Dissection of *in vitro* behavior and microtubule association of centriolar satellites

by

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A Dissertation Submitted to the
Graduate School of Sciences and Engineering

in Partial Fulfillment of the Requirements for

the Degree of

Master of Science

in

Molecular Biology and Genetics

KOÇ
ÜNİVERSİTESİ

August 2023

Dissection of *in vitro* behavior and microtubule association of centriolar satellites

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To my ever supportive and loving family…

ABSTRACT

Dissection of *in vitro* behavior and microtubule association of centriolar satellites

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Master of Science in Molecular Biology and Genetics

August 10, 2023

Centriolar satellites are membrane-less granules that localize around the main microtubule organizing center of animal cells, the centrosome. Centriolar satellites interact with microtubules and molecular motors and move around centrosomes in a microtubule-dependent way. They are scaffolded by pericentriolar material 1 (PCM1) protein, which interacts over 200 proteins including the ones mutated in ciliopathies, primary microcephaly and schizophrenia. In agreement with their disease links, they have been reported to regulate a diverse array of cellular processes such as primary cilium assembly, Hedgehog signaling, ciliary motility, autophagy and proteostasis. They mediate their functions by acting as cellular machines that store, traffic, modify and assemble their residents. Although centriolar satellite functions and molecular mechanism of action are evident, little is known about how they are assembled and maintained. To address this question, I dissected the biophysical the biophysical properties of PCM1 because it is the only essential protein for centriolar satellite assembly and maintenance. Phase separation has emerged as a mechanism by which a variety of membrane-less organelles assemble. Therefore, my central hypothesis was that PCM1 phase separates to form centriolar satellites. Supporting my hypothesis, centriolar satellites are spherical in shape in cells, dissolve during mitosis and exhibit fusion and fission events. To test my hypothesis, I used condensation assays, removal of solubility tag and treatments of manipulating the external conditions of the solvent. Results from these experiments showed that PCM1 assembles solid spherical condensates and scaffold-like mesh networks in vitro. To gain further insight into the PCM1 domains that contribute to its assembly; localization and microtubule association experiments identified a 923-1200 aa. region in central PCM1 that binds and bundles microtubules *in vitro* and *in vivo*. The mutant PCM1 lacking the microtubule binding domain was compromised for microtubule association. The microtubule binding fragment of PCM1 formed condensates upon disruption of microtubule network in cells. In in vitro assays, this fragment formed liquid-like condensates in the absence of microtubules. Taken together, these results identify phase separation as a mechanism that contributes to the assembly of the centriolar satellite scaffold and provide insight into how centriolar satellites interact with microtubules.

ÖZETÇE

Sentriyolar Satelit *in vitro* davranışı ve mikrotübül ilişkisinin teşrihi

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Sentriyolar satelitler, hayvan hücrelerinin ana mikrotübül düzenleme merkezi olan sentrozom çevresinde lokalize olan zarsız granüllerdir. Sentriyolar satelitler, mikrotübüller ve moleküler motorlarla etkileşime girer ve sentrozomların etrafında mikrotübüllere bağımlı bir şekilde hareket eder. Siliyopatiler, primer mikrosefali ve şizofrenide mutasyona uğramış olanlar da dahil olmak üzere 200'den fazla proteinle etkileşime giren pericentriolar materyal 1 (PCM1) proteini iskeleti sentriyolar satelitler oluşmaktadırlar. Hastalık bağlantılarıyla uyumlu olarak, birincil silyum düzeneği, Hedgehog sinyal yolağı, siliyer motilite, otofaji ve proteostaz gibi çeşitli hücresel süreçleri düzenledikleri bildirilmiştir. Sakinlerini depolayan, aktaran, değiştiren ve bir araya getiren hücresel makineler gibi hareket ederek işlevlerine aracılık ederler. Sentriyolar satelit fonksiyonları ve hücre bazındaki moleküler etki mekanizması açık olmasına rağmen, bunların nasıl bir araya getirildiği ve sürdürüldüğü hakkında çok az şey bilinmektedir. Bu soruyu ele almak için, PCM1'in biyofiziksel ve biyofiziksel özelliklerini karakterize ettim çünkü PCM1 proteini, sentriyolar satelitler oluşumu ve devamlılığı için gerekli tek proteindir. Faz ayrımı, çeşitli zarsız organellerin oluşmasına dair bir mekanizma olarak ortaya çıkmıştır. Bu nedenle, ana hipotezim, PCM1 proteinin faz ayrımı davranışı sergileyerek sentriyolar satelitleri oluşturmak için sitoplazmadan ayrıldığıydı. Sentriyolar satelit özellikleri olarak hücrelerde küre şeklindedirler, mitoz sırasında çözünürler ve füzyon ve bölünme olayları sergilerler. Bu özellikler, PCM1 proteinin faz ayrımına giderek oluşan zarsız bir granül yapı olan sentriyolar satelit oluşumunu sağladığına dair olan hipotezimi destekleyen faktörlerdir. Hipotezimi test etmek için, yoğunlaşma deneyleri, çözünürlük etiketinin çıkarılması ve çözücünün dış koşullarını manipüle etme işlemlerini kullandım. Bu deneylerden elde

edilen sonuçlar, PCM1'in katı küresel kondensatları ve iskelet benzeri ağ yapılarını in vitro olarak bir araya getirdiğini gösterdi. Sentriyolar satelit oluşumuna katkıda bulunan PCM1 etki alanları hakkında daha fazla bilgi edinmek için yapılan lokalizasyon ve mikrotübül ilişkilendirme deneyleri, PCM1 proteinin merkezi bölgesinde bulunan 923-1200 amino asit sekansının mikrotübülleri in vitro ve in vivo olarak bağlayan ve demet haline getiren bir mikrotübül bağlanma bölgesi olduğunu göstermiştir. Mikrotübül bağlama bölgesine sahip olmayan mutant PCM1, mikrotübül bağlanma özelliğinden yoksundur. PCM1'in mikrotübül bağlanma fragmanı, hücrelerde mikrotübül ağının bozulması üzerine yoğunlaşır ve faz ayrımına giderek küresel kondensatlar oluştur. İn vitro deneylerde, PCM1 proteinin 923-1200 amino asit bölgesi, mikrotübüllerin dağılımı sonrası sıvı benzeri kondensatlar oluşturdu. Birlikte ele alındığında, bu sonuçlar, sentriyolar satelit yapısının oluşumuna katkıda bulunan ve sentriyolar satelitlerin mikrotübüllerle nasıl etkileşime girdiğine dair fikir veren bir mekanizma olarak faz ayrımını tanımlar.

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CHAPTER 1: INTRODUCTION

1.1 Microtubules

Cellular cytoskeleton network is categorized into three distinct structures; actin filaments, intermediate filaments and microtubules that participates in determination of many features such as cell morphology, structure, motility, and rigidness. The main role of microtubules in this complex system are mainly the motility of the cell, division and trafficking and intracellular organization and regulation of organelles. The dynamic structure and rearrangement of microtubules is facilitated through polymerization and depolymerization of conserved alpha/beta tubulin heterodimers. The heterodimers of alpha/beta tubulin form polymerize to form hollow cylindrical structures of outer dimeter of 25 nm constructed from thirteen protofilaments (Cooper, 2000). The minus end of microtubules undergo catastrophe whereas, the plus end undergoes polymerization for growth of microtubule filament. The state of hydrolysis of GTP protein on alpha-tubulin monomer determines the polymerization/depolymerization state. Scarce tubulin where the GTP is hydrolyzed drives depolymerization on minus end while the presence of GTP cap where tubulin is abundant drives polymerization (Wade *et al.*, 2009; Cooper, 2000). Microtubules facilitate formation of unique structures, centrioles, mitotic spindle, primary and motile cilia, and flagella (Alberts et al., 2002).

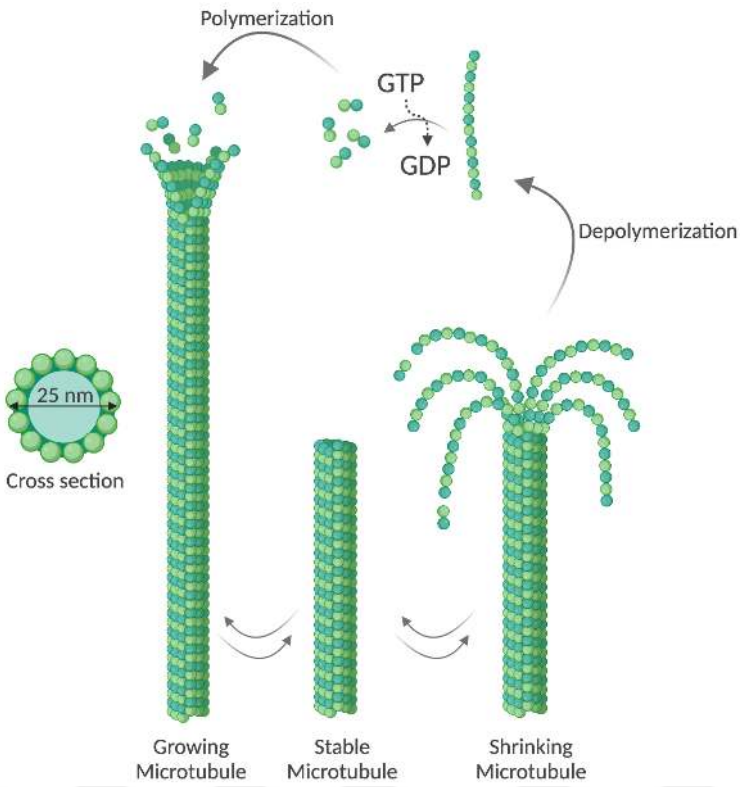


Figure 1.1. Microtubule structure and dynamics. The cross section of a microtubule filament is 25 nm in diameter. Upon GTP binding of beta-tubulin, the polymerization occurs and the hydrolysis of the GTP to GDP drives depolymerization.

1.2 Microtubule Based Structures

1.2.1 Centrosome

Centrosomes are the main microtubule organizing center (MTOC) of animal cells which participate in many cellular processes ranging from cell polarity, adhesion, motility, and cell division. Centrosomes are membrane-less organelles that consist of two microtubule-based cylindrical structures termed centrioles and associated pericentriolar material (PCM) (Gomes & Shorter, 2019). Centrioles are cylindrical microtubule-based structures that recruit PCM to form the centrosomes as well as template the formation of the cilia in quiescent cells. PCM is a dynamic matrix consisting of structured macromolecular protein complexes. It concentrates tubulin heterodimers, gamma-tubulin ring complex, and microtubule associated proteins (MAPs) to facilitate the nucleation, polymerization, and regulation of organization of microtubules.

Centrioles are conserved among ciliated protists to humans. All centrioles share a conserved 9-fold symmetry and proximal-distal polarity. Their size vary across different cell types in the same organism as well as across different organisms. Centriole size is maintained over successive cell divisions. It is about 250 nm in diameter and 500 nm in length in human cells. They are composed of 9 compound microtubules that are arranged circumferentially with respect to each other (Wang & Stearns, 2017). The inherent polarity of the centriole's microtubule scaffold is rendered from the proximal end governing the minus end and, the distal end governing the plus end. The proximal end of centrioles is composed of triplet microtubules that consists of a complete inner A-tubule where the assembly of the partial B-tubules and C-tubules occur. In contrast, the distal end contains 9 compound doublet microtubules. Based on the maturation level and the organism, centriole distal end may carry the appendage sites whereas, the proximal end may govern the cartwheel. The central core resides between the proximal and distal ends which accommodate Y-shaped linkers (Hamel et al., 2017; Le Guennec et al.,2020). The older centriole inherited from the mother cell is referred as the mother centriole where the younger centriole is referred as the daughter centriole.

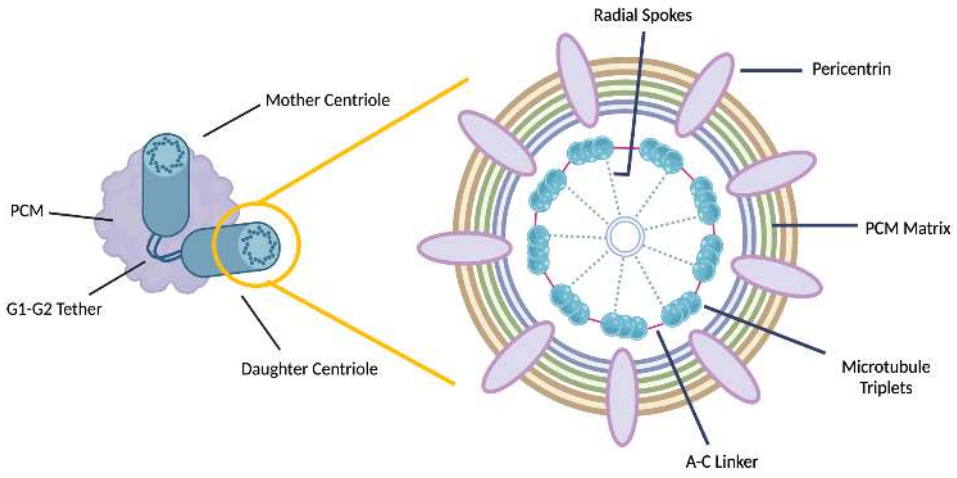


Figure 1.2. The structure of centrosome and overview of the centriole cross section.

Like DNA, centrioles duplicate once and only once during cell cycle. They undergo duplication in S phase, elongate and mature by G2 phase and are segregated equally to daughter cells by the mitotic spindle in dividing cells (Blanco-Ameijeiras *et al.*, 2022). Although majority of healthy cells have a pair of centrioles, a hallmark of cancer cells is supernumerary centrioles, which indicates the presence of more than 2 centrioles in cells. Centrosome amplication was shown to cause cancer by leading to aneuploidy, invasive behavior and deregulated cellular signaling (Song *et al.*, 2023). In addition to centriole duplication, pericentriolar material also undergoes modifications as cells enter mitosis. In a process called centrosome maturation, the amount of pericentriolar material around the centrioles increases, which leads to enhanced microtubule nucleation required for the formation of the mitotic spindle in dividing cells (Woodruff *et al.*, 2014). Microtubules are essential to alignment of chromosomes during metaphase and their equal segregation to daughter cells during anaphase. Accordingly, defects in centrosome maturation and consequently microtubule nucleation and organization lead to aneuploid and is prevalent in cancer (Song *et al.*, 2023).

1.2.2 Primary Cilium

Primary cilium serves as a cellular antenna and signaling hub for response to a variety of external stimuli. Primary cilium has distinct compartments that can be distinguished through their unique functions and structure. The distal and subdistal appendages of the mother centriole renders the function of basal body which serves as a template for assembly of primary cilium. The other compartments of primary cilium are the transition zone, the axoneme and the tip.

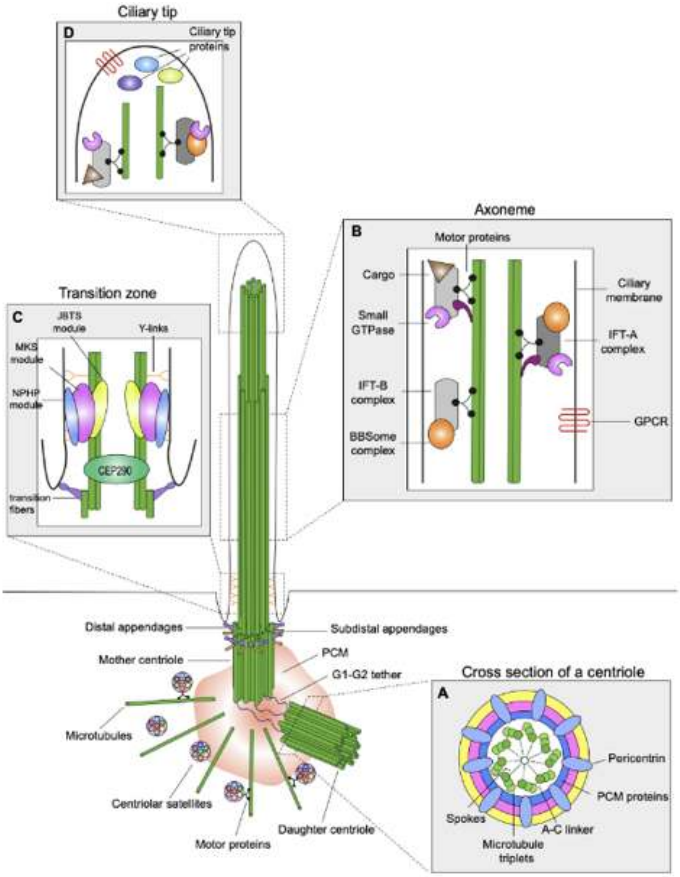


Figure 1.3. The structure of primary cilia: The primary cilium compartments include the basal body, ciliary axoneme, ciliary membrane and the ciliary tip. These compartments are separated through distinct protein pools and the functions attributed to the complexes formed (adapted from Arslanhan).

Ciliary axoneme resides at the core of the primary cilia which is composed of nine radially arranged microtubule doublets abbreviated to be 9x2+0 where the number nine represents the nine peripheral doublets, 2 represents doublets and 0 represents the lack of central singlet microtubules. The axoneme of motile cilia is abbreviated as 9x2+2 where the +2 represents the additional central microtubules allowing the motile cilia beating. Each doublet is composed of a A-tubule (termed complete) consisting of 13 protofilaments with an adjacent B-tubule (termed incomplete compared to A-tubule) consisting of 10 protofilaments. The protofilaments consist of polymers of alpha-beta tubulin heterodimers polymerized by the presence of GTP. Ciliary tubulins can carry many distinct post translational modifications such as acetylation, glutamylation and

detyrosination, collectively referred as the tubulin code (Gadadhar et al., 2017, Janke et al., 2020). The ciliary axoneme is surrounded by the ciliary membrane where many signaling molecules and target receptors of pathways regulated. Primary cilia mediated signaling and sensing mechanisms through receptors render it crucial for organismal development. For instance, sonic Hedgehog signaling pathway orchestrate the trafficking of Patched (PTCH1), Smoothened (SMO), GPR161, SUFU, and Gli transcription factors antero- and retro- grade with respect to ciliary axoneme. SMO recruitment to primary cilium by activation of hedgehog pathway activation upregulates the expression of downstream components through Gli transcription factors. In addition to Hedgehog signaling pathway, many other cascades such as Wnt, Notch, Hippo, G-protein coupled receptors (GPCRs), platelet-derived growth factor (PDGF), mammalian target of rapamycin (mTOR) and TGF-β are also mediated by the primary cilia. Multi systemic disorders that are collectively termed as ciliopathies are linked to structural and functional defects of cilium as primary cilia mediated signaling is crucial for development of embryo and maintenance of tissue homeostasis. In addition to centrosomes contribution to primary cilium assembly, the third component of the complex, namely the centriolar satellites also participate in primary cilium function, assembly, regulation, and maintenance (Khan & Scholey, 2018; Mitchell, 2007; Mitchison & Valente, 2017).

1.3 Centriolar Satellites

Centriolar satellites were first described as 70-100 nm electron dense granules that are clustered around the centrosomes of vertebrate cells. Their clustering around the centrosomes led to their nomenclature as “centriolar satellites”. After their initiatl discovery by electron granules, centriolar satellites remained as enigmatic structures for a long time until the discovery of their molecular marker PCM1(Kubo *et al.*, 1999). Immunogold staining of PCM1 confirmed that centriolar satellites localize around centrosomes in epithelial cells. Fluorescent staining of PCM1 in different tissues showed that its localization varies across different cell types, which indicates that they are differentially regulated and have different functions in different contexts (Odabaşı, 2019). Live imaging of fluorescent fusions of PCM1 relative to microtubules and in

cells manipulated for microtubules and molecular motors showed that they move along microtubules in c microtubule- and molecular motor-dependent manner (Conkar, 2019. The relative movement of these granules can reach maximum rate of 0.8 μm/s which reflect the motor dependent motion as the speed matches the rate of dynein movement which is also supported from the fact that their motility deteriorates drastically upon inhibition of dynein (Kubo *et al*. 1999, Odabasi et al., 2020, Prosser & Pelletier, 2020). Some granules display speed lower than 0.5 μm/s indicating diffusion-based motility (Conkar *et al.*, 2017).

Depletion or deletion of PCM1 in cells and organisms led to complete loss of centriolar satellites and relocalization of centriolar satellite residents to the centrosome or cytoplasm (Conkar, 2019; Aydın *et al.*, 2020). These studies identified PCM1 as the essential scaffolding protein for centriolar satellites and led to elucidation of specific centriolar satellite functions and mechanisms. Depletion of PCM1 by siRNA or shRNA led to defects in protein targeting to centrosome, microtubule network organization in interphase cells, progression of cell cycle, cell proliferation, cilium formation and deficiency in proliferation of neuronal progenitor cells and migration of them during cortical development (Dammermann *et al.*, 2002; Kim *et al.*, 2008; Nachury *et al.*, 2007; Zhang *et al.*, 2016; Ge *et al.*, 2010). After development of CRISPR/Cas9 mediated genome editing, PCM1-/- cells were generated and characterized, which was an important milestone in our understanding of centriolar satellites. While PCM1-/- retinal epithelial cells did not form primary cilium, PCM1-/- kidney epithelial cells formed cilia, but less efficiently. Importantly, the cilia formed by these cells were delayed in responding to Hedgehog stimuli. By determining which ciliogenesis factors are regulated by PCM1, characterization of these cells showed that PCM1 in part confers its functions at the primary cilium via regulation of the IFT-B component IFT88, centriole cap protein CP110 and ubiquitin ligase MIB1. Furthermore, characterization of PCM1-/- cells in other contexts led to identification of their functions during aggressome formation and autophagy (Joachim et al., 2017). In addition to cellular PCM1-/- models, mouse PCM1-/- models were generated and characterized, which provided insight into organismal functions of centriolar satellites. One of the PCM1-/- mouse models exhibited impairment in brain development,

behavioral abnormalities and developmental issues (Zoubovsky *et al*., 2015). The other also had ciliary integrity problems but specifically displayed symptoms of deficient embryonic development, underdeveloped cortical cortex and overall body and early death of the animal (Hall *et al.*, 2023). Additionally, morpholino treatment of PCM1 in zebrafish exhibit progressive decline in ciliary integrity in addition to adult onset of anatomical and psychomotor deficiencies (Monroe *et al.*, 2020). In agreement with animal models, mutations affecting PCM1 were implicated in schizophrenia and leukemia (Monroe *et al.*, 2020; Lee *et al*., 2018). Centriolar satellite proteome was described by a combination of proximity-based proteomics and biochemical pulldowns. It is composed of over 200 proteins which include proteins linked to many diseases, namely the ciliopathies, primary microcephaly, schizophrenia and Huntington's disease (Fırat-Karalar *et al*., 2014; Quarantotti *et al*., 2019). PCM1 baited affinity-based purification of satellites identified 223 proteins that represent the stable interactome of PCM1 as the satellite proteome containing proteins involved in centrosome organization, cilium assembly and cell division (Quarantotti *et al*., 2019). Proximity-dependent biotin labeling of 22 satellite proteins for determination of interactome by mass spectrophotometry-based proteomics approach identifies 660 proteins that reflect the transient interactions of centriolar satellites which also represents proteins involved in centrosome organization, cilium assembly and cell division in addition with proteins related to different biological processes including regulation of transcription and centrosome duplication (Quarantotti *et al*., 2019). Consequently, the integrity of centriolar satellites is crucial for development and organ physiology due to their function fundamental cellular processes of centrosome and cilia assembly, and function. In addition to their centrosome/cilia complex regulation function, they also facilitate autophagy, nucleation and organization of actin filament structure (Joachim *et al*., 2017; Odabaşı *et al.*, 2019).

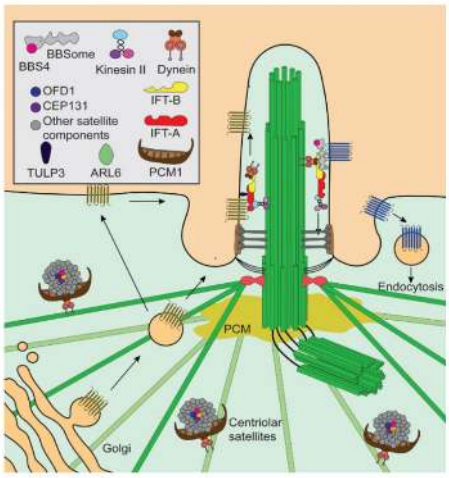


Figure 1.4. Ciliary Compartmentalization and Transport of Ciliary Proteins by Centriolar Satellites (Adapted from Arslanhan).

The main scaffolding protein of centriolar satellites PCM1, also has unique post translational modification sites that enable regulation of centriolar satellite abundance, stability and distribution. MIB1 E3 ubiquitin ligase mediated monoubiquitylation of PCM1 in proliferating cells arrest cilia formation which enables progression from quiescent phase to mitosis. The ubiquitination process is inhibited upon cellular stress inducing conditions such as ultraviolet irradiation or heat which consequently leads to initiation of cilia formation. PCM1 is also a substrate of a variety of kinases that phosphorylate their targets. For instance, phosphorylation of PCM1 by DYRK3 on multiple threonine and serine residues during the transition from G2 phase to mitosis after the breakdown of nuclear membrane initiates disassembly of pericentriolar material. Upon exit of cell from mitosis, DYRK3 is degraded through ubiquitination by APC complex. As DYRK3 is degraded, PCM1 scaffolding of centriolar satellites reassemble around the centrosome. In contrast to DYRK3 mediated PCM1 regulation, PLK4 phosphorylation at the 372 amino acid position (serine) facilitates maintenance of centriolar satellite integrity. Future studies will hopefully dissect the mechanism for regulation of assembly of satellites, their remodeling and number control. (Rai et al., 2018)

The main functions of PCM1 protein, thus the centriolar satellites are centrosome assembly and function through mediation of correct localization of

centrosomal proteins such as CEP250, CETN3, PCNT and NEK2. Such function encompasses cilium biogenesis through recruitment of ciliary protein complexes necessary for biogenesis such as BBSome. PCM1 is also required for anchoring the microtubule network to the centrosome as well as centrosome and cilium stability through recruitment of tubulin glutamylase complex (TPGC) to centriolar satellites. As mentioned above, the post translational modification of glutamylation is a part of the tubulin code which renders specific function to microtubule-based structures (Kubo & Tsukita, 2003).

1.4 Membrane-less Organelles

Compartmentalization enables the cell to spatiotemporally regulate the biochemical processes necessary for distinct function through organelles. Organelles are lipid bilayer enclosed compartments with their unique homeostasis. There are also membrane-less organelles; the centrosome and the ribosome which are rigid structures and are not only composed of condensed state of proteins. In addition to these exceptions, another class of membrane-less organelles have been defined through their ability to form by phase separation of their unique scaffold proteins. By phase separation, a condensate consisting of enriched scaffold material is formed which is surrounded by the cytoplasm where the concentration of the scaffold is much lower compared to that of the condensate. Such membrane-less organelles possess the ability to rapidly respond to cellular events by regulation of their internal and external dynamics by different means (Wheeler and Hyman, 2018). Formation of membranelles organelles is facilitated through the phenomena of phase separation of protein condensates. Condensation of proteins in aqueous solutions by phase separation can be mediated through means of crystallization, liquid-liquid phase separation aggregation and gelation for solid state.

Characterized cytoplasmic membranelles granules within the cytoplasm are stress granules, processing bodies (p-bodies) and germline p-granules while the nuclear membranelles granules include nuclear speckles, nucleoli, nuclear stress bodies, histone locus bodies, cajal bodies and Promyelocytic leukemia nuclear bodies (PMLs). Membrane-less organelles are considered to act liquid-like due to their ability of

wetting-relaxation dynamic into spherical condensates upon fusion in addition to their internal dynamicity. The formation of a membrane-less granule is facilitated through protein-protein and protein-RNA interaction partners. The condensates formed upon phase separation creates a state within the intracellular fluid that is different in terms of biophysical and biochemical properties thus, enables compartmentalization without the need for a rigid boundary of a membrane (Li et al., 2012).

1.5 Mechanism of Membrane-less Organelle Formation

The homotypic interaction between two of the same molecules and the heterotypic interaction between two different molecules contribute to the process of phase separation in aqueous solution. Homotypic interactions require higher energy compared to heterotypic interactions which favors entropy to maintain the system in a phase separated regime of lower energy. The analogy of the two types of molecules in this system represents the cytoplasm and the polymer of protein/nucleic acids (Bansil, 1993).

1.5.1 Phase Separation

Biochemically, phase separation is the state where a homogenous solution of a polymer, which would be the scaffolding protein within the cytoplasm in cellular context, separates to form two phases co-existing together: one enriched with the polymer and one depleted of the polymer. The polymer enriched state is referred as the condensates. For the condensation to be favorable for the cell, they must be regulated in terms of the timing of assembly and disassembly as well as the composition of proteins within the condensate (Wheeler and Hyman, 2018). The condensates may exist in different physical states: solid, gel like or liquid like which is determined by the nature of the polymer protein, scaffold. The phase separation of the condensates facilitates biochemical isolation of target reactions in a reversible and regulated manner (Gomes and Shorter, 2019).

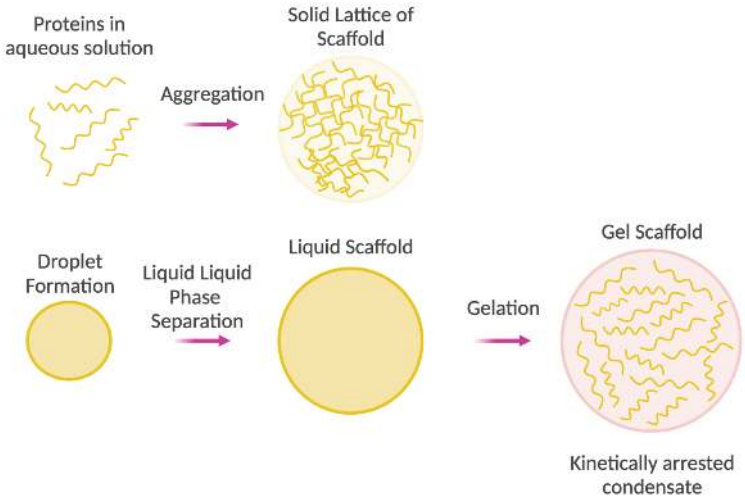


Figure 1.5. Phase separation of proteins, the mechanistic of condensate formation and the physical states of condensates.

1.5.2 Scaffolding proteins and residents

The physiological state of the condensate: solid, gel like or liquid like is determined by the nature of the polymer protein, scaffold and the other proteins that are partitioned within the scaffold condensate, the residents or clients. Client proteins of the condensate are the solutes as they are the target of the biochemical reactions within the condensate. They can alter the state of the condensate based on the multivalency they provide to the scaffolding protein, the solvent (Wheeler and Hyman, 2018).

1.5.3 Concentration Dependency

Partitioning of the scaffolding protein of the condensate to phase separate from the cytoplasm requires a certain concentration where the protein is saturated enough to thermodynamically favor the equilibrium to form the condensate structure (Wheeler & Hyman, 2018). The concentration of the scaffold can be regulated by macromolecular crowding of the cytoplasm for sequestration which creates an environment where the solute of scaffold can phase separate and convert to the condensate form which can be expressed in terms of partitioning co-efficient, referred as: k, of the phase separation reaction.

1.5.4 Multivalency

Multivalency is the phenomena of strong and reversible interactions among two units (protein-protein, protein-RNA) which enables functional molecular structures in a selective manner. It also enables formation of controlled interaction among surfaces of these structures creating scaffold structure (Haag, 2015). The formation of such phase separated architectures rely on the multivalency of the scaffold and the interaction partners. Such multivalency and affinity among proteins can be mediated by many factors including repeat motifs of interaction sites, intrinsically disordered regions that fold flexibly to facilitate binding of fitting surfaces. The phase separation of scaffold and the residents can mediate formation of liquid-like or gel-like droplets (Li et al., 2012).

1.5.5 Intrinsically disordered regions

In addition to interaction between proteins through ordered repeat domains, intrinsically disordered regions (IDRs) can also serve as template for multivalency. IDRs are especially important for RNA binding ability of proteins which facilitate formation of RNA binding proteins referred as ribonucleoproteins (RNPs). Examples of RNPs include FUS, TDP-43, TAF15, EWSR1, hnRNPA1, hnRNPA2, and TIA-1 which from phase separated condensates in vivo and malfunctions in their folding are associated with neurodegenerative diseases emphasizing the importance of phase separation in functionality (King et al., 2012). The molecular grammar buried in the sequence information creates the phase transition ability of such proteins.

1.5.6 Domain oligomerization

Multivalency being a means of phase separation not only includes interaction between two different molecules but interaction between molecules of the same kind which is not oligomerization of different proteins but self-oligomerization. The naturally occurring example of such cases is N-terminal domain oligomerization of TDP-43. In a study represented by Shin *et al.*, 2017, they utilized fusion of Cry-2 with the intrinsically disordered regions of several RBPs which enables Cry-2 to artificially undergo phase separation proving that IDRs and oligomerization domains can mediate phase separation alone.

1.5.7 Post Translational Modifications (PTMs)

Post translational modifications (PTMs) regulate phase separation by either facilitating or antagonizing the phase transition of the target protein. PTMs can affect the valency of the protein by altering the charge, folding, interaction partners. For instance, the SUMOylation of main scaffold protein of PML bodies, PML enables recruitment of binding partners for formation of the membrane-less granule (Zhong *et al.*, 2000) (Shen *et al.*, 2006). Tyrosine phosphorylation of NCK, serine phosphorylation of FUS are examples of mediation of phase separated droplet formation while arginine methylation of Ddx4, hnRNPA2, FUS and Dyrk3 mediated phosphorylation inhibit phase separation (Gomes *et al.*, 2019). Initiation or antagonizing effect of phase separation can be modulated through PTMs.

1.5.8 Nucleation and Seeding Mechanisms

Phase transition into a proteinaceous condensate relies on reaching a certain concentration of the required protein or proteins which is referred as the critical concentration. For a protein to reach the critical concentration at a certain region from being dispersed homogeneously throughout the cytoplasm requires a nucleation and seeding mechanism for crowding and concentrating. Cells take advantage of biomolecular nucleators for seeding the proteins to the critical concentration necessary for phase separation. These nucleators are RNA, poly-ADP ribose and polyphosphates.

1.5.8.1 RNA Dependent Nucleation

RNA binding proteins (RBPs) display phase separation behavior that is biologically relevant for their function as they form membrane-less organelles. The RBPs of the relevant membrane-less granules contain domains that enable multivalency as well as IDRs that modulate phase separation. For instance, hnRNPA1 protein of stress granules undergoes liquid-liquid phase separation to form protein-rich droplets in vitro mimicking the in vivo liquid behavior of stress granules (Molliex et al., 2015). Another example is the ALS-associated FUS protein undergoing liquid-liquid phase separation

in vivo and in vitro which can then form aggregates related to ALS phenotype upon mutation on domains that renders the phase transition ability of FUS (Patel et al., 2015). LAF-1 protein found in P granules also phase separates into liquid droplets (Garfinkle et al., 2015). RNA binding protein TDP-43 also undergoes liquid-liquid phase separation to form membrane-less ribonucleoprotein granules and the malfunction in phase separation of TDP-43 to aggregates is associated with ALS and Alzheimer's Disease (Wang et al., 2018). All the mentioned proteins above can undergo phase separation through either their IDRs or their RNA binding domains rendering them functional.

1.5.8.2 RNA-like molecule (poly ADP ribose) Dependent Nucleation

Poly-ADP ribose, shortly PAR, has been demonstrated as a key driving force of phase separation of stress response related membrane-less organelles (Schreiber *et al.*, 2006). It constitutes the stress granules in addition to FUS and TDP-43 scaffolded condensates (Patel *et al.*, 2015, Altmeyer *et al.*, 2015).

1.5.8.3 Polyphosphate Dependent Seeding

Nucleation of amyloids is dependent on polyphosphate-based seeding. Additionally, polyphosphate-based granules itself are assembled in bacteria upon stress response to starvation (Cremers *et al.*, 2016, Racki *et al.*, 2017). Assembly of polyphosphate granules is like RNP-granules in terms of their partitioning into phase separation and are specifically liquid-liquid phase separation dependent.

1.6 Advantages of Phase Separation

1.6.1 Fine tuning of biochemical reactions

Microdroplets of membrane-less organelles accelerate biochemical reaction rate by creating the conserved specific microenvironment within the phase regime through crowding the constituent's concentration in an order of two times the magnitude of the cytoplasm (Li et al., 2012). The phase separated droplet can also serve as a selective filter for the molecules that are required for the reaction and eliminate the molecules that are not needed.

1.6.2 Cellular fitness and response to external stimuli

The protein rich cytoplasm and the lack of separation from the crowded environment can cause misfolding of proteins. To overcome the lack of control in terms of the constituents of the cytoplasm, phase-separated condensates are formed which are reversible in terms of sequestering, storing and releasing proteins. The tunability of phase separated condensates and their reversible nature of formation according to environmental stimuli can serve the function of stress sensing and response (Boeynaems et al., 2018). Upon induction of stress, the temporarily stored proteins and RNA constituents of condensates can be rapidly released in response necessary for recovery. Such response mechanism enables the cell to adapt to environmental conditions and escape deleterious situations. One example case would be the yeast prion protein Sup35 which acts as a sensor of pH that forms liquid-like condensates that transition phase from liquid to gel-like upon decrease in pH of cytoplasm. The Sup35 based gel condensates protect the proteome of the yeast and enable recovery (Franzmann et al., 2018).

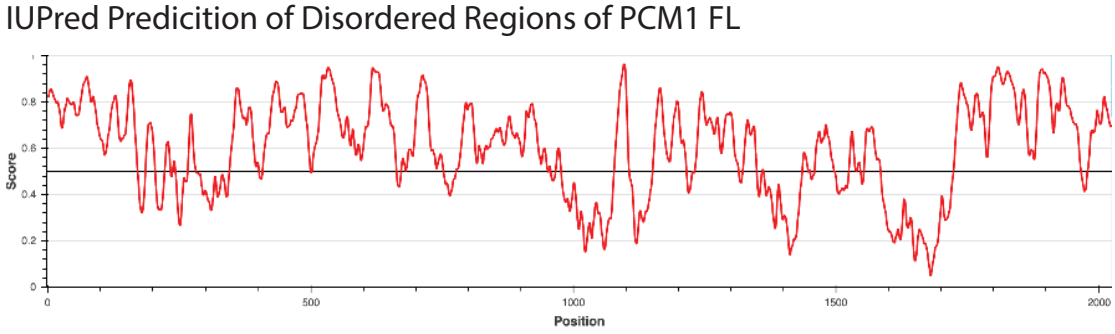
CHAPTER 2: RESULTS

2.1 Main scaffold of centriolar satellites: PCM1 is a highly disordered protein

Centriolar satellite granules are highly dynamic in terms of their localization and distribution across cellular processes. PCM1, being the main scaffolding protein of centriolar satellites, forms membrane-less granules of centriolar satellites and its behavior regulates the assembly and disassembly of centriolar satellites. Given the fact that membrane-less granules have been demonstrated to form by phase separation, elucidating the potential of PCM1 to phase separate by investigating the existence of factors that facilitate phase separation is crucial. As the presence of IDRs are a key indicator of phase separation, in slico sequence-based prediction tools, IUPred and PONDR was utilized to analyze PCM1 protein sequence in terms of ordered and

disordered regions. It was revealed that PCM1 protein contains many disordered regions (Figure 2.1A and Figure 2.1B). Proteins that contain IDRs are collectively referred as intrinsically disordered proteins (IDPs). The alphafold structure prediction represents the IDRs of PCM1 which are regions with an undefined folding and three-dimensional structure that can exist as heterogenous confirmations (Figure 2.1C). IDRs contribute to face separation of a protein by means of interaction regions and solvating regions (Borcherds *et al.*, 2021). Another key factor that drives phase separation is the multivalency of protein which is defined as the potential sites of interaction (Martin *et al.*, 2020). The interaction between proteins is facilitated through flexibility of their confirmation to bind their interaction partners. Lack of defined three-dimensional structure and folding pattern of the IDR sequence renders the protein capable of interacting with its partners and intra-cellular environment to reach the most energetically favorable confirmation of phase separation by increased solubility (Martin *et al.*, 2020). Consequently, the existence of IDRs renders PCM1 a great candidate for a phase separating protein as a mechanism for membrane-less centriolar satellite granule formation.

A



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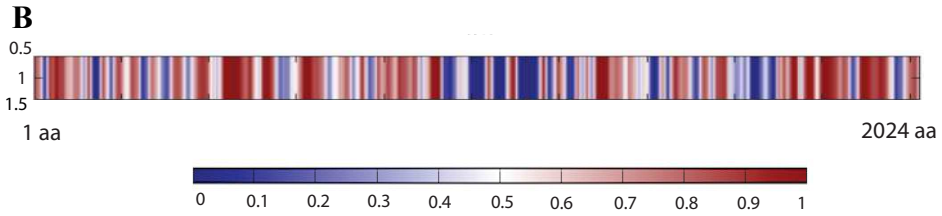

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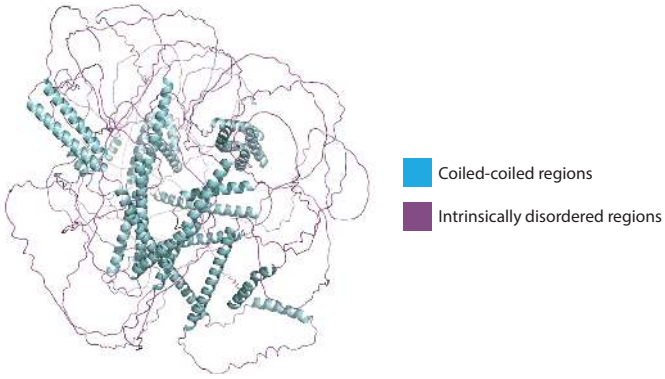


Figure 2.1. PCM1 is a higly disordered protein according to structural analysis in slico. **(A)**IUPred Disorder predication of PCM1shows that it is highly disordered as score higher than 0.5 predicts intrinsically disordered regions of PCM1. **(B)** In the heat map representation of PONDR tool, red indicates region with IDRs while the blue indicates ordered regions of the 8 coil-coils of PCM1 Protein between amino acids 218-301, 400-424, 487-543, 651-682, 726-796, 824-858, 1063-1089, 1510-1539). **(C)** Alphafold prediction of PCM1 protein folding where the blue regions represent the structured coiled-coil domains, and the purple regions of strings represent the IDRs.

2.2 PCM1 protein undergoes phase separation *in vitro*

To test the behavior of PCM1 in terms of phase separation, different buffers that mimic the cellular environment have been tested. In both buffer (HEPES based: 10 mM HEPES, 150 mM NaCl, 0.1 mM EDTA, 2 mM DTT, 10% PEG, pH 7.4 and Tris-based: 50 mM Tris-HCl, 50 mM NaCl, 10% PEG), His MBP PCM1 FL formed droplets and mesh-like scaffold structure *in vitro* (Figure 2.2A). HEPES based buffer have been used for all *in vitro* assays and is referred as LLPS Buffer as both droplets and mesh-like scaffold structure have been observed and the protein did not aggregate and was soluble which is a requirement for *in vitro* assays. As PCM1 is assessed for its phase separating behavior, dissecting the potential solid scaffold structure is important to characterize its physiological state.

In order to eliminate artifacts that can be caused by the fusion protein tag and the crowding agent polyethylene glycol (PEG), the native condition of the protein was also assessed for phase separation. PEG is a crowding agent and is used to mimic the biomolecular crowded environment of cytosol however, phase separating protein in literature have been reported to from condensates without crowding agent addition as well. Additionally, the fusion tag can also interfere with phase separation of a protein as MBP is known to affect the solubility of the fusion protein (Raran-Kurussi *et al.*,

2015). In order to eliminate such artifacts and to test if the native PCM1 protein can phase separate, TEV cleavage assay was performed without addition of any crowding agent in HEPES based buffer (10 mM HEPES, 150 mM NaCl, 0.1 mM EDTA, 2 mM DTT, pH 7.4). There is a linker region between the His MBP tag and PCM1 FL protein of sequence Glu-Asn-Leu-Tyr-Phe-Glu-Gly-Ser (ENLYFQ∗G/S) which is the recognition site for TEV protease cleavage. The proteolytic cleavage occurs between the Glu and Gly/Ser residues (Figure 2.2B). For control case of no TEV cleavage where the fusion protein is intact with its tag, the protein forms droplets and in the case of TEV addition, the number of droplets formed increase significantly compared to control case demonstrating that PCM1 FL alone without the His MBP tag can phase separate (Figure 2.2C and Figure 2.2D).

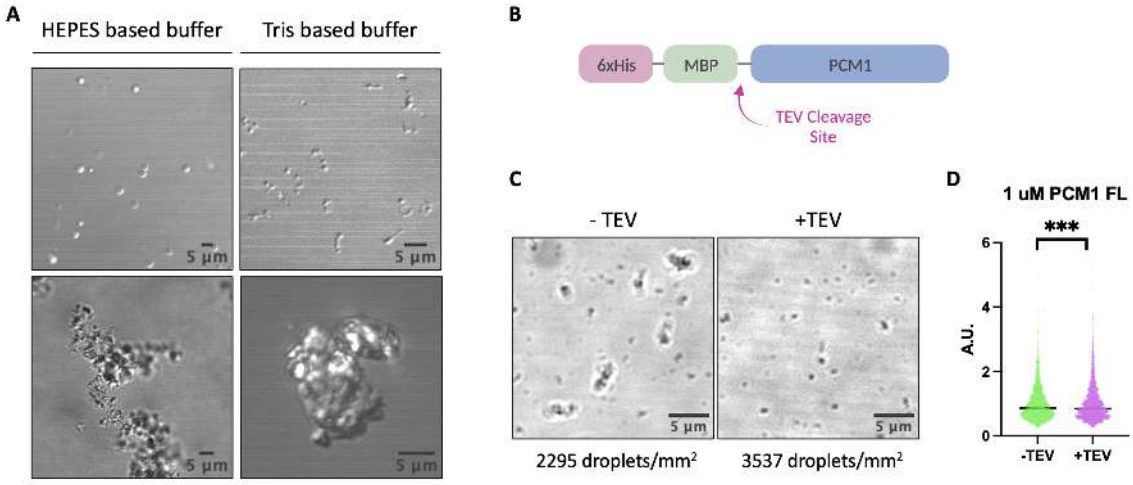


Figure 2.2. PCM1 phase separates into condensates and forms mesh like scaffold structure. **(A)** His MBP PCM1 FL His MBP PCM1 FL phase separates into spherical shape condensates or forms scaffold-like mesh network. Scale bar 5 µM. **(B)** Schematic representation of the His MBP PCM1 FL for the TEV cleavage site. MBP tag increases solubility of a protein thus, tag removal by TEV cleavage enables observation of the protein itself and is used as a method to induce phase separation. **(C)** For the case of no TEV cleavage, the scaffold like network of PCM1 is observed and there exists 2295 droplets/mm^2 on average of two replicates while upon cleavage by TEV and consequent removal of solubility tag, the scaffold like network of PCM1 FL phase separates into spherical condensates and there are 3537 droplets/mm^2 on average of two replicates. **(D)** Quantification of structures observed from two biological replicates showing significant drop in size upon phase separation of large scaffold-like network into smaller condensates (*p-value* < 0.0001).

2.3 His MBP PCM1 FL phase separates to a homogenous mixture of condensates/mesh-like structure that do not dissolve upon *1,6-hexanediol* treatment

As phase separating behavior of PCM1 have been demonstrated, we wanted to address the properties of the phase separating structures of PCM1 in terms of their biophysical features. To assess the state of condensates and scaffold-like network formed by PCM1 FL, *1,6-hexanediol* treatment was applied. His MBP PCM1 FL forms droplets and scaffold-like network that is resistant to *1,6-hexanediol* treatment. We can conclude that the phase separation of PCM1 forms solid structures of condensates and mesh-like structure that is rigid.

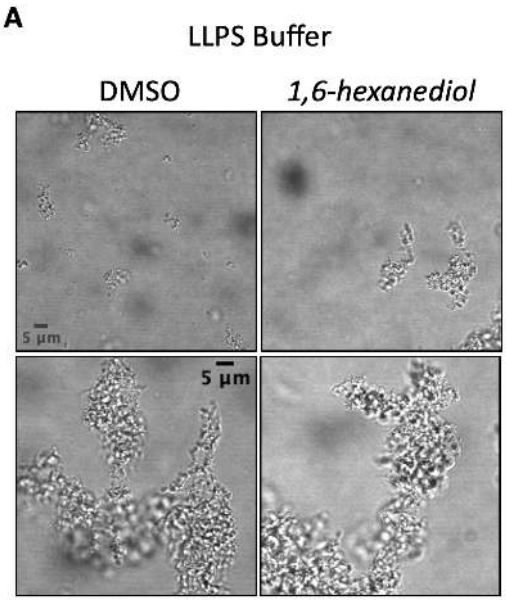


Figure 2.3. PCM1 scaffold and condensates are hexanediol resistant thus, are solid-like. His MBP PCM1 FL scaffold does not dissolve upon *1,6-hexanediol* treatment which shows that it is solid like as seen in upper panel whereas the condensates of His MBP PCM1 dissolve upon *1,6-hexanediol* treatment indicating that they are liquid-like. Scale bar 5 µM.

To further understand the effect of ionic interactions and their contribution to PCM1 behavior, the salt concentration of the buffer was manipulated to assess behavior of PCM1 under different ionic conditions that alter the folding of the protein thus, contribute to phase separation. In condition of high salt (500 mM), His MBP PCM1 forms mesh-like scaffold structure and condensates which upon *1,6-hexanediol* treatment, the scaffold structure remains intact verifying that is solid like and resistant to high salt concentrations indicating a wide range of ionic environment where it can phase separate (Figure 2.4A). In low salt conditions where the solubility of protein is expected to drop as per compared to cytosolic ionic charge (25 mM), His MBP PCM1 forms mesh-like scaffold structure and condensates. Upon *1,6-hexanediol* treatment, mesh-like scaffold structure and the condensates persist demonstrating solid behavior.

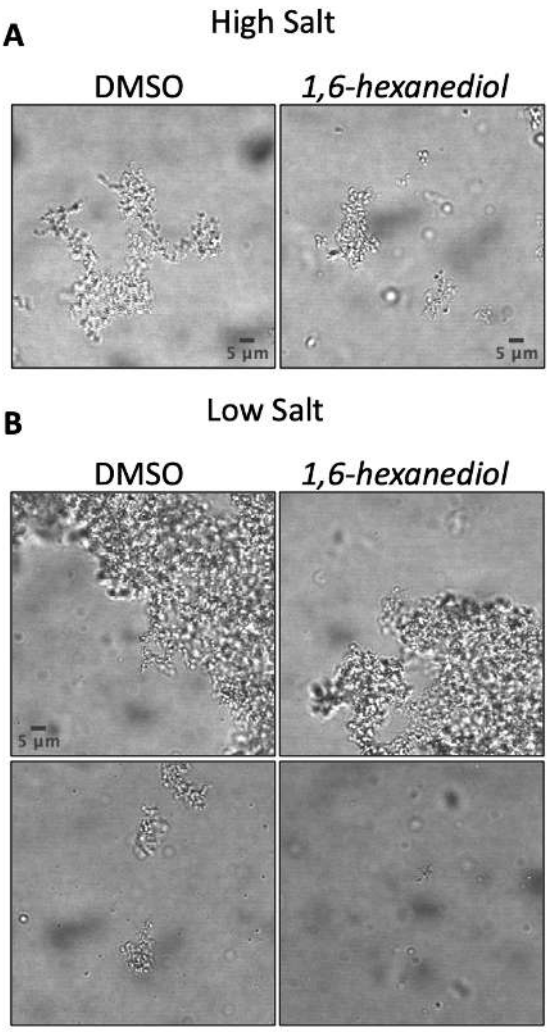


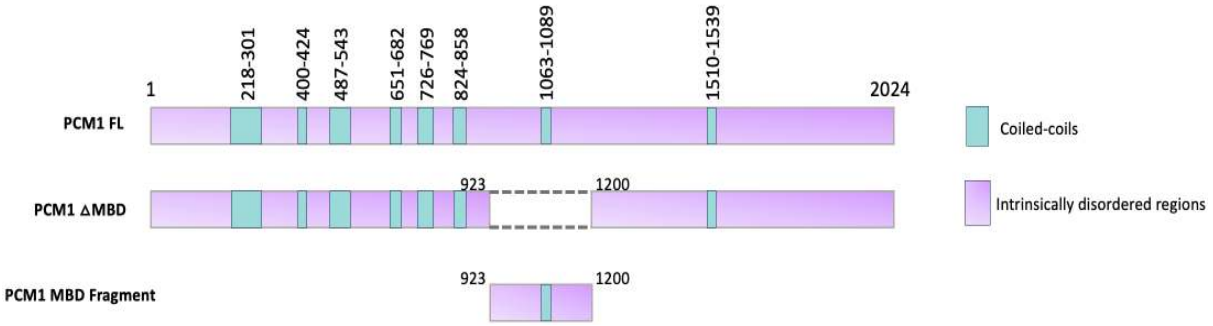
Figure 2.4. PCM1 scaffold and condensates are hexanediol resistant thus, are solid-like in varying conditions of ionic strength. In high salt concentration of 500 mM NaCl, His MBP PCM1 FL forms mesh-like scaffold network and condensates. Upon *1,6-hexanediol* treatment both the scaffold network and the droplets persist indicating that it is solid. In low salt concentration of 25 mM NaCl, His MBP PCM1 forms mesh-like scaffold network and condensates and both structures are *1,6-hexanediol* resistant indicating that it acts solid like.

Conclusively, PCM1 protein phase separates into condensates and mesh-like scaffold structure that exhibit solid or gel like behavior which is the proposed mechanism for formation of membrane-less granules.

2.4 PCM1 protein has contains a microtubule binding domain

For further dissection of PCM1 characteristics, PCM1 was fragmented into domains, and it was observed that the 923-1200 aa fragment of PCM1 displays a unique pattern of localization unlike the FL and all the previously reported fragments of N terminal long (1-1200 aa), N terminal short (700-1200 aa) and C terminal (1200-2024 aa) which either form granules like FL or behave cytoplasmic (Lopes *et al.*, 2011). 923-1200 aa fragment of PCM1 binds and bundles microtubules. The deletion mutant PCM1 ΔMBD forms centriolar satellite granules upon expression in U2OS PCM1 KO cells therefore, it can be concluded that the 923-1200 aa region of PCM1, which will be referred as microtubule binding domain (MBD), does not impair the ability of PCM1 to form satellite granules (Figure 2.4).

A


1
218-301
400-424
487-543
651-682
726-769
824-858
1063-1089
1510-1539
2024
PCM1 FL
923
1200
PCM1 ΔMBD
923
1200
PCM1 MBD Fragment
Coiled-coils
Intrinsically disordered regions

B

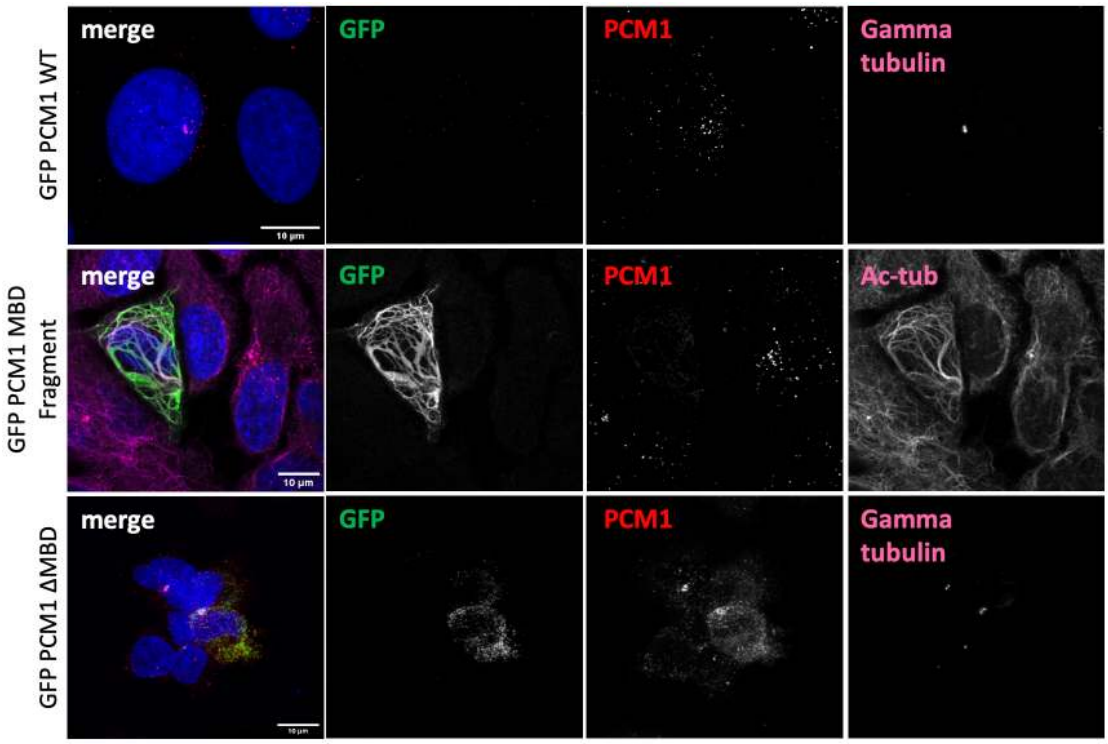


Figure 2.5. PCM1 has a microtubule binding domain that binds and bundles microtubules in vivo. **(A)** The representation of the constructs PCM1 FL which is the wild-type sequence of the 2024 aa length protein, PCM1 ΔMBD which has a deletion of the microtubule binding domain between 923-1200 aa and the PCM1 MBD Fragment which is the fragment of PCM which binds microtubules. **(B)** PCM1 staining of U2OS WT cells show the typical structure of granular centriolar satellites clustered around the centrosome stained with gamma tubulin as well as scattered throughout the cytosol. Upon fragmentation of PCM1, it was found that the 923-1200 aa region of PCM1 binds and bundles microtubules *in vivo* instead of forming centriolar satellite granules as seen in transfection of GFP PCM1 MBD Fragment and the colocalization acetylated tubulin staining. PCM1 ΔMBD is the microtubule binding site deletion mutant and can form granules like the PCM1 FL which shows that the microtubule binding domain is not necessary for granule formation in U2OS PCM1 KO cells. Scale bar 10 μM.

2.5 PCM1 ΔMBD deletion mutant does not bind to microtubules

In order to test the ability of PCM1 FL to bind microtubules and whether the deletion of MBD impairs the ability of PCM1 to bind microtubules, GFP PCM1 FL and GFP PCM1 ΔMBD mutant was transfected to HEK293T cells along with GFP control. The microtubule pelleting assay performed by incubation of Taxol stabilized microtubules with the *ex vivo* cell lysate and centrifugation at high speed over cushion shows that GFP PCM1 FL pellets with microtubules while the GFP PCM1 ΔMBD mutant does not pellet compared to control of no microtubule case. The GFP control also does not pellet with microtubules as a control of the tag's interference with binding and specificity of the PCM1 and PCM1 ΔMBD affinity towards microtubules (Figure 2.5).

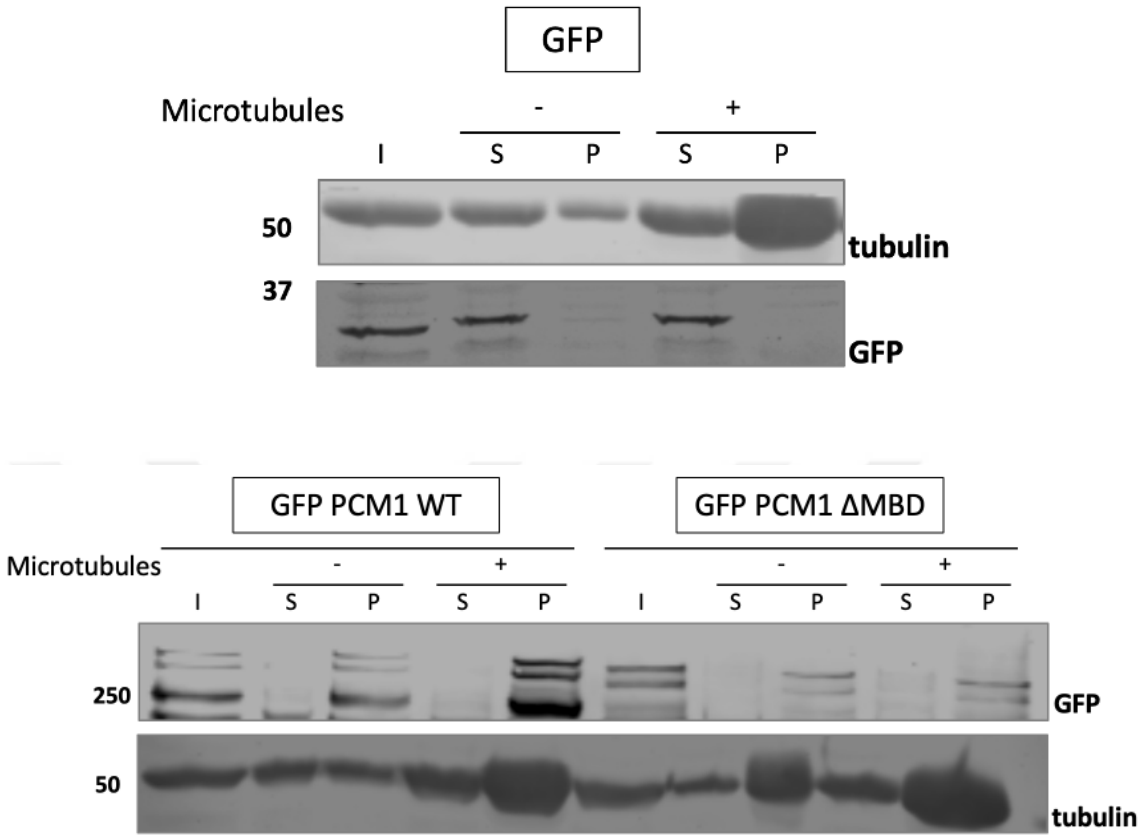


Figure 2.6. PCM1 binds microtubules while the PCM1 ΔMBD mutant does not bind. GFP is used as control as a protein that does not interact with microtubules as well as a control of the tag of PCM1 WT and PCM1 ΔMBD. GFP alone remains in the supernatant and does not pellet with microtubules showing that the binding of PCM1 WT is specific. PCM1 WT pellets with microtubules upon comparison of no microtubule and addition of microtubule while PCM1 ΔMBD does not pellet more compared to no microtubule control case demonstrating that deletion of the microtubule binding domain of PCM1 impairs its ability to interact with microtubules form the *ex vivo* cell lysate coming from HEK293T cells transfected with GFP, GFP PCM1 WT and GFP PCM1 ΔMBD.

2.6 PCM1 FL co-sediments with microtubules *in vitro*

After showing that GFP PCM1 FL co-pellets with microtubules and GFP PCM1 ΔMBD mutants' affinity towards microtubules is impaired due to deletion of MBD site, the interaction of PCM1 FL was verified with *in vitro* microtubule sedimentation assay. Purified His MBP PCM1 FL protein was co-pelleted with microtubules, and it was shown that for the control case of no microtubules it mainly remains in supernatant however gets almost completely depleted from the supernatant and co-pellets with microtubules (Figure 2.6). Therefore, it can be concluded that PCM1 FL itself has affinity towards microtubules and co-sediments with them directly verifying the previous result of ex-vivo sedimentation assay. Moreover, by the *in vitro* microtubule pelleting assay of purified His MBP PCM1 protein, the direct interaction was demonstrated as the *ex vivo* results might have been caused by a mediator of interaction such as a protein that has interacts with both PCM1 and microtubules causing them to co-pellet together in a complex.

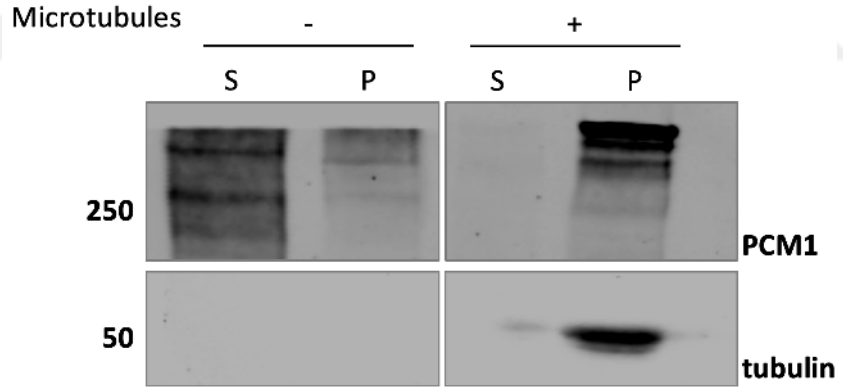


Figure 2.7. PCM1 co-sediments with microtubules in vitro. His MBP PCM1 FL remains mostly in supernatant and does not pellet with microtubules however, upon addition of microtubules, it gets depleted from the supernatant pellets with Taxol stabilized microtubules. This shows that PCM1 FL itself has a certain affinity to microtubules itself.

2.7 PCM1 MBD Fragment co-sediments with microtubules *in vitro*

To dissect and characterize the ability of MBD fragments' ability to bind and bundle microtubules, *in vitro* assays were performed to show direct interaction and the effects of MBD fragment on microtubules in terms of bundling. To do so, His MBP PCM1 MBD Fragment was expressed and purified from Rosetta Bl21 cells, and the purification was verified through Coomassie staining (2.7).

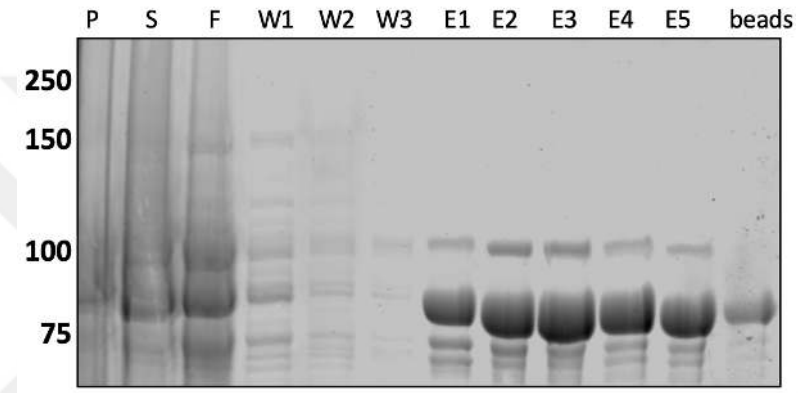


Figure 2.8. Coomassie of the His MBP PCM1 MBD Fragment showing that it is soluble and is purified for in vitro assays of microtubule binding as well as phase separation assays.

For biochemical investigation of MBD Fragments affinity towards microtubules *in vitro* microtubule sedimentation assay was performed along with bovine serum albumin (BSA) protein control as the globular BSA protein does not have affinity towards microtubules and is used to demonstrate the specificity of interaction between MBD fragment and microtubules. BSA protein remains in the supernatant and does not co-pellet with microtubules while the His MBP PCM1 MBD Fragment co-sediments with microtubules upon increased concentration of microtubules compared to no microtubule control case where the protein only pellet much less due to aggregation and high-speed centrifugation. To conclude, His MBP PCM1 MBD fragment was demonstrated to have specific affinity towards microtubules and can bind microtubules *in vitro* (Figure 2.8).

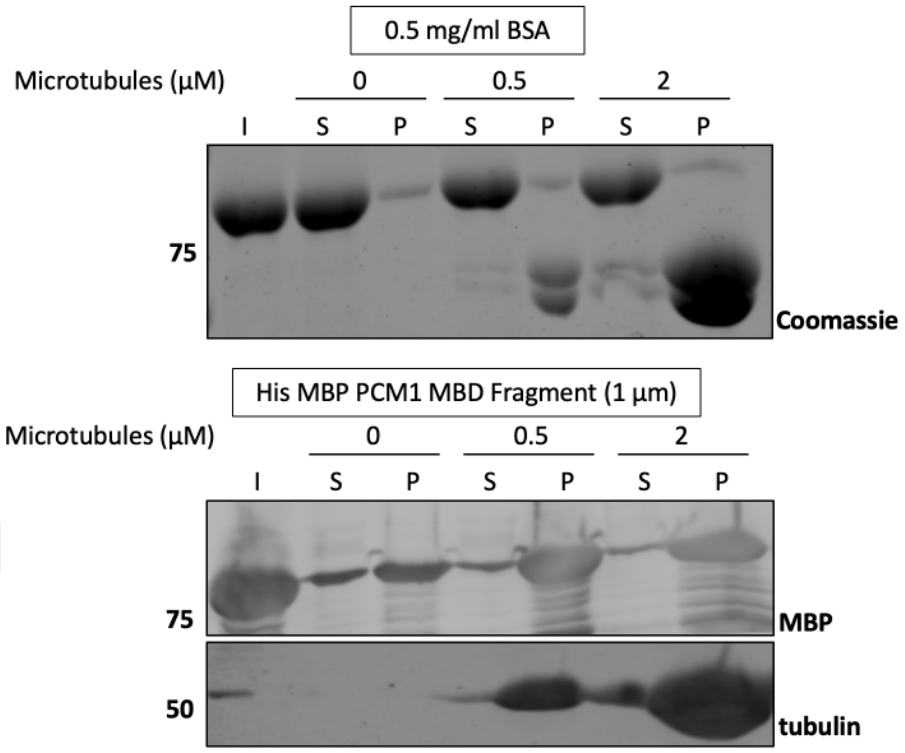


Figure 2.9. His MBP PCM1 MBD Fragments pellets with microtubules. BSA control does not pellet upon sedimentation of microtubules at high speed and remains in the supernatant while the His MBP PCM1 MBD fragment pellets with microtubules which shows that it has specific affinity towards microtubules.

2.8 PCM1 MBD Fragment directly binds and bundles microtubules *in vitro*

The biochemical assays of microtubule sedimentation to test affinity of MBD Fragment demonstrated that PCM1 MBD Fragment binds microtubules *in vitro*. As MBD Fragment binds and bundles microtubules *in vivo* and directly interacts with them based on *in vitro* co-sedimentation assays, we sought to further investigate and verify their direct interaction by an imaging-based approach to elucidate and resolve the nature of the interaction as microtubule binding proteins (MAPs) are known to have different effects on microtubules. For instance, TPX-2 is a microtubule associated protein that directly binds free tubulin from its N-terminal and nucleates microtubules (Roostalu *et al.*, 2015). Unlike TPX-2, MAP4 does not contribute to nucleation of microtubules but their stability as its regulation by phosphorylation alters its affinity towards microtubules and enables MAP4 to bind microtubules for stability as demonstrated by

nocodazole resistance of microtubules upon expression of MAP4 and MAP4 phospho-deficient mutant (Chang *et al.*, 2001). To do so, Total Internal Reflection Fluorescence (TIRF) microscopy-based imaging was performed. By this method, fluorescently labelled (rhodamine) microtubules are incubated with GFP His MBP PCM1 MBD Fragment as well as microtubules alone for control and are flown into flow chambers and were imaged. The TIRF assay demonstrates the control case where the Rhodamine labelled microtubules alone remain as individual filaments of microtubules while the addition His MBP GFP PCM1 MBD Fragment binds and creates parallel and anti-parallel bundles of microtubules *in vitro* (Figure 2.9). It does not form condensates on microtubules but binds the whole lattice.

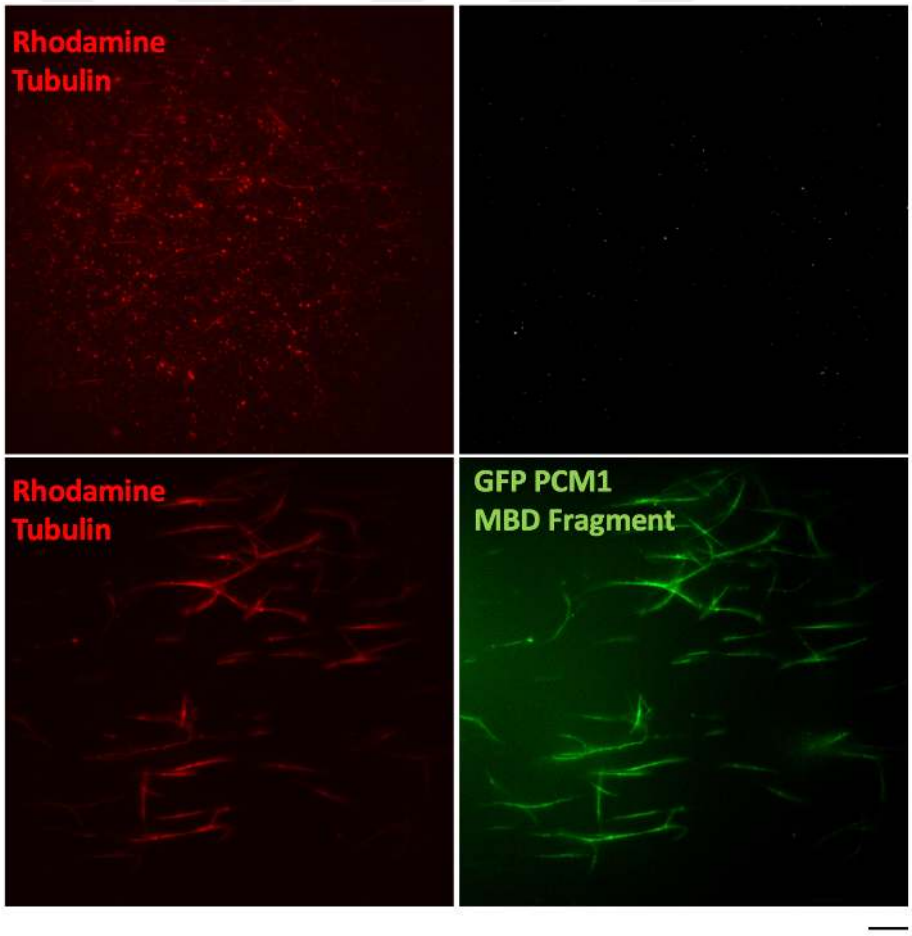


Figure 2.10. GFP PCM1 MBD Fragment binds and bundles microtubules *in vitro*. TIRF imaging of Taxol stabilized rhodamine microtubules alone show individual filaments of microtubules while the addition of GFP PCM1 MBD Fragment creates

parallel and anti-parallel bundles of microtubules and shows colocalization. Scale bar 10 μM.

2.9 PCM1 MBD Fragment forms condensates upon depolymerization of microtubule network

After demonstrating the microtubule binding and bundling ability of PCM1 MBD Fragment, it was investigated whether its behavior in cell changes upon disruption of microtubule network as for the case of PCM1 WT, upon disruption of microtubule network, the distribution of centriolar satellite granules is affected and they become distributed across cytoplasm instead of their described localization clustering around the centrosome (Conkar *et al.*, 2019). U2OS PCM1 KO cells were transfected with GFP PCM1 MBD Fragment and the cells were treated with nocodazole and DMSO for control. The control case of DMSO treatment displays microtubule binding and bundling as previously described, while upon depolymerization of microtubules, GFP PCM1 MBD Fragment formed granules that are scattered around the cytoplasm. It can be concluded that PCM1 MBD Fragment binds microtubules due to its high affinity and does not undergo phase separation but, upon loss of intact microtubule network the released PCM1 MBD Fragment into the cytoplasm forms granules.

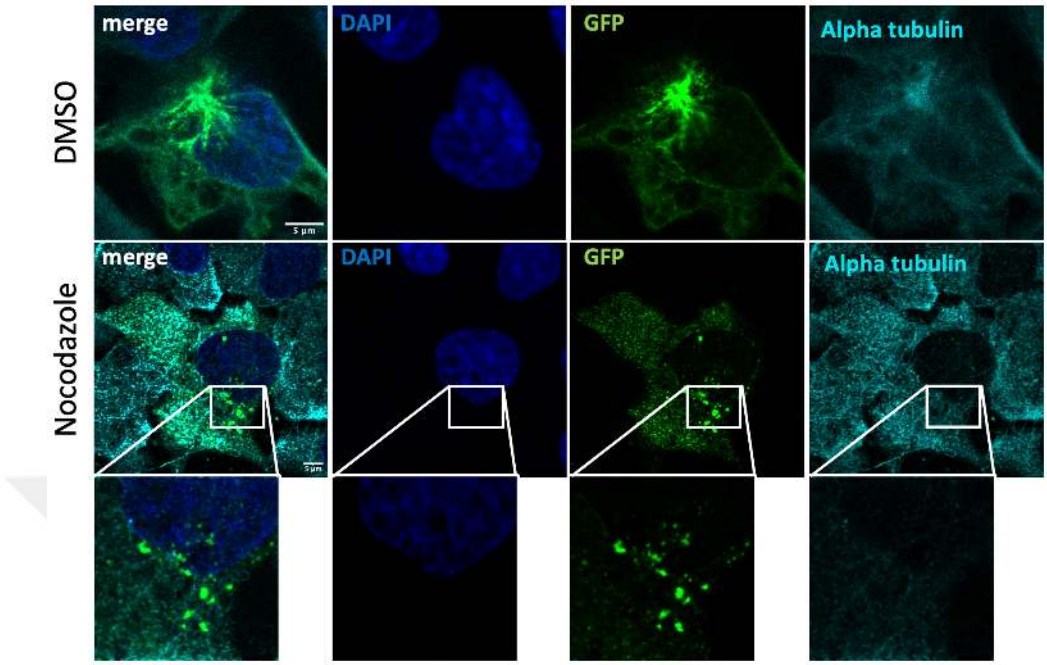


Figure 2.11. PCM1 MBD Fragment forms condensates upon disruption of microtubule network. The control case of DMSO (solvent of nocodazole; microtubule depolymerizing agent) shows colocalization of GFP PCM1 MBD Fragment with alpha tubulin staining as demonstrated previously in. Upon depolymerization of microtubules with nocodazole treatment, GFP PCM1 MBD Fragment that is released from the microtubules forms granular structures like centriolar satellites indicating potential of phase separation. Scale bar 10 μM.

2.10 PCM1 MBD Fragment phase separates *in vitro*

In vivo, PCM1 MBD Fragment binds microtubules as it favors due to its high affinity towards microtubules. Upon disruption of the network and the loss of microtubules, PCM1 MBD Fragment that gets released into cytoplasm forms granules which leads to the question of its potential of phase separation. As PCM1 FL have been described to undergo phase separation *in vitro* to form condensates and mesh-like scaffold network and PCM1 MBD Fragment showed a similar behavior to FL, we sought to dissect the *in vitro* behavior of PCM1 MBD Fragment in the context of how it behaves without microtubules. To do so, firstly the phase separation ability of His MBP PCM1 MBD Fragment was tested *in vitro* by utilizing poly-ethylene-glycol mediated crowding to replicate macromolecular crowding present in the cellular cytoplasm. It was observed

that His MBP PCM1 MBD Fragment underwent phase separation and formed condensates *in vitro* (Figure 2.11).

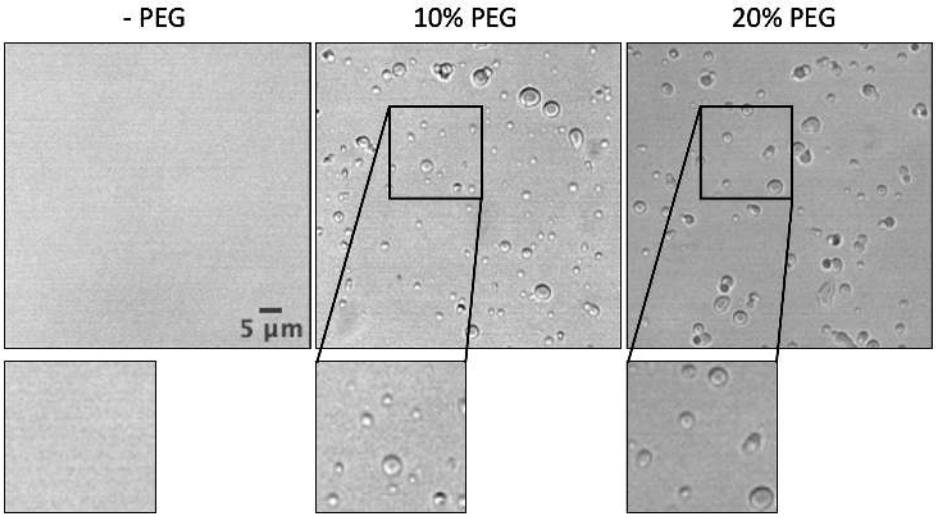


Figure 2.12. His MBP PCM1 MBD Fragment undergoes phase separation. Upon addition of polyethylene glycol (PEG) which acts as a crowding agent that mimics the biomolecular crowding present in cytoplasm, His MBP PCM1 MBD Fragment undergoes phase separation and forms spherical droplets. Scale bar 5 µM.

2.11 PCM1 MBD Fragment forms liquid-like condensates *in vitro*

After showing that His MBP PCM1 MBD Fragment undergoes phase separation to form condensates, the physical properties of the condensates in terms of liquid- or solid-like behavior were tested by *1,6-hexanediol* treatment. In the control case of DMSO, His MBP PCM1 MBD Fragment forms condensates approximately 1 µM in terms of area. Upon *1,6-hexanediol* treatment which dissolves condensates formed by liquid-liquid phase separation, it was observed that the condensates dissolved. It can be concluded that PCM1 MBD Fragment undergoes phase separation however, differently from the FL the formed condensates exhibit liquid behavior.

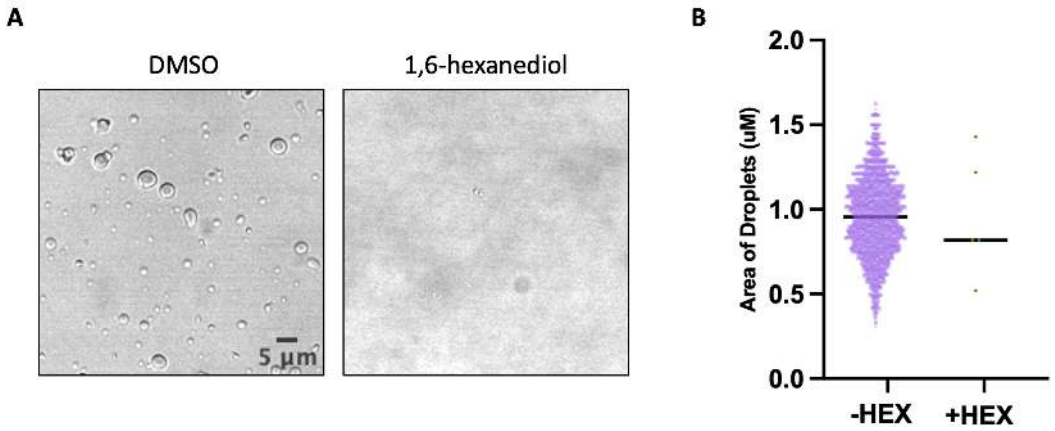


Figure 2.13. PCM1 MBD Fragment condensates dissolve upon *1,6-hexanediol* treatment and are liquid-like. **(A)**His MBP PCM1 MBD Fragment droplets that are formed upon molecular crowding of the protein dissolve upon addition of *1,6-hexanediol.* The phase separated condensates of His MBP PCM1 MBD Fragment dissolve. This shows that the condensates are liquid-like in nature and are undergoing liquid-liquid phase separation. **(B)** Quantification of area of droplets where each dot represents a single condensate. Scale bar 5 µM.

Since liquid-liquid phase separation of a protein is dependent on ionic interactions, change in environmental conditions in which the protein is in, can alter its phase separation. To assess the ionic charge state by which the PCM1 MBD Fragment can undergo phase separation, the salt concentration of the condensation assay buffer was manipulated to extreme conditions of low and high salt concentrations which can favor the folding of protein and its self-dimerization ability. His MBP PCM1 MBD Fragment was able to form condensates in extreme conditions like its behavior in LLPS buffer salt concentration of 150 mM NaCl (Figure 2.13A). In high salt concentration of 500 mM NaCl, the condensates also exhibited wetting ability which is an indicator of liquid-like behavior where the phase separated condensate wets the surface by which it is in contact with (Figure 2.13A).

In order to assess the physical state of condensates, *1,6-hexanediol* treatment was administered under the same salt concentration extremities. The condensates formed in low salt concentration of 25 mM NaCl acts exhibit solid-like behavior and do not dissolve indicating that low salt concentration favors phase separation of PCM1

MBD Fragment into gel or solid-like state. However, the condensates that are formed in higher salt concentrations (physiologically relevant and extremely high) exhibit liquid-like behavior and dissolution upon *1,6-hexanediol* treatment. The high salt concentration drives liquid phase separation of PCM1 MBD Fragment (Figure 2.13B).

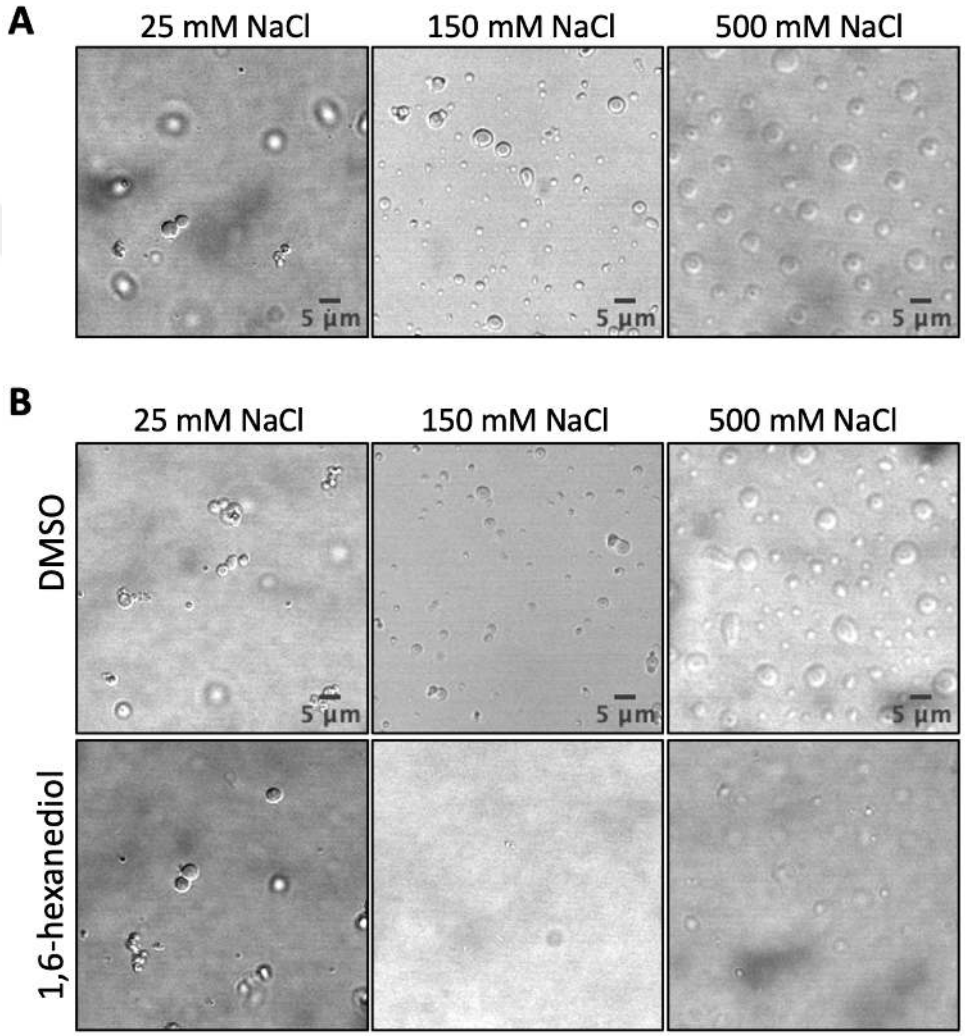


Figure 2.14. Phase separation behavior and material state of PCM1 MBD Fragment (**A)** In low salt concentration and high salt concentration His MBP PCM1 MBD Fragment forms condensates. In high salt concentration of 500 mM NaCl, the condensates that are formed also shows wetting behavior where they settle on the surface of the cover slide and spread over which is a key indication of liquid-like behavior. **(B)** The condensates that are formed at low salt concentration does not dissolve upon *1,6-hexanediol* treatment indicating that they are solid-like while the

condensates formed at 150 mM and 500 mM salt concentration dissolve which means they are liquid like. Scale bar 5 μM.

In comparison to solid condensates and mesh-like scaffold network of PCM1 FL, PCM1 MBD Fragment exhibits liquid-like behavior. The solid behavior of condensates in low salt concentration depends on the solubility of the protein and regulation of phase separation by multivalency.

CHAPTER 3: DISCUSSION

Membrane-less organelles play critical roles during development and physiology and their deregulation leads to various human diseases including neurodegenerative and developmental disorders. Therefore, it is essential to understand how these organelles assemble with a specific composition and structure without having a membrane around them. A membrane-less organelle that has come to spotlight in the recent years is centriolar satellites, which localize and move around centrosomes in most animal cells. Centriolar satellites were recently described as trafficking machines that assemble, modify, store and traffic their residents and regulate a diverse array of functions such as primary cilium assembly, Hedgehog signaling, ciliary motility, autophagy and proteostasis. Centriolar satellites are scaffolded by pericentriolar material 1 (PCM1) protein, which interacts over 200 proteins including the ones mutated in ciliopathies, primary microcephaly and schizophrenia. Depletion and deletion of PCM1 in cells and organisms leads to loss of centriolar satellites. Mouse knockout of PCM1 exhibited symptoms similar to the ones observed in ciliopathies and schizophrenia, which highlights their critical roles during development and physiology (Zoubovsky *et al.*, 2015; Monroe *et al.*, 2020). Although centriolar satellite functions and molecular mechanism of action are evident, little is known about how they are assembled and maintained. To address this question, I dissected the biophysical the biophysical properties of PCM1, which identified phase separation as mechanism by which PCM1 scaffold forms. Moreover, deletion analysis of PCM1 identified the microtubule-binding domain of PCM1, which provides insight into the microtubule-

based behavior of centriolar satellites as well as the mechanism by which centriolar satellites assemble.

Another membrane-less structure that shares almost 40% of the centriolar satellite proteome is the pericentriolar material (PCM) of the centrosome, which gives leads to further dissection of centriolar satellite biogenesis. PCM is a spherical compartment that sequesters tubulin heterodimers and mediates microtubule nucleation and organization. How it sequesters tubulin heterodimers have been studied through the scaffold protein of C. elegans PCM, which is SPD-5. Core scaffold proteins of PCM; pericentrin, SPD-5 are phosphorylated by polo kinase and mutations of phosphorylation sites by polo kinase impairs assembly of PCM suggesting that the phosphorylation of pericentrin and SPD-5 is necessary (Woodruff *et al.*, 2014). Additionally, Aurora A phosphorylation regulates recruitment of γ-tubulin and ZYG-9 thus, regulates recruitment of microtubule nucleators and microtubule binding protein. Independently, inhibition of Aurora A does not exhibit any effect on localization of core PCM components such as SPD-5 (Hannak, 2001). Upon these findings, it can be concluded that the initial assembly of PCM is driven by PLK-1 whereas Aurora A phosphorylation regulates downstream components that render PCM functional in terms of microtubule nucleation. Later, *In vitro* studies of SPD-5 demonstrated that it phase separates into spherical condensates that mimic PCM of cells in terms of morphology and dynamicity. Strikingly, co-condensation of SPD-5 with the microtubule polymerase ZYG-9 and microtubule stabilizer TPXL-1 recapitulated *in vivo* microtubule nucleation ability of PCM. After establishment of an assay for SPD-5 scaffold formation, addition of different PCM components to the scaffold reaction uncovered the molecular mechanisms by which PCM nucleates microtubules and led to in vitro reconstitution of microtubule nucleation at the PCM. Motivated by this study, it is essential in the future to add different centriolar satellite proteins to the PCM1 scaffold in vitro to determine how they regulate the scaffold properties such as material state, size and dynamic behavior. Although RNA has yet not been defined in centriolar satellites, RNA plays a major role in phase separation of many membrane-less organelles from the cytoplasm, which include stress granules and P bodies (Gao *et*

al., 2021). Future studies that aim to determine whether and if so how RNA contributes to phase separation of PCM1 in vitro and in cells will address this outstanding question.

A characteristic of a group of membrane-less organelles including the centriolar satellites and stress granules is that they dissolve as cells undergo mitosis. Strikingly, DYRK3 was identified as the major dissolves that mediates dissolution of these organelles. Inhibition of DYRK3 activity leads to inhibition of their dissolution and impaired mitotic progression, suggesting that dissolution of centriolar satellites and other membrane-less organelles during mitosis is required for proper mitotic progression and chromosome segregation. DYRK-3 inhibition in mitotic cells leads excessive condensation of PCM1 while the opposite effect of PCM1 dissolution is observed upon overexpression of DYRK3 WT but not the kinase dead DYRK-3 suggesting DYRK-3 to be a potential candidate for regulation of PCM1 material state and phase separation (Rai *et al.*, 2018). This study also indicates that phosphorylation contributes to the condensation behavior of centriolar satellites. Given the critical role of PTMs in general in regulation of protein behavior, it is important in the future to address the role of phosphorylation and other PTMs in regulation of biophysical properties of PCM1 in vitro and in cells.

Identification of the microtubule-binding domain of PCM1 highlights the importance of characterizing the in vitro and cellular behavior of deletion mutants of scaffolding proteins such as PCM1 as microtubule associated proteins have been demonstrated to undergo phase separation with the contribution of their interaction with microtubule lattice. Phase separation of tau is regulated by microtubule lattice which gatekeeps tau condensation by regulating self-association leading to formation of dynamic condensates. Condensates formed along the microtubule lattice act as a selective barrier that regulate availability of lattice to microtubule severing enzymes and molecular motor movement highlighting the mechanism of microtubule lattice dependent phase separation (Hinrichs *et al.*, 2012).

In preliminary studies, we found that N-terminal fragment (1-1200 aa) of PCM1 that contains the MBD fragment form granules in cells, which its C-terminal (1200-2024) region is cytosolic. Strikingly, the granules formed by N-terminal PCM1 were much bigger and heterogenous than the ones formed by full length PCM1. These results

suggest that N-terminal region of PCM1 is required for assembly of granules while its C-terminus regulates size of centriolar satellites. In future studies, I aim to develop in vitro and cellular assays for different PCM1 fragments in order to gain a better understanding of how different regions of PCM1 cooperate during the assembly of the centriolar satellite scaffold.

CHAPTER 4: MATERIALS AND METHODS

4.1 Cell culture, transfection

Human embryonic kidney (HEK293T) cells were cultured with DMEM medium (Pan Biotech) supplemented with 10% FBS and 1% penicillin-streptomycin. All cell lines were tested for mycoplasma by MycoAlert Mycoplasma Detection Kit (Lonza). HEK293T cells were transfected with the plasmids by 1 µg/µl polyethylenimine, MW 25 kDa (PEI, Sigma-Aldrich, St. Louis, MO). Microtubule depolymerization experiments, were performed by treating cells with 5 µg/ml nocodazole (Sigma-Aldrich) for 1 hour at 37°C.

4.2 Immunofluorescence, antibodies and microscopy

Cells were grown on coverslips, washed twice with PBS and fixed in either ice cold methanol at -20°C for 10 minutes. After rehydration with PBS, cells were blocked with 3% BSA (Capricorn Scientific, Cat. # BSA-1T) in PBS + 0.1% Triton X-100. Then the cells were incubated with primary antibodies in blocking solution for 1 hour at room temperature. Cells were washed with PBS three times and incubated with secondary antibodies at 1:2000 and DAPI (Thermo Scientific, cat#D1306) at 1:5000 for 1 hour at room temperature. Followeed by three PBS washes, cells were mounted using Mowiol mounting medium containing N-propyl gallate (Sigma-Aldrich).

High resolution images were taken by using HC PL APO CS2 63x 1.4 NA oil objective and HC PL APO CS2 63x 1.4 NA water objective with Leica SP8 confocal microscope.

4.3 Immunoblotting

For immunoblotting, equal quantities of cell extracts were resolved on SDS-PAGE gels, transferred onto nitrocellulose membranes, blocked with TBST in 5% milk for 1 hour at room temperature. Blots were incubated with primary antibodies diluted in 5% BSA in TBS-T and sodium azide overnight at 4°C, washed three times with TBST for 5 minute cyccles and blotted with secondary antibodies for 1 hour at room temperature. After washing blots with TBS-T three times for 10 minutes, they were visualized with the LI-COR Odyssey® Infrared Imaging System and software at 169 µm (LI-COR Biosciences).

4.4 *In vitro* microtubule sedimentation assay

For sedimentation assays performed with ex vivo cell lysates, HEK293T cells transfected with GFP, GFP PCM1 FL and GFP PCM1 ΔMBD. The cells were lysed in in BRB80 buffer (80 mM PIPES pH 6.8, 1 mM EGTA, 1m M MgCl2) supplemented with protease inhibitors (10 µg/ml LPC, 1 mM PMSF and 10 µg/ml aprotinin) at 4°C for 30 minutes and centrifuged at 13.000 R.P.M. for 15 minutes. Lysate was further cleared by centrifugation at 90.000 R.P.M. for 5 minutes at 4°C with TLA100 rotor (Beckman). 100 µg cell lysate was supplementd with 1 mM GTP. Lysate was incubated with 25 µM taxol stepwise at 30°C for 25 minutes according to Mitchison Lab tubulin guide protocols. Lysate was centrifuged at 55.000 R.P.M. for 10 minutes at 30°C in a TLA100 rotor (Beckman) through a 80 ul 40% glycerol cushion prepared from BRB90 buffer supplemented with 1 mM GTP. Pellets were resuspended in the same final volume as the input and equivalent volumes of pellet and supernatant were separated by SDS-PAGE and processed by immunoblotting.

For sedimentations assays performed with purified proteins, pFastBac His MBP PCM1 FL bacmid was transfected to Sf9 cells seeded at 1 million cells the day prior to transfection with FuGENE (Promega, Cat. No: E5911) according to users guide in 6-well plate for making of the P0 virus. The P0 virus was used for infection of Hi5 cells in T75 adherent culture and the virus was expanded to until P3 with infection of 50 ml suspension cultures of Hi5. The His MBP PCM1 FL was purified from the large-scale infections of Sf9 and Hi5 cells. The cells were lysed by sonication in lysis buffer (50 mM NaH2PO4, 500 mM KCl, 10 nM Imidazole, 1% Trition-X, 0.5 mM Arginine, 0.33 mM CHAPS, 10% glycerol, 10 µg/ml LPC, 1 mM PMSF and 10 µg/ml aprotinin and 1 mM 2-mercaptoethanol; pH = 7.5) and proteins were purified by Ni-NTA Agarose beads (QIAGEN, Cat. No: 30210), washed in wash buffer (50 mM NaH2PO4, 500 mM KCl, 30 mM Imidazole; pH = 7.5) and eluted with elution buffer (50 mM NaH2PO4, 500 mM KCl, 300 mM Imidazole; pH = 7.5). The eluted protein was exchanged to storage buffer depleted of Imidazole and concentrated on columns (MERCK, Amicon Pro Purification). For purification of His MBP and His MBP PCM1 MBD Fragment, the proteins were expressed in Rosetta cells at 30 °C for 18 h. Bacteria expressing MBP-fusion proteins was lysed by sonication with 1mg/ml lysozyme in lysis buffer (50 mM NaH2PO4, 500 mM KCl, 10 nM Imidazole, 1% Trition-X, 0.5 mM Arginine, 0.33 mM CHAPS, 10% glycerol, 10 µg/ml LPC, 1 mM PMSF and 10 µg/ml aprotinin and 1 mM 2-mercaptoethanol; pH = 7.5) and proteins were purified by Ni-NTA Agarose beads (QIAGEN, Cat. No: 30210), washed in wash buffer (50 mM NaH2PO4, 500 mM KCl, 30 mM Imidazole; pH = 7.5) and eluted with elution buffer (50 mM NaH2PO4, 500 mM KCl, 300 mM Imidazole; pH = 7.5). The eluted protein was exchanged to storage buffer depleted of Imidazole and concentrated on columns (MERCK, Amicon Pro Purification). Purified bovine brain tubulin and purified proteins were precleared at 90.000 R.P.M. for 5 minutes at 4°C with TLA100 rotor. Cleared tubulin was polymerized by first incubating with 20 µM taxol stepwise at 37°C for 30 minutes. 0.75 nmol of purified protein was brought to final taxol concentration of 20 µM, mixed 1:1 with polymerized tubulin and incubated at 30°C for 30 minutes. Samples were loaded onto 40% glycerol BRB80 cushions and centrifuged at 55.000 rpm for 10 min with TLA100 rotor. Equivalent volumes of supernatant and pellet fractions were resolved by SDS-PAGE.

4.5 *In vitro* microtubule bundling assay

Fluorescent MTs were polymerized at 2 mg/ml by incubating tubulin (bovine) and rhodamine labeled tubulin (Cytoskeleton Inc.) at 10:1 ratio in BRB80 with 1 mM DTT and 1mM GTP for 5 min on ice, then preclearing by centrifuging for 10 min at 90000 rpm at 2°C. Cleared tubulin mixture was polymerized at 37°C by adding taxol and increasing concentration stepwise, with final concentration of 20 µM according to Mitchison Lab tubulin guide protocols. Microtubules were pelleted over warm 40% glycerol BRB80 cushion at 30 °C in TLA100 rotor at 70000 rpm for 20 min. The pellet was resuspended in 80% of the starting volume of warm BRB80 buffer with 1mM DTT and 20 µM Taxol. In short, 100 nM His-MBP-PCM1 FL or His MBP PCM1 MBD Fragment or His MBP protein was mixed with 2 µM MTs and 20 µM taxol in BRB80 buffer (80 mM PIPES pH 6.8, 1 mM EGTA, 1m M MgCl2) for 30 min rocking at room temperature. The reaction mixtures were transferred into a flow chamber under a HCl-treated coverslip. Bundling of fluorescent MTs was observed with Zeiss Elyra 7 with lattice structured illumination SIM microscope with alpha Plan-Apochromat 100x / 1.46 oil TIRF objective.

4.6 *In vitro* condensation assay

His MBP PCM1 FL and His MBP PCM1 MBD Fragment were thawed on ice and precleared at 90.000 RPM for 5 minutes at 4°C with TLA100. The proteins were incubated in LLPS buffer (10 mM HEPES, 150 mM NaCl, 0.1 mM EDTA, 2 mM DTT, 10% PEG) for 30 minutes at room temperature to induce phase separation. The reaction mixtures were flown into a flow chamber under a HCl-treated coverslip.

4.7 *In vitro* condensation assay treatments

After incubation of His MBP PCM1 FL and His MBP PCM1 MBD Fragment in LLPS buffer for induction of phase separation, 1,6-hexanediol (ChemCruz, Lot#B2519) was dissolved in the LLPS buffer at 5% concentration for 10 minutes and the reaction mixtures were flown into a flow chamber under a HCl-treated coverslip.

The TEV protease is stored at 2 mg/ml concentration and is diluted to working concentration of 1 unit of TEV protease for 100 units of protein to cleave weight wise, incubated overnight at 4°C. The reaction mixtures were flown into the flow chamber under a HCl-treated coverslip.

4.8 Statistical tests

No statistical method was used to estimate sample size. Statistical results, average and standard deviation values were calculated and represented as graphs using Prism 7. Comparison of two groups was done using a two-sided Student's t-test. The comparisons of more than two groups were performed using one- or two-way ANOVAs. n indicates the number of experimental replicates for each sample and condition. Two-tailed t-tests were applied to compare measurements. The graphs with error bars indicate Standard Deviation (SD) and the significance level were denoted as *P < 0.05, **P < 0.01, ***P < 0.001, **** P < 0.0001.

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