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Abstract

Centriolar satellites are 70-100nm sized cytoplasmic particles concentrated around centrosomes and cilia in vertebrate cells. Previous work has linked satellites to various processes including centrosome and cilium biogenesis; however, the mechanisms underlying their function are still poorly understood. Centriolar satellites were originally identified as transport modules for delivering centrosomal and ciliary cargo proteins to the centrosome. The main scaffolding component, PCM1, is essential for their formation and loss of PCM1 causes loss of certain components from the centrosome. However, the majority of satellites show diffusive rather than directed motion. While centriolar satellites share almost half of their proteome with centrosomes and cilia, understanding their functional contribution to these cellular structures is essential. Recent studies have identified orthologs of the satellite protein PCM1 outside of vertebrates, enabling us to investigate putative conserved roles and mechanisms. Thus, the aim of my PhD thesis was to characterize the fruit fly (*Drosophila melanogaster*) ortholog of PCM1, Combover, in order to identify conserved features, functions and insights into the role of satellites at an organismal level. By studying Combover mutants I identified phenotypic similarities; loss of Combover caused both centrosome and cilia signature phenotypes. Defects at the light-and electron microscopy level could be examined in different cells and tissues of the fly. Careful examination of Combover localization showed that the protein localized to cytoplasmic foci similar to PCM1 in human cells with many known centrosomal proteins co-localizing to these structures. Surprisingly, however, Combover distribution was random and did not recapitulate the concentration around centrosomes known from vertebrate cells, indicative of a function other than transport. In addition, I performed proteomic studies of Combover containing centriolar satellites in fly cells and tissues. Comparing these results to previous published datasets revealed overlap with both the centrosome-cilium and RNA -associated proteomes. The presence of several eukaryotic translation initiation factors led us to hypothesize that centriolar satellites could be sites of translation. Indeed, I found first evidence for this hypothesis in *Drosophila* and vertebrate cells. In vertebrate cells, some centrosomal mRNAs associated with centriolar satellites while depletion of PCM1 phenocopied inhibition of translation. Altogether, my work shows conserved features of satellites and suggests that centriolar satellites might act as translational hubs for centrosomal and ciliary proteins.

Zusammenfassung

Zentrioläre Satelliten sind 70-100nm große, im Cytoplasma liegende Partikel, die sich in Zellen von Wirbeltieren um Zentrosomen und die Zilienbasis konzentrieren. Bisher wurden Satelliten mit verschiedenen Prozessen assoziiert, darunter der Aufbau von Zentrosomen und Zilien. Die molekularen Mechanismen, die der Funktion der Satelliten unterliegen, sind jedoch kaum verstanden. Sie wurden ursprünglich als Transportmodule für zentrosomale und ziliäre Proteine identifiziert. Das Protein, das ihr Gerüst bildet und essentiell für ihre Bildung ist, ist PCM1; ohne PCM1 können sich viele Proteine nicht am Zentrosom anlagern. Nichtsdestotrotz kann das Transport-Modell nicht erklären warum die meisten Satellitenpartikel sich nicht aktiv bewegen. Da zentrioläre Satelliten die Hälfte ihres Proteoms mit Zentrosomen und Zilien teilen, ist es essentiell zu verstehen welchen Beitrag Satelliten leisten. Eine vorherige Studie hat orthologe Proteine von PCM1 außerhalb von Wirbeltieren identifiziert und damit ermöglicht dessen evolutionär konservierten Rollen und Mechanismen zu studieren.

Ziel meiner Doktorarbeit war es das orthologe Protein von PCM1, Combover, in der Fruchtfliege (*Drosophila melanogaster*) zu charakterisieren. Durch Studien an Combover Mutanten konnte ich Ähnlichkeiten zu PCM1 feststellen, inklusive zentrosomaler und ziliärer Phänotypen. Defekte in verschiedenen Zellen und Geweben der Fliege konnten durch Licht- und Elektronenmikroskopie charakterisiert werden. Die Analyse der Lokalisation von Combover ergab, dass dieses Protein Partikel formt die ähnlich zu PCM1 sind und zentrosomale Proteine beinhalten. Überraschend jedoch war, dass die Verteilung dieser Partikel in der Zelle gleichmässig verteilt ist, ohne jegliche Konzentration um die Zentrosomen. Dieses deutet auf eine andere Funktion als Transport hin. Außerdem analysierte ich das Proteom der Satelliten in Zellkulturen und Geweben der Fliege. Der Vergleich meiner Ergebnisse mit den bisher publizierten Datensätzen für Wirbeltiere ergab eine Überschneidung mit dem Proteom von Zentrosomen, Zilien und RNA-assoziierten Proteinen. Die Präsenz mehrerer eukaryotischer Translationsfaktoren brachte uns dazu zu spekulieren dass Satelliten Orte lokaler Translation sein könnten. Tatsächlich fanden wir erste Beweise für diese Hypothese in der Fliege und in menschlichen Zellen. In diesen Zellen konnte ich bekannte zentrosomale mRNAs in Satelliten lokalisieren und zeigen, dass der Verlust von PCM1 ähnliche Folgen hat wie die Inhibierung der Proteinsynthese. Zusammengefasst zeigt meine Studie evolutionär konservierte Eigenschaften, insbesondere dass zentrioläre Satellitenproteine Zentren der Synthese von zentrosomalen und ziliären Proteinen sein könnten.

List of abbreviations

Pericentriolar Material	PCM
Microtubule	MT
Microtubule organising center	MTOC
Microtubule inner protein	MIP
Structured illumination microscopy	SIM
Stochastic optical reconstruction microscopy	STORM
Multiciliated Cells	MCC
Basal body	BB
Transition zone	TZ
Distal appendage	DAP
Subdistal appendage	sDAP
Intraflagellar transport	IFT
BBsome	BB
Centriolar satellites	CS

1 Introduction

1.1 From Centrosomes and Cilia to Centriolar Satellites - A Perspective

To convey the relevance of my study I would like to first explain the importance of studying centrioles, centrosomes and cilia and later describe how centriolar satellites contribute to fundamental aspects of centrosome/cilium biology. In the following, I will shed light on centrosomes from various angles. First, I will briefly review the early days of centrosome research that have laid the groundwork for modern centrosome studies. Then I will address centrosome structure, provide an overview of its functions and highlight its role in development and disease. Last but not least I will summarize the current debate about the importance of centrosomes.

1.2. Centrosomes -The origin

The discovery of the centrosome dates back to 1887 when Theodor Boveri and Edouard van Beneden independently identified that spindle poles of fertilized eggs of the nematode *Ascaris megalocephala* (now *Parascaris equorum*) are cellular organelles that act as the main organizing center of the spindle during mitotic cell division (Boveri, 1887; Scheer, 2014; Van Beneden, 1887). It was at that time, when Boveri discovered that these structures duplicate and separate and set the cell division plane. Hence, he termed these cell bodies ‚centrosomes‘, as they appear to define the centre of the cell (Boveri, 1887). Even though it was soon clear that the core of the centrosome is comprised of a denser material, it took another 12 years to identify what has since become known as the ‚centriole‘ (Boveri, 1900). Boveri’s studies on *Ascaris* and cleaving sea urchin eggs have laid the foundation for much of today’s centrosome research (Boveri, 1900; Scheer, 2014).

Theodor Boveri, often seen as the ‚father‘ of centrosomes, has inspired many cell biologists to work on fundamental questions of centrosome biology and cell division and to take advantage of nematodes as model organisms (if not *Ascaris*). Among them was Sydney Brenner, who established the non-parasitic soil nematode *Caenorhabditis elegans* (*C. elegans*) as an experimental model in the 1960s (Schwarz et al., 2018). The transparency of *C. elegans* allows easy visualization of individual cells; centrosomes, spindles and nuclei can be readily detected using differential interference contrast microscopy (DIC). Until today, *C. elegans* has been and continues to be extensively used for cell biology studies.

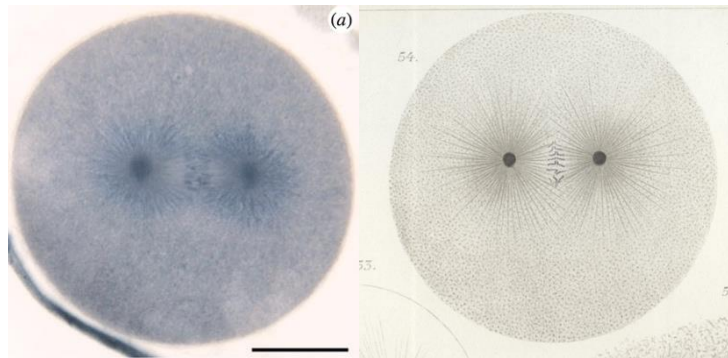


Figure1. First images of the centrosome during the cleavage of fertilized sea urchin eggs (*Echinus microtuberculatus*). Photograph of a modern microscopic re-examination of Boveri's original preparations (left) (Scheer, 2014) and drawing (right) from his Monograph „Über die Natur der Centrosomen“ in 1900 (Boveri, 1900).

1.2.1 Components of the centrosome

The structure of the centrosome as we know it today also has its roots in Theodor Boveri's observations. He followed the centrosome over the course of the cell cycle and noticed that it is a dynamic cellular organelle. In addition, he not only identified that both centrosomes and centrioles replicate but also that each centrosome contains at least one centriole. He described the centriole as a magnet that attracts surrounding protoplasm to form a sphere (Schwarz et al., 2018). Today we know that each centrosome contains a pair of barrel-shaped centrioles surrounded by the pericentriolar material (PCM).

Centrioles are essential for PCM recruitment and hence to form a fully functional centrosome. This has been demonstrated by induced disassembly of centrioles following microinjection of antibodies to polyglutamylated tubulin and the subsequent dispersal of the PCM in animal cells (Bobinnec et al., 1998). Through their function in centrosome assembly, they are determinants of centrosome number; failure in centriole duplication results in failure of centrosome duplication and the formation of monopolar spindles (O'Connell et al., 2001).

The PCM is comprised of several hundred proteins, including those involved in microtubule nucleation. The major protein required for microtubule nucleation is γ -tubulin, part of the small (γ -TuSC) and large gamma-tubulin ring (γ -TuRC) complexes (Kollman et al., 2011).

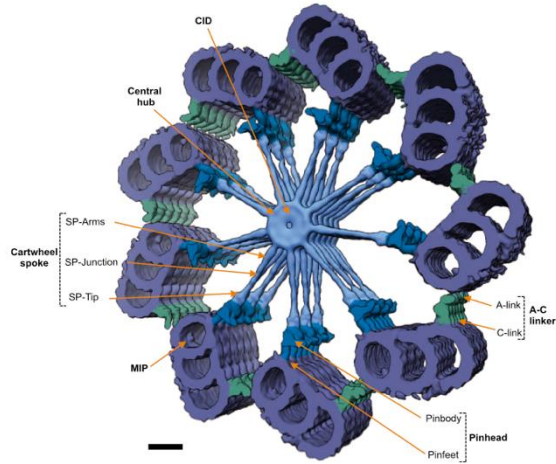
100 years after Boveri's initial discoveries and with the advancement of technologies, particularly transmission electron microscopy and electron tomography it was possible to refine centrosome structure. Ultrastructural analysis of centrioles contributed greatly to our understanding of centriole structure. In contrast, the organization of the surrounding PCM is still not fully resolved.

1.2.2 Centriole structure

Centrioles are microtubule-based structures with a 9-fold symmetry, approximately 250nm wide and 450nm long in eukaryotes (Jana, 2021). Centrioles just 150nm in size can be found in *C. elegans*, while *Drosophila* sperm centrioles can reach up to 2.5µm. Centrioles in humans are comprised of triplet MTs arranged in a radially symmetric order and each triplet is composed of A, B and C tubules as well as non-tubulin components. The A tubule with 13 protofilaments is complete, however B and C tubules are only partially assembled (10 protofilaments). The protofilaments are made up by α/β tubulin, while non-tubulin structures include microtubule inner proteins (MIP), the A-C linker, the pinhead, the Y/L-linker and inner scaffold (Jana, 2021) (Figure 2). Considerable variations in centriole architecture exist, including in embryos of the fruit fly, *Drosophila melanogaster* and in *C.elegans*, which contain centrioles with doublet and singlet microtubules, respectively (Winey and O'Toole, 2014). MIPs are a relatively recently recognized feature, identified as electron densities within the lumen of the microtubules (Guichard et al., 2013; Li et al., 2012).

The A-C linker, connecting the A and C-tubules as the name suggests, provides stability between microtubule triplets (Winey and O'Toole, 2014) and additional structures such as the Y-shaped linker, joining A and B tubules have been recently identified and possibly contribute to centriole stability as well. Within the centriole lumen is the so-called cartwheel, consisting of a 20-25nm central hub and 9 radial spokes. The main cartwheel component is the coiled-coil protein Sas-6 that sits within the hub and extends towards the spokes (Winey and O'Toole, 2014). Additional spoke components include Bld10/Cep135 that connects the cartwheel hub to the A-tubule of a MT-triplet. At this connection another density was identified, called the pinhead, crucial for centriole formation and stability and within this, the proteins CPAP (SAS-4) and STIL (SAS-5). Further variations to the described cartwheel architecture exist; however, the molecular components involved and the purpose they have stay the same (see also function).

A



B

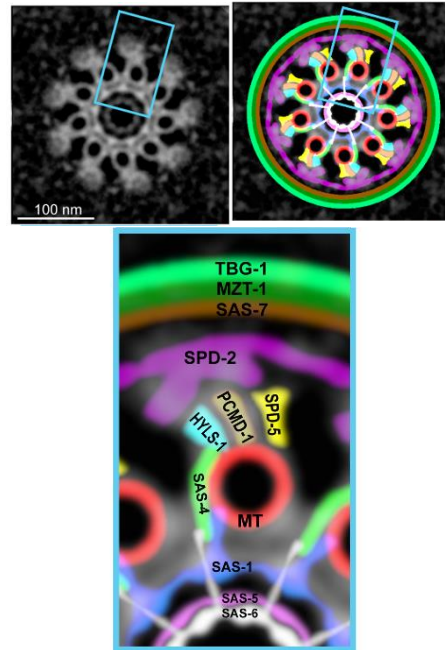


Figure 2. Structure of centrioles. (A) 3D structure of the proximal region of a centriole. Scale bar is 20nm (Guichard et al., 2013). (B) EM image of a *C.elegans* centriole (9-fold symmetrized) and overlay with schematic representation. Localization of components within the centriole based on expansion microscopy (Woglar et al., 2022).

An additional feature of older centrioles in vertebrates are the distal and subdistal appendages. Distal appendages are fibrous extensions radiating out from the distal end of the centrioles containing several proteins involved in primary cilium formation. Subdistal appendages, found proximal to the distal appendages, are triangular in shape and attach laterally to the sides of the microtubule scaffold. These have been found to be involved in microtubule anchoring. Analogous to appendages, basal bodies have similar structures that are termed basal feet and transition fibers (Tateishi et al., 2013).

Recent technological advances such as cryo-electron tomography and subvolume averaging have paved the way to fully uncover centriole architecture and its complexity. Super-resolution microscopy and expansion microscopy to dissect localization of centriole components will further help to refine structural details.

1.2.3 Pericentriolar material (PCM) structure

Surrounding the pair of centrioles is a complex network of proteins called the pericentriolar material (PCM), whose detailed organization has yet to be fully resolved. Electron micrographs from the 1960s showed that the PCM is a dense, amorphous mass (Robbins et al., 1968). Follow-up analysis using EM tomography and immunofluorescence microscopy further identified γ -tubulin containing ring structures within the PCM of isolated *Drosophila* centrosomes (Moritz et al., 1995). Schnackenberg et al. confirmed the existence of similar ring complexes (γ -TuRCs) in centrosomes isolated from surf clam (*Spisula*) oocytes (Schnackenberg et al., 1998). They further showed that high salt treatments with potassium iodide removed these γ -TuRCs and most of the PCM, leaving behind an insoluble scaffold that resembles a filamentous network (Schnackenberg et al., 1998) (Figure 3A). In the following years, several core PCM components could be identified and interestingly a common feature was identified: the presence of coiled-coil domains (Andersen et al., 2003; Hamill et al., 2002; Pelletier et al., 2004; Varmark et al., 2007). Superresolution microscopy (SIM and STORM) allowed a mapping of PCM proteins to distinct locations within the PCM, revealing a concentric-toroid organization during interphase (Figure 3B). Metaphase centrosomes, however, appear less structured. The study by Schnackenberg et al. has led to the following model: the PCM is first assembled onto a scaffold onto which other client proteins are then assembled (Woodruff et al., 2014).

The *C. elegans* scaffold protein required for PCM assembly is SPD-5 (Hamill et al., 2002). It was shown that recombinant SPD-5 could polymerize to form micron-sized networks in vitro, capable of scaffolding the recruitment of other PCM proteins (Woodruff et al., 2015). Thus, a single protein can drive PCM assembly through homo-oligomerization of its coiled-coil domains. In *Drosophila*, the coiled-coil protein Centrosomin (Cnn) is underlying PCM assembly (Megraw et al., 2001). Similar like SPD-5 it self-assembles into micron-scale structures; this self-assembly is mediated through an interaction of the leucine zipper (LZ) with the Cnn-motif 2 (CM2) domain (Feng et al., 2017). In vertebrates, the coiled-coil protein Pericentrin acts as a scaffolds for anchoring of numerous proteins in the PCM (Doxsey et al., 1994). Similarly, in *Drosophila*, the C-terminal Pericentrin-AKAP450-centrosome-targeting (PACT) domain is required and sufficient to target the Pericentrin ortholog, Plp, to centrosomes and has also been implicated in the recruitment of several proteins to the PCM (Martinez-Campos et al., 2004). Depletion of Pericentrin and Cdk5rap2 disrupts centrosome maturation, suggesting that these two proteins form the PCM scaffold in vertebrates (Lattao et al., 2017).

In *Drosophila* Plp mutant flies display defects in PCM assembly in mitosis, however less severe as Cnn or Spd-2 mutants (Martinez-Campos et al., 2004).

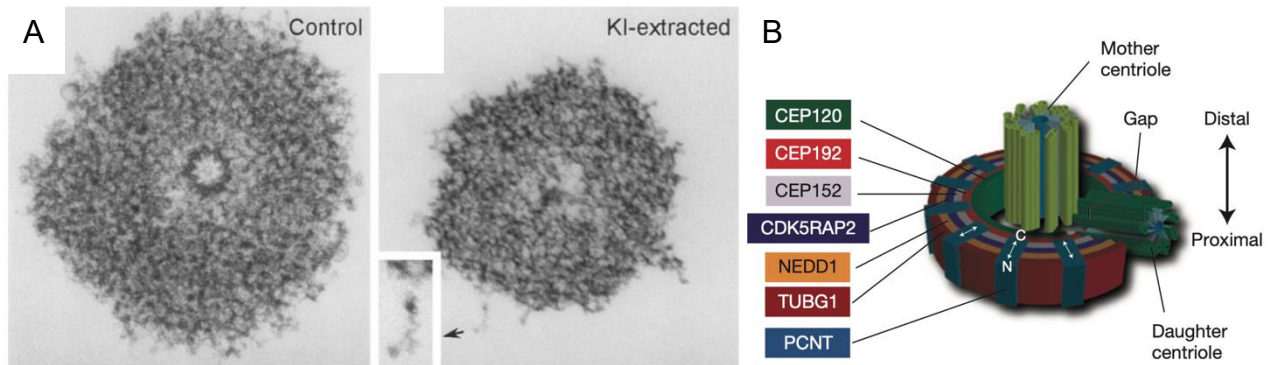


Figure 3. Structure of the PCM. (A) Transmission electron microscopy of isolated *Spisula* centrosomes. The PCM forms an amorphous network around the centrioles (left), salt treatment with KI extracts the centrioles and leaves behind an insoluble remnant (Schnackenberg et al., 1998). (B) Organization of components within the interphase centrosome based on superresolution microscopy (Lawo et al., 2012).

1.3 The centrosome cycle

The number of centrosomes within a cell has to be tightly controlled to ensure spindle bipolarity and hence mitotic fidelity. Aberrations in centrosome number can have deleterious effects; centriole loss can result in cell cycle arrest or death via the p53 pathway, while centrosome amplification can lead to spindle defects which may contribute to human disease (Godinho et al., 2014). To avoid this, centrioles have to duplicate exactly once and only once per cell cycle. This cycle involves three different stages: centriole duplication, centriole elongation and centriole disengagement/centriole-to-centrosome conversion (Nigg and Raff, 2009). In cycling cells, centrioles duplicate in S-phase and stay engaged with the newly formed procentriole (daughter centriole). They elongate during S and G2 phases of the cell cycle, reaching full size as cells enter mitosis. The replicated centriole pairs and associated PCM move to opposite sites to form the two poles of the mitotic spindle (Nigg and Raff, 2009).

1.3.1 The molecular events underlying centrosome duplication

How are centrioles formed and duplicated? Studies have shown that the pathway of centriole assembly is remarkably conserved. The process starts with Plk4 (Sak in *Drosophila*, ZYG-1 in *C. elegans*) which is recruited to parental centrioles by Cep152 and Cep192; in *Drosophila* this recruitment is mediated by the Cep152 ortholog Asl, in *C. elegans* by the Cep192 ortholog SPD-2. Once Plk4 is activated it phosphorylates STIL (Ana2 in *Drosophila*, SAS-5 in *C. elegans*) and together with Sas-6 initiates formation of the centriolar cartwheel with its characteristic nine-fold symmetry, involving the oligomerization of the universally conserved centriole protein Sas-6 (Dammermann et al., 2004)(Kitagawa et al., 2011; van Breugel et al., 2011). At the tip of each cartwheel spoke CPAP/Sas-4 together with γ -tubulin assembles the centriolar microtubule wall (Dammermann et al., 2008). Plk4 is a key mediator of centriole duplication, loss of Plk4 results in failure of centriole duplication while overexpression results in multiple daughter centrioles per parent (Cunha-Ferreira et al., 2013; Kleylein-Sohn et al., 2007). Levels of Plk4 are maintained through the action of the SCF/Slimb ligase complex, which limits the amount of Plk4 by catalyzing Plk4 ubiquitination and thus degradation by the proteasome (Rogers et al., 2009). In addition, the activity of Plk4 is important, since Plk4 autophosphorylation has been shown to control Plk4 self-destruction and thus centriole number (Cunha-Ferreira et al., 2013).

The newly formed centrioles gradually elongate throughout S and G2. The proteins Cpl10 and Cep97 localize to the distal end of centrioles and limit centriole elongation by their capping function (Kleylein-Sohn et al., 2007). Loss of Cpl10 or Cep97 both result in elongated centrioles, while Cep97 has additionally been shown to provide centriole stability (Dobbelaere et al., 2020; Schmidt et al., 2009). Additional proteins implicated in centriole elongation are Cep135/Bld10, Poc1 and Bug22. Cep135 and Poc1 mutants display shorter centrioles, while Bug22 leads to longer centrioles (Lattao et al., 2017). Furthermore, Cep135, Asl and Ana1 act on centriole elongation *in vivo* and are involved in the process of centriole-to-centrosome conversion, enabling PCM recruitment by the newly formed centriole (Fu et al., 2016).

1.3.2 The molecular events underlying PCM assembly and maturation

PCM assembly is initiated at the G2/M transition and is primarily governed by the kinase Polo/Plk1. The interphase PCM is organized in thin concentric layers around the mother centriole with Plk1 largely inactive. At the onset of mitosis, Plk1 is activated and starts

phosphorylating scaffolding components (Cnn in *Drosophila*, Spd-5 in *C.elegans*, PCNT in humans). Inhibition of Polo prevents γ -tubulin recruitment at centrosomes and mitotic PCM expansion in humans, flies and worms (Dobbelaere et al., 2008; Lee and Rhee, 2011; Woodruff et al., 2014). In *Drosophila*, perturbing Polo activity impairs localization of Cnn to the centrosome at mitotic onset (Conduit et al., 2014). Only a few proteins form the PCM scaffold: PCMD-1, SPD-2, and SPD-5 in *C. elegans*, PCNT/D-PLP, Asterless/Cep152 and Cnn/CDK5RAP2 and in *Drosophila*/humans. Despite a lack of sequence conservation among the main scaffolding proteins, they share structural similarity as they are comprised of several coiled-coil domains (Conduit et al., 2014). In flies, there is a positive feedback loop during centrosome maturation between Spd-2 and Cnn with Spd-2 helping in the recruitment of Cnn and Cnn helping in maintaining Spd-2 at the PCM (Conduit et al., 2014). Similarly, in worms SPD-2 has been shown to be involved in PCM assembly by promoting SPD-5 loading to the centrosome (Pelletier et al., 2004). Recently, the coiled-coil protein pericentriolar matrix deficient-1 (PCMD-1) was identified as a novel player in PCM assembly in *C. elegans*, necessary for efficient recruitment of SPD-2 and SPD-5 (Erpf et al., 2019).

Following phosphorylation of scaffolding proteins, the PCM starts expanding. A kinase necessary for PCM maturation is Aurora A kinase, which regulates recruitment of microtubule nucleating complexes and MAPs. Loss of Aurora A impairs accumulation of γ -tubulin and ZYG-9 in *C. elegans* embryos (Hannak et al., 2001) and Msps and D-TACC in *Drosophila* S2 cells (Giet et al., 2002). Thus, while Plk1 mediated the assembly of the core structure of the PCM, Aurora A drives the addition of ‘client’ proteins/downstream proteins to the scaffold. PCM maintenance is thought to be governed by the total amount of a limiting component and the rates of phosphorylation and dephosphorylation reactions (Woodruff et al., 2014).

1.3.3 The molecular events underlying pericentriolar material disassembly

As centrosomes exit mitosis, they rapidly disassemble in a process that requires a combination of dephosphorylation and microtubule-pulling forces (Enos et al., 2018). The protein phosphatase PP2A and the protein phosphatase P4 (PPH4) have been reported to promote PCM dissolution; however, additional proteins are likely to be involved and are yet to be identified. In *C. elegans*, depletion of the regulatory subunit of PP2A phosphatase, SUR-6, slowed down disassembly of SPD-5 and PP2A complexes dephosphorylated SPD-5 *in vitro*, suggesting that the PCM disassembles through active dephosphorylation and subsequent

fragmentation of the SPD-5 scaffold (Enos et al., 2018). Furthermore, cortical forces could help in disassembling the centrosome. Enos et al. showed that knockdown of the cortical dynein anchor GPR-1/2 by RNAi reduced the rate of SPD-5 disassembly. This phenotype was even stronger when combined with PP2A/SUR-6 depletion, suggesting that combinatorial effects of PP2A dephosphorylation and microtubule pulling forces drive PCM disassembly (Enos et al., 2018). Detailed analysis of PCM disassembly further revealed that different PCM proteins disassembled with different kinetics and different behaviors and it was suggested that dephosphorylation events would first lead to PCM dissolution and microtubule forces that would then rupture the PCM proteins into packets (Magescas et al., 2019).

1.4 Centrosome function

Centrosomes act as the main microtubule organizing center (MTOC) in animal cells; however, the requirement of centrosomes for successful cell division varies between species and developmental stages (Nigg and Raff, 2009). In most animal cells, such as the fertilized embryo of the frog *Xenopus laevis* (Klotz et al., 1990), the fruit fly *Drosophila melanogaster* (Stevens et al., 2007) or the nematode *C.elegans* (Kirkham et al., 2003) centrosomes are absolutely essential for cell division and embryonic development. Likewise, centrosomes can be experimentally perturbed in many somatic cells without affecting bipolar spindle assembly (Gadde and Heald, 2004; Khodjakov et al., 2000). Here, spindle assembly is ensured via a centrosome-independent pathway involving Ran GTPase and microtubule motor proteins (Kalab and Heald, 2008). While bipolar spindle assembly can occur through other means, through their astral microtubules centrosomes are important for spindle positioning as well as for the proper segregation of cell fate determinants during asymmetric cell division (Basto et al., 2006).

As MTOCs centrosomes depend on their ability to nucleate, anchor and release microtubules. This is achieved through a combination of PCM components. Apart from the proteins forming the scaffold, additional PCM proteins help in forming a docking station of molecules that aid the nucleation of MTs (Zheng et al., 1995). The most important protein needed for MT nucleation is γ -tubulin, which forms part of two different complexes: the γ -tubulin small complex (γ TuSC) and the γ -tubulin ring complex (γ TURC). The γ TUSC is a tetrameric complex comprised of two γ -tubulin molecules and one molecule each of GCP2 and GCP3 (*Drosophila* DGRP84 and DGRP91) (Bettencourt-Dias and Glover, 2007). Several

γ TUSC complexes are forming a ring joined by additional γ TURC-specific proteins to create the γ TURC. γ TURC is more complex than γ TUSC, containing 5 different variants of GCP proteins and it is the key mediator of microtubule nucleation and anchorage (Moritz et al., 2000). γ -tubulin is very abundant and mostly found in the cytoplasm, however only when recruited to the centrosome or other MTOCs does it become a microtubule nucleator (Stearns and Kirschner, 1994).

While a few microtubules are also found attached to the centriolar subdistal appendages, microtubules are primarily being anchored in the PCM (reviewed in (Badano et al., 2005), (Bornens, 2002) and (Bettencourt-Dias and Glover, 2007)). Ninein is localized to the subdistal appendages of mother centrioles and connects with the centriole on one and with the γ TURC on the other end and earlier studies have suggested that they anchor MTs (Delgehyr et al., 2005). Other proteins associated with microtubule anchoring are EB1, CEP135 and p50, whose depletion leads to anchoring defects and a delay in microtubule nucleation (Askham et al., 2002; Louie et al., 2004; Uetake et al., 2004). However, more recently the prevailing model is that MTs are primarily anchored by γ -tubulin itself (Vineethakumari and Lüders, 2022).

Phosphorylation by the protein kinases Polo-kinase-1 (Plk1) and Aurora A during centrosome maturation promotes microtubule nucleation. It is Plk1/Polo that promotes the recruitment of γ TURC (Glover, 2005) and phosphorylation of SPD-5 by Plk1 which enable it to bind to γ -tubulin (Ohta et al., 2021). In *Drosophila*, Aurora A phosphorylates the centrosome protein TACC which in turn interacts with a MT-associated protein, Minispindles (MSPS) to regulate astral MT behavior (Barros et al., 2005; Brittle and Ohkura, 2005).

Besides nucleating and organizing the mitotic spindle, centrosomes also play a role in directing intracellular trafficking and organelle positioning in interphase by guiding microtubule motors (Nigg and Raff, 2009). These microtubule motors are shuttling between the minus ends, embedded within the centrosome, and the plus ends of microtubules, extending outwards into the cytoplasm. Motor proteins of the kinesin family include both plus and minus end directed motors, whereas motor proteins of the dynein family contain only minus end directed motors. The retrograde transport of secretory cargo towards the Golgi as well as organelle positioning of the nucleus and Golgi in the center of the cell is mediated by dynein (Yadav and Linstedt, 2011), while anterograde transport and the dispersal of the ER throughout the cell volume is mediated by attachment to plus end-directed kinesin motors.

1.5 Centrosomes in health and disease

The idea that centrosome abnormalities are linked to tumorigenesis and cancer emerged as early as 1914 when Theodor Boveri suggested that erroneous chromosome segregations, caused by irregular centrosome number, would lead to cancer (Nigg and Raff, 2009). Centrosome number must indeed be tightly regulated so that every centriole duplicates once and only once during the cell cycle. Failure in centriole duplication control likely leads to centrosome amplification which could alter cell shape, polarity and motility, influencing tumor architecture and the ability to metastasize (Godinho et al., 2014). By now centrosome amplification are established hallmarks of cancer, occurring in different types such as breast, prostate, ovarian, colon and pancreatic cancer. Furthermore, they are known to be one of the leading causes of chromosomal instability (CIN), itself a common feature of many cancer types (Godinho et al., 2014). However, not only numerical but also structural centrosome aberrations have been detected in cancers, frequently showing unusual large centrosomes and an increased amount of PCM components (Nigg and Raff, 2009). Tumor suppressor genes and oncogenes when deregulated often lead to a change in centrosome number, triggering the debate whether centrosome aberrations are a cause or consequence of tumor progression (Godinho et al., 2014).

Centrosomes are also linked to developmental disorders, including primary autosomal recessive microcephaly, a disorder characterized by a smaller brain size and (mild) mental retardation. Up to date of the nine genes known to be involved in microcephaly five encode core centrosomal components (CPAP, CEP152, CEP135, STIL and CDK5RAP2) and two are mitotic spindle proteins (WDR62 and ASPM) (Gilmore and Walsh, 2013). Microcephalic primordial dwarfism (PD) are disorders with pre-and postnatal growth failure that are accompanied by microcephaly (Klingseisen and Jackson, 2011). Genes implicated in PD encode centrosome proteins CEP152, CPAP, PCNT and CEP63 which lead to aberrations in centrosome number or structure that ultimately affect brain development (Chavali et al., 2014).

1.6 Cilia- An Overview

Cilia are microtubule based cellular protrusions that perform multiple signaling and motile functions. They can be found in almost all tissues and cells of the human body and can be broadly divided into motile and non-motile/primary cilia. Motile cilia can be found on spermatozoa, in the respiratory system, the reproductive system and the ventricles of the brains (Mitchison and Valente, 2017). Non-motile cilia are more ubiquitous, found in diverse cell

types such as stem cells, muscle cells, epithelial and endothelial cells, connective tissue and neurons (Venkatesh, 2017). In vertebrate cells, some cells contain several cilia, known as multiciliated cells (MCC). MCCs are found in specialized cell types of the human body, where they beat in a coordinated fashion to drive fluid across an epithelium. They are found in the spinal cord and ventricles of the adult brain, in the airway and in the oviduct/fallopian tubes (Brooks and Wallingford, 2014).

1.7 Cilia-Origin and Evolution

Cilia are found in almost every branch of the eukaryotic tree of life (Hodges et al., 2012). They were first described by Antoni van Leeuwenhoek around 1674/1675, who observed motile cilia and their motion under light microscopy (Leeuwenhoek, 1677) (reviewed in (Satir, 2017)). The observation of these swimming protists had led to the idea that cells use cilia for motility (Leeuwenhoek, 1677). Non-motile primary cilia in contrast are only found in metazoans. Besides the well-known example of sensory organs such as the photoreceptors of the eye, they were largely considered to be vestigial until their important signaling function became appreciated from the 1990s onwards with the identification of the first non-motile ciliopathies (see below).

In an evolutionary big bang -a billion years ago- new superclades (unikonts and bikonts) and clades were defined, among them the opisthokonts, comprised of metazoa, fungi and choanoflagellates (Chernikova et al., 2011). Based on the presence of ciliary features across these distantly related organisms it can be deduced that the last eukaryotic common ancestor (LECA) already possessed centrioles and cilia, structurally similar to cilia today (see structure of cilia) (Carvalho-Santos et al., 2011). These early cilia presumably had both motile and sensory functions, since both signature features are found in multiple branches of the eukaryotic tree of life. The co-existence of centrioles and cilia in all known species further indicates that the primary and ancestral function of centrioles is to template cilia (Carvalho-Santos et al., 2011). In contrast, centrosomes appears to be a much later opisthokont phenomenon (Azimzadeh, 2014). Due to its ancestral origin, many features and functions of cilia are shared among extant species, as will be discussed in the following sections.

1.8 Cilia structure

Cilia are membrane-bound projections that are templated by centrioles. The microtubule cytoskeleton upon which they are built is called the axoneme (Figure 4). Axonemes are surrounded by the ciliary membrane, an extension of the plasma membrane, and extend from the mother centriole (reviewed in (Reiter et al., 2012)). They have one of the two characteristic arrangements: either 9+2, where nine peripheral microtubule doublets surround a single pair of microtubules, or 9+0, in which the central pair is missing (reviewed in (Satir and Christensen, 2007)). Motile cilia generally follow a 9+2, whereas non-motile/primary cilia follow a 9+0 pattern. In general, 9+0 cilia also lack molecular motors and axonemal dyneins needed for motility. Nevertheless, in recent years it has become evident that ciliary architecture and in particular axonemal structure are not always following this canonical distinction between motile and primary cilia and many more intermediate or mixed forms of cilia do exist (reviewed in (Mill et al., 2023)). During ciliogenesis, the mother centriole in mammalian cells docks to the plasma membrane to form the ciliary basal body (BB). Basal bodies have further accessory structures: transition fibers, basal feet and ciliary rootlets. Transition fibers and basal feet are analogous to distal and subdistal appendages respectively (reviewed in (Kobayashi and Dynlacht, 2011)). Distal appendages help during anchorage of the mother centriole, whereas subdistal appendages promote microtubule nucleation and anchorage and position the basal body. Ciliary rootlets reach from the proximal end of the basal body into the cytoplasm, aiding in structural stability of the cilium. Connecting the basal body and the axoneme is the transition zone (TZ), a gating structure where axonemal MT doublets are closely linked to the ciliary membrane. As a diffusion barrier between cell and cilium, the transition zone plays a key role in controlling transport to and from the cilium (reviewed in (Mill et al., 2023)). The ciliary membrane is distinct from the plasma membrane and are enriched in receptors that respond to external cues (Nachury and Mick, 2019).

quiescent cells and in G1 phase of proliferating cells, while they are disassembled during S or G2 phase and usually absent in mitosis (Avasthi and Marshall, 2012). Cilia in vertebrates form via one of two pathways. In the intracellular pathway of ciliogenesis, the mother centriole after being uncapped attaches to a vesicle, the ciliary vesicle (CV), within the cytoplasm. The ciliary vesicle itself is formed from smaller vesicles (pre-ciliary vesicles, PCVs) that fuse and dock to DA, building so-called distal appendage vesicles (DAVs) (Zhao et al., 2023). Next, the CVs start reorganizing to form a double membrane sheath around the budding axonemal structures. The extracellular membrane channel joins the developing intracellular cilium membrane with the plasma membrane, and the axoneme and ciliary membrane extend to form the final structure of the cilium, with a ciliary pocket leaving the mature cilium embedded within the cell. In the extracellular pathway, no fusion with vesicles is observed and the mother centriole directly docks to the cell surface via DAPs, inducing uncapping of the centriole (Zhao et al., 2023). Ciliary pockets are typically missing. While the intracellular pathway has been studied extensively and many proteins involved are known, the extracellular pathway and especially how centrioles are transported to the cell surface is much less understood. This latter pathway is known to occur in epithelial cells, sperm and multiciliated cells.

1.9 Cilia function

Cilia are primarily known for their function as sensory and signaling hubs and contain multiple receptors for downstream signaling pathways. One such pathway is Hedgehog (HH) which involves G protein-coupled receptor (GPCR)-based signaling (Reiter and Leroux, 2017). Proteins of the GPCR family are involved in diverse processes, from light detection in photoreceptor cells, odor sensation in olfactory sensory neurons, cognitive processes in the brain to communication between cells, tissues and organs (Mill et al., 2023). In addition, some Wnt signaling pathways require cilia for their function. Since both HH and Wnt signaling modulate embryonic development and patterning, ciliary functions extend to development and tissue homeostasis. Furthermore, motile cilia have a well described role in calcium signaling. They contribute to mechano-, osmo- and chemosensory functions, responding to flow by opening calcium channels and allowing a rapid influx of calcium ions into the cell (Delling et al., 2013). Another function of motile cilia (nodal 9+0 cilia) is that they create a leftward flow of morphogens, crucial for left-right asymmetry and positioning of organs during development (Nonaka et al., 2002).

1.10 Cilia in health and disease

Due to the widespread functions of cilia in animals it is not surprising that the consequences of ciliary dysfunction are highly pleiotropic. Mutations in genes that encode for ciliary proteins lead to a range of disorders collectively termed ciliopathies (Reiter and Leroux, 2017). Defects in motile cilia can cause Kartageners syndrome with symptoms such as chronic bronchitis, sinusitis, male sterility and situs inversus (Afzelius, 1976). These conditions are referred to Primary Cilia Dyskinesias (PCDs), which despite the name are caused by defects in motile and not primary cilia. Primary cilium dysfunction is associated with a broad range of pathologies such as polydactyly, cranio-facial abnormalities, brain malformation, situs inversus, obesity, diabetes, and polycystic kidney disease (Nigg and Raff, 2009). All of these conditions can be caused by a single ciliopathy, Bardet-Biedl syndrome (BBS), a rare autosomal recessive inherited disorder with a prevalence of approximately 1:100 000 within Europe. Other ciliopathies include polycystic kidney disease (PKD), nephronophthisis (NPHP), Joubert syndrome (JBTS), Alström syndrome (ALMS) and Meckel syndrome (Hildebrandt and Otto, 2005). Ciliopathies can be divided into first-order ciliopathies, with defects in ciliary proteins, and second-order ciliopathies with non-ciliary proteins required for cilia function. An example that exists in both forms would be PCD, which can be caused by mutations in cilia genes but also mutations in cytoplasmic proteins like DNAAF2, DNAAF3 and DYX1C1 needed for the pre-assembly of axonemal dynein complexes (Reiter and Leroux, 2017). Besides these classical ciliopathies, a group of ‘disorders with ciliary contribution’ has recently emerged. According to the Human Phenotype Ontology database >300 additional disorders have been found with phenotypes that might arise through ciliary defects (Mill et al., 2023).

1.11 Centriolar satellites- An Overview

Additional structures regulating centrosome and cilium biology are multiprotein assemblies called centriolar satellites. For a long time centriolar satellites remained enigmatic and much of the early literature on ‘satellites’ in fact described appendages. This, however, changed with the identification of the main scaffolding component pericentriolar material 1 (PCM1). Further studies focused on identifying additional satellite components by

colocalization with PCM1, characterizing their composition, dynamics and function. Recently, centriolar satellites have been put more into focus in part due to their association with a plethora of different biological processes. In the following section I will review the major events in centriolar satellite biology from the identification of seemingly simple protein granules to the complex multiprotein assemblies involved in diverse processes known today. I will highlight important advances towards understanding their function and molecular, cellular and biochemical properties and provide a comprehensive summary of the current state of knowledge. Finally, I will look at the gaps in our understanding about centriolar satellites, setting the stage for this PhD project as well as future research.

1.12 Centriolar satellites – The origin of centriolar satellites and the history of PCM1

The discovery of centriolar satellites dates back to the 1950/60s, when electron dense roughly spherical granules around the centrosome were first seen by electron microscopy and the term “satellites” was first introduced (Bernhard and de Harven, 1960; Bessis and Breton-Gorius, 1958; Theg, 1964). These structures appeared 70-100nm in diameter and were occasionally found to be associated with microtubules. However, due to the lack of information about their molecular nature and architecture they remained understudied for some time. The discovery of the first centriolar satellite protein, pericentriolar material 1 (PCM-1), prompted scientists to study these novel non-membranous granules in more depth. PCM-1 was originally identified as an antigen of human autoimmune sera (Balczon et al., 1999) but soon thereafter its homolog in *Xenopus* oocytes was characterized (Kubo et al., 1999). This study revealed that PCM-1 constitutes distinct cytoplasmic granules that show cell-cycle dependent localization and for a subset of satellites movement towards the centrosome was observed in an ATP-dependent manner in live cells and in vitro reconstituted asters (see section centriolar satellite dynamics). Furthermore, they showed that PCM-1 is also concentrated in bigger electron-dense spherical granules, fibrous granules (~75-400nm in diameter) that appear in the cytoplasm of ciliated cells once ciliogenesis is induced (Kubo et al., 1999; Sorokin, 1968). Fibrous granules were associated with basal body replication in differentiating multiciliated cells; however, their precise role is not known. The first attempt towards understanding the function of centriolar satellites came from studies in human cells, where depletion of PCM1 -besides disrupting the radial microtubule network organization- lead to failure to properly localize centrosome components such as centrin, pericentrin and ninein (Dammermann and Merdes, 2002). In addition, overexpression of a PCM-1 truncation caused endogenous PCM-1 to form large

cytoplasmic aggregates containing mislocalized centrin and pericentrin, while microinjection of antibodies against PCM-1 caused aggregation of these proteins. Thus, centriolar satellites seemed to be somehow involved in regulating centrosomal protein levels.

Centriolar satellites display a high degree of heterogeneity and context-dependent localization, composition and subcellular distribution, as was first reported by Kubo et al., in 2003 in cultured fibroblasts, epithelial cells, hepatocytes and nerve cells (Kubo and Tsukita, 2003). While PCM-1 was present in all cells analyzed, the number of foci and centrosomal concentration of satellites varied greatly. There was also a pronounced cell cycle-dependent relocalization, with a prominent centrosomal localization in prophase and prometaphase, followed by dispersal at later mitotic stages (Dammermann and Merdes, 2002; Kubo and Tsukita, 2003). Recently, this cell-cycle dependent accumulation and dispersal was attributed to the ability of PCM-1 to self-aggregate and it was suggested that some molecular mechanisms likely suppress the self-aggregation of PCM1 and thus disassemble PCM-1 granules during mitosis (Rai et al., 2018). Interestingly, PCM1 reappeared at the end of mitosis when cells re-entered interphase (Dammermann and Merdes, 2002; Kubo and Tsukita, 2003).

1.13 PCM1- a scaffolding protein

PCM1 is not only the first centriolar satellite protein that was identified, it is also essential for centriolar satellite assembly; in the absence of PCM1 satellites dissociate and cargo proteins disperse into the cytoplasm and in some cases relocate to the centrosome (Dammermann and Merdes, 2002; Stowe et al., 2012). The terminology pericentriolar material 1 (PCM1) is somewhat misleading, since unlike many other satellite proteins it is actually absent from the centrosome. Components of centriolar satellites are routinely identified by interaction or colocalization with PCM1; making it an important molecular marker for centriolar satellites; however, we still don't know the underlying molecular mechanism of centriolar satellite assembly. Most other centriolar satellite components are found in other cellular structures such as centrosomes, cilia, Golgi or microtubules (Gheiratmand et al., 2019; Quarantotti et al., 2019). Other proteins like Odf1 and BBS4 are required for the maintaining centriolar satellite integrity but not centriolar satellite assembly; however, they do localize to the centrosome and exhibit important centrosome functions. Therefore, PCM1 is a bona fide scaffolding component, providing a structural platform for centriolar satellites (Lopes et al., 2011; Romio et al., 2003). PCM1 shows differential localization in a cell and tissue-specific

manner. During skeletal muscle differentiation, PCM1 is initially observed around the centrosome in myoblasts but later found as part of an insoluble matrix around the nuclear envelope of myotubes (Srsen et al., 2009). Intriguingly, in a recent study PCM1 has been identified as a component of a meiotic spindle-like domain (LISD) in mouse oocytes, a novel structure thought to form by liquid-liquid phase separation (LLPS) (So et al., 2019). PCM1 is ubiquitously expressed but shows especially high expression in the brain, suggesting that PCM1 might play a role in neurogenesis and related disorders.

From a structural biology perspective PCM1 has been studied to some extent, however its secondary structure can for most parts of the protein only be predicted with low confidence. In humans, PCM1 encodes a large protein of 2024 amino acids (228kDa). Like the known scaffolding proteins of centrosomes in several organisms, PCM1 contains multiple coiled-coil domains throughout the entire structure, interspersed with regions of low complexity. The self-assembly properties of PCM1 mentioned previously could be assigned to two distinct regions (aa 201-494 and 745-1128) using yeast-two-hybrid (Y2H) analysis (Kubo and Tsukita, 2003). Deletion of the C-terminal region of PCM1 (aa 1372-1990), evolutionarily the most conserved region, causes the formation of massive protein aggregates, up to ten times the size of normal satellites (Dammermann and Merdes, 2002). Multiple binding sites between PCM1 and other proteins have been established (e.g. PLK4, Odf1, PLK1, BBS4, CEP131, MIB1), one important interaction that led to the link of PCM1 with autophagy is the interaction of PCM1 with the ATG8-family protein GABARAP via the LIR-motif near the C-terminus (Joachim et al., 2017; Wirth et al., 2019). Another feature of PCM1 is the presence of several intrinsically disordered regions (IDRs), in line with its putative phase separation properties.

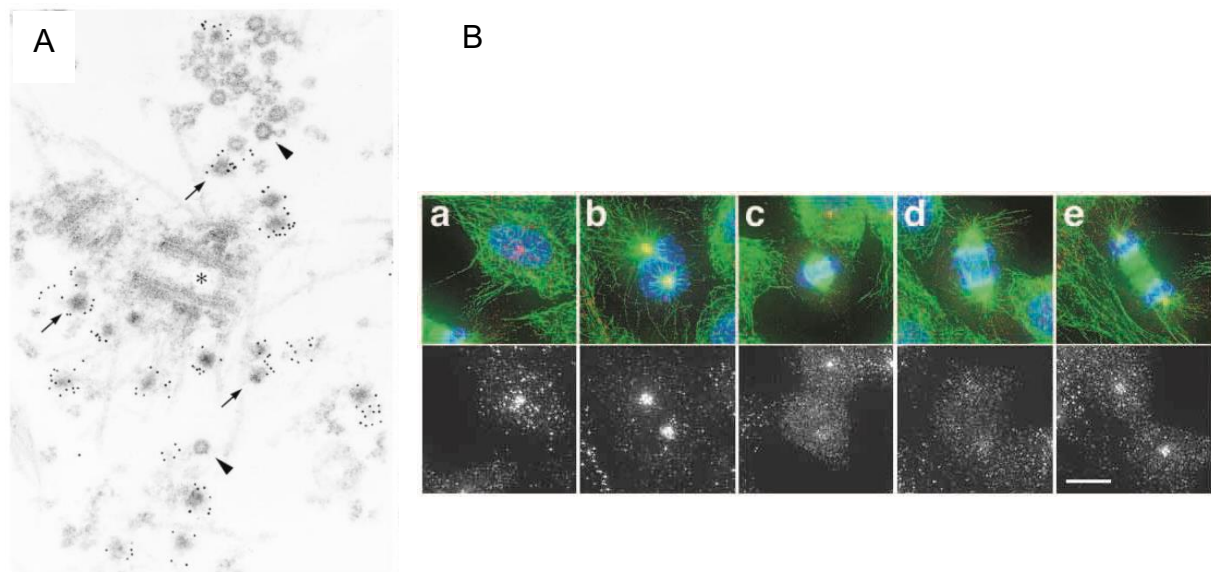


Figure 5. PCM1, the scaffolding component of centriolar satellites. (A) Electron microscopy of A6 cells treated with Triton-X 100 and labeled with anti-XPCM1 antibody. Satellites appear as electron dense granules (arrows) around the centrosome (asterisk) (Kubo et al., 1999). (B) A6 cells stained with XPCM1 antibody (red), FITC-conjugated anti- α -tubulin (green) and DAPI (blue). PCM1 containing centriolar satellites display cell-cycle dependent assembly and disassembly (Kubo and Tsukita, 2003).

1.14 Centriolar satellite dynamics and responses to environmental stresses

With the development of live imaging techniques, scientists for the first time reported on the movement of centriolar satellites towards the minus ends of microtubules, thus towards the centrosome in cultured cells (Balczon et al., 1999; Kubo et al., 1999). This movement seemed to depend on the molecular motor dynein, and independent of kinesin. Additionally, *in vitro* in reconstituted asters a similar behavior was observed, where foci associated with microtubules were moving towards the centrosome. Interestingly, the behavior of some of these granules was peculiar, switching between microtubule tracks before ending up on the centrosome. Despite these reports of microtubule-dependent movement it is worth noting that centriolar satellites were only occasionally found on microtubules and a big subset seemed to be localized randomly in the cytoplasm. Yet, these early observations have led to the assumption that centriolar satellites are highly mobile structures.

In more recent years however centriolar satellite dynamics have been analyzed in a more quantitative manner, showing that centriolar satellites exhibit little directed motility

(Conkar et al., 2019). This study was performed using single particle tracking of PCM1 and another known centriolar satellite protein, CCDC66 in RPE1 cells. Importantly, the majority of centriolar satellites display diffusive behavior, while only a small fraction was seen to undergo directed movement. Analysis of instantaneous speed revealed that these directed movements match the *in vivo* speeds reported for dynein and kinesins (speeds > 0.5µm/s). Direct interactions of PCM1 with motor proteins such as dynein and kinesin have not been found yet; however, in this study CCDC66 was shown to interact with the dynein complex by Co-IP. Collectively, we can conclude that only a minor part of satellites is indeed moving in a directed manner, with the majority either entirely static and moving by classical Brownian motion.

There is, however, a dynamic response that centriolar satellites undergo upon certain stresses and changes in environmental conditions. Low-temperature treatments and cold incubations at 4°C result in a coalescence into larger centrin-positive satellites in PtK2 cells and thus a decrease in the number of foci (Baron et al., 1994). In contrast, elevating intracellular calcium levels by ionomycin treatment lead to an increase in pericentrosomal satellite foci. Other stresses such as ultraviolet (UV) radiation, heat shock and transcription block cause a dramatic reorganization of centriolar satellite distribution and mostly abolish the cytoplasmic fraction of most centriolar satellite proteins (Villumsen et al., 2013). Proteotoxic stress induced not only ciliation but also the dissociation of several key centriolar satellite components (CEP290, CEP131) from satellites. This displacement of centriolar satellite components was further investigated and found to be dependent on the activity of the p38, MK2 and 14-3-3 regulatory proteins, which target CEP131, sequestering it in the cytoplasm and thereby inhibiting the formation of centriolar satellites (Tollenaere et al., 2015). Thus, satellite reorganization requires the MAPK pathway. Moreover, cellular stresses inhibit an E3 ubiquitin ligase and centriolar satellite component, Mib1, in a p38-independent way, thereby promoting ciliogenesis (Villumsen et al., 2013).

Intriguingly, recent observations have provoked speculation as to whether centriolar satellites are phase-separated compartments. A subset of foci imaged by live imaging were undergoing fusion and fission, a common feature of many proteins that are able to undergo phase separation (Banani et al., 2017)(Conkar et al., 2019).

Other reported liquid-like properties like fast (internal) rearrangement upon FRAP, sensitivity towards 1,6-Hexanediol (Kroschwald et al., 2017) were not or only to a minor extent observed for satellite proteins. Specifically, it was shown that GFP-PCM1 in live mouse ependymal cells (mEPCs), as part of fibrogranular material and associated deuterosomes,

undergoes only very slow recovery upon photobleaching (Zhao et al., 2021). However, in an attempt towards characterizing the potential liquid-like properties of PCM1 they expressed and purified three different fragments of PCM-1 with a high disorder probability in *E. coli*, with one fragment forming liquid droplets in the presence of crowding agents. How the full-length version of PCM1 would behave in vitro has yet to be determined.

Another emerging concept is that specific kinases could act on and promote phase transitions. DYRK3, a dual specificity kinase that regulates specificity of P-bodies, was recently shown to interact with centrosomal proteins as well as PCM1 (Rai et al., 2018). Even though not yet proven, it is tempting to speculate that DYRK3 is modulating PCM1 and thereby centriolar satellite phase separation behaviour to allow the reported cell cycle dependent condensation and dissolution of these structures.

1.15 The role of centriolar satellites in centrosome assembly and function

In 2002, loss of function experiments on PCM1 showed for the first time a role for PCM1 and thus centriolar satellites in the assembly of centrin, pericentrin and ninein (Dammermann and Merdes, 2002). This has led to the idea that satellites could be involved in the recruitment of centrosomal proteins and consequently in anchorage of microtubules, as mislocalized ninein caused a loss of microtubule anchorage. The loss of centriolar satellites furthermore was found to impair only radial microtubule organization and not microtubule nucleation capacity of the centrosome. Satellites do not impact microtubule nucleation; however, it was shown that knockdown of one centriolar satellite component CEP72 led to decreased levels of γ -TURCs at the centrosome and subsequent reduction in the nucleation capacity of centrosomes (Oshimori et al., 2009; Oshimori et al., 2006). This was accompanied by reduced stability of spindles poles and hence led to compromised spindle assembly. The observation that centriolar satellites are involved in the recruitment of components to the centrosome and base of the cilium has led to the idea that they act as carrier-proteins that mediate the transport of both centriolar and pericentriolar material proteins from the cytoplasm to the centrosome along microtubules. While some proteins are recruited to the centrosome in a PCM-dependent manner others, such as γ -tubulin, are not (Dammermann and Merdes, 2002). Moreover, depletion of the centriolar satellite protein Par6 α leads to mitotic defects and decreased levels of ninein, PCM1 and centrin at the centrosome in a dynein-dependent manner, further corroborating the recruitment model of centriolar satellites (Kodani et al., 2010).

Collectively, all of these studies show that the assembly of centrosomal proteins and microtubule organization depends on centriolar satellites, and thus on PCM1.

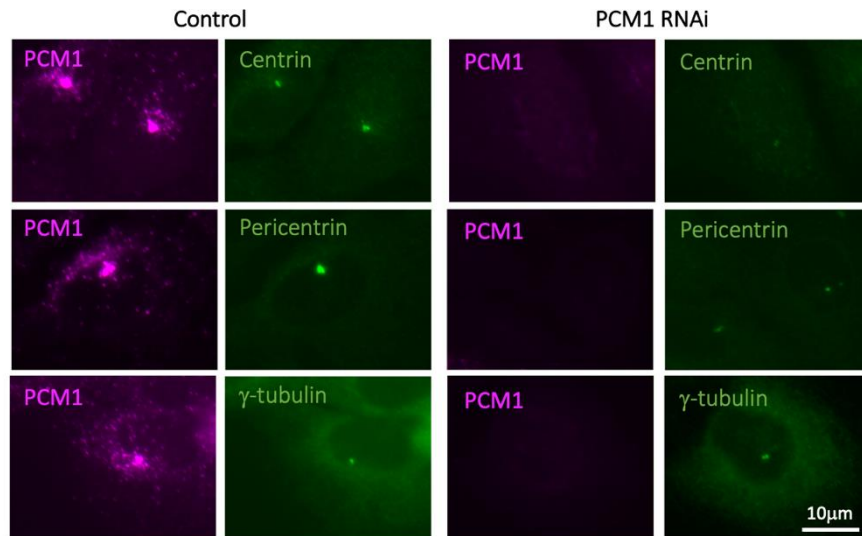


Figure 6. PCM1 is required for recruitment of some centrosomal components. siRNA mediated depletion of PCM1 in U2-OS cells reveals that PCM1 is required for the recruitment of Centrin, Pericentrin and Ninein to the centrosome. Adapted from (Dammermann and Merdes, 2002)

1.16 The role of centriolar satellites in cilium formation and function

Many proteins localized to centriolar satellites are ciliary proteins, some of them known interactors of PCM1. BBS4 and CEP290 for example, are common cilia proteins that are found on centriolar satellites and have an established interaction with PCM1 (Kim et al., 2008; Stowe et al., 2012). Satellites were initially linked to ciliogenesis in studies showing that acute depletion of PCM1 in mammalian epithelial cells and PCM1-targeting morpholinos in zebrafish led to a reduction in the number and length of primary cilia (Kim et al., 2008; Nachury et al., 2007; Stowe et al., 2012). In zebrafish, this caused typical cilia phenotypes such as abnormal body axis curvature, ectopic otoliths and shorter cilia (Stowe et al., 2012). The role of satellites in cilia assembly is best characterized in human retinal epithelial cells, where (CRISPR-) knock outs of PCM1 cause a total lack of ciliation (Odabasi et al., 2019; Quarantotti et al., 2019; Wang et al., 2016). In mouse kidney epithelial cells, cilia were still formed upon

PCM1 deletion but with reduced efficiency as well as delayed Hedgehog signaling (Odabasi et al., 2019). Acute inhibition of centriolar satellites by mispositioning to the cell periphery resulted in reduced ciliation and the formation of shorter cilia (Aydin et al., 2020). In many of the cells where PCM1 was depleted or inhibited, core ciliogenesis factors needed for different steps in the process were mispositioned or their levels at the centrioles and cilia altered. During early ciliogenesis *Talp3* which promotes ciliary vesicle formation was affected as well as CP110, a centriole distal cap protein to inhibit cilium formation, and components of the IFT machinery (Hall et al., 2023; Odabasi et al., 2019; Wang et al., 2016). Aurora kinase A (AURKA) predominately localizes to the basal body and phosphorylates BB components upon loss of PCM1, causing premature cilium disassembly and hence impaired ciliation (Arslanhan et al., 2021). Thus, many core ciliogenesis proteins depend on PCM1 for their recruitment to the basal body/cilium for proper function of primary cilia. In a motile cilia system such as multiciliated cells, loss of PCM1 does not abolish ciliogenesis or reduce the number of centrioles or cilia; however, it causes typical ciliary phenotypes including ciliary immotility and abnormal rotatory beat pattern potentially due to ultrastructural defects in basal bodies and axonemes (Zhao et al., 2021). PCM1 knock out mouse ependymal cells showed delayed centriole amplification and impaired multiciliogenesis (Hall et al., 2023). In contrast, tracheal multiciliated cells (mTECs) depleted of PCM1 exhibit normal basal body biogenesis, ciliogenesis and ciliary beat frequency (Hall et al., 2023). In differentiating mTECs depleting PCM1 resulted in lower levels of many motile ciliary proteins, including dynein motor proteins, assembly factors and docking factors in the early steps of ciliogenesis (Hall et al., 2023). Once again, these studies show that the phenotypic characterization varies greatly between different cell types and organisms. Yet a common feature of PCM1 loss is, that at least one part of cilia structure or function is compromised; an observation that applies to both, primary and motile cilia.

A

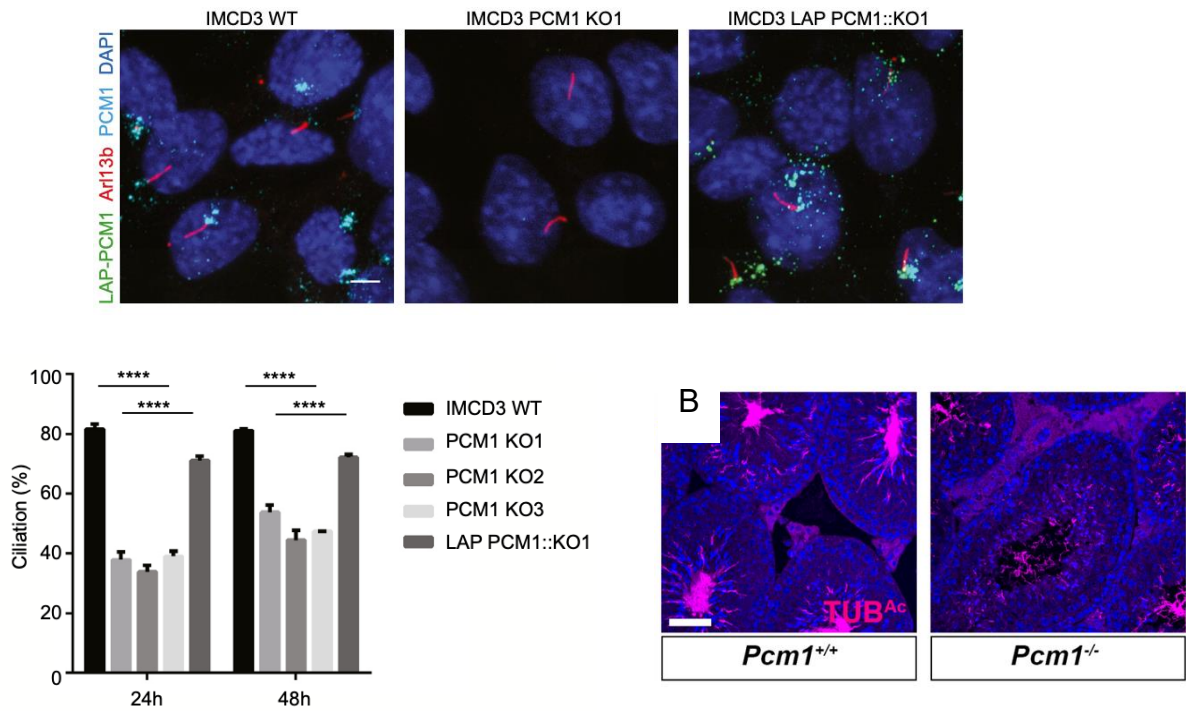


Figure 7. Ciliary phenotypes upon loss of PCM1. (A) Immunofluorescence images of wild-type and PCM1 knockout IMCD3 cells. Loss of PCM1 results in reduced ciliation (Odabasi et al., 2019). (B) Immunofluorescence images of wild-type and PCM1 knockout seminiferous tubules. Deletion of PCM1 leads to a reduction in the number of sperm per tubule (Hall et al., 2023).

1.17 The role of centriolar satellites in proteostasis

Mass spectrometry based studies have identified many proteostasis proteins as putative interactors of PCM1, among them, E3 ligases and antagonistic deubiquitinating enzymes (DUBs) as well as autophagy proteins (Gheiratmand et al., 2019; Quarantotti et al., 2019). The link of centriolar satellites to ubiquitination and protein quality control is best described for the E3 ubiquitin ligase Mindbomb1 (Mib1), which dissociates centriolar satellites, ubiquitinates PCM1 and Cep131 and marks them for proteasomal degradation, thereby suppressing ciliogenesis (Lecland and Merdes, 2018; Wang et al., 2016). PCM1 has been suggested to prevent polyubiquitination and destruction of centriole and centrosome components (such as Talpid3) by sequestering Mib1 to satellites (Wang et al., 2016). In a more recent study CYLD, a DUB, was identified to be part of satellites and to counteract Mib1 activity by removing ubiquitin from Mib1 and suppressing centriolar satellites dissociation and promoting

ciliogenesis (Douanne et al., 2019). In another study the DUB USP9X was found to antagonize PCM1 ubiquitylation to protect it from proteasomal degradation and thereby help to stabilize PCM1 and centriolar satellite integrity (Han et al., 2019). Thus, it was suggested that the interplay between ubiquitination and deubiquitylation is regulating centriolar satellite homeostasis and fine-tuning ciliogenesis. Additional E3 ligases and DUBs are likely involved in maintaining centriolar satellite homeostasis or perhaps have redundant functions (Mib2?), since previous proteomic studies identified an enrichment of several proteins involved in the ubiquitin-proteasome system (Quarantotti et al., 2019). One of the first studies identifying a link between centriolar satellites and proteasomal degradation demonstrated that proteasome inhibitors led to an accumulation of centrosomal components in the PCM and an enrichment of satellites around the centrosome (Didier et al., 2008).

PCM1 and centriolar satellites have also been linked to autophagy. Autophagy describes a conserved, cellular recycling process where degradation of old, damaged or surplus cytoplasmic organelles, proteins and macromolecules contributes to cell survival and maintenance. The process of autophagy takes place under normal conditions but its levels increase in response to a multitude of cellular stresses (Zaffagnini and Martens, 2016). How have satellites been linked to autophagy? A study showed that autophagy deficiency (upon depletion of autophagy regulators ATG7 and ULK1) led to the formation of larger abnormal centriolar satellite structures through accumulation of satellite components as well as dysregulation of centrosome composition (Holdgaard et al., 2019). Further investigation showed that PCM1 and Cep131 interact with one ATG8 family member and satellite component, GABARAP, however not with GABARABPL2 or LC3B. The interaction between PCM1 and GABARAP occurs through its C-terminal LIR motif, promoting selective centriolar satellite turnover (doryphagy). Hence, centriolar satellites are thought to be autophagic substrates critical for maintaining centrosome integrity. Apart from destruction by autophagy, a second mechanism has been proposed in which centriolar satellites are sequestered into aggresomes, big inclusions concentrating misfolded proteins, ubiquitin, molecular chaperons and proteasomes (Johnston et al., 1998; Prosser et al., 2022). Proteasome inhibition, depletion of PCM1 or other satellite components impaired aggresome assembly, causing smaller ring-like aggregates (Prosser et al., 2022). A similar phenomenon has been observed in PCM^{-/-} cells, where smaller aggregates formed by mutant huntingtin (HTT and HAP1) indicating that satellites could play a role in protein misfolded disorders such as Parkinson's, Alzheimer's or Huntington's disease.

Furthermore, the ciliary and centriolar satellite components BBS4 and OFD1 both physically interact with the proteasome and are thought to regulate its composition and activity (Liu et al., 2014). Loss of BBS4 or OFD1 altered the abundance of signaling mediators of the Wnt, Hedgehog and Notch pathways (Gerdes et al., 2007; Liu et al., 2014). Interestingly, OFD1 levels are reportedly regulated by autophagy, thereby representing a link between autophagy and the ubiquitin proteasome system (UPS) (Prosser and Pelletier, 2020; Tang et al., 2013). Taken together, an alternative model for centriolar satellite function is that they represent sites of cellular proteostasis.

1.18 The role of centriolar satellites beyond centrosomes and cilia

The identification of hundreds of proteins enriched in the centriolar satellite proteome has revealed further clusters of biological processes that could potentially provide novel functions for PCM1 or centriolar satellites. Among these are tubulin modifiers, components of P-bodies and the microtubule-nucleation-mediating HAUS complex (Gheiratmand et al., 2019). While the centrosome proteome remains unchanged upon loss of PCM1, the global proteome is altered significantly (Odabasi et al., 2019). Among the most enriched processes/pathways are those related to the actin cytoskeleton such as cell migration, cell adhesion and endocytosis. If and to what extent PCM1 is involved in these processes still needs to be elucidated. A previous report, however, suggested that satellites target actin assembly factors to the centrosome to allow it to act as an actin-nucleating center (Farina et al., 2016). While actin filaments could not be observed on satellites in cells, *in vitro* actin nucleating filaments that lacked acetylated tubulin were positive for PCM1 in approximately 20% of spots analyzed, raising the question if satellites could act as actin nucleating centers or if they are just carrier of the actin nucleation machinery.

Moreover, centriolar satellites have been functionally linked to neurogenesis. Even though the underlying mechanisms are not known so far, it is possible that centriolar satellites exhibit similar or the same function in neurogenesis as for MTOCs. Several proteins of the neuronal body or extensions, postsynaptic membranes or axon guidance were enriched in centriolar satellite proteomic studies. Mutations in PCM1 have been associated with schizophrenia, however mechanisms are unclear and genome-wide studies could not confirm the association (Lam et al., 2019; Ripke et al., 2014). Furthermore, PCM1 and the centriolar satellite constituents HOOK3, DISC1 and SDCCAG8 are involved in neuronal progenitor cell maintenance and neuronal migration during cortical development (Insolera et al., 2014; Prosser

and Pelletier, 2020). A PCM1-MIB1 interaction has also been described in neuronal progenitors where PCM1 regulates the localization of MIB1 to centrioles, which is required for cell fate determination (Tozer et al., 2017). Further corroborating the link between PCM1 and neurogenesis is the fact that PCM1 mutant mouse models show defects at different developmental stages and brain regions: The PCM1 CRISPR knock out in mice led to a reduced brain size, cerebellar hypoplasia while the PCM1 gene trap caused cognitive defects primarily in the postnatal brain (Hall et al., 2023; Monroe et al., 2020).

1.19 Models explaining centriolar satellite function-The transport model

The early studies on PCM1 and its involvement in the recruitment of centrosomal proteins has led to a model in which PCM1 and centriolar satellites are acting as transport modules for centrosomal cargo (Dammermann and Merdes, 2002). This was the prevailing model for a long time, as many follow-up studies were providing further data supporting this observation. Pivotal to this idea was the identification of long-range directed movement of centriolar satellites to and from the centrosome (Kubo et al., 1999). The trafficking was suggested to occur in a microtubule- and dynein-dynactin dependent manner, since dynactin inhibition by dynamitin and prolonged treatment of nocodazole led to reduced levels of centrosomal proteins (ninein, centrin and pericentrin) at the centrosome (Dammermann and Merdes, 2002). Similar experiments in ciliated cells have extended the trafficking function towards the cilium and the delivery of its components.

How did the transport model arise? This concept is based on previous data showing that dynein and dynactin play important roles in microtubule organization at centrosomes with dynactin being required for γ -tubulin assembly (Quintyne et al., 1999). Dynein, a minus-end directed multi-subunit motor, works together with dynactin, a multiprotein complex, which works as an adaptor that allows dynein to bind cargo proteins. The interaction between dynein and dynactin occurs via the dynactin sidearm subunit, p150glued. The so-called shoulder/sidearm consists of p150Glued, dynamitin and p24 subunits and overexpression of these subunits results in microtubule disorganization, altered centrosome organization and delayed microtubule nucleation. Thus, as PCM1 was reported to dock onto the dynein-dynactin complex it is not surprising that PCM1 depletion phenocopies dynactin inhibition, perturbing radial microtubule organization.

When comparing centriolar satellite trafficking to ciliary intraflagellar transport (IFT, see above) it becomes noticeable that despite sharing some features, they lead to different

phenotypes when perturbed. Both systems work by microtubule-based transport of cargo proteins to their respective destination, the tip of the cilium or the basal body in case of IFT, and centrosomes and basal bodies in case of centriolar satellite trafficking. However, what distinguishes them, are the differential effects they have on the structure, their cargo proteins and their function. Mutations affecting IFT proteins or motors showed that they are required for the assembly and maintenance of cilia (Rosenbaum and Witman, 2002). Moreover, disruption of the IFT system leads to mislocalization of cargo proteins and impairs their function, leaving the cilium non-functional or with impaired function (Pedersen et al., 2006). Thus, proteins rely on active transport via the IFT machinery and the molecular motors (kinesin and dynein) that enable the shuttling inside and out of the cilium, while only very few, small proteins and molecules are reported to move by diffusion inside the cilium. In contrast, perturbations affecting centriolar satellites or the dynein-dynactin complex, result in much milder phenotypes. While client proteins in some cases show reduced localization to centrosomes/basal bodies, the consequences of satellite disruption are largely limited with only minor or no effects on their cargo proteins and in turn little effect on centrosome and cilium structure and function.

1.20 A model for centriolar satellites in proteostasis

In more recent years, researchers began to think of satellites not as facilitators of cellular transport but as structures involved in maintaining protein homeostasis. This was primarily triggered by the identification of several satellite components with links to centrosomes, cilia but also autophagy and protein degradation (Gheiratmand et al., 2019; Quarantotti et al., 2019). In addition, not all centriolar satellite proteins depend on PCM1 for their recruitment to the centrosome/cilium; Cep131 for example is found on centriolar satellites but is localizing to the centrosome in the absence of PCM1 (Gheiratmand et al., 2019). Thus, the trafficking model fails to explain all phenotypes observed upon loss of PCM1. An opposing model is that centriolar satellites are sequestering proteins to centriolar satellites, potentially providing a favorable environment for proteins that do not (yet) fulfill their function. In this model centriolar satellites act as modulators of cellular homeostasis/proteostasis by tethering proteins into centriolar satellites and potentially promoting or suppressing their degradation.

The concept of centriolar satellites as sites of protein sequestration and homeostasis has been put forward by several groups. Interestingly, two labs showed that centriole depletion left satellite composition largely unaffected, suggesting that centrosomes and centriolar satellites

form independent of each other despite sharing a large subset of components (Gheiratmand et al., 2019; Quarantotti et al., 2019). Thus, centriolar satellites could provide a reservoir of centrosomal proteins in conditions where transcription or translation of centrosomal proteins are limited (Quarantotti et al., 2019). Furthermore, disruption of the microtubule network did not alter satellite composition, with most client proteins remaining in close association with PCM1 (Gheiratmand et al., 2019). These observations are unexpected when thinking of centriolar satellites as microtubule-based transport modules that contribute to centrosome/cilium assembly and function. Taken together, these studies support a model for satellites in mediating protein homeostasis.

1.21 PCM1 in different cells and organisms/Evolutionary conservation of PCM1

As described above, PCM1 and centriolar satellites are very heterogeneous in their localization, composition and potentially their function. Due to the assumption that satellites exist only in vertebrates, they have largely been studied in vertebrate tissue culture cells including ciliated cells such as RPE1 or IMCD3 cells as well as non-ciliated cells. However, since vertebrates harbor powerful checkpoints that monitor centrosome integrity and cause a p38-dependent cell cycle arrest when satellites are depleted the observed effects could also be direct or from indirect consequences of perturbed cell cycle progression or a cell culture artefact (Srsen et al., 2006; Vladar and Stearns, 2007). Furthermore, studies of satellites in vivo have been very limited and only until recently a CRISPR knockout mouse has been made (Hall et al., 2023).

Even though the evolutionary conservation of centriolar satellites was thought to be limited to vertebrates, a recent study gave reason for speculation. Heptad repeats that form the basis of coiled-coils hinder conventional sequence alignments by overestimating sequence similarities between these repeats, resulting in false positives. Hence, coiled-coil sequences are usually masked from standard similarity searches, complicating the identification of true homologs. However, a refined sequence alignment technique optimized for coiled-coil proteins showed 17% more reciprocal best hits than conventional BLAST for centrosomal proteins (Kuhn et al., 2014). PCM1, itself a coiled-coil protein was included in this analysis and was shown to be present outside of vertebrates, across opisthokonts. Interestingly, the study identified orthologs in the model organisms *Drosophila melanogaster* (CG10732 or Combover) and *C. elegans* (Y56A3A.7), suggesting that the evolutionary distribution of PCM1

and potentially centriolar satellites in general is wider than previously thought. This raised the question to what extent PCM1 and satellites in these organisms are similar, if they exist and if so, if they share the same function.

1.22 Aims of my PhD Project

The aim of my PhD project was to study PCM1 in *Drosophila* to gain insights into the role of PCM1 (and centriolar satellites) at an organismal level. Taking advantage of *Drosophila* as a model organism to examine centrosome and cilium function we set out to investigate different aspects of centriolar satellite biology, for the first time outside of vertebrates. Using *Drosophila* as a model system we could also look at PCM1 at an organismal level under physiological conditions, providing important in vivo data. With this we intended to broaden our understanding of the complex role of satellites in the context of centrosome and cilium biology as well as potentially identify putative conserved features and functions. Comparative approaches ultimately help in narrowing down the diverse functional associations or as Theodosius Dobzhansky, an Ukrainian-American geneticist, once said: ‘Nothing in biology makes sense except in the light of evolution’ (Dobzhansky, 1973). To identify putative common themes, we aimed to answer the following questions:

- Do satellites exist in flies and what is their localization?
- Is Combover (Cmb) involved in centrosome/cilium function?
- What are the dynamics of Cmb-containing centriolar satellites?
- What is the composition of Cmb-containing centriolar satellites?
- What is the function of satellites in *Drosophila*?
- Which aspects of satellite composition, dynamics and function are conserved?

2 Methods

The following experimental models, methods and materials are as described in the preprint Pachinger et al., 2024 (Pachinger et al., 2024) with minor clarifications in the text and including additional experiments.

2.1 Experimental models

2.1.1 *Drosophila melanogaster* stock and husbandry

All flies were maintained on standard media at 25°C and flipped to new bottles/vials every 3-4 days. For RNAi experiments, 4 UAS-hairpin RNAi males and 5-8 GAL4 driver line females were crossed in standard vials and allowed to mate for 4 days at 25°C, then shifted to 29°C to induce GAL4 expression. W¹¹¹⁸ flies were used as controls for all experiments, white^{RNAi} as controls for RNAi experiments. All strains used in this study are listed in Table 1. Cmb mutants and flies overexpressing Cmb-RA under GAL4/UAS control were a kind gift from Andreas Jenny. Flies expressing RFP-Cnn, H2A-RFP and Asl-GFP are from Jordan Raff, Bam-GAL4 (Chen and McKearin, 2003) and Pnr-GAL4 (Calleja et al., 2000) driver lines a gift from Helen White-Cooper and Jürgen Knoblich, respectively. The GFP nanobody-TurboID construct under eggless promoter was cloned from an NLS-containing nanobody-Turbo construct from the lab of Julius Brennecke to eliminate the NLS and flies generated by integration into the attP40 landing site on Chromosome 2 by the IMBA Fly Facility. All other fly strains were acquired from the Vienna *Drosophila* Resource Center (VDRC) or Bloomington *Drosophila* Stock Center (BDSC) or generated by genetic crosses as detailed in the key resources table. Detailed information on strains is available on FlyBase (<http://flybase.org>), as well as on the websites of the two stock centers (BDSC, <https://bdsc.indiana.edu>, VDRC, <https://stockcenter.vdrc.at/control/main>).

2.1.2 Insect cell culture

Drosophila Schneider S2 cells were obtained from Life Technologies and cultured in Schneider's medium containing 10% Fetal Bovine Serum (FBS), penicillin (50 units/ml), and streptomycin (50 µg/ml). Cells were kept in incubators at 25°C at atmospheric CO₂ and passaged every 3-4 days. For proximity-based interaction analysis of Sas4 by BioID in S2 cells, a construct expressing myc-BirA*(R118G) under the copper-inducible metallothionein promoter (pMt-myc-BirA*) was made by inserting the myc-BirA* coding sequence (derived

from a plasmid obtained from Kyle Roux) into the pMt-V5-6xHisB vector backbone (Invitrogen). The coding sequence of Sas4 was then inserted into that construct by PCR and restriction cloning (Gibson cloning). For Cmb TurboID, the coding sequence of Cmb-RA was cloned into the pDONR Zeo entry vector (Invitrogen) and then transferred into the pMT-TurboID-V5 vector (a gift from Norbert Perrimon) by Gateway cloning. To follow Cmb dynamics in S2 cells, the coding sequence of Cmb-RA was cloned into pMt-V5-6xHisB by Gibson cloning. Clonal cell lines expressing GFP/biotin ligase fusions were generated using TransIT transfection reagent (Mirus) by co-transfection of 0.6µg/ml expression plasmid with 0.06µg/ml pCoBLAST selection plasmid (Invitrogen) and using serum-free medium. Cells were grown for 3-5 days before selection with 25µg/ml blasticidin. GFP/biotin ligase expression was induced by addition of 500/100µM of CuSO₄ for 24h before imaging or harvesting.

Puromycin labeling in S2 cells was performed as described in Hao et al. (Hao et al., 2021). Cells were incubated with 10µg/ml puromycin for different incubation times (30sec, 1min, 3min, 5min, 10min). Control cells were treated with DMSO for the same period of time or pre-treated with 10µg/ml Cycloheximide (CHX) for 30min to stop translation prior to the addition of puromycin. After treatment cells were immediately fixed in -20°C methanol and processed for immunofluorescence as described below.

2.1.3 Vertebrate cell cultures

HeLa CCL-2 cervical carcinoma cells and their derivative, *PCNT* mutant cell line ODCL0025(Watanabe et al., 2020), were a kind gift from Karen Oegema and cultured in DMEM containing 10% FBS, 25mM Hepes (Gibco) and non-essential amino acids (Gibco). Human osteosarcoma U2-OS HTB-96 cells are from the American Type Culture Collection (ATCC) and were cultured in Dulbecco's Modification of Eagles Medium (DMEM) containing 10% FBS, penicillin (100 units/ml), and streptomycin (0.1mg/ml). Cells were grown at 37°C in 5% CO₂ and passaged every 3-4 days. All cell lines were regularly tested for mycoplasma contamination. RNAi-mediated depletion of PCM1 was performed by using a mixture of two siRNA oligonucleotides ((Dammermann and Merdes, 2002), see key resources table), purchased from Sigma-Aldrich and introduced into cells by Lipofectamine RNAiMAX-mediated transfection following manufacturer's instructions. Phenotypes were assessed 48 hours after transfection.

2.2 Method Details

2.2.1 Identification of orthologs of centrosomal/ciliary genes across opisthokonts

PCM1 orthologs and orthologs of centrosomal/ciliary genes across different phyla were identified by reciprocal BLAST search (BLAST+ 2.13.0, (Camacho et al., 2009)) performed locally using the human protein as the starting point, with bidirectional best match at an E-value threshold cutoff of 0.1 as a simple but robust (Kristensen et al., 2011) method to infer orthology. Where direct comparisons failed to identify a clear ortholog, indirect searches were carried out using less divergent related species as intermediates, as well as by applying hidden Markov models with HMMER (Eddy, 2011) using alignments of known representatives, constructed with MAFFT (Katoh et al., 2019). Conserved motifs within PCM1 were identified with the help of Meme (<https://meme-suite.org/meme/>) and sequences aligned using MUSCLE within Jalview (<https://www.jalview.org>). The presence of centrosomes and cilia within fungi, Nematomorpha and Platyhelminthes was inferred from the conservation of core centriolar (STIL/Ana2, SASS6/Sas6, CENPJ/Sas4, CEP135/Bld10), centrosomal (CDK5RAP2/Cnn, CEP192/Spd2) and ciliary (transition zone, IFT and BBS components, inner and outer dynein arm components, dynein assembly factors, nexins, N-DRC, radial spoke and central apparatus components (Dobbelaere et al., 2023)) proteins, as well as literature reports (Azimzadeh et al., 2012; Grell and Benwitz, 1981).

2.2.2 *Drosophila* behavioral assays

To examine fly coordination, 10 three-day-old adult males were collected in 8cm graduated, flat bottom tubes. Flies were left for 30min to recover from the anesthetic and acclimatize, then banged to the bottom of the tube and filmed climbing back upward. Videos were analyzed to establish the time at which all flies had crossed the half-way mark. Experiment was repeated 3 times per condition.

To assess male fertility, single three-day-old males were crossed with four control virgin females in standard culture vials. After mating for 1 hour, males were removed and females kept in the vial for an additional 24 hours and the number of offspring counted prior to hatching. Experiment was repeated 3 times with 10 males per condition.

To assess embryonic viability, two virgin females and one male were put into cages with apple juice plates and allowed to lay eggs for 24 hours at 25°C. Agar plates were

transferred into a humid chamber and the number of hatched and unhatched embryos determined after 24 and 48 hours.

2.2.3 *Drosophila* immunofluorescence staining

Rabbit polyclonal antibodies against the N-terminus (amino acids 1-150) of *Drosophila* Cmb were raised using a GST fusion as antigen and purified over the untagged cleaved antigen as described (Oegema et al., 2001). The following primary antibodies were used for immunofluorescence in *Drosophila*: mouse anti-puromycin (clone 12D10, Merck Millipore) at 1:200, rabbit anti-Cmb antibody (this study), 1:300, mouse anti-NompC (Liang et al., 2011), rabbit anti-Ana1 (Conduit et al., 2010), rabbit anti-Cep97 (Dobbelaere et al., 2020), rabbit anti-Sas4 (Basto et al., 2006), all at 1:500, rabbit anti-Cnn (Dobbelaere et al., 2008), 1:600, rabbit anti-CP110 (Franz et al., 2013), 1:700, rabbit anti-Spd2 (Cabral et al., 2019), 1:800, rabbit anti-Ana2 (Cabral et al., 2019), 1:900, mouse anti- γ -tubulin (GTU88; Sigma-Aldrich), 1:1000. Secondary antibodies (Jackson ImmunoResearch) were used at 1:100.

For immunofluorescence staining in S2 cells, cells seeded on Concavalin A-coated coverslips were fixed in 4% formaldehyde for 15min at room temperature (or -20°C methanol for 20min in the case of puromycin labeling), permeabilized with PBS 0.2% Tween-20 for 15min and blocked 20min in 5% BSA in PBS 0.1% Triton-X100. Cells were incubated with primary antibodies in blocking solution for 1 hour, washed 3x 5min with PBS 0.1% Triton-X100 and incubated with secondary antibodies in blocking solution for 1 hour. Following staining with 1 μ g/ml Hoechst in PBS for 5min cells were washed 3x 5min with PBS 0.1% Triton-X100 and once with PBS before being mounted in Vectashield.

For immunofluorescence staining in testes, adult males were dissected in ice-cold PBS, the testes transferred to a microscope slide, covered with a coverslip and flash-frozen in liquid nitrogen. After recovering slides and removing the coverslip, samples were incubated for 5min in -20°C methanol and 1-2min in -20°C acetone. Samples were then rinsed with PBS, rehydrated in PBS 1% Triton X-100 for 10min and washed 2x with PBS for 5min, followed by blocking with 1% BSA in PBS for 45min. Primary antibodies in blocking solution were then added and samples incubated overnight at 4°C. The next day, samples were washed 2x with PBS for 5min before incubation with secondary antibodies in blocking solution for 1 hour. Finally, after 2x washes with PBS for 5min, samples were stained with 1 μ g/ml Hoechst in PBS for 5min and mounted in Vectashield.

For immunofluorescence staining of chordotonal neurons, leg chordotonal organs were dissected out of 36 hour old male pupae, placed between a microscope slide and coverslip and

gently pressed to remove them from the cuticle. Fixation and staining were performed as described for testes above.

For immunofluorescence staining of neuroblasts, brains from third-instar larvae were dissected in PBS and placed in 4% paraformaldehyde in PBS for 20min, treated with 45% acetic acid for 15s, followed by 60% acetic acid for 3min, then covered with a coverslip and squashed between coverslip and slide before flash-freezing in liquid nitrogen. After recovering slides and removing the coverslip, samples were fixed in methanol for 5-8min at -20°C, washed 4x 15min with PBS 0.1% Triton-X100 and incubated in primary antibody in PBS 0.1% Triton-X100 overnight at 4°C. The next day, brains were washed 3x 5min with PBS 0.1% Triton-X100 and incubated with secondary antibody overnight at room temperature for 3-4 hours. Finally, samples were washed 3x 15min with PBS 0.1% Triton-X100, stained with 1µg/ml Hoechst in PBS for 5min and mounted in Vectashield.

For embryo immunofluorescence, flies were put into cages on apple-juice plates with yeast-paste 3 days before embryo collection. Early embryos (0-2 hours) were collected from plates, washed and quickly treated with 5% bleach to dechorionate them and fixed in heptane/4% paraformaldehyde in PBS for 2min. Heptane was replaced by methanol and samples incubated for 1min. Embryos were rehydrated with PBS 0.1% Triton X-100 for 15min rotating, blocked 2x with 5% BSA in PBS for 30min before incubation with primary antibody in blocking buffer overnight at 4°C. The next day, embryos were washed 3x with PBS 0.1% Triton X-100 for 20min and incubated on the rotator with secondary antibodies in blocking buffer for 1 hour. Finally, embryos were washed in PBST, stained with 1µg/ml Hoechst in PBS for 5min, washed again and mounted in Vectashield.

2.2.4 *Drosophila* wholemount testes staining

For Hoechst/Phalloidin wholemount testes staining, adult males were dissected in PBS and testes transferred to Nunc MicroWell MiniTrays containing PBS and fixed in 4% paraformaldehyde in PBS for 25min. Testes were washed 3x with PBS 0.1% Triton-X100 for 5min and stained with 5µg/ml Hoechst and 1:50 Phalloidin Alexa Fluor 568 in PBS overnight at 4°C. The next day, testes were washed 3x with PBS, carefully separated from the rest of the abdomen and placed in a drop of Vectashield on a multi well slide and covered with a coverslip.

2.2.5 Immunofluorescence staining in vertebrate cells

The following primary antibodies were used for immunofluorescence in vertebrate cells: rabbit anti-MIB1 (Sigma-Aldrich), 1:100, rabbit anti-AZI1 (Abcam), 1:250, mouse anti-hsPCM1 (G-6, Santa Cruz), rabbit anti-PCM1 (Dammermann and Merdes, 2002), both 1:300, rabbit anti-CDK5RAP2 (Merck Millipore), rabbit anti-CEP290 (Novus Biologicals), rabbit anti-PCNT (Abcam), all 1:400, rabbit anti-OFD1 (Sigma-Aldrich), 1:500, mouse anti- γ -tubulin (GTU88; Sigma-Aldrich), 1:1000. Secondary antibodies (Jackson ImmunoResearch) were used at 1:100.

For immunostaining in vertebrate cells, cells were fixed in -20°C methanol for 20min, then washed and rehydrated in PBS 2x 5min. Permeabilization was done in PBS + 0.1% Tween-20 for 5min, followed by blocking in 0.5% BSA in PBS-Tween-20 for 5min. Cells were subsequently incubated with primary antibodies in blocking buffer for 40min, washed 2x 5min with PBS and once with PBS-Tween-20. Cells were then incubated with secondary antibodies for 20min and stained with 1 μ g/ml Hoechst in PBS for 5min. After 2 further washes with PBS (5min each) and once with PBS-Tween-20, coverslips were mounted on slides with Vectashield. For immunofluorescence experiments including the mouse anti-hsPCM1 (G-6, Santa Cruz) antibody, we followed the protocol described in (Chen et al., 2022).

2.2.6 Single-molecule inexpensive fluorescence in situ hybridization (smiFISH)

SmFISH was performed according to Tsanov et al. (Tsanov et al., 2016) with the following adaptations: FISH probes were designed using Stellaris Probe designer (www.biosearchtech.com/stellarisdesigner) and 5' conjugated with FLAP-X sequence (5'- CCTCCTAAGTTTCGAGCTGGACTCAGTG-3') and produced and purchased in 96-well plates (low scale synthesis 25nmol, 100 μ M, resuspended in TE buffer) from Sigma Aldrich. Secondary probes contain the FLAP-Y sequence, the reverse complement of the FLAP-X sequence (5'- CACTGAGTCCAGCTCGAACTTAGGAGG-3') conjugated with Quasar-570, Quasar-670 or Cy5 at both the 5' and 3' end. Secondary probes were generated and purchased from Biosearch Technologies or Sigma Aldrich. FLAP hybridization was performed according to Tsanov et al. and probes used for smFISH (-IF) as described below. Sequences and transcript accession numbers are provided in Table.

2.2.7 Sequential single molecule fluorescence in situ hybridization (smFISH) and immunofluorescence staining in vertebrate cells

Fluorescence in situ hybridization probes against the coding sequence of *CEP290* (transcript variant 1, Accession: NM_025114) and *PCNT* mRNA (transcript variant 1, Accession: NM_006031) were designed using Stellaris Probe Designer (www.biosearchtech.com/stellarisdesigner) and ordered conjugated with Quasar 570. Sequences can be found in the key resources table. Probes were resuspended in TE buffer at 25mM. The protocol for sequential smFISH-IF was adapted from (Bayer et al., 2015) and (Trcek et al., 2017). Cells were fixed in 4% paraformaldehyde for 15min, washed 3x 10min with PBS 0.05% Triton-X100, then incubated in warmed pre-hybridisation buffer/FISH wash buffer (10% formamide, 2x SSC) at 37°C for 20min before incubation in hybridization buffer (10% formamide, 10mg/ml sheared salmon sperm DNA, 10% dextran sulfate, 10mM Vanadyl ribonucleoside complexes solution) containing 300nM smFISH probe overnight at 37°C. The next day cells were permeabilized and blocked with 1% BSA in 2x SSC 1% Triton-X100 for 2 hours (solution exchanged twice during incubation). Cells were then washed 2x with 2x SSC and incubated with primary antibody in 2x SSC and 1% Triton-X100 for 1 hour, washed 3x with IF wash buffer (2x SSC, 0.5% Triton-X100 in ddH₂O), and incubated with secondary antibody solution in 2x SSC for 2-4 hours at room temperature. Finally, cells were washed 3x 10min with IF wash buffer, stained with 1µg/ml Hoechst in PBS for 5min and mounted in Vectashield.

2.2.8 Fixed imaging

For imaging of fixed samples (except wholemount testes staining), 0.2µm 3D widefield datasets were acquired using either a 60x 1.42NA UPLanX Apochromat or a 100x 1.4NA UplanS Apochromat lens on a DeltaVision Ultra Epifluorescence Microscope equipped with a 4-Megapixel sCMOS camera and 7-Color SSI module, computationally deconvolved using SoftWorx and imported into Fiji for post-acquisition processing. Exposure settings were held constant across conditions.

For wholemount testes staining, samples were imaged on a Zeiss LSM 700 confocal laser scanning microscope equipped with 40× 1.3NA Plan Apochromat oil immersion objective. Image stacks of approximately 20 z-planes were acquired at 4 µm increments for each of the six testes. Maximum projections from 4 z-stacks were stitched together using ZEN software (Zeiss) to cover the whole testis.

2.2.9 Live cell imaging

For live cell imaging of *Drosophila* syncytial-stage embryos, 0-2 hour embryos were collected on apple juice agar plates, dechorionated on double-sided tape, immobilized on glass-bottom dishes coated with Scotch tape adhesive dissolved in heptane and mounted with Voltalef oil. Embryos were imaged on a Yokogawa CSU-X1 spinning disk confocal mounted on a Zeiss Axio Observer Z1 inverted microscope equipped with a 63x 1.4NA Plan Apochromat lens, 100mW 488nm and 200mW 561nm solid state lasers and a Hamamatsu ImageEM X2 EM-CCD camera and controlled by VisiView software (Visitron Systems). 14x0.75 μ m GFP/mCherry z-series were acquired at 15s intervals, with manual focus adjustment between intervals if needed, using low laser illumination to minimize photobleaching. Image stacks were imported into Fiji for post-acquisition processing.

For live cell imaging in S2 cells, GFP expression was induced with CuSO₄ 24hours before imaging. On the day of imaging, cells were plated on glass bottom dishes and allowed to settle for at least 30min. Imaging was performed on a Yokogawa CSU X1 spinning disk confocal mounted on a Zeiss Axio Observer Z1 inverted microscope equipped with a 100x 1.3NA EC Plan-Neofluar lens, 100mW 488nm solid-state laser and an Evolve EM512 EMCCD camera (Photometrics) and controlled by VisiView software (Visitron Systems). 0.5 μ m GFP z-series were acquired at 5s intervals, using low laser illumination to minimize photobleaching. Image stacks were imported into Fiji for post-acquisition processing.

For live cell imaging of sperm motility, seminal vesicles of 3-day old males were dissected in PBS and immediately transferred into a drop of Schneider medium on a microscope slide. The seminal vesicle was pierced with a sharp tungsten needle, mineral oil used to encircle the drop, a coverslip placed on top and sperm motility imaged immediately using dark field settings on a Zeiss Axio Imager Z2 microscope equipped with a 63x 1.4NA Plan-Apochromat lens at 12 frames/s. Image sequences were imported into Fiji for post-acquisition processing.

2.2.10 Quantification of centrosome intensity in the early embryo

Analysis of centrosome intensity in the early embryo was done as described in (Alvarez-Rodrigo et al., 2019) with modifications described hereafter. Maximum intensity projections of z-stacks were generated and image sequences bleach-corrected using the ImageJ plugin 'Bleach correction' (Miura, 2020) applying the exponential fitting method. Centrosome intensity (RFP:Cnn channel) was analyzed at nuclear envelope breakdown (NEBD). A fixed-sized ROI (4.88 μ m x 4.88 μ m) was placed manually around the center of each centrosome and

the mean intensity of this centrosome ROI computed using Fiji. For background quantifications, we analyzed mean intensities within the same sized ROI placed in three cytoplasmic regions close to the centrosome, calculated the average and subtracted this value from the centrosome mean intensity. For each embryo, all centrosomes that were fully captured within the z-stack were used for quantification and included in the statistical test. The final average mean intensity per embryo was calculated and plotted in GraphPad Prism.

2.2.11 Colocalization analysis

To assess colocalization of Cmb/PCM1 with proteins of interest the Fiji colocalization Plugin JaCoP(Bolte and Cordelières, 2006) was used for both image channels. A cytoplasmic region containing satellite foci away from the nucleus of size $3.5\mu\text{m} \times 3.5\mu\text{m}$ was defined in Fiji for both channels. To assess colocalization at the centrosome, the ROI for both channels was positioned such that the centrosome was located not in the center but at one extreme of the ROI. The 3D z-stack was cropped and segmented using the built-in threshold option. To exclude random colocalization, one of the channels was rotated by 90° . The Pearson's correlation coefficient was calculated and used as an indicator of the degree of overlap. Any difference between experimental and randomized condition was assessed by statistical testing.

2.2.12 Quantification of number of foci

For quantification of number of satellite or mRNA foci, maximum projections were generated in Fiji. Pixel-based segmentation of foci was carried out using Ilastik with the simple segmentation method. A custom-made Fiji macro was used to convert the background and signal image classes into 0 and 1s, respectively. The segmented images were analyzed in Matlab with a custom-made script. In brief, the maximum intensity projections of DAPI, centrosome and foci channels were combined into a single image, the user given the opportunity to adjust brightness and contrast to make the outline of the cells visible and the outline of individual cells manually delineated using the CROIEditor.m function (<https://github.com/aether-lab/prana/blob/master/CROIEditor.m>). Segmented images were post-processed using the watershed algorithm and the area and intensity of foci quantified using regionprops. The cell background intensity was calculated, results for intensities, number of foci per cell and foci size imported into a .csv table and analyzed in Excel.

2.2.13 Proximity analysis for mRNA and protein

To assess proximity of mRNA and protein the object-based Fiji plugin DiAna(Gilles et al., 2017) was used. While DiAna is capable of proximity measurements in 3D, a 2D measurement was chosen in order to take advantage of the ‘shuffle’ function to test for non-random proximity. Deconvolved, single-plane images for the two respective channels (mRNA and protein) were loaded into Fiji and segmented using global thresholding and Gaussian filtering implemented in the plugin. Distance to the first nearest object (center-center distance) was calculated. To assess statistical significance, distances between pairs of proximal particles was compared to their counterparts in randomized images. For this purpose, a mask was applied using Fiji ‘create mask’ to manually outline each cell (excluding the nucleus, mitotic and apoptotic cells). In randomized conditions, the ‘shuffle’ function was used to randomly distribute objects within the mask created for each cell and distances calculated as described before.

2.2.14 Quantification of satellite signal around centrosomes

To determine the subcellular distribution of PCM1 and Cmb relative to the centrosome, radial profiles were quantified. First, maximum intensity projections were created, thresholded and the center of mass of each centrosome based on γ -tubulin signal identified using the ‘analyze particles’ function in Fiji. A circular ROI was placed around this point and the plugin Radial Profile Plot (<https://imagej.nih.gov/ij/plugins/radial-profile.html>) used to obtain a radial intensity profile of satellite foci centered around each centrosome. Concentric rings and corresponding integrated intensities were quantified within 5 μ m of the centrosome. For background quantifications, three smaller sized circular ROIs in distant cytoplasmic regions without foci were used to subtract from the mean intensity value. Intensities were then normalized to the central ring, all intensities were combined, averaged and mirrored to plot a single symmetric radial profile around the centrosome.

2.2.15 Single particle tracking and analysis of satellite dynamics

Time-lapse recordings of Cmb foci were first exported and processed using Fiji to create maximum intensity projections. Segmentation of Cmb foci was performed using Ilastik (v1.4.0-OX)(Berg et al., 2019) and the simple segmentation method(Geisler et al., 2023). Particles exceeding 700nm in diameter or exceeding a mean fluorescence signal intensity two-

fold higher than average were excluded from analysis to avoid artefacts of overexpression. Single particle tracking of Cmb-GFP satellites in S2 cells was performed using Trackmate (v7.11.1)(Tinevez et al., 2017) with the following settings: Ilastik detector, simple linear assignment problem (LAP) tracker, maximum linking distance of 5 μ m, maximum gap-closing distance of 5 μ m and maximum gap-closing of 2 frames. The resulting spots and tracks features were exported and further analyzed in Excel. Instantaneous speed, average speed and mean squared displacement were computed from coordinates of satellites from trajectory analysis. Tracks were further characterized using TraJClassifier (v0.8.1)(Wagner et al., 2017), a Fiji plugin that classifies trajectories into normal diffusion, subdiffusion, confined diffusion and directed/active motion by a random forest approach. Parameters used were as follows: minimum track length of 11 frames, window size (positions) of 10, segment length of 10, resample rate and pixel size 0 and frame rate of 0.2 frames per second.

2.2.16 Transmission electron microscopy

Transmission electron microscopy was carried out as previously described in (Dobbelaere et al., 2020). For EM of chordotonal organs, legs from 36hour old pupae were cut off with microscissors and fixed in 2% glutaraldehyde and 2% paraformaldehyde in 0.1mol/l sodium phosphate buffer (pH 7.2) in a desiccator for 2 hours at room temperature and then overnight on a rotator at 4°C. The next day, legs were rinsed with sodium phosphate buffer, post-fixed in 2% osmium tetroxide in buffer on ice, dehydrated in graded series of acetone on ice and subsequently embedded in Agar 100 resin. 70nm sections were cut and post-stained with 2% uranyl-acetate and Reynolds lead citrate. Sections were examined on a Morgagni 268D microscope (FEI) operated at 80kV. Images were acquired with a 11 megapixel Morada CCD camera (Olympus-SIS).

For EM of testes, late pupal testes from third instar larvae were dissected in PBS and fixed using 2.5% glutaraldehyde in 0.1mol/l sodium phosphate buffer, pH7.2 for 1 hour at room temperature. Samples were then rinsed with sodium phosphate buffer, post-fixed in 2% osmium tetroxide in ddH₂O on ice, dehydrated in a graded series of acetone and embedded in Agar 100 resin. 70nm sections were then cut, processed and imaged as described above.

2.2.17 BioID and TurboID in *Drosophila* S2 cells

BioID and TurboID pulldown was performed as detailed in (Roux et al., 2012), with minor modifications. Cells expressing pMt-myc-BirA*, pMt-Sas4-myc-BirA*, pMt-V5-

TurboID-GW, or pMt-V5-TurboID-Cmb were incubated overnight with 50µm biotin, washed 3x with PBS and lysed in ELB+ buffer (150mM NaCl, 50mM Hepes pH7.5, 5mM EDTA, 0.3% NP-40, 6% Glycerol) supplemented with Roche cOmplete Mini EDTA-free Protease-Inhibitor-Cocktail (1 tablet/10ml lysis buffer), 1mM PMSF and 1mM Benzamidine. Lysates were clarified by brief centrifugation at 200g for 3min at 4°C in a benchtop centrifuge before pelleting insoluble cellular material including centrosomes by centrifugation at 20000g for 30min at 4°C. To perform TurboID on the detergent-soluble cytoplasmic fraction, supernatant was used directly for pulldown. For the detergent-insoluble cytoskeletal fraction, pellets were resuspended in 2% SDS, 1% beta-mercaptoethanol in PBS and boiled for 30min at 95°C with intermittent vortexing. Subsequently, the SDS concentration was reduced to 0.2% by dilution with PBS and Triton-X-100 added to a final concentration of 2%. Samples were then sonicated by tip sonication (3x 30s pulses using a Bandelin Sonopuls GM70 sonicator at 60% continuous output, with brief cooling on ice between pulses), the SDS concentration reduced to 0.1% SDS with PBS and samples sonicated once more for 30s at 60% output before centrifuging at 20000g for 30min at 4°C and recovering the supernatant. Pierce Streptavidin-coated magnetic beads were equilibrated with PBS containing 0.1% SDS before incubating with the detergent-soluble cytoplasmic fraction or solubilized cytoskeletal fraction on a rotator overnight at 4°C. For Cmb TurboID samples, beads were further acetylated to reduce background(Hollenstein et al., 2023) by incubation with a mixture of 190µl 50mM HEPES-NaOH pH7.8 containing 0.2% Tween-20 and 10µl 100mM Pierce Sulfo-NHS-Acetate in for 1 hour at room temperature for 1h prior to incubation with lysate. Following the overnight incubation, unbound lysate was removed and beads washed for 8min each on a rotator at room temperature, first with 2% SDS in ddH₂O, then 0.1% deoxycholic acid, 1% Triton X-100, 1mM EDTA, 500mM NaCl and 50mM HEPES pH7.5, and finally 0.5% deoxycholic acid, 0.5% NP-40, 1mM EDTA, 500mM LiCl and 10mM Tris pH7.5. Beads were then washed 5x for 3min with 50mM Tris pH7.4 before being sent for on-bead protein digestion and mass spectrometry analysis.

2.2.18 Nanobody TurboID in *Drosophila* testes

Lysates were prepared as previously described for *C. elegans* in (Holzer et al., 2022). Briefly, adult flies were washed 3x with PBS, recovering flies by centrifugation for 3min at 300g, quickly ground in RIPA buffer (1% Triton X-100, 1mM EDTA, 0.5% sodium deoxycholate, 0.1% SDS, 150mM NaCl, 50mM Tris-HCl pH7.4) supplemented with Roche cOmplete Mini EDTA-free Protease-Inhibitor-Cocktail (1 tablet/10ml lysis buffer), 1mM

PMSF and 1mM Benzamidine using a mortar and pestle and drop-frozen in liquid nitrogen. Frozen fly ‘popcorn’ was further ground by cryogenic milling using a SPEX 6875 cryogenic mill (5 cycles, 1min pre-cool, 2min run-time, 1min cool-time; 12 cps). Lysates were thawed and SDS and DTT added to a final concentration of 1% and 10mM, respectively, boiled at 95°C for 5min and sonicated by tip sonication (2x 1min at 20% continuous output, with brief cooling on ice between pulses). Urea solution (8M urea, 1% SDS, 50mM Tris-Cl, 150mM NaCl) was added to a final concentration of 2M urea and lysates centrifuged at 100000g for 45 min at 22°C. Lysates were desalted over Zeba spin desalting columns (7K MWCO, Thermofisher) to remove free biotin and incubated with streptavidin magnetic beads by incubating on a rotator overnight. Following incubation, beads were washed twice with 150mM NaCl, 1mM EDTA, 2% SDS, 50mM Tris-HCl, pH7.4, once with 1x TBS buffer pH 7.4, twice with 1M KCl, 1mM EDTA, 50mM Tris-HCl, 0.1% Tween-20, pH7.4, twice with 0.1M Na₂CO₃, 0.1% Tween-20, pH 11.5, twice with 2M urea, 10mM Tris-HCl, 0.1% Tween-20 pH 8.0 and finally 5x with 1x TBS buffer. Beads were finally resuspended in 1X TBS buffer and sent for on-bead protein digestion and mass spectrometry analysis.

2.2.19 Sample preparation for mass spectrometry analysis

Beads were resuspended in 50µl 1M urea and 50mM ammonium bicarbonate. Disulfide bonds were reduced with 2µl of 250mM DTT for 30min at room temperature before adding 2µl 500mM iodoacetamide and incubating for 30min at room temperature in the dark. Remaining iodoacetamide was quenched with 1µl of 250mM DTT for 10min. Proteins were digested with 150ng LysC (mass spectrometry grade, FUJIFILM Wako chemicals) in 1.5µl 50 mM ammonium bicarbonate at 25°C overnight. The supernatant without beads was digested with 150ng trypsin (Trypsin Gold, Promega) in 1.5µl 50mM ammonium bicarbonate followed by incubation at 37°C for 5 hours. The digest was stopped by the addition of trifluoroacetic acid to a final concentration of 0.5%, and the peptides desalted using C18 Stagetips (Rappsilber et al., 2007).

2.2.20 Liquid chromatography separation coupled to mass spectrometry

Peptides were separated on an Ultimate 3000 RSLC nano-flow chromatography system (Thermo-Fisher), using a pre-column for sample loading (Acclaim PepMap C18, 2cm × 0.1mm, 5µm, Thermo-Fisher), and a C18 analytical column (Acclaim PepMap C18, 50cm ×

0.75mm, 2 μ m, Thermo-Fisher), applying a segmented linear gradient from 2% to 35% and finally 80% solvent B (80% acetonitrile, 0.1% formic acid; solvent A 0.1% formic acid) at a flow rate of 230nl/min over 120min. The peptides eluted from the nano-LC were analyzed by mass spectrometry as described below.

For Sas4 S2 cell BioID, a Q Exactive HF Orbitrap mass spectrometer (Thermo Fisher) coupled to the column with a nano-spray ion-source using coated emitter tips (PepSep, MSWil), was used with the following settings: The mass spectrometer was operated in data-dependent acquisition mode (DDA), survey scans were obtained in a mass range of 380-1650m/z with lock mass activated, at a resolution of 120k at 200m/z and an AGC target value of 3E6. The 10 most intense ions were selected with an isolation width of 2.0m/z without offset, fragmented in the HCD cell at 27% collision energy and the spectra recorded for max. 250ms at a target value of 1E5 and a resolution of 30k. Peptides with a charge of +2 to +6 were included for fragmentation, the peptide match and the exclude isotopes features enabled, and selected precursors were dynamically excluded from repeated sampling for 30s.

For Cmb S2 cell TurboID, a Q Exactive HF-X Orbitrap mass spectrometer (Thermo Fisher) coupled to the column with a nano-spray ion-source using coated emitter tips (PepSep, MSWil), was used with the following settings: The mass spectrometer was operated in data-dependent acquisition mode (DDA), survey scans were obtained in a mass range of 375-1500m/z with lock mass activated, at a resolution of 120k at 200m/z and an AGC target value of 3E6. The 8 most intense ions were selected with an isolation width of 1.6m/z with offset 0.2m/z, fragmented in the HCD cell at 28% collision energy and the spectra recorded for max. 250ms at a target value of 1E5 and a resolution of 30k. Peptides with a charge of +2 to +6 were included for fragmentation, the peptide match and the exclude isotopes features enabled, and selected precursors were dynamically excluded from repeated sampling for 30s.

For Cmb testes TurboID, an Exploris 480 Orbitrap mass spectrometer (Thermo Fisher) coupled to the column with a FAIMS pro ion-source (Thermo-Fisher) using coated emitter tips (PepSep, MSWil), was used with the following settings: The mass spectrometer was operated in DDA mode with two FAIMS compensation voltages (CV) set to -45 or -60 and 1.5s cycle time per CV. The survey scans were obtained in a mass range of 350-1500m/z, at a resolution of 60k at 200m/z and a normalized AGC target at 100%. The most intense ions were selected with an isolation width of 1.2m/z, fragmented in the HCD cell at 28% collision energy and the spectra recorded for max. 100ms at a normalized AGC target of 100% and a resolution of 15k. Peptides with a charge of +2 to +6 were included for fragmentation, the peptide match feature was set

to preferred, the exclude isotope feature was enabled, and selected precursors were dynamically excluded from repeated sampling for 45s.

2.2.21 Mass spectrometry data analysis

The RAW MS data were analyzed with FragPipe (20.0), using MSFragger (3.8)(Kong et al., 2017), IonQuant (1.9.8)(Yu et al., 2021), and Philosopher (5.0.0)(da Veiga Leprevost et al., 2020). The default FragPipe workflow for label free quantification (LFQ-MBR) was used, except “Normalize intensity across runs” was turned off. For Sas4 BioID, the “MBR top runs” parameter was set to 1 to address batch measurements of sample replicates. Cleavage specificity was set to Trypsin/P, with two missed cleavages allowed. The protein FDR was set to 1%. A mass of 57.02146 (carbamidomethyl) was used as fixed cysteine modification; methionine oxidation and protein N-terminal acetylation were specified as variable modifications. MS2 spectra were searched against the *D. melanogaster* 1 protein per gene reference proteome from Uniprot (Proteome ID: UP000000803, release 2023.03), concatenated with a database of 382 common laboratory contaminants (release 2023.03, <https://github.com/maxperutzlabs-ms/perutz-ms-contaminants>).

Computational analysis was performed using Python and the in-house developed Python library MsReport (version 0.0.23). Only non-contaminant proteins identified with a minimum of two peptides and being quantified in at least two replicates of one experiment were considered for the analysis. LFQ protein intensities reported by FragPipe were log₂-transformed and normalized across samples using the ModeNormalizer from MsReport. This method involves calculating log₂ protein ratios for all pairs of samples and determining normalization factors based on the modes of all ratio distributions. For the data presented in the figures, missing values were imputed by deterministic lowest of detection (LOD) after filtering out contaminants and proteins with less than two razor and unique peptides, an approach we previously found to yield superior results for centrosomal proteins which are frequently absent in one or all control samples but also not highly abundant in the experimental samples(Holzer et al., 2022). Also included in Table S2 is the more standard approach of imputation by drawing random values from a left-censored normal distribution modeled on the whole dataset (data mean shifted by -1.8 standard deviations, width of distribution of 0.3 standard deviations). Likely proximity interactors largely passed the significance threshold with both methods of imputation. These were defined as a log₂ fold change of >1 and a p-value

in an unpaired t-test of <0.05 . GraphPad Prism was used to prepare bar graphs and volcano plots using LFQ values imported from Microsoft Excel.

2.2.22 Comparisons to published human datasets and Gene Ontology (GO) analysis

To compare the proteins significantly enriched in our Cmb proximity interactome analyses in flies with those reported in published datasets of centrosomal, ciliary and centriolar satellite proteins as well as cytosolic mRNA-associated proteins in vertebrates (Gheiratmand et al., 2019; Gupta et al., 2015; Quarantotti et al., 2019; Youn et al., 2018) we first generated a proteome-wide matrix of all conserved proteins based on reciprocal BLAST analysis as described above, then performed a pairwise comparison between datasets using Flybase IDs in *Drosophila* and Ensembl gene IDs in humans. Venn diagrams presented in the figures report both the number of conserved proteins common to both datasets and those unique to each, as well the larger number of unique proteins including those proteins not detectably conserved across species.

Gene Ontology analyses were performed using the PANTHER 18.0 (Mi et al., 2019) and GO Ontology database release 2023-10-09, comparing the protein IDs of the orthologs of proteins enriched in our Cmb proximity interactome analyses against the human proteome, identifying GO annotation terms statistically enriched based on Fisher's Exact Test, applying Bonferroni correction for multiple testing. Annotation terms displayed in the figures are the top 8 terms for 'cellular compartment' and 'biological process' based on fold enrichment.

2.3 Quantification And Statistical Analysis

2.3.1 Statistical analysis

Statistical analysis was performed in Excel and GraphPad Prism 10. Each dataset was tested for normal distribution using D'Agostino-Pearson (omnibus K2) test. Where data was displaying a normal distribution, an unpaired, two-tailed Student's t-test with Welch's correction was used for comparison of two groups. When data failed the normality test for at least one of the datasets examined, an unpaired, two-tailed Mann-Whitney U-test and a Kruskal-Wallis test with Dunn's multiple comparisons test was used for two groups or more than two groups, respectively. If n was too small to test data for normal distribution a Student's t-test was used. For all experiments the significance threshold was taken as $P < 0.05$.

Significance levels are defined as follows: ****P< 0.0001, ***P< 0.001, **P< 0.01, *P< 0.05, NS, not significant. Error bars display mean with standard deviation unless otherwise stated.

2.3.2 Table 1/ KEY RESSOURCE TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Donkey anti-Mouse IgG (H + L) Cy2-conjugated antibody	Jackson ImmunoResearch	Cat# 715-225-150, RRID: AB_2340826
Donkey anti-Mouse IgG (H + L) Cy3-conjugated antibody	Jackson ImmunoResearch	Cat# 715-165-150, RRID: AB_2340813
Donkey anti-Mouse IgG (H + L) Cy5-conjugated antibody	Jackson ImmunoResearch	Cat# 715-175-150, RRID: AB_2340819
Donkey anti-Rabbit IgG (H + L) Cy2-conjugated antibody	Jackson ImmunoResearch	Cat# 711-225-152, RRID: AB_2340612
Donkey anti-Rabbit IgG (H + L) Cy3-conjugated antibody	Jackson ImmunoResearch	Cat# 711-165-152, RRID:AB_2307443
Donkey anti-Rabbit IgG (H + L) Cy5-conjugated antibody	Jackson ImmunoResearch	Cat# 711-175-152, RRID:AB_2340607
Mouse monoclonal anti-dmNompC antibody	(Liang et al., 2011)	N/A
Mouse monoclonal anti-gamma Tubulin antibody	Sigma-Aldrich	Cat# T6557, RRID: AB_477584
Mouse monoclonal anti-hsPCM1 antibody	Santa Cruz Biotechnology	Cat# sc-398365, RRID: AB_2827155
Mouse monoclonal anti-Puromycin	Merck Millipore	Cat# MABE343, RRID: AB_2566826
Rabbit polyclonal anti-dmAna1 antibody	(Conduit et al., 2010)	N/A
Rabbit polyclonal anti-dmAna2 antibody	(Cabral et al., 2019)	N/A
Rabbit polyclonal anti-dmCep97-N-ter antibody	(Dobbelaere et al., 2020)	N/A
Rabbit polyclonal anti-dmCmb antibody	This study	N/A
Rabbit polyclonal anti-dmCnn antibody	(Dobbelaere et al., 2008)	N/A
Rabbit polyclonal anti-dmCP110 antibody	(Franz et al., 2013)	N/A
Rabbit polyclonal anti-dmSas4 antibody	(Basto et al., 2006)	N/A

Rabbit polyclonal anti-dmSpd2 antibody	(Cabral et al., 2019)	N/A
Rabbit polyclonal anti-hsCDK5RAP2 antibody	Merck Millipore	Cat# 06-1398, RRID: AB_11203651
Rabbit polyclonal anti-hsCEP131/AZI1 antibody	Abcam	Cat# ab84864, RRID: AB_1859791
Rabbit polyclonal anti-hsCEP290 antibody	Novus Biologicals	Cat# NB100-86991, RRID: AB_1201171
Rabbit polyclonal anti-hsMIB1 antibody	Sigma-Aldrich	Cat# M5948; RRID: AB_1841007
Rabbit polyclonal anti-hsOFD1 antibody	Sigma-Aldrich	Cat# HPA031103, RRID: AB_10602188
Rabbit polyclonal anti-hsPCM1 antibody	(Dammermann and Merdes, 2002)	N/A
Rabbit polyclonal anti-hsPCNT antibody	Abcam	Cat# ab4448, RRID: AB_304461
Chemicals, Peptides, and Recombinant Proteins		
Agar 100 Resin	Agar Scientific	Cat# AGR1031; CAS: 25038-04-4
Cycloheximide	Sigma-Aldrich	Cat# C1988; CAS: 66-81-9
25% Glutaraldehyde, EM grade	Agar Scientific	Cat# AGR1020; CAS: 111-30-8
Hoechst 33258	Thermo Fisher	Cat# H1398; CAS: 23491-45-4
Lysyl Endopeptidase (LysC)	FUJIFILM Wako chemicals	Cat# 129-02541
Osmium Tetroxide 4% Solution	Electron Microscopy Sciences	Cat# 19170; CAS: 20816-12-0
Paraformaldehyde	Roth	Cat# O335.1; CAS: 30525-89-4
32% Paraformaldehyde, EM grade	Electron Microscopy Sciences	Cat # 15714
Phalloidin Alexa Fluor 568	Thermo Fisher	Cat# A12380
Pierce Streptavidin Magnetic Beads	Thermo Scientific Pierce	Cat# 88817
Puromycin dihydrochloride	Sigma-Aldrich	Cat# P8833
Reynolds lead citrate	Delta Microscopies	Cat# 11300; CAS: 512-26-5
Trypsin Gold Mass Spec Grade	Promega	Cat# V5280
Uranyl acetate	Science Services	Cat# E22400; CAS: 541-09-3
Vanadyl ribonucleoside complexes solution	Sigma-Aldrich	Cat# 94742
Vectashield Plus	Vector Laboratories	Cat# H-1900

Critical Commercial Assays		
Lipofectamine RNAiMax	Invitrogen	Cat# 13778100
TransIT Insect Transfection reagent	Mirus Bio	Cat# MIR 6104
Experimental Models: Cell Lines		
<i>D. melanogaster</i> : S2 cells	Life Technologies	Cat# R69007, RRID:CVCL_Z232
Human: HeLa cells	ATCC	Cat# CCL-2, RRID:CVCL_0030
Human: HeLa <i>PCNT</i> KO cells	(Watanabe et al., 2020)	ODCL0025
Human: U2-OS cells	ATCC	Cat# HTB-96, RRID:CVCL_0042
Experimental Models: Organisms/Strains		
Asl RNAi/ P{TRiP.HMS01453}attP2	BDSC	BDSC ID: 35039, RRID: BDSC_35039
Asl-GFP & H2A-RFP; CmbΔ/ P{His2Av-mRFP1}; P{UAS.asl-asl-GFP.B}; CmbΔ/Tmb6 SbTb	This study	N/A
Asl-GFP & H2A-RFP/ P{His2Av-mRFP1}; P{UAS.asl-asl-GFP.B}	This study	N/A
Asl-GFP/ P{UAS.asl-asl-GFP.B}	(Blachon et al., 2008)	Flybase ID: FBtp0040947
Bam-GAL4 & Nanos-GAL4/ P{GAL4-nos.NGT}40; P{bam-GAL4:VP16}	(Dobbelaere et al., 2023)	N/A
Cmb OE/ P{UAS-cmb.RA}	(Fagan et al., 2014)	Flybase ID: FBtp0095481
Cmb RNAi 1/ P{KK103563}VIE-260B	VDRC	VDRC ID: 109767, RRID: Flybase_FBst0481420
Cmb RNAi 2/ P{GD7077}v18666	VDRC	VDRC ID: 18666, RRID: Flybase_FBst0453208
Cmb-GFP rescue/ PBac{fTRG01063.sfGFP-TVPTBF}VK00002; CmbΔ	This study	N/A
Cmb-GFP/ PBac{fTRG01063.sfGFP-TVPTBF}VK00002	VDRC	VDRC ID: 318739, RRID: Flybase_FBst0491914
CmbΔ/ CmbΔ/Tm6 SbTb	(Fagan et al., 2014)	Flybase ID: FBal0298847
Cnn RNAi/ P{GD1726}v44526	VDRC	VDRC ID: 44526, RRID: Flybase_FBst0465625
Control RNAi/ P{TRiP.HMS00045}attP2	BDSC	BDSC ID: 33644, RRID: BDSC_33644
Deficiency 1/ w[1118]; Df(3L)BSC614/TM6C, cu[1] Sb[1]	BDSC	BDSC ID: 25689, RRID: BDSC_25689
Deficiency 2/ w[1118]; Df(3L)BSC737/TM6C, Sb[1] cu[1]	BDSC	BDSC ID: 26835, RRID: BDSC_26835

Elav-GAL4/ P{GawB}elav[C155]	BDSC	BDSC ID: 458, RRID: BDSC 458
Frtz RNAi/ P{GD9437}v40088	VDRC	VDRC ID: 40088, RRID: Flybase FBst0463379
GFP nanobody-TurboID/ P{egg-TurboID.vhhGFP.3xHA}	This study	N/A
H2A-RFP/ P{His2Av-mRFP1} P{UAS.asl-asl-GFP.B}	(Pandey et al., 2005)	Flybase ID: FBtp0056035
In RNAi/ P{GD10892}v27252	VDRC	VDRC ID: 27252, RRID: Flybase FBst0456836
Plp RNAi/ P{GD9625}v20667	VDRC	VDRC ID: 20667
Pnr-GAL4/ P{GawB}pnr[MD237]	(Calleja et al., 2000)	Flybase ID: FBti0004011
RFP-Cnn; CmbΔ/ P{Ubi-p63E-cnn.RFP}; CmbΔ/Tmb6 SbTb	This study	N/A
RFP-Cnn/ P{Ubi-p63E-cnn.RFP}	(Conduit et al., 2010)	Flybase ID: FBtp0056991
Sas4 RNAi/ P{GD6852}v17975	VDRC	VDRC ID: 17975, RRID: Flybase FBst0452943
Spd2 RNAi/ P{KK110116}VIE-260B	VDRC	VDRC ID: 101882, RRID: Flybase FBst0473755
Wild-type/ w[1118]	BDSC	BDSC ID: 6326, RRID: BDSC 6326
Oligonucleotides		
siRNA targeting sequence: PCM-1 #1: GGCUCUAAACGUGCCUCC	(Dammermann and Merdes, 2002)	N/A
siRNA targeting sequence: PCM-1 #2: UCAGCUUCGUGAUUCUCAG	(Dammermann and Merdes, 2002)	N/A
<i>RanBP10</i> RNA FISH probe 1 ggagtctcttgctggttgac	Biosearch technologies	N/A
<i>RanBP10</i> RNA FISH probe 2 gtagttgtatttgccttg	Biosearch technologies	N/A
<i>RanBP10</i> RNA FISH probe 3 aataaatgccacagcgagca	Biosearch technologies	N/A
<i>RanBP10</i> RNA FISH probe 4 agagtcctattcccatgtaa	Biosearch technologies	N/A
<i>RanBP10</i> RNA FISH probe 5 cataggaatgtttgtcccaa	Biosearch technologies	N/A
<i>RanBP10</i> RNA FISH probe 6 ccgaggagcagaacgaatgc	Biosearch technologies	N/A
<i>RanBP10</i> RNA FISH probe 7 tggtgaatgtgggaccatag	Biosearch technologies	N/A

<i>RanBP10</i> RNA FISH probe 8 ccattgatgaggttgacaca	Biosearch technologies	N/A
<i>RanBP10</i> RNA FISH probe 9 gaggtctgtgaaggctatac	Biosearch technologies	N/A
<i>RanBP10</i> RNA FISH probe 10 ctacggtggggtagaggttg	Biosearch technologies	N/A
<i>RanBP10</i> RNA FISH probe 11 caaagttggcgtccacaatc	Biosearch technologies	N/A
<i>RanBP10</i> RNA FISH probe 12 gtctcaatgtcaaacagga	Biosearch technologies	N/A
<i>RanBP10</i> RNA FISH probe 13 actgatggggaagcagtgga	Biosearch technologies	N/A
<i>RanBP10</i> RNA FISH probe 14 atgaaacctgttctgcagc	Biosearch technologies	N/A
<i>RanBP10</i> RNA FISH probe 15 caatacccatgatgcacgag	Biosearch technologies	N/A
<i>RanBP10</i> RNA FISH probe 16 gagcaaaagccgtggctgtg	Biosearch technologies	N/A
<i>RanBP10</i> RNA FISH probe 17 tgaatcggggtttcagtcac	Biosearch technologies	N/A
<i>RanBP10</i> RNA FISH probe 18 ttctttatggacgcctgttc	Biosearch technologies	N/A
<i>RanBP10</i> RNA FISH probe 19 cagcttctggatcttttgc	Biosearch technologies	N/A
<i>RanBP10</i> RNA FISH probe 20 gagcatgaagaggaggttg	Biosearch technologies	N/A
<i>RanBP10</i> RNA FISH probe 21 cattcaccatctccaaaac	Biosearch technologies	N/A
<i>RanBP10</i> RNA FISH probe 22 tcaaactcggacactcactg	Biosearch technologies	N/A
<i>RanBP10</i> RNA FISH probe 23 tgtgcatgtgggaactactg	Biosearch technologies	N/A
<i>RanBP10</i> RNA FISH probe 24 cattgctacagctgggactg	Biosearch technologies	N/A
<i>RanBP10</i> RNA FISH probe 25 cagggtatttactgtggttc	Biosearch technologies	N/A
<i>RanBP10</i> RNA FISH probe 26 gagtctgttgagttggactc	Biosearch technologies	N/A
<i>RanBP10</i> RNA FISH probe 27 aacaatgcgtgtggacatgc	Biosearch technologies	N/A
<i>RanBP10</i> RNA FISH probe 28 ggccaaacagaatgatcctt	Biosearch technologies	N/A
<i>RanBP10</i> RNA FISH probe 29 aactgctcactcaatgcctg	Biosearch technologies	N/A
<i>RanBP10</i> RNA FISH probe 30 ctgtgtgggccaattcttg	Biosearch technologies	N/A

<i>RanBP10</i> RNA FISH probe 31 agtatgccagcaggctgaag	Biosearch technologies	N/A
<i>RanBP10</i> RNA FISH probe 32 taaaatggcgctgttgaggg	Biosearch technologies	N/A
<i>RanBP10</i> RNA FISH probe 33 tgagccggagacactcagat	Biosearch technologies	N/A
<i>RanBP10</i> RNA FISH probe 34 caaaggagcaagaaccagg	Biosearch technologies	N/A
<i>RanBP10</i> RNA FISH probe 35 gtgcaagtagtcatcaactc	Biosearch technologies	N/A
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<i>Nin</i> RNA FISH probe 3 CCTCCTAAGTTTCGAGCTGGACTCAGTG tgatcccaaatcttcacaa	IDT	N/A
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<i>Nin</i> RNA FISH probe 7 CCTCCTAAGTTTCGAGCTGGACTCAGTG gtccactctcatcgaaagac	IDT	N/A
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<i>Nin</i> RNA FISH probe 9 CCTCCTAAGTTTCGAGCTGGACTCAGTG cagtattctctccacagatg	IDT	N/A
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<i>Nin</i> RNA FISH probe 12 CCTCCTAAGTTTCGAGCTGGACTCAGTG ggatttcagccttaaagctg	IDT	N/A

<i>Nin</i> RNA FISH probe 13 CCTCCTAAGTTTCGAGCTGGACTCAGTG aagctcattttccagacgac	IDT	N/A
<i>Nin</i> RNA FISH probe 14 CCTCCTAAGTTTCGAGCTGGACTCAGTG tcatattctgccacttctc	IDT	N/A
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<i>Nin</i> RNA FISH probe 16 CCTCCTAAGTTTCGAGCTGGACTCAGTG atttctcatctgtgtcagtc	IDT	N/A
<i>Nin</i> RNA FISH probe 17 CCTCCTAAGTTTCGAGCTGGACTCAGTG gagttcatctacttggtctt	IDT	N/A
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<i>CEP290</i> RNA FISH probe 3 tttgaagagccaattgctc	Biosearch technologies	N/A
<i>CEP290</i> RNA FISH probe 4 acgtttgttctctcttcta	Biosearch technologies	N/A
<i>CEP290</i> RNA FISH probe 5 ctgtctgatgcacaatagct	Biosearch technologies	N/A
<i>CEP290</i> RNA FISH probe 6 ttctcccttaggttatgtaa	Biosearch technologies	N/A
<i>CEP290</i> RNA FISH probe 7 ttatcagcatcaagctgagc	Biosearch technologies	N/A
<i>CEP290</i> RNA FISH probe 8 tctttgattgtgcttcactc	Biosearch technologies	N/A
<i>CEP290</i> RNA FISH probe 9 gactcgcatcaaagattcct	Biosearch technologies	N/A
<i>CEP290</i> RNA FISH probe 10 taagctgatcaacttgggct	Biosearch technologies	N/A
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<i>CEP290</i> RNA FISH probe 12 attagccagttctagtctcag	Biosearch technologies	N/A
<i>CEP290</i> RNA FISH probe 13 caggtgttccaagtacttg	Biosearch technologies	N/A
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<i>CEP290</i> RNA FISH probe 18 cattctgagggactctactt	Biosearch technologies	N/A

CEP290 RNA FISH probe 19 tgggtgcaacttggcaatgag	Biosearch technologies	N/A
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CEP290 RNA FISH probe 22 ccactaaactgtcgtcgtag	Biosearch technologies	N/A
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CEP290 RNA FISH probe 26 cctgtagcttcttcaaactt	Biosearch technologies	N/A
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Recombinant DNA		
Blasticidin selection vector: pCoBlast	Thermo Fisher	Cat# K515001
GST expression vector: pGEX-6P-1 Cmb (aa1-150)	This study	N/A
BioID control vector: pMt-myc-BirA*	This study	N/A
BioID Sas4 vector: pMt-Sas4-myc-BirA*	This study	N/A
S2 cell expression vector: pMt-V5-6xHis B	Invitrogen	Cat# V412020
Cmb GFP vector: pMt-V5-6xHis-Cmb-GFP	This study	N/A
TurboID Cmb vector: pMt-V5-TurboID-Cmb	This study	N/A
TurboID control vector: pMt-V5-TurboID-GW	Norbert Perrimon	N/A
Indirect TurboID fly expression vector: pUASz-TurboID-vhhGFP-3xHA	This study	N/A
Indirect TurboID expression vector (+NLS): pUASz-TurboID-vhhGFP-3xHA-NLS	(Baumgartner et al., 2022)	N/A
Gateway vector: pDONR Zeo	Thermo Fisher	Cat# 12535035
BioID source vector: pcDNA3.1 mycBirA*	Kyle Roux	N/A
Software and Algorithms		
Fiji v 2.0.0	NIH	https://imagej.net/software/fiji/
FragPipe v 20.0	University of Michigan Medical School	https://github.com/Nesvilab/FragPipe

GraphPad Prism 10	GraphPad	https://www.graphpad.com/scientific-software/prism
HMMER v 3.4	(Eddy, 2011)	http://hmmer.org/
Ilastik v 1.4.0	(Berg et al., 2019)	https://www.ilastik.org
IonQuant v 1.9.8	(Yu et al., 2021)	https://github.com/Nesvilab/IonQuant
Jalview v 2.11.1.4	(Waterhouse et al., 2009)	https://www.jalview.org/
MAFFT v 7	(Kato et al., 2019)	https://mafft.cbrc.jp/alignment/server/
Matlab v R2023a	MathWorks	https://www.mathworks.com
MSFragger v 3.8	(Kong et al., 2017)	https://github.com/Nesvilab/MSFragger
MsReport v 0.0.23	Max Perutz Labs Mass Spectrometry Facility	N/A
NCBI BLAST+ v 2.13.0	(Altschul et al., 1997)	ftp://ftp.ncbi.nlm.nih.gov/blast/executables/blast+/LATEST/
Philosopher v 5.0.0	(da Veiga Leprevost et al., 2020)	https://github.com/Nesvilab/philosopher

3 Results

3.1 Contribution to this study

The author of this thesis was primarily responsible for the design, acquisition, analysis and interpretation of the experiments described here and in the preprint Pachinger et al., 2024 (Pachinger et al., 2024). In particular, gross phenotypic and behavioral analysis of the fly mutant, light microscopy experiments in fly cells and tissues, as well as in vertebrate cells, and proteomics experiments were conducted by the author. In addition, the author was closely involved in the overall study design and conception, as well as in the preparation and writing of the resulting manuscript.

3.2 The satellite scaffolding component PCM1 is conserved beyond vertebrates

We identified Combover, the PCM1 ortholog in *Drosophila*, as a proximity interactor of the core centriolar component Sas-4 in *Drosophila* S2 cells as well as in mass spectrometry analysis of isolated centrosomes. This finding and its reported homology to PCM1 by Kuhn et al. led us to believe that Combover (Cmb) could be a *bona-fide* ortholog of vertebrate PCM1. To examine the evolutionary distribution of PCM1 in more detail we used reciprocal BLAST analysis with less divergent related species as intermediates, as well as Hidden-Markov Models in cases where direct reciprocal BLAST failed to identify a clear ortholog. We could recapitulate the identification of PCM1 orthologs in *Drosophila* and *C. elegans* as well as extend its distribution to further members of the opisthokont group including choanoflagellates and ciliated fungi (Blastocladales, chytrids). PCM1 is absent in higher fungi (Dikarya), where both centrioles and cilia have been secondarily lost and in the parasitic phylum nematomorpha, which lack most ciliary genes. In *Schmidtea mediterranea*, a planarian that lacks centrosomes but retains centrioles and cilia, PCM1 is present. Thus, the presence and absence pattern of PCM1 across >1000 million years of opisthokont evolution correlates perfectly with the evolutionary conservation of centriole-based structures (Figure 8).

While PCM1 is clearly conserved in insects, the sequence similarity of the *Drosophila* ortholog to vertebrate PCM1 is very low (Figure 9). Combover is a protein with 4 different isoforms and a length of 1657 amino acids (for isoform 1 Cmb-PA) with the highest sequence

similarity in insects towards the C-terminus, corresponding to a small stretch before the C-terminus of PCM1. Structural comparisons are difficult since the majority of the structure can only be predicted with low confidence, however both proteins share the presence of many coiled-coil regions, with a fraction of 4% and 21% coiled-coil residues for the human and fly proteins respectively (Kuhn et al., 2014). Based on AlphaFold predictions, no reported direct interactions could be mapped to Combover. Considering the low levels of sequence and structural similarity we were curious to see if and to what extent functions of PCM1 (and centriolar satellites) are conserved.

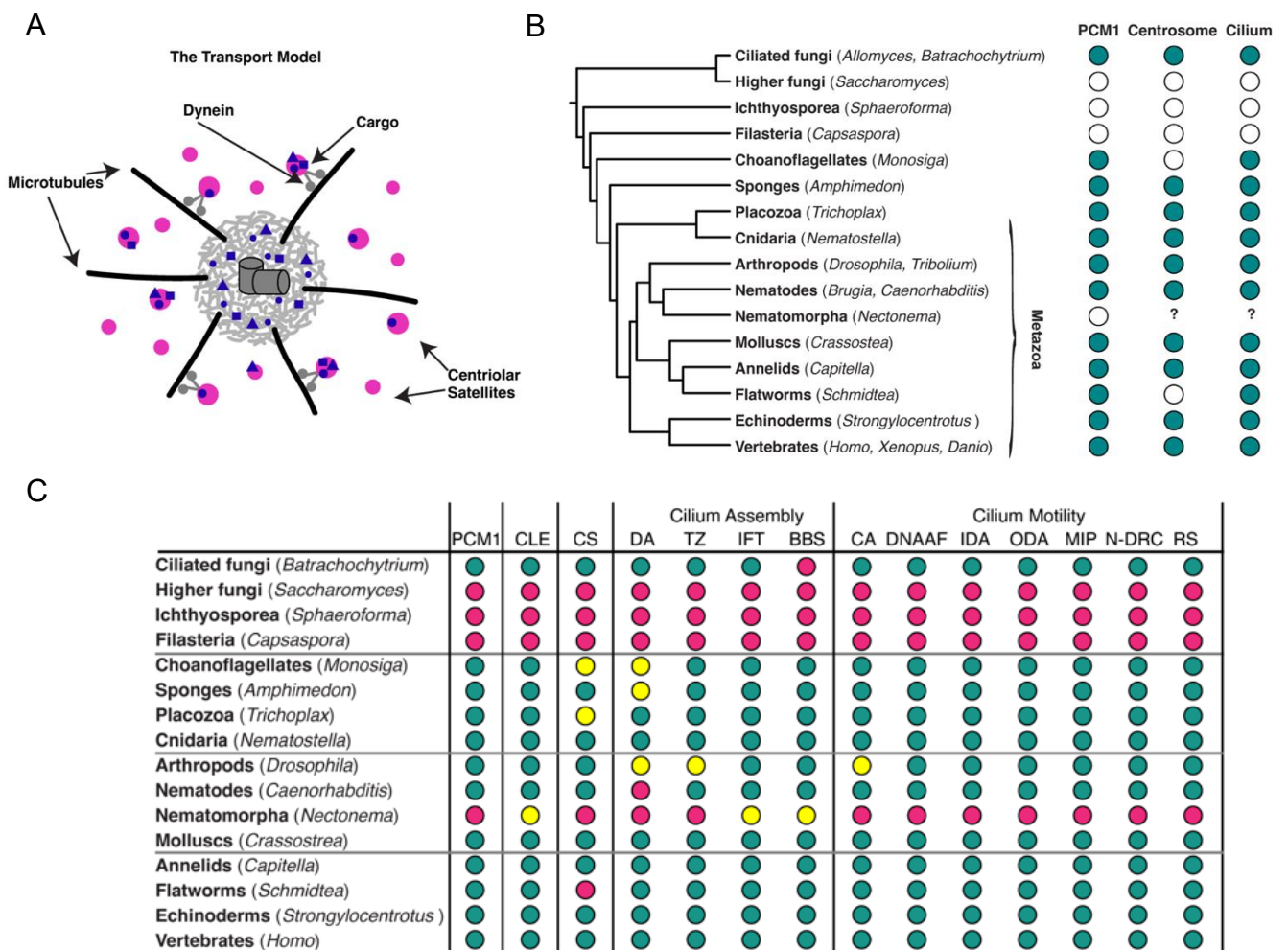


Figure 8. Evolutionary conservation of PCM1. (A) Schematic representation of the transport model for centriolar satellites. Satellites function in dynein-mediated transport of centrosomal and ciliary cargo proteins (blue) for centrosome and cilium biogenesis. (B) Reciprocal BLAST analysis showing the evolutionary distribution of PCM1, centrosomes and cilia. PCM1 is present across opisthokonts and its presence correlates with the presence of centriole-based

structures. (C) Conservation of PCM1 and core centriolar (STIL/Ana2, SASS6/Sas6, CENPJ/Sas4, CEP135/Bld10), centrosomal (CDK5RAP2/Cnn, CEP192/Spd2) and ciliary proteins (transition zone, IFT and BBS components, inner and outer dynein arm components, dynein assembly factors, nexins, N-DRC, radial spoke and central apparatus components, (Dobbelaere et al., 2023)) across opisthokonts, based on reciprocal BLAST analysis and hidden Markov model-based searches. Color code is green > 2/3 of genes in indicated category present, yellow > 1/3 of genes present, magenta < 1/3 present. Adapted from (Pachinger et al., 2024).

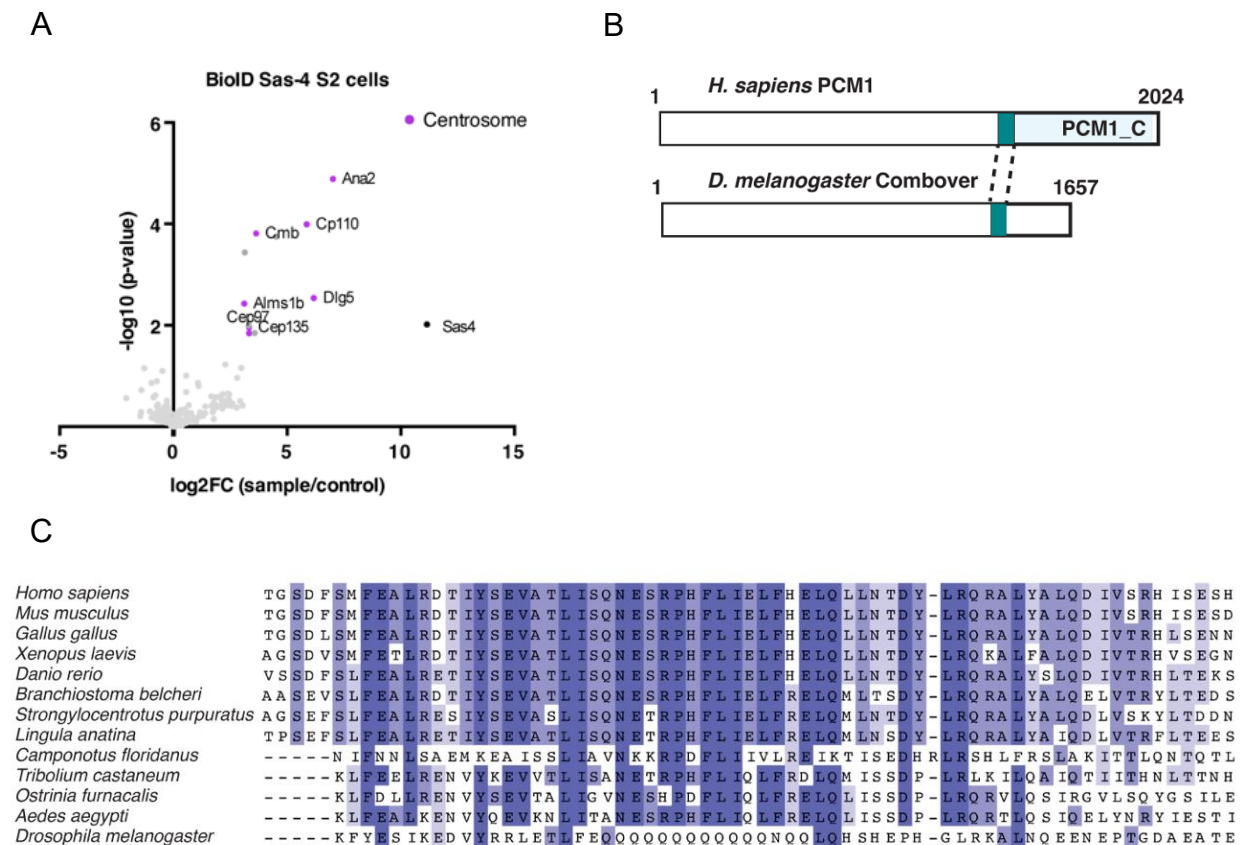


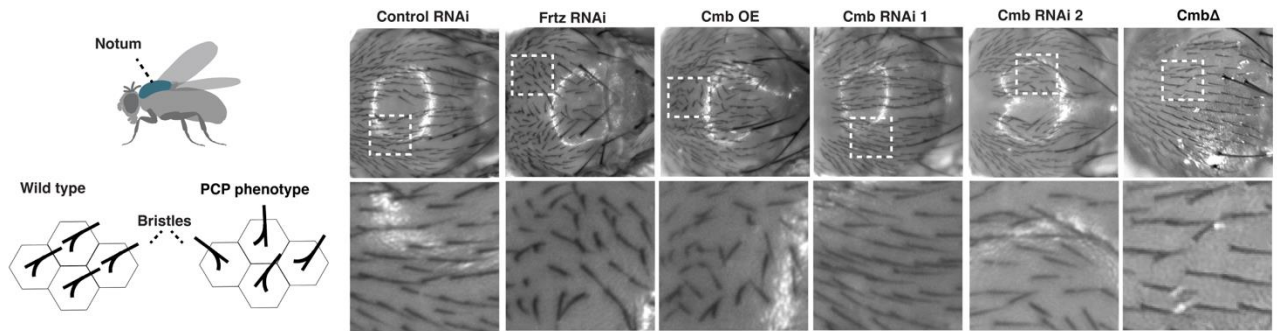
Figure 9. Identification of Combover as the *Drosophila* ortholog of PCM1. (A) Direct proximity labeling experiment (BioID) of the centriole component Sas-4 in *Drosophila* S2 cells. Volcano plot is displaying $-\log_{10}$ p-values against $\log_2$ fold change (sample/control). Significantly enriched proteins ($\log_2$ enrichment >1, p-value <0.05) are presented in dark grey, centrosomal proteins are shown in magenta. Cmb was detected as a high confidence interactor. (B) Schematic of the conserved sequence shown in (C). (C) Multiple sequence alignment of conserved C-terminus (part of pfam15717) of selected PCM1 orthologs, including *D. melanogaster*. The alignment shows only conserved sequences, *Drosophila* Combover (Cmb) appears highly divergent. Adapted from (Pachinger et al., 2024).

3.3 Combover (Cmb) is not required for planar cell polarity (PCP)

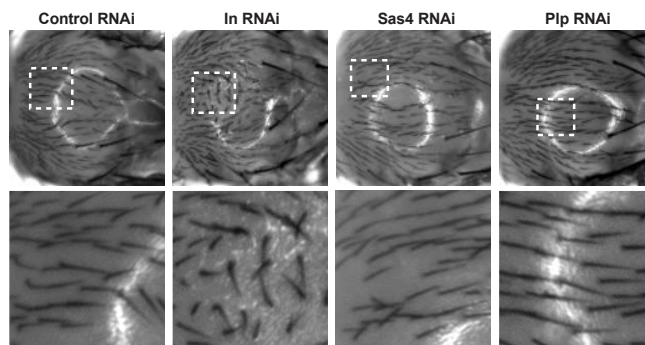
Combover (Cmb) was originally identified as an *in vitro* substrate of Rho-kinase (Rok) and a component of the planar cell polarity pathway (PCP) (Fagan et al., 2014). Overexpression of Combover caused the formation of multiple hair cells in the wing, besides misaligned hair a common result of perturbation of PCP genes such multiple wing hair (Mwh) and Rok (Yan et al., 2008). PCP effector genes themselves, like mwh and rok are required to position hair at the right location (Fagan et al., 2014). Despite this PCP phenotype, it remains unclear how Cmb functions in this pathway and -based on genetic interaction experiments- it could only be speculated that the positive role of Cmb on wing hair formation is antagonized by Mwh and/or Rok (Fagan et al., 2014).

Since PCP and cilia in *Drosophila* are unconnected, we set out to better understand the role of Cmb in this pathway by performing RNAi-mediated depletion of Combover, genes involved in the PCP pathway, centrosome assembly, ciliogenesis as well as control genes. To examine effects on PCP we chose the pannier-GAL4 expression system to drive the expression in the central region of the notum of the fly, due to the availability of the driver line, the easy observable phenotypes and its use in a genome wide bristle screen by Mummery-Widmer et al. (Mummery-Widmer et al., 2009). Tissue-specific depletion of Combover via the GAL4/UAS system in the notum of the fly did not result in multiple wing hair or misaligned bristles, indicating no PCP phenotype similar to what Fagan et al. showed in the wing (Fagan et al., 2014) (Fig. 10A-C). Overexpression of Cmb on the other hand caused defects in the orientation of the bristles, similar to the depletion of PCP effector genes Frtz or other PCP genes (delta, O-fut1, bazooka, starry night), again as reported for the wing (Fagan et al., 2014). Similarly, a Cmb mutant (a null mutant generated by A. Jenny by replacing the genomic DNA with a White⁺ marker via homologous recombination) failed to cause a PCP phenotype, indicating that Combover is not required for PCP. Importantly, depletion of centrosome genes (Sas-4, Plp, Cnn, Spd-2, Asl) did not cause a PCP phenotype. Overall, we were able to recapitulate the expected phenotypes for the PCP genes with a similar score as seen in the bristle screen (Mummery-Widmer et al., 2009). Since neither acute depletion by RNAi nor loss of the protein in Cmb mutants caused a PCP phenotype, we concluded that Cmb is not required for PCP. Instead of a direct role, our data would point towards an indirect role of Cmb in the process of planar cell polarity.

A



B



C

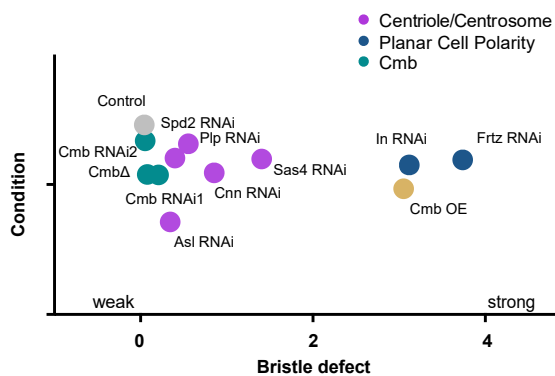


Figure 10. Combover (Cmb) is not required for planar cell polarity (PCP). (A) Tissue-specific RNAi performed using the Pannier-GAL4 driver, with the PCP effector Fritz (Strutt and Warrington, 2008) as positive control to drive the expression in the fly notum. Overexpression but not RNAi-mediated depletion or mutation of Cmb results in a planar cell polarity (PCP) phenotype, as seen by misalignment of bristles in the fly notum. (B) Further characterization of PCP phenotypes of selected genes. RNAi of PCP genes such as Inturned

(In) results in strong phenotypes, while centrosomal genes (Sas4 and Plp) show no or weak phenotypes. (C) Quantitation of bristle defects for selected genes. Phenotypes scored on a scale from 0 (no phenotype) to 4 (strong phenotype), with values shifted slightly to avoid overlap. N=10 flies per condition. Adapted from (Pachinger et al., 2024).

3.4 Combover is required for ciliogenesis

We next asked whether Combover is involved in any cilia-related function, given its homology to PCM1. We noticed abnormal wing posture in adult flies, a phenotype that has been linked to impaired mechanosensation in insects and has been previously reported for some cilia mutants (Dobbelaere et al., 2023; Tuthill and Wilson, 2016) (Fig. 11C). In *Drosophila*, cilia are present only in a few cell types that have specialized functions and allow specific readouts at the behavioral and microscopy level (Lattao et al., 2017). Sensory cilia are part of the type 1 monodendritic sensory neurons of the peripheral nervous system and as such are found in two different sense organs: external sense organs (for mechano- and chemosensation, olfaction) and chordotonal organs (proprioception and hearing) in the legs (Lattao et al., 2017). In initial experiments using the GAL4/UAS system described earlier we showed that tissue-specific depletion of Cmb in neurons (nanos-bam-GAL4) caused uncoordination of flies or pharate lethality. Defects in the mechanosensory cilia of the sensory bristles as well as the chordotonal organs cause defects in coordination and sensing. This behavior can be analyzed in the climbing assay, where flies get knocked to the bottom of the vial and their speed and ability to climb up the wall of a vial is assessed. Cmb mutants were severely uncoordinated, indicative of defects in negative geotaxis (Fig. 11 A,B). This phenotype could be rescued by introduction of a GFP transgene and was neither observed for the depletion of other PCP effector genes (such as Fy) nor the overexpression of Cmb. Instead, the observed phenotype of Cmb mutants strongly resemble those observed for centrosome mutants such as Sas-4 and Asl.

We next examined cilium morphology of Cmb mutants by light and electron microscopy to link the observed phenotype directly to defects in mechanosensory neurons. We observed scolopidia of chordotonal organs using the ciliary marker NompC and found no observable differences between wild-type and mutant cilia (Fig. 11E). However, by DIC imaging we could see subtle defects with an increased distance of the ciliary dilation to the cap cell, as seen for other cilia mutants (Fig. 11F). Electron-microscopy of cilia of the chordotonal organs revealed no gross structural defects in Cmb mutants, showing 9-fold symmetric centrioles in all samples analyzed (Fig. 11G). However, mild defects in the

morphology/organization of cilia could be observed in some of the analyzed samples. These include broken rings or an additional central pair of microtubules that usually is not observed in wild-type cilia. Taken together, the observed uncoordination phenotype could be traced to mild defects in ciliary ultrastructure.

We then investigated if motile cilia/sperm flagella are affected by loss of Cmb. Flagellar motility is required for sperm movement, hence defects in cilia commonly result in male infertility. From initial experiments using tissue-specific, RNAi-mediated depletion of Cmb in testes (Nanos-Bam-GAL4) we observed complete male infertility with both RNAi-lines used. Cmb mutants confirmed this phenotype, with no observable offspring (Fig. 12A). This defect could be rescued by expression of the GFP-transgene, confirming specificity of the phenotype. As with the climbing assay we saw no effect on fertility for Cmb overexpression or depletion of PCP genes, indicating that the phenotype is specific to Cmb and resembles the phenotype of known ciliary mutants. Similarly, placing the mutant over a deficiency showed that there are no non-allelic effects and female fertility was not affected as expected if defects are restricted to centrosomes/cilia (Fig. 11D). The fertility defects could further be corroborated by studying sperm motility. Sperm of Cmb mutant flies showed reduced motility and a failure to reach the seminal vesicle, with seminal vesicles consequently smaller than in wild-type (Fig. 12E). To examine the process of spermatogenesis we used Hoechst-Phalloidin staining to visualize nuclear morphology and actin cytoskeleton (Fig. 12F). While the early stages of spermatogenesis seemed unaffected with normal nuclei shape in the gonialblast, spermatocyte and spermatid stage, the later stage of spermatogenesis showed severe individualization defects. Instead of stacked actin cones appeared much more scattered. A similar but milder phenotype could be seen in the testes of Cmb RNAi flies. Thus, spermatogenesis is more profoundly affected than ciliogenesis in neurons.

We next turned towards transmission electron microscopy to examine sperm ultrastructure. Low magnification overviews showed an abnormal number of axonemes per cyst (Fig. 12C-D). In 8% of cysts there were fewer than 64 axonemes per cyst (surprisingly a few also contained more than 64), suggestive of a failure in axoneme extension or prior cell division defects. Closer examination of early and late spermatids revealed clear axonemal defects, with approximately 25% of defects in early and 15% defects in late spermatids respectively (Fig. 12B). The observed defects, including broken axonemes and missing microtubule doublets have been previously observed for other cilia mutants such as CFAP97 and CFAP299 family members (Dobbelaere et al., 2023). To sum up, loss of Cmb (by both

depletion and deletion of the protein) causes strong motile and weak neuronal cilia defects indicating that Cmb is required for cilium biogenesis and function.

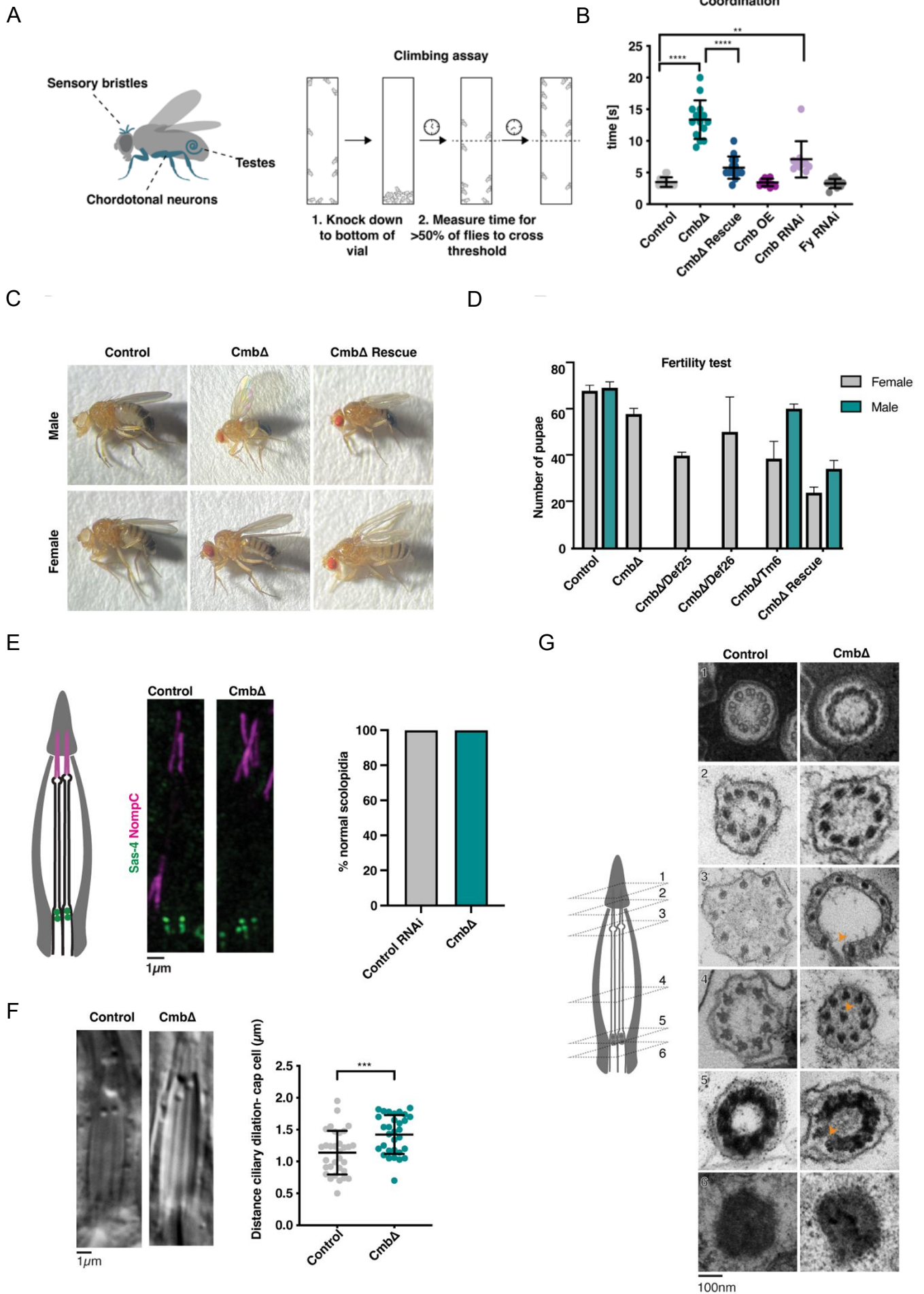
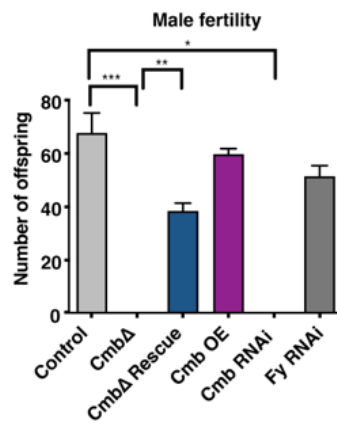
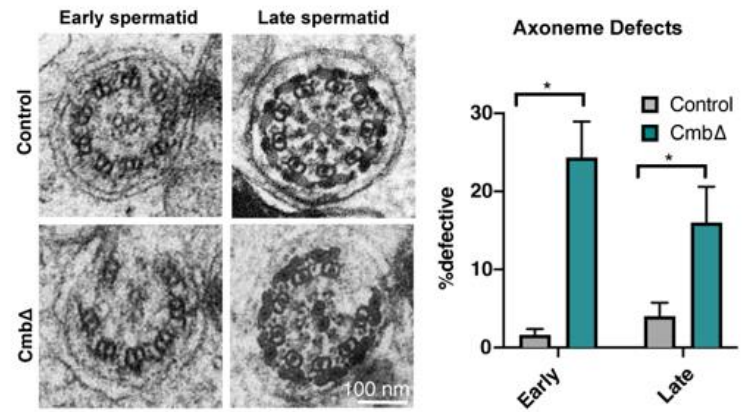


Figure 11. Combover is required for ciliogenesis. (A) Schematic of ciliated tissues in *Drosophila* used for phenotypic analysis. Primary cilia are found in sensory bristles and chordotonal neurons, while motile cilia/flagella are found in testes (Jana et al., 2016). (B) Climbing assay used to assess defects in mechanosensation. Cmb mutant flies are severely uncoordinated, a phenotype rescued by expression of Cmb-GFP. PCP flies (Fy RNAi) show no detectable phenotype. N>10 flies per condition. Kruskal-Wallis test with Dunn's multiple comparisons test was performed; **P< 0.01, ****P< 0.0001. (C) Appearance of Cmb mutant flies, as well as Cmb mutants rescued by expression of a GFP-Cmb transgene. Cmb mutants display abnormal wing posture, a phenotype associated with defective mechanosensation. (D) Fertility test performed on Cmb mutant males and females, Cmb mutants rescued by expression of a GFP-Cmb transgene or maintained over a balancer (Tm6) and Cmb mutants placed over a deficiency that covers the Cmb locus (Def 25, 26). Cmb mutant males but not females exhibit fully penetrant sterility, a defect rescued by expression of the GFP transgene. Placing the mutant over a deficiency does not impact fertility, excluding potential non-allelic effects. N=3 single males, each crossed to 4 virgin females. (E) Schematic and immunofluorescence micrographs of scolopidia in chordotonal organ of the fly. Sas4 and NompC used to visualize basal body (green) and ciliary tip (magenta), respectively. Each scolopidium contains two cilia with their ends embedded in the cap cell. No apparent ciliary structural defects are observed in Cmb mutants. N=63 control scolopidia, 63 Cmb mutant. Statistical test is t-test with Welch's correction. (F) DIC images of scolopidia. Cmb mutants display a larger distance between ciliary dilation and cap cell, indicative of ciliary positioning defects. N=32 control, N=31 Cmb mutants. Student's t-test with Welch's correction used; ***P< 0.001. (G) Cross sectional views of control and Cmb mutant scolopidia by transmission electron microscopy (TEM). Position indicated by numbers in schematic on the left. Cmb mutants show minor structural defects, including broken axonemes and misplaced doublet microtubules. Adapted from (Pachinger et al., 2024).

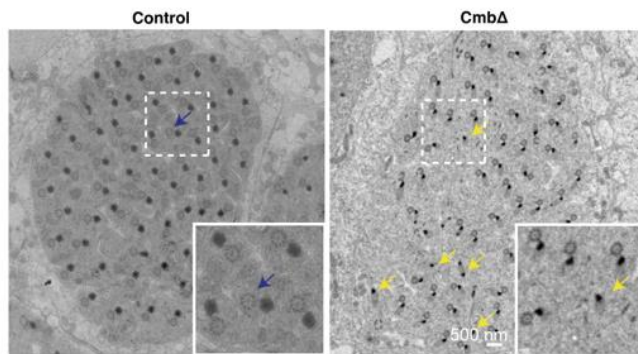
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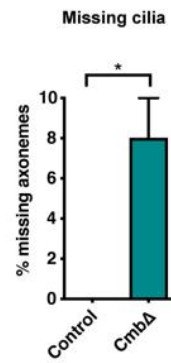
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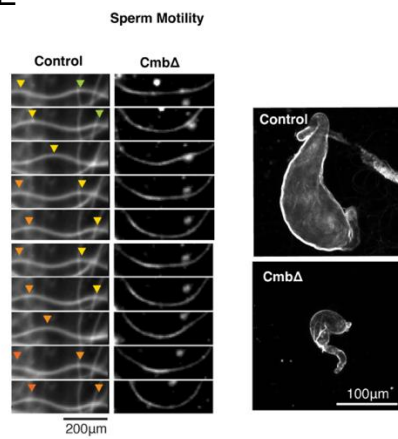
C



D



E



F

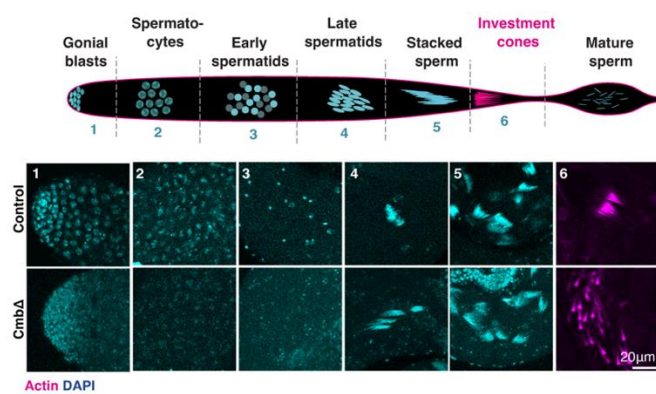


Figure 12. Combover is required for ciliogenesis. (A) Male fertility scored by crossing individual males with WT virgins and assessing the number of offspring. Cmb RNAi/mutant flies are fully male infertile. N=3 single males each crossed to 4 virgin females per condition. Kruskal-Wallis test with Dunn's multiple comparisons test was performed; *P<0.05, **P<0.01, ***P<0.001. (B,C) TEM analysis of sperm axonemes in control and Cmb mutant testes. Cross-sectional views reveal missing axonemal doublets and fragmented axonemes (D) as well as overall lower number of cilia (number per cyst < 64). N=38 cysts control, 38 cysts Cmb mutant. Student's t-test used to assess statistical significance; *P<0.05. (E) Left: Analysis of flagellar movement of control and Cmb mutant sperm by high-speed video capture in dark-field microscopy. Sinusoidal motion can be seen in wild type. Cmb mutant sperm show severely compromised flagellar movement. Right: In contrast to controls, seminal vesicles of Cmb mutant flies are almost devoid of sperm, indicating defective movement of sperm to seminal vesicle. (F) Schematic and immunofluorescence images of *Drosophila* spermatogenesis. In Cmb mutants the early stages of spermatogenesis appear superficially normal; however, in later stages investment cones involved in individualizing sperm fail to form properly. Adapted from (Pachinger et al., 2024).

3.5 Combover is required for proper cell division

Having established a link between Combover and ciliogenesis we wondered if the role of Cmb is exclusively cilia related or if it would extend towards centrosomal functions. To tackle this question we took advantage of the *Drosophila* early embryo, a perfect system for the study of centrosome assembly and function (Fig. 13A). Within this syncytial embryo centrosomes undergo multiple rounds of synchronous duplication and separation, allowing us to trace multiple centrosomes simultaneously over time. First, I crossed markers for centrosomes (Asl-GFP) and DNA (Histone 2A-RFP) into the Cmb mutant and imaged these rapid divisions, in particular nuclear cycle 12-13 (Fig. 13B). I could observe frequent chromosome missegregation events, cell cycle asynchrony and nuclei that eventually got ejected from the cortex in a process called nuclear fallout. Nuclear fallout is a mechanism employed by the embryo to protect the developing embryo from damaged nuclei that would otherwise result in lethality (Sullivan et al., 1993). Cmb mutants exhibited nuclear fallout to a higher degree and more frequently, with approximately 25% of mutant and 3% of wild type embryos being affected. This perhaps also explain the embryonic lethality seen upon crossing homozygous females to heterozygous males (homozygous mutant males being unable to mate and produce offspring), with 50% of lethality among the offspring (being 50% homozygous and 50% heterozygous) (Fig. 13C). If this is indeed the direct consequence of nuclear fallout cannot be determined at this point.

While this shows that Cmb is needed for efficient progression through the cell cycle it does not provide a direct link to centrosome function. From imaging of centrioles (Asl-GFP) in the mutants it became clear that centriole duplication was not affected at least at this level of resolution. However, to be able to look at centrosomes in more detail we then introduced a PCM-marker (Cnn-RFP). Imaging Cmb mutants expressing Cnn-RFP confirmed that there was no duplication defect; however, duplicated centrosomes were oftentimes not separating or not separating properly leading to the appearance of single larger centrosomes with abnormal morphology (Fig. 13D). This centrosome separation defect was observed in all mutant embryos analyzed, affecting approximately 7% of centrosomes within the mutant (as compared to 1.5% in wild type) and could be the cause of the mitotic defects. Quantification of centrosome intensity of fully separated centrosomes at nuclear envelope breakdown (NEBD) in nuclear cycle 12 showed no difference between mutant and control embryos, indicating that PCM recruitment is not affected. Considering that PCM1 has been shown to be required for the recruitment of some centrosomal components it is tantalizing to speculate that other

centrosome proteins might be affected as well in *Drosophila*; however, the PCM scaffolding component Cnn at least is not. Furthermore, PCM defects would tend to cause premature centriole separation and not delayed centriole separation. In conclusion, like PCM1 in vertebrates Cmb is required for proper cell division and cilium function as well as organismal viability and fertility. These phenotypic similarities suggest that Cmb could indeed be the functional homolog of PCM1.

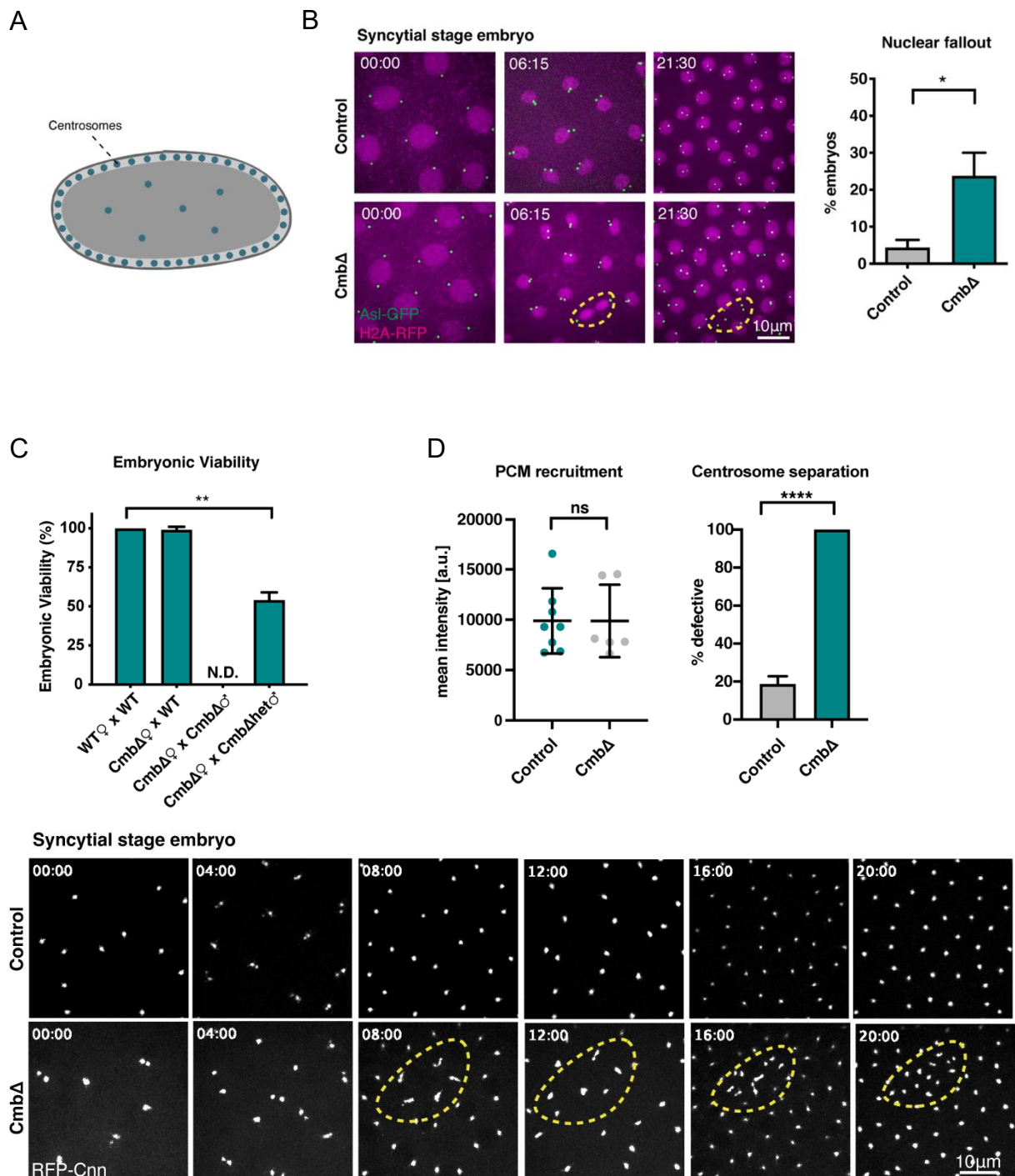


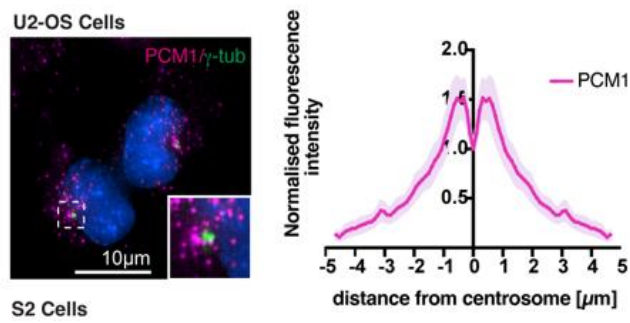
Figure 13. Combover is required for proper cell division. (A) Schematic of *Drosophila* syncytial-stage embryo showing synchronous nuclear divisions occurring close to the egg surface. (B) Live imaging of control and Cmb mutant early embryos (nuclear cycle 12) expressing the centriole marker Asl-GFP and H2A-RFP. Time shown in min:s. Cmb mutants show lagging chromosomes and increased frequency of nuclear fallout (orange dashed circle). N=6 control, 8 Cmb mutant embryos. Student's t-test was used to assess statistical significance; *P< 0.05. (C) Embryonic viability test shows lethality in 50% of offspring of Cmb mutant females with heterozygous mutant males (viability could not be assessed for homozygous males due to their failure to mate and fertilize oocytes). N=2 virgin females crossed to one male per condition. Student t-test used to assess statistical significance; **P<0.01. (D) Quantifications and live imaging of control and Cmb mutant early embryos (nuclear cycle 12) expressing the centrosome marker RFP-Cnn. Time shown in min:s. Cmb mutants show defects in centrosome separation but not PCM recruitment. PCM recruitment is unaffected in Cmb mutants. Cnn centrosome intensity measured at nuclear envelope breakdown (NEBD) in nuclear cycle 12. Dots represent average intensity of all centrosomes in a single embryo. N=8 control, 6 Cmb mutant embryos. Mann-Whitney test used to assess statistical significance. Centrosome separation assessed at nuclear cycle 12 and 13. N=16 control, 12 Cmb mutant embryos. Mann-Whitney test used to assess statistical significance; ****P< 0.0001. Adapted from (Pachinger et al., 2024).

3.6 Centriolar satellites are conserved in *Drosophila* but do not concentrate near centrosomes or cilia

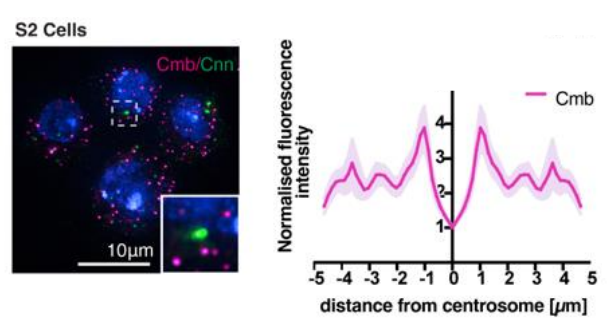
To examine if centriolar satellites in *Drosophila* do exist and if they are cytoplasmic protein assemblies that are similar to vertebrate centriolar satellites we set out to determine the localization of Cmb in different cells and tissues where centrosomes and cilia are present. For this we generated a rabbit polyclonal antibody targeting the N-terminal region of Cmb (amino acids 1-150) by raising a GST-fusion as antigen and purifying as described in the Materials and Methods. We first determined Cmb localization in S2 cells, where Cmb localized as discrete foci in the cytoplasm. Unexpectedly, unlike PCM1 the protein did not concentrate around centrosomes at any cell cycle stage. Radial profile analysis of Cmb in interphase showed no accumulation around the centrosome, in contrast to what is seen for PCM1 in vertebrate (U2-OS) cells (Fig. 14A,B). Further immunofluorescence experiments in early embryos, larval neuroblasts and testes confirmed the previous finding and again revealed no apparent concentration around the centrosome (Fig. 14C). Importantly, we could confirm the absence of signal in Cmb mutant testes, thereby confirming the specificity of the antibody. In testes we additionally looked for characteristic localization patterns during spermatogenesis; however, neither in early stages (gonialblast, spermatocyte) nor in later stages (spermatid, stacked sperm) could we observe any obvious patterns in Cmb localization. Furthermore, we examined localization of endogenous promoter-driven Cmb-GFP flies in several tissues, confirming this localization pattern. This lack of concentration around the centrosome was surprising and let us to believe that Cmb in flies has a function different from transport towards the centrosome and/or that these foci are not equivalent to vertebrate centriolar satellites. However, we could occasionally observe Cmb at the centrosome (in 21.1 +/- 7.4 % of S2 cells in interphase), colocalizing with the centriole protein Sas-4, with whom it was initially pulled down in a proximity-based experiment (Fig. 15A). Therefore, we still believed that there is a relationship to centrosomes despite the lack of clustering and the observed random localization. Cmb distribution was, however, not entirely different from PCM1 since it showed a very similar cell-cycle dependent expression, with a noticeable decrease in the number of foci during mitosis. To summarize, we showed that Cmb does not cluster around the centrosomes suggesting a transport-independent function; however, the similar cell cycle dependent distribution and occasional localization to centrosomes indicate a function related to centrosomes, that might also be applied to vertebrate PCM1 and centriolar satellites in general.

To rule out the possibility that these foci are distinct granules, different from the known protein assemblies, we looked at putative components of Cmb-containing foci. Since many of the proteins localizing to centriolar satellites in human are centrosomal components, we systematically checked the localization of centrosome proteins (centriole and PCM) for which antibodies in *Drosophila* were available in the lab. In S2 cells, which frequently contain extra centrosomes, we evaluated localization in conjunction with γ -tubulin, to distinguish between centrosomes and additional cytoplasmic foci. We could detect colocalization with many centrosome components (interestingly mainly centriole components such as Cpl10, Ana1, Ana2, and Sas-4, but also PCM-markers like Cnn) at cytoplasmic foci, indicating that like PCM1 Cmb-containing foci harbor a set of centrosome proteins (Fig. 14D, 15B). The degree of overlap for most of these proteins is not high, as assessed by quantification of Pearson's correlation coefficient as a proxy for co-localization; however, this correlation coefficient is similar to that for PCM1 in vertebrate cells (Gheiratmand et al., 2019). The absence of γ -tubulin from these granules again is consistent with some reports in vertebrates according to which γ -tubulin is absent from satellites and unaffected by PCM1 perturbations (Dammermann and Merdes, 2002). The absence of γ -tubulin and corresponding lack of microtubule nucleation ability therefore distinguishes PCM1 and Cmb-containing satellites from centrosomes. Certainly, many of the typical centriolar satellite components such as BBS4, SSX2IP, CCDC66 or OFD1 could not be tested, due to the lack of available antibodies or the lack of clear homologs in flies. Nevertheless, the unifying theme, the presence of centrosome proteins, seemed to be the same. In S2 cells, we found that centrosomal proteins seemed to be much less abundant in the cytoplasm compared to the centrosome, as seen by the very weak signal and that only few of the Cmb foci seemed to be colocalizing with cytoplasmic centrosome proteins. Nevertheless, when we evaluated co-localization of Cmb with Sas-4 *in vivo* in early spermatocytes we discovered that many more cytoplasmic Cmb-containing foci colocalized with Sas-4, while few colocalized on centrioles. This suggests that Cmb-containing granules show tissue-specific localization and co-localization patterns, reminiscent of the heterogeneity that is known for satellites in vertebrates. If the discrepancy in localization would be linked to cell-type specific and context-dependent functions remains to be investigated in the future. In summary, our data show that Cmb granules are conserved in flies, that they are indeed analogous to centriolar satellites in vertebrates, forming cytoplasmic foci comprised of centrosomal proteins.

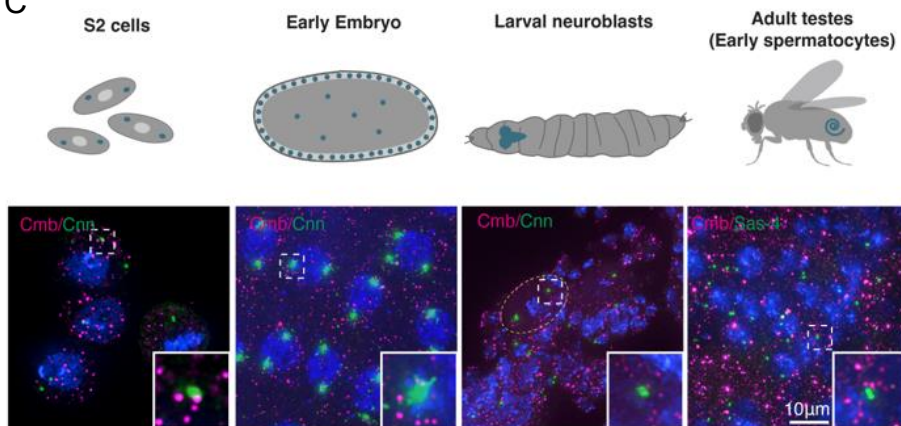
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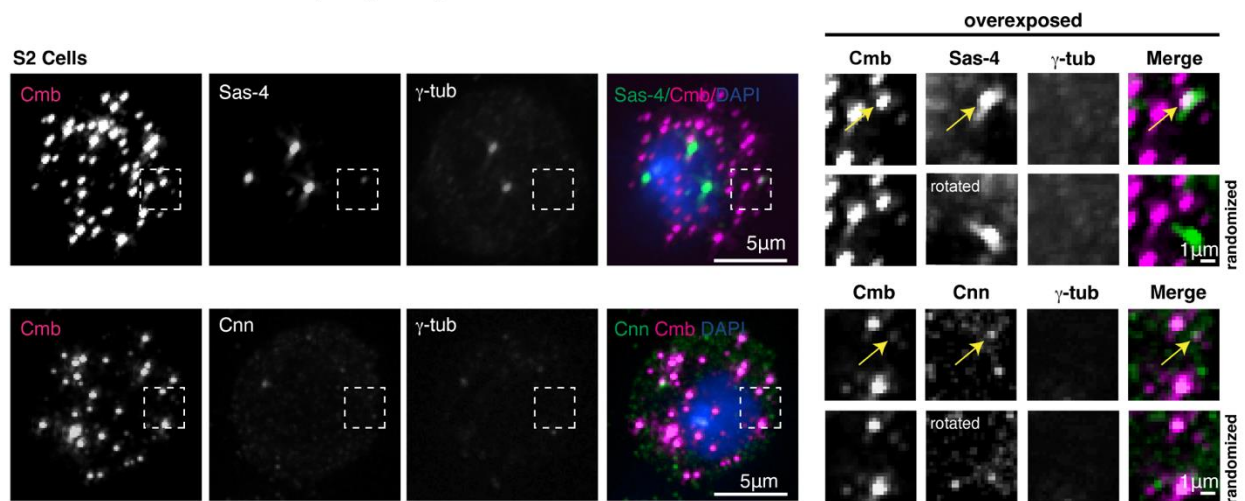
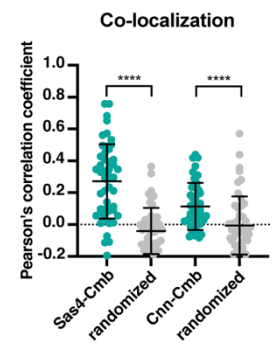
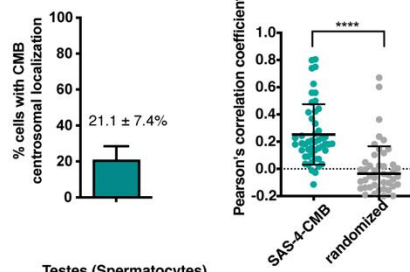
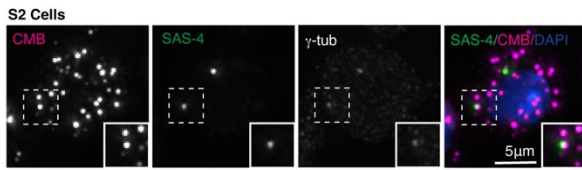
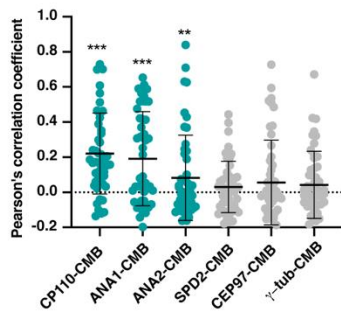
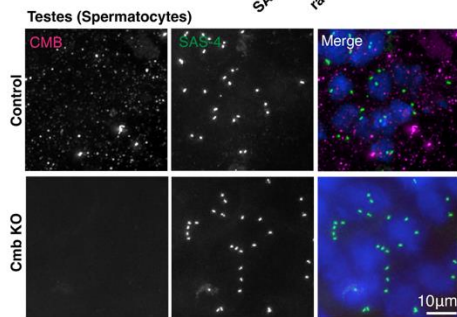


Figure 14. Centriolar satellites are conserved in *Drosophila* but do not concentrate near centrosomes or cilia (A) PCM1 concentrates in the vicinity of centrosomes (marked with γ -tubulin) in vertebrate cells. Radial profile of the normalized distribution of PCM1 (N=27 centrosomes). (B) Cmb localization in *Drosophila* S2 cells. Unlike PCM1, Cmb does not concentrate near the centrosome (marked with Cnn), but is found throughout the cytoplasm. N=39 centrosomes. (C) Top: Schematics showing cells and tissues used to examine Cmb localization/dynamics. Bottom: Immunofluorescence micrographs show Cmb localizing to cytoplasmic foci in all tissues examined. Sas4/Cnn used to visualize centrioles/centrosomes. (D) Centriolar (Sas-4) and centrosomal (Cnn) proteins co-localize with Cmb on cytoplasmic foci, although immunofluorescence signal is weak compared to that at centrosomes (marked with γ -tubulin). Colocalization of Cmb with Sas-4 and Cnn assessed by Pearson's correlation coefficient. Randomized control has one of the channels rotated 90°. Centrosomes excluded from analysis. N=50 cells per condition. Mann-Whitney test used to test statistical significance ;****P< 0.0001. Adapted from (Pachinger et al., 2024).

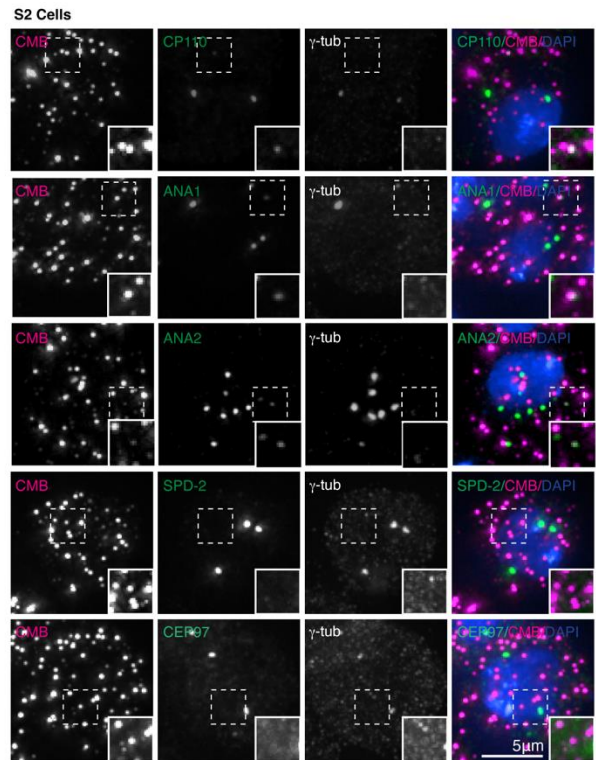
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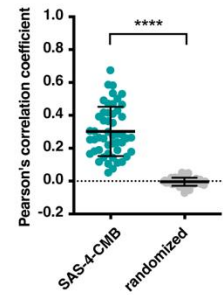
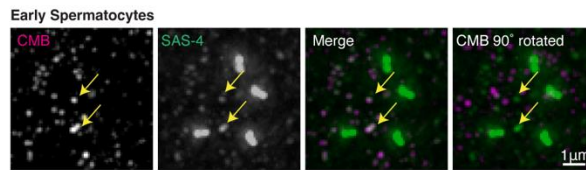


Figure 15. Further analysis of Cmb localization, related to Figure 7. (A) Immunofluorescence micrograph showing Cmb localizing to centrosomes in a subset of cells in S2 cells. Quantitation of Cmb localization reveals centrosome localization in ~20% of cells. Pearson correlation coefficient analysis of centrosomal signal shows significant overlap between Sas-4 and Cmb compared to randomized controls (single channel rotated by 90° with centrosome positioned in the upper right quadrant of the square analyzed). Student's t-test was used; ****P< 0.0001. N=50 cells. **(B)** Specificity of polyclonal antibody raised against Cmb confirmed by absence of immunofluorescence signal in Cmb mutant testes. **(C)** Immunofluorescence micrographs showing some (Cp110, Ana1, Ana2) but not all (Spd-2, γ -tubulin) centrosomal proteins colocalizing with Cmb on cytoplasmic foci. Pearson's correlation coefficient analysis on cytoplasmic signal assessed as in Figure 3G. N=50 cells per condition. Mann-Whitney test used to test statistical significance; ***P< 0.001, **P< 0.01. **(D)** Immunofluorescence micrographs showing Sas4 colocalizing with Comover in the cytoplasm of primary spermatocytes in the testes. Colocalization quantified by Pearson's correlation coefficient analysis. N=12 animals. t-test used to assess statistical significance of colocalization compared to randomized controls (single channel rotated 90°); ****P< 0.0001. Adapted from (Pachinger et al., 2024).

3.7 Centriolar satellites show little sign of directed movement

In order to better understand the nature of centriolar satellites in *Drosophila* we aimed at elucidating the dynamics of these protein assemblies. Given the lack of enrichment around the centrosome we wondered if we would still see signs of transport and thus directed movement. In the early embryo, in flies expressing Cmb-GFP and Cnn-RFP we could observe foci, some of them moving randomly in different directions. To be able to directly compare the dynamic behavior to the one described for PCM1 and other centriolar satellite proteins in vertebrate cells we then investigated the dynamics of Cmb in S2 cells stably expressing Cmb-GFP. Remarkably, only a subset of those foci exhibit long- or short range movements, while the majority of satellites seemed to be rather static (Fig. 16 A-E). Analysis of the distances travelled over time showed that instantaneous speed is mostly below $0.5\mu\text{m}/\text{sec}$ with the majority moving with approximately $0.2\mu\text{m}/\text{sec}$ (Fig. 16C). These values are significantly lower than those reported for motor proteins like kinesin and dynein (speed $>0.5\mu\text{m}/\text{sec}$). Similarly, the mean squared displacement (MSD) and calculated diffusion coefficient points towards minimal movement and a behavior that can be mostly explained by Brownian diffusion, as seen by the linear relationship between MSD and time. Furthermore, we then looked at individual tracks in more detail using TraJClassifier, a machine-learning based Fiji plugin that characterizes different trajectories based on different features and classifies them into different groups (see Materials and Methods). This analysis showed that approximately 90% of tracks can be classified into the group of diffusion (50% Brownian diffusion, linear relationship between MSD and time) and subdiffusion (40% anomalous diffusion, non-linear relationship between MSD and time) (Fig. 16D). The remaining 10% of tracks can be described by confined movement or directed/active movement. If there was movement, we could observe mostly short-range but sometimes also long-range movement and importantly, this movement seemed to be random, in different directions with no obvious signs of kinesin and dynein-driven bidirectional movement along microtubules. Thus, we can conclude that centriolar satellites in *Drosophila* share similarities in their dynamic behavior with vertebrate centriolar satellites. The (almost complete) lack of movement and in particular directed movement strongly suggests that satellite function does not involve transport along microtubules.

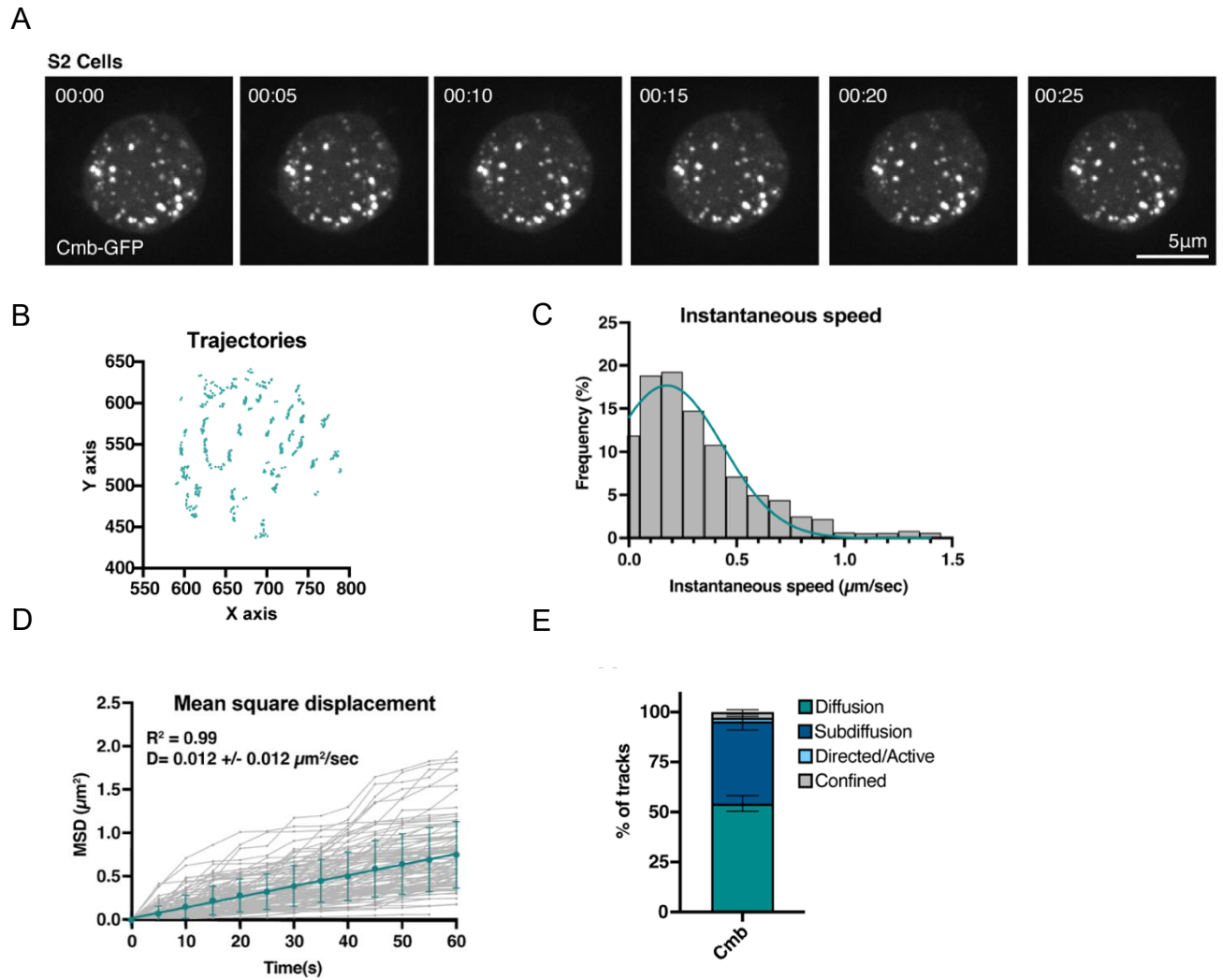


Figure 16. Centriolar satellites show little signs of directed movement. (A) Time-lapse recording of *Drosophila* S2 cell expressing Cmb-GFP. Time is shown in min:s. Cmb particles display little movement. (B) Corresponding trajectory analysis showing position of individual particles over time. (C) Histogram plotting frequency of instantaneous speed ($\mu\text{m}/\text{sec}$) of all particles tracked in 8 different cells. $N=140$ particles. The majority of particles moves at slow speeds ($0.2\text{--}0.3\mu\text{m}/\text{sec}$), consistent with diffusion. (D) Mean-Squared-Displacement (MSD) as a function of time. Trajectories with at least 11 frames from 8 cells were analyzed. All tracks analyzed shown in grey ($N=99$ tracks). Weighted mean square displacement (mean $\pm$ SD) of all diffusive particles follows a linear fit (turquoise line), reflecting overall Brownian diffusion. (E) Stacked column plot (mean $\pm$ SEM) showing all tracks analyzed in (D) assigned to different categories as described in Materials and Methods. The majority of tracks analyzed display Brownian diffusion ($54.2\% \pm 3.9$) or subdiffusion/anomalous diffusion ($41.1\% \pm 4.3$). Only a minor fraction displays directed/active ($1.8\% \pm 0.7$) or confined movement ($2.9\% \pm 1.1$). Adapted from (Pachinger et al., 2024).

3.8 Centriolar satellites are enriched in centrosome and cilia components

After determining phenotypes, localization and dynamics we aimed at elucidating the role of satellites in *Drosophila*. To get more functional insights we turned towards proteomics with the hope of identifying clusters or networks of proteins that would help us guide or analysis. I first generated a construct expressing the proximity ligase TurboID in *Drosophila* S2 cells, stably transfected cells, induced biotinylation and pulled down proteins in close vicinity of Cmb using streptavidin-coated beads (Fig 17A). Since Cmb in some cells did localize to the centrosome, we decided to distinguish between interactions at the centrosome (insoluble/pellet fraction) and in the cytoplasm (soluble fraction). Most of the proteins identified in the pellet fraction (61 out of 73) could be identified in the cytoplasmic fraction as well, whereas only a smaller fraction (approximately 12%) of proteins identified in the cytoplasm could be identified in the pellet fraction. Thus, more interactions happen in the cytoplasmic fraction and no obvious differences are observed. Importantly, using this reciprocal mass spec experiment I was able to confirm Sas-4 as a proximity interactor of Cmb in both cytoplasm and centrosome fractions. We first looked for centrosome components and identified both centriole and centrosome components within the highly enriched genes: in the pellet fraction centriole proteins (Sas-4, Ana1, Cep135, Cnb) and inner PCM proteins (Spd2, Plp) could be detected; in the cytoplasmic fraction mostly the same proteins were identified, as well as a few additional ones (Ana2, Cnn, TACC). This, together with the colocalization of some of these components to centriolar satellites, confirmed that satellites do exist in *Drosophila* as protein assemblies of centrosomal proteins.

Since S2 cells are non-ciliated cells, we did not detect any cilia components. In order to see if cilia proteins are enriched as well in satellite preparations and to extend our analysis to an *in vivo* level, I generated flies expressing a GFP-nanobody-TurboID construct under a germline promoter (cloned from a TurboID construct generated in the Brennecke lab, see material and methods) to investigate proximity interactors within fly testes. Once more, I was able to pull down Sas-4 but additionally many cilia-related proteins, especially components of the ciliary motility machinery (CCDC39, CCDC40) and axonemal dynein assembly factors (DNAAFs). Spermatogenesis in *Drosophila* testes involves a specialized form of cytoplasmic ciliogenesis, in which IFT and BBS proteins do not contribute to cilium biogenesis, and thus were not identified. Comparison of these results to the proteome of the centrosome-cilium interface previously defined in vertebrates (by Gupta et al.) showed that 122 out of 412 Cmb proximity interactors in S2 cells (30%) -and 103 out of 331 for TurboID in testes- are conserved

in humans and a smaller but significant part (15%,16%) overlapped with interactors of the centriolar satellite proteome defined by Gheiratmand et al and Quarantotti et al. (Fig. 17F). Thus, centriolar satellites in *Drosophila* share a conserved set with vertebrate centriolar satellites.

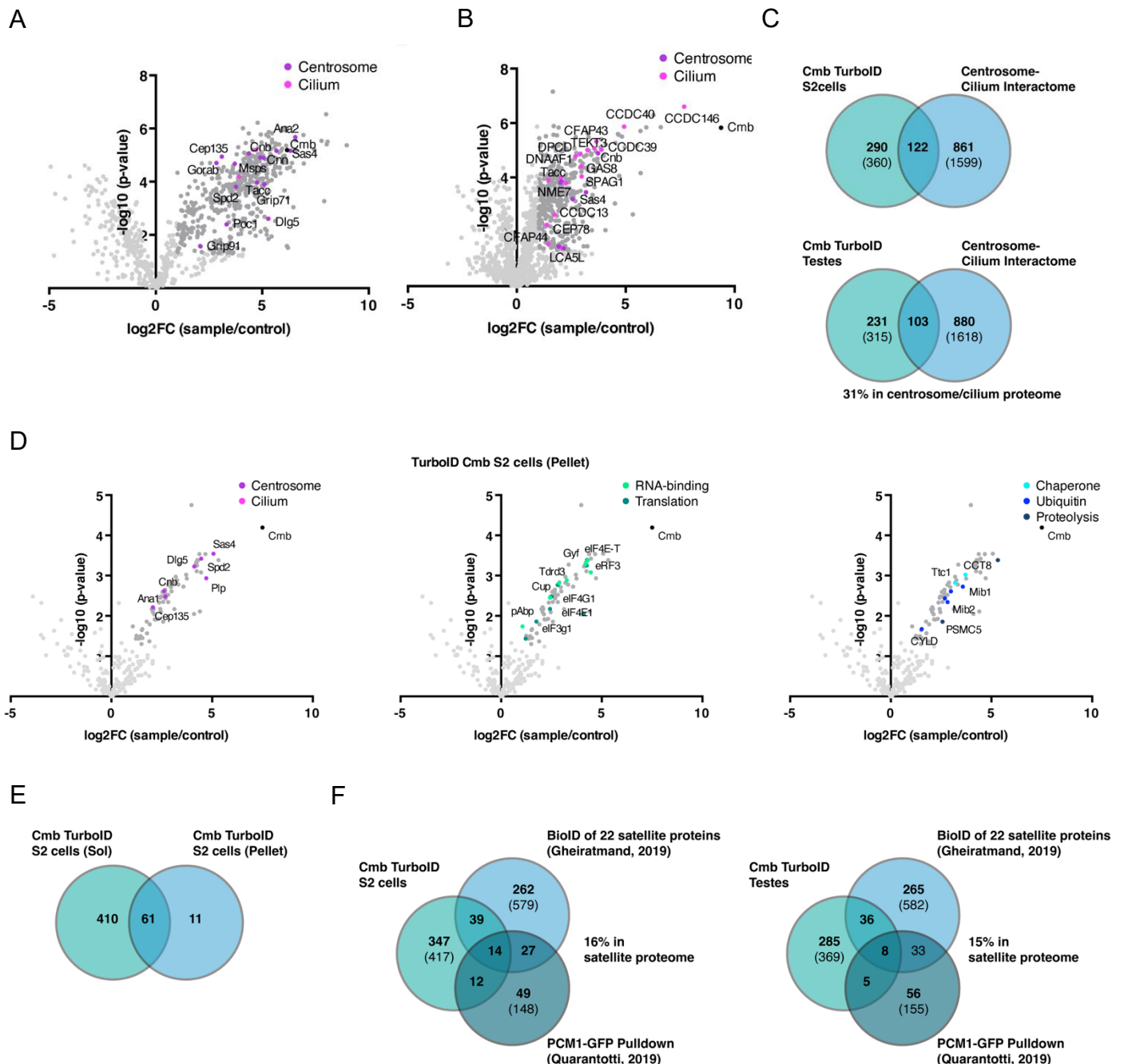


Figure 17. Centriolar satellites are enriched in centrosome and cilia components. (A) Direct TurboID of Cmb in S2 cells (cytosolic fraction, A) and indirect, GFP nanobody-targeted TurboID in fly testes (B) identifies centrosomal (magenta) and ciliary proteins (pink). Volcano plots of $-\log_{10}$ p-values against $\log_2$ fold change (sample/control). Significantly enriched proteins ($\log_2$ enrichment >1 , p-value <0.05) indicated in dark grey, with proteins of the above functional categories highlighted in color. (C) Venn diagrams showing overlap between Cmb S2 cell and testes TurboID interactomes and human centrosome/cilium proteome defined by (Gupta et al., 2015). Comparison for those proteins conserved between human and flies. Numbers in parentheses are total number in each dataset. Results of direct TurboID performed on Cmb S2 cells (detergent-insoluble cytoskeletal fraction). (D) LC-MS/MS analysis reveals centrosomal proteins (magenta), RNA-binding proteins (light green) and proteins involved in translation (dark green), chaperone-mediated protein folding (light blue), ubiquitination (blue) and proteolysis (dark blue). Volcano plots of $-\log_{10}$ p-values against $\log_2$ fold change (sample/control). Significantly enriched proteins ($\log_2$ enrichment >1 , p-value <0.05) indicated in dark grey, with proteins of the above functional categories highlighted in color. See also Table S2C. (E) Venn diagram revealing significant overlap between Cmb proximity interactome obtained from detergent-soluble (cytoplasmic) and detergent-insoluble (cytoskeletal) fractions of S2 cell extracts. See also Table S2E. (F) Comparison of Cmb S2 cell and testes TurboID interactomes with previous published datasets for centriolar satellites: the BioID of 22 satellite proteins mapped by (Gheiratmand et al., 2019) and the PCM1-GFP pulldown performed by (Quarantotti et al., 2019). Comparison for those proteins conserved between human and flies. Numbers in parentheses are total number in each dataset. See also Table S2E. Adapted from (Pachinger et al., 2024).

3.9 Centriolar satellites are associated with cytoplasmic protein synthesis

Closer examination of the conserved set of proteins and all the hits identified in both mass spectrometry experiments revealed additional conserved proteins. Among these, the ubiquitin ligase Mib1 and the deubiquitinase CYLD previously linked to PCM1 (Douanne et al., 2019). Intriguingly, one third of the highly enriched genes in the testes mass spec experiment was linked to protein quality control either via proteostasis or translation and of those another third linked to translation (Fig. 18A,B). For the proteins related to proteostasis, proteins of different pathways could be identified with the most prominent ones being

chaperone proteins, ubiquitin proteins and proteolysis proteins. Apart from Mib1 and CYLD, the presence of Mib2 in both datasets is interesting, and although no direct evidence of a Mib2 PCM1 interaction has so far been reported, it has been suggested in vertebrates to potentially work redundantly to Mib1 (Matsuda and Chitnis, 2009). Members of the family of chaperonin containing T-complex polypeptide (CCT) proteins as well as DnaJ/HSP40 proteins (important for protein translation, folding, unfolding, translocation and degradation) (Grantham, 2020; Qiu et al., 2006) were also present in both *in cellulo* and *in vivo* mass spec datasets.

Since centriolar satellites have been previously linked to proteostasis in a few studies, these results were interesting but not too surprising. Unpredicted however was the presence of the many translation-related proteins. In fact, we could observe a 37-40% overlap of the Cmb interactome conserved in human with the cytosolic RNA interactome defined by Youn et al. (Youn et al., 2018) (Fig. 18C). Here, RNA-binding proteins or transcription factors involved in different steps of translation were most prominent. The presence of RNA-binding proteins Orb2 and Fmr1 is exciting since both have been previously associated with translation of centrosome components (Fang and Lerit, 2022; Ryder et al., 2020). In humans, CPEB proteins (cytoplasmic polyadenylation element-binding proteins, homologous to *Drosophila* Orb and Orb2) localize to the mitotic spindle and centrosome and many centrosomal proteins could be identified as CPEB targets (Stepien et al., 2016), while Fmr1 -besides sharing many targets with CPEB- was linked to regulation of centrosome-related mRNAs (Ryder et al., 2020). Surprisingly, many eukaryotic translation factors and in particular numerous subunits of eukaryotic translation initiation factors were enriched. The many factors involved in the initial steps of translation initiation and the presence of almost all subunits of the EIF4F complex as well as the 43S Pre-initiation complex strongly suggest that Cmb is involved somehow in this process. Indeed, when performing the Gene Ontology (GO) analysis, we identified a striking enrichment of terms related to translation initiation both for the categories ‘cellular component’ and ‘biological process’ (Fig. 18D). An expert in the field of proteostasis we consulted, Della David (Babraham Institute, UK) pointed out that besides the eukaryotic translation initiation factors, many chaperones/chaperonins involved in checking the protein folding in the first step of translation are present in our datasets (eg NAC). Prompted by these observation, we hypothesized that centriolar satellites could be sites of translation specifically for centrosomal and ciliary proteins.

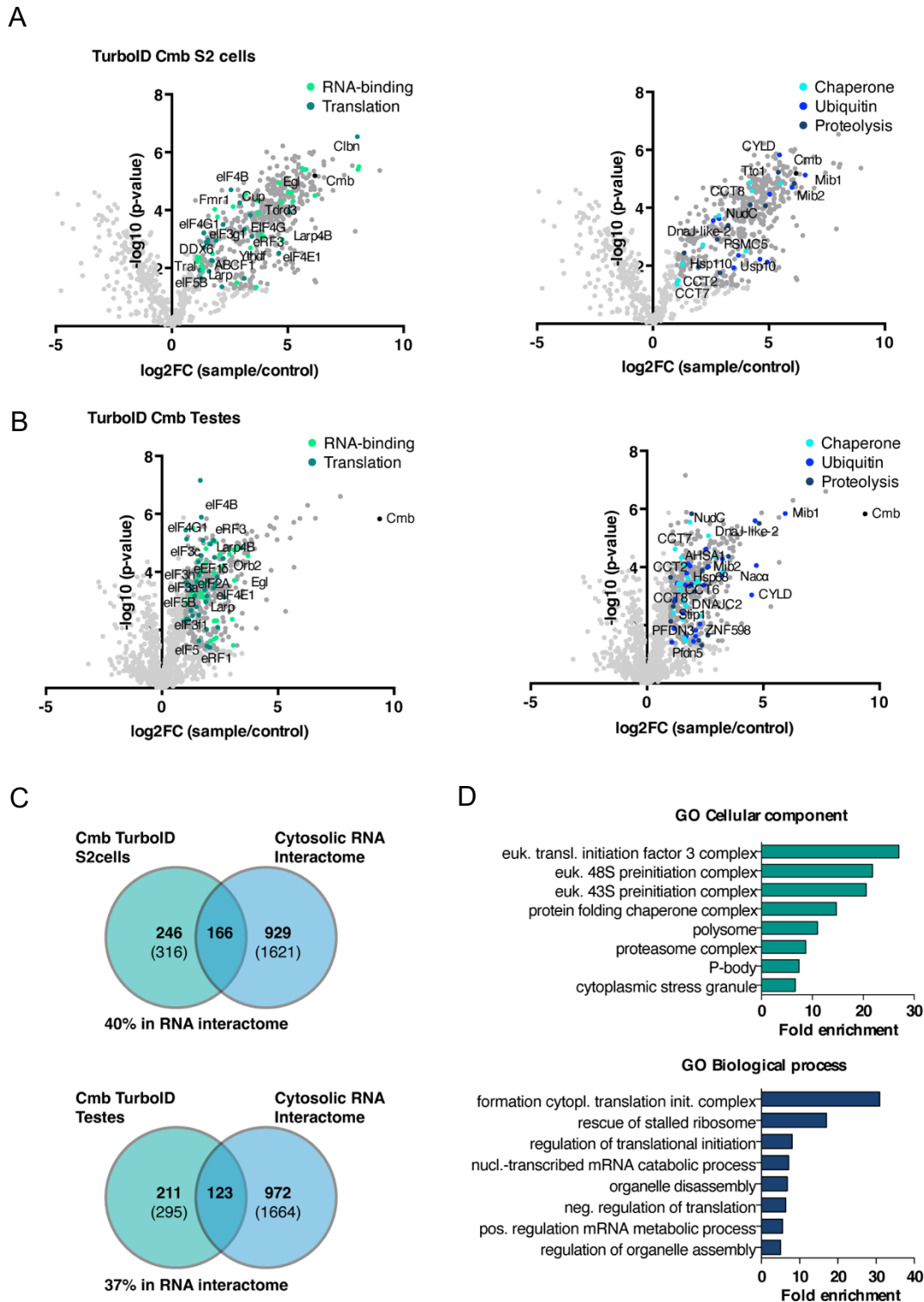


Figure 18. Centriolar satellites are associated with cytoplasmic protein synthesis. (A) Direct TurboID of Cmb in S2 cells (cytosolic fraction, A) and indirect, GFP nanobody-targeted TurboID in fly testes (B) identifies RNA-binding proteins (light green) and proteins involved

in translation (dark green), chaperone-mediated protein folding (light blue), ubiquitination (blue) and proteolysis (dark blue). Volcano plots of $-\log_{10}$ p-values against $\log_2$ fold change (sample/control). Significantly enriched proteins ($\log_2$ enrichment >1 , p-value <0.05) indicated in dark grey, with proteins of the above functional categories highlighted in color. (C) Venn diagrams showing overlap between Cmb S2 cell and testes TurboID interactome and cytosolic RNA interactome defined by (Youn et al., 2018). Comparison for those proteins conserved between human and flies. Numbers in parentheses are total number in each dataset. See also Table S2E. (D) Gene Ontology (GO) enrichment analysis performed on human orthologs of the Cmb TurboID testes dataset. The top eight terms and their fold enrichments are shown for the GO categories ‘cellular component’ and ‘biological process’. Adapted from (Pachinger et al., 2024).

3.10 Centriolar satellites as sites of translation in *Drosophila*

In order to test if centriolar satellites in *Drosophila* could be sites of protein synthesis I employed a method to visualize this process by microscopy. Several approaches have been developed in the past to detect translating ribosomes at a single cell level *in vivo*, some based on click-chemistry (OPP-puro labeling), others for the detection of specific mRNAs in tissues and cells (Puro-PLA)(Aviner, 2020). To look for protein synthesis in general I decided to use an *in vivo* puromycin labeling assay. This approach involves the treatment of cells with puromycin, a translation inhibitor that mimicks tyrosyl-tRNA, binds the ribosomal acceptor site, and labels nascently translated peptides. Subsequently, puromycylated peptides get released and can be detected with specific antibodies against puromycin, allowing rapid detection of protein synthesis (Fig. 19B).

I started out by treating S2 cells with puromycin for different incubation times from short pulses (30s, 1min), to intermediate incubation times (2min, 3min) to longer incubation times (5min, 10min). It quickly became clear that longer incubation times caused unspecific signal, labeling almost the entire cytoplasm due to the rapid diffusion of puromycin. However, short treatments did result in individual foci that could be detected. Importantly, control cells, treated with DMSO instead of puromycin for the indicated time, showed few foci that furthermore did not colocalize with Cmb. Similarly, cells that were pre-treated with the translational inhibitor cycloheximide before incubation with puromycin, showed only diffuse cytoplasmic signal, indicating that puromycin diffuses randomly into the cytoplasm and is

unable to bind to nascent peptides since translation has been stopped beforehand (Fig. 19C). In cells treated briefly with puromycin (30s, 1min, 2min, 3min) I could detect multiple puromycin foci, some of which overlapped with Cmb foci, indicating that those could indeed be sites of translation. Calculating the Pearson's correlation coefficient for Cmb and Puromycin for all these conditions revealed a significant difference between the puromycin condition compared to control cells or cells treated with Cycloheximide prior to puromycin addition. Overall, approximately 15% of Cmb foci per cell were labeled with puromycin. Since in a few cells, only few co-localizing foci could be detected we repeated the experiments including markers for centrosomes to rule out that the labeled foci are centrosomes and not satellites. We could show that puro foci did indeed colocalize with Cmb in the cytoplasm and not on centrosomes, indicating that Cmb containing centriolar satellites are sites of translation. Unfortunately, puromycilation of tissues (testes and embryos) did not work in our hands, potentially due to issues in permeability. Nevertheless, our data suggests that, at least in *Drosophila*, centriolar satellites could be hubs for translation.

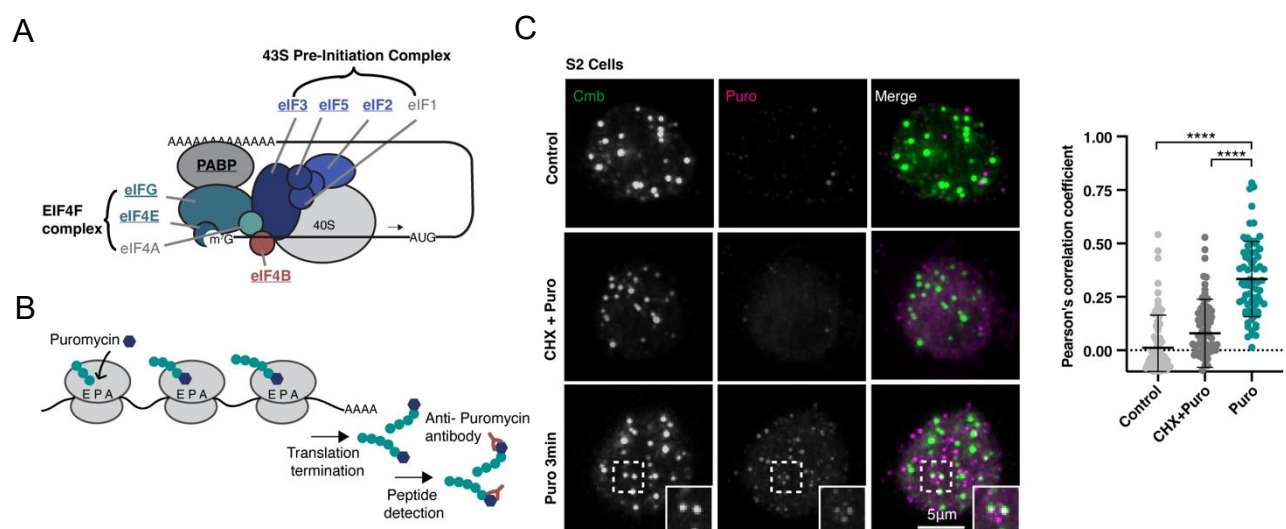


Figure 19. Centriolar satellites are sites of translation in *Drosophila*. (A) Schematic of eukaryotic translation initiation(Jackson et al., 2010). The 43S-preinitiation complex is recruited to the mRNA by the EIF4F complex through interaction of EIF4G with eIF3. Components identified in the Cmb interactome are highlighted in bold. (B) Schematic of puromycin labeling assay. Puromycin mimics tyrosyl-tRNAs and binds the ribosomal acceptor site, blocking translation. Nascent peptide chains labeled with puromycin (puromycylated) are released into the cytoplasm and can be detected using antibodies against puromycin. (C)

Puromycin labeling performed in *Drosophila* S2 cells. Puromycin labels Cmb foci in the cytoplasm after brief incubation with puromycin. No signal is detected in control cells or cells pre-treated with cycloheximide before addition of puromycin. Colocalization of Cmb and puromycin label quantified by Pearson's correlation coefficient analysis. N=70 cells per condition. Kruskal-Wallis test followed by Dunn's multiple comparison test used to assess statistical significance; ****P< 0.0001. Adapted from (Pachinger et al., 2024).

3.11 Centriolar satellites as sites of translation in vertebrate cells

Prompted by this observation, we were wondering if centriolar satellites in *Drosophila* are indeed functioning as translation sites, if this could be a conserved function that has so far been missed in vertebrates. Therefore, we sought to apply the same method for visualization of translation in vertebrate cells. However, in none of the cell lines tested and in none of the conditions with different incubation times could we observe distinct puromycin foci colocalizing with PCM1. In fact, in most cases puromycin did not even form distinct foci but was rather found uniformly dispersed in the cytoplasm, indicating fast diffusion into the cytoplasm. Control cells and pre-treated cells did, however, show the expected outcome, indicating labeling was occurring. We then applied a different version of the puromycin labeling assay that works in the same way, but uses a alkyne analog of puromycin (OPP, O-propargyl-puromycin) that incorporates into newly translated proteins and can be detected using click-chemistry. Again, the signal was primarily found in the cytoplasm and no distinct foci could be detected. Hence, we suspected that either the higher rate of protein synthesis in these cells compared to S2 cells or the rapid diffusion of nascent polypeptides could be the explanation for this method failing in our hands. Indeed, a recent study found that nascent polypeptide chains are instantly released and diffuse away from the ribosome into the cytoplasm, making it difficult to identify true sites of translation (Enam et al., 2020).

Nonetheless, I aimed to further test our hypothesis and took advantage of the fact that many centriolar satellite proteins in vertebrates are well characterized and antibodies to them are commercially available. Therefore, I again used puromycin, but this time for prolonged incubation times (1 hour) to completely abort protein synthesis and look at the effect on several centriolar satellite proteins (Fig. 20A). Centrosome proteins that also localize to centriolar satellites (PCNT, Cep290, Cdk5rap2 and Cep131) were completely depleted from centriolar satellites in the cytoplasm or decreased dramatically in number while the centrosome fraction

for each of the proteins tested remained, indicating that the cytoplasmic centriolar satellite fraction is newly synthesized while the centrosome contains comparably older protein. For PCNT this effect was very pronounced with almost no additional cytoplasmic foci being left after the treatment with puromycin. In addition, for some of those candidates I additionally applied another translational inhibitor, cycloheximide, that works similarly to puromycin with the difference of staying bound to the ribosome, and could again observe a similar decrease in number of cytoplasmic foci or complete elimination from the centriolar satellite fraction (as in the case of PCNT). While this result in principle could also be the result of redistribution of centriolar satellite components to the centrosome, we ruled out redistribution by showing that the levels at the centrosome was not increased. Thus, the satellite population for the centrosomal proteins tested is indeed newly synthesized.

Importantly, proteins of the quality control machinery (Mib1 and Ofd1) as well as PCM1 itself were not abolished from their centriolar satellite fraction, indicating that they are translated differently. However, inhibition of translation did not leave those proteins unaffected, since signal dispersed throughout the cell, with little or no increase in the number of foci. The persistence of PCM1 upon puromycin addition suggests that centriolar satellites still exist (though dispersed) and indicates that loss of centrosome components from the cytoplasm is not due to disassembly of centriolar satellites. In addition, both Ofd1 and Mib1 remained in association with PCM1 even though dispersed, with Pearson's correlation coefficient that were not significantly different from controls (Fig. 20B). This could mean that those proteins are involved in PCM1 function and thus potentially part of the machinery involved in translation. In any case, what the experiment above demonstrates is that while centrosomal proteins are apparently synthesized on satellites, PCM1 and the quality control machinery are not. This then would make centriolar satellites specific for the translation of centrosomal and ciliary proteins.

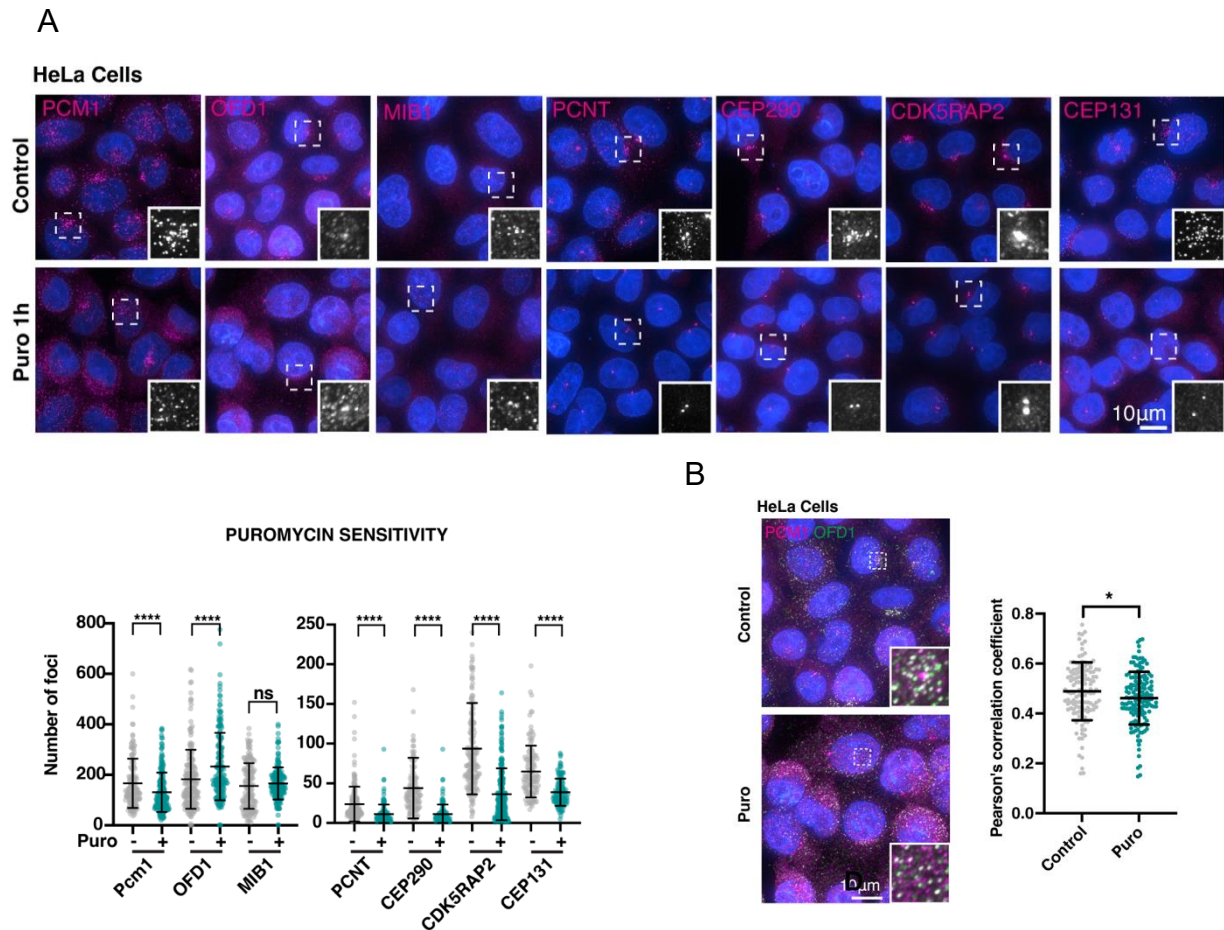


Figure 20. Centriolar satellites are sites of translation of centrosomal and ciliary proteins in vertebrate cells. (A) Immunofluorescence micrographs and quantitation of centriolar satellite protein signal in control and puromycin-treated HeLa cells. Centrosomal and ciliary proteins PCNT, CEP290, CDK5RAP2 and CEP131 are significantly depleted of their cytoplasmic localization upon puromycin treatment, whereas their centrosome localization persists (centrosomes identified using anti- γ -tubulin countermarker, not shown), suggesting the former represents newly-synthesized protein. In contrast, foci of PCM1, OFD1 and MIB1 remain, although now dispersed throughout the cytoplasm. >100 cells analyzed per condition. Mean $\pm$ SD are displayed. Mann-Whitney test used to assess statistical significance; **** $P < 0.0001$. (B) Immunofluorescence micrographs and quantitation of PCM1 and OFD1 distribution by Pearson's correlation coefficient analysis in control and puromycin-treated cells. PCM1 and OFD1 co-localize independent of translation. N=125 cells control, 147 puromycin treatment. Mean $\pm$ SD indicated (Students t-test; * $P < 0.05$). Adapted from (Pachinger et al., 2024).

3.12 Centrosomal/ciliary mRNAs closely associate with centriolar satellites

Next we reasoned that if centriolar satellites are truly sites of translation for centrosomes and cilia we would expect to see centrosomal/ciliary mRNAs on these granules. We were most curious to investigate one particular mRNA, *PCNT* mRNA, for the reason that is a well-established centriolar satellite client protein, because it showed a strong effect in the puromycin sensitivity assay and because of its localization around the centrosome in prometaphase, a stage where PCM1 is mainly found concentrated (Dammermann and Merdes, 2002). Hence, we made use of single molecule fluorescence in situ hybridization (smFISH) in combination with immunofluorescence to visualize mRNA and their localization in regards to centriolar satellites. In Hela cells, *PCNT* mRNA was found in close proximity to PCM1 in interphase, indicating that *PCNT* is translated in the vicinity of centriolar satellites (Fig. 21B). Interestingly, most mRNA foci resided in approximately 400nm away from the center of mass when calculating center-center distances. However, when displaying the results as edge-edge distances it became clear that those 19% of *PCNT* mRNA foci co-localizing (edge-edge distance of 0-0.05 μm), correspond to the fraction localizing up to 400nm away from the center of PCM1 (center-center distance $<0.4\mu\text{m}$). We took advantage of the ‘shuffle’-function, build-in the DiAna plugin, to show that the measured distances are significantly different from a random localization. Here, both foci for *PCNT* mRNA and PCM1 get randomly distributed within a defined mask (the cell excluding nucleus), and distances and cumulative frequencies displayed. When performing this analysis we could show that the measured distances for *PCNT* mRNA-PCM1 are indeed statistically different from a localization by chance. In conclusion, a fraction of *PCNT* mRNA foci (approximately 19%) is colocalizing with PCM1 in interphase cells and could thus mean that it is being translated there.

As expected, in prometaphase both *PCNT* mRNA and PCM1 showed colocalization around the centrosome. This then could mean that *PCNT* is translated on/near centriolar satellites in interphase, and brought to the centrosome at the onset of mitosis. We confirmed the specificity of the mRNA probe (and *PCNT* antibody) by examining *PCNT* KO cells, where both mRNA and protein for *PCNT* could not be detected (Fig. 21A). In wildtype cells, *PCNT* mRNA did colocalize with a C-terminal *PCNT* antibody, demonstrating that translation is indeed happening in the cytoplasm and not on centrosomes as has been suggested previously (Sepulveda et al., 2018) (Fig. 14C). Hence, the co-existence of *PCNT* mRNA and its cognate protein strongly suggest that it is being translated at satellites.

Following the analysis of *pcent* mRNA and PCM1, we evaluated distances for a centriolar/ciliary mRNA and satellite cargo protein, Cep290, using the same method and type of analysis as before. In HeLa cells, Cep290 did form fewer cytoplasmic foci than *pcent* mRNA, however the center-center distances between Cep290 and PCM1 closely resembled the ones for *pcent* mRNA and PCM1. Here, approximately 15% of all *Cep290* mRNA foci co-localized with PCM1 (center-center distances $<0.4\mu\text{m}$ and edge-edge distances of $0-0.1\mu\text{m}$). Once more, the calculated distances were statistically different from a random localization. Taken together, these results demonstrate that two centrosomal/ciliary mRNAs (*PCNT* and *Cep290*) co-localize with PCM1 on centriolar satellites, where also their respective protein resides, strongly suggesting that they get translated at centriolar satellites.

To further reinforce that satellites are sites specific for centrosomes and cilia, we set out to investigate control mRNAs and their localization in respect to PCM1. To do so, we searched for transcripts of housekeeping genes that would show random localization, or rather do not show localization to any particular location within a cell. Classical housekeeping genes (HK genes) like GAPDH, tubulins and actins are supposedly characterized by being constitutively expressed and linked to cell maintenance and are routinely used as controls for gene expression experiments (Warrington et al., 2000). Yet, many of these HK genes show considerable variations between cells, tissues and conditions, rendering them ineffective for this purpose (Dheda et al., 2004). In addition, they are expressed at considerably higher levels, making direct comparisons to centrosomal mRNAs challenging (Dheda et al., 2004). Hence, we capitalized on a web-based tool, the ‘Housekeeping And Reference Transcript Atlas’ (HRT Atlas v1.0, www.housekeeping.unicamp.br), which compiles massive RNA-seq datasets (human and mouse) to accurately determine reference genes and transcripts that are constant and stably expressed among different tissues (Hounkpe et al., 2021). We first collected a list of reference transcripts that showed similar expression level in the same tissue (endocervix, since HeLa cells are derived from cervical cancer cells) to the centrosomal mRNAs used in our study in order to identify transcripts that would be the most comparable. Using the human protein atlas (www.proteinatlas.com) we verified similar expression levels in our cell line (HeLa cells) as well as confirming that the reported localization was only ‘cytoplasm’ or ‘cytoplasm and nucleus’ and no distinct cellular structure. Next we validated that these transcripts have a coding sequence (CDS) $>1\text{kb}$, to enable the design of the minimum number of probes efficient enough for the detection by smFISH. These criteria resulted in a rather small list of genes, out of which we chose RanBP10, APE1 and PP2R5B. Using smiFISH (see Materials and Methods) we looked at localization of these transcripts and decided on RanBP10 as most suitable because

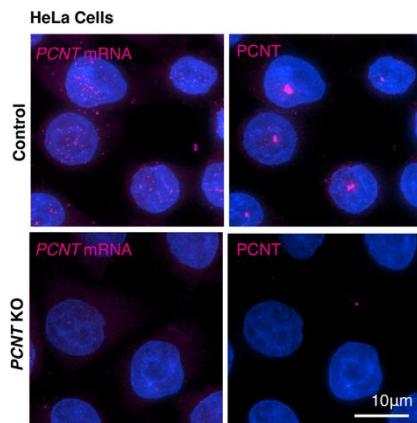
of similar expression level to *PCNT* mRNA (similar number of foci per cell) (Fig. 22A). This construct as well as a directly labeled RanBP10-Quasar570 probe showed only a minimal amount of transcript (2.2% of RanBP10 foci $<0.4\mu\text{m}$) co-localizing with PCM1 and that this localization is not considerably different from a localization by chance ($\sim 1\%$ of RanBP10 foci $<0.4\mu\text{m}$), as determined by randomization (see Materials and Methods). Thus, by showing that a non-centrosomal, random control mRNA is in fact not localizing to centriolar satellites or with significant lower frequency, we further highlight that the localization of centrosomal mRNAs to centriolar satellites is specific.

We included additional centrosomal mRNAs in our study to elucidate their localization and chose Ninein (Nin) and Cdk5rap2, all centriolar satellite resident proteins (Fig. 23A, B). Ninein, one of the first proteins described to localize to satellites caught our attention due to the very same reported localization of its mRNA around the centrosome as *PCNT* mRNA (Safieddine et al., 2021; Sepulveda et al., 2018). Indeed, smiFISH-IF of Ninein mRNA together with PCM1 displayed a comparably high degree of colocalization with approximately 37% of Ninein mRNA foci colocalizing with PCM1 (center-center distance $<0.4\mu\text{m}$, edge-edge distance of 0- $0.05\mu\text{m}$). Cdk5rap2 on the other hand, showed lower but still significant colocalization with PCM1 with 16% of Cdk5rap2 mRNA foci in proximity (center-center distances $<0.4\mu\text{m}$). Thus, Ninein and Cdk5rap2 together with *PCNT* and Cep290 show significant colocalization with centriolar satellites, where also their encoded proteins localize, suggesting that they are translated there.

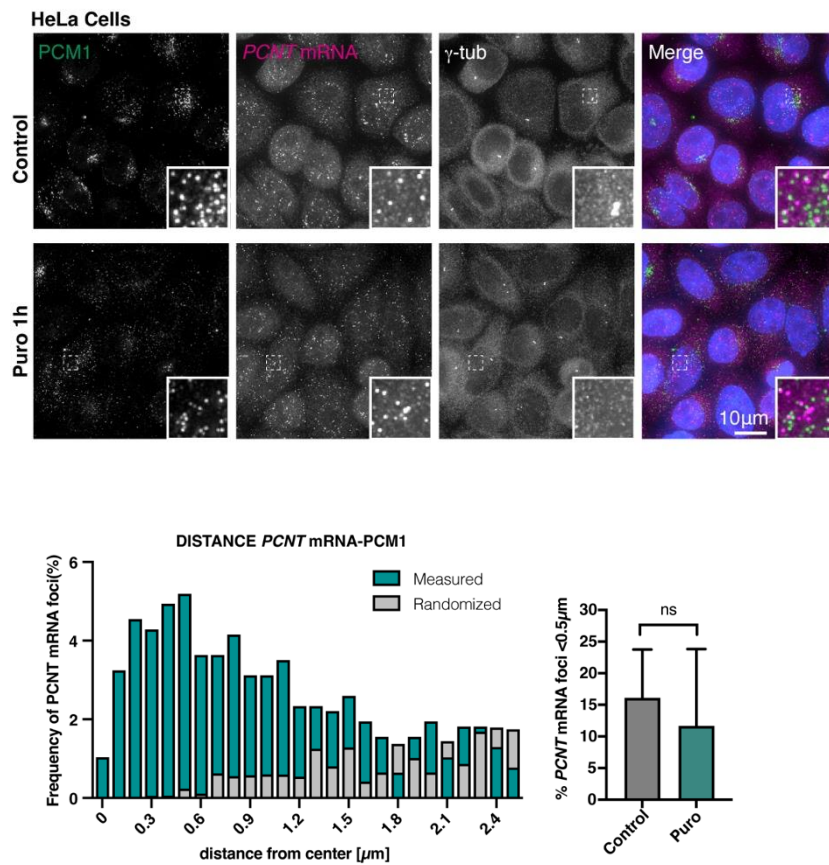
Since all of these transcripts belong to rather large proteins, the questions arises if centriolar satellites have a preference towards synthesizing large proteins. RNA-seq experiments should help shed light on the type of transcripts enriched at centriolar satellites and if they share specific traits such as size.

Having identified centrosomal mRNAs that localize to satellites, I next set out to test the effect of puromycin treatment (1 hour) on the *PCNT* mRNA-satellite interaction (Fig. 21B). While in this condition *PCNT* protein is depleted from satellites as shown before, its mRNA remained in close proximity with PCM1. This came as a surprise, given that both *PCNT* mRNA and PCM1 were found dispersed in the cytoplasm upon puromycin incubation. Thus, we can conclude that the *PCNT* mRNA-PCM1 association is independent of translation and that the mRNA interacts with PCM1 and not (or not only) *PCNT* protein.

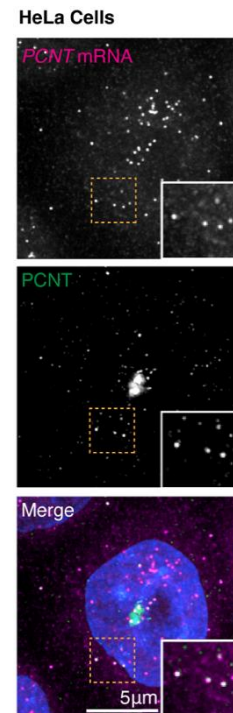
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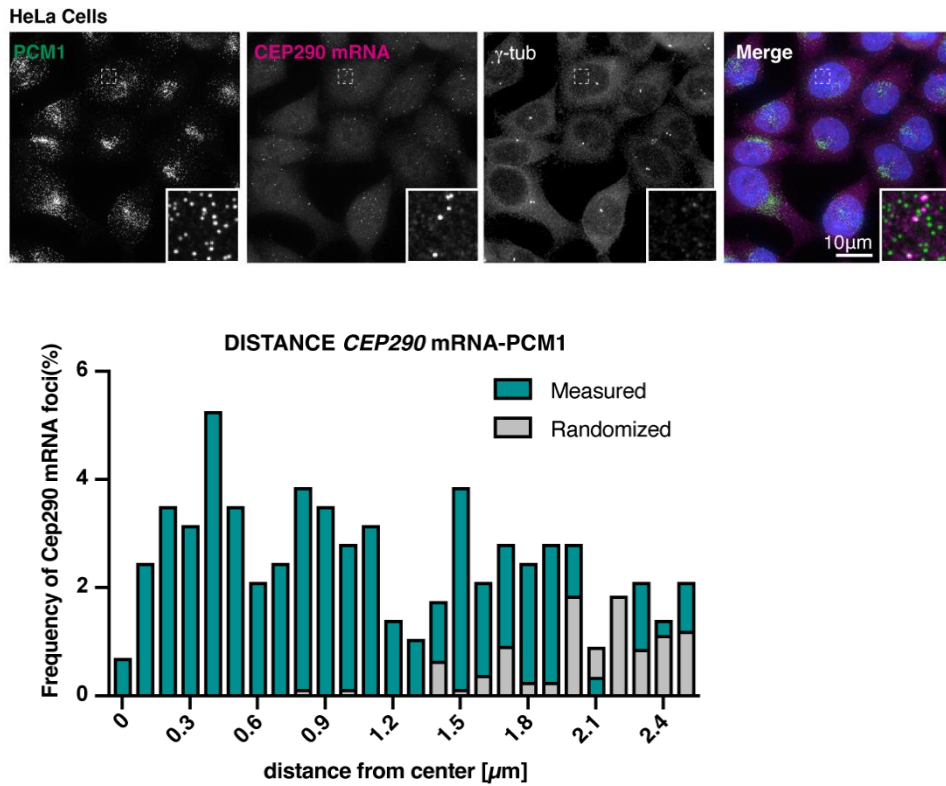


Figure 21. Centrosomal mRNAs closely associate with centriolar satellites. (A) Specificity of *PCNT* mRNA FISH and protein immunofluorescence signal confirmed by absence of signal in *PCNT* KO cells. (B) Single molecule fluorescence in situ hybridization (smFISH) combined with immunofluorescence microscopy in control and puromycin-treated HeLa cells. *PCNT* mRNA localizes in the vicinity of PCM1 independently of ongoing translation. To exclude random co-localization/proximity, distribution was compared with randomized controls. N=77 cells control, >100 cells puromycin. (C) SmFISH combined with immunofluorescence microscopy shows *PCNT* mRNA colocalizing with nascent *PCNT* protein in the cytoplasm. (D) Single-molecule fluorescence hybridization (smFISH) combined with immunofluorescence microscopy in HeLa cells. *CEP290* mRNA localizes in the vicinity of PCM1. To exclude random co-localization/proximity, distribution was compared with randomized controls. N=78 cells. Adapted from (Pachinger et al., 2024).

A

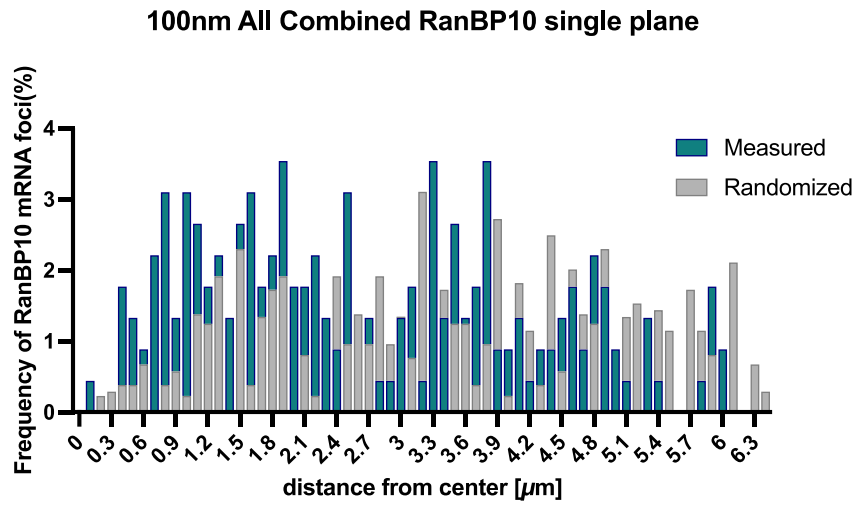
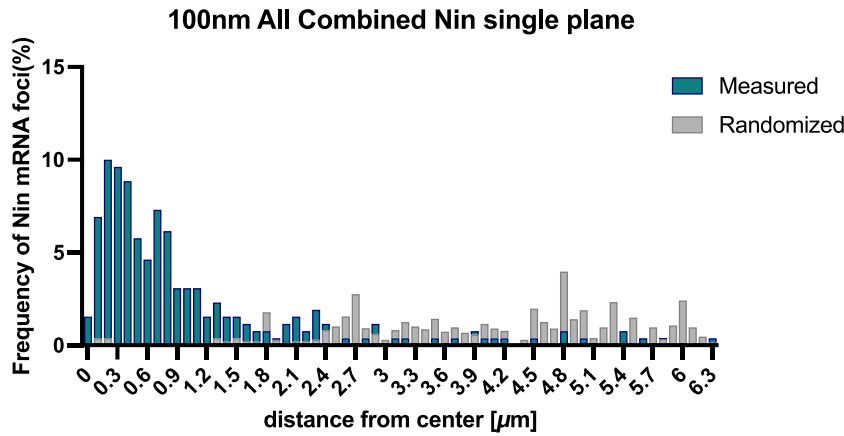


Figure 22. (A) control mRNA (RanBP10) does not localize to centriolar satellites. Single-molecule fluorescence hybridization (smFISH) combined with immunofluorescence microscopy in HeLa cells. *CEP290* mRNA localizes in the vicinity of PCM1. To exclude random co-localization/proximity, distribution was compared with randomized controls. N=78 cells. Adapted from (Pachinger et al., 2024).

A



B

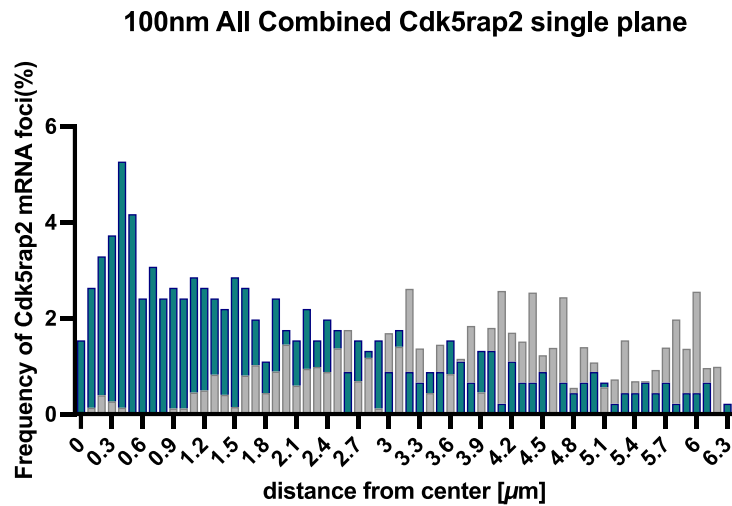


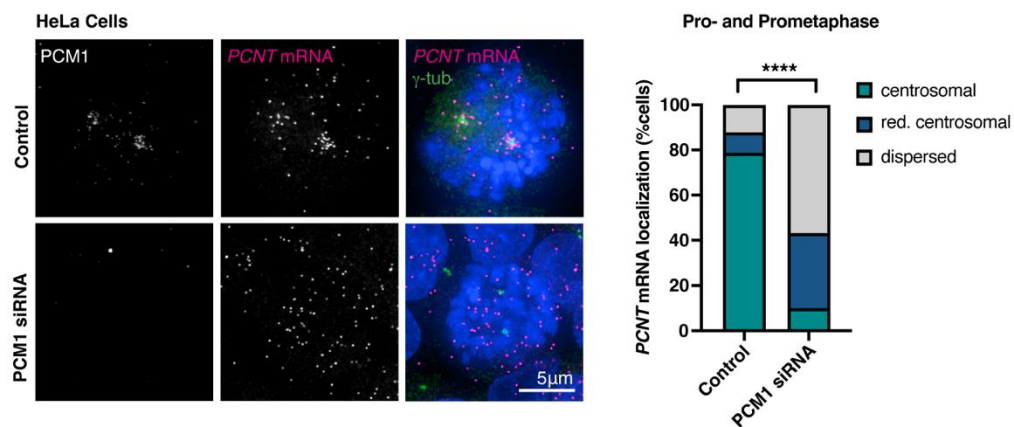
Figure 23. Additional centrosomal mRNAs localize to centriolar satellites. (A) Single-molecule fluorescence hybridization (smFISH) combined with immunofluorescence microscopy in HeLa cells. *Nin* mRNA localizes in the vicinity of PCM1. To exclude random co-localization/proximity, distribution was compared with randomized controls. N=78 cells. **(B)** Single-molecule fluorescence hybridization (smFISH) combined with immunofluorescence microscopy in HeLa cells. *Cdk5rap2* mRNA localizes in the vicinity of PCM1. To exclude random co-localization/proximity, distribution was compared with randomized controls. N=78 cells. Adapted from (Pachinger et al., 2024).

3.13 Depletion of PCM1 phenocopies inhibition of translation

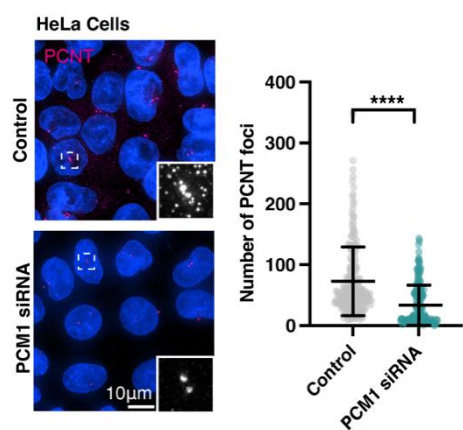
Having established that centriolar satellites harbor newly synthesized cargo proteins, and contain centrosomal/ciliary mRNAs that likely are translated there, we reasoned that the loss of centriolar satellites should affect that process. To test this idea, we depleted PCM1 in HeLa cells using siRNA targeting two different locations as described in earlier studies (Dammermann and Merdes, 2002). After confirming the efficiency of the knock down of PCM1, we assessed number of foci for the same centrosomal and ciliary proteins and proteins involved in protein quality control as before. In agreement with previous reports, we found centrosomal and ciliary proteins PCNT, Cep290, Cdk5rap2 and Cep131 to be significantly depleted from their satellite population with a clear centrosome fraction persisting (Fig. 24D). This is in line with the disassembly of satellites upon PCM1 depletion, and the effects seen upon inhibition of translation described earlier. However, some centriolar satellite client proteins seemed to be differentially affected, since Mib1 albeit being dispersed still remained almost unaffected in terms of number of foci while Ofd1 showed similar behavior to centrosomal proteins, being mostly abolished from the cytoplasm and retaining its centrosome localization. Apart from its association to protein quality control and translation, Ofd1 is best known for its role as a regulator of centriole elongation, a function that Ofd1 might employ in the absence of PCM1.

Furthermore, I discovered that the depletion of PCM1 did not affect the number of *PCNT* mRNA foci; however, those foci appeared now more dispersed, especially at the pro- and prometaphase stage. This again matches the phenotype of inhibition of translation, where several centrosomal mRNAs including *PCNT* mRNA are dispersed throughout the cytoplasm, indicating that mRNA localization is translational dependent. This effect is most pronounced in pro- and prometaphase where the majority of *PCNT* mRNA foci (approximately 80%) are usually concentrated around the centrosome. After PCM1 depletion, however, only less than 10% remained peri-centrosomal with a high number of cells showing either reduced number of foci around the centrosome or foci entirely dispersed in the cytoplasm (Fig. 24A). To sum up, the loss of centriolar satellites induced by the depletion of its main scaffolding component PCM1, strongly phenocopies the inhibition of translation, with multiple proteins being abolished/reduced in the cytoplasm and a dispersion of specific centrosomal mRNAs.

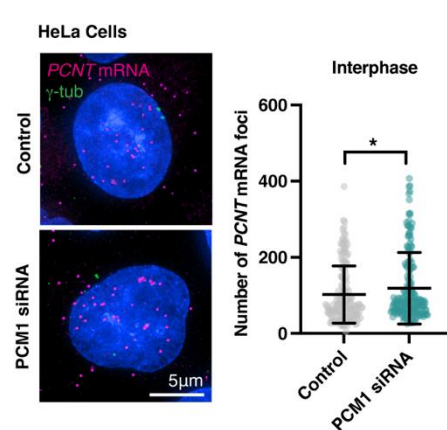
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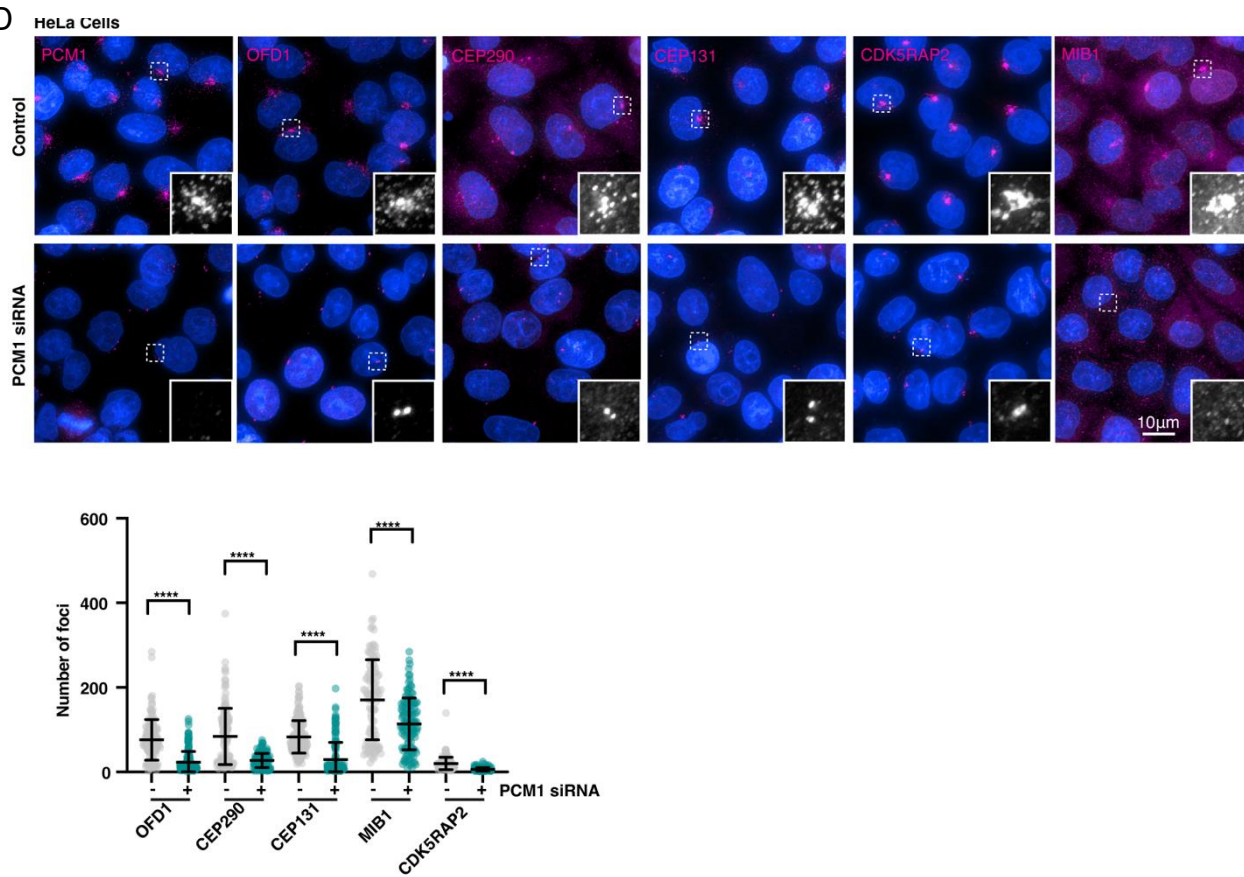


Figure 24. Depletion of PCM1 phenocopies inhibition of translation. (A,C) smFISH combined with immunofluorescence microscopy in control cells and cells depleted of PCM1. PCM1 depletion impairs *PCNT* mRNA localization around the centrosome in prometaphase, reflecting a loss of co-translational targeting. However, the total number of *PCNT* mRNA foci assessed in interphase remains almost unchanged (C). N= 33 prometaphase-stage and 189 interphase cells control, 30 and 196 cells PCM1 siRNA. Mann Whitney test used to assess statistical significance; ****P< 0.0001, *P< 0.05. (B) Immunofluorescence micrographs and quantification of cytoplasmic PCNT signal in control and PCM1 siRNA-treated cells. Depletion of PCM1 leads to a significant decrease in number of PCNT foci (N=211 control cells and 171 PCM1 siRNA cells). Data analyzed using Mann-Whitney test; ****P< 0.0001. (F) Immunofluorescence micrographs and quantitation of centriolar satellite signal in control and PCM1 siRNA treated cells. Depletion of PCM1 largely eliminates cytoplasmic foci of OFD1, CEP290, CEP131, CDK5RAP2, while centrosomal signal remains. MIB1 signal is largely unaffected, although foci are now dispersed throughout the cytoplasm. Mean +/- SD indicated. N>100 cells each condition. Statistical test to compare control and PCM1 depletions is t-test with Welch's correction (MIB1), non-parametric Mann Whitney test (others); ****P<0.0001. Adapted from (Pachinger et al., 2024).

3.14 Co-translational targeting of client proteins explains centrosomal enrichment of PCM1

The data shown above point towards a role for centriolar satellites in protein synthesis. The fact that *Drosophila* centriolar satellites are not concentrated, strongly suggest that satellite function in protein synthesis is independent of their localization. If that is the case, why are vertebrate centriolar satellites still clustering around the centrosome and the base of the cilium (in general and specifically at pro-and prometaphase)? One reason for this could be a phenomenon called co-translational targeting, which has first been described for membrane proteins but recently shown for certain centrosomal proteins including PCNT, which is specifically recruited to the centrosomes together with its mRNA at the onset of mitosis (Safieddine et al., 2021; Sepulveda et al., 2018). This subcellular localization was shown to be translation-, microtubule- and dynein-dependent, and at least in the case of PCNT it might be a simple way of maximizing the efficiency of PCNT protein production and delivery to the centrosome to facilitate incorporation into the expanding PCM during mitosis.

As mentioned previously, *PCNT* mRNA is highly enriched around the centrosome in prometaphase, the very same stage where PCM1 appears concentrated. Thus, it seemed plausible based on our hypothesis that co-translational targeting would result in PCM1 protein localizing close to centrosomes. We therefore expected to see an effect on satellite localization if we would delete or deplete PCNT protein. Indeed, in HeLa cells, when PCNT was knocked out, we saw a reduction of PCM1 foci around the centrosome in pro- and prometaphase (from approximately 40% in control cells to 20% in PCNT KO cells) and an increase in cells with complete dispersion (from approximately 25% in control cells to 70% in PCNT KO cells) (Fig. 25A). The localization of PCM1 in interphase cells was not affected by the loss of PCNT, indicating that its localization close to centrosomes at that stage is not dependent on PCNT translation. Importantly, it has been previously shown that PCNT depletion alone does not compromise mitotic spindle assembly nor affect the presence of γ -tubulin, excluding the possibility that this observation is an indirect result of affecting microtubules. The lack of concentration of satellites in pro- and prometaphase, however, shows the involvement of satellites in co-translational targeting and suggests that PCM1 may be passively dragged along towards centrosomes along with *PCNT* mRNA and nascent protein. This is different to its original ascribed role as a targeting module, where loss of PCNT would not change the localization of PCM1 around the centrosome. In conclusion, we propose that PCM1 is involved in the co-translational targeting of PCNT, providing further support for our hypothesis that centriolar satellites are associated with protein synthesis in vertebrates.

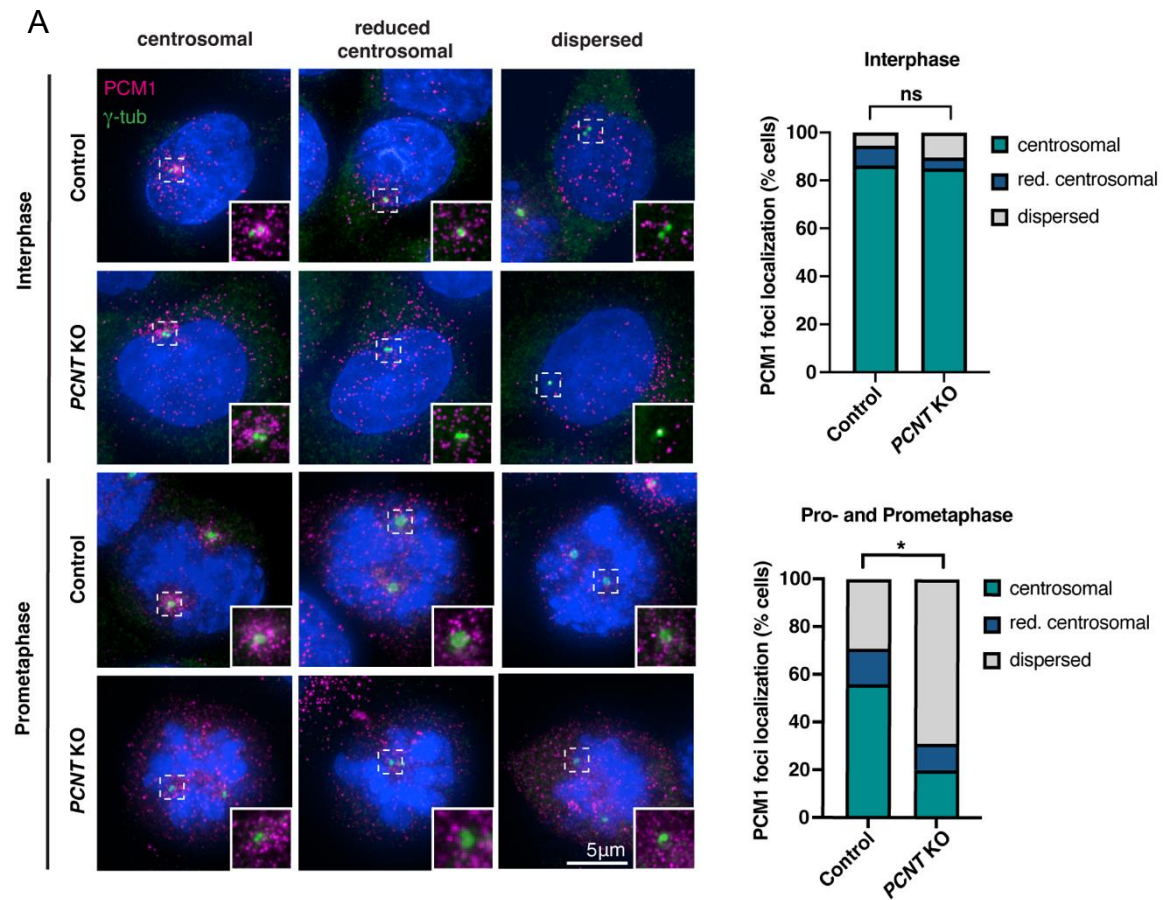


Figure 25. Co-translational targeting of client proteins explains centrosomal enrichment of PCM1. (A) Immunofluorescence micrographs of PCM1 in control and *PCNT* KO cells. Loss of *PCNT* does not impact PCM1 localization in interphase but compromises its concentration near centrosomes in pro- and prometaphase. N= 154 prometaphase and 348 interphase cells control , 161 and 355 cells *PCNT* KO. Localization groups compared using Mann-Whitney test, *P< 0.05. Adapted from (Pachinger et al., 2024).

3.15 The model- Centriolar satellites as sites of translation for centrosomes and cilia

If we combine all these results, we can come up with a model that is different from the original model of satellites as transport modules. The bioinformatic analysis in this study together with earlier studies show that PCM1 is conserved well beyond vertebrates. We characterized the PCM1 homolog in *Drosophila*, showing that centriolar satellites themselves are also conserved, forming cytoplasmic granules containing centrosomal proteins as well as ciliary proteins based on proteomics. However, the lack of an apparent enrichment around centrosomes and their lack of movement argue against a transport function in *Drosophila*. Instead, we find *Drosophila* centriolar satellites in cells and tissues to be associated with protein quality control and in particular protein synthesis. Puromycin labeling in S2 cells support a function of satellites in translation. This function we believe to be conserved in vertebrates, where inhibition of translation showed that centrosomal and ciliary proteins localized to satellites are newly synthesized. Furthermore, we show that satellites closely associate with centrosomal mRNAs (independent of translation), indicating that those could be translated on satellites. Loss of PCM1 had an impact on efficient translation (by dispersal of mRNA around the centrosome) and vice versa, inhibiting efficient translation by removing PCNT protein impaired PCM1 clustering around the centrosome.

In short, we believe that the data provided here point towards a role of satellites in the synthesis of centrosomal and ciliary proteins. The differential impact of puromycin treatment affecting only centrosomal and ciliary proteins and not those involved in protein quality control together with the absence of their mRNAs localizing to satellites strongly suggests that centriolar satellites are sites of protein synthesis specifically for centrosomes and cilia. In this way, specific mRNAs and their proteins perhaps be coming together to form complexes in a coordinated fashion before delivery to their destination. It is worth mentioning that general protein synthesis of centrosomal and ciliary proteins still occurs in the absence of PCM1, with protein levels of several centrosomal/ciliary proteins being unaffected or only mildly affected, both globally and at centrosomes (Quarantotti et al., 2019). Thus, centriolar satellites are ensuring protein homeostasis by fine-tuning the translation and levels of their client proteins at centrosomes and cilia. This concept of protein complex formation might be a more general mechanism applicable also to other non-membranous organelles.

While the main function of satellites in the original model was to deliver proteins to their destination along microtubules, we suggest that satellites are not involved in the targeting

per se, but rather passively moved along while still being engaged in the synthesis. How the newly translated proteins end up at centrosomes and cilia, in other words how the trafficking is mediated remains to be determined. The model proposed here (Fig. 26A) is consistent with the phenotypes that have been previously reported for loss of PCM1 in vertebrate cells and *in vivo*. In particular, the loss of satellites in mitosis could well be explained by the shutdown of protein synthesis at this stage. Future studies aiming at determining the specificity of centrosomal mRNAs and providing further proof/evidence for active translation are required to confirm this hypothesis. Should this model hold true, then I believe that it changes our perspective of centriolar satellites from structures rather peripheral to centrosome/ciliary function to crucial players involved in protein synthesis and homeostasis of centrosomal and ciliary proteins. This study obviously raises many questions and still requires further experiments to allow us to fully understand the idea of satellites as specific hubs for centrosomal/ciliary protein production. Some of these points I am going to address in the next section (discussion). Taken together, I believe that the work presented provides important insights into the evolutionary and *in vivo* aspects of centriolar satellites, that so far have been missed and will hopefully encourage researchers to further investigate to what extent and in which organisms the function of PCM1 is conserved. Additionally, the implication of centriolar satellites in the process of translation should be likewise interesting to understand ‘local’ translation and complex formation as a general theme for organelles to fine-tune gene expression for cellular homeostasis.

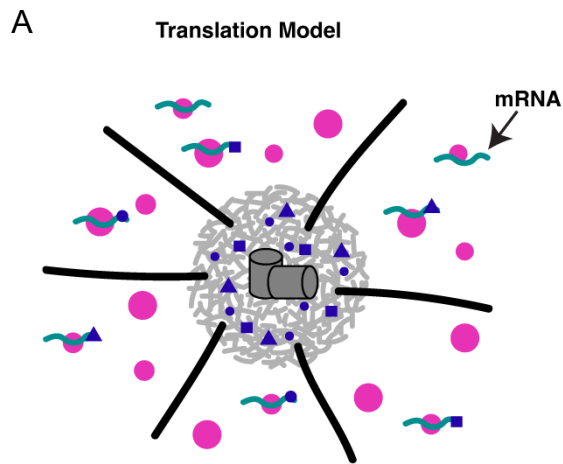


Figure 26. The translational model. (A) Revised model of centriolar satellite function: centriolar satellites are sites of translation of centrosomal and ciliary proteins. Their concentration close to centrosomes in vertebrates is a byproduct of co-translational targeting of certain centrosomal/ciliary clients. Adapted from (Pachinger et al., 2024).

4 Discussion

4.1 Mild but pleiotropic phenotypes underlying loss of Combover

One major outcome of this study is that centriolar satellites exist in *Drosophila*, allowing the comparison of different aspects of centriolar satellites in the context of centrosome and cilium assembly. So, how do centrosomal and ciliary phenotypes compare to the ones known to occur for PCM1 mutants? Our study shows that as for PCM1, loss of Combover is pleiotropic, affecting different cells and tissues with varying degree of severity. The mild neuronal and strong motile ciliary phenotype is similar to the phenotype described in vertebrates. Loss of PCM1 leads to reduced ciliation in vertebrate cells and axonemal defects including missing microtubule doublets (Hall et al., 2023), similar to loss of Combover.

A question that remains unanswered is how these cilia phenotypes come about and how it would fit into the model of satellites being translational sites? It is certainly hard based on the current data to draw a direct link between translation and the phenotypes observed; however, we can hypothesize that perturbed formation of specific protein complexes and subsequent failure in delivery to the cilium are the underlying cause. In the case of axonemal dyneins, it has been shown that -at least in multiciliated cells- dynein axonemal assembly factors (DNAAFs) pre-assemble in the cytoplasm in specialized liquid-like organelles called DynAPs to form distinct complexes that help in the formation of outer (ODAs) and inner dynein arms (IDAs) (Lee et al., 2020). Considering the presence of many of such dynein assembly factors and chaperones in our proximity experiments, it is tantalizing to speculate that in testes *Drosophila* centriolar satellites are similar to *Xenopus* DynAPs. From our electron microscopy images it looks as if ODAs are not affected while IDAs appear to be missing in some cases. Interestingly, another study has proposed that RNP granules exist in *Drosophila* testes, which contain mRNAs for axonemal dynein motor proteins destined for incorporation into the cilium (Fingerhut and Yamashita, 2020). If those RNP granules are the same as Combover foci remains to be determined; however, it underlines the importance of local translation. The *Drosophila* sperm flagellum with its large size and cytoplasmic assembly is certainly a unique case, which makes it possible that particularly efficient means of protein production are required for its formation.

In contrast to PCM1 we have so far failed to observe any obvious defects in centrosomal protein recruitment in *Drosophila*. It is possible that centrosomal levels of some proteins are moderately affected but protein levels could also be not affected at all. The cell cycle

asynchrony and nuclear fallout phenotype is similar to mutants of the centrosomal protein Plp (human PCNT), which together with Cnn has been shown to be important chromosome segregation fidelity in the *Drosophila* early embryo (Lerit et al., 2015). In the absence of Plp, chromosome missegregation and resulting cell cycle asynchrony as well as nuclear fallout contribute to overall genome stability and -like Combover- ultimately cause embryonic lethality. In vertebrates, disruption of PCNT is linked to aberrant mitotic progression (Tibelius et al., 2009). Given the similar phenotypes for Combover and Plp I propose that, as for PCNT in vertebrates, loss of Cmb affects Plp translation and/or complex formation and subsequent delivery to the centrosome. Although co-translational targeting has so far been exclusively described in vertebrates, it could be that a similar mechanism takes place in flies. Consistent with this, plp mRNA has recently been shown to be localized close to centrosomes in flies, regulated by the RNA-binding protein Orb, a homolog of human CPEB proteins (Fang and Lerit, 2022). Interestingly, Orb2, another CPEB protein family member, was highly enriched in our testes mass spec data and thus represents an interesting candidate that could be involved in satellite-mediated translation of centrosomal/ciliary mRNAs such as Plp. Investigation of centrosomal mRNAs and their putative colocalization with Combover will be necessary to determine which mRNAs are being translated, before addressing the molecular mechanisms underlying their translation.

4.2 Centriolar satellites – phase separated compartments?

Centriolar satellites are one of many non-membranous organelles that have been recently added to the list of potential phase separated compartments. A major finding providing evidence for this concept was the identification of PCM1 as a substrate of the dual-specificity tyrosine-phosphorylation-regulated kinase 3 (DYRK3), which has been implicated in the regulation of liquid-liquid phase separation of multiple membrane-less organelles by phosphorylating intrinsically disordered regions (IDRs) that confer multivalent weak interactions (Rai et al., 2018). This mechanism would be a way of explaining the cell-cycle dependent localization of PCM1 with satellites undergoing dissolution during mitosis and reemerging at the end of mitosis. In accordance with this protein levels of DYRK3 have been suggested to increase shortly before mitosis, while in anaphase DYRK3 degradation may enable centriolar satellite to form again. How does our data on PCM1 and Cmb fit into the model of satellites being phase-separated and how can it be coupled to protein synthesis? While

there are obvious differences in satellite distribution during interphase, Combover (at least in S2 cells) follows the same pattern of dissolution in mitosis and condensation in interphase. It is therefore conceivable that the same or similar mechanisms drive this phenomenon. We did, however, not identify or test any kinases that could take on DYRK3's putative function in the fly. Consistent with liquid-liquid phase separation behavior, however, is the presence of mRNAs on centriolar satellites, given the proposed function for RNAs to buffer phase separation behavior (Maharana et al., 2018). In addition, in live imaging experiments on GFP PCM1 we and others could confirm fusion and splitting events that have been described for other phase separated compartments (Conkar et al., 2019). The slow fluorescence recovery after photobleaching behavior for both Combover and PCM1 and the insensitivity of satellites to 1,6-hexanediol that we see in preliminary experiments do not necessarily invalidate the phase separation behavior; however the latter observation indicates that the interactions underlying satellite formation do not depend on hydrophobic interactions. The discovery of mRNAs on satellites as well as RBPs as proximity interactors changes our view of centriolar satellites from protein complexes to cytoplasmic ribonucleoprotein (RNP) condensates. Stress granules and processing bodies are among the most famous cytoplasmic, membranelles, phase-separated RNPs, regulating different aspects of the RNA life cycle (Decker and Parker, 2012). I propose that centriolar satellites are among those biomolecular condensates potentially formed by liquid-liquid phase separation behavior (LLPS). Aberrant phase transitions and aberrant RNP condensates are often detrimental, leading to pathogenic protein aggregates, a hallmark of neurodegenerative diseases like Alzheimer's disease or amyotrophic lateral sclerosis (ALS). Although PCM1 aggregates have so far not been identified as part of a disease/disorder, it seems possible that satellite mediated protein aggregation (due to aberrant phase transition) disorders do exist as the self-oligomerization capability and the resulting bigger aggregates upon deletion of its C-terminus would suggest.

If satellites are indeed phase separated condensates that mediate mRNA translation, how would this property influence protein synthesis and *vice versa*? Firstly, the phase transition leading to dissolution of satellites in mitosis would be consistent with the general shutdown of translation at this cell cycle stage, while the prominent localization to centrosomes at the onset of mitosis would go hand in hand with increased protein synthesis of specific centrosomal proteins required for the assembly and function of mitotic centrosomes. Moreover, stresses like UV or heat are linked to defined integrated stress responses that are able to rapidly turn off protein synthesis, which could explain the stress-dependent dissolution of centriolar satellites. Increased protein production at the onset of mitosis might be a prerequisite for centrosomes to

properly mature into a full grown centrosome during mitosis and possibly apply not only to PCNT but also other proteins. In addition, the phase separation nature of satellites are reminiscent of other RNP granules, where loss of their phase separation properties has been linked to impaired mRNA transport, best exemplified in *Drosophila* embryonic development where loss of liquid like properties prevent localization of *oskar* mRNA (Bose et al., 2022). Hence, dynamic compartmentalization through phase separation might be a way of mediating mRNA trafficking, potentially facilitating long-distance transport.

4.3 Centriolar satellites as a special form of ‘local translation’

The mechanisms of local mRNA translation are probably best described in highly polarized neuronal cells, where axons and dendrites extend several centimeters away from the cell body (Holt et al., 2019). In this case mRNAs are trafficked into specific subcompartments in a dormant state before being locally translated, thus allowing efficient on-site protein production and immediate proteome changes upon certain stimuli. Similarly, in the case of *Drosophila* oocytes, mRNAs (i.e. *bicoid*, *oskar*, *nanos*) are being targeted in a translationally repressed state prior to local synthesis (Lasko, 2012). In the case of centriolar satellites however, protein synthesis seems to be independent of localization. This raises the question why there are organismal changes in satellite distribution and in particular why proteins would be produced not in the immediate vicinity of centrosomes and cilia but rather more distantly. The canonical model of local translation is said to be energetically more favorable than trafficking of nascent proteins (Holt et al., 2019). However, in the case of satellites we suspect that they represent a platform for co-synthesis of specific proteins as well as the assembly of specific multiprotein complexes, thereby avoiding non-specific interactions or protein aggregation. This mode of regulation of protein homeostasis might be more crucial than the location of protein synthesis.

In addition, the clustering of satellites around the centrosome in human cells might be due to co-translational targeting, a mechanism that so far has only been described in vertebrates and only for a small subset of centrosomal proteins (Sepulveda et al., 2018). Large proteins like PCNT (or ASPM) can thereby be efficiently translated and targeted to overcome the kinetic challenges that would otherwise take place. As expected, puromycin impaired mRNA localization, indicating that the nascent polypeptide chain is required for mRNA targeting. This is reminiscent of secretory proteins where SRP-dependent targeting of proteins to the ER stalls ribosomes until the entire complex docks to the SRP-receptor on the ER (Nyathi et al., 2013).

Hence, it will be interesting to further dissect the mechanisms of co-translational targeting and see if ribosome stalling could be involved as well.

How centrosomal/ciliary transcripts are localized and how nascent proteins and protein complexes on satellites end up on centrosomes or cilia remains elusive. In the case of PCNT it has been suggested that dynein-mediated transport along microtubules is underlying co-translational targeting (Sepulveda et al., 2018); however, if this is the mode of transport for all satellite components is questionable. If dynein is indeed involved in the transport it would be interesting to see if the nascent protein, the ribosomes themselves or additional players are interacting with dynein to mediate the trafficking. As for short-range movements, transport along actin filaments would be an option, as well as an interplay between the two cytoskeletal networks. In case of general protein (complex) delivery, transport along microtubules seems plausible, yet evidence supporting this hypothesis is missing. Instead of satellites mediating the transport we believe that they are occasionally passively moved along, nascent proteins could potentially employ ‘organelle hitchhiking’ (Salogiannis and Reck-Peterson, 2017) and move along other organelles that undergo movements (i.e. endosomes, COP1 vesicles) or get incorporated into RNP transport granules, an efficient way to minimize the energy costs. Alternatively, nascent proteins could reach their destination by simple diffusion coupled with anchoring, involving specific local tethering molecules at the landing site. To address some of these questions reporter based assays for live imaging such as the SunTag system (Tanenbaum et al., 2014) together with a marker for centriolar satellites will reveal how translation and movement/diffusion are coupled.

If centriolar satellites are specific sites of protein synthesis for centrosomes and cilia, what distinguishes them from the canonical translation sites within a cell, that likewise assist in the protein synthesis of such proteins? Once again, we believe that the microenvironment as well as how quickly parts of a protein get produced (the translation rate) determines the selection of interacting partners to favor the formation of stable complexes. The location could potentially determine how the same transcript through alternative splicing leads to the production of different isoforms. However, if there are general compositional changes between centriolar satellites and other translational factories for the synthesis of centrosomal/ciliary proteins or specific cues/stimuli remains to be investigated. In any case, my and previous data point towards satellites as a form of compartmentalization, where specific proteins coexist to improve stability and homeostasis.

This concept of compartmentalization of translation has in effect been raised previously, since earlier studies have reported the localization of some transcripts and their

encoded proteins (or together with nascent polypeptide imaging) to cytoplasmic foci that were distinct structures, different from P-granules and other organelles) (Chouaib et al., 2020). Interestingly, these mRNAs and proteins did not localize to the same structures, suggesting that several translational factories exist in a cell. In fact, this study suggests that based on the identification of four translational factories upon screening of 500 mRNA transcripts and extrapolation to the 20 000 human genes would imply several hundred translational hubs are present in a cell (Chouaib et al., 2020). Thus, we propose based on our findings that centriolar satellites could be among those translational factories that together with P-bodies and other general sites of translation regulate RNA metabolism.

4.4 The complexity of centriolar satellite composition

Our centriolar satellite proteomics in *Drosophila* has identified several proteins linked to translation and RNA related processes, some of which have been recently identified in the vertebrate satellite proteome as well. Despite the fact that several eukaryotic translation factors and RNA binding proteins are highly enriched, we still lack an understanding of the proteins involved in interacting with the mRNA and mediating the translation. The previously mentioned RBPs CPEB1 (*Drosophila* Orb1 and Orb2) as well as FMRP (*Drosophila* Fmr1) provide interesting candidates, because they are universally conserved and due to their previous association with centrosomal targets (Fang and Lerit, 2022; Ryder et al., 2020). Hence, if one of these RBPs could directly bind centrosomal/ciliary transcripts and if specific motifs (i.e. ‘zip codes’) (Kislauskis and Singer, 1992) are required to determine specificity will be an exciting avenue. In this regard, RNA-seq experiments (i.e. APEX-seq, RIP-seq) (Fazal et al., 2019; Zambelli and Pavesi, 2015) provide valuable tools for identification of centriolar satellite enriched mRNAs, which not only represent candidates for co-localization studies but also the basis for motif-based searches. Even though we were able to colocalize some mRNAs to centriolar satellites, we did not try to visualize ribosomes or eukaryotic translation initiation factors (EIFs) by immunofluorescence or smFISH due to the high abundance of those proteins and the concomitant high background. Nevertheless, we believe that based on our mass spectrometry experiments that these proteins localize there. The presence of single or multiple ribosomes (polysomes) on centriolar satellites then raises the question if those ribosomes are distinct from other ribosomes in the cell and even within satellites, if specific subpopulations would contain a specific set or not. There is debate on the hypothesis of ‘specialized ribosomes’, to what extent ribosome heterogeneity or distinct ribosome-associated factors exist

and if they would show variations in their activity as a way to regulate gene expression (Genuth and Barna, 2018). Alternatively, all centriolar satellites contain the same (or similar) set of core proteins involved in the translational machinery, yet the demand and rate of synthesis could be cell- and context specific, thereby ensuring spatiotemporal control of gene expression.

The previous observation that the centriolar satellite proteome in vertebrates is largely independent of centrosomes, would fit with satellites being centers of translation, where PCM1 and the translational machinery would be unaffected and likely resume translation, due to their absence from centrosomes. However, it would be interesting to see if cells depleted of centrosomes would show slight changes in the rate of synthesis due to the decreased demand of protein production or higher rates of misfolded proteins if satellites are depleted. If so, those changes would be only detectable on a single cell level rather than globally.

Analysis of the centriolar satellite proteome in vertebrates and *Drosophila* have identified components of processing bodies (P-bodies), RNA granules associated with cell stress and storage of translationally repressed mRNA (Gheiratmand et al., 2019; Quarantotti et al., 2019). This poses the question if centriolar satellites could potentially switch between promotion of translation and degradation of their transcripts. Despite sharing a significant set of proteins with either stress granules or P-bodies, centriolar satellites are likely distinct cellular compartments; immunofluorescence experiments with PCM1 and the P-body specific component DCP1 α or with the stress granule marker G3BP2 in vertebrate cells will be informative. Nevertheless, it is intriguing that satellites seemed to be highly similar to those biomolecular condensates not only in terms of their proteomic landscape (as shown by the 40% overlap with the ‘RNA proteome’ mentioned earlier) but also in terms of their properties (membraneless organelles, putative phase separation behaviour, components with IDRs,...). Thus, future studies should aim at deciphering to what extent centriolar satellites are indeed similar to these granules, which might reveal exciting universal themes underlying their assembly and function.

4.5 Disease relevance of centriolar satellite dysfunction

Mutations in several centrosomal or ciliary genes have been implicated in a variety of disorders (see introduction), their corresponding proteins often localize to centriolar satellites, highlighting the importance of understanding the assembly and function of these structures. The Pericentrin gene (PCNT), located on chromosome 21 and hence additional copies of PCNT are found in trisomy 21, the genetic cause of Down syndrome (Antonarakis et al., 2020). Earlier

studies have shown that elevated Pericentrin expression leads to cilia defects and a decrease in the number of cilia, presumably due to compromised trafficking of ciliary proteins and pericentrosomal clustering (Galati et al., 2018). Conversely, in our study we find that upon PCM1 depletion PCNT protein is mostly abolished from the cytoplasmic centriolar satellite fraction, while *PCNT* mRNA is dispersed. This phenotype can be explained by the loss of translation (and co-translational targeting) but would also be in agreement with the opposite phenotype, PCNT clustering upon increased translation (overexpression) and the resulting elevation in co-translational targeting. In light of satellites as translational modules it seems plausible that overexpression of specific proteins would disturb the balance of certain protein complexes, leading to accumulated proteins and or other potentially detrimental secondary effects. Hence, when seen from this perspective the defects in cilia upon overexpression of PCNT are not due to impaired trafficking but due to disruption of multiprotein complexes and coordinated synthesis.

While Down syndrome is just one example, in general defects in protein complex formation are a common cause of protein aggregation. Huntington's disease (HD) is characterized by inclusions of misfolded proteins due to aggregation of the Huntington protein (HTT). HTT interacts with PCM1 via HAP1, the presence of expanded polyglutamylated tracts (polQ) in HTT results in PCM1 aggregation, a process that was reported to be facilitated by centriolar satellites (Prosser et al., 2022). Thus, I speculate that perturbing PCM1 structure and function might impact satellite protein synthesis and complex formation, thereby leading to protein aggregates implicated in human disorders.

Should our model of satellites as translational sites hold true, it might also change our way of looking at centrosome associated disorders as well as ciliopathies. Therefore, the analysis of the mutated gene and encoding protein together with its interaction partners might show if protein complexes are misformed, potentially providing important insights into potential therapeutic targets.

5 Conclusion

I believe that the identification of the PCM1 orthologs outside of vertebrates, in particular our characterization of the ortholog in flies, Combover, was key to understanding the role of satellites by taking an evolutionary point of view to identify core features and functions. In my work I was able to analyze and describe some of the characteristics and behaviors that satellites have in *Drosophila*; however, detailed analysis of the many cells and tissues where centrioles and centrosomes are present might provide further insights. Likewise, similar studies in other invertebrate model organisms will further highlight conserved aspects. Although we are still far away from a comprehensive understanding of the complex features and functions of centriolar satellites I hope that our proteomics data in flies will be a valuable resource for other centriolar satellite researchers. Furthermore, by providing for the first time a link between centriolar satellites and translation, I hope to have laid the groundwork for future studies to test if and how satellites could mediate this novel function.

6 References

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