



Centriole amplification in brain multiciliated cells: high resolution spatiotemporal dynamics and identification of regulatory mechanisms

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Centriole Amplification in Brain Multiciliated Cells

High resolution spatiotemporal dynamics and
identification of regulatory mechanisms

Adel Al Jord

PhD thesis

2016

Université Pierre et Marie Curie

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Doctoral school: ED3C Cerveau, Cognition, Comportement

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Centriole amplification in brain multiciliated cells

*High resolution spatiotemporal dynamics and identification of
regulatory mechanisms*

Adel Al Jord

PhD thesis in cell and developmental biology

Supervised by Alice Meunier & Nathalie Spassky

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To Olga & Rabih Al Jord

For noticing and nurturing my innate curiosity

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Abstract

Multiciliated mammalian cells play a crucial role in the propulsion of physiological fluids. Their dysfunction can be lethal or cause severe chronic diseases. In contrast to the strict centriole number control in cycling cells, multiciliated cell differentiation is marked by the production of up to several hundred centrioles, each nucleating a motile cilium. The mechanisms of centriole amplification or centriole number control in these cells were unknown and new centrioles were thought to appear *de novo* in the cytoplasm. At the beginning of this PhD project, videomicroscopy combined with correlative super-resolution and electron microscopy has enabled us to determine that all procentrioles are generated via runs of nucleation from the pre-existing progenitor cell centrosome. We show that the daughter centriole of the centrosome is the primary nucleation site for 95% of the new centrioles in multiciliated cells and thus refute the *de novo* hypothesis. Then, we provide evidence of an activation of the Cdk1-CyclinB1 complex along with its mitotic regulatory network during the centriole amplification dynamic. By coupling single cell live imaging with pharmacological modulation of mitosis regulators, we show that the mitosis machinery orchestrates the spatiotemporal progression of centriole amplification in terminally differentiating multiciliated cell progenitors. The fine-tuning of Cdk1 activity prevents mitosis while allowing the timely coordination of centriole number, growth, and disengagement through checkpoint-like phase transitions necessary for subsequent functional motile ciliation. Collectively, these results provide a new paradigm for studying multiciliated cell differentiation, cilia-related diseases and pathological centriole amplification associated with cancer and microcephaly.

Introduction

CENTROSOME OVERVIEW

Almost 130 years ago, a puzzling cellular organelle caught the simultaneous attention of two scientists conducting research on chromosome divisions in nematodes. In 1887, both Edouard van Beneden in Liège and Theodor Boveri in Munich provided independent reports of a centrally localized and self-replicating organelle that seemed to organize the cell as well as its division ¹⁻³. This central cell organizer retained Boveri's nomenclature and became known as the "Centrosome".

The present day centrosome is often referred to as the major microtubule organizing centre (MTOC) of animal cells ^{4,5}. By organizing the cytoskeleton in quiescent cells, the centrosome modulates the foundations for cellular architecture and participates in polarized epithelial tissue design ⁶. The microtubules nucleated by the centrosome position cellular organelles as well as the cellular secretory system by providing the infrastructure necessary for bidirectional cargo and vesicle transport ⁷⁻⁹. The competency of the centrosome to continuously remodel the microtubules supports cellular motility and morphology in cells undergoing migration ^{10,11}. In parallel, the centrosome can dock to the apical plasma membrane and nucleate the primary cilium, a microtubule-based cell projection embedded with receptors that sense the external environment and provide developmental signalling cues ^{12,13}. In addition to conveying signalling molecules through its cilium nucleation capacity, the centrosome itself can act as a signalling hub and modulate cell cycle progression by becoming a platform for the convergence of DNA damage sensing molecules and for the activation of mitosis signalling cascades ¹⁴⁻²². As the cell cycle progresses, the centrosome duplicates and the two centrosomes engage in the organization of the mitotic microtubule bipolar spindle ^{23,24}. During mitosis, the two centrosomes participate in the balanced segregation of genetic material between the future daughter cells by generating pulling and pushing forces on the centromeres and the cell cortex to separate sister chromatids. It is worth noting that age

differences between the duplicated centrosomes can also source asymmetric cell behaviour in the daughter cells ^{25–27}. In the mouse brain for example, preferential inheritance of the older centrosome by neural progenitors maintains their stem cell properties, thus positively regulating embryonic brain development ²⁷. The centrosome can therefore be viewed as a dynamic organelle that modulates cellular signalling in addition to organizing cellular architecture during quiescence and proliferation.

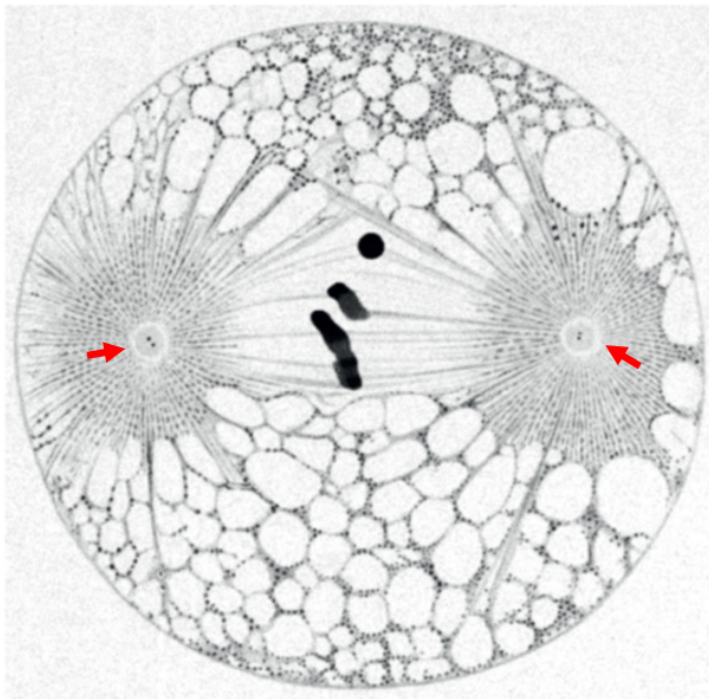


Figure 1 Theodor Boveri's illustration of a mitotic cell from 1900 ²⁹

Note the two spindle poles with centrosomes (red arrows). Each centrosome is illustrated with two centrioles surrounded by a concentric pericentrosomal region that he named "centroplasm". Adapted from ¹⁰⁰.

By the end of the 19th century, Boveri's research focus shifted towards the centrosome's physiological relevance based on his ensuing short report ²⁸ and landmark monograph ²⁹. He makes key observations on paraffin sections of sea urchin embryos, stained with iron haematoxylin, on centrosome's composition, behaviour, and function (Fig. 1). First, he observes that the centrosome is composed of 2 distinct granules, or "centrioles", and a surrounding "centroplasm", a circular cloud of a homogenous slightly fibrillated substance. Then, he highlights the centrosome's dynamic behaviour and reports that centrosomes duplicate in interphase before separating in nuclear contact at the onset of mitosis. Finally, he

states that it is solely the centropasm that acts as an “excitation” and organizing centre of the mitotic astral rays. His pioneering work and impressive observational skills provided a comprehensive foundation for modern day centrosome biology. More than a century of research later, significant advances in microscopy allowed to confirm and to enrich the knowledge of the centrosome and of its composition.

CENTROSOME COMPOSITION

Cells inherit a single centrosome from the previous cell division. The resting G0 phase centrosome is composed of a cloud of satellite proteins that surround a pair of centrioles tethered by interconnecting fibres ⁴ (Fig. 2). Long assumed to be amorphous, the cloud surrounding the centrioles is in fact a highly organized concentric and layered assembly of accessory proteins that constitute the pericentriolar material (PCM) ^{30,31}. Permanent residents of the PCM mainly consist of proteins that mediate microtubule nucleation or mitotic spindle assembly. Other molecules that include the scaffold protein Cep152 or the mitotic kinases Plk1 (Polo-like kinase 1) and Aurora A localize to the PCM to regulate the initiation or the

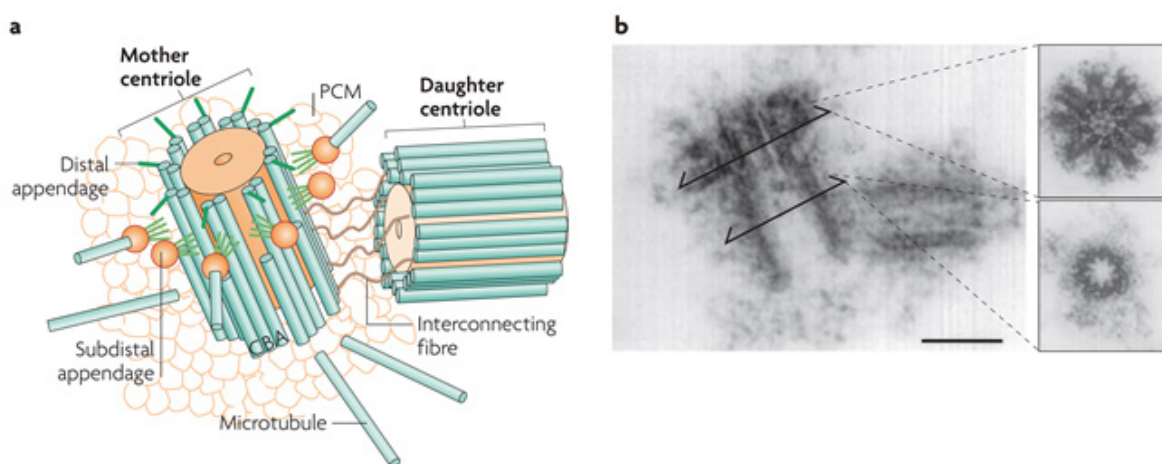


Figure 2 Centrosome illustration and ultrastructure

a, Schematic view of the centrosome composed of a mature mother centriole, a less mature daughter centriole, and the surrounding PCM. **b**, Transmission electron microscopy section of a centrosome. Cross section of the distal portion (top right inset; sub distal appendages) and the proximal portion (bottom right inset; note the microtubule triplets that compose the cylinder) of the mother centriole. Scale bar, 0.2µm. Adapted from ⁹⁵.

progression of the centrosome duplication cycle^{22,32–35}.

The centrally localized pair of centrioles are microtubule based cylinders that measure approximately half a micron long and quarter of a micron wide. Both centrioles are composed of a circular assembly of nine microtubule triplets and display a proximal to distal polarization (Fig. 2b). However, centrosomal centrioles are not equivalent since they inherently present age-related and structural asymmetries³⁶. The older “mature” mother centriole is decorated by sub-distal and distal appendages that are absent from the younger “immature” daughter centriole. Cells use this generational asymmetry of the centrioles composing its centrosome to perform different tasks. Sub-distal and distal appendages allow only the mature mother centriole to nucleate and anchor microtubules or to function as a basal body for the nucleation of the primary cilium^{26,37}.

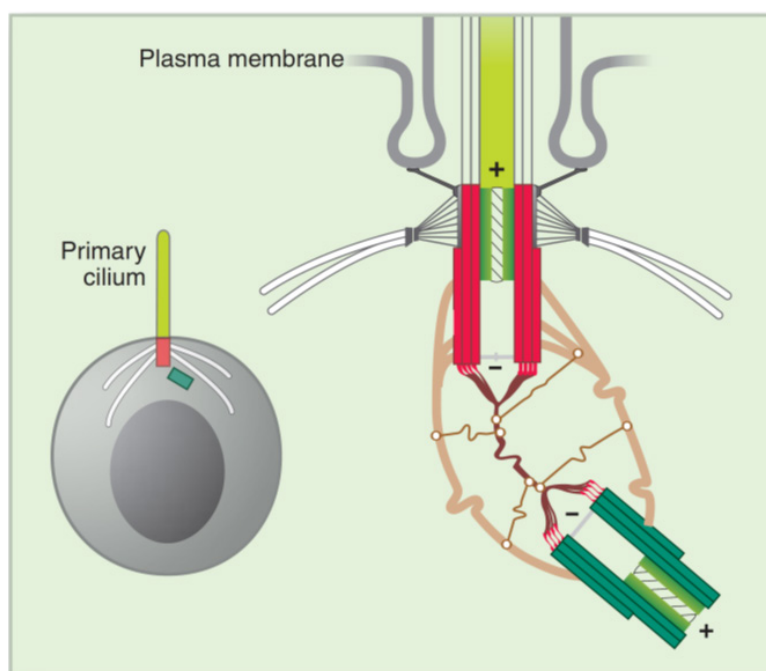


Figure 3 Primary cilium nucleation

Schematic view of the centrosome. The mother centriole (red) docks to the apical membrane to function as a basal body for the nucleation of the primary cilium. Adapted from⁴.

The primary cilium is an antenna-like structure that emanates from the apical surface of the cell to reception chemical as well as mechanical stimuli of the external environment^{13,38,39}. The nucleation of the cilium depends on the attachment of the mother centriole to the apical plasma membrane via distal appendages^{26,40–42} (Fig. 3). Similar to the mother centriole itself,

the ciliary axoneme presents a nine-fold symmetry, in the form of 9 peripheral microtubule doublets. Dynamic intraflagellar transport supports the architecture of the cilium and relays the signaling messages from the cilium into the cell where they can subsequently regulate developmental programs¹³.

CENTROSOME CYCLE

With every cell cycle, the cycling cell replicates its DNA and duplicates its centrosome to pass them on to daughter cells (Fig. 4). Despite the mentioned age and structure asymmetry, the mature mother centriole and the immature daughter centriole are both regarded as equivalent in their ability to form new centrosomes. Their symmetric duplication during the cell cycle results in the formation of two new centrosomes, which is crucial for cell homeostasis and proper genetic partitioning between the daughter cells^{43–47}.

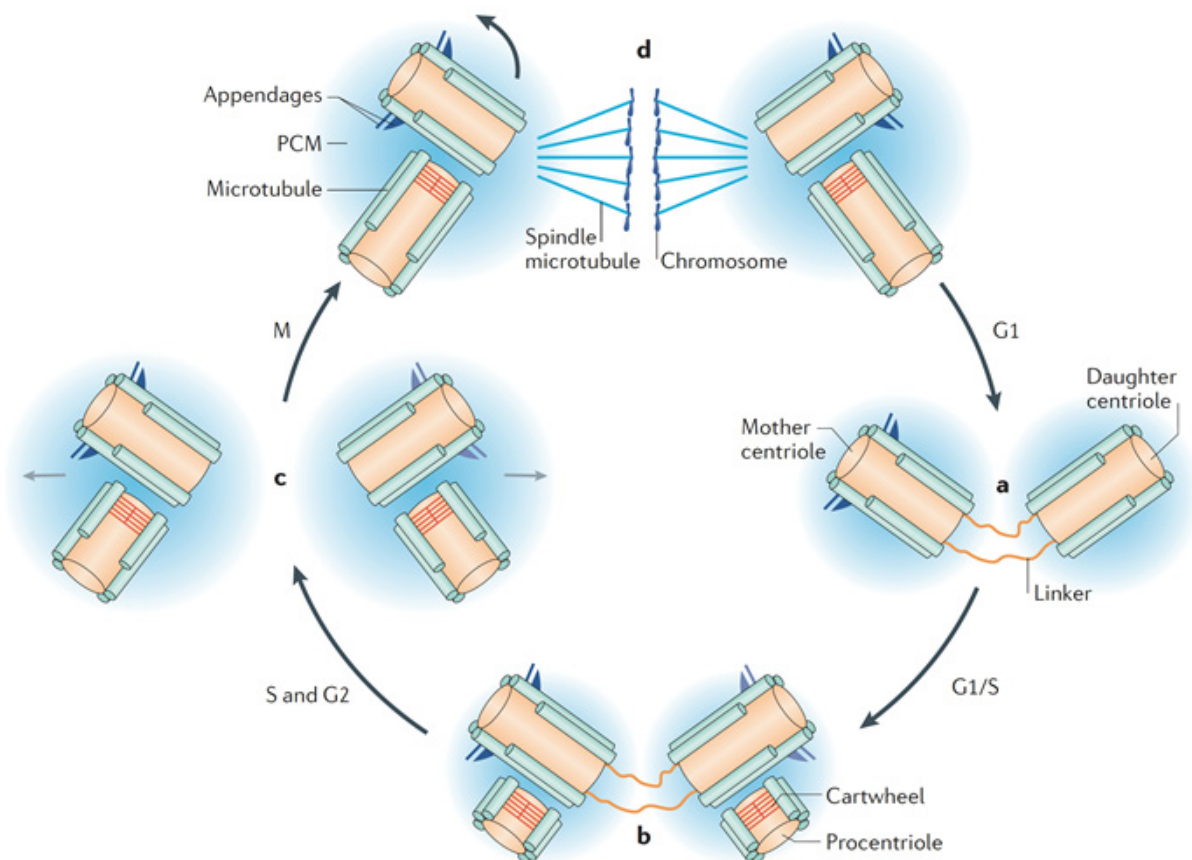


Figure 4 Centrosome duplication cycle

Schematic view of the centrosome duplication cycle in relation to the cell cycle phases. *Adapted from*¹⁰⁰.

At the onset of centrosome duplication, a conserved molecular cascade controls the formation of one procentriole from a single nucleation point focalized on the proximal segment of each existing centriole^{5,24,48,49} (Fig. 5a). In late G1 phase, procentriole biogenesis is initiated from

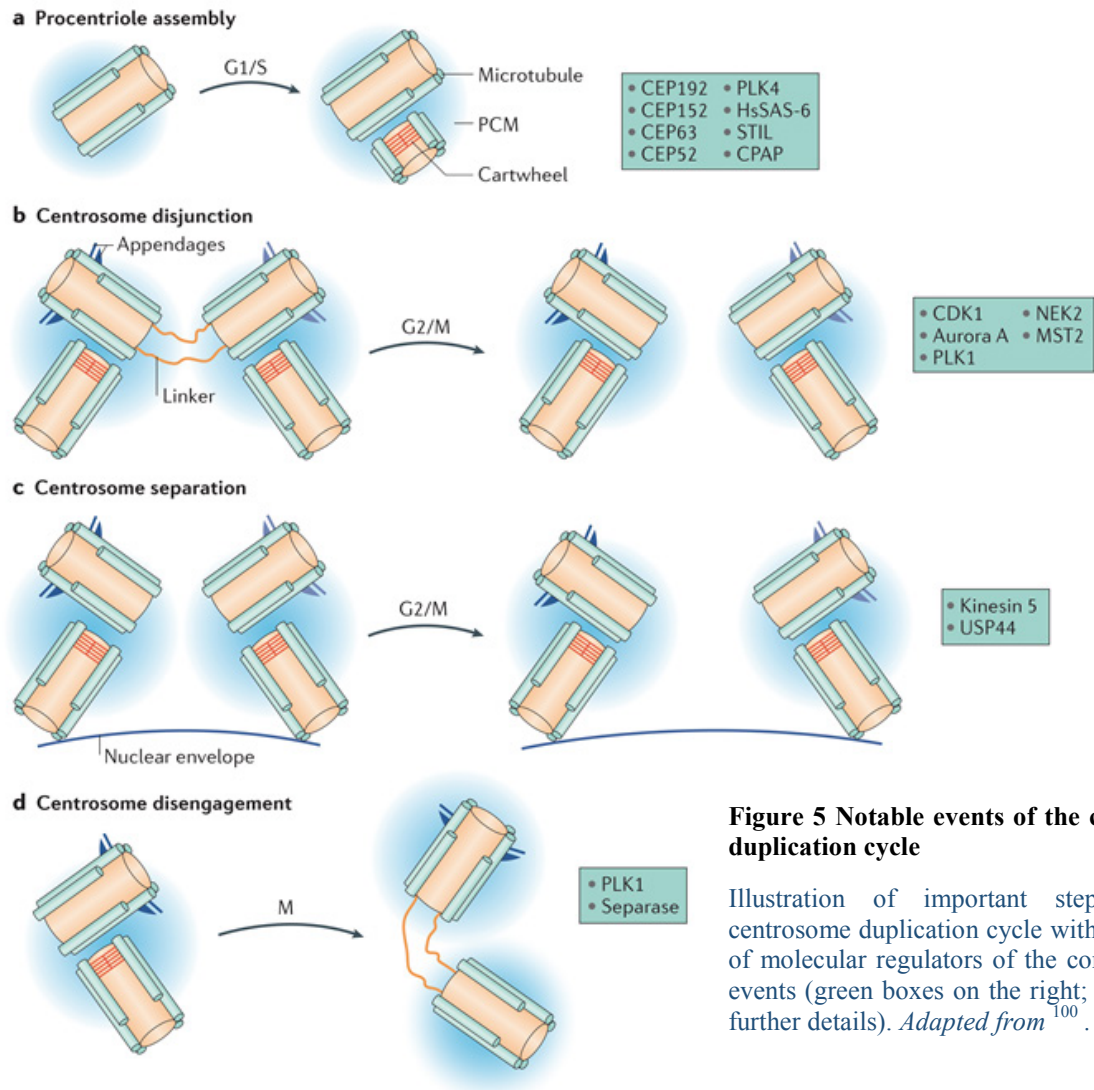


Figure 5 Notable events of the centrosome duplication cycle

Illustration of important steps of the centrosome duplication cycle with a selection of molecular regulators of the corresponding events (green boxes on the right; see text for further details). Adapted from¹⁰⁰.

the mother and daughter centrioles when the PCM scaffold Cep152 recruits the Polo-like kinase 4, Plk4, to the nucleation region^{32,50–53}. Plk4 then phosphorylates Stil that favors the loading of the structural cartwheel protein Sas-6 in charge of scaffolding the future centriolar nine-fold symmetry^{54–58}. In early S-phase, a nascent procentriole is built as microtubules associate onto the cartwheel base and are stabilized by Centrobin^{59,60}. As of this point, the two newly formed procentrioles are linked to and orthogonally aligned with their parental centrioles. This angular centriole-procentriole configuration, known as centriole engagement,

inhibits the potential untimely initiation of additional procentrioles and is maintained until the end of the centrosome cycle ⁶¹. The procentrioles then start elongating through the S and G2 phases by continuous tubulin incorporation while maintaining their engagement. Procentriole elongation is regulated by capping proteins such as Ckap and CP110 ⁶² as core centriolar proteins that include tubulin, Cep120, and Centrin are incorporated ⁶³⁻⁶⁵. At the G2/M transition, Poc5 constructs the distal portion of the procentrioles while polyglutamylation stabilizes the elongated tubulin cylinders ^{66,67}. At the onset of mitosis, resorption of the primary cilium ⁶⁸ in addition to the loss of the interphasic connecting fibers composed of C-nap1 and rootletin allows the separation of the duplicated centrosomes along the nuclear membrane as they migrate to opposite poles of the cell in preparation for mitotic spindle formation ⁶⁹⁻⁷¹ (Fig. 5b-c). In parallel to the separation, the daughter centriole becomes the mother centriole of the newly duplicated centrosome as it acquires appendages and expands its own PCM to enhance microtubule nucleation ⁷². During late mitosis, the tight orthogonal centriole-procentriole configuration is relaxed in both centrosomes during a process called mitotic licensing ^{61,73} (Fig. 5d). Consequently, each newly formed daughter cell inherits a centrosome with disengaged mother/daughter centrioles licensed to initiate a new round of procentriole biogenesis necessary for the consecutive centrosome cycle.

Centrosome duplication is highly synchronized with the progression of DNA replication. Centriole duplication onset parallels the initiation of DNA replication at the G1/S transition ^{74,75}. Later on, in parallel to DNA condensation and dependent on the activity of the G2/M checkpoint regulators, new centrosomes separate along the nuclear membrane ^{34,76,77}. Then, in parallel to sister chromatid separation and dependent on spindle assembly checkpoint (SAC) regulators, mother/daughter centrioles disengage ^{61,73,78}.

Such synchronization is achieved through the use of common regulators for both DNA and centriolar events (Fig. 5). At the G1 to S phase transition, the E2F family of transcription

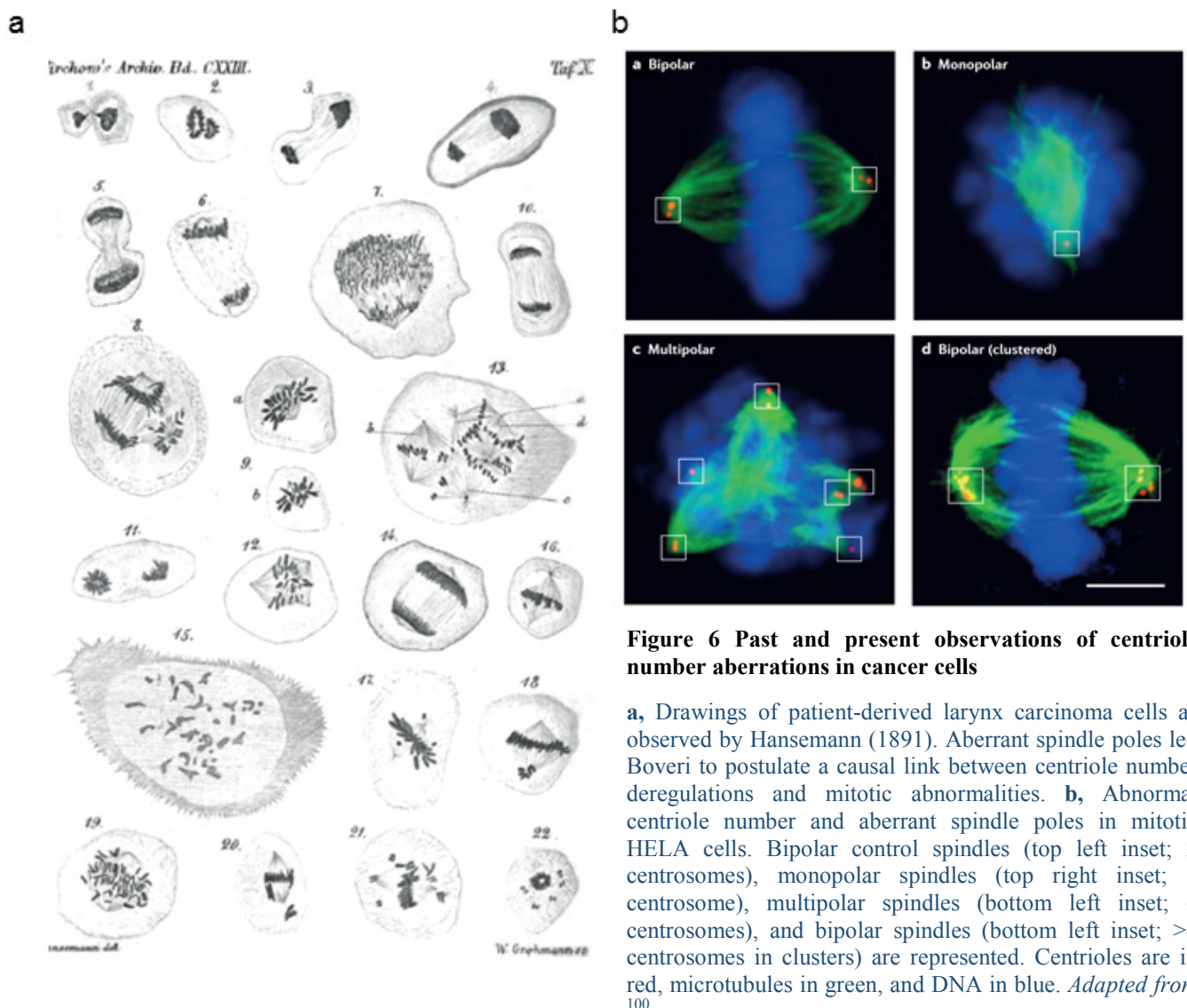
factors and the Cyclin-dependent kinase 2 (Cdk2) trigger the onset of DNA replication and centriole duplication^{74,75}. The master M-phase regulator Cyclin-dependent kinase 1 (Cdk1) associated with G2/M cyclins, along with Aurora A kinase and Plk1, phosphorylate downstream targets to trigger mitosis onset, a process marked by DNA condensation and nuclear envelope breakdown^{19,20,22,79–88}. Regulators involved in M-phase entry are also involved in mitotic cytoskeletal reorganization and in the migration of the centrosomes along the nuclear envelope^{77,89,90}. During the metaphase-to-anaphase transition, the ubiquitin ligase, known as the anaphase promoting complex in association with its activator Cdc20 (APC/C-Cdc20), targets mitotic cyclins for proteasome degradation thereby decreasing Cdk1 activity for mitosis exit. In parallel, Securin is also targeted for degradation and results in the release of an active Separase. The mitotic activity of Plk1 followed by the now active Separase initiate final steps of mitosis by disconnecting replicated chromosomes and duplicated centrosomal centrioles^{73,91–93}.

Cell cycle coupled coordination of centrosome duplication in addition to a tight control of centriolar protein expression ensure a strict spatiotemporal control of centriole number in cycling cells^{56,94–96}. Aberrant numbers of centrioles lead to defects in cytoskeletal organisation and thus to incorrect chromosome segregation and abnormal cell migration^{43–45,97}. Perturbing the mechanistic links between centrosome duplication and DNA replication may result in centriole amplification. For instance, the absence or the premature loss of centriole-procentriole engagement due to Plk1 or Cdk1 dysfunction licenses the formation of more than two centriole copies per cycle. Another mechanism of centriole amplification lies in the upregulation of centriole biogenesis initiators, such as Cep152 and Plk4, or of the cartwheel proteins, Stil and Sas-6^{32,56,78,94,98,99}. Regardless of the various mechanistic origins sourcing supernumerary centrioles in cycling cells, pathological centriole amplification is

continuously attracting scientific attention as numerous reports increasingly link this phenomenon to microcephaly and tumorigenesis^{43–46}.

CENTRIOLE AMPLIFICATION

Late 19th century observations of defective mitosis in carcinomas led Boveri to state that centrosome number deregulations cause mitotic abnormalities and initiate cancer (Fig. 6a). Boveri's hypothesis was disputed and subsequently remained dormant for decades before being revived by increasing documentations of centrosome number aberrations in a large majority of human cancers¹⁰⁰. Whether centriole amplification is a cause or a consequence of mammalian tumorigenesis remains a debatable question. However, an increasing line of evidence points out possible or validated mechanisms through which centrosome amplification may deregulate cell behaviour and proliferation, or contribute to tumorigenesis.



During interphase, additional centrioles excessively recruit PCM proteins resulting in increased centrosomal microtubule nucleation capacities with possible repercussions on cell polarity and secretory activities ^{8,101,102}. Increases in microtubule nucleation renders cell migration more efficient and may provide invasive characteristics that translate experimentally into metastatic behaviour within a tumorigenic context ⁴⁵. Supplementary centrosomes have also been shown to nucleate auxiliary primary cilia ^{103,104}, thus compromising the cilium-dependant Sonic hedgehog signalling pathway ¹⁰⁴. Moreover, the centrosome itself is also a platform for mediating initial Cdk1-CyclinB1 activity propagation ^{17,22,72}. Additional centrosomes could therefore compromise centrosome mediated signalling and potentially promote premature mitotic entry and licensing.

Chromosomal instabilities are hallmarks of cancer that could be generated in mitotic cells with more than two centrosomes ^{105–108}. Cells with excess centrosomes can either form bipolar mitotic spindles or transient multipolar spindles that defectively attach microtubules with kinetochores and cause faltered chromosome separation (Fig. 6b; lower insets). Lagging chromosomes that do not integrate the nucleus on time form cytoplasmic micronuclei, which are more prone to DNA damage due to a lack of a fully protective nuclear membrane ¹⁰⁹. The micronuclei that reintegrate the main nuclear compartment in the following cycle can lead to cancer-associated chromothripsis ^{110,111}. The result of a single catastrophic event, chromothripsis is characterized by shuffled reintegration of shattered DNA back into the nucleus that gives rise to a large number of mutational rearrangements in localized regions of single chromosomes. Chromothripsis can therefore be a generator of DNA replication errors as well as a direct contributor to transcriptional defects.

Another form of chromosomal instabilities induced by abnormal spindles comprises aneuploidy arising from asymmetric segregation of chromosomes ⁹⁷. The loss of tumour suppressor genes or the gain of oncogenes can provide a platform for tumorigenesis in the

subsequent cycles. However, cells with aberrant numbers of chromosomes often undergo checkpoint arrest followed by a programmed suicide. That is the case in the developing brain where neural stem cell aneuploidy caused by divisions in presence of amplified centrioles is countered by p53-dependent checkpoint arrests. The prolonged blockage results in apoptotic death of progenitors leading to tissue degeneration followed by viable microcephaly or organismal death^{46,112}.

To summarize, the formation of supernumerary centrioles in cycling cells may potentially affect numerous centrosome-related functions beyond mitotic spindle establishment and fidelity that include intracellular signalling, polarity, cell migration, as well as asymmetric cell divisions (Fig. 7a-f). Centriole amplification in cycling cells is therefore considered to be contributor to tumorigenesis as well as a cause of tissue degeneration.

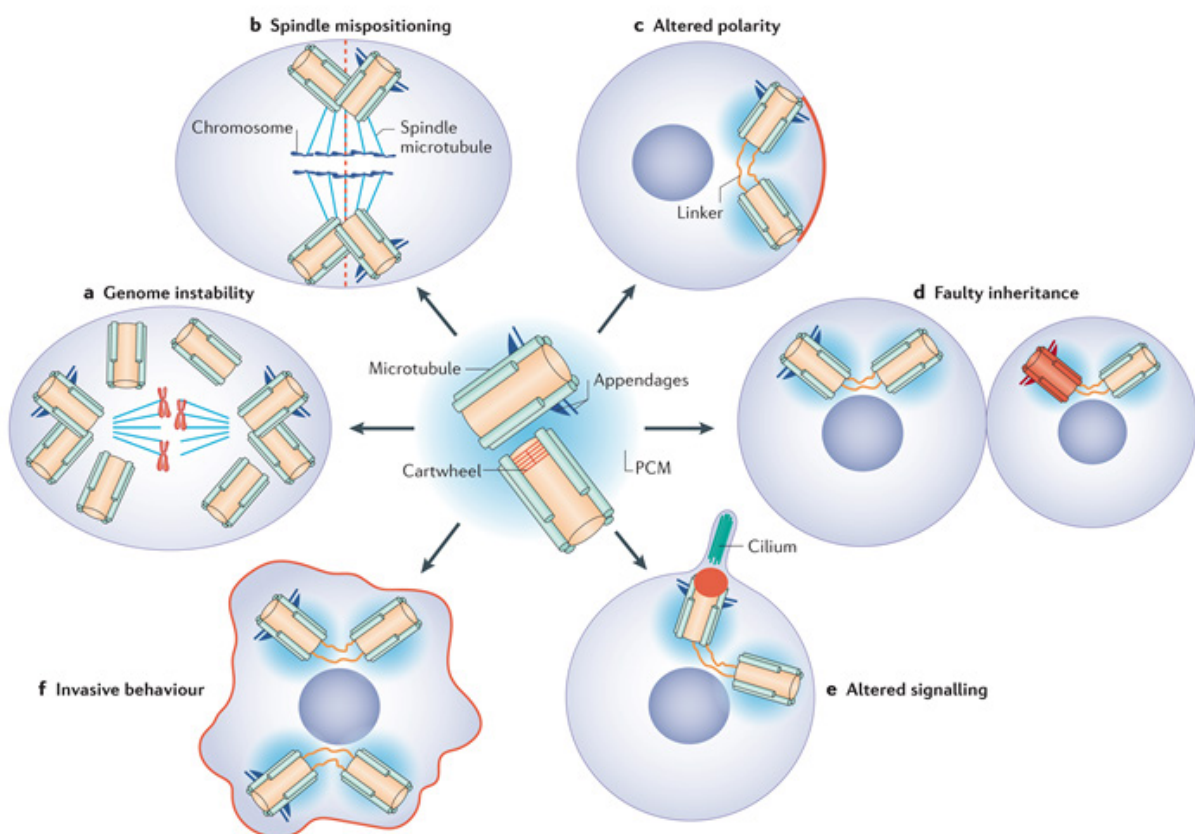


Figure 7 Centrosome defects linked to potential consequences

Schematic overview linking abnormal centrosome function or number with potential consequences on cell homeostasis or proliferation. Adapted from¹⁰⁰.

In stark contrast to the cycling cell context, centriole amplification is part of the physiological differentiation process observed in post-mitotic multiciliated cells (MCC)¹¹³. In the course of differentiation of MCC, the number of centrioles in stem cells shifts from 2 to more than 100 to nucleate the same number of motile cilia required for the efficient propelling of physiological fluids.

MULTICILIATED CELLS

After the invention of the microscope in the 17th century, one of the first cells to be observed and described by Antonie van Leeuwenhoek were multiciliated unicellular organisms known as ciliates. In a communication to the Royal Society of London dating back to 1667, he stated that these protozoans were covered with “little legs [cilia]” which “brought off incredibly quick motions”. The motile cilium is therefore one of the first cellular organelles to be documented in the history of cell biology. We now know that numerous vertebrate tissues are lined by MCC which are specialized epithelial cells with patches of multiple motile cilia protruding from their apical surface^{114–119}. In addition to completing the epithelial barrier function in tissues, MCC employ their motile cilia for the generation of a directional and transporting stream of fluids and particles^{117,120,121}. Depending on the tissue of localization, the patches of cilia in MCC are composed of tens to more than several hundreds of motile cilia that reach an average height of 10 micrometres above the apical surface^{122–124}. Each motile cilium stems from a single centriolar basal body and has a similar width of approximately 250 nanometres^{38,125}. The motile cilium differs functionally from the primary cilium by its motility that is made possible by structural differences in axoneme components. The main structural difference is that the 9 peripheral microtubule doublets, composing the outer axoneme of the motile cilium, are physically bridged to an additional central pair of singlet microtubules via connecting fibres known as radial spokes (Fig. 8a). ATP dependant interactions between the axonemal dynein motor proteins and the peripheral doublets cause

microtubule sliding that is countered by the radial spokes, thus inducing prompt unilateral bending of the motile cilium. The consecutive bendings visually translate into characteristic motile ciliary beatings which are binary: a rapid forward “effective” stroke is always followed by a relatively slower backward “recovery” stroke ^{38,126} (Fig. 8b). Although cilia beat frequencies depend on numerous environmental, experimental, and regulatory factors ^{127–134}, measured beat frequencies of motile cilia can exceed 40Hz (beats per second) and propel fluids above them at speeds ranging from 20 to 500 $\mu\text{m/s}$. To reach such impressive

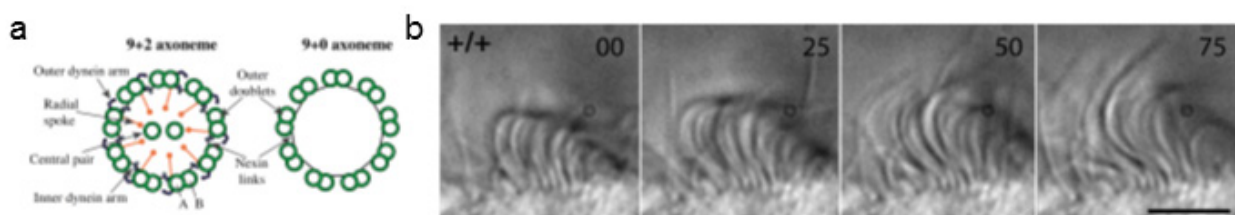


Figure 8 Cilia structure and motile cilia beating

a, Schematic cross-section view of structural differences between a motile cilium (left panel) and a primary cilium (right panel). **b**, Time sequence showing ependymal motile cilia beating that is marked by an effective stroke (00-25) and a recovery stroke (50-75). Time in ms; scale bar, 5 μm . Adapted from ^{271,272}.

propulsion speeds, ciliary beatings are directionally coordinated not only within a single patch of motile cilia, but also along the epithelium that the MCC line ^{38,126,135,136}. In addition, tissue-wide coordination generates energy-efficient polarized and propagated waves of ciliary beatings that can theoretically induce an up to a three-fold increase in the global fluid propulsion velocity on the macroscale of a tissue ¹³⁰. In mammals, MCC execute their functions in epithelial tissues contained within the reproductive, respiratory, as well as the central nervous systems (Fig. 9).

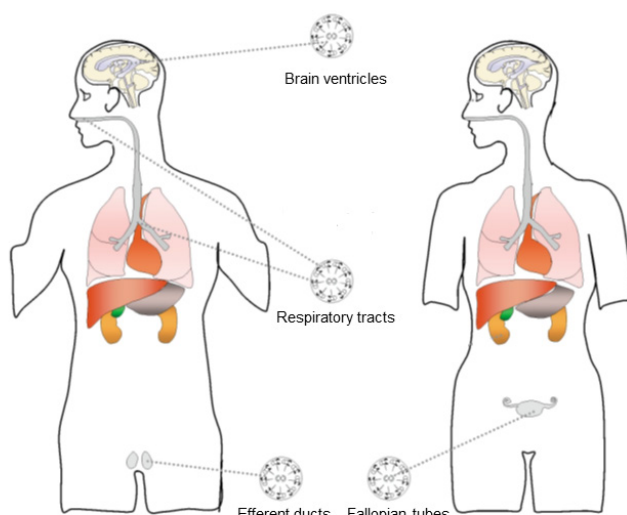


Figure 9 Localization of multiciliated cell epithelia in humans

Schematic view of tissues containing MCC in the human body. Adapted from ²⁰³.

MULTICILIATED CELLS IN THE REPRODUCTIVE SYSTEMS

In the male reproductive system, developing spermatozoa exit the seminiferous tubules via the rete testis in a bath of luminal fluids and accumulate in the proximal regions of multiciliated tubal epithelia known as efferent ducts (Fig. 10a). From there, the motile ciliary beating of an increasing gradient of MCC coupled to surrounding smooth muscle contractions conduct the spermatozoa along the duct and towards the epididymis. In parallel, stirring of the luminal fluids by the motile cilia aids neighbouring complementary non-ciliated cells to absorb up to 90% of the initially deposited fluid volume, therefore increasing concentrations of spermatozoa as they reach the epididymis and acquire their own axoneme motility^{137,138}.

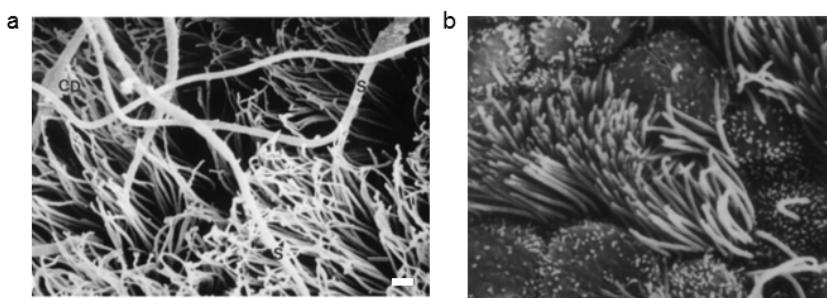


Figure 10 Multiciliated cells in reproductive tracts

Scanning electron microscopy images showing MCC lining vertebrate (ostrich) efferent ducts (a) and mouse fallopian tubes (b). “S” in a indicates maturing spermatozoa in the efferent duct. Scale bar in a, 1 μ m; magnification X5000 in b. Adapted from^{119,273}.

As millions of spermatozoa are inseminated, only thousands bypass the cervical mucus and the uterotubal junction in the female reproductive tract¹³⁹. After passing these first two barriers, the remaining spermatozoa reach the Fallopian tubes, which are multiciliated tubal epithelia (Fig. 10b) bridging the uterus to the two ovaries. Spermatozoa then arrest in the proximal segment of the fallopian tube as sperm heads bind to the ciliated cells via species specific carbohydrate moieties¹⁴⁰. Ciliated cells are thus proposed to form a viable sperm reservoir where spermatozoa remain latent, up to six days in humans, until ovulation^{139–141}. In some species such as reptiles, the duration of spermatozoa storage can surprisingly exceed several months of time¹⁴². After ovulation, the cumulus-oocyte-complex (a.k.a. COC) is first picked up by ciliary adhesions of MCC in the distal segment of the Fallopian tube^{117,143}. As the current of oviductal fluid generated by the ciliary beating guides the COC in direction of

the uterus, the ciliated cells in the proximal segment of the fallopian tube are proposed to release discrete batches of spermatozoa in a sequential manner^{117,139,140,144}. The most motile spermatozoa are able to slither against the oviductal fluid current generated by the MCC until successful fertilization in the central segment of the Fallopian tube. Fertilization induces a significant decrease in the ciliary beat frequency of motile cilia as the ciliated cells along with smooth muscle contractions delicately channel the newly formed zygote outwards through the tube into the uterus for implantation^{117,133}. In addition to transporting and selecting for vital female and motile male gametes, MCC of the fallopian tube thus contribute to directional transport of the embryo in its earliest stages of development.

MULTICILIATED CELLS IN THE RESPIRATORY SYSTEM

At birth, the respiratory system comes in direct contact with airborne biological, chemical, and physical contaminants. To face a lifelong exposition to environmental hazards, the respiratory epithelium (Fig. 11a) continually produces airway liquids that function as physical protective barriers and as a junction for immune defences^{121,145–148}. The liquid consists of two distinguishable layers. The first layer is composed of a less than 10 micrometre band of hydrating fluids filling the periciliary region, whereas the second one is a layer of glycoproteins and salts that form the mucus. The viscous mucus is constantly transported by the tips of motile cilia^{124,147,149}. The airway liquids cover the entire respiratory epithelium that is composed of mucus secreting goblet cells, periciliary fluid-secreting serous

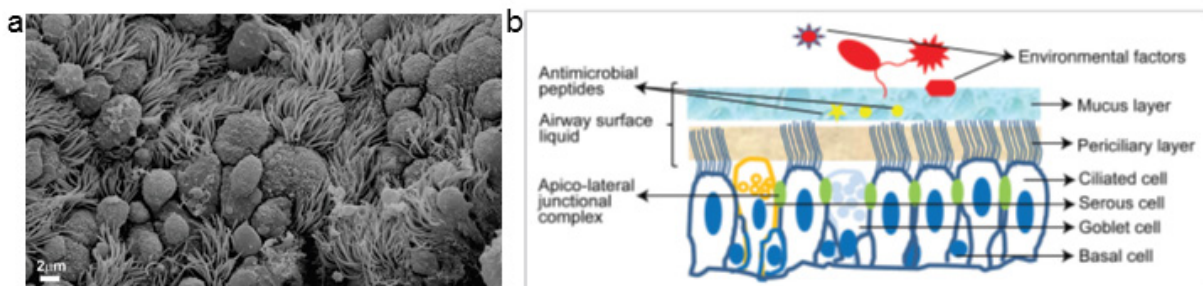


Figure 11 Multiciliated cells in airways

a, Scanning electron microscopy image showing MCC lining the mouse trachea **b**, Schematic view of major components of human airway tract epithelium. Scale bar, 2μm. Adapted from^{147,274}.

and clara cells, and an increasing gradient of MCC from the terminal bronchioles up to the nasal cavity (Fig. 11b). Neutrophils and macrophages associate to the mucus as external bodies accumulate. In parallel, all four types of airway epithelial cells directly contribute to innate immune defences by injection of microbial membrane disruptors and pro-inflammatory cytokines into the periciliary fluid that eventually initiate the adaptive immunity apparatus^{124,145,146}. The coordinated ciliary beating of MCC provides the necessary forces for the displacement of pathogen-filled mucus towards the oropharynx for elimination. Furthermore, MCC dependant mucociliary clearance allows the renewal of the periciliary fluid and thus maintains, in concertation with neighbouring secretory cells, a sustainable and efficient immune response as well as tissue-wide homeostasis.

MULTICILIATED CELLS IN THE CENTRAL NERVOUS SYSTEM

In the adult human central nervous system (CNS), half a litre of a watery vehicle of numerous nutrients and signalling molecules known as the cerebrospinal fluid (CSF) is secreted on a daily basis into the brain ventricular system and onto a specialized ciliated epithelium known as the ependyma. The CSF is composed of hundreds of molecules that, together with mechanical forces of the generated flow, modulate long-term CNS development, locomotion, as well as behaviour by actively participating in the maintenance of CNS homeostasis^{150–152}. During embryogenesis, the CSF delivers growth factors, such as sonic hedgehog and insulin like growth-factors, which stimulate brain progenitor proliferation. In addition, the CSF simultaneously provides the necessary internal pressure to support physical brain enlargement^{151,153,154}. In adults, the CSF contains modulators of appetite, seasonal circadian rhythms, and the sleep/wake cycle¹⁵¹. For instance, the naturally occurring sleep-inducing fatty acid Oleamide accumulates in the CSF as a result of sleep deprivation^{155,156}, whereas presence of the hypothalamic excitatory neuropeptide Orexin-A promotes prolonged wakefulness¹⁵⁷. The CSF composition is additionally supplemented by

physiological metabolic excretions of the CNS, pathological brain toxins, and infectious agents that are deposited into the brain ventricular system ¹²⁰. In addition to its potent clearance function, the circulating CSF counters the deposited waste by channelling antioxidants like melatonin, as well as immune cells that bypass the blood brain barrier in response to infection and inflammation ^{120,150,151}.

CSF originates from the choroid plexus, flows across the walls of the lateral ventricles in direction of the third ventricle, prior to descending towards the fourth ventricle and down the central canal of the spinal cord. The fluid then circulates in the subarachnoid space where it is resorbed back into the lymphatic and blood system. CSF is produced by specialized secretory MCC outlining the choroid plexus, which is a thin epithelial sheet that forms a filtering blood-brain barrier by encapsulating arborescent blood vessels ¹⁵⁰. Due to the continuous secretory activity, the apical surfaces of the choroid plexus MCC have remarkably large amounts of microvilli and relatively low numbers of motile cilia. Consistently, the initial pulsatile CSF flow from the choroid plexus is generated by arterial pulsations instead of ciliary motility. Also, the pressure gradient created by the secretion of CSF at the back of each ventricle promotes CSF outflow at the front. As the CSF is pulsated towards the motile ciliary tufts of ependymal MCC lining the ventricles (Fig. 12a), metachronal waves of ciliary beating create a near-wall directional current of CSF oriented towards the next ventricular cavity ^{136,158}. Instead of being the predominant generators of a directional CSF bulk flow, which is promoted by choroid plexus secretion yet subjected to reversal at each cardiac cycle, ependymal MCC lining the lateral ventricles are proposed to generate an active directional flow at the ventricular surfaces ¹³⁴ and through the thin-sectioned interventricular aqueducts ¹³⁰. In lateral ventricles, the subventricular zone (SVZ), which is the main neurogenic region of the adult mammalian brain, notably lies beneath the ependymal MCC epithelium ^{159–161} (Fig. 12b). Interestingly, a study conducted in mice proposed that the directional CSF flow

propagated by motile ciliary beating of ependymal cells creates a caudo-rostral gradient of chemorepellent Slit molecules that guides the migration, in the parenchyma, of newly formed neuroblasts towards the olfactory bulb ¹⁵⁸. In addition to providing an epithelial barrier, ependymal multiciliated cells in the lateral ventricles may therefore contribute to sustained lifelong development and function of the CNS.

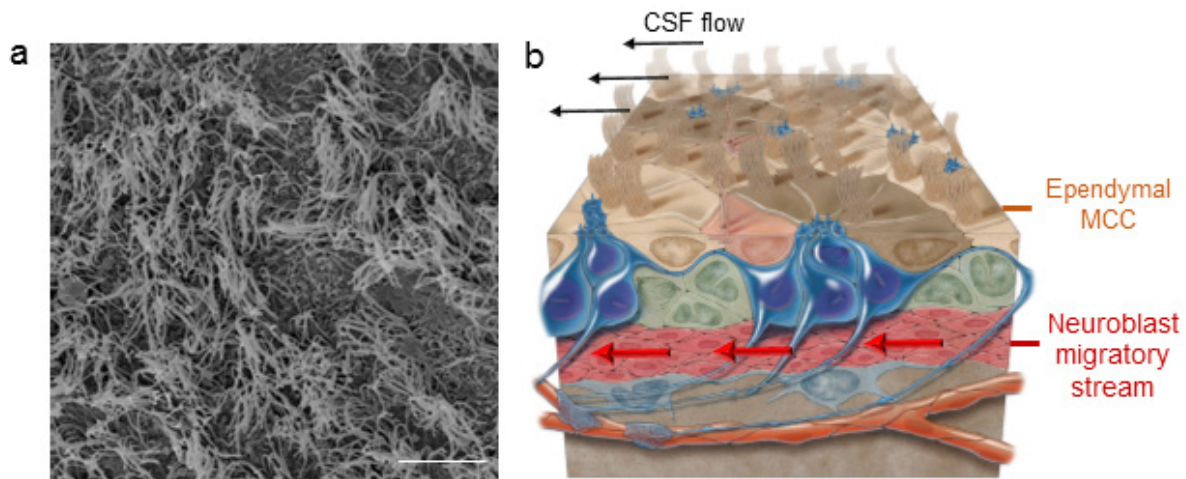


Figure 12 Ependymal multiciliated cells lining the ventricles and the SVZ

a, Scanning electron microscopy image showing ependymal MCC lining the mouse lateral ventricle **b**, Schematic view of the mouse ventricular and subventricular zone (SVZ). Directional CSF flow (black arrows) generated by ependymal MCC guides the migration ¹⁵⁸ of newly formed neuroblasts (red arrows). Scale bar, 10 μ m. Adapted from ^{159,274}.

MULTICILIATED CELLS AND DISEASE

Present in the three biological systems, mammalian MCC support a large array of functions with an outreach beyond their organs of localization that impact global organism homeostasis. Absence of motile cilia or defects in ciliary length or motility cause defective fluid and particle transport that leads to life threatening diseases. In the reproductive tracts, alterations in MCC associate with infertility and can give rise to extra-tubal implantations of the embryo ¹¹⁷. Presence of dysfunctional MCC in respiratory conduits leads to excessive accumulation of mucus and flattening of the periciliary environment resulting in chronic chest infections ¹⁶². In the brain, ciliary dysfunction causes a progressive accumulation of the CSF in the ventricles that increases intracranial pressure and eventually enlarges the ventricles ^{120,163–165}. Hydrocephalus, the medical term designating the enlargement of brain ventricles

due to the accumulation of CSF (Fig. 13), causes functional brain defects affecting locomotion and sources profound neuropsychological anomalies. In a short and long term developmental context, hydrocephalus also affects neurogenesis by modulating the architecture and the function of the SVZ ^{158,166}.

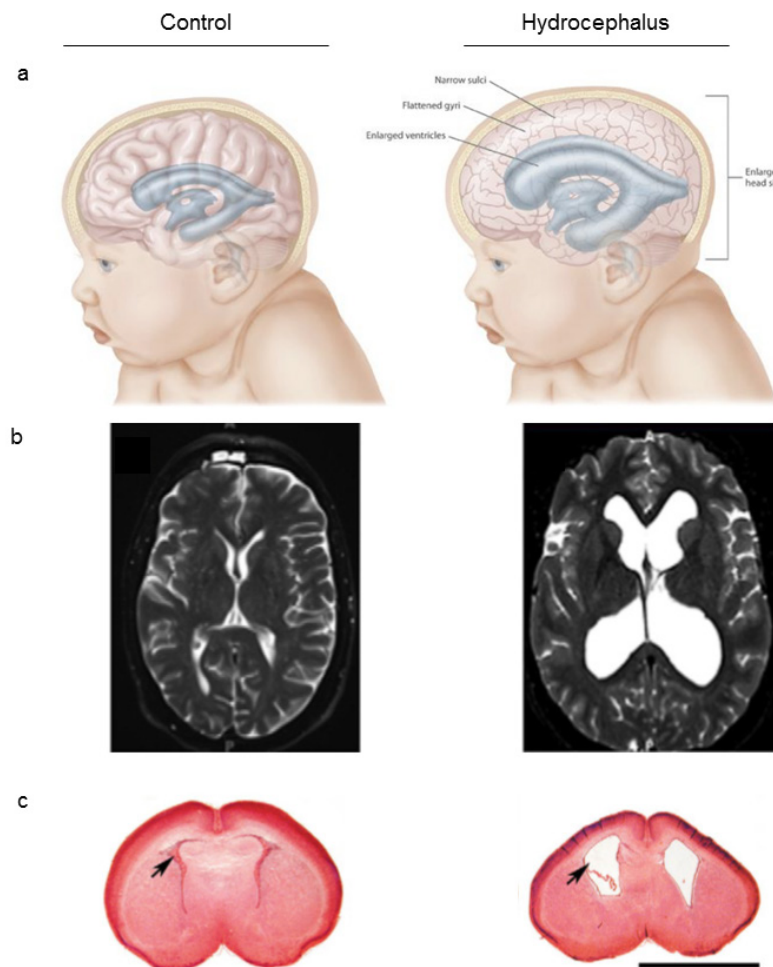


Figure 13 Hydrocephalus in humans and mice

a, Illustration of human infant ventricles in control (left panel) and hydrocephalus case (right panel) with consequences on brain anatomy. **b**, Magnetic resonance imaging comparing human adult ventricle size between a control (left panel) and a hydrocephalic patient (right panel). **c**, Hematoxylin and Eosin stained coronal sections comparing ventricle size between P10 control mouse (left panel) and corresponding motile ciliary mutant mouse (right panel); lateral ventricles indicated by arrows. Scale bar, 4mm. Adapted from ^{163,166,275}.

Infectious agents are also capable of compromising the function of MCC in all three systems. To increase chances of respiratory infection, corona and influenza viruses or pneumolysin, a *Streptococcus pneumoniae* virulence factor, halter mucociliary clearance by inducing ciliary dyskinesia and disrupting epithelial integrity ¹⁶². *Chlamydia trachomatis* infections of the fallopian tube correlate to tubal deciliation and infertility ¹¹⁷, whereas the mumps virus can cause hydrocephalus by damaging multiciliated ependymal cells lining the brain ventricles ^{167,168}.

Genetic mutations of proteins required for the development of MCC or necessary for ciliary beating result in motile ciliopathies that are often systemic. Primary Cilia Dyskinesia is caused in most cases by mutations in outer or inner dynein arms and results in immotile or disorganized ciliary beating ¹⁶⁹. Primary cilia dyskinesia patients are consequently less fertile, have increased risks of ectopic pregnancies as well as respiratory infections, and present a higher risk of hydrocephalic symptoms ¹⁶⁹. Recently, mutations in *mcidas* and *ccno*, genes regulating the initiation of centriole amplification, have been discovered in cohorts of PCD patients ^{170,171}. The mutations result in complete absence of or a significant decrease in the number of motile cilia. This medical condition, named ciliary aplasia and included in PCD before, has been renamed “Reduced Generation of Multiple Motile Cilia” (RGMC). Patients with RGMC present the same symptoms as patient with PCD ¹⁷⁰ and diagnosis is done through centriole and cilia detection by immuno-stainings or electron microscopy.

Other genetic diseases can directly or indirectly affect the physiological function of MCC in a more tissue specific manner. Brain ventricles of patients with the neurodegenerative Huntington disease present disorganized layers and increased lengths of motile cilia as a result of the autosomal dominant genetic mutation. As a consequence of abnormal ciliary beating, altered cerebrospinal fluid flow and hydrocephalus are observed in mouse models of the Huntington disease. In addition, altered MCC in Huntington patients are thought to exacerbate the progression of the disease by disrupting brain homeostasis ¹⁷². In the case of patients with the pulmonary subclass of Cystic Fibrosis ¹⁷³, excessive production of mucus compromises the respiratory epithelial microenvironment by misbalancing the proportion between the mucus and the periciliary fluid ^{149,174}. The strong accumulation of viscous mucus eventually weighs in the periciliary fluid environment and surpasses the mucociliary clearance capacity of airway MCC. Defects in mucociliary clearance then cause chronic infections of the respiratory system that further aggravate the disease outcome.

Clinical interest in the complexity of multiciliated epithelia is on the rise as a result of advances in medically assisted reproduction, pulmonary drug metabolism research, and the therapeutic potential of CSF-mediated drug delivery to the CNS. Consistently, a relatively major progress in the understanding of the molecular network initiating multiciliated cell specification has been achieved in the last 5 years.

MULTICILIATED CELL DEVELOPMENT AND MATURATION

Although post mitotic MCC differentiate terminally, the populations of MCC in the reproductive and respiratory systems undergo renewal. In the fallopian tube, a sub-population of MCC are thought to renew with every proliferative phase of the menstrual cycle ¹¹⁷ whereas airway MCC are, in majority, replaced after chemical or infection-mediated damage ¹⁷⁵. In contrast, brain lateral ventricle ependymal MCC transform at birth from a fixed non-renewable stock of radial glial progenitors ¹¹⁶. For MCC to develop, a conserved signalling cascade induces multiciliated cell specification that is followed by a phase of cellular differentiation and maturation that prepares the cell for motile ciliation.

Studies describing the developmental specification of vertebrate MCC were carried out in various organisms ranging from fish to amphibians and mammals. Although many actors of the MCC differentiation program are evolutionary conserved, differences between organisms as well as differences between ciliated tissues within an organism have been documented ^{113,122,123}. The data on the developmental programs regulating MCC differentiation should be interpreted with care and in consideration of potential divergences. Nevertheless, inhibition of the Notch pathway is proposed to induce the specification of MCC in all three mammalian systems containing ciliated epithelia ^{176–179}. Downstream targets of Notch inhibition, *Gemc1* and *Mcidas* (a.k.a. *Idas* or *Multicilin*) act as differentiation triggers sufficient for the initiation of the multiciliated cell transcriptional program ^{179,180}. Both *Gemc1* and *Mcidas* are relatives of *Geminin*, a protein that positively coordinates cell cycle progression and that is silenced in

cells exiting the cell cycle¹⁸¹. Interestingly, Geminin represses MCC differentiation by binding Mcidas. A subsequent interaction between Mcidas and the cell cycle repressor E2f4 is thought to upregulate the transcription factors necessary for the expression of centriole components and ciliogenic transcription factors^{138,182,183}. Downstream of the Mcidas/E2f4 complex is C-myb^{180,183,184}, a promoter of S-phase entry, in addition to Ccno^{171,185}, a cyclin-like protein with described functions in apoptosis and meiosis resumption^{186,187}. Although the mechanism of action of Ccno in MCC remains to be determined, C-myb is thought to be involved in the initiation of centriole amplification in MCC by regulating the transcription of basal body components. In parallel to the centriole amplification program, the RFX (Regulatory Factor X) family of proteins¹⁸⁸ in addition to FOXJ1 (Forkhead Box J1)^{189,190} transcriptionally activate the ciliogenic network required for basal body migration and docking as well as the expression of core motile ciliary components.

The maturation phase is marked by the amplification of up to several hundred centrioles that dock to the apical membrane synchronously to serve as basal bodies for motile cilia nucleation^{123,191}. From the earliest studies investigating centriole amplification in MCC dating back to the 1960's, large scale centriole production in MCC was thought to occur through two independent pathways known as “centriolar” and “acentriolar”. In the “centriolar” pathway, a limited number of centrioles (less than 6) grow from the lateral

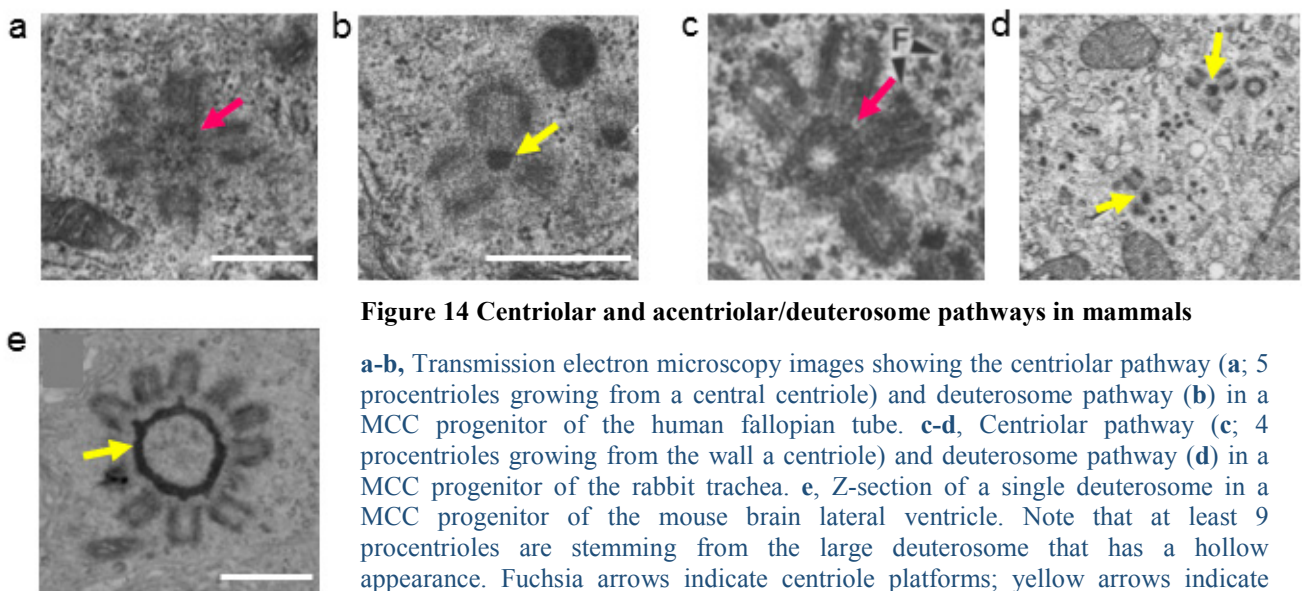


Figure 14 Centriolar and acentriolar/deuterosome pathways in mammals

a-b, Transmission electron microscopy images showing the centriolar pathway (**a**; 5 procentrioles growing from a central centriole) and deuterosome pathway (**b**) in a MCC progenitor of the human fallopian tube. **c-d**, Centriolar pathway (**c**; 4 procentrioles growing from the wall a centriole) and deuterosome pathway (**d**) in a MCC progenitor of the rabbit trachea. **e**, Z-section of a single deuterosome in a MCC progenitor of the mouse brain lateral ventricle. Note that at least 9 procentrioles are stemming from the large deuterosome that has a hollow appearance. Fuchsia arrows indicate centriole platforms; yellow arrows indicate spherical deuterosomes. Scale bars, 0.5μm; magnification X45340 (**c**) and X19600 (**d**). Adapted from^{116,276,277}.

surface of the two centrosomal centrioles^{192,193}, with resemblance to the phenotype of Plk4 overexpression in cycling cells⁵² (Fig. 14a and c). In the “acentriolar” or “deuterosome” pathway, electron-dense spheres or platforms called deuterosomes were assumed to support the nucleation of the remaining and large majority of centrioles^{192,194} (Fig. 14b, d, and e). In this context, the centrioles were believed to form *de novo* because no pre-existing centrioles were shown to be involved^{113,193} (Fig.15).

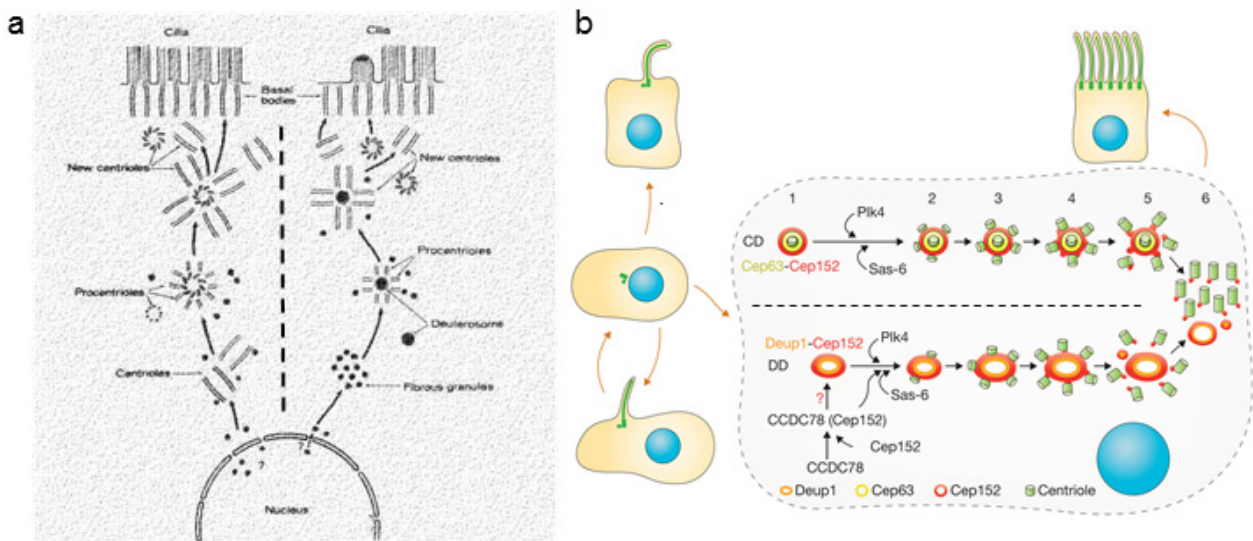


Figure 15 Predominant views of multiple centriole biogenesis in progenitors of MCC

a, Illustration of the centriolar (left) and deuterosome (right) pathway summarizing the 20th century views of centriole biogenesis in mammalian multiciliated cells. Note that deuterosomes were thought to arise from the cytoplasm before supporting “*de novo*” centriole biogenesis. **b**, Schematic view of large scale centriole biogenesis summarizing the findings of the 2013 paper¹⁹³ that described the first deuterosome-specific protein, Deup1. The centriolar pathway (CD; top) was still thought to be independent of the deuterosome/Deup1 pathway (DD; bottom). Note that this PhD project started in 2012. *Unknown source for a; b adapted from*²⁷⁸.

At the start of this study in 2012, the origin of all centrioles, the mechanisms controlling centriole number, as well as the centriole dynamic that allows the subsequent coordinated formation of the ciliary tuft remained obscure. The goal of this PhD project was (i) to uncover the dynamics of centriole amplification occurring in differentiating progenitors of MCC and (ii) to provide mechanistic advances in the understanding of the centriole number control.



“Now, though I have with great diligence endeavoured to find whether there be any such thing in those Microscopical pores of Wood or Piths, as the Valves in the heart, veins, and other passages of Animals, that open and give passage to the contain'd fluid juices one way, and shut themselves, and impede the passage of such liquors back again, yet have I not hitherto been able to say any thing positive in it; though, me thinks, it seems very probable, that Nature has in these passages, as well as in those of Animal bodies, very many appropriated Instruments and contrivances, whereby to bring her designs and end to pass, which 'tis not improbable, but that some diligent Observer, if help'd with better Microscopes, may in time detect.”

Robert Hooke

Micrographia: or, Some physiological descriptions of minute bodies made by magnifying glasses.

Published by J. Martyn and J. Allestry, London, 1665.

Article 1

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Centriole amplification by mother and daughter centrioles differs in multiciliated cells

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The semi-conservative centrosome duplication in cycling cells gives rise to a centrosome composed of a mother and a newly formed daughter centriole¹. Both centrioles are regarded as equivalent in their ability to form new centrioles and their symmetric duplication is crucial for cell division homeostasis^{2–4}. Multiciliated cells do not use the archetypal duplication program and instead form more than a hundred centrioles that are required for the growth of motile cilia and the efficient propelling of physiological fluids⁵. The majority of these new centrioles are thought to appear *de novo*, that is, independently from the centrosome, around electron-dense structures called deuterosomes^{6–8}. Their origin remains unknown. Using live imaging combined with correlative super-resolution light and electron microscopy, we show that all new centrioles derive from the pre-existing progenitor cell centrosome through multiple rounds of procentriole seeding. Moreover, we establish that only the daughter centrosomal centriole contributes to deuterosome formation, and thus to over ninety per cent of the final centriole population. This unexpected centriolar asymmetry grants new perspectives when studying cilia-related diseases^{5,9} and pathological centriole amplification observed in cycling cells and associated with microcephaly and cancer^{2–4,10}.

Large-scale centriole production in multiciliated cells is thought to occur through two independent pathways: a commonly described '*de novo*' pathway, also known as the acentriolar/deuterosome pathway; and a centriolar pathway, where new centrioles are formed adjacent to pre-existing ones in a manner akin to centriole duplication^{6,11,12}. The significance of having two pathways to produce the same organelle is enigmatic and nothing is known about their interplay or their exact contribution to the final population of centrioles. We first established that both pathways occur in the brain ependyma as in other mammalian ciliated epithelia^{7,11–13} (Fig. 1a, b and Extended Data Fig. 1a). Using live imaging, we traced the dynamics of centriole formation in ependymal cells from transgenic mice expressing a GFP-tagged version of the distal core centriolar protein centrin 2 (ref. 14), Cen2-GFP (Cen2 also known as Ctn2). At the onset of centriole amplification, a Cen2-GFP cloud accumulated around the pre-existing centrosome (Fig. 1c, d), which spawned a growing number of Cen2-GFP rings, or 'halos', that accumulated in the cytoplasm (Fig. 1e, f). The Cen2-GFP fluorescence in every halo intensified progressively and adopted a flower-like shape (a ring of fluorescence resembling petals; Fig. 1g). The flowers finally dissociated into tens of Cen2-GFP dots (Fig. 1h). The Cen2-GFP⁺ centrioles then migrated to the apical membrane where they initiated the extension of the motile ciliary tufts (Extended Data Fig. 2a, b and Supplementary Videos 1 and 2; $n = 12$). Together with immunostainings of centriolar markers on fixed developing ependymal cells (Extended Data Fig. 1b–h), these observations show that centriole amplification occurs in the vicinity of the centrosome and that the 'halo stage', which corresponds to the accumulation of centriolar precursors, is followed by a 'flower stage', during which maturing centrioles appear in a synchronized manner.

To characterize the origin and composition of the halos further, we monitored centrosomal centrioles at a higher resolution. Live imaging showed that nascent halos budded from the wall of one centrosomal

centriole in the form of Cen2-GFP⁺ rings that detached and accumulated in the nearby cytoplasm (Fig. 2a, Extended Data Fig. 2c, d and Supplementary Videos 3 and 4; $n = 15$ cells). Use of 3D-structured illumination microscopy (3D-SIM) showed that the halos were composed of Sas-6⁺ (also known as Sass6)/Cen2-GFP⁺ subunits (Fig. 2b, c, Extended Data Fig. 2e and Supplementary Video 5) indicating that they represent spherical assemblies of procentrioles. A generational asymmetry exists between centrosomal centrioles and has been shown to confer different functions to each member of the pair^{15–17}. To determine the contribution of mother and daughter centrioles to procentriole formation, we immunostained the structural protein Sas-6 (ref. 18) and the key regulators of centriole biogenesis Cep152 and Plk4 (refs 19,20). In ependymal cycling progenitors (positive for the cycling cell marker Ki67) duplicating their centrosome, these proteins stained symmetrically on both centrosomal centrioles. However, in postmitotic differentiating cells (positive for the multiciliated cell transcription factor Foxj1) the immunostains were significantly larger and more intense on the daughter than on the mother centriole (Fig. 2d, e and Extended Data Fig. 2f, g). Additionally, conventional and 3D-SIM analyses revealed that 100% of the Cen2-GFP⁺ halos formed on the wall of the daughter centriole ($n = 33$) and were contiguous to the proximal portion of the centriole (Fig. 2f–h), which is the canonical site of procentriole nucleation in cycling cells. Although the onset of differentiation was characterized by the lengthening of the primary cilium (a cell antennae nucleated by the mother centriole²¹), no alteration of centrosome asymmetry was observed in ciliary mutants throughout the amplification process (Extended Data Fig. 9a–d). The centrosome thus switches from symmetric canonical centriole duplication in cycling progenitors to asymmetric centriole amplification in

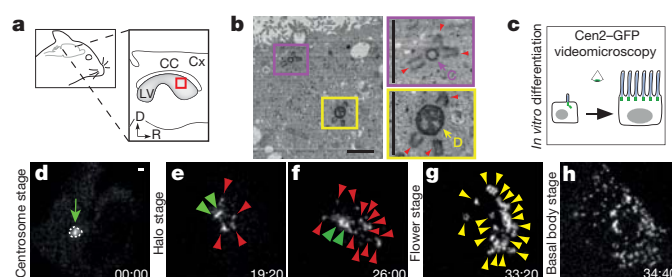


Figure 1 | Centriole amplification dynamics during ependymal cell differentiation. **a**, Sagittal view of the mouse lateral ventricular wall. Square indicates the region analysed in **b**. Cx, cortex; CC, corpus callosum; LV, lateral ventricle; D/R, dorsal/rostral. **b**, Transmission electron microscopy (TEM) on a differentiating P4 ependymal progenitor cell showing procentrioles (red arrowheads) growing from a centriole (C, 'centriolar' pathway) or from a deuterosome (D, 'deuterosome' pathway). **c–h**, Videomicroscopy during Cen2-GFP⁺ ependymal progenitor differentiation. **d–h**, From Supplementary Video 1. Arrow indicates the centrosome; dashed line delineates the Cen2-GFP⁺ cloud; green arrowheads point to centrosomal centrioles; red arrowheads point to halos; yellow arrowheads point to flowers. $t_{\text{amplification}} = 29.3 \pm 6$ h, $t_{\text{halos}} = 21 \pm 5$ h and $t_{\text{flowers}} = 9.7 \pm 2$ h, where $\bar{t}$ = mean time. Time in hh:mm; scale bars, 1 μ m.

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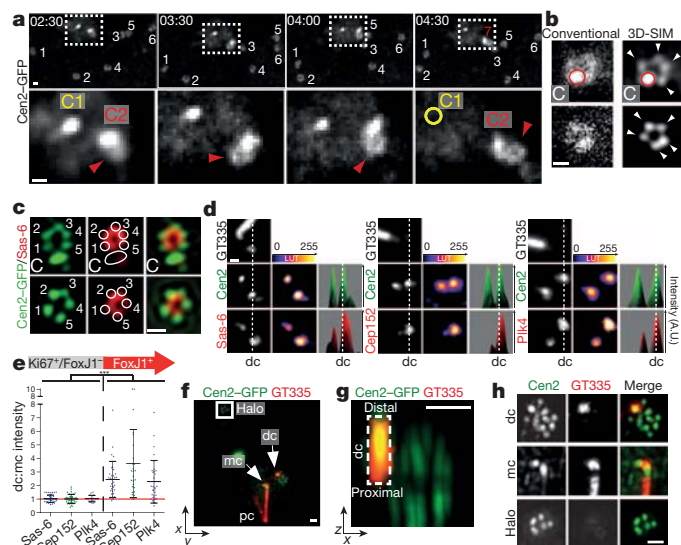


Figure 2 | Halos are a spherical assembly of procentrioles nucleated from the centrosomal daughter centriole. **a**, From Supplementary Video 3. Cen2-GFP dynamics during halo formation. Top: z-projections (4.2 μm) of centrosomal centrioles (C1 and C2) and six halos in the cytoplasm. Bottom: single z-plane (0.7 μm) of the centrosome. Arrowhead points to the seventh halo forming from C2. **b**, Single 0.1 μm z-plane of a Cen2-GFP⁺ centrosome (C; top) and cytoplasmic halo (bottom) imaged by conventional epifluorescence microscopy (left) and 3D-SIM (right). Arrowheads indicate Cen2-GFP⁺ subunits observed only with 3D-SIM. **c**, 3D-SIM z-plane of a Cen2-GFP⁺ centrosome (C; top) or cytoplasmic (bottom) halo immunostained with Sas-6 (red). Circles delineate Cen2-GFP⁺ subunits. **d**, Sas-6, Cep152 and Plk4 stainings on Cen2-GFP⁺ mother (growing GT335⁺ primary cilium) and daughter (dc) centrosomal centrioles at the halo stage. 'Fire' colour look-up table (LUT) and fluorescence intensity profiles are shown. A.U., arbitrary units. **e**, Symmetric to asymmetric switch of centrosomal markers. Daughter:mother centriole (dc:mc) signal ratios in cycling Ki67⁺/Foxj1[−] (left; Sas-6 ($n = 42$), Cep152 ($n = 43$), Plk4 ($n = 18$)) and differentiating Foxj1⁺ ependymal progenitors at the halo stage (right; Sas-6 ($n = 33$), Cep152 ($n = 25$), Plk4 ($n = 32$)). Error bars represent mean $\pm$ s.d.; P values derived from two-tailed Mann-Whitney U -tests, *** $P \leq 0.0003$; red line represents a ratio equal to 1. **f**, 3D-SIM z-projection (1.8 μm) of Cen2-GFP and GT335 immunoreactivity at the halo stage. Centrin aggregates are occasionally observed in Cen2-GFP cells. **g**, Side view of the GT335⁺ daughter centriole from **f** showing a Cen2-GFP⁺ halo contiguous to the proximal portion of the daughter centriole. **h**, z-projection (0.6 μm) magnifications of both centrosomal centrioles (top and centre) and the cytoplasmic halo (bottom) from **f**. pc, primary cilium; time in hh:mm; scale bars, 0.5 μm.

postmitotic ependymal progenitors. The amplification is a recurrent process during which procentrioles are nucleated from the centrosomal daughter centriole to form halos that are released into the cytoplasm.

Deuterosomes are the source of the presumed 'de novo' pathway⁶, and are composed of two newly identified proteins, Deup1 and Ccdc78, which recruit Cep152 and Plk4 to drive centriole amplification^{7,8}. Deup1 and Ccdc78 were localized to the centrosome in ependymal cycling progenitors (Extended Data Fig. 3a). As the cells entered the halo stage, substantial accumulation of both proteins was observed at the daughter centriole (Extended Data Fig. 3b, c). Since high concentrations of Deup1 were shown to trigger deuterosome formation⁷, amplification from the daughter centriole may be driven by the formation of deuterosomes. Consistently, 3D-SIM and live imaging highlighted the presence of a Deup1⁺ cluster in the centre of the halo released by the daughter centriole (Fig. 3a, b, Extended Data Fig. 3d and Supplementary Video 6). To test this hypothesis further, we correlated 3D-SIM observations with transmission electron microscopy on Cen2-GFP⁺ cells (3D-SIM/EM; Extended Data Fig. 3e). 3D-SIM/EM on halo-stage cells showed that the Cen2-GFP⁺ subunits of the halos were actually procentrioles organized around spherical deuterosomes (Fig. 3c). Serial sections of entire cells ($n = 3$ cells)

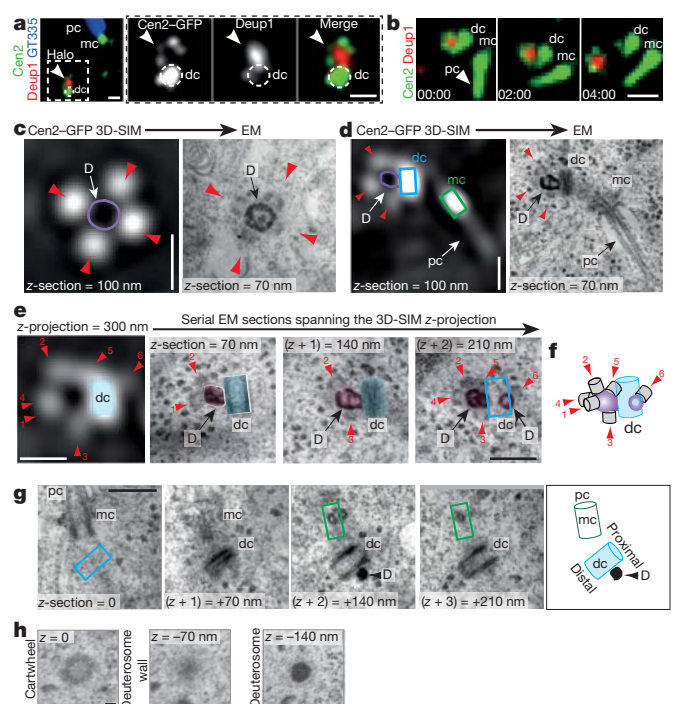


Figure 3 | Immature procentrioles are amplified from the daughter centriole via deuterosome formation. **a**, 3D-SIM z-plane (0.12 μm) of a Cen2-GFP⁺ centrosome during halo (arrowhead) formation from the daughter centriole (circle) immunostained with Deup1 and GT335. Dotted panel shows a z-projection (0.6 μm) magnification of the daughter centriole. dc, daughter centriole; mc, mother centriole; pc, primary cilium. **b**, Cen2-GFP/ Kusabira-Orange-Deup1 time-lapse images during halo release from the daughter centriole. Note that Cen2-GFP occasionally enters the primary cilium. Time in hh:mm. **c**, **d**, Correlative images of 3D-SIM z-plane (left) and TEM (right) of a cytoplasmic halo (c) showing procentrioles on a deuterosome, and of a centrosome (d) showing an incomplete procentriole-loaded deuterosome connected to the daughter centriole. Purple lines delineate TEM deuterosomes. D, deuterosome. **e**, Correlative images of 3D-SIM z-plane of the daughter centriole (blue) from **d** and the corresponding serial EM sections spanning the deuterosome (purple). Rectangle indicates the daughter centriole location on the previous EM section. A second, smaller deuterosome is connected to another site on the proximal wall of the daughter centriole. Spatial resolution of 3D-SIM is insufficient to individualize some of the procentrioles visible by EM. **f**, 3D-reconstruction of **e**. **g**, Serial EM sections of the centrosome in an early differentiating ependymal progenitor showing the first deuterosome without procentrioles adjacent to the daughter centriole. The image on the right shows an illustration of the collated EM images. **h**, Serial EM sections of a procentriole at the halo stage growing from a deuterosome. Red arrowheads, procentrioles; scale bars, 50 nm (**h**) and 0.5 μm (**a–g**).

showed that all Cen2-GFP⁺ procentrioles were associated with deuterosomes and, conversely, that all deuterosomes were loaded with procentrioles (23 out of 23) (Extended Data Figs 4 and 5). Developing halos corresponded to deuterosomes with incomplete spherical shapes attached to the proximal segment of the daughter centriole (Fig. 3d and Extended Data Figs 4 and 5). One or two deuterosomes were observed connected to the daughter centriole (Fig. 3e, f and Extended Data Figs 4, 5 and 6b, c). To visualize the first events of deuterosome formation, we screened early ependymal progenitors, identified by the absence of deuterosomes in the cytoplasm and a small accumulation of electron-dense aggregates⁶, and found cells where the daughter centriole had a single attached deuterosome without detectable procentrioles (Fig. 3g and Extended Data Fig. 6a; $n = 3$ cells). The procentriole-free deuterosomes were smaller than centrosome-bound deuterosomes loaded with procentrioles and, more generally, deuterosome size was correlated with the number of procentrioles they support (Extended Data Fig. 6d, e). Deuterosomes are

thus generated from the proximal segment of the daughter centriole and do not appear *de novo*.

During the halo stage/deuterosome formation, procentrioles displayed a tube-like structure organized around a cartwheel containing microtubule singlets (Fig. 3h). The absence of microtubule triplets indicated that the procentrioles were immature. Consistently, the lengths and widths of procentrioles were significantly smaller than those of mature centrioles. Procentrioles were similar in size both within and among halo-stage cells (Fig. 4g, h, red and grey quantifications; Extended Data Figs 7 and 8e) and were comparable to early S-phase procentrioles in cycling cells²².

Together, these data show that during ependymal cell development the centrosomal daughter centriole greatly amplifies procentrioles by generating intermediate structures, the deuterosomes. Once nucleated at the daughter centriole, procentrioles remain latent at an early stage of their biogenesis on the deuterosomes.

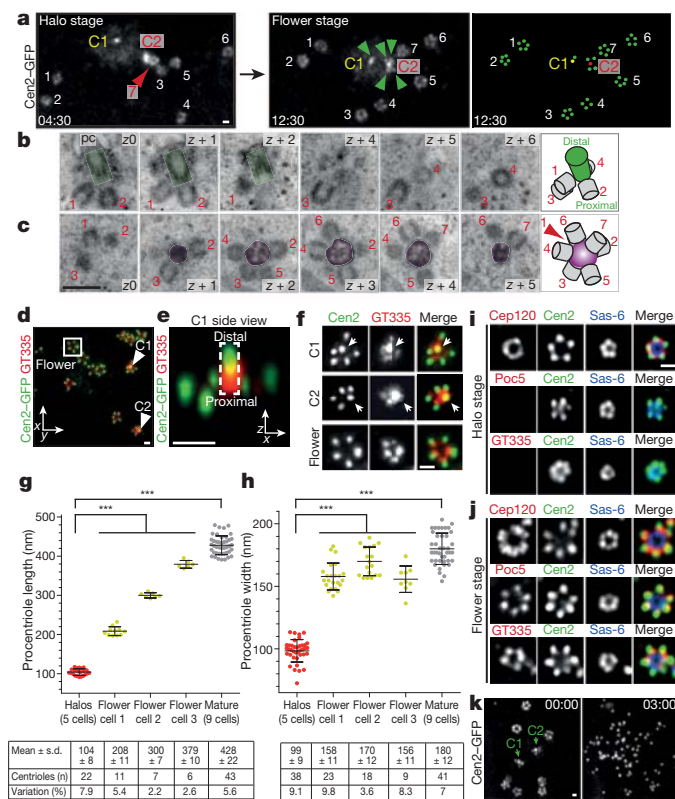


Figure 4 | Procentrioles grow simultaneously from both deuterosomes and centrosomal centrioles after formation of the last deuterosome. **a**, From Supplementary Video 3. Cen2-GFP dynamics showing the transformation of halos (numbered at 04:30) into flowers (numbered at 12:30). Red arrowhead indicates the last halo (seventh) to be formed (same cell as Fig. 2a). Green arrowheads point to procentrioles on the wall of centrosomal centrioles. **b**, **c**, Serial EM sections spanning a centrosomal centriole (**b**; green) and a deuterosome (**c**; purple) in a flower-stage cell. Procentrioles are numbered. Images on the right show 3D-reconstruction schematics. **d**, 3D-SIM z-projection (1.8 μm) of Cen2-GFP and GT335 immunoreactivity at the flower stage. **e**, Side view of C1 showing procentrioles growing from its proximal portion. **f**, z-projection (0.6 μm) magnifications of C1/C2 centrosomal centrioles (white arrows) and deuterosome (flower). **g**, **h**, Lengths and widths of procentrioles from cells at the halo, flower and mature basal body stages; error bars represent mean ± s.d.; *P* values derived from two-tailed Mann-Whitney *U*-tests, ****P* < 0.0001. **i**, **j**, 3D-SIM z-planes (120 nm) comparing Cep120, Poc5 and GT335 immunoreactivities between halos (**i**) and flowers (**j**). **k**, From Supplementary Video 7. Cen2-GFP dynamics showing simultaneous release of procentrioles from centrosomal centrioles and deuterosomes. Time in hh:mm; scale bars, 0.5 μm.

We next visualized the dynamics of centriole growth during the halo-to-flower stage transition. Immediately after the formation of the last halo/deuterosome at the daughter centriole, all of the Cen2-GFP⁺ halos simultaneously transformed into intensely fluorescent flower-like structures. At this stage, up to five procentrioles were also seen growing directly from the proximal portions of both centrosomal centrioles (Fig. 4a–f, Extended Data Figs 6, 8a, b, d and Supplementary Videos 3 and 4; *n* = 30 cells). These ‘centriolar’ procentrioles were probably nucleated during the halo stage, since they were already visible during the late phases of deuterosome formation (Fig. 2h and Extended Data Figs 4, 5 and 6b). Both centrosomal centrioles thus produce procentrioles with a canonical orthogonal configuration, which defines the so-called ‘centriolar’ pathway. However, more than 90% of the centrioles were generated via deuterosomes and less than 10% directly from centrosomal centrioles (14 ± 1 deuterosomes per cell, 4.2 ± 0.4 procentrioles per centrosomal centriole, 5.6 ± 0.3 per deuterosome (mean ± s.e.m.); *n* = 10 cells).

All procentrioles in a given cell grew synchronously during the flower stage, since their sizes were similar in each cell and increased significantly in comparison to the procentrioles in halo-stage cells (Fig. 4g, h and Extended Data Figs 6f, 7 and 8d, e). At the molecular level, halo-stage procentrioles were positive for the early markers of centriole biogenesis (Cep152, Plk4, Sas-6, Stil, Cnap (also known as Cenpj), Cep120, CP110), and flower procentrioles became positive for markers of the late steps of centriole assembly (Poc5 (ref. 23) and GT335 (ref. 24) (an antibody against glutamylated tubulin)) (Fig. 4i, j and Extended Data Fig. 8f, g). Centriole biogenesis in multiciliated cells is therefore a two-step process in which procentriole initiation (halo stage/deuterosome formation) is clearly demarcated from the late phases of centriole assembly (flower stage). Synchronized growth is followed by the simultaneous detachment of procentrioles from both centrosome and deuterosome platforms ($\bar{t} = 2.5 \pm 0.85$ h; *n* = 9 cells; Fig. 4k, Extended Data Fig. 8c and Supplementary Videos 4 and 7). After migration and docking at the apical membrane, Sas-6 disappears from centrioles as motile cilia grow (Extended Data Fig. 8h). Identical steps of centriole amplification and similar centrosome asymmetry were observed in tracheal multiciliated cells *in vivo* (Extended Data Fig. 9e–k), suggesting that all mammalian multiciliated cells might share this mechanism of large-scale centriole biogenesis.

Our results revise the ‘*de novo*’ hypothesis; the so-called ‘centriolar’ and ‘deuterosome’ pathways are the outcomes of a single pathway orchestrated by the centrosome (Fig. 5). The ability of mother and daughter centrioles to differentially control both the number of and the way they generate new centrioles reveals a new centrosome asymmetry. A differential regulation of Deup1 levels at centrosomal centrioles could underlie the ability of the daughter centriole to form deuterosomes and amplify centrioles⁷. The identification of proteins that interact with Deup1 using proximity-dependent biotinylation²⁵ may help uncover the upstream signals controlling centrosome asymmetry in these cells.

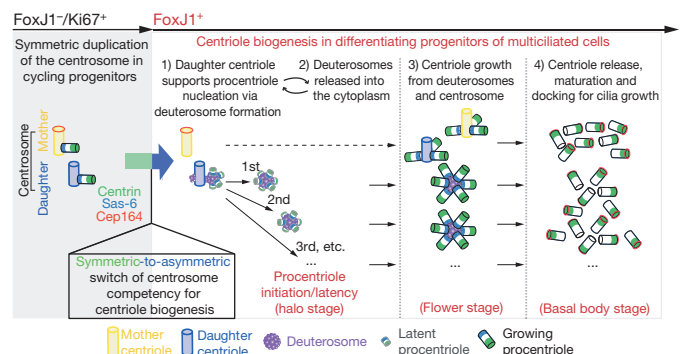


Figure 5 | Model of centriole amplification in multiciliated cells.

Deuterosomes develop from the same proximal region of the centriolar wall from which procentrioles grow during canonical centriole duplication and use known regulators of this process^{7,8} (Extended Data Fig. 10). They thus function as shuttles, displacing the late phases of centriole assembly from the centrosome to the cytoplasm while procentriole maturation is arrested. This mechanism allows massive, albeit centriole-mediated, production of centrioles. Emerging studies showing the involvement of S-phase regulators during multiciliated cell differentiation^{26–30}, together with the phase-like progression of centriole amplification (Fig. 5), suggest that the amplification process may be a facet of the duplication cycle, in which, however, spatial and temporal controls of centriole number are bypassed. It would therefore be interesting to explore the roles of S, G2 and mitosis regulators throughout the amplification stages delimited here.

In this study, we report that, under physiological conditions, the centrosome supports either the duplication of centrioles or their massive amplification. Our finding unifies the mechanisms of centriole biogenesis in cycling and multiciliated cells. Since the daughter centriole of the centrosome is the primary nucleation point for amplification, it could play a major role in the control of centriole production. This newly found centrosome asymmetry thus provides a new paradigm for understanding the regulation of centriole biogenesis and number.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Supplementary Information is available in the online version of the paper.

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Author Contributions A.A.J. performed and analysed experiments; A.-I.L. performed videomicroscopy experiments; N.D. performed and analysed cilia mutant experiments; M.F. contributed to *in vitro* experiments; N.S. designed the study and supervised the project; A.M. initiated the study, designed, performed and analysed experiments, and supervised the project; A.M., N.S. and A.A.J. wrote the manuscript. All authors commented on the manuscript.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to N.S. (nathalie.spassky@ens.fr) or A.M. (alice.meunier@ens.fr).

METHODS

Animals. All animal studies were performed in accordance with the guidelines of the European Community and French Ministry of Agriculture and were approved by the Direction départementale de la protection des populations de Paris (Approval number Ce5/2012/107). The mice used in this study have already been described and include: OF1 (Oncins France 1, Charles River Laboratories); Cen2-GFP¹⁴ (CB6-Tg(CAG-EGFP/CETN2)3-4Jgg/J, The Jackson Laboratory); *Kif3a*^{fl}, *Kif3a*^{ko/+31}; and nestin-Cre. To produce the conditional mutant *Kif3a* (*Kif3a* cKO), we crossed *Kif3a*^{fl/fl}/Cen2-GFP mice with *Kif3a*^{ko/+}/nestin-Cre mice. *Kif3a*^{fl/+}/nestin-Cre mice were used as controls and *Kif3a*^{ko/fl}/nestin-Cre mice as mutants. Videomicroscopy and 3D-SIM experiments were performed with heterozygous Cen2-GFP mice. Other experiments were performed in parallel using OF1 and heterozygous Cen2-GFP mice; no differences between these two strains were observed regarding ependymal differentiation, amplification stages, centrosome asymmetry or number of deuterosomes and centrioles.

Brain and trachea dissections. Whole mounts of developing ventricular walls were prepared from P0–adult mice as previously described³². Developing tracheae were separated surgically from the larynx (cranially) and the bronchi (caudally) of anaesthetized E17–P0 mice. For whole-mount preparations, trachea-associated muscles, vessels, nodes and glands were scraped off in cold L15 medium before cutting the trachea in half cranio-caudally with micro scissors.

Primary ependymal cell cultures and transfections. Newborn mice (P0–P2) were killed by decapitation. The brains were dissected in Hank's solution (10% HBSS, 5% HEPES, 5% sodium bicarbonate, 1% penicillin/streptomycin (P/S) in pure water) and the extracted ventricular walls were cut manually into pieces, followed by enzymatic digestion (DMEM glutamax, 33% papain (Worthington 3126), 17% DNase at 10 mg ml⁻¹, 42% cysteine at 12 mg ml⁻¹) for 45 min at 37 °C in a humidified 5% CO₂ incubator. Digestion was stopped by addition of a solution of trypsin inhibitors (Leibovitz Medium L15, 10% ovomucoid at 1 mg ml⁻¹, 2% DNase at 10 mg ml⁻¹). The cells were then washed in L15 and resuspended in DMEM glutamax supplemented with 10% fetal bovine serum (FBS) and 1% P/S in a Poly-L-lysine (PLL)-coated flask. Ependymal progenitors proliferated for 5 days until confluence followed by shaking (250rpm) overnight. Pure confluent astroglial monolayers were replated at a density of 7×10^4 cells per cm² (corresponding to days *in vitro* (DIV) – 1) in DMEM glutamax, 10% FBS, 1% P/S on PLL-coated coverslips for immunocytochemistry experiments, Lab-Tek chambered coverglasses (Thermo Fisher Scientific) for time-lapse experiments or glass-bottomed dishes with imprinted 50 µm relocation grids (Ibidi, catalogue no. 81148, Biovalley) for correlative 3D-SIM/EM and maintained overnight. The medium was then replaced by serum-free DMEM glutamax 1% P/S, to trigger ependymal differentiation gradually *in vitro* (DIV 0). All primary cultured cells derived from *Kif3a*^{ko/fl}/nestin-Cre were devoid of primary and motile cilia. Transfections were performed in suspension at DIV – 1 using the Jetprime (Polyplus) system.

Plasmids. The full-length complementary DNA for Deup1 (GenBank accession number KC211186) was amplified by PCR from DIV 5 ependymal cell cultures. To express a GFP-tagged version of Deup1, the PCR fragments containing the Deup1 cDNA were cloned into pEGFP-C1. To generate a Kusabira-Orange-Deup1, the EGFP sequence of pEGFP-C1 was replaced by the Kusabira-Orange sequence using the megaprimer technique.

Immunostaining. Lateral brain ventricles and cell cultures were fixed in methanol at –20 °C for 5 min. Tracheae were permeabilized in 0.5% Triton X-100 1× PBS for 3 min before methanol fixation at –20 °C for 7 min. Tissues and cells were pre-blocked in 1× PBS with 0.2–0.5% Triton X-100 and 10% FBS before incubation with primary and secondary antibodies. Tissues and cells were counterstained with DAPI (10 µg ml⁻¹, Sigma) and mounted in Fluoromount (Southern Biotech). The following antibodies were used: rabbit anti-Ccdc78 (1:50; Sigma-Aldrich, AV53233 and HPA041186); rat anti-CD24 (1:100; BD Pharmingen); mouse IgG2b anti-Centrin 20H5 (1:2000; Millipore); rabbit anti-Cep120 (1:2000)³³; rabbit anti-Cep152 (1:2000)⁷; rabbit anti-Cep164 (1:750)³⁴; rabbit anti-CP110 (1:1000, Bethyl laboratories); rabbit anti-Cpap (1:1000)³⁵; rabbit anti-Deup1 (1:2000)⁷; mouse IgG1 anti-FoxJ1 (1:700, eBioscience); mouse IgG1 anti-GT335 (1:2000, Adipogen)³⁶; mouse IgG1 or rabbit anti-Ki67 (1:10, BD Biosciences) or 1:50, Spring Bioscience); human or rabbit anti-ninein (1:100, 2g5 clone, Recombinant antibody platform, Curie Institute or 1:10000³⁷); rabbit anti-Plk4 (1:1000)³⁸; rabbit anti-Poc5 (1:500)²³; mouse IgG2b anti-Sas-6 (1:750, Santa Cruz); rabbit anti-Stil (1:1000, Bethyl Laboratories); and species-specific Alexa Fluor secondary antibodies (1:400, Invitrogen).

Microscopy. Epifluorescence microscopy. Fixed cells and whole-mount ventricles were examined with an upright epifluorescence microscope (Zeiss Axio Observer.Z1) equipped with an Apochromat ×63 (NA 1.4) oil-immersion objective and a Zeiss Apotome with an H/D grid. Images were acquired using Axiovision with 240-nm z-steps.

Videomicroscopy. Cells were filmed *in vitro* using an inverted spinning disk Nikon Ti PFS microscope equipped with oil-immersion ×63 (NA 1.32) and ×100 (NA 1.4)

objectives, an Evolve EMCCD Camera (Photometrics), dpss lasers (491 nm, 561 nm), appropriate filter sets for DAPI/FITC/TRITC, a motorized scanning deck and an incubation chamber (37 °C; 5% CO₂; 87% humidity). To decrease photo-toxicity, laser intensities were decreased to 3% and the image capture time was increased to 1 s per frame. Images were acquired with Metamorph Nx at different time intervals ($\Delta t = 20$ to 40 min). Image stacks were recorded with a z-distance of 0.3 µm to 0.7 µm and subjected to constrained iterative deconvolution (conservative ratio, 1–6 cycles, medium noise filtering, ImageJ software). After image acquisition, to maintain the same cell coordinates for CD24 staining, the medium was removed with a Pasteur pipette without moving the chambered coverglass, and CD24 primary and secondary antibodies were added for 15 min each, in medium supplemented with FBS (10%). The cells were then fixed for 5 min with 0.5% paraformaldehyde (PFA) before the final images were acquired (see Extended Data Fig. 2b). Four-dimensional (x, y, z, t) time-lapse images were analysed with Imaris (Bitplane Scientific Solutions) and Image J.

Transmission electron microscopy. Cultured cells or P4 ventricle slices were fixed in 2.5% glutaraldehyde and 4% PFA, treated with 1% OsO₄, washed and progressively dehydrated. The samples were then incubated in 1% uranyl acetate in 70% methanol, before final dehydration, pre-impregnation with ethanol/epon (2/1, 1/1, 1/2) and impregnation with epon resin. After mounting in epon blocks for 48 h at 60 °C to ensure polymerization, ultra-thin sections (70 nm) were cut on an ultramicrotome (Ultracut E, Leica) and analysed using a Philips Technai 12 transmission electron microscope.

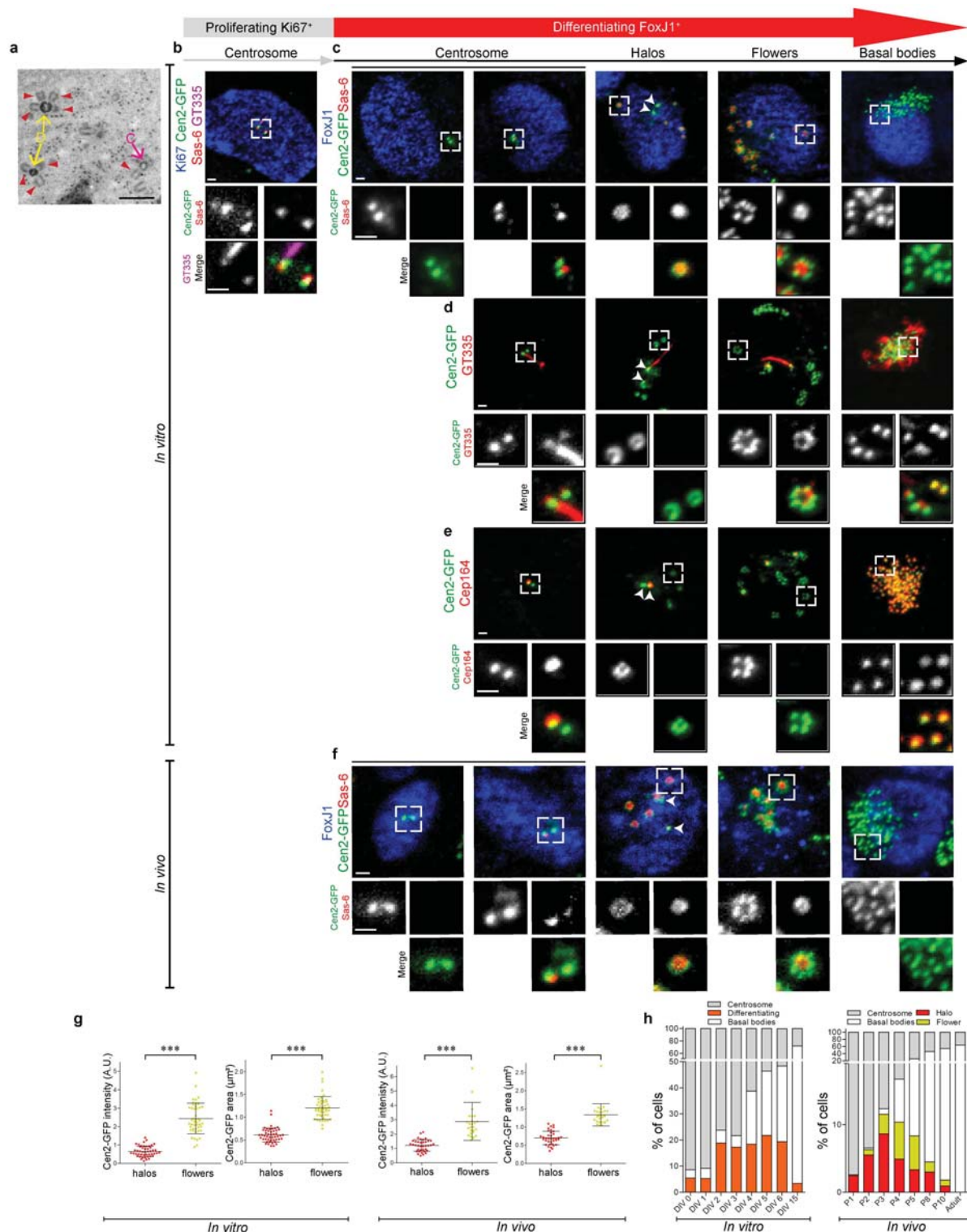
3D structured illumination microscopy. 3D-SIM images were obtained using a Nikon N-SIM Eclipse Ti Inverted Microscope equipped with a MCL Piezo stage, an Apochromat ×100 (NA 1.49) oil-immersion objective and 488/561/647 nm diode lasers. Image stacks were recorded with a z-distance of 100–120 nm, and 3D reconstruction and alignment was performed on an NIS-Elements AR microscope. Conventional images, when shown, were acquired after 3D-SIM acquisitions (see Fig. 2b). Cen2-GFP measurements were made with Image J on single 100 nm axial slices (details in Extended Data Fig. 7b).

Correlative 3D-SIM and electron microscopy. Primary Cen2-GFP ependymal progenitors were grown in 0.17-mm thick glass dishes with imprinted 50 µm relocation grids (Ibidi). At 3 days *in vitro* (DIV 3), cells were fixed with 4% PFA for 10 min and ependymal progenitors undergoing halo formation were imaged for Cen2-GFP and DAPI, in PBS, with a 3D structured illumination microscope (N-SIM - Eclipse Ti Inverted Microscope). Coordinates on the relocation grid of the cells of interest were recorded. Cells were treated for transmission electron microscopy within 2 h and embedded in epon resin. After epon polymerization, resin blocks were detached from the glass dish by several baths in liquid nitrogen. Using the grid pattern imprinted in the resin, 50 serial ultra-thin 70-nm sections of the squares of interest were cut and transferred onto formvar-coated EM grids (0.4 × 2 mm slot). The central position of the square of interest and DAPI staining are used to relocate the cell of interest (See Extended Data Fig. 3e).

Quantification and statistical analyses. Data were obtained from at least three independent experiments and the results presented as the mean ± s.d., unless otherwise stated. Non-parametric two-tailed Mann–Whitney U-tests were used to compare two distributions and two-tailed Kruskal–Wallis tests to compare groups of data, unless otherwise stated. Variances were similar between compared distributions. Distributions were tested for normality when necessary. Pearson's correlation coefficient was calculated to determine the strength of the relationship between the volume of the deuterosomes and the number of procentrioles they hold (Extended Data Fig. 6d, e). Fluorescence intensity ratios (Extended Data Fig. 1g) were obtained by dividing raw integrated densities (RID) of halos or flowers by the average RID of centrosomal centrioles (internal fluorescence control) in the corresponding cell. Daughter:mother centriole signal ratios (centrosomes in Fig. 2d, e; Extended Data Figs 2g, 3b, c and 9c, d, h, i) were calculated from centrosomes in halo-stage FoxJ1⁺ cells containing at least two cytoplasmic halos. In 15–42% of the centrosomes analysed in cells at the halo stage, Sas-6, Cep152, Plk4, Ccdc78 and Deup1 immunoreactivity was observed exclusively at the daughter centriole; these cells were excluded from quantification of the daughter:mother centriole signal ratio. Fluorescence signal intensity was quantified using Image J. All graphs and statistical analyses were obtained using GraphPad Prism software. See corresponding figure legends for P values and further details.

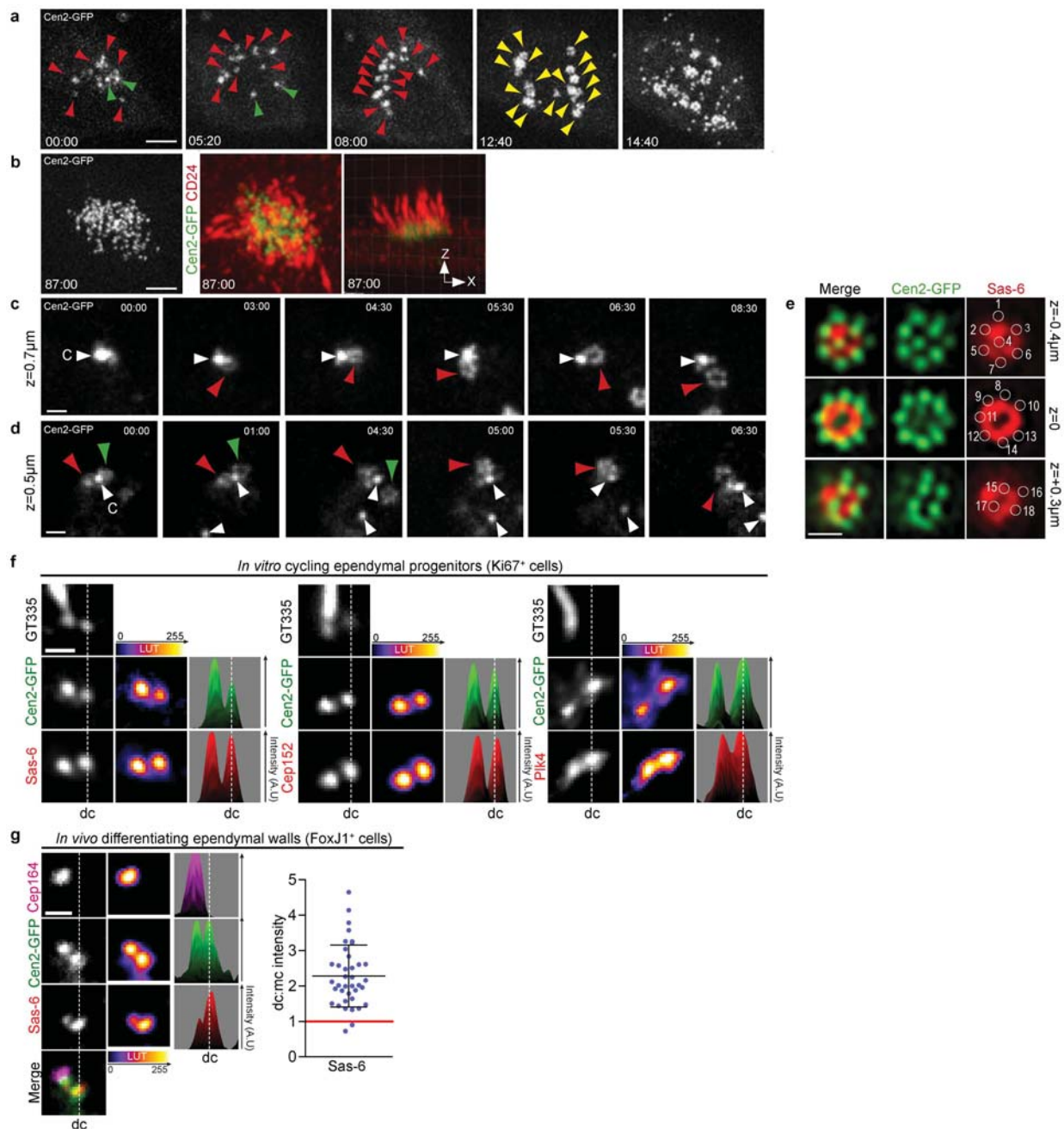
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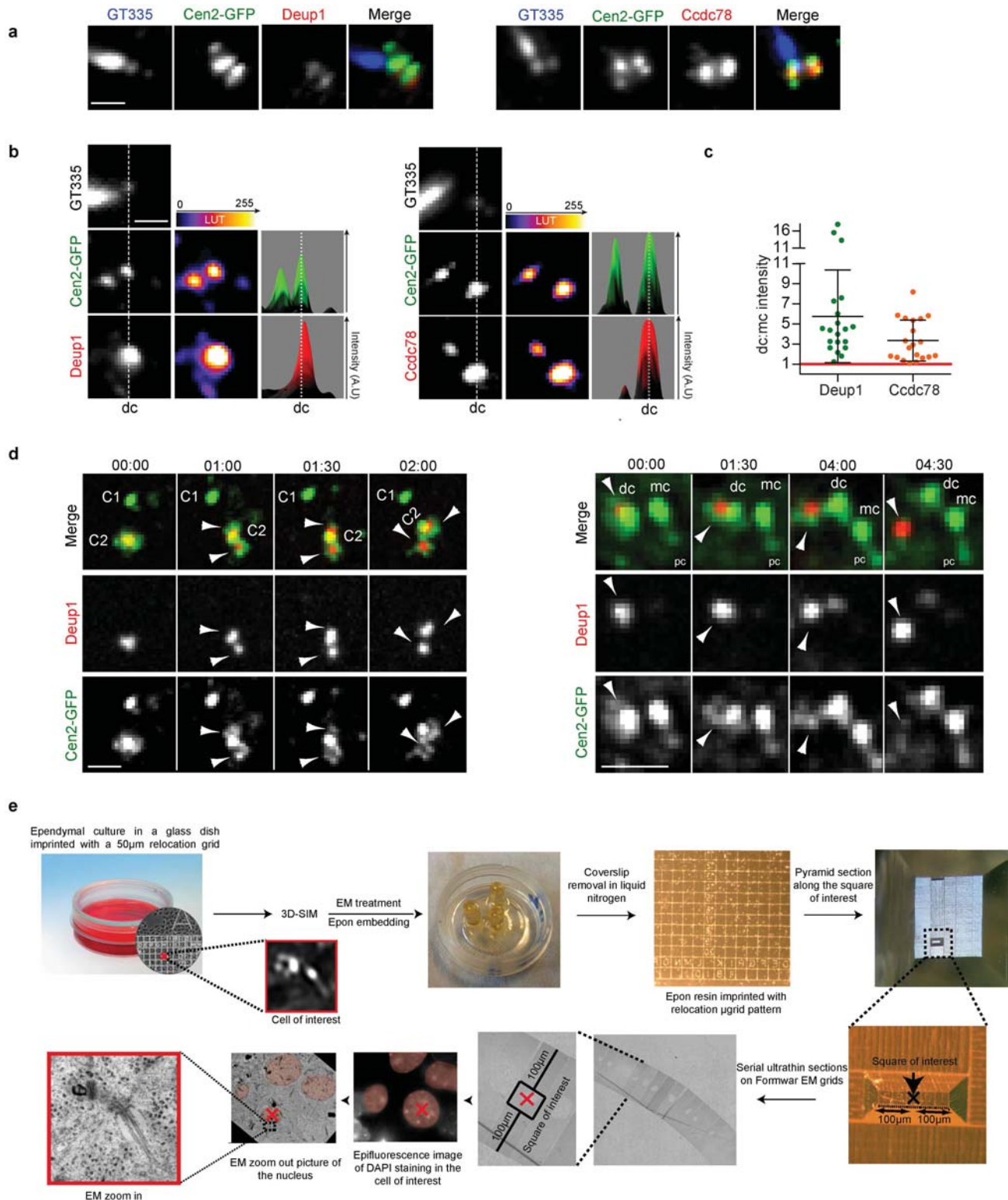
Extended Data Figure 1 | Centriole amplification in ependymal progenitor cells *in vitro* and *in vivo*. **a**, TEM showing the apical surface of a differentiating ependymal progenitor *in vitro* (DIV 3). Procentrioles (arrowheads) are seen growing from deuterosomes (D; 'deuterosome' pathway) or from a centriolar structure (C; 'centriolar' pathway). **b**, Ki67, Sas-6 and GT335 immunoreactivity during centrosome duplication in Cen2-GFP⁺ cycling ependymal progenitors *in vitro* (DIV -2). **c-e**, FoxJ1, Sas-6, GT335 and Cep164 immunoreactivity in Cen2-GFP⁺ differentiating ependymal progenitors *in vitro* (DIV 2 to DIV 5) in order of appearance (see **h**). **f**, *In vivo* immunostainings of Sas-6 and FoxJ1 in P3 to P5 Cen2-GFP⁺ ventricular walls showing that the same stages of amplification exist *in vivo* as *in vitro* (see also **h**). Boxes in **b-f** indicate

magnified structures. White arrowheads indicate the centrosomal centrioles at the halo stage. **g**, Analysis *in vitro* (DIV 3) and *in vivo* (P3-P5 lateral ventricular walls) of Cen2-GFP fluorescence showing an increase in the intensity and area of Cen2-GFP 'flower' fluorescence compared to Cen2-GFP 'halos' (*in vitro*: $n = 48$ halos; $n = 47$ flowers; *in vivo*: $n = 37$ halos; $n = 27$ flowers); error bars represent mean $\pm$ s.d.; P values derived from two-tailed Mann-Whitney U -tests, *** $P < 0.0001$. **h**, Left: percentages of differentiating cells *in vitro* according to DIV ($n = 1,244$ cells from 3 experiments per day). Right: percentages of differentiating cells *in vivo* in the region of the lateral brain ventricle indicated by the red square in Fig. 1a according to post-natal day (P) ($n = 4,025$ cells from 2 to 3 animals per age). Scale bars: 1 μ m.



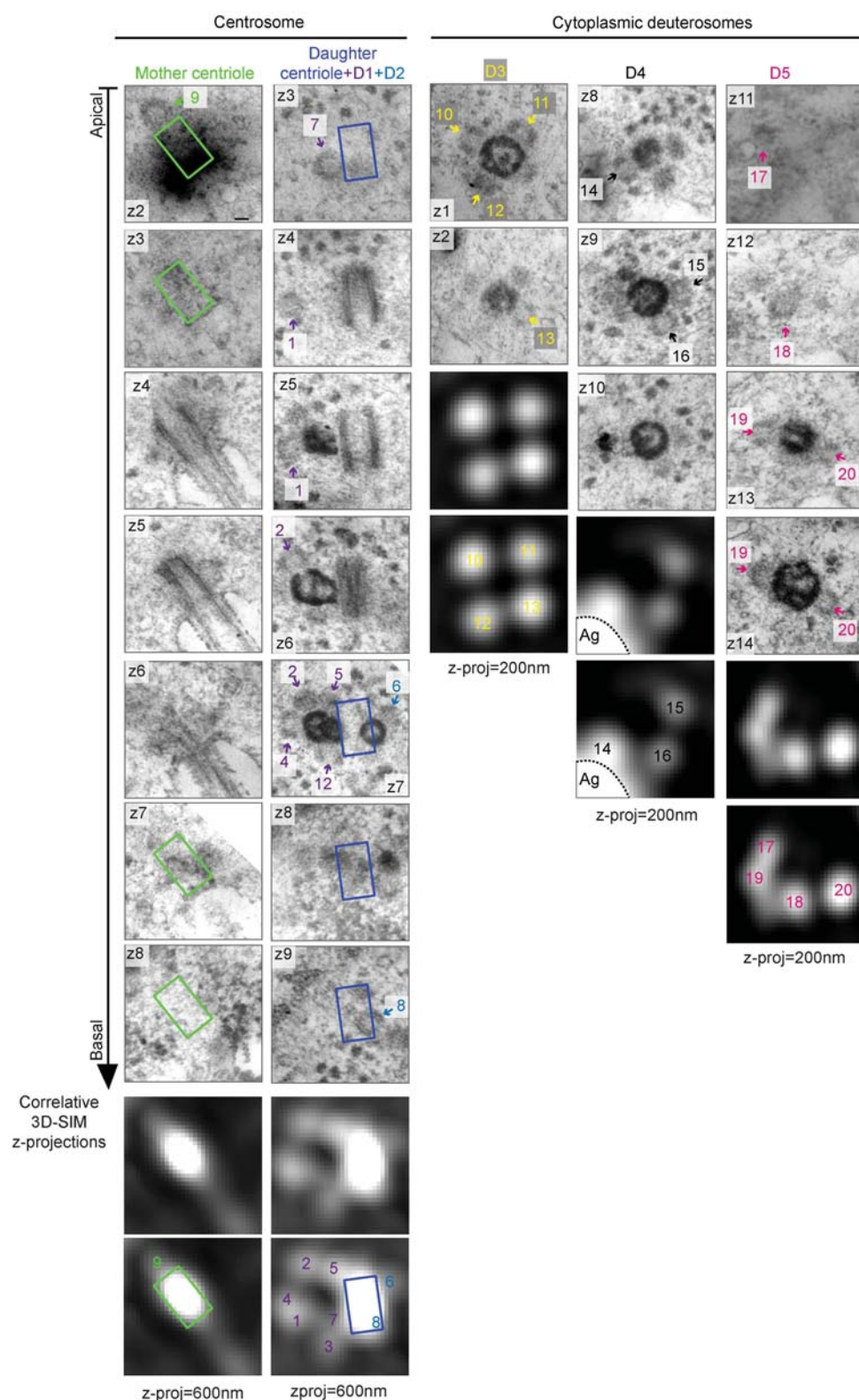
Extended Data Figure 2 | Dynamics of centriole amplification analysed live *in vitro*; centrosomes of cycling endopydymal progenitors *in vitro*; centrosome Sas-6 asymmetry *in vivo*. **a**, From Supplementary Video 2. Dynamics of centriole amplification in a differentiating Cen2-GFP⁺ endopydymal progenitor. Note that the video begins when some halos have already accumulated in the cytoplasm (00:00). Green arrowheads point to the centrosomal centrioles when discernible. Halos (red arrowheads) first accumulate in the cytoplasm (00:00–08:00) then transform into flowers (12:40; yellow arrowheads) before simultaneous centriole release (14:40). All the centrioles then migrate to the apical membrane where they grow cilia (87:00; **b**). **b**, Immunostaining, at the end of the time-lapse experiment shown in **a**, of the endopydymal-specific membrane protein CD24 confirms that Cen2-GFP⁺ structures are docked centrioles (basal bodies) growing motile cilia. **c**, From Supplementary Video 4. Single z-plane images of a Cen2-GFP⁺ centrosomal centriole (white arrowhead) during the formation of a halo (red arrowhead). Note that the second centrosomal centriole is outside the field of observation. **d**, Single z-plane of centrosomal centrioles (white arrowheads) during the concomitant formation of two halos (red and green arrowheads) from one of the two centrosomal centrioles. The halo on the right (green arrowhead) detached at 04:30 then disappeared from the field. Note that the second centrosomal

centriole appears in the field at 01:00 (second white arrowhead). **e**, From Supplementary Video 5. Three 3D-SIM z-planes (0.1 μm) showing a cytoplasmic halo with 20 procentrioles organized in a raspberry-like structure (18 Sas-6⁺/Cen2-GFP⁺ procentrioles are numbered; circles show positions of Cen2-GFP⁺ subunits relative to Sas-6 staining). **f**, Representative images of the quantifications shown in Fig. 2e (Ki67⁺; left). Sas-6, Cep152 and Plk4 immunoreactivity on Cen2-GFP⁺ mother (GT335⁺ primary cilium) and daughter centrosomal centrioles in cycling Ki67⁺ endopydymal progenitors at DIV -2. 'Fire' LUT and fluorescence intensity profiles are shown. **g**, Daughter:mother centriole Sas-6 signal ratios in FoxJ1⁺ differentiating endopydymal progenitors in P4 Cen2-GFP endopydymal walls. Left: Sas-6 staining on mother (Cep164⁺) and daughter (Cep164⁻) centrosomal centrioles in halo-stage cells, their corresponding 'fire' LUT's, and their fluorescence intensity profiles. Right: daughter:mother ratios of Sas-6 fluorescence intensity (error bars represent mean $\pm$ s.d.; 2.3 ± 0.9 ; $n = 38$ cells). The distribution differs significantly from a theoretical mean ratio of 1 (one sample *t*-test, $P < 0.0001$); distributions of Sas-6 ratios in differentiating progenitors *in vitro* (Fig. 2e) and *in vivo* do not differ statistically. Time in hh:mm; scale bars, 5 μm (**a–b**); 1 μm (**c–g**).



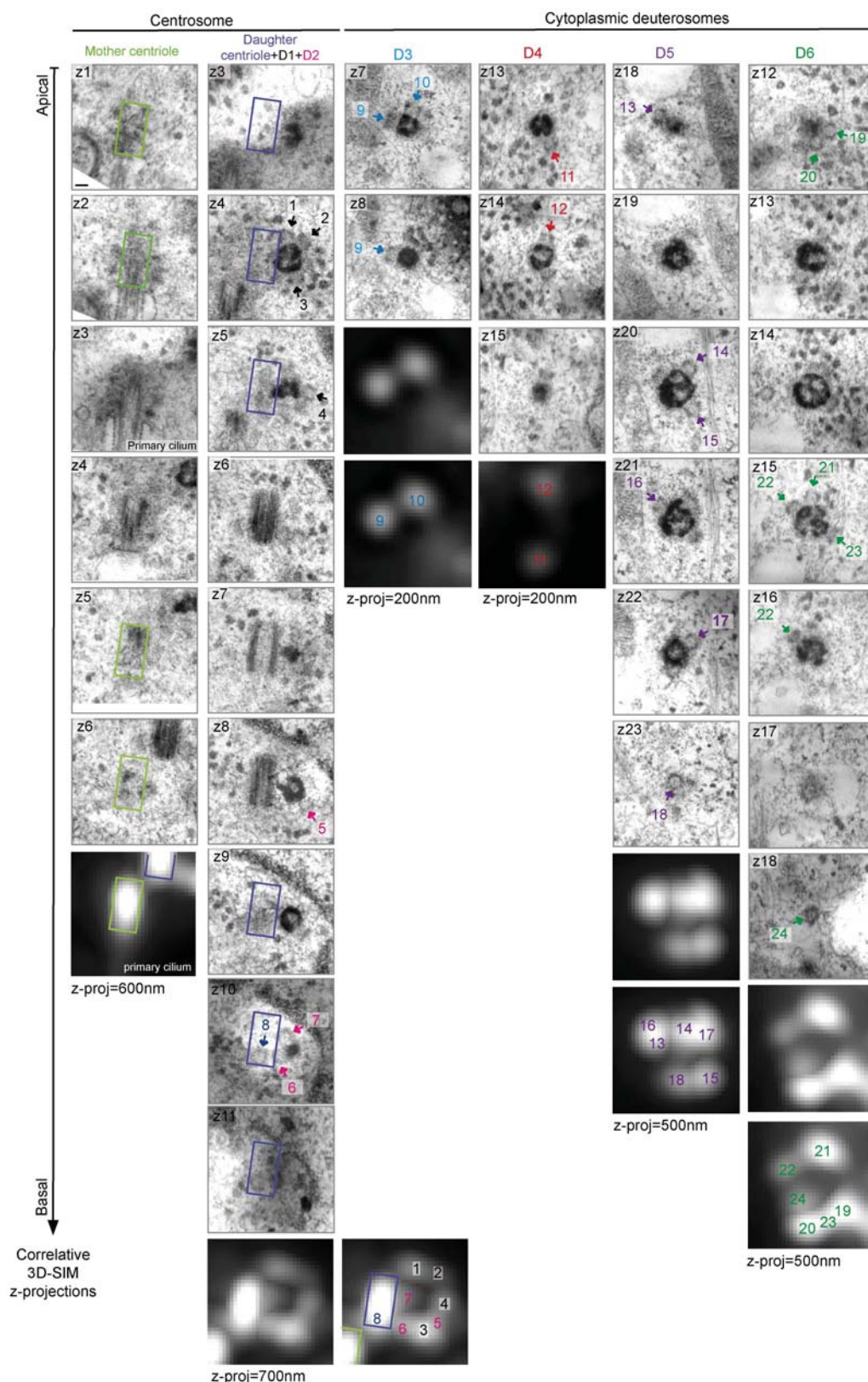
Extended Data Figure 3 | Deup1 and Ccdc78 in cycling and differentiating ependymal progenitors; correlative 3D-SIM and transmission electron microscopy (3D-SIM/EM) protocol. **a**, Representative images of Deup1 and Ccdc78 immunoreactivity in cycling (Ki67⁺) Cen2-GFP⁺ ependymal progenitors. **b**, Deup1 (left panel) and Ccdc78 (right panel) immunoreactivity on Cen2-GFP⁺ mother (GT335⁺ primary cilium) and daughter centrosomal centrioles in halo-stage cells. 'Fire' LUT and fluorescence intensity profiles are shown. **c**, Daughter:mother centriole Deup1 (5.7 ± 4.6 ; $n = 20$ cells) and Ccdc78 (3.4 ± 2 ; $n = 20$ cells) signal ratios in differentiating (FoxJ1⁺) ependymal progenitors at the halo stage; error bars represent mean $\pm$ s.d..

Distributions differ statistically from a theoretical mean ratio of 1 (two-tailed one sample *t*-test, $P < 0.0001$). **d**, Cen2-GFP/KusabiraOrange-Deup1 (K-O-Deup1) $\times 100$ videomicroscopy image sequences showing concentric Cen2⁺/Deup1⁺ halo formation (white arrowheads) and release from one of the two centrosomal centrioles (left panel; concomitant formation of 2 halos from C2) or from the daughter centriole (right panel; Cen2-GFP occasionally enter the primary cilium, distinguishing the mother from the daughter centriole). **e**, Overview of the correlative 3D-SIM/EM protocol (see Methods). C1 and C2: centrosomal centrioles (when mother and daughter centrioles cannot be distinguished). Time in hh:mm; scale bars, 1 μ m.



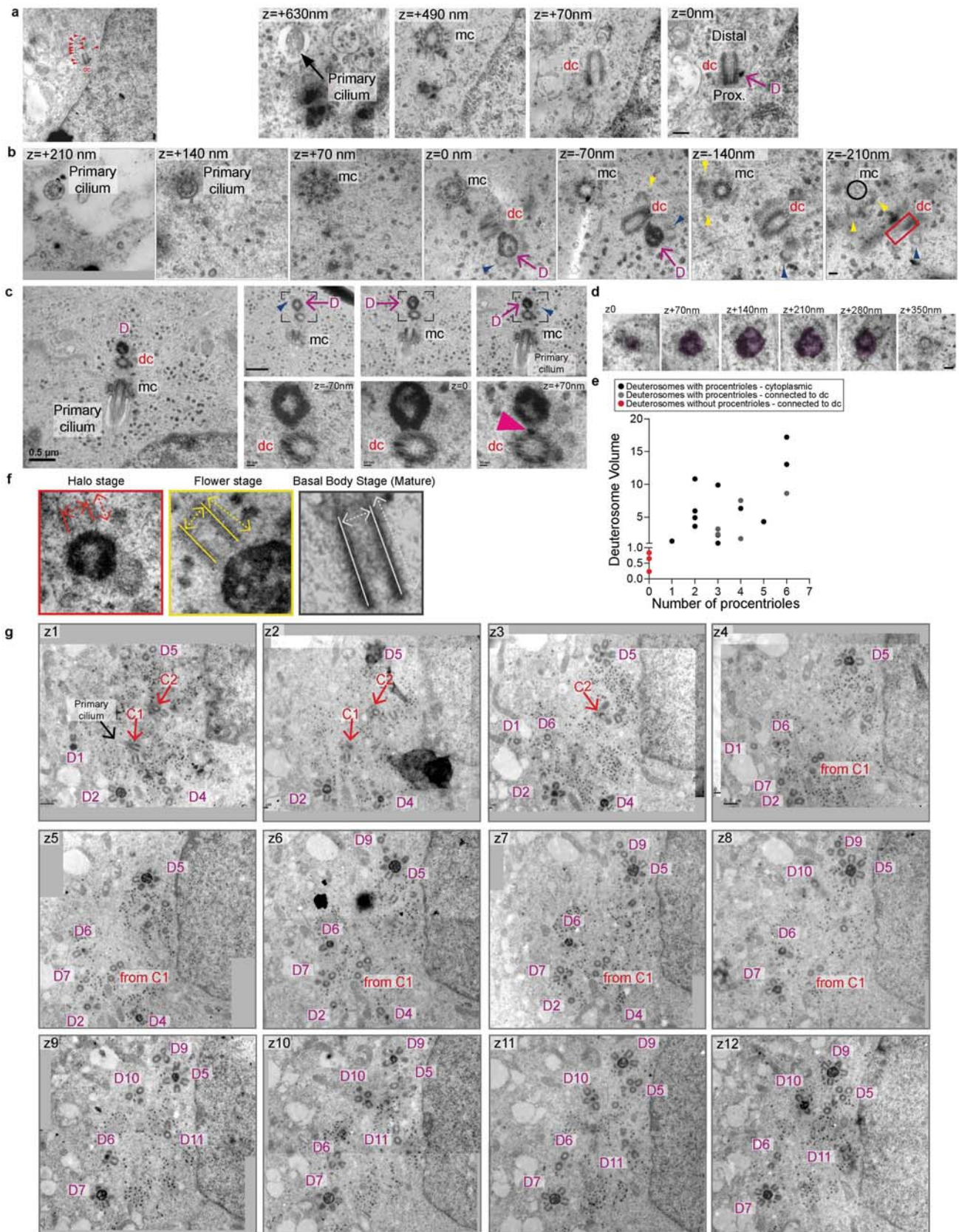
Extended Data Figure 4 | Correlative 3D-SIM and transmission electron microscopy of halo formation in a Cen2-GFP ependymal progenitor (example 1). Consecutive apical-to-basal ultra-thin EM sections (70 nm) and corresponding 3D-SIM z-projections (200 or 600 nm as indicated) are shown for both centrosomal centrioles and three cytoplasmic deuterosomes (D) in the same cell. Procentriole positions are indicated by numbers. Rectangles show the localization of the mother (green) and daughter (blue) centrioles on the

preceding or following EM section. In this cell, two deuterosomes are forming from the daughter centriole. A procentriole is growing directly from the wall of the mother centriole (9). Procentrioles cut in the sagittal plane through the centre have the same appearance (2, 4, 10, 11, 13, 14, 16). In transverse sections, the procentrioles have a tube-like structure (7, 8, 9, 18). Note that the spatial resolution of 3D-SIM is not sufficient to distinguish some of the procentrioles visible by EM (1–4, 3–7, 8). Ag, aggregate of Cen2-GFP; scale bar: 0.1 μ m.



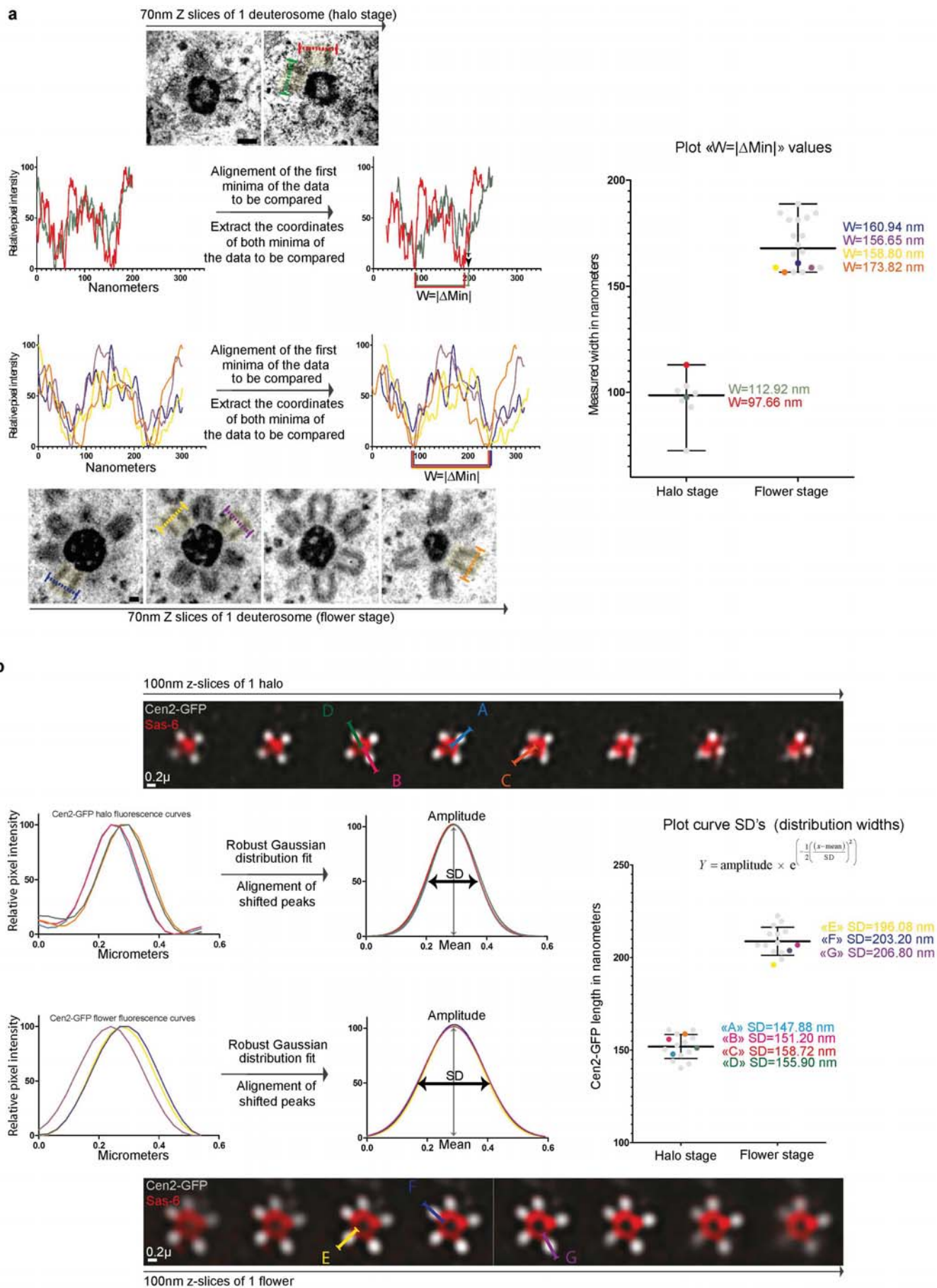
Extended Data Figure 5 | Correlative 3D-SIM and transmission electron microscopy of halo formation in a Cen2-GFP endymal progenitor (example 2). Consecutive apical-to-basal ultra-thin EM sections (70 nm) and corresponding 3D-SIM z-projections (200–700 nm as indicated) are shown for the centrosomal centrioles and 4 cytoplasmic deuterosomes (D) in the same cell. Procentriole positions are indicated by numbers. Rectangles show the localization of the mother (green) and daughter (blue) centrioles on the

preceding or following EM section. In this cell, two deuterosomes are forming from the daughter centriole. A procentriole is growing directly from the wall of the daughter centriole (8). Procentrioles cut in the sagittal plane through the centre have the same appearance (2, 7, 15, 17, 19, 21). In transverse sections, the procentrioles have a tube-like structure (8, 18, 24). Note that the spatial resolution of 3D-SIM is not sufficient to distinguish some of the procentrioles visible by EM (3–5, 6, 8, 13–17, 19, 20–23). Scale bar, 0.1 μm .



Extended Data Figure 6 | Formation of deuterosomes from the daughter centrosomal centriole during the halo stage and growth of procentrioles during the flower stage. **a**, Formation of the first deuterosome (D) in an early differentiating ependymal progenitor (DIV 3 cell). Left: EM image showing the daughter centrosomal centriole. In contrast to the cells presented in **b** and **c**, note the low abundance of electron-dense aggregates (red arrowheads) in the cytoplasm representative of an early step of centriologenesis⁶. Right panels: serial EM sections spanning the centrosome of the same cell (note that the pictures have been rotated to re-orient the daughter centriole). A small deuterosome is attached to the proximal part of the centrosomal daughter centriole. Serial sections covering 2 μm of the apical portion of this cell show the absence of procentrioles and other deuterosomes. Prox., proximal. **b**, Serial EM sections spanning the centrosome of a differentiating ependymal progenitor showing a procentriole-loaded deuterosome attached to the proximal wall of the daughter centriole. Blue arrowheads point to procentrioles extending from the deuterosome. Yellow arrowheads point to procentrioles growing directly from the proximal part of the mother and the daughter centrioles. A rectangle and a circle indicate the position of the daughter and the mother centriole, respectively, in the preceding serial EM section. **c**, Left: centrosome of a cell during deuterosome formation.

Note the microtubule network converging on the mother centriole. Right panels: serial EM sections spanning the centrosome. A deuterosome is attached to the proximal part of the daughter centriole. An electron-dense patch (magenta arrowhead), previously defined as centriolar precursor material in cycling cells²⁰, can be observed on the wall of the daughter centriole adjacent to where the deuterosome contacts the centriolar wall. Blue arrowheads point to procentrioles. **d**, Deuterosome volume calculation protocol. Deuterosomes were outlined interactively (purple surfaces) on consecutive ultra-thin EM slices spanning the entire structure. Areas were summed for each deuterosome and multiplied by the thickness of the ultra-thin sections (0.07 μm). **e**, Deuterosome volume distribution relative to their localization and the number of attached procentrioles; Pearson's correlation coefficient $r = 0.67$ ($P = 0.001$) for 20 deuterosomes from 6 cells. **f**, Representative TEM images of procentrioles at the halo stage (red), the flower stage (yellow), or mature centriole docked at the plasma membrane nucleating motile cilia (grey). **g**, Serial EM sections spanning 910 nm of the apical part of a flower-stage cell (DIV 3). Procentrioles are seen growing from both centrosomal centrioles (C1 and C2) and from deuterosomes (D1 to D11). Note the raspberry-like organization of the centrioles growing from the spherical deuterosomes. Scale bars: 0.2 μm (**a**); 0.1 μm (**b**, **d**), 0.5 μm (**c**, **g**).



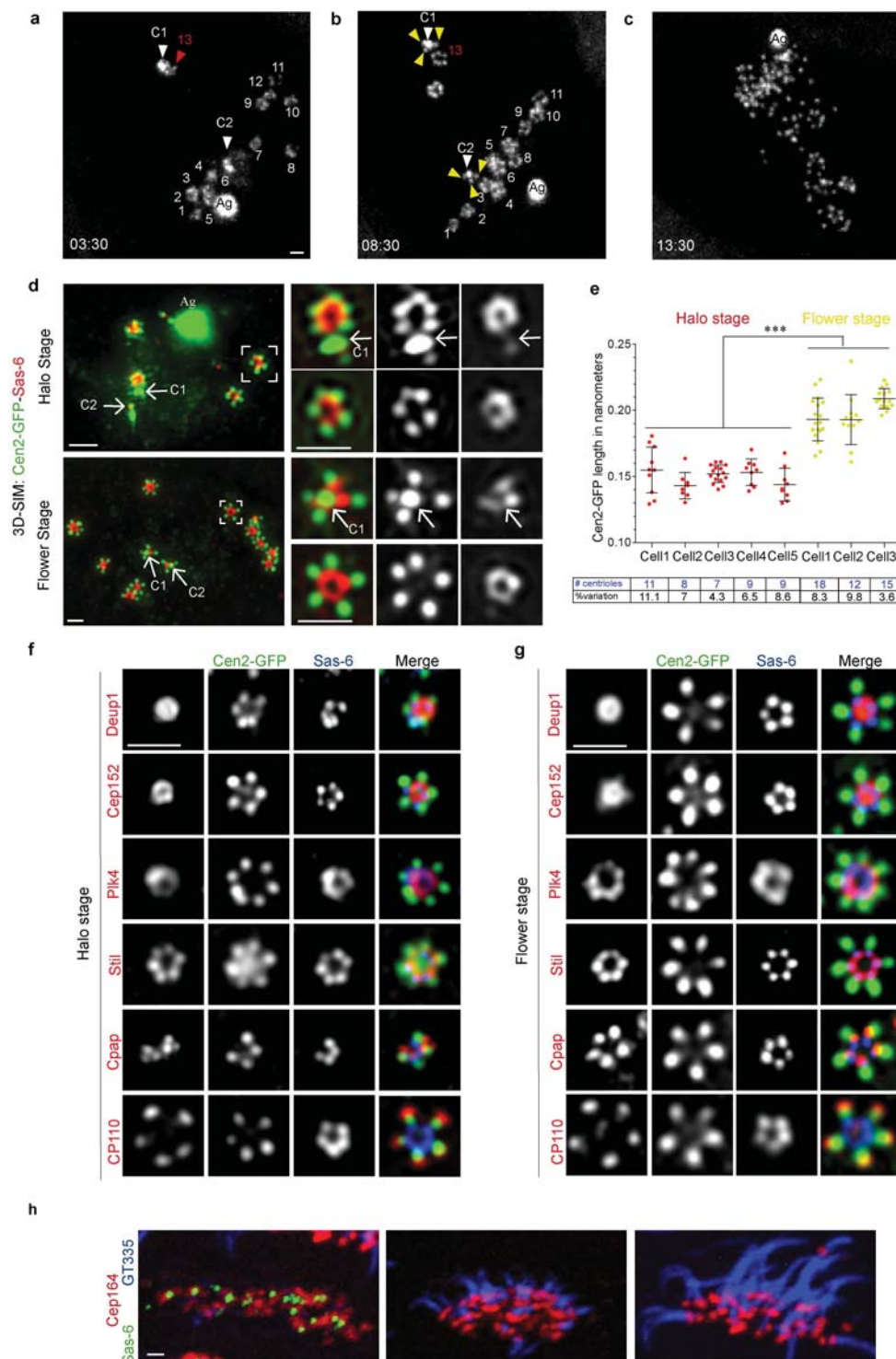
Extended Data Figure 7 | Protocols for measuring procentrioles from EM and 3D-SIM images.

a. Protocol for measuring procentrioles from EM images. Centrioles aligned parallel to the 70-nm *z*-slices were selected for quantification of EM width. Centriole width was quantified using a pixel intensity measurement tool placed at the distal extremities of the centrioles perpendicular to the centriolar walls. The resulting distributions of normalized pixel intensity were characterized by two minima on the *y*-coordinates corresponding to the centre of each of the two centriolar walls. Final plotted widths were obtained using the equation $W = |\Delta \text{Min}|$; where W is the absolute difference in nm between the *x*-coordinates of both *y*-axis minima.

b. Protocol for measuring procentriole lengths from 3D-SIM images. Measurements were made of Cen2-GFP procentrioles, stained with Sas-6, that

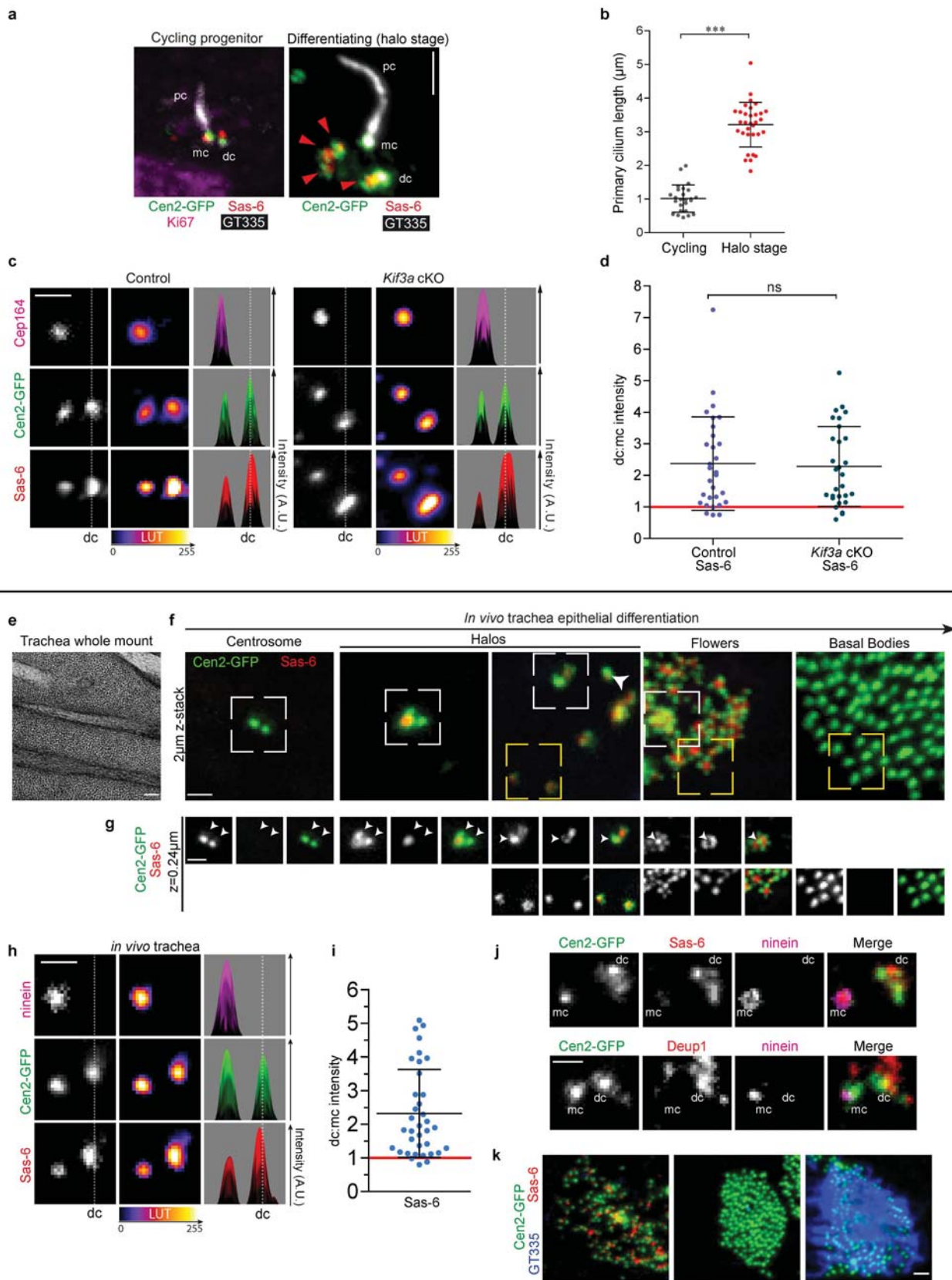
were parallel to the *x,y* plane on single 100-nm *z*-slices. Sas-6 staining was used to determine the longitudinal axis of the procentriole and was excluded from measurements of fluorescence intensity. Distributions of normalized Cen2-GFP fluorescence intensities were fitted using the robust nonlinear regression method, aligned, and their widths were measured to obtain a plot of procentriole lengths in nanometers. Each plotted dot corresponds to the width of the distribution curve obtained using the equation

$Y = \text{amplitude} \times e^{\left(-\frac{1}{2} \left(\frac{x - \text{mean}}{\text{SD}}\right)^2\right)}$, where SD is the width of the distribution, amplitude is the height of the centre of the distribution in *Y* units, and mean is the *x* value at the centre of the distribution.



Extended Data Figure 8 | Halo to flower stage transition in differentiating ependymal progenitors. **a–c**, From Supplementary Video 4. Dynamics of the transformation of Cen2-GFP halos (numbered at 03:30) into flower-like structures (corresponding numbers at 08:30) after the formation of the thirteenth and last halo (red arrowhead, same cell as in Extended Data Fig. 2c). Note that procentrioles become visible on the walls of both centrosomal centrioles at the flower stage (yellow arrowheads). Centrioles detach simultaneously from both centrosome and deuterosome platforms at 10:30. C1 and C2 are centrosomal centrioles. Ag, aggregate of Cen2-GFP. **d**, Sas-6 immunoreactivity on 3D-SIM z-projections (left panel) or single z-slices (120 nm, right panels) of Cen2-GFP⁺ (green) cells at the halo and flower stages. At the halo stage, halos have accumulated in the cytoplasm and a halo is seen forming from the C1 centriole (same cell as in Fig. 2c). At the flower stage,

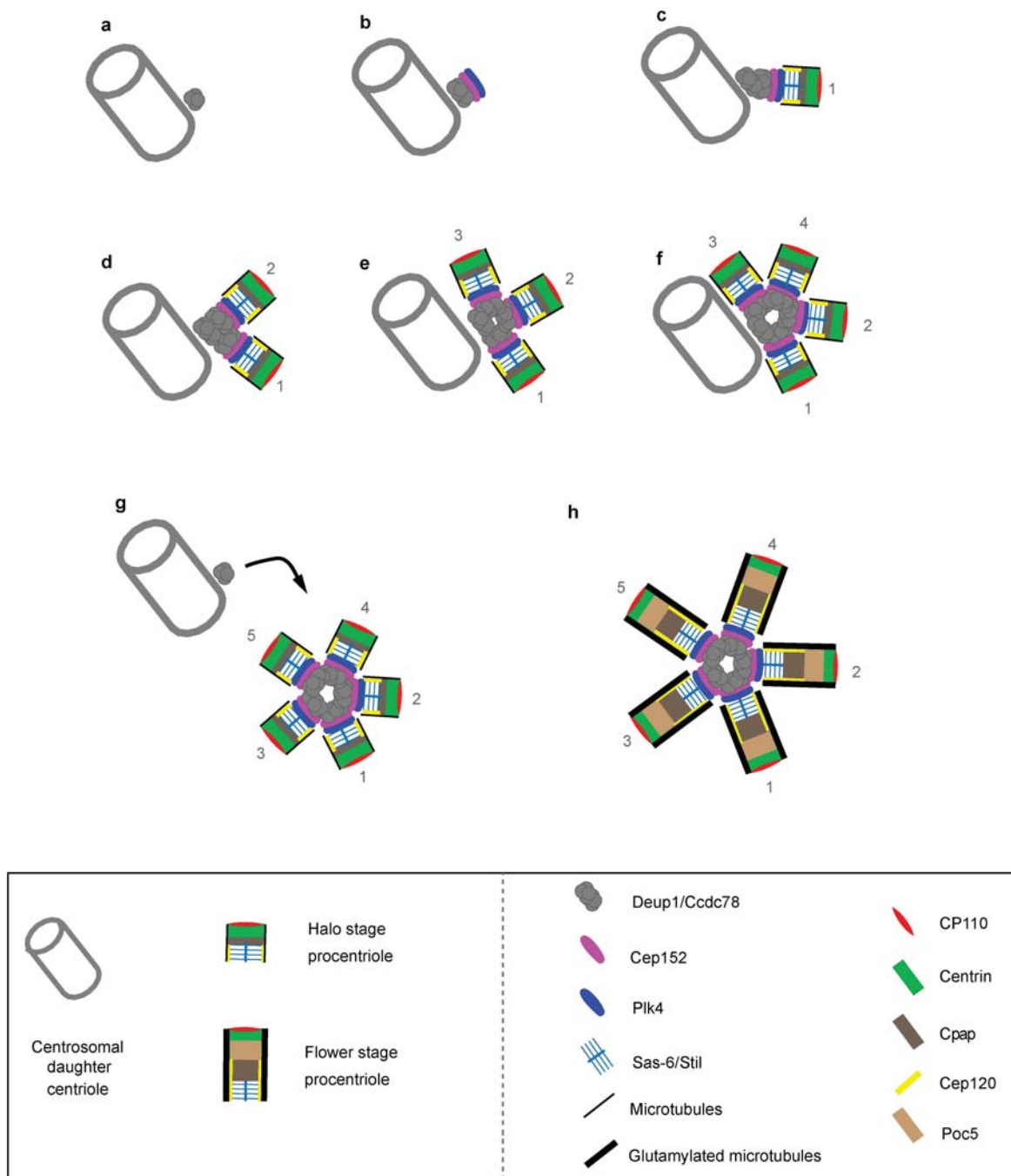
eleven flowers are visible in the cytoplasm and procentrioles are elongating from C1 and C2 centrosomal centrioles and deuterosomes. **e**, Quantification of the length of Cen2-GFP⁺ procentrioles on 3D-SIM images of cells at the halo (red) and flower (yellow) stage. There are no significant differences among cells. The lengths of the procentrioles in flower stage-cells are statistically different from the lengths of the procentrioles in halo-stage cells (error bars represent mean $\pm$ s.d.; two-tailed unpaired *t*-test, ****P* < 0.0001). See Extended Data Fig. 7b for quantification protocol. **f**, **g**, 3D-SIM z-slices (120 nm) showing the immunolocalization of Sas-6, Deup1, Cep152, Plk4, Stil, Cpap and CP110 in Cen2GFP⁺ halo (**f**) and flower (**g**) procentrioles. **h**, Immunostaining of P4 ependymal walls showing that Sas-6 immunoreactivity disappears from Cep-164⁺ centrioles when they begin to grow motile cilia (GT335⁺). Time in hh:mm; scale bars, 1 μ m.



Extended Data Figure 9 | Ciliary mutant analyses *in vitro* and centriole amplification in mouse trachea *in vivo*.

a–d, Ciliary mutant analyses *in vitro*. **a**, GT335 and Sas-6 immunoreactivity in a Cen2-GFP⁺ cycling ependymal progenitor (DIV -2; Ki67⁺) and a differentiating progenitor at the halo stage (DIV3). Arrowheads point to halos. **b**, Quantification of primary cilium length in cycling ($1 \pm 0.4 \mu\text{m}$; $n = 25$ cells) and differentiating progenitors ($3.2 \pm 0.6 \mu\text{m}$; $n = 32$ cells) showing a significant elongation at the halo stage (error bars represent mean $\pm$ s.d.; two-tailed Mann–Whitney *U*-test, *** $P < 0.0001$). **c, d**, The absence of a primary cilium in differentiating ependymal progenitor cells from *Kif3a* cKO mutants does not affect Sas-6 asymmetry. **c**, Representative Sas-6 staining on Cen2-GFP⁺ mother (Cep164⁺) and daughter centrosomal centrioles in control (left panel) and *Kif3a* cKO (right panel) halo-stage cells. Second and third columns show, respectively, the corresponding ‘fire’ LUT signal intensities and fluorescence intensity profiles. **d**, Daughter:mother centriole Sas-6 signal ratios in differentiating (FoxJ1⁺) control (2.4 ± 1.5 ; $n = 30$ cells) and *Kif3a* cKO (2.3 ± 1.3 ; $n = 29$ cells) ependymal progenitor cells at the halo stage. The distributions are not statistically different, but both differ significantly from a theoretical mean ratio of 1 (error bars represent mean $\pm$ s.d.; two-tailed one sample *t*-test, $P < 0.0001$; ns, not significant). **e–k**, Centriole amplification in mouse trachea *in vivo*. **e**, Bright-field image showing a typical cranio-dorsal region of the E17–P0 trachea whole mounts analysed in (f–k). **f**, Merged images of Sas-6-immunostained Cen2-GFP⁺ tracheal multiciliated progenitors showing that the steps of centriole amplification are the same as in brain ependymal walls; the stainings in the white and yellow boxes are shown

respectively in the upper and lower panels of **g, g**. Single $0.24 \mu\text{m}$ z-slices of the boxed regions in **f**: the centrosome images show Sas-6 negative centrosomal centrioles; the halo images show that halos arise from the wall of a centrosomal centriole (upper panels) and accumulate in the cytoplasm (lower panels); the flower images show procentrioles on a centrosomal centriole (centriolar pathway; upper panels) and hollow Sas-6⁺ flowers (deuterosome pathway; lower panels); the basal body images show mature docked Sas-6-negative basal bodies. White arrowheads point to centrosomal centrioles in **f** and **g**. **h**, Sas-6 staining on Cen2-GFP⁺ mother (ninein⁺) and daughter centrosomal centrioles in halo-stage cells in trachea epithelium. Second and third columns show, respectively, the corresponding ‘fire’ LUT signal intensities and fluorescence intensity profiles of the same markers. **i**, Daughter:mother centriole Sas-6 signal ratios (2.3 ± 1.3 ; $n = 36$ cells) in halo-stage cells in E17–P0 trachea. The distribution differs statistically from a theoretical mean ratio of 1 (error bars represent mean $\pm$ s.d.; two-tailed one sample *t*-test, $P < 0.0001$); Sas-6 halo-stage daughter/mother centriole ratios are similar in ependymal walls (Extended Data Fig. 2g) and trachea epithelium. **j**, Upper panels: $0.96 \mu\text{m}$ z-stacked images of a typical Sas-6/ninein immunoreactivity on E17–P0 Cen2-GFP trachea showing that halo structures form exclusively on the daughter centriole ($n = 12$ cells). Lower panels: $0.96 \mu\text{m}$ z-stacked images of a typical Deup1/ninein immunoreactivity on E17–P0 Cen2-GFP trachea showing accumulation of Deup1 on the daughter centriole. **k**, Immunostaining of P0 trachea showing that Sas-6 immunoreactivity disappears from basal bodies when they begin to grow GT335⁺ motile cilia. Scale bars, $1 \mu\text{m}$.



Extended Data Figure 10 | Hypothetical model of centriole amplification through deuterosome formation. **a**, An unknown mechanism leads to Deup1 and Ccdc78 accumulation at the daughter centriole. **b**, Binding of Cep152 and recruitment of Plk4 (refs 7,8) activate nucleation of the first pro-centriole. **c**, Accumulation continues from the same active site and displace the first pro-centriole. **d**, The second pro-centriole arises from the new complex. **e–g**, The process continues until the deuterosome detaches from the centriolar wall. **g**, The process begins again to form another deuterosome and nucleate new

pro-centrioles. During deuterosome formation (halo stage), pro-centrioles remain latent at an early stage of their biogenesis. Several deuterosomes can form concomitantly from different active sites on the daughter centriole proximal wall. **h**, The cycle continues until centriole growth is triggered and pro-centrioles grow from the walls of both centrosomal centrioles and deuterosomes (flower stage). See Fig. 4i, j and Extended Data Fig. 8f, g for 3D-SIM localization of structural and regulatory proteins depicted here.

Supplementary Video Legends

Supplementary Video 1

Apical (upper panel) and side (lower panel) views of a time-lapse sequence showing Cen2-GFP dynamics (63X magnification, $\Delta t = 40$ minutes) during the maturation of an ependymal progenitor *in vitro*. Note that halos appear within the centrosome region (14:00-30:40) and transform into flower-like structures (31:20-34:00) before the simultaneous release and apical docking of all the centrioles (34:00-72:40). White arrowheads point to the centrosomal centrioles of the ependymal progenitor when discernible. See Fig. 1(i-n). Time is in hh:mm. Scale bar: 2 μ m.

Supplementary Video 2

Apical (upper panel) and side (lower panel) views of a time-lapse sequence showing Cen2-GFP dynamics (63X magnification, $\Delta t = 40$ minutes) during the maturation of an ependymal progenitor *in vitro*, starting from the halo formation stage (00:00-04:00). Halos transform into flower-like structures (04:40-12:40) prior to the simultaneous release and apical docking of all the centrioles (12:40-32:40). Cd24 staining at 87:20 shows that basal bodies have nucleated cilia. White arrowheads indicate the centrosomal centrioles of the ependymal progenitor when discernible. See Extended Data Fig. 2(a-b). Time in hh:mm. Scale bar: 2 μ m.

Supplementary Video 3

Apical view of a time-lapse sequence showing Cen2-GFP dynamics (100X magnification, $\Delta t = 30$ minutes) during the process of halo formation. Note that 6 halos have already formed and are numbered in the cytoplasm. White arrowheads indicate centrosomal centrioles. Red arrowhead indicates the 7th and last halo formed from a centrosomal centriole (01:00-04:30).

After the last halo is released, the 7 halos transform into flowers (numbered at 12:30). Centrioles become visible on the walls of both centrosomal centrioles (yellow arrowheads). See Fig. 2a for single 0.7 μm z-planes of halo formation. Time in hh:mm. Scale bar: 2 μm .

Supplementary Video 4

Time-lapse sequence showing Cen2-GFP dynamics (100X magnification, $\Delta t = 30$ minutes) during halo formation. Note that 12 numbered halos have already formed. White arrowheads indicate centrosomal centrioles. Red arrowhead indicates the 13th and last halo formed from a centrosomal centriole (01:00-08:30). All halos then transform simultaneously into flowers (numbered at 08:30). Centrioles become visible on the wall of both centrosomal centrioles (yellow arrowheads). Centrioles detach simultaneously from both centrosome and deuterosome platforms from 10:30. Centrin aggregates are occasionally observed in Cen2-GFP cells. See Extended Data Fig. 2c for single z-planes of halo formation. Time in hh:mm. Scale bar: 2 μm .

Supplementary Video 5

3D-SIM z-planes (0.1 μm) spanning a halo and showing the raspberry-like organization of 20 procentrioles (green: Cen2-GFP, red: SAS-6). Dashed circles (left) outline the Cen2⁺ subunits and dashed rectangles (right) outline the Cen2⁺/SAS-6⁺ procentrioles that are oriented parallel to z-plane. z labeling is in μm .

Supplementary Video 6

Time-lapse sequence of the Cen2-GFP/KusabiraOrange-Deup1 dynamics (100X magnification, $\Delta t = 30$ minutes) during centriole amplification showing that halos and flowers display a Deup1⁺ core. Ten halos are already cytoplasmic, some of which are superimposed due to z-stacking. White arrowheads indicate centrosomal centrioles. Magenta arrowhead indicates the 11th and last halo formed from a centrosomal centriole (00:00-01:30). After the

last halo is formed, all the halos transform simultaneously into flower-like structures by 05:00. Cyan arrowheads indicate procentrioles that become visible on the walls of both centrosomal centrioles. Centrin aggregates are occasionally observed in Cen2-GFP expressing cells. Time in hh:mm. Scale bar: 2 μ m.

Supplementary Video 7

Cen2-GFP time-lapse imaging (100X magnification, $\Delta t = 30$ minutes) at the end of the flower stage showing the synchronized release of procentrioles ($t = 00:00-03:00$) from both centrosomal centrioles and deuterosomes ($\bar{t} = 2.5 \pm 0.85$ h in 9 cells from 5 independent experiments). Released centrioles have migrated to the apical membrane by 20:30. White arrowheads point to the centrosomal centrioles when discernible. Halos appearing at -01:30 in the lower right corner and flowers appearing at 11:00 are from neighboring cells. Time in hh:mm. Scale bar: 2 μ m.



Youssef Nabil - One Lonely Star, Alexandria 1999

Hand coloured gelatine silver print. Courtesy of the Artist and Nathalie Obadia Gallery, Paris/Brussels

***“Analogy points in this direction, and though analogy is
often misleading, it is the least misleading thing we have.”***

Samuel Butler

*The Note-Books of Samuel Butler; Chapter VII; On the Making of Music, Pictures,
and Books; Thought and Word ii.*

Published by Henry Festing Jones, 1912

Article 2

*Please note that the following article is a first version draft manuscript.
Some experiments are still ongoing in order to finalize certain details of the study.*

Mitosis machinery dampening coordinates centriole amplification and prevents mitosis in differentiating multiciliated cells

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Abstract

Mitosis and meiosis are driven by a common regulatory network centered on the activity of the Cdk1-CyclinB1 complex. The protein network coordinates M-phase entry and exit crucial for cell division and meiotic gamete production. Here we provide evidence of an activation of the Cdk1-CyclinB1 complex along with its mitotic regulatory network in a novel non-proliferative cellular context. By coupling single cell live imaging with pharmacological modulation of mitosis regulators, we show that the mitotic machinery orchestrates the spatiotemporal progression of the centriole amplification dynamic in terminally differentiating multiciliated cell progenitors. The fine tuning of Cdk1 activity avoids mitotic entry while allowing the timely coordination of centriole number, growth, and disengagement through checkpoint-like phase transitions necessary for subsequent functional motile ciliation. By defining the mechanism that regulates centriole number and allows the formation of motile cilia, these results establish new outlooks for studies treating cilia-related diseases and pathological centriole amplification associated with cancer and anomalous brain development.

Introduction

The cell cycle is organized around a negative feedback loop involving Cdk1–CyclinB1 protein kinase and the E3 ubiquitin ligase APC/C^{Cdc20}. During cell cycle progression, the gradual synthesis of CyclinB1 with mitotic kinase and phosphatase activity make the cell toggle from the interphase state with inactive Cdk1–CyclinB1 to a mitotic state with active Cdk1-CyclinB1^{17,86,195,196}. Cdk1-mediated phosphoactivations coordinate mitotic entry by inducing cytoskeletal reorganizations, chromosome condensation and nuclear membrane disintegration^{19,20,79,81,85,196}. Cdk1 activity eventually triggers the activation of the Anaphase Promoting Complex (APC/C^{Cdc20}) that tags, among others, mitotic Cyclins and Securin for degradation thus completing the loop by returning the cell to the interphase state^{92,93,197,198}. Anaphase is promoted by the activation of Separase and the activity of Plk1¹⁹⁹ that trigger sister chromatid separation in time for mitosis exit. The decline of Cdk1 activity finally promotes the unwinding of chromosomes and the reformation of the nuclear envelope.

The centriole duplication cycle is mechanistically linked to cell cycle progression by a shared pool of master regulators^{61,73,74,91,200}. Centrioles are microtubule based structures that pair up to form the core of the centrosome, the major microtubule organizing center of animal cells⁴. The duplication of centrioles during interphase forms two centrosomes that participate in bipolar spindle organization during mitosis to support symmetric separation of chromosomes at each cell division²⁴. In interphase and at the G1/S phase transition, a procentriole is formed next to each preexisting centriole of the centrosome. The orthogonal, a.k.a. engaged, configuration of each duplicated daughter centriole relative to its mother centriole and their proximity is maintained during interphase to prevent unscheduled biogenesis of additional centrioles⁶¹. However, Plk1 and Cdk1 kinase activity at the G2/M phase transition induce the distancing of mother and daughter centrioles within each centrosome, as centrosomes separate along the nuclear envelope to opposite sides of the cell^{77,89,200,201}. To prevent additional

centriole formation as a result of mitotic entry, Cdk1 displaces the centriole cartwheel protein Stil⁷⁸ from the centrosome to the cytoplasm, and blocks its interaction with the centriole biogenesis initiator Plk4⁹⁶. At the metaphase-to-anaphase transition, APC/C^{Cdc20} targets Stil for degradation in parallel to the decrease in Cdk1 activity⁷⁸. Plk1 activity followed by Separase activation after Securin degradation disengage the centrioles to license a new centriole duplication cycle in the newly formed daughter cells^{73,202}.

Aberrant coordination of the centriole duplication cycle with the cell cycle may source supernumerary centrioles that are linked to tumorigenesis and tissue degeneration^{43–46}. In contrast to the strict centriole number control in the cycling cell context, post mitotic and terminally differentiating progenitors of multiciliated cells must amplify up to several hundred centrioles to nucleate an equal number of motile cilia¹⁹⁴. Mature mammalian multiciliated cells employ their motile cilia to transport vital fluids and particles along epithelia in the reproductive, respiratory, and central nervous systems¹¹³. Defects in the centriole amplification dynamic lead to ciliopathies responsible for infertility, irreversible bronchial damages, and anomalous brain development^{170,171,203}.

By investigating the centriole amplification dynamic in differentiating progenitors of multiciliated cells, our previous work has established that all amplified centrioles originate from the preexisting centrosome via multiple rounds of procentriole seeding²⁰⁴. A paralogue of the core centriole duplication regulator Cep63¹⁹³, Deup1, supports the multiple rounds of procentriole seeding by materializing into deuterosomes, which are centrosome auxiliary spherical structures that carry the newly formed procentrioles. The centrosomal origin of all amplified centrioles²⁰⁴ and the shared molecular cascade initiating centriole biogenesis^{193,205} suggest that multiciliated cells seize a differential control of the centriole duplication program to bypass the centriole number control for the purpose of multiple cilia formation. However, after several cycles of amplification the whole procentriole population goes through a

sequential and highly synchronized progression of events organized around two abrupt transitions²⁰⁴. The first “amplification-to-growth phase” (A/G) transition is marked by a halt in deuterosome formation and centriole amplification coupled to a synchronous growth of the whole procentriole population from both centrosome and deuterosome platforms (Fig. 1a). During the growth phase, microtubules of the newly formed centrioles become polyglutamylated and centrioles acquire the distal marker Poc5. At the second “growth-to-disengagement phase” (G/D) transition, the growing centrioles disengage synchronously from centrosome and deuterosome platforms before migrating toward the plasma membrane to drive motile ciliation (Fig. 1a). After this second transition, centrioles acquire distal appendages and lose procentriole proteins Sas-6 and Stil, markers of immaturity²⁰⁴.

Together with the progressive acquisition of centriole maturity markers, the two abrupt transitions marking the centriole amplification dynamic are reminiscent of centriole behavior in cycling cells at the G2/M and Metaphase/Anaphase transitions. We therefore sought to test the hypothesis that, as for centriole duplication, mitotic regulators coordinate the timely progression of centriole biogenesis in brain multiciliated cell progenitors.

Results

Mitotic network activation correlates to the progression of centriole phase transitions

Multiciliated ependymal cells covering lateral ventricles are derived from radial glial cells during brain development. Their post-mitotic maturation, marked by a massive centriole production, starts at early postnatal stages^{116,204}. We first immunostained Centrin2-GFP (Cen2-GFP) lateral ventricles for the key mitosis initiating complex proteins Cdk1 and Cyclin B1. Maturing progenitors of multiciliated cells stain positive for both Cdk1 and Cyclin B1 from the amplification phase up to the centriole disengagement phase before a gradual staining decrease in mature multiciliated cells (Fig. 1a-b and Extended Data Fig. 1a). A

parallel phosphorylation of cyclin B1 on the Cdk1-Cyclin B1 autophosphorylation site ¹⁷ is observed (Fig. 1c and Extended Data Fig. 1b and 2a-b) along with a decrease in the nuclear staining of the Cyclin-dependent kinase inhibitor 1B known as p27^{Kip1} (Extended Data Fig. 1c and 2c) ^{206,207}. Nuclear histone H3 Ser10 (pH3) and lamin A-C Ser392 phosphorylation (pLamin), normally phosphorylated at the onset of mitosis, starts after the A/G transition. Vimentin Ser55 phosphorylation (pVim), normally phosphorylated in late mitosis, is observed from the G/D transition (Fig. 1d-e and Extended Data Fig. 1d and 2d). These results suggest a temporary loss of quiescence and a cell cycle like entry of non-cycling ependymal progenitors timely correlated with the two transitions of centriole amplification. The immunostaining profiles and sequential mitosis-like phosphorylations together with the expression of mitotic cyclins known to bind to Cdk1 (Extended Data Fig. 2e) suggest that Cdk1 is activated during the centriole amplification process.

To test whether Cdk1 activity is functionally responsible for the observed mitosis-like phosphorylations, we treated differentiating cultures with pharmacological modulators of the Cdk1 mitotic regulatory network. Direct inhibition of Cdk1 using RO-3306 ²⁰⁸ abolished vimentin Ser55 phosphorylation in all differentiating cultures of multiciliated cell progenitors (Extended Data Fig. 3a-b). Inactivating the nuclear and cytoplasmic Cdk1 inhibitors Wee1 ²⁰⁹ and Myt1 ²¹⁰ using PD 166285 ²¹¹ boosted pH3 and pVim stainings in cells during the growth and disengagement phases. Chromosome condensations could even be observed at these stages (Fig. 1f, PD 166285, second and third panels). We then focused on APC/C, the complex responsible for mitotic cyclin degradation and anaphase onset. Cells incubated with the partial inhibitor of APC/C activity, proTAME ²¹², showed pH3 and pVim hyperphosphorylations only at the disengagement phase. They were accompanied by prophase-like chromosome condensations as well as prometaphase-like and telophase-like figures (Fig. 1f, proTAME, third panel). The percentage of cells undergoing proTAME-

mediated chromosome condensation was null when co-incubated with RO-3306 (2 independent experiments) and decreased 2.7 fold in cells with siRNA depletion of CyclinB1 (siScramble n=49 cells and siCyclinB1 n=18 cells at the disengagement phase with condensed chromosomes per differentiating culture at DIV4), suggesting that proTAME-induced chromosome condensation is dependent on Cdk1-CyclinB1 complex activity. Coupling proTAME-mediated APC/C disruption with MK 1775²¹³, an inhibitor of the nuclear Cdk1 regulator Wee1, advanced the pH3 and pVim hyperphosphorylations as well as prophase-like chromosome condensation to the growth phase (Fig. 1f, proTAME+MK 1775, second and third panel). Similar to proTAME treatment alone, prometaphase as well as telophase-like figures were observed during the disengagement phase (Fig. 1f, proTAME+MK 1775, third panel). In total, an increase of 79% of cells with condensed chromosomes was observed in proTAME+MK 1775 treatment (n=23/30 cells) compared to proTAME treatment alone (n=3/7 cells). These collective results show that Cdk1 activity is responsible for the sequential mitosis-like phosphorylations observed during the centriole amplification dynamic. The development of pseudo-mitotic events after drug-induced hyperactivation of Cdk1 and inhibition of APC/C activity show that differentiating ependymal cells are soaked with all main mitosis actors. More precisely, we can conclude from the timing of drug-induced pseudo-mitosis events that Cdk1 activity is dampened by Myt1 and Wee1 at the A/G transition and by APC/C at the G/D transition. The delimitations of activity timings presented here further support our hypothesis concerning the parallel between the A/G and the G2/M transitions, as well as between the G/D and the metaphase/anaphase transitions. Altogether, the data suggest that the activity of Cdk1 and of its mitotic regulatory network correlates to the progression of the centriole amplification dynamic.

Analogies between the centriole amplification dynamic and mitosis events

Along with Cdk1 activity during ependymal progenitor differentiation, the monitoring of centriole kinetics through spinning disk live cell imaging as differentiating cells passed across the two transitions highlighted additional features of mitosis. During the amplification phase, centrosomal centrioles remain in proximity and deuterosome platforms carrying newly formed procentrioles stay within a close centrosomal radius. At the A/G transition, both centrosomal centrioles and deuterosome platforms pull away (Fig. 2a-b) while migrating towards the nucleus to assume a three-dimensional perinuclear distribution (Fig. 2c-d and Extended Data 4a). At the onset of the disengagement phase, the primary cilium resorbs and centrioles collectively disengage from their growing platforms in an isotropic manner along the nuclear envelope (Fig. 2e-f, Extended Data Fig. 4b and Supplementary Video 1). During this phase, individualized centrioles colocalize with a drastically reorganizing tyrosinated microtubule network (Fig. 2g-h) ²¹⁴. After the disengagement phase, all centrioles migrate towards the apical membrane to dock and nucleate motile cilia (Extended Data Fig.4b, Supplementary Video 1, and Article 1 Extended Data Figure 2). Separation of centrosomal centrioles and procentriole-bearing deuterosomes along the nuclear envelope recall centrosome separation during mitotic prophase thus reinforcing the analogies between A/G and G2/M transitions. Then, the subsequent disengagement of newly formed centrioles from their growing platforms further evokes the mother-daughter centriole disengagement occurring around mitotic anaphase thus reinforcing the analogies between the G/D and metaphase/anaphase transitions. Together with the activation of mitosis regulators, the results suggest that differentiating progenitors may employ the mitotic machinery to orchestrate the phase transitions observed during the centriole amplification dynamic.

Cdk1 timely triggers centriole growth and disengagement in addition to regulating final centriole number

To test whether the activity of the mitotic network is responsible for the coordination of the centriole amplification dynamic, we coupled single cell live imaging of differentiating Cen2-GFP progenitors with pharmacological inhibition of mitosis regulators and monitored the centriole amplification phase transitions. Inhibition of Cdk1 by RO-3306 significantly delayed both A/G and G/D phase transitions (Fig. 3a-b and Supplementary Videos 2 and 3). This leads to a perturbation of centriole migration and subsequent motile cilia nucleation (Extended Data Fig. 5a and Supplementary Video 3). Interestingly, prolonging the amplification phase by displacing the A/G transition significantly increases the number of deuterosomes produced (Fig. 3c-d). Inversely, PD 166285-mediated inhibition of negative regulators of Cdk1 activity, Myt1 and Wee1, halts the amplification phase and significantly accelerates both phase transitions (Fig. 3e-f and Supplementary Video 4). Shortening the amplification phase decreases, dose dependently, deuterosome (Extended Data Fig. 5b-c) and final centriole number (Fig. 3g-h). Inhibition of Wee1 alone by MK 1775 also leads to a reduction of final centriole number (Extended Data Fig. 5d-e). Both PD 166285 and MK 1775 treatments resulted in cultures with dose dependant increases in the percentage of multiciliated cells that are marked by a reduced generation of multiple motile cilia (Extended Data Fig. 5f-g). These results show that fine-tuning of Cdk1 activity by its mitotic modulators Myt1 and Wee1 timely triggers centriole amplification arrest and controls the number of centrioles. After centriole growth, Cdk1 triggers the subsequent synchronous disengagement of centrioles for correct migration and ciliation.

APC/C-Cdc20 and Plk1 activity trigger centriole disengagement

In cycling cells, the metaphase/anaphase transition is promoted by the activation of APC/C^{Cdc20} complex due to mitotic Cdk1-Cyclin B1 activity¹⁹⁸. Continuing the parallel with the cell cycle, we further tested whether Cdk1 triggers G/D transition through APC/C activation. To proceed, we screened the centriole amplification dynamic in presence of proTAME. Disrupting APC/C activity did not affect the A/G transition but significantly delayed the G/D transition, thus

perturbing centriole apical migration and ciliation (Fig. 4a-b, Supplementary Video 5). Consistent with the development of pseudo-mitosis observed by immunocytochemistry (Fig. 1d, proTAME, third panel), 60% of cells incubated with proTAME that passed the G/D transition condensed their chromosomes and underwent karyokinesis during the centriole disengagement phase (n=33 cells entering pseudo-mitosis out of 55 cells initiating centriole disengagement; Fig. 4c, Extended Data Fig. 6a and Supplementary Videos 6,7, and 8; cells excluded from centriole amplification dynamic analysis in Fig. 4a-b). This pseudo-mitosis is characterized by mitotic chromosome condensation (Fig. 4c and Extended Data Fig. 6a-b), nuclear envelope breakdown (Extended Data Fig. 6b) and the formation of monopolar, bipolar, as well as multipolar spindles (Extended Data Fig. 6c). Although microtubule spindles are organized from patches of newly formed centrioles (Extended Data Fig. 6c), the direction of chromosome migration suggests that they are incapable of attaching chromosomes (Fig. 4c, Extended Data Fig. 6a, and Supplementary Videos 7 and 8). No EdU incorporation after 24-hour incubation time periods is observed in control or proTAME treated cells (data not shown). Consistently, centromere immunostainings confirmed the presence of single chromatids in pseudo-mitotic cells (n=40 centromeres per pseudo-mitotic cell; Extended Data Fig. 6d). Condensed chromosomes separated after an average of 1.9 ± 0.9 hours (Fig. 4d) before cells exited pseudo-mitosis and reformed one or more nuclei (Extended Data Fig. 6a and d) while the disengaged centrioles continued to migrate apically. Lagging chromosomes were occasionally observed and resulted in the formation of micronuclei (arrowheads in Fig. 4c and Extended Data Fig. 6c). The exit from the pseudo-mitosis state suggested that proTAME-mediated APC/C inhibition is partial as in mitotic cells^{212,215,216}, and that hyperactivation of Cdk1 eventually leads to APC/C activation. To increase APC/C^{Cdc20} inhibition efficiency, we incubated proTAME treated cells with Apcin, a synergistic APC/C inhibitor²¹⁶. Co-incubation with the two inhibitors resulted in a significant delay of the time spent in pseudo-mitosis (Fig. 4d), confirming partial inhibition of APC/C activity by proTAME and suggesting that Cdc20 is the activator of

APC/C in this context^{215,216}. Collectively, these results show that centriole disengagement is dependent on both Cdk1 and APC/C. Although further validation is required, one could hypothesize that, like in cycling cells at the metaphase/anaphase transition¹⁹⁸, Cdk1 maximizes APC/C^{Cdc20} activity which in turn triggers centriole disengagement through the targeting of Securin and Cyclin B1 for proteasome degradation, thereby promoting Separase-mediated centriole disengagement and Cdk1 de-activation for terminal G0 state differentiation. These results also show that, in addition to Myt1 and Wee1, Cdk1 activity is fine-tuned by APC/C at the G/D transition to coordinate centriole amplification without entering mitosis.

Since APC/C^{Cdc20} activity along with Plk1 coordinate the disengagement of centrioles and chromosomes in cycling cells at the metaphase/anaphase transition^{73,91}, we also tested the implication of Plk1 in centriole disengagement during massive centriole production. Plk1 inhibition by BI 2536²¹⁷ resulted in a blockade of the centriole G/D transition (Fig. 4e-f and Supplementary Video 9). Consistent with the role of Plk1 in mitosis initiation via Cdk1-CyclinB1 complex activation through the inhibition of Myt1/Wee1 and the activation of Cdc25 as well as Cyclin B1^{86,87,218}, pharmacological inactivation of Plk1 also delayed the A/G transition (Fig. 4e-f and Supplementary Video 10). As with direct inhibition of Cdk1 by RO-3306, this amplification phase prolongation is accompanied by a significant increase in deuterosome number (Fig. 4g-h). The transition delays resulted in a dose dependant decrease of the number of multiciliated cells formed within a 24-hour incubation time period (Extended Data Fig. 6e). Longer incubation periods with both BI 2536 and RO-3306 would be of interest in order to evaluate to what extent the final centriole number is affected by these treatments. Comparable to the role of Plk1 in the control of the G2/M and metaphase/anaphase transitions, these results show that Plk1 is involved in the control of A/G and G/D transitions in ependymal cells. It further confirms the presence and functional implication of a mitosis regulatory network in the maturation of differentiating multiciliated progenitors.

Discussion

In this report, we complete the spatiotemporal description of the centriole amplification dynamic and highlight that a minimal mitosis molecular network is coordinating this process in developing ependymal cells. We show that centriole growth and disengagement are timely regulated by a network involving Plk1, Cdk1 and APC/C in order to control centriole number and a synchronous release of centrioles for motile ciliation (Fig. 5). This study further completes the picture of the cellular and molecular mechanisms that bridge multiple centriole biogenesis to the establishment of multiciliated cell and tissue polarity^{116,191,204}.

Our drug experiments reveal that Cdk1 activation is the trigger for centriole amplification arrest and subsequent centriole growth. Studies of centriole duplication in cycling cells have shown that Cdk1-Cyclin B1 prevents supernumerary centriole biogenesis from the G2/M transition until mitosis exit by displacing Stil and preventing Stil-Plk4 interactions^{78,96}. One can hypothesize that the same Cdk1-mediated action on Stil stops the amplification in multiciliated cells. The causal link between the centriole amplification arrest and centriole growth still needs to be assessed.

The same stages of centriole amplification are also observed in multiciliated cells covering the trachea²⁰⁴ (Article 1; Extended Data Fig.9), suggesting that all mammalian multiciliated cells may share the same molecular mechanism to orchestrate the centriole amplification dynamic. However, the number of motile cilia significantly differs in between multiciliated tissues of a single organism^{122,123}. In mice, ependymal cells usually generate less than 70 centrioles *in vivo*, whereas airway multiciliated cells produce up to 300 centrioles. Although a differential regulation of the Cdk1 network at the amplification-to-growth phase transition may explain final centriole number variability across mammalian multiciliated epithelia, several studies suggest that the number of centrioles correlates to multiciliated cell surface size^{123,205}. It

would thus be interesting to test whether Cdk1 also coordinates multiciliated cell surface growth as in cell cycle contexts²¹⁹.

By designating components of the mitotic machinery as the master regulators of the two phase transitions, we further unify the mechanisms of centrosome duplication in cycling cells and large-scale centriole production in multiciliated cells. However, the centrosome duplication cycle in cycling cells is tightly coupled to the DNA cycle, whereas multiciliated cell progenitors uncouple centriole biogenesis from DNA synthesis. The network of regulators curbing Cdk1 activity along with immunostaining profiles of mitotic phosphorylations, microtubule reorganization, and mRNA levels suggest that basal activity levels of Cdk1 during the centriole amplification dynamic are purposely kept low beneath a mitosis commitment threshold level. Developing ependymal cells may therefore implement an alleviated version of mitosis to manage centriole amplification using mitosis tools while uncoupling centriole from chromosome dynamics. This consequently suggests that centriolar events may require less Cdk1 activity than DNA events.

We provide evidence of physiological and thresholded mitotic phosphorylations of vimentin, histones, and lamins during the centriole amplification dynamic of non-proliferative progenitors of multiciliated cells. Phosphorylation of Histone 3 (Ser10) suggests that Aurora B kinase is expressed and active during the centriole phase transitions²²⁰. It remains to be determined whether these mitotic phosphorylations are simply downstream readouts of mitotic kinase activity for the coordination of the centriole dynamic, or if they have additional functional purposes. In cycling cells and from within the nucleus, both Cdk1 and Aurora B kinase phosphorylate histones and condensins to promote chromosome condensation at mitotic entry^{81,85,221,222}. Lamin phosphorylation by Cdk1 disassembles nuclear pore complex proteins before disintegrating the nuclear membrane, thus exposing condensed chromosomes to the cytoplasm^{79,88,223}. Since our results suggest that Cdk1 is active in both the cytoplasm

and the nucleus, it raises the question whether the curbed mitotic phosphorylations during the centriole amplification dynamic permeabilize the nuclear membrane or initiate chromosome condensation by nuclear recruitment of condensins. Assessing the spatiotemporal distribution of condensins, the integrity of the nuclear membrane, and the potential activation of DNA damage pathway components during the centriole amplification dynamic may clarify this question as well as provide insights into how developing multiciliated cells maintain DNA integrity in presence of an active mitosis machinery.

Radial glial cells, which are progenitors of ependymal multiciliated cells ¹¹⁶, seem to retain the capacity to reactivate cell cycle regulators, albeit specific to their cycling state, for the purpose of terminal differentiation. Hyperactivation of Cdk1 or APC/C disruption in differentiating ependymal progenitors leads to abrupt pseudo-mitosis entry and exit marked by aberrant chromosome segregation and fusion of decondensing chromosomes into one or more nuclei. Interestingly, radial glial cells are also the main source of deadly childhood ependymal tumors known as ependymomas ²²⁴. Since notable causes of ependymomas are rapid oncogenic gene fusions ²²⁵ or epigenetic modifications ²²⁶ in their radial glial progenitors, further assessment of pseudo-mitosis consequences on differentiating radial glial cells may prove to be of interest.

Supplementary Materials

Methods

Legends for Figures 1 to 5

Legends for Extended Data Figures 1 to 6

Legends for Supplementary Videos 1 to 10

Figures 1 to 5 and Extended Data Figures 1 to 6

Methods

Animals

See Article 1, Methods section.

Brain dissections

See corresponding figure legends and Article 1, Methods section.

Electroporation of ventricular walls

The electroporation protocol was adapted from Boutin et al.²²⁷ for Arl13b-GFP/Centrin2-RFP experiments (see Figure 2e). New born (P0) Arl13b-GFP transgenic mice were anesthetized by 3 minutes of hypothermia. A suture needle is pierced through the cranium and a glass capillary is introduced into the newly formed orifice (at a 1mm depth to reach the ventricle) that injects a solution of plasmid DNA Centrin1-RFP (gift of Xavier Morin) at 2 $\mu\text{g}/\mu\text{L}$ and 1/20 Fast Green dye into the right ventricle. Electrodes (CUY650P5) covered in conductive gel (Aquasonic 100, Parker, ref 01-08) are placed from both sides of the skull with the + bourne on the side of the injected hemisphere and 5 pulses (100V for 50ms every 850ms) are applied. The mice are heated up to 37°C and left overnight with their mother. The mice are then sacrificed and only parts of the ventricular walls positive for the Fast Green dye are scalped and cultured by using the same protocol described below.

Primary endymal cell cultures and transfections

See Article 1, Methods section.

RT-PCR

The RNeasy Qiagen kit (74004) was used to extract mRNA at corresponding days of *in vitro* differentiation. cDNA was obtained with the MasterMix 5 PRIME solution and protocol. The following oligonucleotide sequences were used for the PCR: Cdk1 (F:ACAGAGAGGGTCCGTCGTAA,R:ATTGCAGTACTGGGCACTCC); Deup1 (F:AGGAATTAAGCAAGGCTGTGGA,R:TGAAGTCGGTGTGTGTCTCTGC); Cyclin O (F: CGCTGGATCTCCAGACCTTC,R:GATTCGGCAGTCAGTCACTTGTGG); Cyclin A1 (F:CAGCAGGCTGTGGCTTACTA,R:CTGTGATCTCCTGGCCACA); Cyclin A2 (F:TCGCTGCATCAGGAAGACCA,R:AAGAGGAGCAACCCGTC); Cyclin B1 (F:CTCTCCAAGCCCGATGGAAA,R:TGACTGCTCTTCCTCCAGTTG); and Cyclin B2 (F:GCTAGCTCCCAAGGATCGTC,R:CTGCAGAGCTGAGGGTTCTC).

Immunostaining

For Centrin, Foxj1, Sas-6 and GT335 protocol and references, see Article 1, Methods section. Lateral brain ventricles and cell cultures were fixed without permeabilization in PFA for 2 hours and 10 minutes, respectively. Tissues and cells were pre-blocked in PBS 1X with 0.2% Triton X-100 and 10% FBS before incubation with primary then secondary antibodies before counterstaining with DAPI (10 µg/ml, Sigma) and mounting in Fluoromount (Southern Biotech). The following antibodies were used: mouse IgG1 Cdk1 (1:100; Santa Cruz), mouse IgG2a Cyclin B1 (1:50; GNS-11; BD Pharmingen), rabbit Cyclin B1 (pSer126; 1:100; Abcam), rabbit Histone 3 (pSer10; 1:100; Cell Signaling), mouse IgG2b Vimentin (pSer55; 1:400; Abcam), rabbit p27^{kip1} (1:200; Santa Cruz), mouse IgG1 p27^{kip1} (1:200; BD Biosciences), human centromere (1:100; Antibodies Inc.), rabbit Lamins A+C (pSer392; 1:200; Abcam), rat tyrosinated tubulin (YL1/2; 1:200; Abcam), mouse IgG1 Nuclear pore

complex proteins Mab414 (1:500; Eurogentech), and species-specific Alexa Fluor® secondary antibodies (1:400; Invitrogen).

For confocal microtubule visualization, a fixation protocol was adapted from Mathieu Piel. Fixation and detergent solution [3% PFA, 0.2% Nonidet P-40 “NP-40” in Brinkley Buffer 1980 “BRB80” 1X (80mM PIPES, 1mM MgCl₂ hexahydrate, 1mM EGTA; pH 6.8)] was added for 1 minute at 37°C, then replaced by a detergent free fixation solution (without NP-40) and incubated for 10 minutes at 37°C. Cells were permeabilized for 10 minutes (NH₄Cl 0.1M in BRB80) and then washed twice with BRB80 1X for 5 minutes. Blocking buffer (0.2% NP-40, 3% FBS in BRB80 1X) was added for an hour, before incubating with the primary antibodies for one hour at room temperature. Cells were then washed twice with 0.2% NP-40 in BRB80 1X for 5 minutes and incubated with the secondary antibodies for an hour at room temperature. Cells were washed twice with 0.2% NP-40 in BRB80 1X for 10 minutes, and once with DAPI for 5 minutes.

Chemical inhibitors

See figure legends for final concentrations. The following inhibitors were used: RO-3306 (Calbiochem), PD 166285 (Tocris Bioscience), MK 1775 (Axon Medchem), proTAME (Boston Biochem), Apcin (Boston Biochem), and BI 2536 (SelleckChem).

Microscopy

Epifluorescence microscopy.

See Article 1, Methods section.

Confocal microscopy

A Confocal Leica TCS SP5 microscope equipped with a X63 objective was used for microtubule and nuclear pore complex visualization.

Videomicroscopy.

See Article 1, Methods section.

Videomicroscopy with inhibitors.

Inhibitors were diluted in DMEM glutamax, 0% FBS, 1% P/S and added directly into cell chambers 5 to 7 minutes before the start of image acquisition.

Transmission electron microscopy.

See Article 1, Methods section.

Quantification and statistical analyses

Data were compiled from at least 2 independent experiments except for RT-PCR results in Extended Data Figure 2e. Fluorescence intensity was quantified with Image J and compared images were acquired with the exact same acquisition conditions. For centriole number quantifications, an in-house built ImageJ macro plugin customized for 40X magnification was used to automatically detect Cen2-GFP⁺ centrioles in 3D stacks. GFP signal detection in 3D stack uses sequential processing comprising of 3D Gaussian filtering for noise removal, 3D LoG filtering²²⁸ for enhancing GFP signal size and 3D objects while suppressing the rest, and ‘Fast Filter 3D maxima detector’²²⁹ for finding the location of GFP signal in 3D. All the parameters are tuned based on assumed size (0.25 μm diameter) of GFP signal in 3D with a 40X magnification. Detected GFP signal per cell is then processed by the cell profiler software²³⁰ using batch processing to obtain final plotted values. All graphs and statistical analyses were obtained using GraphPad Prism software. See corresponding figure legends for p-values (P) and further details.

Figure legends

Figure 1 Mitotic network activation correlates to the progression of centriole phase transitions

a, Illustration of the procentriole amplification phase (grey bar), the procentriole growth phase (orange bar), and the centriole disengagement phase (green bar). A/G represents the amplification-to-growth phase transition, and G/D represents the growth-to-disengagement phase transition. **b-e**, Cdk1, Cyclin B1, Cyclin B1 (pSer126), Histone H3 (pSer10), and Vimentin (pSer55) immunostainings on differentiating Cen2-GFP⁺ ependymal progenitors *in vivo*. Cen2-GFP⁺ mouse lateral ventricles from P3 to P6 were used. Cells under grey bars are at the centriole amplification phase; cells under orange bars are at the centriole growth phase; cells under green bars are at the centriole disengagement phase. Nuclear DAPI staining is outlined in **c-d**. **a-c** are crops from Supplementary Fig. 1a-b and d. **f**, Representative immunostainings of Histone H3 (pSer10) and Vimentin (pSer55) in differentiating Cen2-GFP⁺ ependymal progenitors *in vitro* (DIV 4) at the amplification (grey bars, panel 1), growth (orange bars, panel 2), and disengagement (green bars, panel 3) phases. Nuclei are stained with DAPI. Cells were incubated for 3 hours with DMSO, PD 166285 0.5μM, proTAME 12 μM, or proTAME 12μM with MK1775 1μM. Scale bars, 5μm.

Figure 2 Progression of the centriole amplification dynamic is analogous to early mitosis events

a, Evolution of the distance between centrosomal centrioles (left panel) or the occupied surface area of deuterosomes (right panel) in 28 Cen2-GFP⁺ filmed cells transitioning from the amplification phase to the growth phase; P values derived from two-tailed Wilcoxon matched-pairs signed rank test, ****P<0.0001. **b**, Filmed Cen2-GFP⁺ cell #15 from **a** transitioning from the amplification phase (grey box) to the growth phase (orange box).

Yellow arrowheads indicate deuterosomes and blue arrowheads indicate centrosomal centrioles. **c**, From Article 1 Supplementary Video 1. Profile view of Cen2-GFP dynamics in a cell transitioning from the amplification (grey bar) to the growth (orange bar) phase. Note the perinuclear distribution of deuterosomes in the cell at the growth phase. Right panel is an apical view single z-plane ($0.7\mu\text{m}$) at the growth phase. **d**, Single confocal z-plane ($0.25\mu\text{m}$) showing immunostainings of Centrin and nuclear pore complex proteins *in vitro* in a cell at the growth-to-disengagement phase transition. **e**, Primary cilium (Arl13b-GFP⁺) and centriole (Cen2-RFP⁺) dynamics from the growth phase through the disengagement phase until the multiple basal body stage. White arrowheads indicate the Arl13b-GFP⁺ primary cilium when present. **f**, Apical and profile view of Cen2-GFP dynamics showing the perinuclear disengagement of 136 centrioles. Apical view of the nucleus region is shaded in blue. **g**, Tyrosinated tubulin and Centrin immunostainings *in vitro* showing the evolution of tyrosinated tubulin networks and centriole localization in cells at the centrosome stage (left panel), the growth-to-disengagement phase transition (central panel), and the mature multiple basal body stage (right panel). Note the loss of microtubule polarization in the cell at the growth-to-disengagement phase transition. DNA is stained with DAPI. **h**, Single confocal basal z-plane ($0.25\mu\text{m}$) from the z-stack ($5.8\mu\text{m}$) in **e** (central panel) showing colocalization of disengaged centrioles with tyrosinated tubulin at the growth-to-disengagement (G/D) phase transition. White boxes in **d**, **f**, and **h** correspond to the magnified regions in lower panels; time in hh:mm; scale bars, $1\mu\text{m}$ (d and h) and $5\mu\text{m}$ (b-c and e-g).

Figure 3 Cdk1 timely triggers centriole growth and disengagement and regulates final centriole number

a, Centriole amplification phase transition analysis of filmed differentiating Cen2-GFP⁺ ependymal cells in presence of DMSO (n=71 cells, left panel) and RO-3306 $9\mu\text{M}$ (n=74 cells, right panel). **b**, Tukey box plot comparing the time cells from **a** spent in the centriole

amplification, growth, and disengagement phases; *** $P=0.0008$, **** $P<0.0001$. **c**, Quantification of the number of Sas-6⁺ deuterosomes in DIV4 Cen2-GFP⁺ cells at the amplification phase (GT335⁻) after 24-hour incubation with DMSO (n=20 cells) or RO-3306 (n=22 cells). * $P=0.0172$. To avoid bias of cells in early stages of the amplification phase, cells with less than 4 deuterosomes were excluded from the quantification. **d**, Sas6 immunostaining showing deuterosomes of DIV4 Cen2-GFP⁺ cells at the amplification phase (GT335⁻) after 24 hours of incubation with DMSO (left panel) or RO-3306 (right panel). **e**, Centriole amplification phase transition analysis of filmed differentiating Cen2-GFP⁺ ependymal cells in presence of DMSO (n=54 cells, left panel) and PD 166285 0.5 μ M (n=53 cells, right panel). **f**, Tukey box plot comparing the time cells from **e** spent in the centriole amplification, growth, and disengagement phases; * $P=0.0301$, **** $P<0.0001$. **g**, Quantification of the number of centrioles in DIV4 Cen2-GFP⁺ cells at the disengagement phase (Sas-6⁺) after 24-hour incubation with DMSO (n=49 cells), PD 166285 0.25 μ M (n=21 cells) and PD 166285 0.5 μ M (n=21 cells). * $P=0.0198$, **** $P<0.0001$. **h**, Immunostainings showing disengaging Sas6⁺ centrioles in Cen2-GFP⁺ cells after 24 hours of incubation with DMSO (left panel), PD 166285 0.25 μ M (central panel), and 0.5 μ M (right panel). Plus signs in **b** and **f** indicate the means; error bars in **c** and **g** represent mean $\pm$ s.d.; all P values derived from two-tailed Mann-Whitney U-tests; scale bars, 5 μ m.

Figure 4 APC/C-Cdc20 activity triggers centriole disengagement and Plk1 activity regulates both centriole amplification phase transitions

a, Centriole amplification phase transition analysis of filmed differentiating Cen2-GFP⁺ ependymal cells in presence of DMSO (n=98 cells, left panel) and proTAME 12 μ M (n=63 cells, right panel). **b**, Tukey box plot comparing the time cells from **a** spent in the centriole amplification, growth, and disengagement phases; *** $P=0.0005$, **** $P<0.0001$. **c**, Centriole (Cen2-GFP⁺) and nucleus (H2B-RFP⁺) dynamics starting from the growth phase until the

multiple basal body stage in DMSO and proTAME 12 μ M treated cells. Pseudo-mitosis initiates during the disengagement phase at 10:40. White arrowheads indicate micronuclei formation after exit from proTAME-mediated pseudo-mitosis. **d**, Tukey box plot comparing the time cells spent in pseudo-mitosis in presence of proTAME 12 μ M (n=33 cells) or proTAME 12 μ M coupled to Apcin 25 μ M (n=6 cells); ***P=0.0014. **e**, Centriole amplification phase transition analysis of filmed differentiating Cen2-GFP⁺ ependymal cells in presence of DMSO (n=61 cells, left panel) and BI 2536 200nM (n=66 cells, right panel). **f**, Tukey box plot comparing the time cells from **e** spent in the centriole amplification, growth, and disengagement phases; **P=0.0054, ****P<0.0001. **g**, Quantification of the number of Sas-6⁺ deuterosomes in DIV4 Cen2-GFP⁺ cells at the amplification phase (GT335⁻) after 24-hour incubation with DMSO (n=27 cells), BI 2536 50nM (n=49 cells), and BI 2536 200nM (n=67 cells). *P=0.0198, **P=0.004. Cells with less than 4 deuterosomes were excluded from the quantification. **h**, Sas6 immunostaining showing deuterosomes of DIV4 Cen2-GFP⁺ cells at the amplification phase (GT335⁻) after 24 hours of incubation with DMSO (left panel), BI 2536 50nM (central panel), and BI 2536 200nM (right panel). Plus signs in **b**, **d**, and **f** indicate the means; error bars in **g** represent mean $\pm$ s.d.; all P values derived from two-tailed Mann-Whitney U-tests; time in hh:mm; scale bars, 5 μ m.

Figure 5 Model of the mechanism regulating the centriole amplification dynamics in maturing multiciliated cells

The centriole amplification-to-growth transition is triggered by Plk1 and Cdk1 activity (green arrows). Wee1/Myt1 regulate activity levels of Cdk1 that seems to associate with Cyclin B1 but probably includes other M-Phase cyclins. Plk1 may potentially participate in Cdk1-Cyclin B1 activation indirectly via Wee1/Myt1 inhibition or directly via Cyclin B1 phosphorylation (dotted grey line and arrow) as in cycling cells at the G2/M transition^{86,218}. The centriole growth-to-disengagement transition is triggered by the Cdk1-APC/C activity axis and Plk1

activity (green arrows). Plk1 may hypothetically promote indirect APC/C activation (grey dotted arrow) as in cycling cells^{231,232}.

Extended Data Figure 1 Immunostaining profiles of mitosis regulators in differentiating ependymal lateral ventricles *in vivo*

a-d, P3 to P6 differentiating Cen2-GFP⁺ lateral ventricles immunostained for Cdk1, Cyclin B1, Cyclin B1 (pSer126), p27^{kip1}, Histone H3 (pSer10), and Vimentin (pSer55). Grey boxes (amplification phase), orange boxes (growth phase), green boxes (disengagement) phase indicate magnified cells in Fig.1a-c. **a** and **c**, Fuchsia arrows indicate FoxJ1⁺ cells at the centrosome stage and fuchsia arrowheads indicate mature ependymal cells at the multiple basal body stage. **c**, Grey arrows indicate cells at the amplification phase; orange arrows indicate cells at the growth phase; green arrow indicates cell at the disengagement phase. **d**, yellow arrowhead indicates a cycling progenitor in mitosis. Scale bars, 5μm.

Extended Data Figure 2 Immunostainings of mitosis regulators in differentiating ependymal cells *in vitro*; Semi quantitative RT-PCR of Cdk1 and mitotic cyclins

a,c-d, Representative immunostainings of Cyclin B1 (pSer126), p27^{kip1}, and Lamins A+C (pSer392) on differentiating Cen2-GFP⁺ ependymal progenitors *in vitro* (DIV 3-5) at the amplification (grey bars), growth (orange bars), and disengagement (green bars) phases. Nuclei are stained with DAPI. **b**, Quantification of nuclear Cyclin B1 (pSer126) immunostainings *in vitro*; left panel, representative stacked image (4.6μm) of Cen2-GFP⁺ ependymal culture with outlined nuclei used for mean fluorescence intensity quantifications of Cyclin B1 (pSer126); right panel, mean fluorescence intensity of nuclear Cyclin B1 (pSer126) signal in cells at the amplification phase (n=6 cells), growth phase (n=6 cells), disengagement phase (n=5 cells), and multiple basal body stage (n=10 cells) relative to nuclear mean signal intensity in cells at the centrosome stage (n=48 cells). Error bars

represent mean $\pm$ s.d.. **e**, Graphs showing cDNA levels of Cdk1 and mitotic cyclins (Cyclin A1, A2, B1, and B2) relative to Cyclophylin cDNA (internal sample loading control). mRNA was extracted from cultures with cycling ependymal progenitors (DIV-2), differentiating ependymal progenitors (DIV1 until DIV10, shaded in grey), and differentiated cultures (DIV15). Deup1¹⁹³ and Cyclin O¹⁸⁵ were used as controls of culture differentiation. Scale bars, 5 μ m.

Extended Data Figure 3 Immunostainings of mitosis regulators in differentiating ependymal cells *in vitro*; Relative cDNA levels of Cdk1 and mitotic cyclins

a, Representative Vimentin (pSer55) immunostainings and corresponding signal surface plots of DIV4 ependymal cultures after 24-hour incubations with DMSO (left panels) or RO-3306 (right panels). **b**, Control and RO-3306 treated Cen2-GFP⁺ ependymal progenitors at the disengagement phase immunostained for Vimentin (pSer55). Nuclei are stained with DAPI. Scale bars, 5 μ m.

Extended Data Figure 4 Transmission electron microscopy (TEM) of centrioles at the growth phase; Cen2-GFP dynamics from the growth phase up to the multiple basal body stage

a, TEM section (70 nm) of a differentiating ependymal progenitor (DIV4) at the growth phase showing centrioles on deuterosomes in nuclear contact; C, Cytoplasm; N, Nucleus. **b**, Apical and profile view of Cen2-GFP dynamics from the growth phase showing the perinuclear disengagement of centrioles followed by the apical migration of all centrioles; nucleus is shaded in blue and white arrowheads indicate centrosomal centrioles. Time in hh:mm; scale bars, 1 μ m (**a**) and 5 μ m (**b**).

Extended Data Figure 5 Analysis of centriole amplification phases post-treatment with RO-3306, PD 166285, and MK 1775

a, Proportion of centriole amplification phases in DIV4 Cen2-GFP⁺ ependymal cultures stained for Sas6 and GT335 after 24 hours of incubation with DMSO (n=143 cells) or RO-3306 9μM (n=112 cells). **b**, Quantification of deuterosome number in DIV4 Cen2-GFP⁺ ependymal progenitors at the amplification phase and the growth phase treated for 24 hours with PD 166285. Cultures were immunostained for Sas-6 and GT335. Amplification phase: DMSO n=66 cells, PD166285 0.25μM n=16 cells, PD 166285 0.5μM n=8 cells; Growth phase: DMSO n=33 cells, PD166285 0.25μM n=10 cells, PD 166285 0.5μM n=3 cells; *P=0.0385, ***P=0.0009. **c**, Example of differentiating Cen2-GFP⁺ ependymal progenitors (DIV4) immunostained at the growth phase (Sas-6⁺, GT335⁺) after 24 hours of incubation with PD 166285 0.25μM. White boxes indicate magnified deuterosomes in the lower panel. **d**, Quantification of deuterosome number in DIV4 Cen2-GFP⁺ ependymal progenitors at the amplification phase and the growth phase treated for 24 hours with MK 1775. Cultures were immunostained for Sas-6 and GT335. Amplification phase: DMSO n=66 cells (same cells as in **b**), MK 1775 0.5μM n=18 cells, MK 1775 1μM n=20 cells; Growth phase: DMSO n=33 cells (same cells as in **b**), MK 1775 0.5μM n=18 cells, MK 1775 1μM n=6 cells. *P=0.0468. **e**, Quantification of the number of centrioles in DIV4 Cen2-GFP⁺ cells at the disengagement phase (Sas-6⁺) after 24-hour incubation with DMSO (n=49 cells; same cells as in Fig.3g), MK 1775 0.5μM (n=16 cells), and MK 1775 1μM (n=20 cells); ****P<0.0001. **f**, Proportion of centriole amplification phases in DIV4 Cen2-GFP⁺ ependymal progenitors stained for Sas6 and GT335 after 24 hours of incubation with DMSO (n=337 cells) MK 1775 0.5μM (n=172 cells), MK 1775 1μM (n=188 cells), PD166285 0.25μM (n=164 cells), PD 166285 0.5μM (n=180 cells). **g**, Representative example of differentiated Cen2-GFP⁺ ependymal cells (DIV4) immunostained for motile cilia (GT335⁺) after 24 hours of incubation with MK 1775 1μM (central panel) or PD 166285 0.5μM (right panel). Error bars represent mean ± s.d.; P

values derived from two-tailed Mann-Whitney U-tests (**b** and **e**) or from Kruskal-Wallis tests (**b** and **d**); scale bars, 2 μ m (**c**) and 5 μ m (**g**).

Extended Data Figure 6 proTAME-mediated pseudo-mitosis and analysis of centriole amplification phases after BI 2536 treatment

a, Centriole (Cen2-GFP⁺) and nucleus (H2B-RFP⁺) dynamics starting from the centriole growth phase until the multiple basal body stage in control (DMSO) and proTAME 12 μ M treated cells. Pseudo-mitosis with proTAME can be observed as of 14:00. **b**, proTAME-mediated chromosome condensation and nuclear membrane disintegration in pseudo-mitotic differentiating ependymal progenitors immunostained for Centrin, Histone H3 (pSer10) and nuclear pore complex proteins. White arrowheads indicate micronuclei. **c**, proTAME-mediated chromosome condensation and the different spindles observed in pseudo-mitotic differentiating ependymal progenitors immunostained for Centrin and tyrosinated tubulin. **d**, Representative centromere immunostainings of proTAME mediated pseudo-mitosis in differentiating Cen2-GFP⁺ ependymal progenitors and showing 40 centromeres. **c-d** DNA is counterstained with DAPI. **e**, Proportion of centriole amplification phases in differentiating Cen2-GFP⁺ ependymal cultures (DIV4) stained for Sas6 and GT335 after 24 hours of incubation with DMSO (n=150 cells), BI 2536 50nM (n=146 cells), and BI 2536 200nM (n=145 cells). Scale bars, 5 μ m.

Supplementary Video 1 Cen2-GFP live imaging in an ependymal progenitor from the centriole growth phase

Apical (upper panel) and profile (lower panel) views of the centriole amplification dynamic in a Cen2-GFP⁺ ependymal progenitor starting from the growth phase (100X magnification, $\Delta t=30$ minutes). Nucleus is shaded in blue in the upper panel. Sequence shows the perinuclear distribution of centrioles at the growth phase (00:00-01:30), their isotropic and synchronized

Figure 1

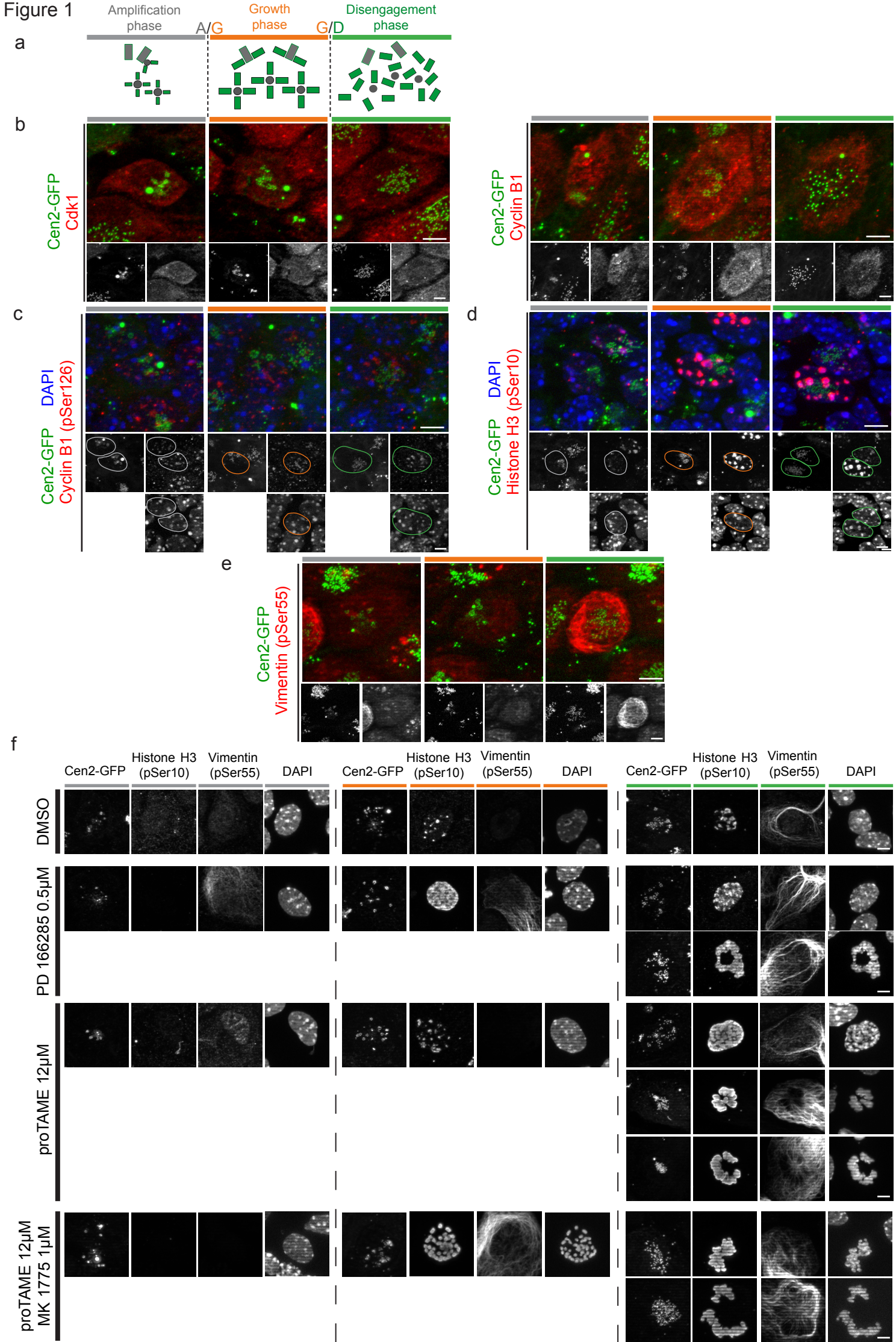


Figure 2

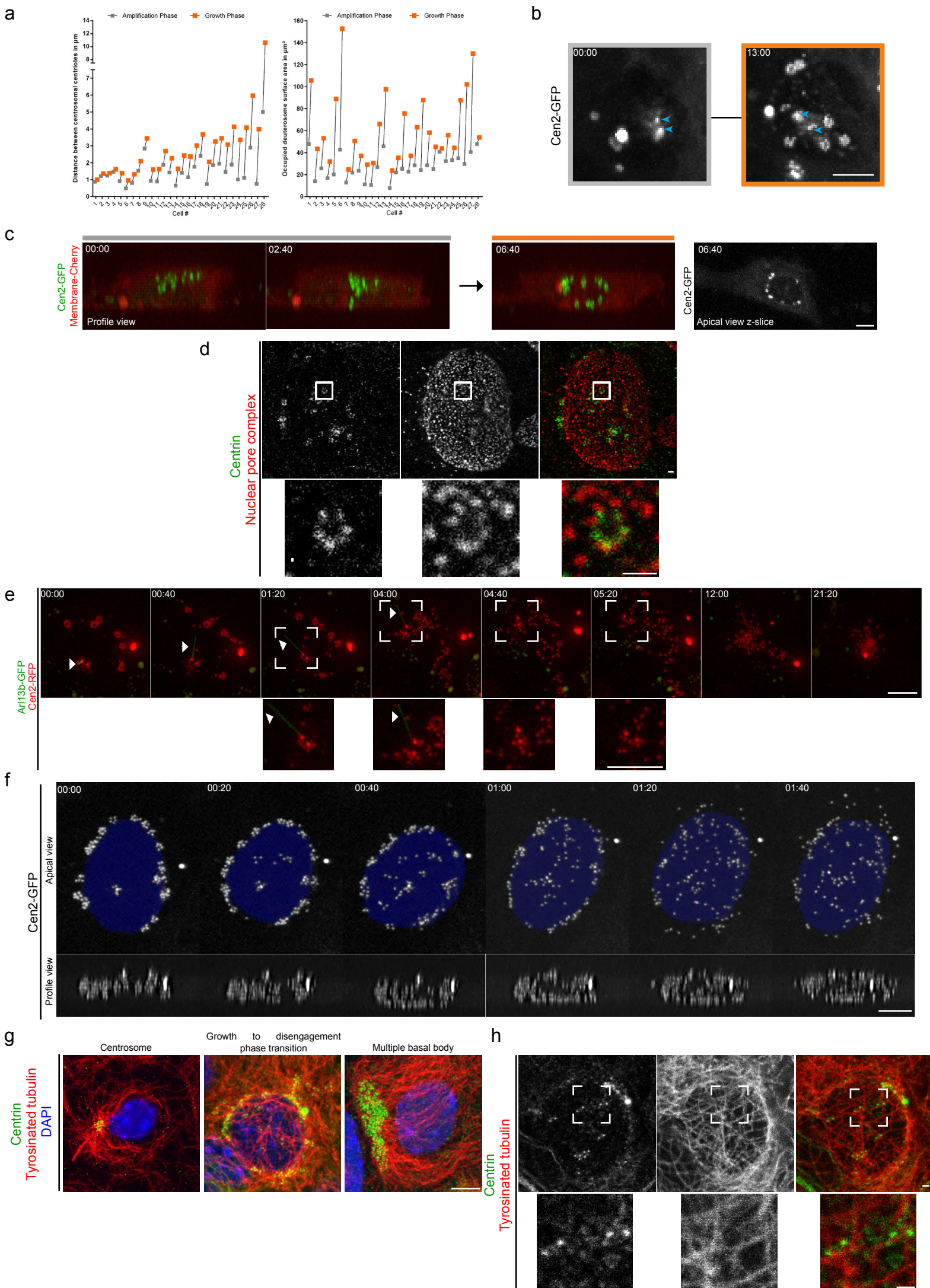


Figure 3

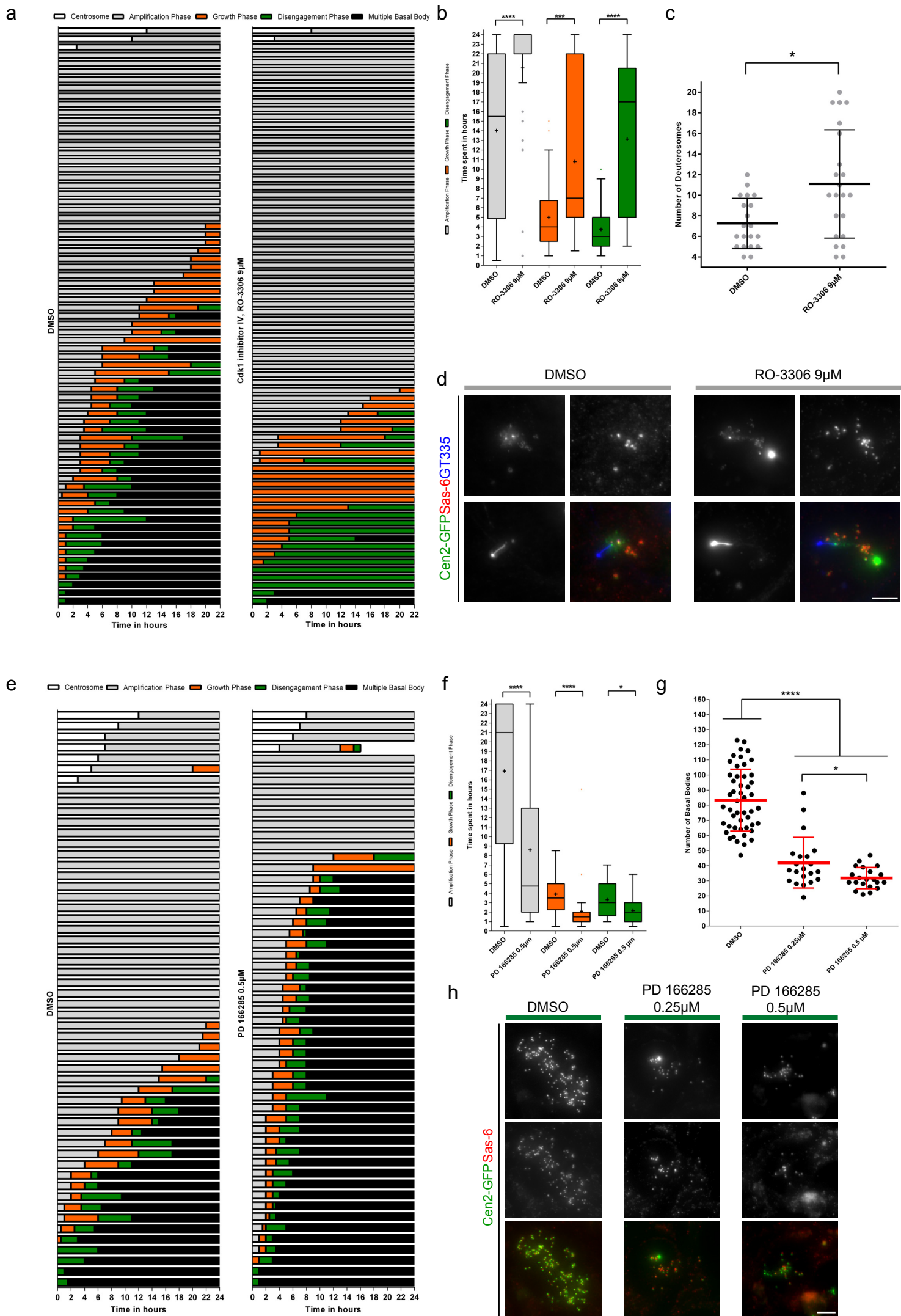


Figure 4

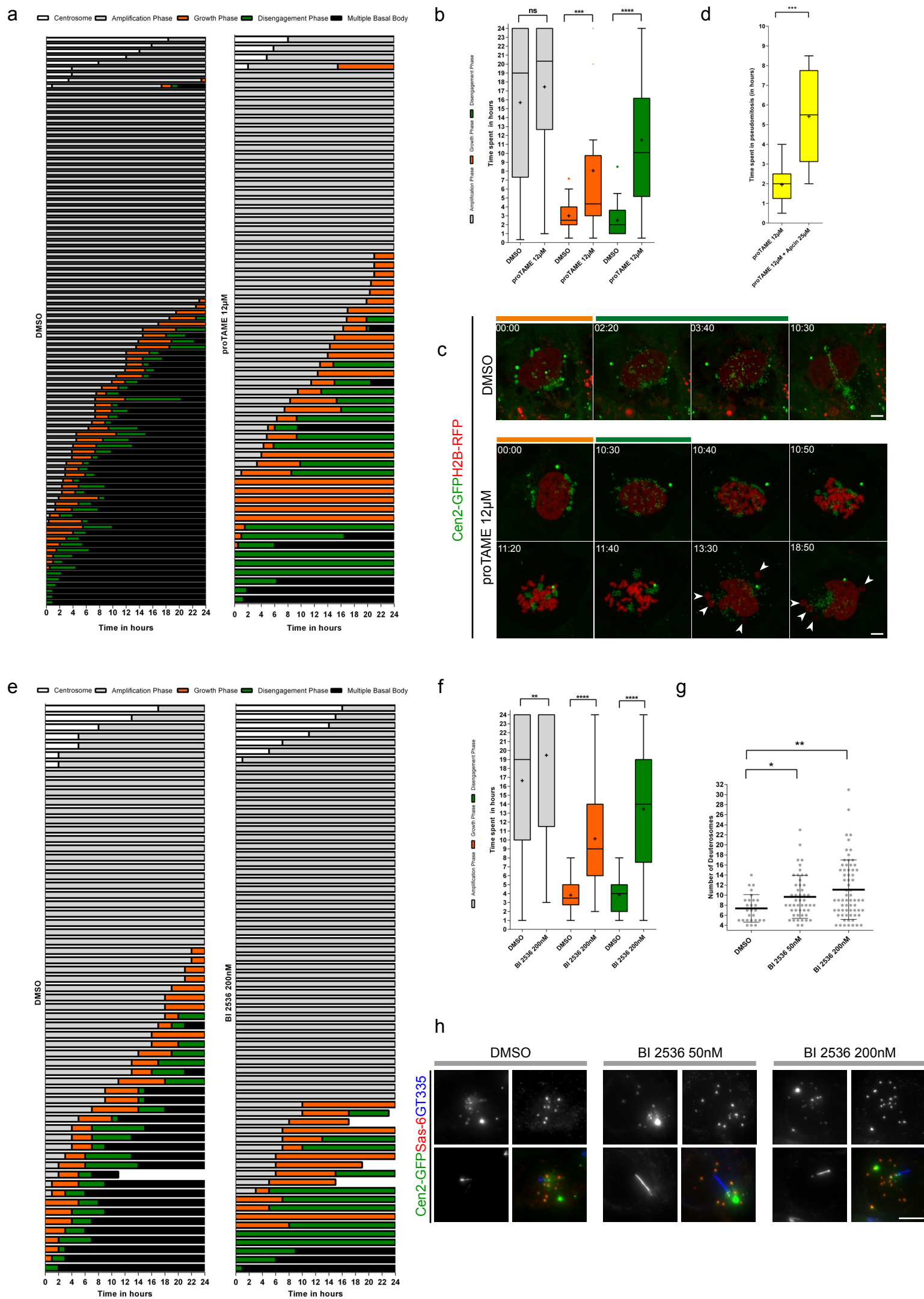
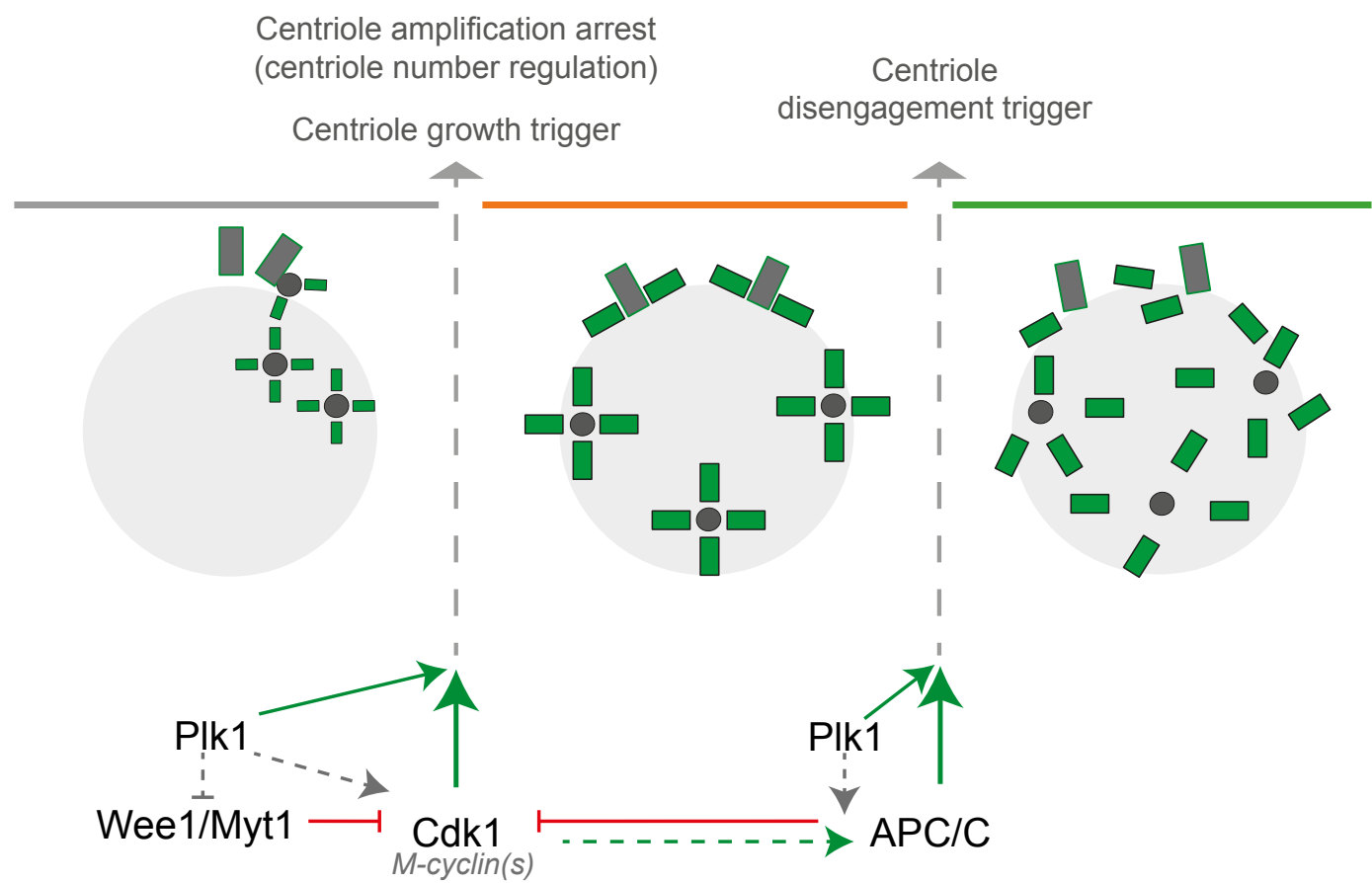
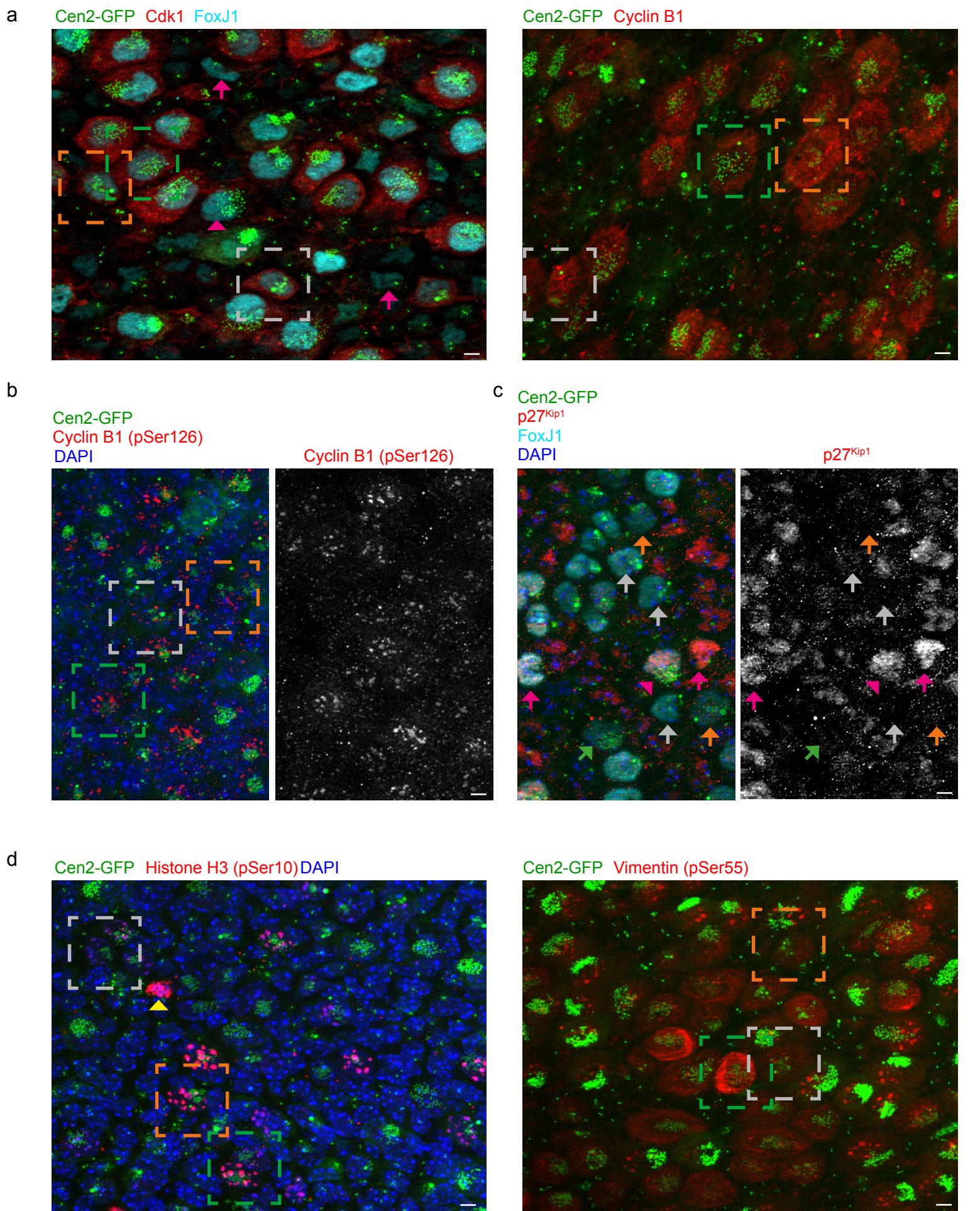
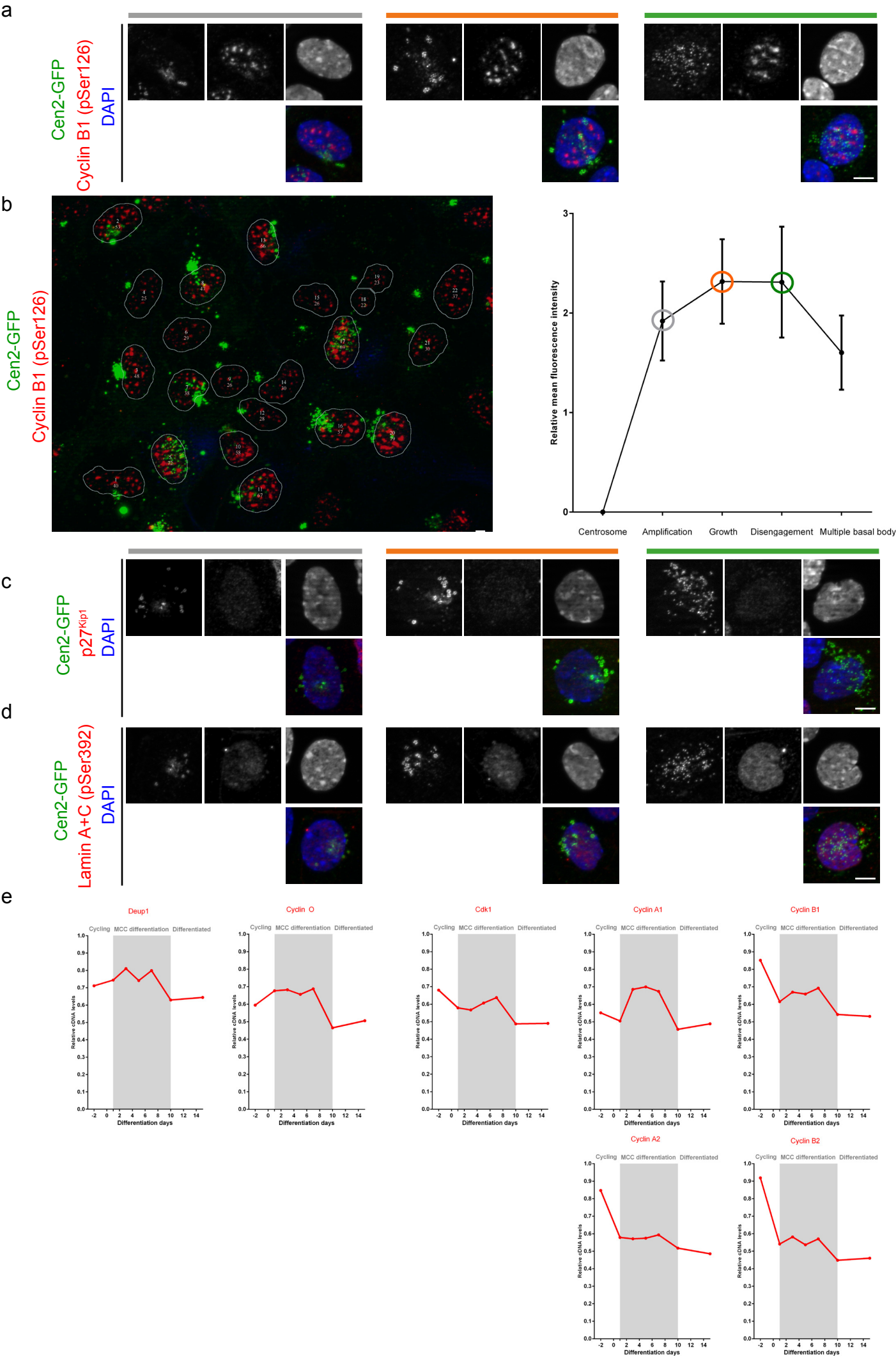


Figure 5

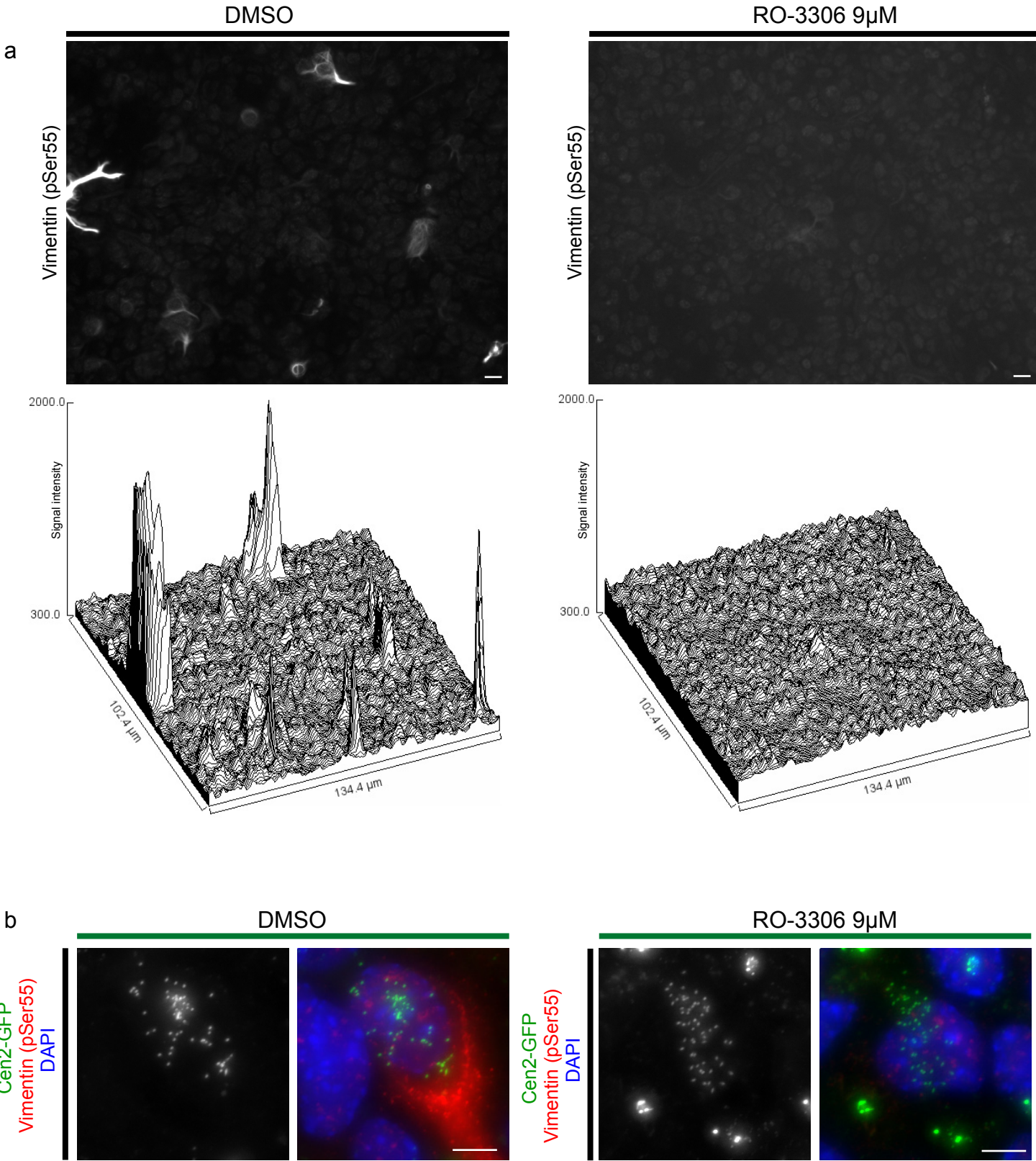




Extended Data Figure 2

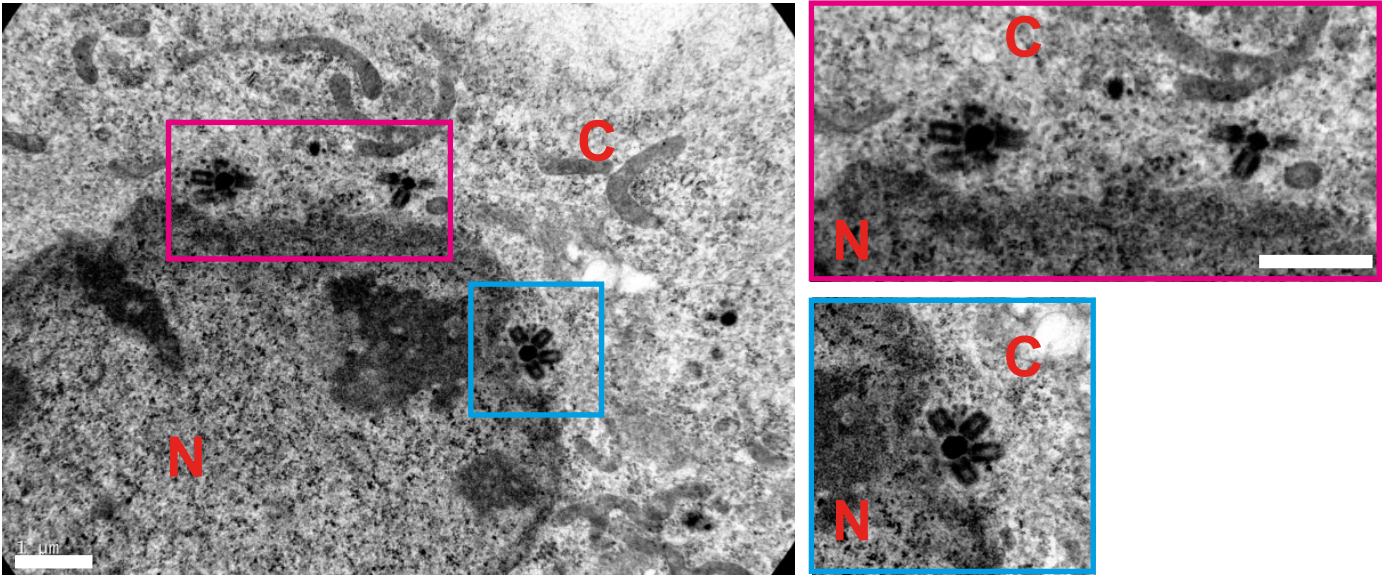


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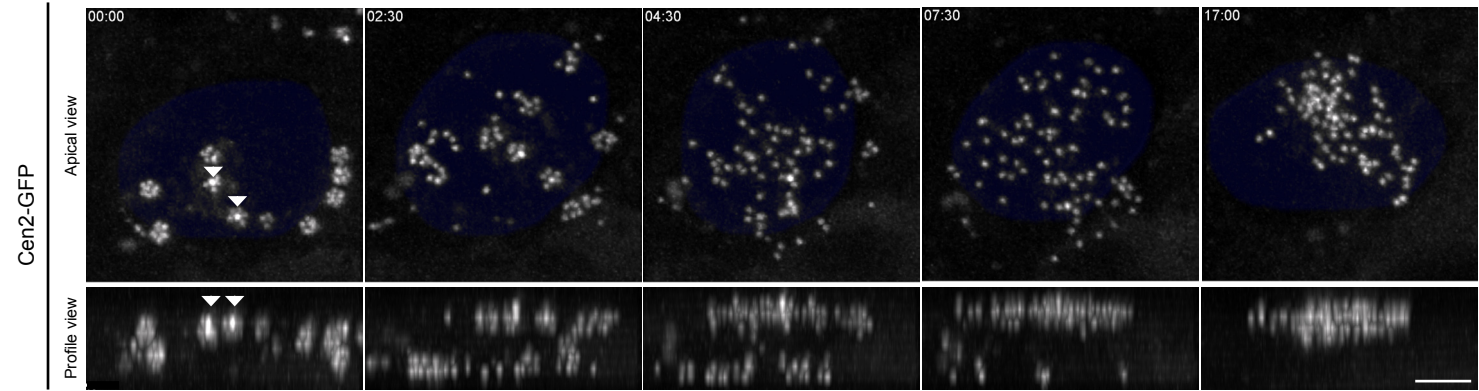


Extended Data Figure 4

a



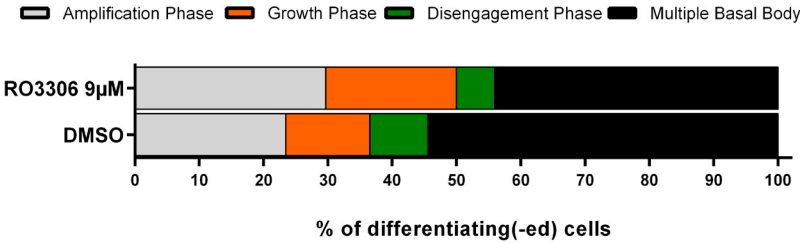
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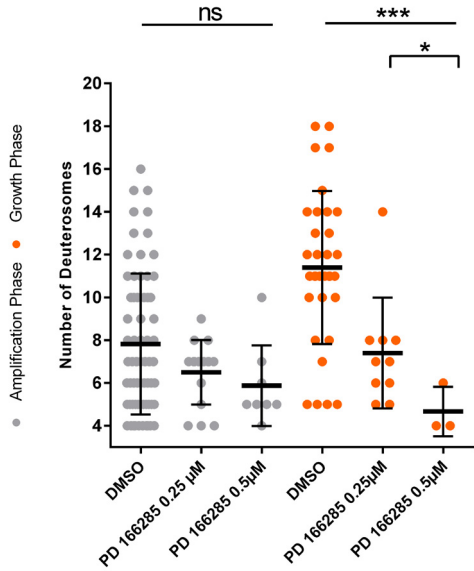
Extended Data Figure 5

Proportion of centriole amplification phases at DIV4 after 24hours of treatment

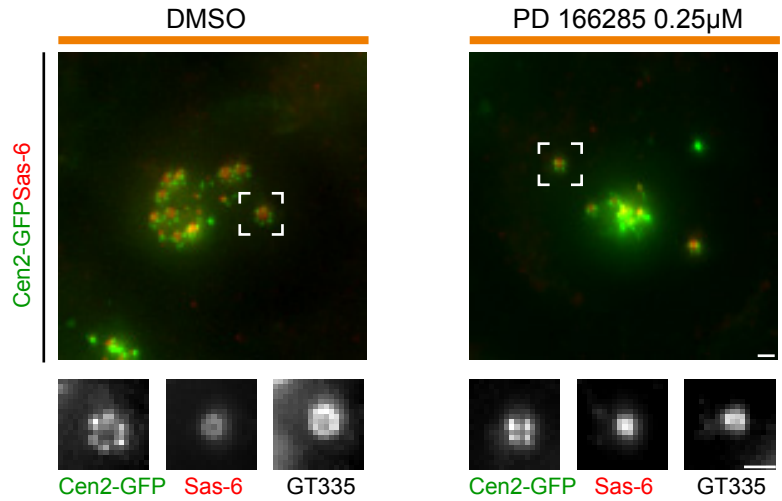
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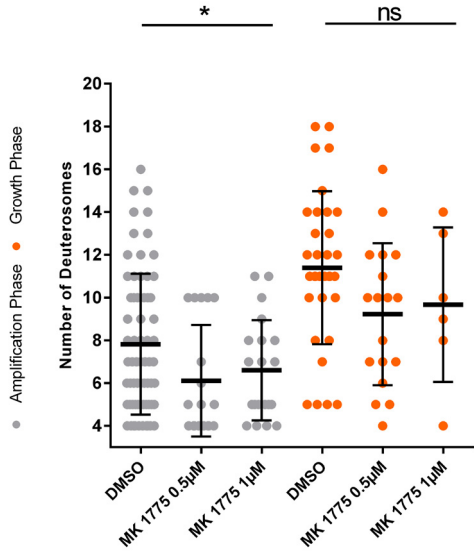
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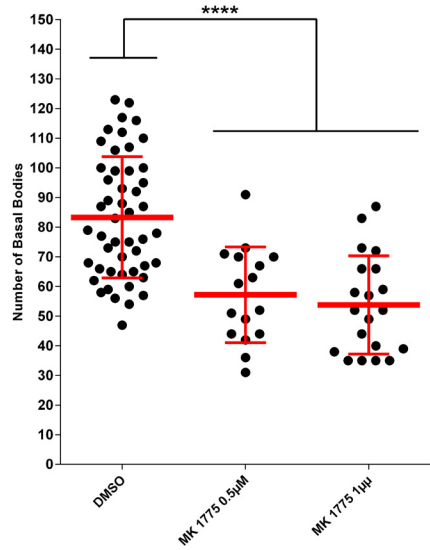
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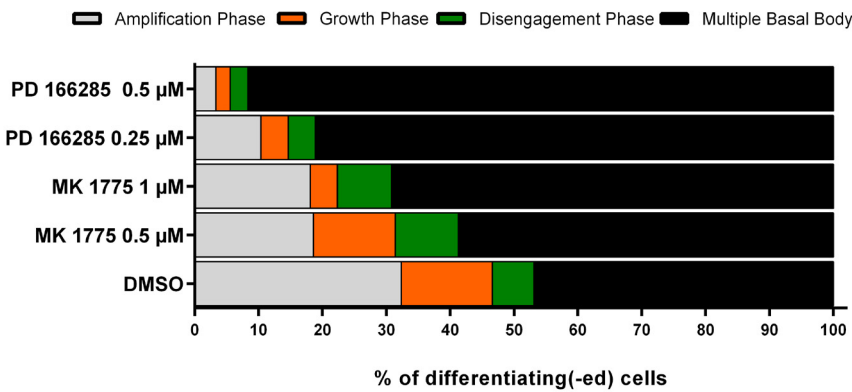


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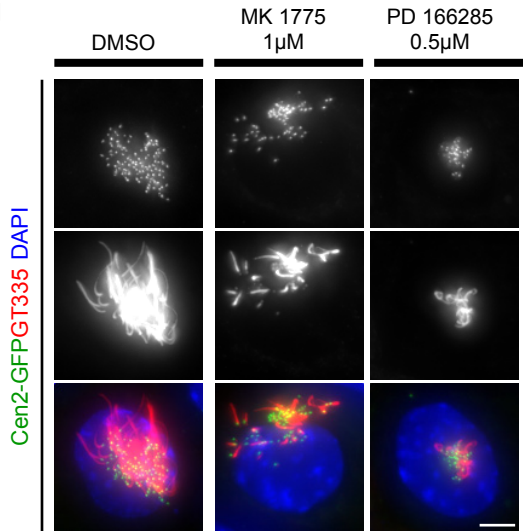


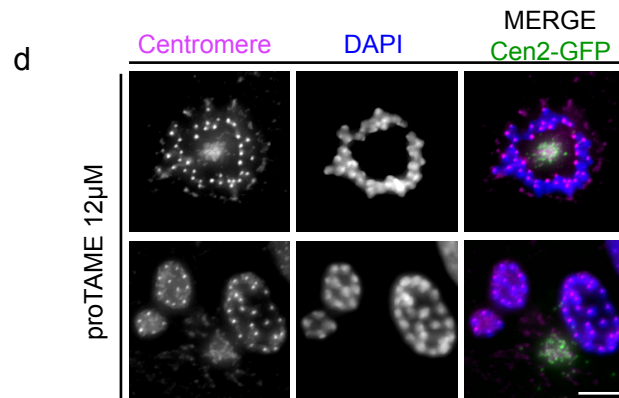
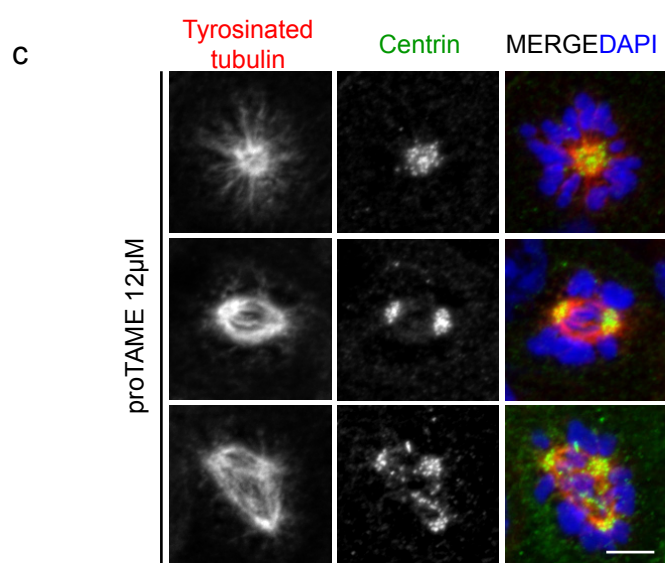
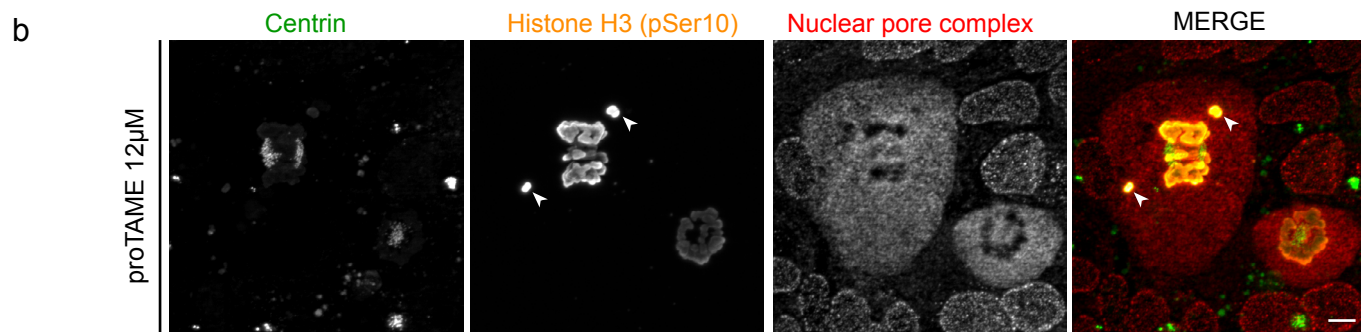
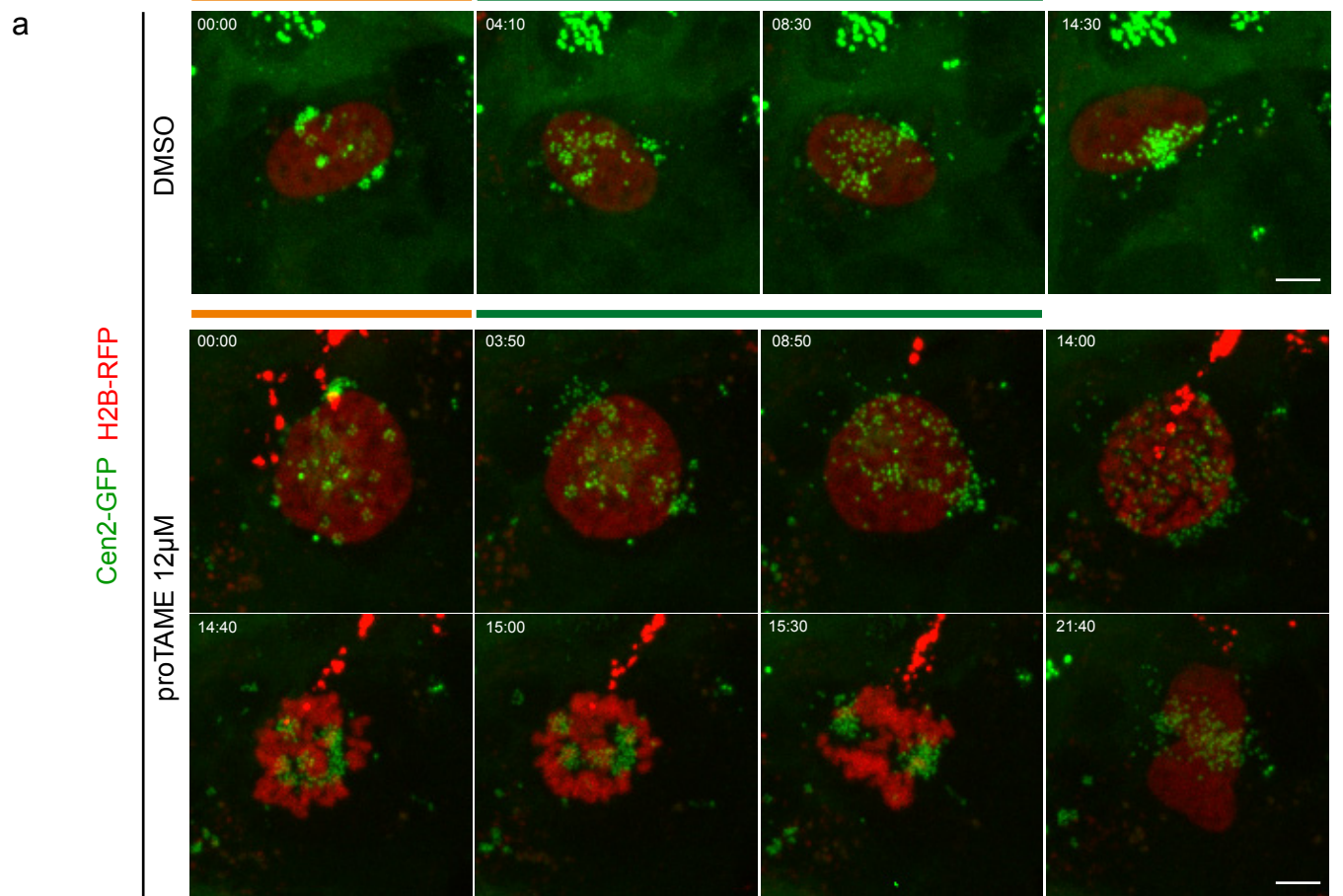
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Proportion of centriole amplification phases at DIV4 after 24hours of treatment

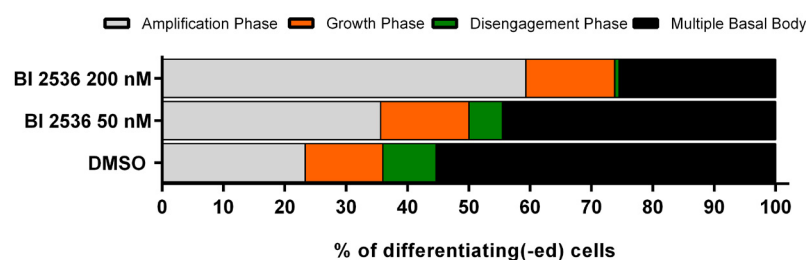


g





e Proportion of centriole amplification phases at DIV4 after 24hours of treatment



CONCLUDING REMARKS

This study provides the first dynamical description of physiological centriole amplification that leads to multiple motile cilia nucleation in ependymal cells. The descriptive work uncovered the cellular origin of all the amplified centrioles and highlighted their highly coordinated progression through two abrupt transitions that precede apical membrane docking (Article 1) ²⁰⁴. First, massive amplification of procentrioles in this subtype of epithelial cells originates from the centrosome, and surprisingly, it is the immature daughter centriole of the centrosome that serves as the nucleation point of more than 95% of the newly formed procentrioles. Then, when the final procentriole number is reached, centriole amplification stops, the whole procentriole population synchronously grows (amplification-to-growth transition) and subsequently, all centrioles simultaneously disengage from their growing platforms (growth-to-disengagement transition). Functional approaches point out the regulatory machinery of mitosis to be mechanistically responsible for the coordination of both transitions and for the consequent tuning of centriole number, control of centriole growth and individualization (Article 2). Although some experiments are still lacking to confirm certain interactions, our model suggests that Cdk1 triggers centriole amplification arrest/procentriole growth and eventually bring about APC/C activation thus triggering a timely disengagement of nearly mature centrioles for subsequent motile ciliation. The dampening of Cdk1 activity by its mitotic regulators Myt1, Wee1 and APC/C allows centriole amplification orchestration while avoiding mitotic commitment of multiciliated progenitors.

CENTRIOLE AMPLIFICATION AND CENTROSOMAL CENTRIOLES

Centrosome mediated centriole amplification refutes the long withstanding hypothesis that multiciliated progenitors are the only centriole-bearing cells assumed to generate centrioles *de novo* ^{193,194}. Inducing *de novo* centriole biogenesis in cycling cells is thought to lead to the formation of structurally aberrant centrioles ^{233,234}. It is therefore proposed that preexisting centrosomal centrioles template the formation of procentrioles during the centriole

duplication cycle^{234,235} (Fig. 1a). This templating mechanism may be the reason behind the nucleation of a hundred centrioles from a single centrosomal centriole in multiciliated cell progenitors (Fig. 1b). Laser ablation^{236–238} or pharmacologically-mediated depletion²³⁹ of the centrosomal centrioles in differentiating progenitors could clarify whether the physical presence of the daughter centriole is required for the amplification of procentrioles or their structural integrity.

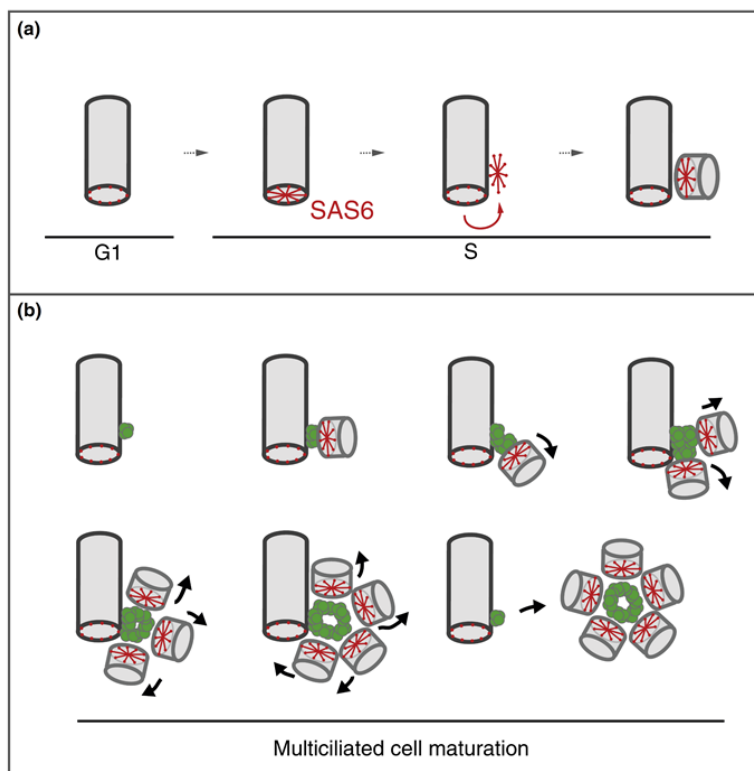


Figure 1 Proposed templating model for centriole biogenesis

a, During centriole duplication in cycling cells, SAS-6 dimers (red) are proposed to be recruited to the lumen of parental centrioles. This interaction is thought to give rise to the correct 9-fold cartwheel configuration. After assembly, the cartwheel relocates to the proximal portion of the centriole where it serves as a template for the biogenesis of the procentriole^{234,279}. **b**, Model for daughter centriole-mediated centriole amplification in multiciliated progenitors²⁰⁴. A similar mechanism to the one depicted in **a** may be responsible for the formation of up to several hundred procentrioles from one centrosomal centriole. Adapted from²⁸⁰.

During the amplification phase, deuterosomes form exclusively from the daughter centrosomal centriole suggesting that the mother centriole has reached a maturity state which inhibits deuterosome formation. The mother centriole is distinguished by two sets of appendages allowing the nucleation of a primary cilium and the anchoring of cytoplasmic microtubules (Article 1, Extended Data Fig. 6a-c)^{26,37,240}. In addition, the pericentriolar material (PCM) can be asymmetrically distributed on centrosomal centrioles²³⁸. The differential micro-environment provided by the two centrosomal centrioles may account for a

disparate activity of the regulatory kinases or a contrasting recruitment of the structural precursors, both necessary for centriole biogenesis. The Cep63 paralogue Deup1, which is able to trigger both “centriolar” and “deuterosome” pathways, is necessary for deuterosome formation and its capacity to form deuterosome structures depends on its concentration ¹⁹³. A differential control of Deup1 quantity at the mother and daughter centrioles could explain why deuterosomes form only from the daughter centriole whereas both centrosomal centrioles undergo the “centriolar” pathway. Perturbing the asymmetric accumulation of Deup1 by fusing it with PACT ²⁴¹, a centrosome localization sequence, may clarify whether the mother centriole mature vicinity is restrictive to deuterosome formation.

Consistent with the existence of a permissive micro-environment provided by the daughter centriole, we observed that Cep164 ⁴¹, Odf2 ⁴⁰, and Ninein ²⁴² invariably localize to the daughter centriole after the formation of the last deuterosome (data not shown). At this amplification-to-growth phase transition, mother and daughter centrioles are not distinguishable anymore by classic molecular markers thus suggesting that the daughter centriole has reached a state of maturity similar to that of the mother centriole. One might hypothesize that the immaturity of the daughter centriole renders it permissive to centriole amplification and that loss of generational mother/daughter appendage asymmetry may contribute to the halt in centriole amplification. Impeding daughter centriole maturation using a siRNA approach or forcing the maturation of the daughter centriole by overexpressing Tau tubulin kinase 2 (TTBK2), a Cep164 recruiter ⁴², may elucidate whether the maturity state of the daughter centriole determines its competency to drive massive centriole amplification.

In Article 2, we show that premature activation of Cdk1 halts the amplification process and triggers procentriole growth. In parallel, we show that inhibition of Cdk1 or Plk1 prolongs amplification and delays growth. Furthermore, our preliminary results suggest that with pharmacological modulations of Cdk1 or Plk1 activities, daughter centriole maturation is still

temporally correlated with procentriole amplification arrest and procentriole growth (data not shown). It therefore seems that the three events, i.e. daughter centriole maturation, procentriole amplification arrest and procentriole growth, are always concurrent. During centriole duplication in cycling cells, Plk1 notably acts on two generations of centrioles: Plk1 triggers both pre-existing daughter centriole maturation and newly formed procentriole growth²⁰⁰. Continuing our cell cycle analogy, it may be that Plk1 in multiciliated cell progenitors also triggers both daughter centriole maturation and procentriole growth explaining why the two events are synchronous. Our results with RO-3306, PD 166285, and MK 1775 suggest that Plk1 functions through Cdk1 activation to proceed.

Cdk1 in cycling cells is also known to prevent centriole overduplication by displacing Stil and preventing Stil-Plk4 interactions^{78,96}. Hypothetically, the same Cdk1-mediated action on Stil could stop the amplification phase in multiciliated cells. In this case, procentriole amplification arrest could be independent from daughter centriole maturation and procentriole growth. The three events may be synchronous due to common upstream regulators that include Plk1 and Cdk1. In addition, procentriole growth trigger from the daughter centriole (centriolar pathway) may participate in the amplification arrest by engaging the daughter centriole, a process known to inhibit further centriole nucleation⁶¹.

Substantial work still needs to be accomplished to highlight the molecular cascade downstream of Plk1 and Cdk1 that drives the tight coordination between procentriole number control and subsequent centriole growth.

CENTRIOLE AMPLIFICATION DYNAMIC AND POLARITY

Mature multiciliated ependymal cells generate directional cerebrospinal fluid flow in mammalian brain ventricles. Amplified centrioles in ependymal progenitors become basal bodies by acquiring appendages and by docking at the apical membrane to nucleate “9+2” motile cilia¹⁹¹. Previous work from our laboratory has shown that this docking is followed by

the accurate alignment of basal bodies in response to a coupling between the Wnt/PCP pathway and hydrodynamic forces created by neighbouring ciliary beating ¹⁹¹. The precise orientation is attained within a limited developmental time frame and thus requires synchronized basal body docking and ciliary growth. The resulting basal body alignment, known as the rotational polarity, is necessary for an efficient cerebrospinal fluid outflow ¹⁹¹ and for the creation of local currents suggested to control signalling molecule dispersion ¹³⁴. Ependymal multiciliated cells have an additional unique “translational” polarity that is due to the anterior migration of docked centriole patches with respect to cerebrospinal fluid flow (CSF). Mature ependymal ciliary tufts are therefore anteriorly positioned in bundles ^{122,159,243} (see illustration of cilia patches in Introduction Fig. 12b). Even though the role of this translational polarity remains speculative with proposed roles in neurogenesis ¹⁵⁹, it was clearly shown to depend on the primary cilium of ependymal progenitors ¹²², and therefore suggests a role for the centrosome in the migration of the future basal body patch.

At the centriole growth-to-disengagement phase transition, a potential contribution of the microtubules and the nucleus is highlighted (Article 2, Fig. 2). The microtubule network reshaping may contribute to centrosomal centriole and deuterosome dispersion around the nucleus after Cdk1 and Plk1 activation, similarly to described mechanisms for centrosomes in cycling cells ^{49,77}. As for the nucleus, nuclear pore complex proteins in cycling cells are involved in nuclear tethering of the centrosomes at mitosis onset ²⁴⁹, a process coordinated by Cdk1-mediated phosphorylations ²⁵⁰ and important for optimal centrosome positioning during spindle pole formation ⁷⁶. Our additional videomicroscopy data show that all deuterosomes come in nuclear contact prior to centriole disengagement (n=50/50 deuterosomes from 3 cells; graph not shown). In addition, once disengaged from their platforms, centrioles migrate isotropically along the nuclear membrane before distancing away from the nucleus into the cytoplasm and migrating to the apical membrane (data not shown but visible in Article 2, Fig.

2f). Centrioles could therefore be attached to the nuclear membrane before their individualization. Tight proximity of centrioles with nuclear pore complex (NPC) proteins at the growth-to-disengagement phase transition is indeed observed by immunostainings (Article 2, Figure 2c-d and i). Altering centriole tethering to the nuclear envelope by depleting Nup133, a protein critical for centrosome positioning in nuclear contact at mitotic entry ⁷⁶, or Nesprins, mediators of centrosome-nucleus coupling during mitosis ⁹⁰, may enable to test whether positioning deuterosome/procentriole around the nuclear membrane before centriole disengagement is a pre-requisite for the disengagement itself or for the positioning of centrioles for a subsequent optimal polarized migration.

Before the migration phase, centrioles indeed do colocalize with tyrosinated microtubules suggesting that microtubules may contribute to, or be the driving force of, migration of centrioles towards the apical membrane (Article 2, Fig. 2g-h). Consistently, electron microscopy studies of developing quail oviduct multiciliated cells in presence of cytoskeletal modulators show that both actin and microtubules are required for late stages of centriole migration as well as ciliation ²⁴⁴. In addition, we observe that pre-existing centrosomal centrioles are always apically localized until the end of centriole migration (Article 2; Extended Data Fig.4b, white arrowheads), and that the whole population of newly disengaged centrioles seems to migrate in their direction. To test the putative convergence of newly formed centrioles towards the pre-existing centrosome, we analysed the directionality of multi-centriole migration from the late disengagement phase to the end of apical migration. Automated global centriole segmentation presents a distribution shift of centrioles towards the centrosome vicinity (Fig. 2a-b, 235 centrioles from 3 cells). In addition, manual single centriole 3D tracking shows a time-correlated decrease of centriole-centrosome distance, suggesting that newly formed centrioles collectively converge towards the centrosome region during their apical migration (Fig.2 c-d, 12 tracked centrioles in one cell). Together with the

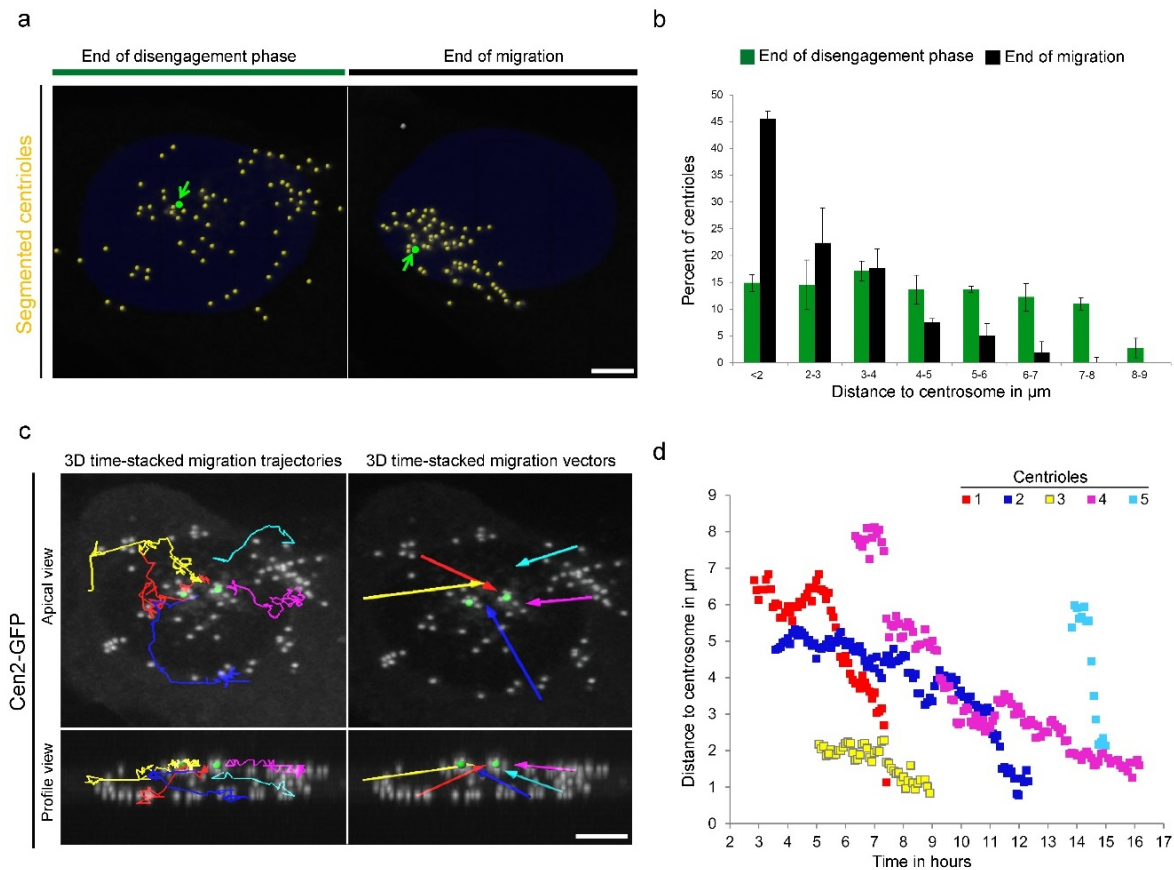


Figure 2 Disengaged centrioles migrate apically and converge towards the preexisting-centrosome region

a, Images illustrating 3D Imaris software automated segmentation of Cen2-GFP⁺ centrioles (yellow spheres) at the end of the disengagement phase and at the end of their apical migration. Green arrow indicates a centrosomal centriole (green sphere). Signal threshold filter was set at a particle diameter of 0.25 μm . **b**, Histogram of centriole proportions comparing 3D centriole distance relative to the centrosome at the disengagement phase (green bars) and at the end of their apical migration (black bars); Note the heterogeneous distribution of centrioles relative to the centrosome at the end of the disengagement phase, and that 65% of centrioles after apical migration are located < 3 μm away from the centrosome; $n=235$ segmented centrioles from 3 cells, error bars represent percentages $\pm$ sd, P -value <0.0001 derived from a one-tailed Chi-square test. **c**, Apical and profile view of 3D Cen2-GFP⁺ Centriole time-stacked trajectories (left panel) and mean vector directionalities (right panel) from the disengagement phase ($t=0$ hours) to the end of apical migration ($t=16$ hours); $n=12$ centrioles tracked manually during 194 time points with a Δt of 5 minutes; 5 centriole trajectories are represented on snapshots from $t=0$ hours, green spheres mark the centrosomal centrioles. **d**, Time-correlated 3D displacements of 5 tracked centrioles from **c** relative to the centrosomal centrioles during the centriole migration phase. Scale bars, 2 μm .

documented role of microtubules in centriole migration^{244,245} and the primary cilium resorption at the growth-to-disengagement phase transition (Article 2, Fig.2e), the convergence of newly formed centrioles towards the centrosome suggests a role for the centrosome as a microtubule organizing center (MTOC) during apical centriole migration. Analyzing centriole disengagement and migration in cells depleted for Ninein, a component of centrosomal sub-distal appendages linked to microtubule anchoring and nucleation²⁴², or

in cells differentiating on micropatterns that control cellular geometry and centrosome position ^{246,247} may clarify if newly formed centrioles, as chromosomes in the context of cycling cells at the anaphase-to-telophase transition, are pulled towards the pre-existing centrosome. Moreover, tyrosinated microtubules are the dynamic subtype which are recruited to the spindle for correct positioning ^{214,248}. Since microtubule plus-end tracking proteins such as Clip170 bind specifically to tyrosinated tubulin ²⁴⁸, filming double transfected Centrin2-GFP/Clip170-RFP maturing ependymal cells could further uncover microtubule implications in the final stages of the centriole amplification dynamic.

Collectively, these descriptive and functional approaches would further complete the picture of the cellular and molecular mechanisms that bridge initial centrosome/primary cilium position in the ependymal progenitor to the establishment of multiciliated cell and tissue polarity ^{122,136}.

CENTRIOLE AMPLIFICATION COORDINATION BY MITOSIS ENZYMES

The implication of mitosis promoters, Cdk1 and Plk1, or inhibitors, Wee1 and Myt1, in the regulation of the two transitions observed during the centriole amplification dynamic raises the question whether all essential kinases and phosphatases of the mitosis regulatory network are active. In the cycling cell context, the mitosis network of regulators is complex and includes numerous components in the likes of the Aurora family of kinases or the Cdc25 family of phosphatases. For instance, Aurora A contributes to mitosis progression by phosphoactivating Plk1 ^{251,252} and Cdc25b ²², a positive regulator of Cdk1-Cyclin B1 complex, in addition to promoting centrosome separation ³⁴. Aurora B kinase also promotes M-phase entry by condensing chromosomes via Histone 3 Serine 10 phosphorylation ^{220,222,253} as well as M-Phase exit in a Plk1 dependent-manner before proceeding with anaphase ^{254,255}. Consistently, treating differentiating cultures of ependymal progenitors with an Aurora B inhibitor ²⁵⁶ abolishes Histone 3 Serine 10 phosphorylation (Fig. 3a). As for the centriole

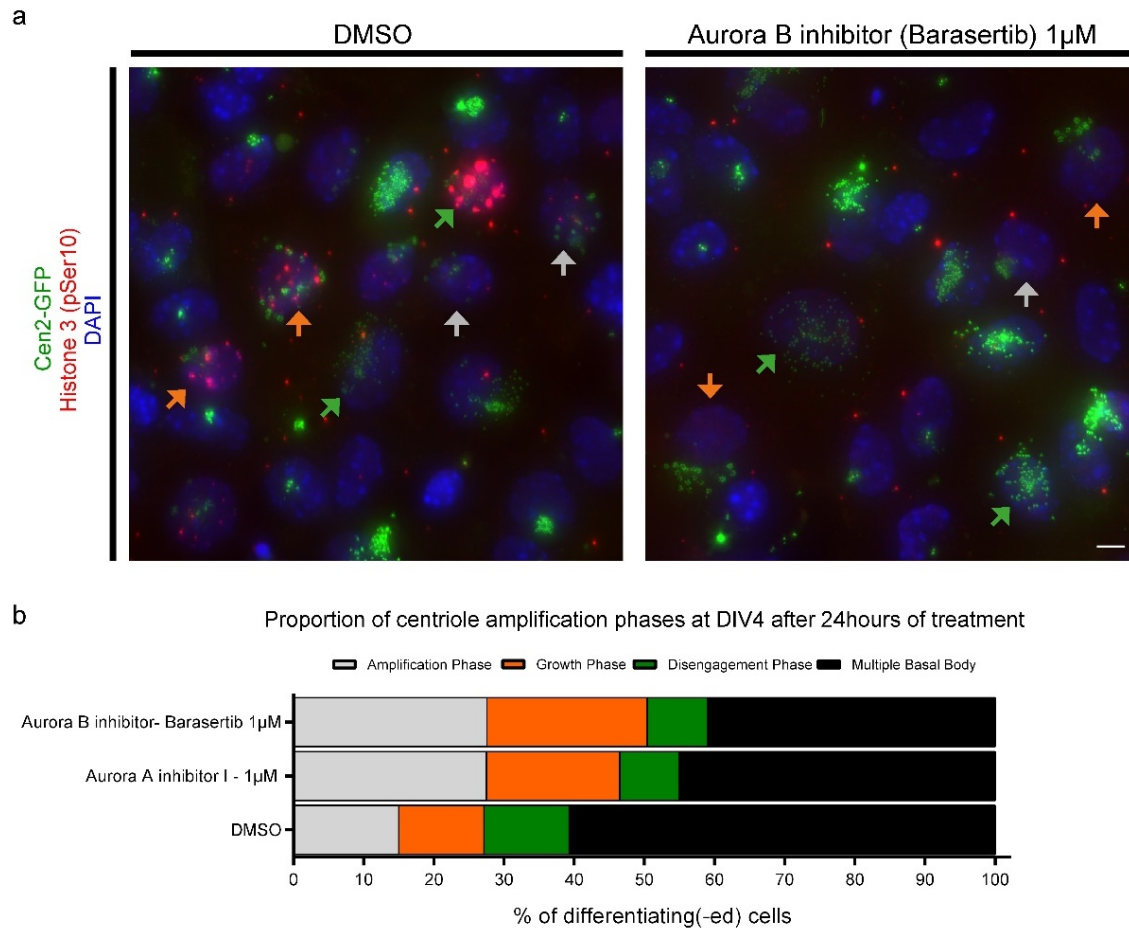


Figure 3 Pharmacological inhibition of Aurora A and Aurora B kinases

a, Representative Histone 3 (pSer10) immunostainings of DIV4 ependymal cultures after 24-hour incubations with DMSO (left panel) or Aurora B inhibitor Barasertib (1 μ M, right panel). Grey arrows indicate cells at the amplification phase; orange arrows indicate cells at the growth phase; green arrows indicate cells at the disengagement phase. **b**, Proportion of centriole amplification phases in DIV4 Cen2-GFP⁺ ependymal cultures stained for Sas6 and GT335 after 24 hours of incubation with DMSO (n=140 cells), Aurora A Inhibitor I 1 μ M (n=142 cells), or Aurora B inhibitor Barasertib 1 μ M (n=127 cells); quantification from a single experiment. Scale bar, 5 μ m.

amplification dynamic, both Aurora A²⁵⁷ and Aurora B inhibition results in diminished proportions of multiciliated cells in favor of cells at the amplification and growth phases (Fig. 3b), suggesting that they are required for the initiation of both centriole amplification phase transitions. Our preliminary results covering Aurora A and Aurora B kinases further strengthen the hypothesis that all mitotic kinases and phosphatases may be activated for the progression of the centriole amplification dynamic.

The growth-to-disengagement phase transition is made possible by the activity of the Cdk1-APC/C axis. The delay between Cdk1 activation at the amplification-to-growth phase

transition and maximal APC/C activity seems to correspond to the centriole growth phase (3 ± 1.5 hours; $n=49$ cells), a duration significantly longer than what is proposed to occur in cycling cells²⁵⁸. The increased delay can be explained by the continuous moderation of Cdk1 activity that avoids mitotic commitment and consequently stretches the duration of the centriole growth phase. Our results, however, are not conclusive concerning APC/C's coactivators or centriolar substrates⁹² during the centriole amplification phase transitions. Although Cyclin B1 stabilization in cycling cells in presence of the APC/C modulator, proTAME, is dependent on APC/C^{Cdc20}²¹⁵ and is consistent with our pseudo-mitosis rescue results (Article 2, text associated with Fig. 1d), proTAME was initially shown to affect APC/C coactivation by both Cdh1 and Cdc20²¹². The dynamism of p27^{kip1} staining profiles in differentiating ependymal progenitors (Article 2, Extended Data Fig.1c) suggests that both APC/C coactivators, Cdh1 and Cdc20, are present^{198,259,260}. APC/C-Cdh1 may be the predominant complex in early stages of differentiation when progenitors, positive for both p27^{kip1} and Foxj1 nuclear stainings, have not begun to amplify procentrioles. During the amplification-to-growth and growth-to-disengagement phase transitions, the decrease in p27^{kip1} levels, mitotic kinase activity¹⁹⁸, primary cilium resorption⁶⁸, and the synergistic effect of Apcin+proTAME on pseudo-mitosis exit²¹⁶ suggest that Cdc20 is the cofactor of APC/C allowing the regulation of centriole disengagement. After the disengagement and migration of centrioles, p27^{kip1} nuclear staining reappears and parallels the loss of Sas6 from the docking centrioles (Article 1, Extended Data Fig. 8h and 9k; Article 2, Extended Data Fig.1c). Cdh1-mediated APC/C activity, as during mitosis exit of cycling cells^{99,261}, may be responsible for these events occurring in the final steps of the dynamic that precede motile ciliation and marks the beginning of a "true" G0 state of multiciliated cells. In addition to Plk1 activity, Separase degrades Cohesin to induce centriole disengagement in mitosis of cycling cells^{73,91}, Securin may therefore be the candidate substrate that APC/C targets for the

initiation of a potentially Separase-dependent centriole disengagement phase in ependymal progenitors. Together with the assessment of Securin's localization coupled to its potential accumulation in presence of proTAME, the expression of a non-degradable form of Securin^{262,263} and Separase depletion may provide insights into the mechanisms of how APC/C triggers the growth-to-disengagement phase transition in differentiating ependymal cells.

To conclude, the results stemming from this PhD project provide a new paradigm for future molecular dissection of terminal multiciliated cell differentiation or other, somehow analogous, physiological or pathological developmental contexts. Differentiating ependymal progenitors seem to employ a variant of mitosis that is continuously alleviated in order to handle amplified centrioles with all the mitosis tools normally available in their cycling progenitors. This suggests that a conserved network of mitosis coordinators is shared by cycling and differentiating progenitors, yet seemingly differentially regulated both at transcriptional and post-translational levels. The use of elements of the mitosis regulatory network has been reported in developmental contexts such as neuronal apoptosis in the developing brain^{264,265}, or in other terminal non-proliferative differentiation contexts that include monociliated cochlear hair cells²⁶⁶ and lens fiber cells²⁶⁷. In lens fiber cells for instance, progenitors degrade the nucleus and its DNA content for the creation of an organelle free zone. The degradation requires the activity of the tissue-specific DLAD (lysosomal nuclease DNase II β) and is dependent on transient Cdk1-CyclinA/B activity²⁶⁷⁻²⁷⁰ that renders the DNA accessible via nuclear membrane disintegration. Consistently, studies report destabilization of nuclear p27^{kip1} for Cdk1 activation along with Cdk1-mediated Histone 1 and Lamins A/C phosphorylation in this specific context^{267,269,270}. However, it remains unclear if other components of the minimal mitosis regulatory network are expressed and activated in differentiating lens fiber cells. In the case of differentiating ependymal progenitors, they do

seem to conserve the whole mitotic network, yet dampen it, to mimic, all along their differentiation, the step-wise regulation of centriole biogenesis in cycling cells in order to tune centriole number and to timely trigger centriole growth and disengagement. Hence, in spite of the large number of centrioles produced in multiciliated cells, the events outlining centriole biogenesis on the scale of a single centriole are comparable to the ones that define the centriole duplication cycle in cycling cells. The redeployment of such a robust network of cell cycle controllers for multiciliated cell differentiation may present an immense economy for the cell given the complexity of centriole biogenesis regulation.

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Abstract in French

Chez les mammifères, les cellules multiciliées jouent un rôle essentiel dans la propulsion des fluides physiologiques. Leur dysfonctionnement peut être létale et provoque des maladies chroniques. Contrairement à la plupart des cellules de mammifères qui possèdent un centrosome composé de deux centrioles, les cellules multiciliées possèdent une centaine de structures centriolaires qui servent de base à la nucléation des cils motiles. Les mécanismes d'amplification de centrioles ou de régulation du nombre de centrioles dans ce type cellulaire étaient jusque-là inconnus. Les centrioles nouvellement formés étaient considérés comme apparaissant « *de novo* », indépendamment des centrioles préexistants. En première partie de ce projet de thèse, une approche de vidéomicroscopie et de microscopie de super-résolution corrélative nous a permis de déterminer que tous les pro-centrioles sont générés à partir du centrosome préexistant. Nous démontrons que le centriole fils du centrosome est le site principal de nucléation de 95% de centrioles nouvellement formés dans les cellules multiciliées. Ces résultats réfutent par conséquent l'origine « *de novo* » des centrioles dans ce type cellulaire. Puis, nos résultats révèlent l'activation du complexe Cdk1-CyclinB1 avec son réseau de régulateurs mitotiques pendant la dynamique d'amplification de centrioles. En couplant la vidéomicroscopie avec l'inhibition pharmacologique des régulateurs mitotiques, nous montrons que la machinerie mitotique orchestre la progression spatio-temporelle de la dynamique centriolaire dans ces cellules post-mitotiques et en phase terminale de différenciation. L'amortissement de l'activité de Cdk1 empêche la rentrée en mitose tout en permettant la coordination du nombre de centrioles, leur croissance, et leur désengagement par des transitions phasiques nécessaires à la nucléation de cils motiles. Ces résultats fournissent un nouveau paradigme pour mieux comprendre la différenciation des cellules multiciliées, les ciliopathies, ainsi que l'amplification centriolaire pathologique associée avec le cancer et la microcéphalie.

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Amplification des centrioles

Quand le centriole fils du centrosome prend le contrôle

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► Chez les mammifères, les épithéliums multiciliés sont essentiels à la propulsion de certains fluides physiologiques. Dans le cerveau, les cils motiles des cellules épendymaires qui bordent les ventricules permettent l'écoulement du liquide céphalo-rachidien ; un dysfonctionnement de leur battement provoque des hydrocéphalies létales. Le battement orienté d'assemblées de cils motiles est également nécessaire dans d'autres organes comme l'épithélium respiratoire et l'oviducte, où il permet l'évacuation de mucus et le transport de l'œuf vers l'utérus, respectivement. Dans ces organes, des défauts de motilité ciliaire peuvent mener au développement de bronchiectasies, de grossesses extra-utérines et/ou à des problèmes d'infertilité [1, 2]. Contrairement à la plupart des cellules de mammifères qui possèdent un centrosome composé de deux centrioles, les cellules multiciliées possèdent une centaine de structures centriolaires, appelées corps basaux, qui sont ancrés à la membrane plasmique et servent de base à la nucléation des cils motiles¹. Un des événements majeurs de la différenciation d'une cellule progénitrice en cellule multiciliée est donc la biogenèse massive de centrioles (Figure 1A). Deux voies parallèles d'amplification centriolaire ont été décrites. Dans la voie « ac centriolaire », qui est majoritaire, les centrioles se forment sur des structures sphériques denses aux électrons, appelées deuterosomes. Dans cette voie, les

centrioles sont considérés comme apparaissant « *de novo* », indépendamment des centrioles préexistants. Dans la voie « centriolaire », qui est minoritaire, les centrioles se forment directement à partir des centrioles du centrosome. Depuis la description de ces deux voies dans des études de microscopie électronique datant des années 1970 [3], l'origine des deuterosomes ainsi que l'interaction entre ces deux voies étaient restées inconnues.

L'amplification centriolaire débute dans la région centrosomale

Afin de mettre au jour la dynamique d'amplification centriolaire, notre équipe a mis au point un système de culture permettant de visualiser en temps réel et en trois dimensions la différenciation de cellules souches neurales en cellules épendymaires multiciliées. L'observation de progéniteurs provenant de souris transgéniques exprimant la protéine centriolaire centrine 2 fusionnée à la GFP (Cen2-GFP, GFP : *green fluorescent protein*) a révélé un processus débutant dans la région centrosomale et marqué par deux étapes (Figure 1B). Au cours de la première étape, une dizaine de foyers de précurseurs Cen2-GFP, ou « halos », apparaissent séquentiellement dans la région centrosomale avant de s'accumuler plus profondément dans le cytoplasme. Au cours de la seconde étape, on observe une intensification progressive et collective du signal Cen2-GFP. L'ensemble des halos se transforment en structures organisées ressemblant à des fleurs. Ces « fleurs » se disso-

cient ensuite de façon synchronisée pour laisser apparaître de multiples centrioles qui migrent à la membrane apicale et nucléent des cils motiles.

Afin de préciser la provenance des précurseurs centriolaires, nous nous sommes focalisés sur la région centrosomale en augmentant la résolution spatiotemporelle de nos expériences de vidéomicroscopie. De manière inattendue, nous avons observé que les halos bourgeonnent séquentiellement à partir d'un des centrioles du centrosome avant de se détacher pour s'accumuler dans le cytoplasme. L'utilisation d'un microscope à super-résolution (3D-structured illumination microscope, 3D-SIM) nous a permis de révéler qu'un halo est composé d'un assemblage sphérique de plusieurs pro centrioles disposés d'une manière tridimensionnelle, à la façon d'une morula (Figure 1C).

Le centriole fils du centrosome amplifie les pro centrioles par l'intermédiaire de deuterosomes

Pour évaluer la contribution de chaque centriole du centrosome à la formation des halos, nous avons distingué les centrioles père et fils en marquant le cil primaire (nucléé par le centriole père), et nous les avons observés par super-résolution. De façon inattendue, nous avons constaté que 100 % des halos se forment sur la partie proximale du centriole fils, mettant ainsi en évidence une asymétrie jusque-là inconnue des centrioles centrosomaux dans la biogenèse des centrioles. Afin de comprendre par quel processus le centriole fils du centrosome produit de nouveaux centrioles, nous avons

¹ Voir le numéro thématique « Cils primaires et ciliopathies » publié par *médecine/sciences*, m/s n°11, vol. 30, novembre 2014.

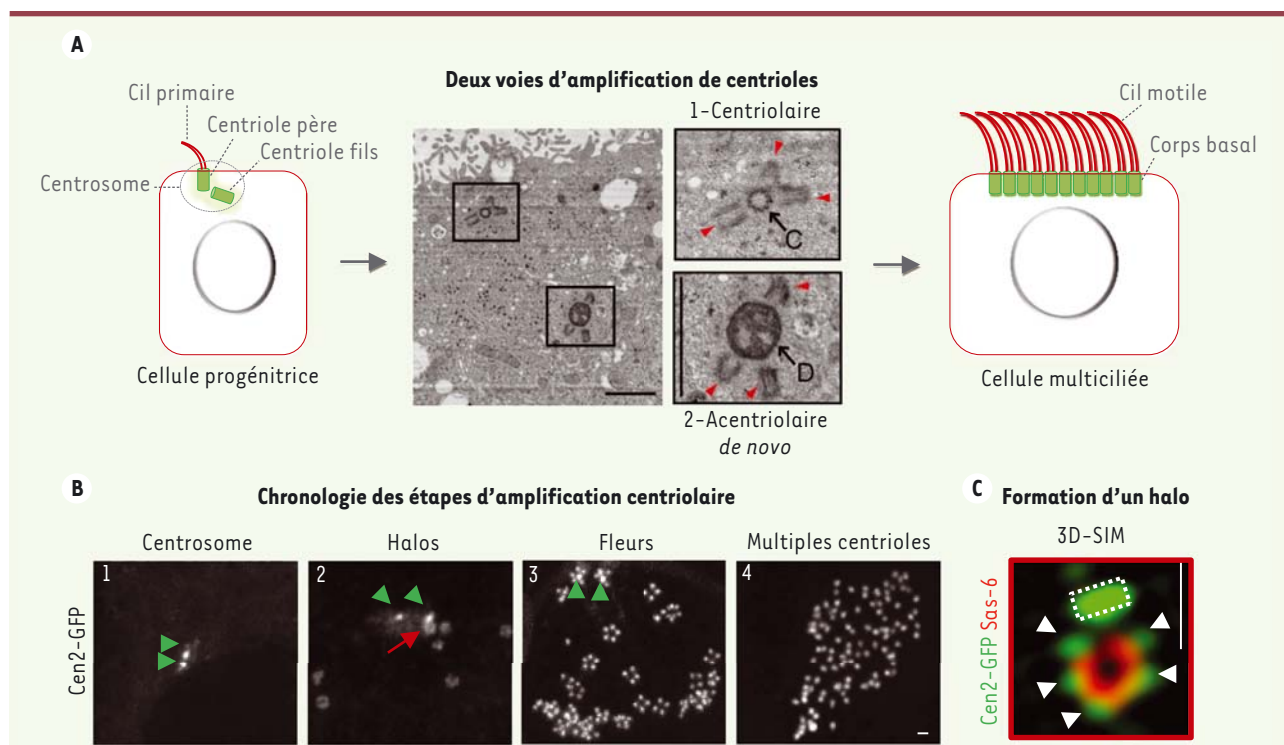


Figure 1. Voies et dynamique d'amplification de centrioles dans les cellules multiciliées. **A.** À gauche, schéma d'un progéniteur d'une cellule multiciliée possédant un centrosome composé d'un centriole père, qui nucléé un cil primaire, et d'un centriole fils. Panneau central, images en microscopie électronique à transmission qui montrent les deux voies d'amplification de centrioles présentes dans le progéniteur d'une cellule épendymaire multiciliée en cours de différenciation. Des procentrioles, indiqués par des têtes de flèches rouges, poussent sur un centriole du centrosome préexistant (C ; voie centriolaire), ou sur un deuterosome (D ; voie acentriolaire ou « *de novo* »). À droite, schéma d'une cellule dotée de multiples centrioles matures, ou corps basaux, servant à la nucléation des cils motiles. **B.** Images de vidéomicroscopie illustrant la chronologie des étapes d'amplification centriolaire au cours de la différenciation d'une cellule épendymaire multiciliée Cen2-GFP. Les 2 centrioles du centrosome sont indiqués par des têtes de flèches vertes. La flèche rouge indique un halo en cours de formation. **C.** Image de super-résolution 3D-SIM du bourgeonnement d'un halo Cen2-GFP composé de 5 procentrioles (têtes de flèches blanches), à partir d'un centriole du centrosome (rectangle en pointillé). Le marquage immunofluorescent Sas-6 (rouge) est spécifique des centrioles immatures. Barres d'échelle : 1 μ m.

marqué Deup1, une protéine impliquée dans la formation des deuterosomes [4] et connue pour recruter Cep152 et Plk4, protéines contrôlant les premières étapes de la nucléation des centrioles au cours de la duplication du centrosome dans les cellules en cycle. Alors qu'au cours de la duplication du centrosome dans les cellules progénitrices des cellules multiciliées Deup1, Cep152 et Plk4 sont distribuées symétriquement sur les deux centrioles centrosomaux, dans les cellules en cours de différenciation, ces protéines se distribuent de façon asymétrique en s'accumulant fortement au centriole fils (Figure 2A). Ces expériences suggérant que le centriole fils amplifie

les centrioles en formant des deuterosomes, nous avons mis au point une technique permettant d'observer les mêmes cellules en super-résolution et en microscopie électronique à transmission (EM). Cette approche, qui permet d'accéder à l'ultrastructure des cellules, a permis de révéler que les halos en formation sur le centriole fils correspondent bien à des procentrioles émanant de deuterosomes en bourgeonnement (Figure 2B). Ces expériences ont ainsi révélé que le centriole fils du centrosome est le centre amplificateur des centrioles dans les cellules multiciliées. Elles réfutent par conséquent l'origine « *de novo* » des centrioles dans ces cellules.

Les deux voies d'amplification centriolaire sont orchestrées par le centrosome

Nous avons ensuite analysé la transition entre l'étape « halos » et l'étape « fleurs » par microscopie super-résolutive et microscopie électronique. Nous avons remarqué que pendant l'étape de « halos », tous les procentrioles ont une taille identique et sont immatures, indiquant la présence d'une phase de latence au cours de cette étape d'amplification par les deuterosomes. Toutefois, après la formation du dernier halo/deuterosome, la microscopie électronique nous a permis de voir que tous les procentrioles s'allongent et mûrent simultanément sur l'ensemble

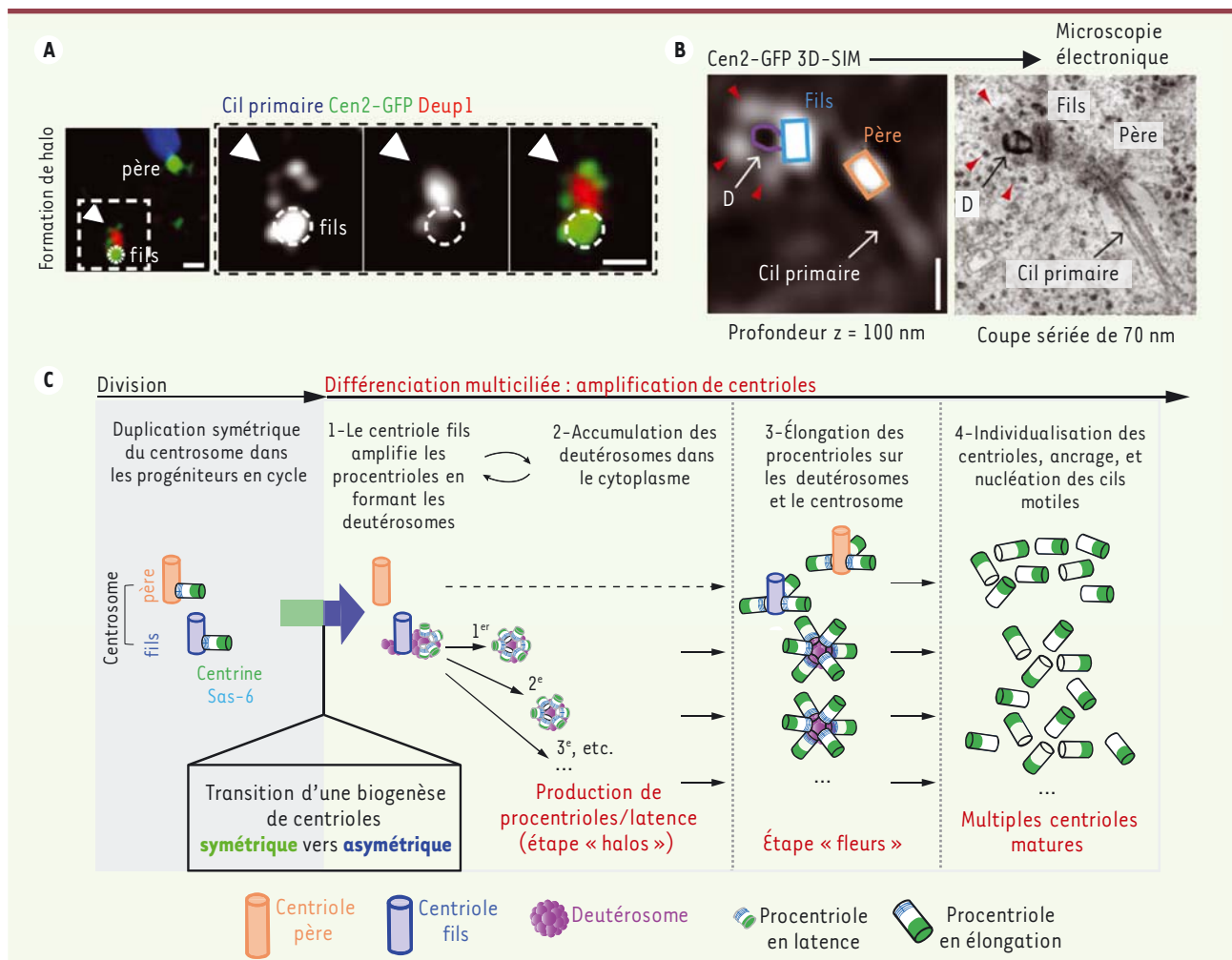


Figure 2. Le centriole fils du centrosome amplifie les procentrioles par l'intermédiaire de deuterosomes. **A.** À gauche, image en super-résolution 3D-SIM d'un centrosome Cen2-GFP marqué avec Deup1 (rouge), pendant la formation d'un halo indiqué par une tête de flèche blanche. Le centriole père est reconnu par la présence d'un cil primaire (bleu ; tubuline glutamylée). Panneau de droite : agrandissement du centriole fils. **B.** Images de microscopie corrélative 3D-SIM/microscopie électronique à transmission, qui montrent un halo en formation à partir du centriole fils. Le halo (gauche) correspond à des procentrioles émanant d'un deuterosome (D) en bourgeonnement (droite). Les têtes de flèches rouges indiquent les procentrioles. **C.** Modèle de biogenèse de centrioles dans les cellules multiciliées. Barres d'échelle : 0,5 μ m.

des deuterosomes. Nous avons également constaté qu'au même moment, une dizaine de procentrioles s'allongent de façon orthogonale, directement à partir des portions proximales des centrioles centrosomaux, constituant ainsi la voie « centriolaire » décrite dans la bibliographie. Ainsi, nos expériences montrent qu'au cours de la différenciation des cellules multiciliées, les deux voies d'amplification « centriolaire » et « de novo » sont en fait une seule et même voie orchestrée par le centrosome (Figure 2C).

Conclusion et perspectives

Cette étude [5] montre que dans un contexte physiologique, le centrosome peut se dupliquer lorsqu'il est dans une cellule en division, mais également orchestrer une amplification massive de centrioles lorsqu'il est dans une cellule en cours de différenciation multiciliée. Le centriole fils du centrosome s'avère jouer un rôle clef dans la biogenèse des centrioles, en tout cas lorsque ceux-ci sont massivement amplifiés (Figure 2C). La mise en lumière de cette asymétrie du centrosome pourrait permettre

de mieux appréhender l'amplification pathologique des centrioles associée aux microcéphalies [6] et aux tumeurs invasives [7-9]. Nous avons par ailleurs décrit une dynamique globale de différenciation dont la progression est phasique. L'amplification centriolaire est en effet marquée par des étapes distinctes et des transitions synchronisées (Figure 2C). De façon intéressante, des études émergentes soulignent l'implication des régulateurs de la phase S du cycle cellulaire dans l'amplification centriolaire des cellules multiciliées



[10-12]. Le processus d'amplification centriolaire pourrait donc être vu, non plus comme un processus particulier aux cellules multiciliées, mais plus comme une facette de la duplication centriolaire dans laquelle la régulation spatiotemporelle du nombre de centrioles a été contournée. L'exploration des rôles potentiels d'autres régulateurs du cycle cellulaire pourrait contribuer à mieux comprendre les anomalies de la différenciation multiciliée observées dans certaines ciliopathies. ♦

Centriole amplification ?

#DaughterCentriole

LIENS D'INTÉRÊT

Les auteurs déclarent n'avoir aucun lien d'intérêt concernant les données publiées dans cet article.

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NOUVELLE



Un nouvel outil pour le traitement de la myopathie de Duchenne : les tricyclo-ADN

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➤ Les maladies neuromusculaires regroupent un ensemble de plusieurs centaines de maladies, principalement d'origine génétique, définies par une perte de force par défaut de commande du muscle ou par destruction du tissu musculaire lui-même. Ces maladies diffèrent dans leurs causes, leur âge d'apparition, leur sévérité et leur évolution. Chacune de ces maladies est peu fréquente (maladies rares), mais conjointement, elles affectent plusieurs dizaines de milliers de personnes en France, constituant un enjeu majeur de santé publique et sociétal. La plus emblématique d'entre elles, la dystrophie musculaire de Duchenne (DMD), concerne un garçon sur 3 500 à la naissance ; ses spécificités génétiques et physiopathologiques en font un véritable cas d'école et un défi pour la conception

et la mise au point de traitements pour des maladies où il faut traiter simultanément l'ensemble de la musculature squelettique, le cœur et, incidemment, le système nerveux central.

Approche thérapeutique par saut d'exon

Caractérisée depuis le milieu du XIX^e siècle par un médecin français, Guillaume Benjamin Duchenne de Boulogne, cette myopathie très invalidante ne bénéficie encore d'aucun traitement satisfaisant. Un quart de siècle après l'identification du locus morbide *DMD* (gène codant la protéine dystrophine) par les équipes de L. Kunkel [1] et K. Davies [2], l'essor des recherches en biotechnologies laisse enfin entrevoir un espoir tangible de corriger certaines des anomalies du gène *DMD* par des

approches de chirurgie de l'ARN (modulation sélective de l'épissage de l'ARNm de la dystrophine – saut d'exon) à l'aide d'oligonucléotides antisens (AON). Le principe de cette thérapie issue de la connaissance des gènes est fondée sur l'utilisation de petites séquences oligonucléotidiques antisens, capables de s'hybrider spécifiquement avec leurs ARN messagers cibles afin de moduler leur maturation et permettre la synthèse de la protéine manquante. Plusieurs études sont en cours avec différents AON modifiés, telles que les 2'O-méthyl phosphorothiate (2'OMe), ou encore les morpholinos (PMO) pour la synthèse des AON destinés à agir sur la production de dystrophine. Malgré les résultats encourageants de certains essais cliniques [3, 4], le bénéfice thérapeutique demeure difficile à démontrer du fait des limites

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“Why do we do basic research?

To learn about ourselves.”

Walter Gilbert, PhD

Multiciliated mammalian cells play a crucial role in the propulsion of physiological fluids. Their dysfunction can be lethal or cause severe chronic diseases. In contrast to the strict centriole number control in cycling cells, multiciliated cell differentiation is marked by the production of up to several hundred centrioles, each nucleating a motile cilium. The mechanisms of centriole amplification or centriole number control in these cells were unknown and new centrioles were thought to appear *de novo* in the cytoplasm. At the beginning of this PhD project, videomicroscopy combined with correlative super-resolution and electron microscopy has enabled us to determine that all procentrioles are generated via runs of nucleation from the pre-existing progenitor cell centrosome. We show that the daughter centriole of the centrosome is the primary nucleation site for 95% of the new centrioles in multiciliated cells and thus refute the *de novo* hypothesis. Then, we provide evidence of an activation of the Cdk1-CyclinB1 complex along with its mitotic regulatory network during the centriole amplification dynamic. By coupling single cell live imaging with pharmacological modulation of mitosis regulators, we show that the mitosis machinery orchestrates the spatiotemporal progression of centriole amplification in terminally differentiating multiciliated cell progenitors. The fine-tuning of Cdk1 activity prevents mitosis while allowing the timely coordination of centriole number, growth, and disengagement through checkpoint-like phase transitions necessary for subsequent functional motile ciliation. Collectively, these results provide a new paradigm for studying multiciliated cell differentiation, cilia-related diseases and pathological centriole amplification associated with cancer and microcephaly.
