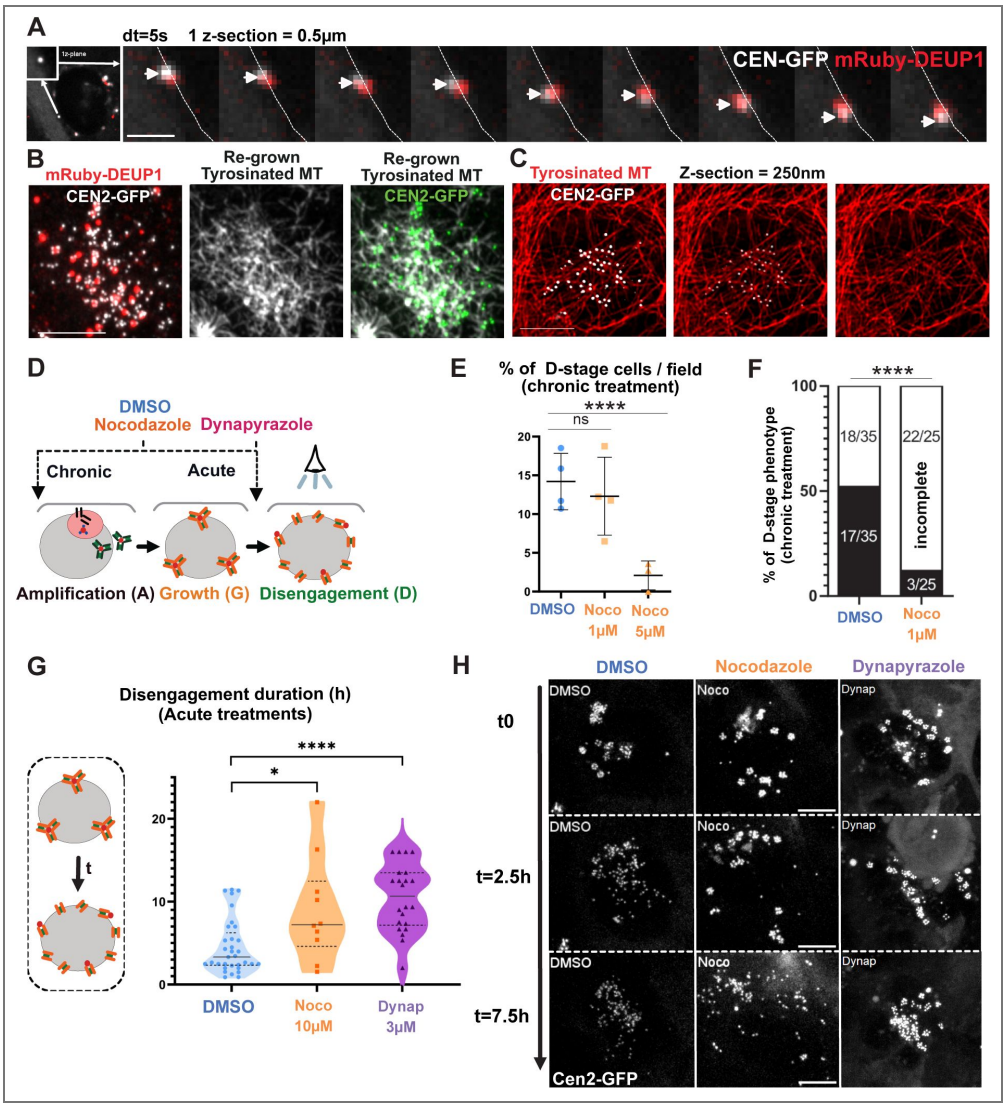

Figure 7. Disengagement of centrioles is assisted by MT and dyneins

(A) Disengaged procentriole associated to a deuterosomal subunit migrating on the nuclear envelope. White arrow head indicates the centriole, the white dot line outlines the shadow of the nuclear membrane seen in CEN2-GFP. dt=5s; Single z-section of 0.5 µm, scale bar, 2 µm. See *Video 23* to see zoom out and whole movie. **(B)** Representative picture of tyrosinated microtubules (YL1/2) and DEUP1 immuno-reactivity in brain CEN2-GFP MCC during D-stage after MT depolymerization through nocodazole treatment (10 µM, 24h) and subsequent nocodazole whashout (4 h). Scale bar, 5 µm. **(C)** Tyrosinated microtubules and deuterosomes immuno-reactivity profiles in brain CEN2-GFP MCC in D-stage. These pictures show one z-section (0.25 µm) in the basal part of the cell. Tyrosinated MTs were stained with (YL 1-2). CEN2-GFP signal intensity is decreased from left to right to show that centrioles are located next to MT. Scale bar, 5 µm. See *Video 24* to see all z-sections and DEUP1 staining. **(D)** Schematic representation of brain MCC differentiation depicting the timing of drug treatments. The eye shows the amplification stage monitored. **(E)** Quantification of the proportion of D-stage cells per fields among amplifying cells under chronic DMSO and Nocodazole treatments (48h, 1 µM and 5 µM). At least three independent experiments were quantified, n=2626 DMSO cells, n=1483 Noco 1 µM cells, n=909 Noco 5 µM cells; error bars represent mean ± SD. ns, not significant; ****p<0,0001; non-parametric Mann Whitney test. **(F)** Quantification of D-stage quality phenotype under chronic DMSO and Nocodazole (48h, 1 µM). Total disengagement in black, incomplete disengagement in white. At least three independent experiments were quantified, n=35 DMSO cells analyzed, n=25 Noco 1 µM cells analyzed. ****p<0,0001; Chi-2 test with Yates' correction. **(G)** Quantification of disengagement duration in CEN2-GFP brain MCCs under DMSO and acute Nocodazole (10 µM) or Dynapyrazole (3 µM) treatments. >4 independent experiments were quantified, n=32 DMSO cells, n=10 Nocodazole cells and n=22 Dynapyrazole cells. *p<0,05; ****p < 0/0001 non-parametric Mann Whitney test. **(H)** Representative movie of CEN2-GFP dynamics in D-stage of brain MCCs under DMSO and acute Nocodazole (10 µM) or Dynapyrazole (3 µM) treatments. (t-1) represents the timepoint before drug treatments. scale bar, 5 µm. See *Video 25* for whole movies.

7D-F). Interestingly, within the cells showing incomplete disengagement, we often saw SAS6 negative procentrioles still engaged to deuterosomes, a phenotype rarely observed in controls (Fig. 7 Supplementary 1B-C, white arrows). Indeed, SAS6 normally disappears from procentrioles when centrioles are docked, just before ciliation (Al Jord et al., 2014). This suggests that, despite an active APC/C (responsible for SAS6 degradation in cycling cells (Arquint & Nigg, 2016), and involved in centriole disengagement in MCC (Al Jord et al., 2017)), centrioles failed to disengage from deuterosomes. Then, with higher dose of nocodazole (5µm), "D"-stage cells are rarely observed (Fig. 7E). These observations suggest that microtubules are required for efficient centriole disengagement. To confirm and to further assess the role of dyneins, we performed acute nocodazole (10 µM) and dynapyrazole (3 µM) treatments on G-stage cells, and live-monitored the dynamics of disengagement. We observed, in both cases, a detachment of procentriole-loaded deuterosomes from the nuclear membrane and a long, unsynchronized and sometimes incomplete disengagement (Fig. 7 G-H, *video 25*).

Altogether these data suggest that the acquired ability of the newly formed centrioles to nucleate microtubules at the A-to-G transition, allows their dynein-dependent nuclear attachment, which assists mechanically their disengagement during the following D-stage.

Disengaged centrioles finally converge apically with the former centrosome

After disengaging, centrioles detach from the nuclear membrane (Fig. 6A; 10:00) and migrate from all over the nucleus to the apical cell surface (Fig. 6 Supplementary 1A; 10:00-14:00) to dock and nucleate motile cilia (Boisvieux-Ulrich et al., 1987, 1990; Boisvieux-Ulrich et al., 1989). This is followed by an acto-myosin dependent planar basal body constriction (Hirota et al., 2010). Dynamics of this collective migration process has never been analyzed and is tedious since MCCs produce several dozens of centrioles that are therefore difficult to track. To begin, we first analyzed the global 3D displacement of all centrioles in individual cells, by segmenting the CEN2-GFP signal throughout their apical migration over time-lapse imaging. As a cellular reference, we took one of the centrosomal centrioles, trackable using their higher CEN2-GFP fluorescence, in cells where the 2 centrosomal centrioles stay close together. This global quantification showed that, at the end of disengagement, centrioles are evenly distributed within a mean radius of 8 µM with respect to the tracked centrosomal centriole (in the range of the nucleus radius). Then, by the end of apical migration and basal body constriction, centrioles are predominantly (65%) located in a radius of 3 µM around this reference point (Fig. 8A). This significant histogram shift (Chi² test, $p<0.0001$) suggests a collective movement of newly formed centrioles that bridges them together and with the centrosomal centrioles. Consistently, centrosomal centrioles finally dock within the newly formed basal body patch and also nucleate cilia (Liu et al., 2020).

To then analyze the displacement dynamics of individual centrioles, we performed live imaging with high temporal resolution (dt=5min) over the entire migration process. This allowed us to manually track a subset of migrating centrioles and catch their individual trajectories before they reach the group (Fig. 8B, *Video 26*). Single centrioles move at 0,12+/-0,09 µm.min^{-1}. Consistent with the global 3D gathering of the whole centriole population, they are seen converging apically toward the centrosomal centriole reference point as reflected by the time-correlated decrease of their distance and by the mean vector directionalities of individual trajectories (Fig. 8B-C, *Video 26*). Although single centrioles display a global directional migration, analysis of the shape of the trajectories, speed of displacement and directionality with regard to the centrosome reference point, show that their migration is complex and marked by back-and-forth movements oscillating between diffusive-like and more processive steps (Fig. 8D, Fig. 8 Supplementary 1A). As steps toward the centrosome are more numerous and more rapid than steps away (Fig. 8 Supplementary 1B), centrioles finally slowly converge at 0,01+/-0,03 µm.min^{-1}. Interestingly, within this complex behavior, the vertical z-motion, when centrioles below the nucleus migrate up around

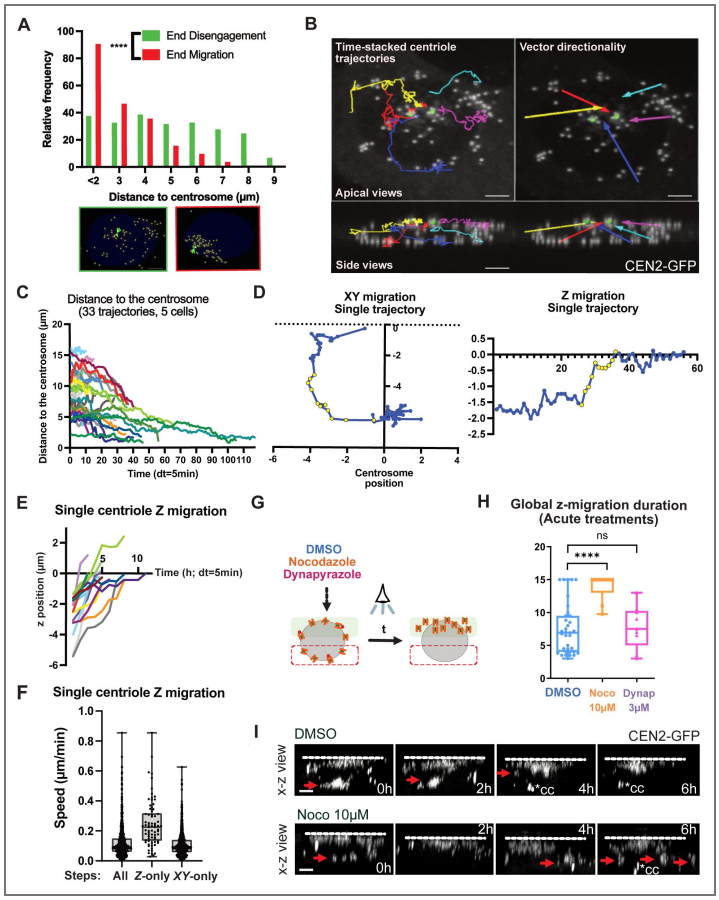

Figure 8. Disengaged centrioles finally converge with the former centrosome

(A) Quantification of the relative frequency of the centriole position regarding the centrosomal centriole (CC) at the end of centriole individualization (green) and the end of apical migration (red). Bottom: representative apical projections of segmented CEN2-GFP+ centrioles at the end of centriole individualization (green frame) and the end of apical migration (red frame). Green arrow shows the centrosome which served as the reference point; the nucleus is in dark blue. n=3 cells; scale bar, 5 µM. **(B)** Manual single centriole 3D tracking reveals convergence of centrioles towards the centrosome region. Top left panel: apical view of manually tracked migrating centrioles trajectories. Top right panel: apical view of the directionality vectors of the migrating centrioles. Bottom panel: corresponding profile views of the top panels. dt=5min; scale bar, 5 µM. See *Video 26*. **(C)** Evolution of individual centriole distance to centrosome over time. dt=5min. Three independent experiments were analyzed, n=33 centriole trajectories in n=5 cells. **(D)** Example of the single blue centriole trajectory from (B) towards the centrosome in the XY plane and in the Z plane. Yellow dots represent the steps of apical migration. The origin represents the position of the centrosome. See *Video 26*. **(E)** Evolution of the Z coordinates during the apical migration of 16 centrioles from 5 cells. Three independent experiments were analyzed. The origin represents the Z position of the centrosome. dt=5min. **(F)** Speed of migration plotted from all 953 steps of 33 migrating centrioles versus the 74 extracted steps of apical migration of the 16 centrioles basally located in the cells. Box and whiskers plots (2,5-97,5 percentile). See distribution in Fig. 8 Supplementary 1C. **(G)** Schematic representation of brain MCC differentiation depicting the timing of MT depolymerisation treatment. The eye shows the amplification stage monitored. **(H)** Quantification of apical migration duration in DMSO and acute nocodazole (10 µM) or dynapyrazole (3 µM) in brain MCCs. Eight independent experiments were scored for n=32 DMSO cells, 3 experiments for n=10 nocodazole cells and 5 experiments for n=8 dyanpyrazole cells. Error bars represent min to max ± median. ****p<0,0001; non-parametric Mann Whitney test. **(I)** Profile view of centriole apical migration after D-stage in CEN2-GFP+ DIV2 brain MCCs treated with acute DMSO and Nocodazole 10 µM. At t-1, cells display late G-stage/early D-stage procentrioles and are not treated with any drug. DMSO and Nocodazole 10 µM were added right after the first timepoint acquisition. In DMSO, 2 centriole patches are above and below (red arrow) the nucleus (t+0h). The patch below the nucleus progressively joins the apical one (t+2h) to become one apical patch (t+4h) and stabilizes (t+6h). In 10 µM Nocodazole, procentrioles start to scatter after 1h, and the group of centrioles below the nucleus travel up and down (t+2h, t+4h and t+6h) and does not succeed to become one apical group of centrioles (t+6h). Red arrowheads point to group of centrioles that we consider as basally localized. *CC corresponds to the centrosome of a neighboring cell. dt=5min; scale bar, 5 µm. See also *Video 27*.

the nuclear membrane (Fig. 8B; light blue, dark blue and red trajectories), is clearly processive (Fig. 8D, yellow dots; Fig. 8E) and more rapid (0,25+/-0,16 µm.min^{-1}, Fig. 8F, Fig. 8 Supplementary 1C), than the steps in the XY plane (0,11+/-0,08 µm.min^{-1}).

To define the involvement of microtubules and dyneins in this collective migration process we compared the global dynamics of migration in control and cells treated with nocodazole (10 µM) or dynapyrazole (3 µM) from the end of D-stage. Under these treatments, the centrosomal centrioles moved too much to be traced. In the absence of a reference point, we restricted our analyzis to the quantification of the baso-apical migration. This z-migration was defined as being the duration between the take-off of the first centriole of the group below the nucleus, until the last one is reaching the apical side of the cell (Fig. 8G). While in the control, global z-migration duration lasted 7,5 +/- 3,9h, under nocodazole, complete migration was generally not reached during the course of the 15h live imaging experiments (migration duration = 14 +/- 2h) and was marked by up and down migration of groups of centrioles (Fig. 8H-I, *video 27*). Interestingly, under dynapyrazole treatment, even if D-stage centrioles detach from the nuclear membrane, they subsequently succeed in migrating up, with a dynamics and a duration comparable to the controls (Fig. 8H, migration duration = 7,8 +/- 3,2h). This suggests that, like for apical centrosome migration during primary ciliogenesis, the dynamics is MT dependent, but dynein-independent (Pitaval et al., 2017). Interestingly though, when waiting longer by treating the cells chronically with nocodazole for 48h (1-5 µm), centrioles seem to finally succeed in migrating up (Fig. 8 Supplementary 1D), even if they fail to gather accurately in the XY plane (Fig. 8 Supplementary 1E-F). This may explain why the first report on quail oviduct MCCs failed to detect any effect of MT depolymerization on final centriole apical positionning (Boisvieux-Ulrich et al., 1989).

Altogether, these data show that centrioles migrate collectively, following a complex and slow motion, from all over the nuclear membrane, to gather with the former centrosome. The migration is characterized by fluctuations between forward and backward directionalities and between processive and diffusive-like movements, but switches to a more processive and rapid motion when directed toward the apical pole. This migration is altered under microtubule depolymerization but seems independent from dynein motors. Wether the apical migration is dependent on actin and Cep164-dependent MT stabilization pushing the centrioles apically, like proposed for primary ciliogenesis, requires further examination (Pitaval et al., 2017).

Discussion

Mature centrioles can be produced in less than an hour in amoebae differentiating into flagellates (Fritz-Laylin et al., 2016). However, in the large majority of vertebrate cells, centriole biogenesis is curbed within a double iteration of the cell cycle, allowing to meet the need of a single primary cilium at the end of cell division. Here, we show that the recently described MCC cell cycle variant is an accelerated version of the cell cycle which superposes two centriole biogenesis cycles (elongation and maturation), to obtain multiple mature centrioles within a single cycle iteration. The precocious maturation of amplified procentrioles is even determinant for their spatial self-organization, disengagement and apical migration.

A pericentrosomal nest

Using live imaging on brain MCC, we highlight that concentration of core deuterosome and centriole proteins around the centrosome precedes the biogenesis onset of deuterosomes and procentrioles. We named this transitory compartment a “nest” since deuterosomes and procentrioles emerge specifically in this region and grow while moving away from it. We highlight that this nest is composed, at least, of DEUP1, PCM1, PCNT and CENTRIN2 and is dependent on MT and dyneins. Its dissolution through MT depolymerization or dynein inhibition is correlated with an alteration -or even a block- in the formation of deuterosomes and procentrioles, suggesting that concentration of core elements of deuterosome and centriole organelles permit their efficient massive production.

This concentration of proteins can result from the retrograde transport of the proteins themselves, as suggested by the movements of DEUP1 or CEN2-GFP foci. Non exclusively, it could result from retrogradely transported mRNA and local translation at centriolar satellites. In fact, the core component of centriolar satellites PCM1 (i) is highly enriched in the pericentrosomal nest, (ii) diffuse when MT are depolymerized, and (iii) has been recently shown to be sites of local translation of centrosomal and ciliary proteins in vertebrate cells (Pachinger et al., 2025). Local translation of centriolar and ciliary proteins, notably at the centrosome, has been shown to be involved in both centriole (over)duplication and primary ciliation (Fang & Lerit, 2022; Martinez et al., 2025; Pachinger et al., 2025; Villa et al., 2025). In brain and airway MCC, PCM1 depletion alters deuterosome formation and centriole production (Hall et al., 2023; Zhao et al., 2021). Such concentration of centriolar proteins by a retrograde MT transport would explain why, (i) in DEUP1 KO MCC where deuterosomes are absent, centrioles are emerging on, and around, the centrosomal centrioles (Mercey et al., 2019), and (ii) when both centrosome and deuterosomes are absent, procentrioles emerge in a cloud of PCM, at the convergence of the microtubule network (Mercey et al., 2019) . Our observations are also consistent with theoretical modeling, simulations and *ex cellulo* experiments suggesting that the place where a critical threshold level of PLK4 activation is surpassed determines procentrioles' apparition spot (Nabais et al., 2021; Yamamoto & Kitagawa, 2019).

Whether the existence of such a pericentrosomal nest exists in respiratory and reproductive cells is unclear. Live imaging is difficult to perform because cells are in a stratified epithelium. In addition, the number of centrioles produced is much greater than in brain cells, and their production seems to be rapid. Consequently, the early dynamics of amplification is difficult to describe. However some studies in fixed cells at early stages observed a pericentrosomal onset of procentrioles and deuterosomes emergence in chick and mouse airway MCCs (Al Jord et al., 2014; Kalnins et al., 1972; Lu et al., 2025; Mori et al., 2017). Also, our observations in non-tissue specific micro-patterned MEF-MCCs suggest that, at least the onset of amplification, occurs in the pericentrosomal region, in a MT-dependent manner. One can hypothesize that, in airways MCC producing more centrioles, the concentration of centriolar proteins may reach the threshold for centriole biogenesis in the pericentrosomal region first, and then rapidly in the rest of the cytoplasm. Also, pericentrosomal localization of pericentriolar satellites is not a conserved feature (Pachinger et al., 2025) and may differ in airway MCC.

Dynamics in the pericentrosomal nest and earliest steps of deuterosome formation

The pericentrosomal nest hosts the formation of early deuterosomal structures whose size and shape are diverse. Correlative light and electron microscopy reveals that early deuterosomes can appear as horse-shoe or spherical shaped structures, not larger or 4-times bigger than a procentriole. In all cases, they bear very few or no centriole, suggesting that the size of young deuterosomes does not correlate with the number of centrioles they associate with. Consistently, size and number of deuterosomes are not changed when PLK4 is depleted and centrioles absent (LoMastro et al., 2022). Further live imaging suggests that small structures become spherical and grow over A-stage progression, and large structures resize into smaller entities. We still don't know which mechanisms (fusion, accretion, splitting, etc.) drive (i) the homogenization of deuterosome size/shape and (ii) the scaling of deuterosome size with centriole number, both observed when arriving at G-stage.

Our data suggest that the pericentrosomal nest is a particular micro-environment characterized by the convergence of MT, the accumulation of centriolar satellites, a concentration of centriole and deuterosome proteins (and maybe mRNA), and the emergence of multiple new organelles. Whether this leads to regional changes in the rheology of the cytoplasm which could facilitate/stabilize the formation of deuterosomes and centrioles is challenging but would be worth studying (Delarue et al., 2018; Fernandes-Mariano et al., 2025; Kalbfuss & Gönczy, 2023).

Interestingly, our live imaging identifies that mRuby-DEUP1+ and CEN2-GFP+ foci (or deuterosomes) are moving with stochastic back and forth fluctuations to the centrosome in a MT- and dynein dependent manner. Random change of cargoes direction alternating between runs and very long pauses exists in numerous cellular contexts. They are driven by complex mechanisms ranging from the binding of cargoes to dyneins and kinesins which can compete or activate each-others to the detachment of motor-cargoes complexes from MT due to their interaction with the surroundings (Hancock, 2014; Shen et al., 2025). Alternative to a direct transport of protein complexes by molecular motors, minus-end directed streaming flows were shown to be produced by the motion of dyneins on MT, and could indirectly drag, or contribute to drag, protein complexes toward the centrosome (Shinar et al., 2011). Consistent with a passive viscous drag of pericentrosomal material, the CEN2-GFP+ aggregates, frequently present in cells over-expressing CEN2-GFP, can also be attracted to the centrosome and subjected to back and forth movements (see *Video 4*).

Mother/daughter asymmetries at the centrosome during centriole amplification

Deuterosomes are frequently seen connected to the daughter centriole during A-stage (Al Jord et al., 2014; Khoury Damaa et al., 2025). Live imaging mRuby-DEUP1 now suggests that this connection can last for hours and be marked by back and forth fluctuations (Fig. 2 Supplementary 1C, E, *Video 2*, *6*, *7*). In addition to DEUP1, PLK4 and SAS6 also accumulate to the daughter centriole both in WT and DEUP1 KO cells (Al Jord et al., 2014; Mercey et al., 2019b, this study). In DEUP1-KO cells, this is accompanied by a larger number of centrioles growing on the daughter versus mother centriole (Mercey et al., 2019b). This asymmetry has never been explained and was not altered when primary cilium growth was inhibited (Al Jord et al., 2014). Given the back and forth fluctuations we observe to one centrosomal centriole, one could consider the existence of asymmetric hydrodynamic forces at the mother versus daughter centriole.

Studying flow fields at the convergence of MT is a technical challenge. Outside cell biological contexts and for engineering purposes, flow fields existing around macroscopic or microscopic (50µm) cylinders and subjected to a directional flow are studied. It reveals the existence of horse-shoe vortical / mixing flow structures upstream of the cylinder, which can lead to horse-shoe shaped material sedimentation at the bottom of the cylinder (Cuéllar et al., 2009; Dargahi, 1989; Ozawa et al., 2023). One could make a parallel with the horseshoe deuterosomes we frequently observe in the pericentrosomal region or connected to the proximal part of the daughter centriole. Since the daughter centriole does not nucleate MT at the beginning of A-stage, it represents a microscopic cylinder subjected to a directional flow toward the mother centriole. Live imaging mRuby-DEUP1 now suggests that the connection of deuterosomes to the daughter centriole can last for hours, explaining the frequency of previous observations, and be marked by back and forth fluctuations (Fig. 2 Supplementary 1C, E, *Video 2*, *6*, *7*). Although the viscosity of the cytoplasm is much higher than the viscosity of water solutions, it is tempting to hypothesize that some vortical forces would contribute to the “sedimentation” of DEUP1 at the bottom of the daughter centriole (Al Jord et al., 2014; Khoury Damaa et al., 2025). Such asymmetric hydrodynamic contexts could explain why deuterosomes are never seen connected to the mother centriole, and seem mostly forming on the side of the daughter centriole opposite to the mother centriole. Such forces could also lead to the sedimentation of other proteins such as PLK4 and SAS6 (Al Jord et al., 2014; Mercey et al., 2019b). This mother/daughter asymmetry which does not seem to have a function in MCC progenitors (Mercey et al., 2019a), may be worth studying as it could have implications on both the control of centriole number and cell fate decision (Tozer et al., 2017).

Physical property/ies of DEUP1-composed structures

Using live imaging, we show that DEUP1 exists first as a dissolved state organized in a pericentrosomal cloud during A-stage, then, form deuterosomes which eventually split and dissolve in D-stage. They can reform spherical dense aggregates before definitively disappear in

the terminally differentiated MCC. Since mature deuterosomes are spherical membrane-less organelles, it would have been appealing to explain their formation through liquid-liquid phase separation. However, evidences accumulate in favor of a solid-like state: (i) DEUP1 first accumulates as a dissolved -yet concentrated- state in a PCM-like manner (ii) we and other did FRAP experiments on mature centriole-loaded deuterosomes composed of exogenous or endogenously tagged DEUP1 and did not observe recovery (Fig. 8 Supplementary 2; (Yamamoto et al., 2021)), (iii) we never observed fusion of mature G-stage deuterosomes, that frequently bump into each other, in the tens of different movies we analyzed (*Video 30*), (iv) consistently, the number of deuterosomes seems stable during G-stage as previously quantified in single cell live experiments (Mercey et al., 2019a). These evidences are consistent with the hypothesis of Sorokin in 1968 proposing that deuterosome results in solid crystallization of a super-saturated solution of deuterosome components (Sorokin, 1968). However, before deuterosomes disappear during D-stage, they frequently merge or split in/into amorphous structures (Fig. 6G), suggesting a change in the physical properties of the organelle between G- and D-stage, in favor a liquid-like state. Such change could facilitate deuterosome dismantlement and could result from post-translational modifications during the APC/C dependent G-to-D transition (Al Jord et al., 2017). Given that disengagement is dynein and MT dependent, the change in physical properties may also involve a dynein dependent transport of molecular actors such as separase.

The debate is intense for identifying the physical properties of the centrosome, which is massively studied organelle (Raff, 2019). We will probably have to wait better tools to assess this question for the different stages of deuterosome structures, keeping in mind that a cellular structure may probably not behave like any of the solid or liquid ideal state.

A 2-in-1 centriole biogenesis cycle

Using CEN2-GFP live imaging and immunostainings, we previously described that procentrioles forms sequentially during A-stage, and accumulate in a latency immature state until they synchronously acquire POC5 and get MT wall polyglutamylated (GT335+) during G-stage. CLEM further showed that this A-to-G transition was also marked by an increase in procentriole diameter, accompanied by the completion and lengthening of MT walls (Al Jord et al., 2014). Such widening has been also recently described, and called blooming, in cycling cells (Laporte et al., 2024). Now, the use of UExM and the identification of a spatio-temporal progression of centriole biogenesis, allowed us to further decompose the events occurring during A-stage. In the earliest stages, where only a small pericentrosomal nest is present, PLK4 and SAS6 accumulate at the parent centrioles and inside the nest. Later on, nascent PLK4+/SAS6+ procentrioles deprived of a microtubule wall grow on (or associate with) small DEUP1+ structures in the vicinity of the centrosome (0-3um). Such “naked” cartwheel assembly was also recently revealed as the earliest step of centriole biogenesis during centriole duplication in cycling cells (Laporte et al., 2024). Then, MT walls begins to appear and lengthen as procentriole migrate away from the nest, to reach a plateau at around 150 nm. This lengthening of MT walls is accompanied by a widening of the procentriole diameter that grows from 120 nm to reach a plateau at around 220 nm. The range and correlation of procentriole length and width increases are consistent with what was recently described in cycling cells during S-stage (Laporte et al., 2024). Then, during radial migration and elongation, MT walls progressively acetylate. As previously documented, the A-to-G transition is then marked by lengthening of procentrioles and further widening which falls in the range recently documented for G2 stage procentrioles and is consistent with the documented acquisition of POC5 – a G2 protein also acquired during G-stage – when procentrioles exceed 160 nm in length (Laporte et al., 2024). Finally, procentrioles, which had reached their final width during G-stage, continue to elongate during D-stage and reach a length of typical M-stage procentrioles (Al Jord et al., 2014; Laporte et al., 2024). In early MCC stage, SAS6 is degraded, as during late M-stage (Al Jord et al., 2014). Altogether, this shows that in the MCC cell cycle variant, the formation of centriole barrels - e.g. core molecular architecture, MT wall acquisition, blooming and elongation- follows the same step-wise dynamics, correlated with cell cycle stages, as in the canonical cell cycle (Fig. 9).

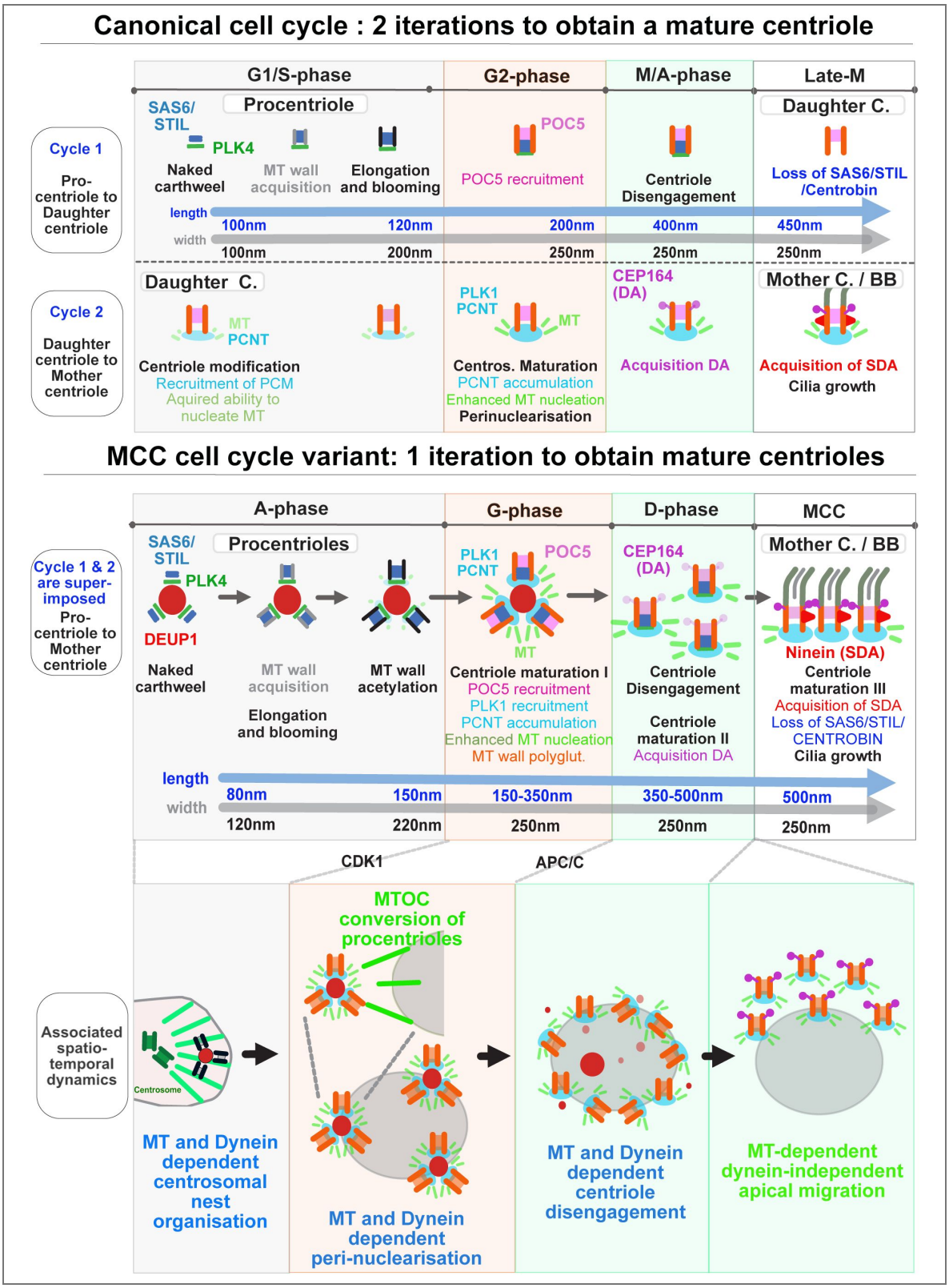

Figure 9. The MCC cell cycle variant incorporates a 2-in-1 centriole biogenesis cycle.

Parallel between centriole biogenesis during the canonical and the MCC cell cycle variants showing that centriole elongation and maturation cycles are conserved but superimposed during the MCC cell cycle. The “daughter centriole” step is skipped and prematurely mature centrioles participate in their own dynamics of sub-cellular organization disengagement and migration. DA: distal appendages, SDA: sub-distal appendages, MTOC: microtubule organizing center.

Importantly, using immuno-stainings, single cell RNA sequencing and MT regrowth experiments, we found that the acquisition of maturity features, such as enhanced MT nucleation capacities and centriole appendage recruitment, also follows a canonical step-wise dynamics correlated with cell cycle stages. However instead of occurring over two successive cycle iterations, as in the canonical cell cycle, maturation occurs on elongating procentrioles within a single iteration of the MCC cell cycle variant (Fig. 9). As a consequence, the "daughter centriole stage" is skipped for the amplified procentrioles. Their maturation is superposed with their own elongation, and is nearly concomitant with the maturation of the MCC daughter centriole (Fig. 9 Supplementary 1). Since centriole elongation and maturation in consecutive cell cycles are controlled by some common key players (CDK1, PLK1, APC/C), one can hypothesize that a molecular break is just lifted in MCC to accelerate centriole biogenesis by allowing elongation and maturation to occur concomitantly on the same centriolar structures. Such maturation of nascent procentrioles over a single iteration of the canonical cell cycle has been experimentally obtained by enhanced PLK1 activity (Kong et al., 2014). This suggests that the decomposition of centriole biogenesis in two cycle iterations is not mandatory, and may have been adopted in dividing cells to ensure the growth of a solitary primary cilium.

Precocious maturation of procentrioles allows their spatial self-organization and disengagement

The superposition of centriole elongation and maturation allows procentrioles to become strong MT nucleators from G-stage. This is accompanied by a dynein-dependent spatial switch in procentriole organization, where the focalized emergence of immature procentriole-loaded deuterosomes, characteristic of A-stage, is followed by their migration and perinuclear distribution around the nuclear membrane during G-stage. This migration and physical distancing of centrosomes and deuterosomes reminds the dynein-dependent nuclear migration of new centrosomes at mitosis onset and their consecutive separation along the nuclear membrane (Agircan et al., 2014). Since A-to-G and G2-M transitions are regulated by PLK1 and CDK1, one can suppose that the same mechanisms are at work in both cycling cells and brain MCC. Consistently, core players involved in centrosome maturation (PLK1, AURKA, CEP192, PCNT), dynein-dependent centrosome-nuclear migration (BICD2, CenpF) and centrosome dysjunction (TPX2, AurA, Nek2A-6-7-9, Mst2) are expressed with comparable dynamics in the canonical and MCC cell cycle variants (Agircan et al., 2014) (Fig. 5 Supplementary 1C).

As during centriole duplication, this perinuclearisation is followed by centriole disengagement, and is known to be PLK1 and APC/C dependent in both canonical and MCC cell cycle variants (Al Jord et al., 2017; Kim et al., 2022; Ruiz García et al., 2019). However, by contrast to the cell cycle where the nuclear disassembly precedes centriole disengagement, in MCC progenitor, the disengagement is tightly correlated with nuclear association. First, the migration of procentriole loaded deuterosomes systematically precedes centriole disengagement. Second, disengagement proceeds through centriole separation from deuterosomes and deuterosome splitting along the nuclear membrane. Third, disengagement is followed by the migration of individualized procentrioles along the nuclear membrane which frequently results in their isotropic distribution before nuclear detachment. Finally, both microtubule depolymerisation and dynein inhibition detach disengaging centrioles from the nucleus and hinders centriole disengagement. Interestingly, a strong effect of diazepam on centriole disengagement from deuterosomes in quail MCC progenitors was shown long ago, and diazepam was later shown to alter MT nucleation (Boisvieux-Ulrich et al., 1987; Spurck & Pickett-Heaps, 1994). Altogether this strongly suggests that the same mechanical forces that pulled procentrioles to the nuclear membrane, also assist their disengagement. One could therefore hypothesize that, once the molecular switch of the mitotic oscillator is 'on', the splitting and/or separation of deuterosomes and centrioles are assisted by dynein-dependent microtubule mechanical forces that pull centrioles away from each other's.

A complex baso-apical migration dynamics

Disengagement around the nuclear membrane is followed by the migration of centrioles to the apical cortex to dock and nucleate cilia. Characterizing this process has never been done and is very challenging since individual centrioles move in close proximity with tens of other centrioles. Following individual trajectories requires high resolution spatio-temporal live imaging while avoiding excessive light exposure which disturbs centriole migration (Boudjema et al., 2024). Our trackings reveal a global apical convergence of new centrioles around the former centrosome. Using high temporal resolution microscopy, we further identify that individual dynamics is complex and can be split at least between the baso-apical migration of centrioles and their motion once they have reach the apical side of the nucleus. The baso-apical migration is processive and more rapid than the apical motion, marked by alternance between back and forth movements and between diffusive-like and processive steps. Such intermittent movement is probably multifactorial. It may result from (i) the resistance of a crowded environment on a massive population of large organelles that congregate (Vale, 1985), (ii) the binding and release of centrioles by molecular motors (Ananthanarayanan et al., 2013), (iii) binding to molecular motors going in opposite directions (Hancock, 2014) (iv) a combined action of microtubules with the acto-myosin network which is known to be concentrated apically in terminally differentiated MCC and involved in the gathering of basal bodies in brain MCC (Al Jord et al., 2014; Hirota et al., 2010; Kunimoto et al., 2012; Lemullois et al., 1988), and (v) an asynchronized docking of basal bodies nucleating cilia, leading to their association with the nuclear membrane.

The rate of pure baso-apical migration we quantify is in the range of the microtubule-dependent centriole migration described in multiciliated mouse olfactory neurons (Ching et al., 2022). It seems also comparable to the more extensively characterized dynamics of centrosome migration for primary ciliogenesis in serum-starved RPE1 cells (Pitaval et al., 2017). In these cells, even if the morphology is standardized by micro-patterning, apical migration duration is highly variable and stands in the same range (0,03-0,3 µm/min) as what we describe here (Fig. 8F). Interestingly, in this case also, the migration is dependent on MT but independent from dyneins, probably explaining why centriole migration for ciliogenesis is 2 order of magnitude slower than centrosome repositioning at the T-lymphocyte synapse, where it relies on dynein pulling forces (Yi et al., 2013). It now remains to study whether multiple centriole apical migration in MCC relies on the same mechanisms as centrosome migration for primary ciliogenesis, e.g. pushing forces exerted by the stabilization of MT polymerized from the newly formed centrioles, combined with actin contractions. Several old and more recent studies on different organisms (ctenophore *Beroë*, *Xenopus* and quail) suggest the involvement of actin in final centriole migration in MCC (Boisvieux-Ulrich et al., 1990; Ioannou et al., 2013; Tamm & Tamm, 1988).

Data availability

Previously published scRNAseq data sets were used (Serizay et al., 2025). Briefly, raw sequencing data have been deposited at the NCBI Gene Expression Omnibus under the accession ID GEO: GSE201773 (GEO; *https://www.ncbi.nlm.nih.gov/geo/*) and are publicly available as of the date of publication. Accession numbers are listed in the key resources table. Processed scRNA-seq data are also available for interactive investigation using the CELLxGENE platform (collection # 33f48a52-31d8-4cc8-bd00-1e89c659a87f) at *https://cellxgene.cziscience.com/* collections/33f48a52-31d8-4cc8-bd00-1e89c659a87f as of the date of publication. All original code has been deposited at Zenodo (record #14105247) and is publicly available at *https://doi.org/10.5281/zenodo.14105247* as of the date of publication. Any additional information required to reanalyze the data reported in this paper is available from the corresponding author upon request.

Acknowledgements

We thank all the members of the Spassky lab who contributed in the elaboration of this research work, Chris Kintner for sharing the adenoviral vectors for the expression of MCIDAS-E2F4-Dp1 and Xavier Morin for sharing the MEFs cells. We are thankful to the IBENS administrative team

and imaging platform for their support, and to the IBENS Animal Facility for animal care. We thank Camille Noûs, a collective of researchers, who encouraged us to maintain open, honest and slow science (*https://www.cogitamus.fr/indexen.html*). The team was funded by the Agence Nationale de la Recherche Investissements d'Avenir (ANR-10-LABX-54 MEMO LIFE and ANR-11-IDEX-0001-02 PSL Research University). The team is supported by Inserm, the CNRS, the École Normale Supérieure (ENS), the ANR (ANR-19-CE13-0027, ANR-20-CE45-0019, ANR-21-CE16-0016, and ANR-22-CE16-0011), PSL (Q-Life program), the Fondation pour la Recherche Médicale (FRM EQU202103012767), and the European Research Council (ERC Consolidator grant 647466). A-R.B received a fellowship from La ligue and the Labex MEMOLIFE. A. AJ is supported by the Spanish Ministry of Science and Innovation through the Agencia Estatal de Investigación grant PID2024-157329NA-I00, the Centro de Excelencia Severo Ochoa (CEX2020-001049-S, MCIN/AEI /10.13039/501100011033), and by the Generalitat de Catalunya CERCA programme. This work was also funded by the Damon Runyon Cancer Research Foundation (C. E. J is a Merck Fellow DRG-2478-22); a Hartwell Foundation Fellowship to C. E. J; R01GM133897, R01GM114119, R01CA266199 to A.. J. H.

Additional information

See supplementary materials for methods, 20 supplementary figures and their captions and 28 movie captions. Video files are available on:
https://hub.bio.ens.psl.eu/index.php/s/NnQS5PEGJXDTTok

Author contributions

A-R. B, R. B. designed, performed and analyzed the majority of the experiments, with C. E. J performing all the U-ExM and FRAP experiments, O. M. providing the first data on the role of microtubules in centriole amplification, A. A. J. having performed some of the movies analyzed in this study and M. F. providing general cell culture support. G. L. M., C. E. J. and A. J. H. designed, produced and validated the PLK4 antibody and the mRuby-DEUP1 mice. All the experiments of MEFs on micropatterns were performed in the lab of M.T with the support of A. S. A-R. B., R. B. C. E. J, N. D., N. S., A. J. H. and A.M. analyzed the data. C.N is a collective of researchers, who encouraged A.M. to maintain open, honest and slow science. A. M. conceived and supervised the study. A-R. B., R. B, C. E. J., A. M. co-wrote the manuscript.

Funding

Funder	Grant reference number	Author
Agence Nationale de la Recherche (ANR)	ANR-19-CE13-0027	Alice Meunier
Agence Nationale de la Recherche (ANR)	ANR-21-CE16-0016	Nathalie Spassky
Fondation pour la Recherche Médicale (FRM)	EQU202103012767	Nathalie Spassky
MEC | Agencia Estatal de Investigación (AEI)	PID2024-157329NA-I00	Adel Al Jord
Centro de Biología Molecular Severo Ochoa (CBMSO)	CEX2020-001049-S	Adel Al Jord
Centro de Biología Molecular Severo Ochoa (CBMSO)	MCIN/AEI /10.13039/501100011033	Adel Al Jord
Hartwell Foundation (HARTWELL)	R01GM133897	Andrew Jon Holland
Hartwell Foundation (HARTWELL)	R01GM114119	Andrew Jon Holland
Hartwell Foundation (HARTWELL)	R01CA266199	Andrew Jon Holland
Ligue Contre le Cancer (laliguecancer)	IP/SC-16052	Amélie-Rose Boudjema
Damon Runyon Cancer Research Foundation (DRCRF)	Merck Fellow DRG-2478-22	Cayla E Jewett
EC | European Research Council (ERC)	https://doi.org/10.3030/647466	Nathalie Spassky

Author ORCID iDs

Cayla E Jewett: https://orcid.org/0000-0002-8406-0814
Andrew J Holland: https://orcid.org/0000-0003-3728-6367
Alice Meunier: https://orcid.org/0000-0001-6131-7747

Additional files

Supplemental material with figures

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Peer reviews

Reviewer #1 (Public Review):

The manuscript by Boudjema et al. describes the cellular events underlying centriole amplification and apical migration to allow the assembly of hundreds of motile cilia in multiciliated cells. For this, they use cell culture models in combination with fixed and live cell imaging using antibody staining and fluorescence from endogenously tagged centriole and deuterostome markers, respectively. The work is largely descriptive and functional analyses are restricted to treatment with the microtubule depolymerizing drug nocodazole. The imaging is state-of-the-art including confocal microscopy, live imaging with optical sectioning and high optical and temporal resolution, as well as super-resolution imaging by ultra-expansion microscopy.

The study does a good job of providing a very detailed description of the dynamics of centrioles and deuterostomes that lead to centriole amplification and apical migration in multiciliated cells. This detailed view was missing in previous work. It also reveals the involvement of microtubules at multiple steps: the formation of a cloud of deuterostome precursors, the nuclear envelope tethering of newly formed centrioles, their separation, and their migration to the apical surface.

It would have been useful to expand the analysis of the role of microtubules by including analyses of the requirement for specific microtubule motors, for a better understanding and additional evidence that microtubule-based transport is involved. A weak point is that there is no visualization of microtubules together with deuterosomes and centrioles at the different steps of centriole amplification and migration, to directly address how these structures may interact with and move along microtubules.

Overall, apart from experimental aspects and since this is largely a descriptive study, the manuscript would benefit from more precise language and a better description of the complex events underlying centriole amplification and movements.

Comments on revised version.

The authors have significantly improved the manuscript, by refocusing it, introducing text and figure changes, and by adding new data including functional analyses. The revised version now has convincing data that support the claims. All my remaining concerns have been addressed.

https://doi.org/10.7554/eLife.96584.2.sa2

Reviewer #3 (Public review):

Summary:

In this manuscript, Boudjerna and Balagé et al. aim to elucidate the spatial origin of centriole amplification and the mechanisms behind the formation of an apical basal body patch in multiciliated cells (MCCs). To this end, they focused on the role of microtubules and developed new tools for spatiotemporal and high-resolution analysis of different stages of centriole amplification, including the centrosome stages, A-stage, G-stage, MCC-stage. Among these tools, the MEF-MCC cells grown on micropatterns stands out for its versatility as it is not tissue-specific and does not require epithelial cell-to-cell contact for differentiation. Additionally, the Cen2-GFP; mRuby-Deup1 knock-in mouse model was used to study different stages of centriole amplification in physiological brain MCCs. This model offers an advantage over the previously described Cen2-GFP model by enabling the resolution of early events in

centriole amplification through the visualization of Deup1-positive structures and their dynamics. Finally, the authors leveraged powerful imaging techniques, including super-resolution microscopy, the U-ExM and high-resolution live cell imaging in order to detect and track centriole amplification, elongation, disengagement, and migration.

By combining the MEF-MCC and knock-in mouse model with spatiotemporal imaging in control and nocodazole-treated cells(treated acutely or chronically), the authors define the sequence of events during centriole amplification, revealing the critical roles of microtubules for the first time. Initially, the centrosome-mediated microtubule network forms, organizing a pericentrosomal nest from which procentrioles and deuterosomes emerge. Their findings indicate the importance of microtubules in recruiting and maintaining pericentriolar material clouds that contain DEUP1, PCNT, SAS6, PLK1, PLK4, and tubulins. Following the amplification stage, the procentrioles mature, leading to cells displaying numerous MTOCs, as demonstrated by regrowth experiments. Mature centrioles then disengage from deuterosomes, attach to the nuclear envelope, and migrate to the apical surface facilitated by microtubules.

Strengths:

The manuscript provides new insights into the regulatory function of microtubules and microtubule-based transport in different stages of differentiation in brain MCCs. Addressing the role of microtubules during different stages of centriole amplification required development of new tools to study brain MCCs, which will be useful in future studies of MCCs. A notable strength of this manuscript is the authors' thorough and quantitative spatiotemporal analysis of highly dynamic processes in MCCs. The precision and detail in describing these dynamic events are impressive and are further strengthened in the revised version through additional analysis and adoption of new methods. This comprehensive analysis advances our understanding of MCC biology regarding the involvement of microtubules.

Comments on revised version.

The revised manuscript is substantially improved, and given the scope, it is appropriate that it primarily establishes a detailed spatiotemporal framework. That said, a few points would further strengthen clarity and impact. First, several observations naturally raise follow-up mechanistic questions, for example whether additional cytoskeletal systems such as actin contribute to steps like centriole apical migration. A slightly more detailed framing of these open questions would help guide future work. Second, some terminology introduced to label observed microtubule-based structures (for example "nest") may not be essential. Finally, while the authors have increased quantification, some analyses would benefit from super plot-style displays with replicate-level comparisons, particularly for intensity-based readouts.

https://doi.org/10.7554/eLife.96584.2.sa1

Author response:

The following is the authors' response to the original reviews.

We have carefully addressed the insightful comments provided by the reviewers which thoroughly increased our comprehension of the dynamics of centriole amplification. The manuscript has been revised accordingly and put in the context of the two papers we published since our last submission, showing that MCC differentiation is a genuine cell cycle variant. A point by point answer to allreviewer comments is provided below.

Briefly:

We have streamlined terminology and nomenclature in text and figures / better define experimental conditions with nocodazole

We have tested the role of dyneins in the dynamics of centriole amplification

We have done correlative light and electron microscopy on the early stages of centriole amplification

We have analyzed a new single cell RNA seq dataset comparing canonical and MCC cell cycle variants in mouse brain progenitors

Collectively, this allowed us to make a clearer parallel with what occurs during centriole duplication and to demonstrate that centriole biogenesis in the MCC cell cycle is marked by the superimposition of 2 canonical centriole cycles.

We believe the manuscript will interest a broader readership since it now provides more fundamental insights on the mechanism of centriole biogenesis.

> ***Public Reviews:***
>
> ***Reviewer #1 (Public Review):***
>
> *The manuscript by Boudjema et al. describes the cellular events underlying centriole amplification and apical migration to allow the assembly of hundreds of motile cilia in multi-ciliated cells. For this, they use cell culture models in combination with fixed and live cell imaging using antibody staining and fluorescence from endogenously tagged centriole and deuterostome markers, respectively. The work is largely descriptive and functional analyses are restricted to treatment with the microtubule depolymerizing drug nocodazole. The imaging is state-of-the-art including confocal microscopy, live imaging with optical sectioning and high optical and temporal resolution, as well as super-resolution imaging by ultra-expansion microscopy.*
>
> *The study does a good job of providing a very detailed description of the dynamics of centrioles and deuterostomes that lead to centriole amplification and apical migration in multiciliated cells. This detailed view was missing in previous work. It also reveals the involvement of microtubules at multiple steps: the formation of a cloud of deuterostome precursors, the nuclear envelope tethering of newly formed centrioles, their separation, and their migration to the apical surface.*
>
> *It would have been useful to expand the analysis of the role of microtubules by including analyses of the requirement for specific microtubule motors, for a better understanding and additional evidence that microtubule-based transport is involved. A weak point is that there is no visualization of microtubules together with deuterosomes and centrioles at the different steps of centriole amplification and migration, to directly address how these structures may interact with and move along microtubules.*
>
> *Overall, apart from experimental aspects and since this is largely a descriptive study, the manuscript would benefit from more precise language and a better description of the complex events underlying centriole amplification and movements.*

We have streamlined terminology and nomenclature, clarified the description of the complex events, and test the role of dyneins in centriole amplification. Microtubules density in MCC does not allow to extract information from imaging. In addition, we have done correlative light and electron microscopy on the early stages of centriole amplification and analyzed a new single cell RNA seq dataset comparing canonical and MCC cell cycle variants in mouse brain progenitors. We also replied points by points to the reviewer specific comments.

Altogether, our new data allowed to demonstrate that centriole biogenesis in the MCC cell cycle is marked by the superimposition of 2 canonical centriole cycles. We believe the manuscript will interest a broader readership since it now provides more fundamental insights on the mechanism of centriole biogenesis.

> ***Reviewer #2 (Public Review):***
>
> *This important work will be of interest to centriole and cilia cell biologists. It describes in detail how microtubules control multiple aspects of centriole amplification in brain multiciliated cells. This study provides a greater time-resolved and molecular proteomic mapping of the different steps involved, with or without microtubule disruption. Boudjema et al. show that microtubules are important throughout the centriole amplification process, from the early stages, where the procentrioles emerge from a pericentriolar "nest", through the growth stage where microtubules maintain the perinuclear localisation, to the detachment stage, where microtubules assist in perinuclear disengagement and apical migration. The results are generally well supported by the evidence, but the manuscript would benefit significantly from some heavy editing to introduce more niche terms, standardize abbreviations in text, and labels on figures to help bring the readers, especially non-specialists, along with them - increasing the accessibility of their work.*

We thank the reviewer for his/her enthusiasm. We have streamlined terminology and nomenclature and clarified the description of the complex events to increase the accessibility of our work. We also replied points by points to his/her specific comments.

> ***Reviewer #3 (Public Review):***
>
> *Summary:*
>
> *In this manuscript, Boudjerna and Balagé et al. aim to elucidate the spatial origin of centriole amplification and the mechanisms behind the formation of an apical-basal body patch in multiciliated cells (MCCs). To this end, they focused on the role of microtubules and developed new tools for spatiotemporal and high-resolution analysis of different stages of centriole amplification, including the centrosome stages, A-stage, G-stage, and MCC-stage. Among these tools, the MEF-MCC cells grown on micropatterns stands out for its versatility as it is not tissue-specific and does not require epithelial cell-to-cell contact for differentiation. Additionally, the CEN2-GFP; mRuby-DEUP1 knock-in mouse model was used to study different stages of centriole amplification in physiological brain MCCs. This model offers an advantage over the previously described CEN2-GFP model by enabling the resolution of early events in centriole amplification through the visualization of DEUP1-positive structures and their dynamics. Finally, the authors leveraged powerful imaging techniques, including super-resolution microscopy, the U-ExM, and high-resolution live cell imaging in order to detect and track centriole amplification, elongation, disengagement, and migration.*

By combining the MEF-MCC and knock-in mouse model with spatiotemporal imaging in control and nocodazole-treated cells (treated acutely or chronically), the authors define the sequence of events during centriole amplification, revealing the critical roles of microtubules for the first time. Initially, the centrosome-mediated microtubule network forms, organizing a pericentrosomal nest from which procentrioles and deuterosomes emerge. Their findings indicate the importance of microtubules in recruiting and maintaining pericentriolar material clouds that contain DEUP1, PCNT, SAS6, PLK1, PLK4, and tubulins. Following the amplification stage, the procentrioles mature, leading to cells displaying nµMerous MTOCs, as demonstrated by regrowth experiments. Mature centrioles then disengage from deuterosomes, attach to the nuclear envelope, and migrate to the apical surface facilitated by microtubules.

Strengths:

The manuscript provides new insights into the regulatory function of microtubules in centriole amplification. Addressing the role of microtubules during different stages of centriole amplification required the development of new tools to study brain MCCs, which will be useful in future studies of MCCs. A notable strength of this manuscript is the authors' thorough and quantitative analysis of highly dynamic processes in MCCs. The precision and detail in describing these dynamic events are impressive. This comprehensive analysis advances our understanding of MCC biology.

Weaknesses:

The role of microtubules and other molecular players during different stages of centriole amplification in brain MCCs can be further studied and strengthened using the tools developed in the manuscript. A more quantitative description of some of the analysis performed in the manuscript is required to strengthen the conclusions.

We thank the reviewer for his/her enthusiasm. We have tested the role of dyneins in the dynamics of centriole amplification, done correlative light and electron microscopy on the early stages of centriole amplification and analyzed a new single cell RNA seq dataset comparing canonical and MCC cell cycle variants in mouse brain progenitors. We also replied points by points to the reviewer specific comments.

Recommendations for the authors:

As you will see, all reviewers felt that the analyses of the involvement of microtubules should be strengthened by including controls and additional experiments. Also, they agree that significant text editing would help to improve the manuscript's accessibility and readability.

Specifically, they would suggest (1) streamline terminology and nomenclature in text and figures; (2) better define experimental conditions with nocodazole (concentrations used, effect on microtubules, effect on canonical centriole duplication); and (3), in the absence of other complementary genetic perturbation experiments, add a limitations paragraph in the discussion about conclusions drawn from nocodazole treatment alone.

Reviewer #1 (Recommendations For The Authors):

Main issues:

(1) The authors use variable terminology to describe the same or similar events/structures. For example, in Figure 1 they refer to "centrosome stage" where they observe a pericentrin "cloud", which they later refer to as a "nest". In all other figures the first stage is not referred to as the "centrosome stage" but as the "cloud stage". Again, they also describe the "cloud" as a "nest" occasionally, but not always. In the cartoon, the

> *nest is termed "centrosome cradle". The variable and inconsistent use of terms is confusing and the authors do not provide any explanation for the use of one vs. another.*

The text is now corrected. The centrosome stage corresponds to the stage preceding the beginning of centriole amplification in MCC progenitor. The pericentrosomal cloud of centriole and deuterosome elements forms later on, during the amplification A-stage. The formation of this cloud marks the beginning of A-stage, and persists up to G-stage where it dissolves. When we show that the cloud hosts the first stages of centriole biogenesis, we defined it as a "nest". We do not use anymore the term craddle.

> *(2) What prompted the authors to use the term "nest"? It gives the impression that they describe aspecific physical entity/structure (also depicted in this way in Figure 3P, with microtubules outside of this structure), but what is the evidence for this?*

The cloud is the spatial entity and the term "nest" is used to define a function of this transient compartment. We decided to keep the term "nest" as we now identified it with correlative light and electron microscopy, in addition to U-ExM, and show that the accumulation of centriole and deuterosome elements is accompanied by the formation of immature procentrioles, deprived of MT walls, as well as immature and empty deuterosomes. The scheme with MT outside the cloud/nest is misleading as we see MT organized by the mother centriole. We have now changed this.

> *(3) The "nest" may simply be a dynamic accumulation of precursor particles around the centrosome, similar to what has been described for centriolar satellites. Rather than proposing a new entity, I suggest testing whether the "nest" particles may colocalize with PCM1 and thus may be related to centriolar satellites. Based on the data, the nest would simply be the centrosomal MTOC that organizes a radial microtubule array on which particles move around its center. In the absence of other evidence, I am not convinced that a new term is needed.*

We totally agree with the reviewer: the centrosome, as MTOC, concentrates centriolar and deuterosome components. This cloud is consistently dissolved when MT are depolymerized or dyneins inhibited. So, the physical entity is a "cloud". We used the term "nest" to propose one function for this cloud which is to form deuterosomes and centrioles, before they move away for maturation. In fact, deuterosome and centriole formation are hindered when the cloud is dissolved. We have tried to edit the text all over the manuscript to make it clearer.

> *(4) Role of MTs: are microtubules required or do they just facilitate some of the investigated events?*

The reason why the role of MT has not been tested yet during centriole amplification is probably because MT not only constitute the cell cytoskeleton on which molecular motors ride to transport cargos or distribute forces, they are also the core component of the structures we are studying. This is why we have tested a range of nocodazole concentrations and used concentrations where MT are perturbed but not entirely depolymerized, allowing centrioles to be produced (Fig. 4 Supplementary 1A-B). This may lead to an underestimation of the role of MT but we cannot study the role of MT on centriole amplification if centrioles cannot be formed.

> *Does multi-ciliation in these models eventually occur normally under the concentrations and treatment conditions used here? This should be tested and discussed in the context of whether microtubules are indeed required and at what step of the entire process (amplification, migration, ciliogenesis) they may be critical.*

We did both chronic and acute treatments.

Chronic treatments were done to test the overall efficiency of centriole amplification when MT (or dyneins) are perturbed. Chronic treatments were used to assess the role of MT (or dyneins) on the global efficiency of centriole and deuterosome formation (number of cells able to amplify, number/size/loading of deuterosomes, final number of centrioles (Fig. 4H-I, Fig. 4 Supplementary 2 B-D). In these chronic treatment, we focused on centriole amplification and not ciliation since it was the scope of this study. Also, we did not take ciliation as a readout of amplification because ciliation is relying on MT polymerization.

Then, we also did acute treatments to test the role of MT (or dyneins) at each stage of amplification (A-amplification, G-growth, D-disengagement, M-migration; Fig. 4, 5, 7, 8 and associated supplementary figures). Since one stage is dependent on the precedent one, this enabled us to decipher the direct role of MT (or dyneins) on each single stage. We have now edited text, methods, legends and pictograms to be clear on whether acute or chronic treatment was done.

> *(5) Can the authors include control (non-amplifying) progenitors in their analyses? It would be useful to know what the signal and distribution of each specific marker are before differentiation begins (before the cloud stage).*

Non amplifying progenitors are analyzed and constitute the so-called “centrosome stage”. We have now precised it and called it the “progenitor stage”.

> *(6) Figure 2: Again, the terminology is confusing, since the authors describe that DEUP1 forms a "cloud" with centrin during the A stage.*

Corrections have been done as explained in point 1.

> *(7) Description Figure 3: the authors introduce yet another term: "halo" A-stage. Is this the early A stage? Again, this is not explained and confusing. More systematic and consistent description is needed.*

Corrections have been done as explained in point 1. The term halos is used un the lab as it was the first term we used in our Nature paper in 2014 in reference to the halo described by Erich Nigg when they overexpressed Plk4. It was an error to use it in the manuscript.

> *(8) Nocodazole treatments: the used concentrations are quite high.*

MCC develop a very dense and stable MT network that is not comparable to cycling cells. MT are very difficult to depolymerize entirely (Fig. 4 Supplementary 1A-B).

> *(a) To avoid non-specific effects the authors should test what the minimal concentration is that completely depolymerizes microtubules in their cell model and perform analyses at this concentration.*

We have of course tested a range of nocodazole concentrations at the beginning of the study (Fig. 4 supplementary 1A-B), and used concentrations where MT are perturbed but not entirely depolymerized, allowing centrioles to be produced (see answer to point 4). In case it was not clear, we refer to this now several time and more clearly in the text and methods.

> *(b) They should demonstrate depolymerization of microtubules by microtubule staining in the acute and chronic noc treatments and at the different noc concentrations used.*

This is, and was, in supplementary material (same, Fig. 4 supplementary 1A).

> *(c) The authors should demonstrate that the used nocodazole concentrations do not impair normalcentriole biogenesis during the cell cycle in these cells; if so, impaired assembly of centriole wall MTs may contribute to the observed effects in Figure 4.*

As mentioned in point 8b, we have of course tested a range of nocodazole concentrations at the beginning of the study (Fig. 4 supplementary 1A), and used concentrations where MT are perturbed but not entirely depolymerized, allowing centrioles to be produced (see answer to point 4). The ability of the cells to form centrioles during chronic treatments were always assessed using immunostainings of SAS6 and/or CEN2-GFP signals (now exemplified in Fig. 4 Supplementary 1B). We also did EM analysis on cells treated with the highest doses of nocodazole (Nocodazole 10 uM for 24h) and this showed that centrioles can form with, what seems to be MT walls, in cells totally deprived of cytoplasmic MT fibers (Fig. 4 Supplementary 3-4). However, this does not show that all the cells can, because the number of cells that can be analyzed by EM are not sufficient to conclude. Also, one cannot assess whether MT walls are properly polymerized. However, the absence of MT walls should not change the results of the Figure 4, which are based on DEUP1, SAS6 or CEN2-GFP signals for deuterosomes and centrioles. Also MT depolymerization affects the formation of deuterosomes, which should not be altered by MT wall defects as it is not affected, even when centriole formation is blocked (LoMastro et al., 2024). Last but not least, we now show that blocking dyneins, as a comparable and even greater effect, on the formation of the cloud, deuterosomes and centrioles (Fig. 4C-I and Supplementary Fig. 4), which confirms that MTOC function, rather that MT wall formation, explain the centriole biogenesis alteration shown in Figure 4.

> *(9) The authors repeatedly refer to the centriole-to-centrosome conversion of amplified centrioles and how this resembles centriole-to-centrosome conversion during the cell cycle. However, they incorrectly claim that this occurs at the G2/M transition. PLK1-dependent modification occurs at this stage, but conversion and PCM recruitment only occur after mitosis (see original work by the Tsou lab, which needs to be cited here).*

We agree with the reviewer. We have now added additional data to show clearly that centriole biogenesis, which requires two cell cycles to proceed in cycling cells, is accelerated during the MCC cell cycle variant where the elongation and maturation cycles are superimposed. This is now clearly shown in Fig. 3, 5, 9 and discussed.

> *(10) Figure 6H-J: the authors claim that at low noc concentration, more D-stage cells showed incomplete disengagement than in controls, but the effect is shown only for the highest 10 µM concentration. Do any eof the phenotypes in Figure 6 also occur at the lowest noc concentration (assuming it depolymerizes MTs)? Again, it is crucial to demonstrate this, to exclude unspecific effects not linked to MT depolymerization.*

An error was made on the figure (but not in the legend). In Figure 6, chronic treatments are at 1 or 5 µM. Only acute treatments were done using 10 µM. In both cases, MT are not entirely depolymerized in these experiments (Fig. 4 supplementary 1A).

> *(11) Disengagement, Figure 7: The authors describe that DEUP1 signal spreads all over the cytoplasm and becomes diffuse during this process, but one cannot see a diffusive signal throughout cells in the figures.*

We pushed the contrast to make it clearer but the deuterosomes are still bright at this stage and it is difficult to have both signal clear (now in Fig. 6B). We have also changed the example in video (now *video 19*) to show it more clearly with DEUP1 channel alone.

> *(12) Figure 7: localization of disengaged centrioles at microtubule "nodes" is not clear from the images. There are many centrioles and random colocalization may be expected simply based on the high number. Higher resolution and/or magnification and quantification would be needed.*

We have edited and now say that centrioles “colocalize” with MT which, since centrioles nucleate MT, seems normal. We agree that it could be random, but given the density of MT,

and the number of centrioles, it does not seem opportune to us to quantify. We can just say that we never see centrioles is regions that are deprived of MT.

> *(13) The term "diffusive" to describe slow centriole movements in Figure 8 suggests that it is not motor or force-dependent, but there is no evidence for that. Movement based on opposing forces could produce a similar result, but would not be considered diffusive.*

We agree. We have changed “diffusive” by “diffusive-like”.

> *(14) The manuscript would greatly benefit from the analysis of some candidate motor activities that may drive the movement and migrations of centrioles in this system. This would support the importance of the microtubule network for the specific steps in these processes, and better define its role beyond "being required". Dynein may be a candidate or minus end-directed kinesins. Since chemical inhibitors are available, these types of experiments would be straightforward.*

We formerly tested ciliobrevin but had hard time because of the small stability of the drug. Since our submission to elife, we tested dynapyrazol and dynarestin and found dynapyrazol very efficient in dissolving the Golgi, a good readout of dynein inhibition. We sought to test the role of dyneins, using dynapyrazol, on (i) the formation of the pericentrosomal cloud in A-stage, (ii) the oscillation of DEUP1+ structures during A-stage, (iii) the number, size, loading of deuterosome, (iv) the final number of centrioles, (v) the migration to the nuclear membrane and (vi) the final apical migration of centrioles. The results are now inserted in main and associated Fig. 4, 5, 7, 8, 9.

> *(15) Discussion:*
>
> *"the role microtubules" lacks "of"*

This is now edited.

> *"This lack is..." Lack of what?*

This is now edited.

> *"reflexive link" - meaning of "reflexive" is not clear in this context*

We have removed it.

> *In my opinion, the study does not identify a nest composed of DEUP1, PCNT, and Centrin2; it only shows that these components accumulate as particles around the centrosome, which functions as MTOC. Consequently, it seems that the "nest" does not exist when MT is depolymerized. One could consider the center of the centrosomal MT array as a nest in this context, but there is no evidence of a specific new structure as suggested by the way the term is used in the manuscript.*

This is what we want to say: the center of the MT array become a nest in this context. We do not state that there is a specific new structure. We just say that MT and dynein dependent concentration of centriole and deuterosome components exists and that this region nests the birth of centrioles and deuterosomes. Also, this compartment is restricted in time and space, which justifies to use a specific term. The MTOC exists in the progenitor cell, while this compartment, marked by DEUP1, Centrin, PCNT accumulation, appears at the beginning of amplification and grows during A-stage to be dissolved at G-stage when all the deuterosomes and centrioles have moved away.

> *What is the evidence that "DEUP1 is a centrosomal protein before building deuterosome structures"? It would be good to refer to the specific experiment. Does DEUP1 localize at*

> *centrioles also in the absence of microtubules? If not, I would not consider it a centrosomal protein.*

We have removed this statement to avoid misinterpretation.

> *"This reminds the centriole-to-centrosome conversion..." the sentence is missing an "of"; also, again the authors confuse the order of events during the cell cycle, where centrosome conversion occurs after completion of mitosis, not at G2/M transition.*

We have removed this statement to avoid misinterpretation. Also, see Point 9.

> *"microtubule dependent nuclear migration" should be rephrased; it sounds as if the nucleus migrates.*

This has been changed

> *The following discussion of disengagement being linked to association with the nuclear envelope and resembling the process in cycling cells is misleading. In cycling cells movement of centrioles along the nuclear envelope occurs at G2/M and drives centrosome separation (separation of centriole pairs) in preparation for mitosis, not centriole disengagement.*

We are now clearer. We compare centriole-loaded deuterosome organization around the nuclear membrane to the migration of new centrosomes during early prophase (Fig. 5F-H, Fig. 5 Supplementary 2G-K).

> *Regarding the possibility that forces by microtubules generated by the daughter centriole drive disengagement also in cycling cells, I would argue that this is unlikely since the daughter centriole can only nucleate microtubules after disengagement has occurred (and conversion to centrosome/PCM recruitment). Once this happens, it may physically separate the disengaged centrioles, which is a different type of activity. Indeed, originally the term "disengagement" was coined to specifically describe the loss of the perpendicular engagement of daughter centrioles with their mothers (Tsou and Stearns, Nature, 2006).*

We have removed this statement to avoid misinterpretation. The perpendicular engagement is difficult to assess on deuterosomes but we do see by live imaging, that attachment changes during D-stage, before centrioles detach clearly from deuterosomes.

> *"high resolutive" should be "high resolution"*

Edit done.

> *"splitted" should be "split"*

Edit done.

> *"Consistently, when the mitotic oscillator is dis-inhibited and cells enter pseudo-mitotic events, centrioles show clear and rapid cell-cycle like clustering" This sentence is not understandable without further explanation; what does mitotic oscillator refer to? What are pseudo-mitotic events? What is cell cycle-like clustering?*

We have removed this statement.

> *Minor:*
>
> *(1) Abstract: "Centriole nμMber must be restricted to two..." Since cells are born with two centrioles and have 4 centrioles (2 pairs) when they enter mitosis, this sentence is inaccurate.*

The sentence has changed.

> *(2) Abstract: "reflexive link"; I am not sure what the term "reflexive" refers to?*

We have removed this statement to avoid misinterpretation.

> *(3) Figure 1C, D: it should be described better that the larger magnification panels represent overlays of many cells and what marker they show. This is not obvious since the smaller single-cell panels always show two different markers. Also, it would be more useful to show also single cells in the magnified view. The overlay does not allow us to see if a marker forms a cloud or a single dot, which is as important as the cell-to-cell variation in distribution.*

We have clarified this in the text and the legend. The cell-to-cell variation cannot be estimated with the overlay, but the projection from several cells (number precised) allows to see that the signal is confined in a restricted region. Or not. Which is what we wanted to analyze.

> *Related to the above, the authors say that pericentrin forms a cloud at the top left in panel D, but there is only one confined centrosomal dot in the single-cell panel.*

The sentence has changed.

> *(4) Results, Figure 2F; video 4 : The authors claim connection and disconnection of DEUP1 aggregates with centrosomal centrioles; can the authors comment on the spatial resolution including in z in this movie to support this claim? Can they exclude that the structures are in proximity of each other rather than "connected"?*

This is a single z-section of 500nm. The resolution in xy is 128nm/pixel. Given the sizes of deuterosomes and a mature centriole, and given the fact that we observed this dynamics in several cells in live, we can state that the structures are connected. This is consistent with deuterosomes frequently observed “kissing” the daughter centriole by EM in the present manuscript (Fig. 2D, Fig. 2 supplementary 3 and 4 and Fig. 4 Supplementary 3-4). One has to look carefully at the daughter centriole (marked “dc”) and span in on the serial sections to see the connected deuterosome (marked by a star): this is at very early stage and therefore it is small. We have not zoomed in since previous manuscript have already described this at later stages with bigger deuterosomes. You can refer to main or supplementary figures in previous manuscripts (Al Jord 2014, Khoury Damaa 2024) where serial sections span the entire deuterosomes and daughter centrioles and show, with nanometric resolution, that both structures are frequently sticked to each others on tens of nanometers.

> *(5) The term "dynamics" as used in the manuscript should be plural.*

It has been used plural, except when for “dynamic microtubules” and “dynamic attachment to the nucleus”, which we think is ok? We have not found any other singular uses in our manuscript.

> *(6) Figure 5: what does "YL1/2 procentriole intensity" refer to in panel F? This should be the intensity of microtubule asters.*

This has been modified.

> *(7) Figure 6 - supplement 1B: contrary to the claim in the text, one cannot see tight colocalization with the nuclear pore marker. This seems to be a very small subset of particles and even in those cases colocalization is not tight. Also, what is the relevance of nuclear pore colocalization?*

We edit and change the phrasing as ‘colocalization with NPC’ is not the good term. What we want to say is that there is a tight connection with the nuclear envelope as shown by the

localization of NPC on the same z-section as centrioles. This is why we present a single z, to show that centrioles and NPC are on the same z-plane of 500nm. NPC are stained to outline the nuclear membrane. This is also clearly visible for G-stage centrioles in the XY plane. We have now added an entire z-stack on *video 18*.

> ***Reviewer #2 (Recommendations For The Authors):***
>
> *To improve accessibility of their manuscript, we would suggest making the following edits:*
>
> *(1) Define 'specialist' or 'niche' terms each time you introduce them, such as 'pericentrosomal nest', or 'flower-like structures'.*

This has been clarified.

> *(2) Have a think about abbreviations, again ones that work for people outside the project- this paper uses 'PC' for 'procentriole' but for many 'PC' is 'Parental centriole' or Figure 6J talks about 'D total' or 'D partial', leaves readers confused.*

This has been clarified.

> *(3) Standardize your abbreviations throughout particularly for your treatments- sometimes Noco sometimes, NOCO, or your imaging experiments sometimes Cen-GFP, sometime CEN2-GFP (Figure 7A, D vs. Figure 6) or DEUP1- mRuby, DEUP1-mRuby3 or mRuby3-DEUP1?*

We now use Nocodazole or Noco in the text and the figure respectively, CEN2-GFP and mRubyDEUP1.

> *(4) About 10% of the population, including several key figures in this field, are red-green color blind. Although 4 colour fluorescence is difficult to get right for everyone, choosing palettes (especially for two colour panels) is inclusive. More so, greyscale or inverted monochrome images make it easier for everyone to visualize changes in localization, size, and intensity. Red on blacksmall foci is particularly difficult to discern. For example, Figure 3 - more individual channels in grayscale with arrows to mc, dc, and cilia would be helpful - difficult to distinguish stainings.*

We thank the reviewer for this comment and for this recommendation of being more inclusive. We have done the changes.

> *To improve the conclusions drawn, we suggest some revisions below:*
>
> *(1) Since the paper really hangs on it, a clearer description of the rationale for when, how long and how much nocodazole treatment was done is needed. The logic currently is difficult to follow seemingly random jumps 10x concentration are used. Microtubules control many aspects of cell biology and could be impacted. For example, I particularly found Figures 6D and H difficult to follow i.e. the timing for 6H seems off.*

MCC develop a very dense and stable MT network that is not comparable to cycling cells. MT are very difficult to depolymerize entirely. We have of course tested a range of nocodazole concentrations at the beginning of the study and shown the extent of MT depolymerization under each treatment. We used concentrations where MT are perturbed but not entirely depolymerized, allowing centrioles to be produced (see answer to point 4 reviewer 1). The level of perturbation of MT and consequences on centriole formation at the different timings and doses were done for each experiment and are exemplified in Fig. 4 supplementary 1A-B. This figure was already present in the first version of the manuscript but we have now edited text, methods and pictograms to clarify this.

> *(2) Perhaps an extension of this point- in general how interdependent are the processes? If there is a defect at the nest stage, how much are the later defects secondary to this, or do MTs genuinely play direct roles at all stages or are these knock-on effects? How do the authors rule this out? Defects in the nest, lead to smaller and more DEUP1+ foci, with defects in concentrating procentriole factors and centrin, which lead to... For example, Figure 4B looks like centrin is reduced upon noco treatment? Does noco treatment affect Cetn2GFP levels globally? Individual channels grayscale would help visualise this better.*

See also our answer to reviewer 1 point 8c.

The stages are indeed interdependent. This is why we did both chronic and acute treatments. Chronic treatments were done to test the overall efficiency of centriole amplification when MT are perturbed. We typically used low dose of 1µM because nocodazole remains 48h in the culture medium. Acute treatments were done to test the role of MT at each stage of amplification (Aamplification, G-growth, D-disengagement, M-migration). Most of the acute treatments were done live and nocodazole was applied after the first time point of live monitoring. We used 10µM to have a rapid effect, and because nocodazole remains only several hours in the culture medium. This allowed to monitor the stage “n”, in cells where the stage “n-1” was completed without any drug which allowed to analyze a stage without having perturbed the precedent one.

We now also test the consequences of dynein inhibition using both acute and chronic dynapyrazole treatments. We show that except for centriole migration, dynein inhibition phenocopies MT depolymerization (centriole number, perinuclear organization and disengagement as well as deuterosome number/loading/size).

Nocodazole chronic treatments do affect intensity of CEN2-GFP at G-stage centrioles suggesting an altered A-to-G transition. In D-stage, CEN2-GFP signal seems normal. We now mention this in the text and in the Fig. 4 Supplementary 1B.

> *(3) The authors nicely show the importance of MTs in the structure of the nest from which procentrioles and DEUP1 positive structures emerge. They suggest this nest may be what supports procentriole generation in the absence of DEUP1 and parental centrioles. Firstly how does this nest look in the absence of DEUP1 and/or parental centrioles (centrinone treatment)? This may be what they are trying to show in Figure 5 Supplement 1 but it currently is very difficult to digest what it is showing relative to controls and whether this is significant in the way it is plotted.*

The nest is conserved in the DEUP1KO with or without centrosomal centrioles, as shown by accumulation of Centrin and PCNT at the center of the self-organised MT network (Mercey et al., 2019). This is in fact what motivated our study on the role of MT in centriole amplification. We have edited the legend to precise the quantification done, which is not related to this question. In this quantification, we show that the increased propensity to accumulate PCNT by centriole-loaded deuterosomes between A and G-stage is maintained in the absence of deuterosomes, indicating that centrioles themselves accumulate/recruit PCNT.

> *(4) Can you do CLEM on DEUP1-Ruby and these early foci at the cloud stage to see if they are visible at the ultrastructural level, relative to procentrioles, microtubules, and other electron-dense structures?*

We thank the reviewer for this question. We have done CLEM on the pericentrosomal cloud during very early steps of centriole amplification. This showed that DEUP1 early accumulation at the centrosome corresponds to a region rich in fibro granular aggregates, suggesting that DEUP1 may be translated here, through locally concentrated centriolar sattelites, known to be involved in local translation. Then, small deuterosomes and immature centrioles are formed, within this cloud of sattelites, confirming that the pericentrosomal

cloud is a nest for centriole biogenesis (Fig. 2C-D + Fig. 2 Supplementary 2-6 for control and Fig. 4 Supplementary 3-4 for nocodazole treated cells). This also shows that immature deuterosomes are not necessarily round shaped, and can be deprived of centriole loading.

> *(5) Check the scale bars- see Fig 4E. Check throughout.*

Done.

> *(6) Figure 3 Supplement 1 and 2 don't match the legend and are likely reversed - which one is right?*

Done.

> *(7) Technical issue - I couldn't play videos 6 or 16? Check these work.*

Done.

> *(8) Nomenclature mammalian proteins- mouse or hμMan- should be all caps DEUP1, PLK4, SAS6,etc. Watch your units- space between nμMber and unit.*

This has been done.

> *(9) Many of the graphs involve three biological replicates but why not plot the mean of each of the three experiments and do stats? The number of events measured may conflate the significance. Try using Superplots.*

Here is how we proceed: we count the number of occurrence of the phenotype we monitor, and the total number of cells. We apply a X^2 to test whether there is a significative difference between our replicates in each condition. If not, we pool the number of occurrence of the phenotype we monitor and the total number of cells for the 3 replicates, and for each condition. Finally we apply a X^2 between the different conditions. This is how we usually proceed to avoid comparing a mean of percentages. This is now explained in the methods.

> *Minor points:*
>
> *(1) "DEUP1 is a centrosomal protein and assembles deuterosomes in the pericentrosomal region in brain MCC". I am not sure you have evidence that DEUP1 is a centrosomal protein. You don't seem to study the relationship between centrosomes and DEUP1? Rewrite this title and tone down this claim.*

This has been modified.

> *(2) Why the crossbow micropattern (versus some other shape) - seems very specific but not discussed?*

We wanted a shape where centrosome is not localized at the center of mass of the nucleus. Among the corresponding patterns, the crossbow was the one where differentiating cells had less propensity to detach.

> *(3) Figure 2 - are the foci of DEUP1 at the cloud stage smaller than at A stage? How do they grow? Measure the diameter at cloud stage, just after they leave the cloud and then once they move away from centrosomal cloud and each other. If so, and they do indeed grow in size from the cloud stage to the growth stage which I think your images suggest - do you envision this happening with the gradual addition of DEUP1 rather than fusion?*

Early deuterosomes are not easy to detect by light microscopy, because of accumulation of DEUP1 in the cloud. We did CLEM on the cloud of early A-stage cells to resolve the earliest deuterosomes which are often very small (see Fig. 2D, Fig. 2 Supplementary 2-6) suggesting that they grow, either by fusion, which we never observe in our movies at later A-stage, or by

accretion of DEUP1. However, by light microscopy, we can detect very early but big deuterosomes, which we see splitting later on into smaller ones. So, we cannot conclude on the mechanism that regulate deuterosome size. This is now discussed in the discussion of the manuscript.

> *You say in the discussion:*
>
> *"Consistently, we never observed fusion events of DEUP1 condensates in our time-lapse experiments. More importantly, we did FRAP experiments on endogenously tagged mRuby-DEUP1 in cells at the different stages of centriole amplification, and did not find significant recovery, supporting that centrosomal DEUP1+ foci and deuterosomes are not liquid-like structures (Figure 8 Supplementary 2)." How do you prove there is no fusion of deuterosomes?*

It is always difficult to prove the absence of something, we agree! But we did tens of movies with high temporal resolution and never observed fusion events. But, as we say in the previous question, the very early deuterosomes can be very small and we do not distinguish them from the DEUP1+ cloud by live imaging. So at this stage, we cannot say. But later on, during A- or G-stage and when deuterosomes are outside the cloud to be easily observed, we very often observe deuterosomes bumping into each others and stay in close contact for minutes, but then moving away. This, for us, supports the lack of fusion properties. But the question remains open. We now explain this in the manuscript and have added an example in *video 28*.

> *If they are getting bigger as I think your imaging suggests from cloud to growth stage, then how is this happening?*

MT depolymerisation and dynein inhibition leads to the formation of very small deuterosomes. Dynein inhibition can even lead to a block in the formation of new deuterosomes suggesting that DEUP1 concentration is a crucial parameter for condensation into deuterosomes. Deuterosome growth may happen through oligomerization of DEUP1 molecules allowed by their dyne-independent concentration. Sorokin in 1968 proposed that a supersaturation of deuterosome components may lead to their solid crystallization into deuterosomes. Deuterosome size can also be regulated by a more complex molecular cascade, involving post-translational modifications of DEUP1 or PCM, such as phosphorylations driven by the cell cycle machinery. This would be consistent with the fact that deuterosomes are very big in the absence of CCNO, a cyclin required for entering the MCC cell cycle variant. This will need further investigations.

> *I'm not sure FRAP actually proves fusion doesn't happen.*

Agreed, this is not what we wanted to say, we clarified. The FRAP experiment just suggests that it is not liquid-like.

> *It is technically difficult to laser ablate individual or only subsets of deuterosomes...*

This is what was done but anyway, FRAP does not firmly show that deuterosome compartments are not liquid-like as we now precise.

> *(4) How do you fix your cells for expansion as you have no preservation of cytoplasmic microtubules? You are saying that there is a "nest" of MTs but beta tubulin ONLY stains the cilia and centriole - why is this? Tyrosinated tubulin on regular confocal shows strong cytoplasmic staining. See Figure 3.*

Cytoplasmic microtubules do not preserve well through the expansion process. We did try a few different fixations and pre-extraction methods but they come at a trade-off to preserving

centrioles. i.e. we could either preserve cytoplasmic tubes or centrioles but not both with the same processing method.

> *(5) "PCNT puncta partially overlap with centrin (Figure 3 Supplementary 2C). At this stage, PLK4, the master regulatory kinase, and SAS6, one of the first centriolar components are either absent or present as small foci within the cloud, often on the wall of the parent centrioles (Figure 3B-C)." some arrows to highlight this would be useful - difficult to see?*

We have tried to make arrows on what is now Fig. 3 Supplementary 1 G, but there is to many CENTRIN colocalizing with PCNT. We have enhanced the contrast of the merge to make it more visible.

> *(6) Figure 3I legend - what are the arrows pointing at? Yellow and white on inserts? ". Around the same time as tubulin, centrin is also recruited to procentrioles (Figure 3I). This stage is probably the stage that we previously documented as A"*
>
> *However you see centrin at DEUP1 foci in D, and you don't show any eg. SAS6 or PLK4 positive DEUP1+ structures lacking centrin specifically, centrin seems to be present on all the procentrioles in Figure 3I. Did I miss it where you show centrin negative procentrioles in the cloud?*

Fig. 3I (now Fig. Supplementary 1J), yellow arrows are pointing at centrioles with non-acetylated MT while white arrows point at acetylated MT. This is now indicated in the legend.

Regarding CENTRIN, it is present as a diffuse staining around the centrosome since the very beginning of amplification (now in Fig. 3 Supplementary 1A with different contrasts), in addition to compose the parental centrioles. This staining can therefore overlap with DEUP1 staining when DEUP1 appears (Fig. 3 Supplementary 1B, E) but not necessarily. In live we observe that CENTRIN and DEUP1 foci can move independently at early stages (Fig. 2 Supplementary 1B, *video 2*). This is later on, as shown now in Fig. 3 Supplementary 1J (previously Fig. 3I), that procentrioles are all strongly positive for CENTRIN.

A new paper (Laporte et al., Cell 2024) recently showed that the recruitment of CENTRIN on duplicating procentrioles first occurs at the distal end, visible by a small dot, and then appears gradually at the level of the inner scaffold when procentriole reach 160nm, the stage where POC5 appears, which corresponds to the A-to-G transition in our MCC progenitors (Al Jord et al., 2014). One can therefore consider that the same is happening in our cells, and that, with the CENTRIN cloud, we have difficulties to detect the distal CENTRIN dot. We have changed the text to add this reference and discuss CENTRIN apparition in MCC procentrioles.

> *(7) " The DEUP1 asymmetry previously described at the centrosomal daughter centriole (Al Jord etal., 2014) becomes visible in some cells during the cloud stage (Figure 3B, N; Figure 3 Supplementary 2B) and in a majority of cells" difficult to see - maybe enlarge and single channel from Figure 3F-H in the supplemental Figure 3 to emphasise this?*

We have either changed the pictures or the contrast to be more representative with the quantifications. This is visible in Fig. 3A, D, E, G; Fig3. Supplementary 1E and now using correlative light and EM in Fig. 2 Supplementary 2, 3, 4 and Fig. 4 Supplementary 3-4. One has to look carefully at the daughter centriole (marked “dc”). We have not zoomed in since previous manuscript have already described this at later stages with bigger deuterosomes. You can refer to main or supplementary figures in previous manuscripts (Al Jord 2014, Khoury Damaa 2024) where serial sections span the entire deuterosomes and daughter centrioles and show, with nanometric resolution, that both strutures are frequently sticked to each others on tens of nanometers.

> *(8) Do you have videos of DEUP1 oscillations with nocodazole to show a lack of oscillations?*

We have now added videos of DEUP1 oscillations under nocodazole and dynapyrazole treatments.

> *(9) "In addition, co-staining of centrioles and nuclear pore proteins show a tight colocalization(Figure 6 Supplementary 1B)." I see the colocalisation in panel 1 but less obvious with panel 2 maybe have some more zoomed in panels and some quantification of the colocalization? Is it more striking at the G stage than the D stage?*

We edit and change the phrasing as 'colocalization with NPC' is not the good term. There is too many centrioles and NPC, they cannot do otherwise than colocalize… What we want to say is that there is a tight connexion with the nuclear envelope. This is why we present a single z, to show that centrioles and NPC are on the same z-plane. This is also clearly visible for centrioles that are loaded on deuterosomes that are around the nuclear membrane in the XY plane. We also added a video to show an entire z-stack of this kind of staining.

> *(10) "Indeed, SAS6 normally disappears from procentrioles when centrioles are docked, just beforeciliation (Al Jord et al., 2014). This suggests that centrioles were able to degrade SAS6, a process also dependent on APC/C (Strnad et al., 2007), but failed to disengage from deuterosomes." Figure 6 Supplement 1E-F - are you sure it wasn't that Sas6 wasn't loaded correctly at the earlier stage and so is reduced recruitment rather than premature disengagement of Sas6? If it is indeed premature disengagement of Sas-6 - what about CP110 - does the CP110 get loaded and is it still present in noco treated cells arrested in the D phase?*

We do not observe SAS6-negative procentrioles on deuterosomes at G-stage but only on deuterosomes in D-stage cells (cells with partly disengaged procentrioles). This is why we hypothesize that, because of the long duration of D-stage and knowing that SAS6 is finally degraded at the end of amplification (Al Jord et al., 2014), we are in the presence of cells where SAS6 has been degraded but where centrioles did not manage to disengage. This is now clarified in the text.

> *(11) Can you track deuterostome splitting live? Maybe not enough spatial or time resolution?*

One has to monitor in 3D (multiple z because deuterosomes move a lot), 2 colors, high temporal resolution (dt=2-5'; to be able to track a single deuterosome), and long duration (deuterosomes are sometimes touching each other and then moving away, giving the impression that they split). This eventually leads to the bleaching of the mRuby fusion protein… We have put an example of what we think is a deuterosome splitting in Fig. 6E (former Fig. 7D). But we decided to finally monitor with low temporal resolution (dt=40') to avoid photobleaching, and analyze numerous deuterosomes and cells to quantify the number and size of deuterosomes over time in single cells.

> *(12) The MT nodes - can you segment the tyrosinated MTs and define nodes and then quantify theDEUP1 presence on them?*

Please see answer to reviewer 1 regarding this point.

> *(13) Figure 8 supp 1 (E): Representative XY distribution of CEN2-GFP+ centrioles at the end of migration (Sas6 negative) in brain MCCs treated with DMSO, Nocodazole 1µM and 5µM (48h). Scale bar, 5µm Bit more detail on how you define fully migrated vs still migrating centrioles in z. You say you are using Sas-6 negativity to define fully migrated cells in the legend, yet you say noco treatment leads to premature sas-6 negativity, and yet the apical migration takes longer upon noco treatment?*

Nocodazole does not lead to premature SAS6 negativity but to a partial disengagement which lead to SAS6 negative “mature” centrioles being still connected to deuterosomes. We define complete migration when all the centrioles are on the apical side of the nucleus. We now clearly define what “apical” migration stands for in the main text and changed the pictograms in Fig. 8G to clarify this.

> *(14) Figure 8H and video 18 - it isn't obviously clear to me that the noco-treated cells are "more erratic" or how you decide what counts as apically migrated successfully. How do you control for drift in z? Can you track individual centrioles as you did in untreated and define what is "erratic about their movement?*

Erratic means that the centrioles are moving away from each others, and back, in a non-predictable way, instead of migrating up and gathering. The drift in z of the whole cell is visible because there is always some centrioles, that are apically located at the beginning, that remains on the apical membrane, probably because they are already docked.

We have indeed followed the centrioles individually in the nocodazole condition. However, in the control, the XYZ coordinates of one of the centrioles of the centrosome, which normally don’t move, are substracted to the coordinates of all the other centrioles as explained in the method section. This allows to have a subcellular reference, and to circumvent the movements of the cell, which are non-negligible at all at this timescale. In the nocodazole treated cells, the centrosomal centrioles share the erratic movements of the other centrioles and can migrate up and down, which exclude them as a reference. Since the nucleus is also moving a lot, we were left with no reference point.

> *(15) Figure 8 supplement 1E can you quantify the final area of centriole patch in XY upon noco treatment?*

It was in main Fig. 8J and is now in Fig. 8 Supplementary 1F.

> *(16) Figure 8J legend- MBB is never defined as an acronym.*

Thank you for pointing this.

> *(17) Define what is the frequency and how is it calculated - Figure 8J.*

This is the MBB patch area in µm²

> *Text edits:*
>
> *(1) "Altogether, these results suggest that, in this non-tissue-specific proxy of MCC progenitors, microtubules organize the onset of centriole amplification in the pericentrosomal region."*

Sentences have changed.

> *(2) "Increasing the temporal resolution to 5-15s reveals that DEUP1+ foci observe an exhibit oscillatory dynamics to at the centrosome (Figure 2E, colored arrows, Video 3, 5/10 cells observed for 1-4min)."*

Sentences have changed.

> *(3) "stage procentrioles were involved in this perinuclear migration and distribution. In fact, this dynamic is reminiscent of the centrosome migration that occurs during the G2-to-M progression in cycling cells in preparation for mitotic spindle organization. In cycling cells, this" Grammar - maybe change to "stage procentrioles were involved in this perinuclear migration and distribution. This is reminiscent of the centrosome migration that occurs during the G2-to-M".*

Sentences have changed.

> *(4) "We then wondered whether these microtubule-dependent dynamics was were required for an efficient subsequent centriole disengagement during the following D-stage."*

Sentences have changed.

> *(5) "Then, monitoring tens of disengagement movies, we identified a transient stage during which disengaging procentrioles redistribute isotropically in the 3 dimensions, along the nuclear membrane (Figure 6A, 4:30, Video 7) before losing its contact to migrate to the apical surface (Figure 6A, 6:30 to 14:00)."*

Sentences have changed.

> *(6) Discussion: "Since pioneer electron microscopy studies on basal body production in quail oviduct MCC 35 years ago (Boisvieux-Ulrich et al., 1987, 1990; Boisvieux-Ulrich et al., 1989), this work is the first to assess the role of microtubules in the now finely described centriole amplification process. This"*

Sentences have changed.

> *(7) "Using live imaging on brain MCC, we highlight the existence of a nest composed of DEUP1, PCNT and Centrin2, pre-assembled before the onset of centriole amplification onset. "*

Sentences have changed.

> *(8) "Recently, formation of DEUP1 pure condensates in solution as well as FRAP experiments after overexpression of DEUP1 in MCC progenitors suggested that deuterosomes where are not liquidlike structures (Yamamoto & Kitagawa, 2019). Consistently, we never observed fusion events of DEUP1."*

Sentences have changed.

> *(9) "This reminds is reminiscent of the centriole-to-centrosome conversion occurring at the G2-M transition followed by the associated microtubule dependent nuclear migration of new centrosomes at mitosis onset (Agircan et al., 2014)."*

Sentences have changed.

> *(10) "Following individual trajectories requires high resolutive resolution spatio-temporal live imaging while avoiding excessive light exposure which disturbs centriole migration (Boudjema et al., 2024)."*

Sentences have changed.

> *(11) " Using high temporal resolution microscopy, we further identify that individual dynamics is are complex and can be splitted between divided into the baso-apical migration, where centrioles move in a processive and more..."*

Sentences have changed.

> ***Reviewer #3 (Recommendations For The Authors):***
>
> *(1) Growing MEF-MCCs on micropatterns has successfully mimicked the dynamics of centriole amplification in brain MCCs, allowing the authors to study the spatial origin of procentrioles. Since this is a powerful system, a more quantitative description of the system will be informative and beneficial for future studies. For example: What is the efficiency of this system? Do the cilia that form in MEF-MCCs motile?*

The system of MEF-MCCs has been described in a previous paper from the Kintner lab. It seems that growing the MEF-MCCs on micropatterns did not ameliorate the ciliation which is partial, probably due to the absence of an apico-basal polarity.

> *(2) Figure 2: The analogy drawn by the authors between DEUP1 oscillatory dynamics and centriolar satellites is intriguing. In early amplifying cells within the cloud, do these DEUP1 structures co-localize with the satellite marker PCM1?*

We have added immuno stainings of PCM1 in mRuby-DEUP1 / CEN2-GFP cells in Fig. Supplementary 2E. Within the centrosomal cloud, DEUP1 colocalizes with PCM1. Interestingly, this PCM1 concentration at the centrosome is dependent, at least in part, on dyneins. Then, PCM1 can localize around the deuterosomes, but it is never colocalized with deuterosomes (not shown). This is also showed by immuno-EM in Zhao et al., 2019. Although it was shown that PCM1 is a proximity interactor of DEUP1 (called ccdc67 at that time) by Firat-Karalar et al., 2014., absence of PCM1 staining on deuterosomes does not favor the hypothesis of PCM1 and DEUP1 being part of the same entities. One could hypothesizes that DEUP1 is transcribed locally within the satellites, explaining the colocalization of the 2 proteins and the + BioID results, and then form PCM1negative deuterosomes.

> *(3) The authors propose a physical link between deuterosomes and centrosomes based on their oscillatory behavior. How are the oscillatory dynamics of DEUP1 affected by nocodazole treatment or inhibition of microtubule motors (i.e ciliobrevin treatment)?*

These oscillations are inhibited by nocodazole (Fig. 4D). They are also inhibited by dynapyrazole (Fig. 4D). We never succeeded in having a nice disruption of the Golgi apparatus with ciliobrevin and therefore we did not used it.

> *(4) In addition to nocodazole treatment, it would be important to determine the consequences of microtubule stabilization by taxol and inhibition of microtubule motors during critical stages of centriole amplification where microtubules are reported to play a role for the first time in this manuscript. Another interesting area of investigation will be to study the extent to which microtubule PTMs contribute to these processes.*

We now blocks dyneins during the different stages of amplification. The results are in main and associated Fig. 4, 5, 7, 8. The role of microtubule PTM, is not in the scope of this manuscript.

> *(5) Describing microtubule dynamics along with Centrin/DEUP1 dynamics will be informative in assessing whether these structures associate and/or move along microtubules? Have the authors performed their imaging experiments with SIR tubulin?*

Yes, we have tried hard! But we have encountered different obstacles:

3-color video microscopy is phototoxic,

siRTubulin is bleaching very rapidly

The density of microtubules in MCC makes the observation hardly informative

> *(6) Figure 5: The role of PLK1 in centriole-centrosome conversion and generation of multiple MTOCs can be tested with a PLK1 inhibitor for further confirmation.*

We have also tried but inhibiting Plk1 blocks the A-to-G and G-to-D transitions so it was not possible to uncouple the role of Plk1 in stage transitions versus centriole maturation.

> *(7) Figure 6: The tight co-localization of nuclear pore proteins with centrioles poses questions about the role of nuclear pore proteins or other nuclear proteins that are associated with centrioles during centriole disengagement and migration. Considering the existing literature on centrosome-nucleus attachments, can there be a way to test this question within the scope of this manuscript?*

We have tried to deplete Nup133 but it's killing the cells. Our additional experiments now show that the nuclear migration of centrioles during G-stage is dynein dependent, reinforcing the parallel with centrosome migration in prophase. We also added results from our scRNA sequencing (Fig. 5 Supplementary 1) showing that some key players of centriole migration to the nuclear membrane are conserved in the MCC cell cycle variant, and expressed with a comparable dynamics as to the canonical cell cycle.

> *(8) Figure 8: Manually tracking a subset of migrating centrioles to define their dynamics during centriole migration and docking provides valuable analysis for determining the molecular mechanism of these processes. In addition to microtubules, does actin contribute to this process? Since centrioles eventually migrate to the apical side in nocodazole-treated cells, there should be other molecular players involved in this process.*

We did block actin polymerization but we found that the different stages were affected and that it would be better to dedicate a whole manuscript on the role of actin during each stage of amplification. We discuss the migration mechanism, and the putative role of actin, in the discussion.

> *(9) The legends for Supplementary Figures 1 and 2 in Figure 3 are mixed and need correction.*

Figures have been remodelled.

> *(10) In Figure 3P, the term "PLK4+" is labeled in bright green, which is not clearly visible. It maybe beneficial to change the color of this label for better visibility.*

We have tried to correct this.

> *(11) Figure 6F quantifies "% tethered flowers" on the nuclear membrane. When quantifying, is the3D localization of DEUP1 flowers in both DMSO- and Noc-treated cells considered? A flower may appear to be on the nucleus in 2D, but it could be detached from the membrane in a 3D view.*

The quantifications are done in 3D. However, flowers that are below or above the nucleus are not quantified since the space is confined and the resolution in z to small to see whether they are connected or not. This is now precised in the legend.

> *Before the editors proceed with an updated assessment, they've requested that we pass on some of the comments that have arisen as part of the evaluation of your revised manuscript. They feel that these concerns should be addressed before we proceed with issuing a formal assessment and publishing the revised Reviewed Preprint:*

We thank the reviewers and the editors for the corrections and insighfull comments. We apologize for our delayed answer and hope our corrections in the main text and some of the figures will give them satisfaction.

> *The revised manuscript is greatly improved with nice new data regarding the role of microtubules. It also has changed quite a bit including the title. The new focus is on the cell and centriole cycle variants in MCC. While this helped to focus the study, there remains an important issue related to the interpretation of the data and the proposed 2-in-1 cycle model. Before providing the final updated assessment, we ask you to address the following points (which were raised already in the first round of review): The manuscript still contains statements that are not aligned with published work and the current view in the field regarding the timing of events during canonical centriole biogenesis. These timings are in conflict with your model that 2 centriole cycles are "superposed" in the MCC cell cycle variant, as currently presented. An alternative straightforward interpretation would be that multiciliogenesis uses an accelerated centriole duplication cycle where key steps occur concomitantly or in short succession instead of being separated by mitotic divisions as in the canonical cycle.*

We do agree with the acceleration of all steps into only one cycle, this is actually what we think we have proposed. When correcting our confusions as regard to centriole-to-centrosome conversion (as explained below) and putting the events in a scheme, this reveals that the events of the two canonical cycles nicely superpose, both in term of molecular composition and dynamics (corrected Fig. 9). We therefore maintain that the null hypothesis is that the acceleration is done through a superposition of events that; although driven by the same molecular machinery, are normally occuring in two consecutive cell cycle. We explain ourself briefly in two paragraphs, before answering point by point to the questions of the reviewers.

As regard to centriole-to-centrosome conversion:

We thank the reviewer for pointing out that we used “MTOC conversion” for what is normally called “centrosome maturation”. We have removed the term “centriole-to-centrosome conversion” during the first round of revision but we now realize that “MTOC conversion” leads to the same misinterpretation as regard to the literature on centriole duplication.

The reviewer asks us to refer to the work of the Tsou lab (Wang 2011, reference now added in the manuscript) showing that daughter centrioles are “modified” (e.g. recruit PCM, become competent for MT nucleation and duplication) during late M/early G1. This “centriole-to-centrosome conversion” can’t occur for our procentrioles at this stage since they are not even born during the mitosis that precedes MCC differentiation. Also, in our cells, such modification does not include the capacity to become competent for duplication since we know that procentrioles become basal bodies without making any round of duplication (Al Jord et al., 2014).

Also, we have not done the experiments to tackle the question on when our centriole become “modified-like”. What we can say is that during A-stage, they become progressively positive for PCM (Fig. 5 Supplementary 2) and a weak signal shows that some MT are seen emerging from them (Fig. 5 and Fig. 5 Supplementary 2, and see point by point answer).

What we do see is that, at the A-to-G transition, they increase their PCM recruitment, show clear and strong MTOC ability (sometimes as strong as the centrosomal centrioles), and that this is associated with migration and separation of centrosome/deuterosomes around the nuclear membrane (Fig. 5). We therefore connect this to what occurs at the G2/M transition which is an increased recruitment of PCM protein, an increased ability to nucleate MT, associated with centrosome migration and separation at the nuclear membrane. Since this

process in the canonical cell cycle is called “centrosome maturation”, we therefore should refer to this term in our study. However, centrioles in the MCC variants are not organized in centrosomes, so we now compare what we see to the “centrosome maturation” of the canonical cell cycle with an associated reference (Joukov et al., 2018), but name it “centriole maturation”.

We have modified the text (track changes visibles) and the schemes (Fig. 5, Fig. 5 Supplementary 1 and 2, Fig. 9, Fig. 9 Supplementary S1; new versions uploaded) accordingly.

As regard to 1.5 or 2 cell cycles

Except for the “MTOC conversion” that we have now changed, as explained above, we think our work does suggest (depicted on Fig. 9) what the reviewer states for centriole duplication: “In the current view, centriole biogenesis starts in early S, elongation proceeds through G2/M and by early G1 it is complete. During M/early G1 centrioles disengage and newly formed daughters recruit PCM (centrosome conversion). Then these centrioles go through another complete cell cycle and when they reach early G1 again they have acquired DAs and SDAs. Key here is that biogenesis and disengagement/centrosome conversion are separated by the first mitosis (ensuring duplication occurs only once), and acquisition of DAs and SDAs is separated by another mitosis (ensuring that cells only form a single cilium)”.

We feel that going from early S to a G1 phase, after 2 mitosis, is what one can call “2 cell cycles”. One of the paper that inspired us a lot when studying how the cell cycle machinery can drive centriole amplification in MCC is a paper from Jadranka Loncarek team (Kong et al., 2014) where they also state that “nascent centrioles gradually mature through 2 cell cycles”. Very interestingly, in this study they show that when they enhance Plk1 activation, they could erase centriole age and new procentrioles are able to recruit PCM and appendages within only 1 cell cycle, without mitotic progression, like what we see in MCC. We have added the reference in our discussion.

Point by point answer

> *(1) Original work on canonical centriole disengagement and centriole-to-centrosome conversion should be cited (e.g. PMID: 16862117, PMID: 21576395)*

As explained earlier, we used the wrong term since the begining. We do not speak about the centriole-to-centrosome (nor MTOC) conversion since we do not test when centriole modification (Wang et al., 2011) occurs in the MCC cell cycle variant. We know that PCNT is present on the procentrioles during A-stage (as shown in Fig. 5 Supplementary 2B), but we do not know when it is recruited (UExM did not work properly with this antibody). We quantify a weak MT staining in regrowth experiment during A-stage and see that procentrioles can be connected to MT in both brain MCC and MEFs (as shown in Fig. 5D, E for brain MCC and Fig. 5 Supplementary 2F for MEFs) , but we do not know when during A-stage they become competent for nucleation. We therefore did not speak about this process that we do not document. What we clearly document/quantify is the enhanced MT nucleation capacities at the A-to-G transition, concomitent with the nuclear migration (easily defined with Cen2-GFP or GT335 stainings) and that we compare to centrosome maturation occuring at the canonical G2/M transition.

> *(2) The authors state in several places that canonical centriole formation and maturation takes two iterations of the canonical cell cycle. This is imprecise. Based on the above work and work by others, the broadly accepted view is that it takes 1.5 cell cycles. This difference matters for the final proposed model (see below). Reviewed e.g. here: PMID: 20869612; PMID: 30601682*

Our answer is in the preamble.

> *(3) "Centriole maturation cycle superposes with centriole elongation cycle in the MCC cell cycle variant": Your description of the canonical cycle differs from the current view in the field. In the current view, centriole biogenesis starts in early S, elongation proceeds through G2/M and by early G1 it is complete. During M/early G1 centrioles disengage and newly formed daughters recruit PCM (centrosome conversion). All this occurs in 0.5 cycles. Then these centrioles go through another complete cell cycle and when they reach early G1 again they have acquired DAs and SDAs (total of 1.5 cell cycles). Key here is that biogenesis and disengagement/centrosome conversion are separated by the first mitosis (ensuring duplication occurs only once), and acquisition of DAs and SDAs is separated by another mitosis (ensuring that cells only form a single cilium).*
>
> *(4) Fig 5A, B and Fig. 9*
>
> *(a) Are 2 separate figures needed for the model? They seem redundant.*

We find it easier not to wait Fig. 9 to have the first part depicted.

> *(b) The model shows loss of SAS6 throughout G1, but this already occurs during M/early G1*

Thanks. It was already ok in Fig. 9, we have modified for Fig. 5.

> *The model shows "MTOC capacity/conversion" during S phase, but this occurs during early G1*

Thanks a lot, as explained earlier, we used the term MTOC conversion occurring in G1 for what is normally called centrosome maturation occurring in G2/M, as explained earlier. We do not speak anymore of MTOC conversion since we have not tackled this question (explained above). We have therefore removed MTOC conversion in the texts and the schemes and replaced it by “centrosome maturation” for the duplication cycle, and by “enhanced MT nucleation capacity” for the MCC cycle. To be clearer and schematize that procentrioles are competent for MT nucleation before G2/M or A/G transitions, we have added some MT nucleated from G1 procentrioles during the canonical cycle, and from late A-stage procentrioles during the MCC cycle.

> *The model shows disengagement only in the second M phase, but this occurs already at the first M phase, directly following centriole biogenesis, right before centosome conversion.*

This is a big edition error in both Fig. 5 and 9. Of course the daughter centriole disengage during the first M-phase. This has been changed. Thanks a lot for spotting it. This, however does not contradict the hypothesis of superposition.

We also added the acquisition of distal appendage which was written in Fig. 5 but not in Fig.

9 for duplication during the second M-phase.

> *When the correct timings are incorporated in the figure, the proposed superposition of two cycles is not an accurate description of the events. Instead, your data seem consistent with a model where MCC incorporates all steps in one cell cycle variant that lacks mitoses, so that disengagement and MTOC conversion occur together with centriole elongation, followed immediately by acquisition of DAs and SDAs.*

We do agree with the acceleration of all steps into only one cycle, this is actually what we tried to propose. When putting the events in a scheme, this reveals that the events of the two canonical cycles nicely superpose, both in term of molecular composition and dynamics (Fig. 9). We therefore maintain that the null hypothesis is that the acceleration is done through a

super opposition of events that; although driven by the same molecular machinery, are normally occurring in two consecutive cell cycle. This is notably consistent with the findings of Kong et al., 2014 cited previously.

> *(5) While all reviewers felt that there was no need to introduce the new term "nest", they leave it to the authors to keep it. However, the authors may want to consider that the term is still not introduced and explained properly, which may confuse readers. For example, while this section reads like an introduction to the term: "Correlative DEUP1 live-imaging and EM highlights the existence of a pericentrosomal "nest" in brain MCC", the term is already used two times before without explanation. The first mentioning is at the beginning of the results section and is followed by citations, which gives the impression that these studies describe the nest, which is not the case.*

The first mention of “nest” is in the end of introduction resuming the findings of the paper where the term is in the following context: “we found that centriole amplification emerges in a pericentrosomal “nest” concentrating core centriole/deuterosome elements”. We looked at nest definition in the Collins Dictionnary : “a structure or other place where creatures, esp. birds, give birth or leave their eggs to develop”, we felt this was clear. We added quotation marks around the term nest.

Then, the result section opens with this sentence: “The origin of amplified centrioles in MCC remains controversial. Some live imaging experiments and electron microscopy suggest that the centrosome could constitute a nest for centriole and deuterosome biogenesis (Al Jord et al., 2014; Kalnins et al., 1972; Mori et al., 2017), but others have proposed that procentriole-loaded deuterosomes emerge independently from the centrosome location, all over the cytoplasm (Nanjundappa et al., 2019; Sorokin, 1968; Zhao et al., 2013, 2019).”. Here, the term nest is again used as a place of birth for centrioles and deuterosomes which is what is actually proposed in these papers. First, Kalnins el al., in 1969 (we made an error on the reference date, this has been changed), resume in their abstract “This observation suggests that all of the clusters may form initially in close association with the diplosomal centrioles”. Then, not to mention Al Jord 2014 which comes from our lab, the title of Mori et al. is “Cytoplasmic E2f4 forms organizing centres for initiation of centriole amplification during multiciliogenesis”, and in the paper, they show that E2F4 accumulates at the centrosome. This is now also proposed by collaborators for MCIDAS (Lu et al., 2025). We feel that these references, which are often omitted, are appropriated at this location.

Then we continue with: “To test whether microtubules drive the organization of a centrosomal nest from which procentrioles emerge”, which keeps the notion of the place of birth.

Then the title "Correlative DEUP1 live-imaging and EM highlights the existence of a pericentrosomal "nest" in brain MCC" arrives. In this section we first speak about a pericentriosomal cloud on which we zoom in using CLEM, to then conclude at the end of the section “Altogether live imaging mRuby-DEUP1/CEN2-GFP during early A-stage suggests that core deuterosome and centriole components are concentrated in a primordial cloud around the centrosome, which constitutes a nest where centrioles and deuterosomes concomitantly form before they move away from the centrosomal region (Fig. 2F)”.

Finally, we begin the discussion section regarding the nest by: “We named this transitory compartment a “nest” since deuterosomes and procentrioles emerge specifically in this region and grow while moving away from it.”

During the first revision, we tried to make it clearer. If this is still not the case after and the reviewer has another proposition of definitions/phrasing, we will be glad to consider it.

As replied to the other reviewer, the term “nest” does not need to be retained as a new

terminology. It is just a way for us to identify the transitory region and to best define one of its function/characteristic which is to host the birth of new deuterosomes and centrioles.

> *The following comments from Reviewer #3 may also provide further context regarding the editors' remaining concerns:*
>
> *The authors have done an excellent job addressing the points I raised overall, and the revision is substantially improved in focus and clarity. That said, some concerns raised by other reviewers, particularly regarding terminology and statistical analysis, could have been addressed more fully. One issue remains insufficiently resolved. Several quantitative analyses (for example Fig. 5C and 5E) still appear to rely on pooled single-event measurements collected across three independent experiments. This approach can overstate statistical significance. The authors indicate in their rebuttal that they use chi-square tests to compare proportions and to justify pooling across replicates. However, I am not convinced this addresses the issue for the intensity-based and single event distributions shown in the panels specified above. I recommend that these key analyses be represented with biological replicates shown explicitly (superplot-style, with replicates distinguished).*

Our reply was for the comparison of proportions and not the intensity-based and single event distributions shown in the panels Fig. 5C and Fig. 5E. We have now changed our plots to represent biological replicates explicitly (superplot-style, with replicates distinguished). As for the statistical analysis: we evaluated differences in marker intensity between A-stage and G-stage samples using a linear regression model, with stages as the main effect and replicate as a fixed covariate, to account for batch variation. Statistical significance was assessed using Type II ANOVA.

> *Separately, I continue to feel that some newly introduced terminology (for example, the "nest") may not be necessary at this stage. It may be sufficient to describe these structures and focus on their spatiotemporal behavior, composition, and measurable features, rather than assigning new names. Having read the authors' response, I understand that they would like to retain this terminology, which is acceptable; however, it may not be readily adopted by the field.*

The term “nest” does not need to be retained as a new terminology. It is just a way for us to identify the region and to best define one of its function/characteristic which is to host the birth of new deuterosomes and centrioles.

> *Minor correction (remove "in MCCs" part from the following sentence):*
>
> *In MCC, PCM1 depletion alters deuterosome formation and centriole production in brain and airway MCC (Hall et al., 2023; Zhao et al., 2021).*

Done

https://doi.org/10.7554/eLife.96584.2.sa0