Elucidation of the mechanisms that underlie centriolar satellite biogenesis and dynamics

by

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Koç University

Graduate School of Sciences and Engineering

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Date: 11. August. 2022

My mum and dad;
Couldn't done this without your genetic material and inspiration.
Couldn't done this without your support and patience.
Couldn't done this without your love.

ABSTRACT

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Dila Gülensoy
Master of Science in Molecular Biology and Genetics
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Centriolar satellites are the third novel component of the mammalian centrosome/cilium complex. They are membrane-less electron-dense granules clustered around the main microtubule organizing center. These granules are heterogeneous in terms of composition, size, and shape and have a variety of roles in different cellular processes such as cilium assembly/disassembly, centriole duplication, stress response, and autophagy. Therefore understanding the centriolar satellite composition, dynamics and behavior are essential to elucidate their implications for health and diseases such as cancer and ciliopathies. The main scaffolding protein of the centriolar satellites is Pericentriolar Material 1 (PCM1), which maintains the satellite integrity since depletion of PCM1 leads to loss of centriolar satellites. Therefore understanding the biochemical and biophysical properties of the PCM1 is an essential first step to uncover the biochemistry of centriolar satellites. I found that PCM1 is a highly disordered protein, suggesting that it might undergo liquid-liquid phase separation (LLPS) to form centriolar satellite granules. Even though they are spherical and dynamic, which aligns with the membrane-less granule properties that go under the LLPS, mature satellite granules are in a gel-like state or become gelified over time. The global RNA depletion leads to the dispersal of the PCM1 granules showing that they have a stable RNA core that helps with the granule integrity. Structure-function analysis of PCM1 showed that three different fragments within the PCM1 N-terminus form granules, whereas the fragments from the C-terminal act cytoplasmic. This result shows that N-terminal has a role in the granule formation, whereas the C-terminal is more likely to have a regulatory role. The PCM1 fragments that form granules exhibit dynamic events including fusion, fission, kiss, and split events. Lastly, the de novo PCM1 disassembly experiment shows that with decreasing protein concentration, the granules are broken into smaller granules until no visible granule is seen. Our findings show that PCM1 goes under the LLPS and shows liquid-like behavior however there is an equilibrium between both the liquid and gel-like state.

ÖZETÇE

Sentriyolar Satelit Biyojenez ve Dinamik Davranışlarında Rol Alan Mekanizmaların Belirlenmesi
Dila Gülensoy
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Sentriyol satelitler memelilerde sentrozom/silyum kompleksinin üçüncü yeni özgün bileşenidir. Membranı olmayan bu elektron-yoğun yapılar hücrelerde mikrotübül düzenleme merkezlerinde kümelenmiş şekilde dururlar. Bu granüller kompozisyon, büyüklük ve şekil açısından heterojen olup; silyum morfogenezi, sentriyol duplikasyonu, stres yolağı ve otofaji gibi hücresel süreçlerde rol almaktadırlar. Bu yüzden sentriyol satelitlerin kompozisyon, dinamik ve davranışlarının anlaşılması, sağlık açısından işlevinin gözlenmesine, hatta kanser, siliopati gibi hastalıklarda olası rollerinin ne olacağının açıklanmasına yardımcı olacaktır. Sentriyol satelitlerin ana iskelet proteinin perisentriyol materyal bir (PCM1) olduğu ve yapısal bütünlüğünün korumasına yardımcı olduğu, tükenmesi durumunda satelitlerin yol olmasıyla kanıtlanmıştır. PCM1'in biyokimyasal ve biyofiziksel özelliklerinin anlaşılması, sentriyol satelitlerin biyokimyasal özelliklerinin açığa çıkarılması için yapılması gereken ilk ve elzem bir adımdır.PCM1'in içsel olarak düzensiz bir protein olduğunu ve likit-likit faz ayrışımı sayesinde sentriyol satelit granülleri oluşturabileceğini gösterdim.Küresel ve dinamik formda olmaları likit-likit faz ayrışımı ile oluşan membranı olmayan granüllerin özellikleri ile uyuşsa da, olgunlaşmış satelit granülleri ya jelimsi bir yapıda yada zamanla jelleşmektedir. Hücre içi RNA tüketme deneylerinin PCM1 granüllerinin dağılmasına sebep olması, PCM1'ın, satelitlerin yapısal bütünlüğünü korumaya yardımcı olan RNA özlü ve kararlı bir yapısı olduğunu göstermektedir. PCM1'in yapı-işlev analizleri proteinin, N-terminalinde bulunan üç farklı bölümünün granül oluşturabilirken, C-terminalinin çoğunlukla sitoplazmik davrandığını göstermiştir. Sonuçlar proteinin N-terminalinin granül oluşturmakla görevli olduğunu gösterirken, C-terminalinin ise daha düzenleyici bir rolü olduğunu göstermektedir. Granül oluşturan PCM1 bölümleri füzyon, fisyon ve birleşip-ayrılma gibi dinamik olayları sergiler. Son olarak, PCM1 üzerinden protein konsantrasyonunu zamanla azaltarak yaptığımız yenilikçi ayrıştırma deneyleri; protein granüllerinin küçülerek görünür olmayan granül kalana kadar parçalandığını gösterdi. Elde ettiğimiz bulgulara göre, PCM1, likit-likit faz ayrışımı altında likite benzer davranışlar gösterse de, hem likit hem de jel halinin arasında denge olduğu gözlenmiştir.

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ABBREVIATIONS

a.a.	Aminoacid
AID	Auxin Inducible Degron
ANA 1	Anastral Spindle 1
APC/C	Anaphase-promoting complex
APS	Ammonium persulfate
BBS	Bardet-Biedl Syndrome
BSA	Bovine Serum Albumin
C-ter	C Terminus
C2cd3	C2 Domain Containing 3 Centriole Elongation Regulator
Cas	CRISPR-associated
CC	Coiled-coil domain
Cep 152	Centrosomal Protein 152
CEP135	Centrosomal Protein 135
Cep164	Centrosomal Protein 164
CEP170	Centrosomal Protein 170
CEP192	Centrosomal Protein 192
CEP295	Centrosomal Protein 295
CEP63	Centrosomal Protein 63
CEP83	Centrosomal Protein 83
Cep89	Centrosomal Protein 89
CEP97	Centrosomal Protein 97
CP110	centriolar coiled-coil protein 110
CPAP	centrosomal P4.1-associated protein
CRISPR	clustered regularly interspaced short palindromic repeats
DAPI	4’,6-diaminodino-2-phenylindole
DDX4	DEAD-box helicase 4
DLD-1	Human colorectal adenocarcinoma cell line
DMEM	Dulbecco’s Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
DTT	1,4-Dithiothreitol
DYRK3	Dual specificity tyrosine-phosphorylation-regulated kinase 3
EB1	End Binding Protein 1
FBF 1	Fas Binding Factor 1
FBS	Fetal Bovine Serum
FL	Full length
FRAP	Fluorescence Recovery After Photobleaching
FUS	FUS RNA Binding Protein
G0	Gap 0
G1	Gap 1
G2	Gap 2

gRNA	guide RNA
HnRNAP1	Heterogeneous nuclear ribonucleoprotein
hPOC5	human POC5
Hsp	Heat-Shock Protein
IDP	Intrinsically Disordered Proteins
IDR	Intrinsically Disordered Regions
IFT	Intraflageller Transport
IFT	Intraflageller Transport
kDa	Kilo-dalton
Kif 3a	Kinesin Family Member 3a
LLPS	Liquid-Liquid Phase Separation
LPC	Leupeptin, Pepstatin, Chymostatin
M	Mitosis
MAP	Microtubule Associated Proteins
mRNA	messenger RNA
MT	Microtubule
MTOC	Microtubule Organizing Center
N-ter	N Terminus
N-WASP	Neural Wiskott-Aldrich syndrome protein
NCK	non-catalytic region of tyrosine kinase
ncRNA	non-coding RNA
NICD	Notch intracellular domain
ODF2	Outer dense fibrin protein 2
OFD1	Oral-facial-digital syndrome 1 protein
PAI-1	Plasminogen inhibitor activator protein
PAR	Poly- ADP Ribose
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline, 0.1% Tween®
PCM	Pericentriolar material
PCM1	Pericentriolar Material 1
PEI	Polyethylenimine
PLK4	Polo-like kinase 4
PML Bodies	Promyelocytic Leukemia Protein Bodies
PrLD	Prion-like Domain
PRM	Proline-rich motif
PTB	Polyprimidine tract binding protein
PTM	Post-translational modifications
RBPs	RNA Binding Proteins
RNA	Ribonucleic Acid
RNAse A	Ribonuclease A
ROI	Region of Interest
RPM	Revolutions Per Minute

RRM	RNA Recognition Motif
RT	Room Temperature
S	Synthesis
SCF	Skip, Cullin, F-Box Containing Complex
SCLT1	Sodium Channel And Clathrin Linker 1
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
SPD-2	Spindle Defective Protein -2
SPICE 1	Spindle and centriole Associate protein 1
STIL	STIL Centriolar Assembly Protein
TBS	Tris-Buffered Saline
TBST	Tris-Buffered Saline, 0.1% Tween®
TDP 43	TAR DNA-binding protein
TEMED	Tetramethylethylendiamine
TIR 1	Transport-inhibitor Response 1
U-ExM	Ultrastructure Expansion Microscopy
U2OS	Human osteosarcoma cells
WT	Wild-type

CHAPTER 1: INTRODUCTION

1. The Mammalian Centrosome/Cilium Complex

The mammalian centrosome/cilium complex comprises the centrosome, the primary cilium, and its third novel component, centriolar satellites. The highly regulated and dynamic communication between these structures is required for this complex's proper assembly and functioning (Arslanhan et al., 2020; Breslow & Holland, 2019; Odabasi et al., 2020; Prosser & Pelletier, 2020).

1.1 The Centrosome

Centrosomes serve as the main microtubule-organizing center (MTOC) in animal cells and have essential roles in cellular processes such as cell motility, adhesion, polarity, and division. They are membrane-less organelles formed by two orthogonally positioned centrioles and the surrounding electron-rich matrix named pericentriolar material (PCM). The centriole is a microtubule (MT)-based and barrel-shaped structure, which is evolutionary conserved with nine-fold symmetry (Breslow & Holland, 2019; Odabasi et al., 2020; Prosser & Pelletier, 2020). Nine connected triplet microtubules establish the nine-fold symmetry. Triplet MTs contain 13 protofilament A-tubule and 10 protofilament B- and C-tubules. Each nine triplets is connected with A-C linkers between the neighboring A and C-tubules (Wang & Stearns, 2017). These cylindrical structures are ~230 nm in diameter and ~420 nm in length, polarized along the proximal-distal axis for distinct functions (Breslow & Holland, 2019). The proximal end spans ~200 nm of the centriole, and the rest is the distal end. The distal end not only differs in length compared to the proximal end but also has a distinct type of an A-C linker, incomplete C-tubule in size, and narrow diameter (Wang & Stearns, 2017). (Figure 1.1)

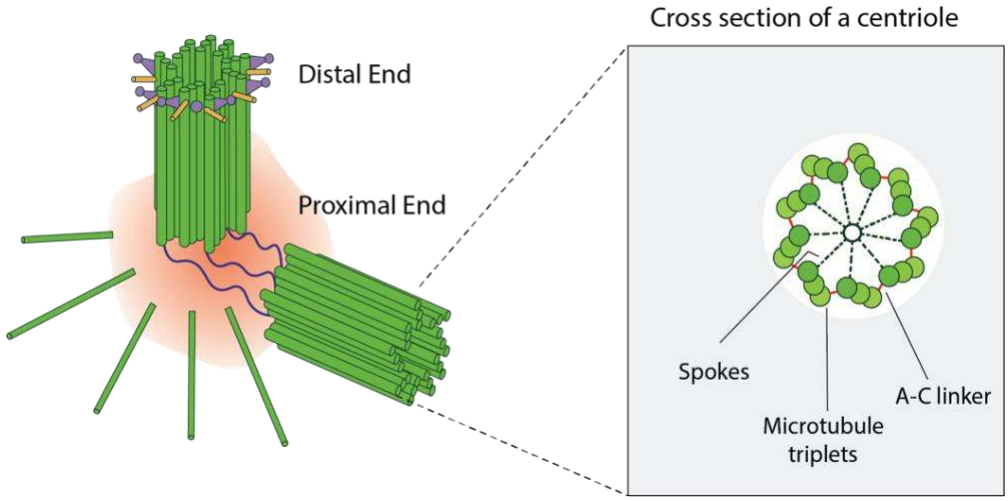

Figure 1.1: The structure of the centriole in different cell cycle stages. Centrosome structure in interphase cells consists of two centrioles (distal appendages of the mother centriole are shown in purple and yellow spikes) and surrounding pericentriolar material (orange) (Adapted from (Arslanhan et al., 2020).

Centrioles inherently have distal-proximity polarity due to the MT scaffold. While the minus ends of the MTs define the proximal end of the centriole, the plus ends to determine the distal end (Azimzadeh & Marshall, 2010; Loncarek & Bettencourt-Dias, 2018). In interphase cells, the centrioles that form the centrosome are tethered together from their distal ends with a loose proteinaceous tether called the "G1-G2" tether (Agircan et al., 2014; Giet et al., 1999; Nigg & Stearns, 2011). During this phase, the centrosome functions as the only MTOC since the cell possesses one centrosome composed of two centrioles (Bettencourt-Dias & Glover, 2007). Centrosomes are also duplicated only once per cell cycle when the cell enters mitosis, just like DNA. The centriole duplication, which controls the centrosome duplication, is initiated at the interphase. After the loss of orthogonal confirmation, the procentriole assembly starts near the adjacent centriole to which it is connected with a linker named the "S-M" linker (Nigg & Stearns, 2011).

Polo-like kinase 4 (PLK4), the master driver of the centriole duplication, is a serine/threonine kinase and the earliest marker site for the procentriole assembly (Bettencourt-Dias et al., 2005; Habedanck et al., 2005; Sonnen et al., 2012). Initial recruitment of the PLK4 to the centrioles in the vertebrates occurs during the G1 phase by CEP63, CEP152 (Asterless in Drosophila), and CEP192 (SPD-2 in C. elegans)(Cizmecioglu et al., 2010; Hatch et al., 2010; Kim et al., 2013; Park et al., 2014; Sonnen

et al., 2013). They specifically encircle the proximal end of the mother centriole. Even though PLK4 receptors are localized around the mother centriole as ring throughout the cell cycle; upon binding to STIL, its localization turns into a single dot at the G1-S transition (Dzhindzhev et al., 2017; Kim et al., 2013; Ohta et al., 2014; Sonnen et al., 2012).STIL is one of the critical binding partners of the PLK4, and the PLK4 phosphorylates it in multiple sites. The phosphorylation of STIL allows the binding to SAS-6, which initiates the centriole formation by the cartwheel assembly. The cartwheel is the centriole's structural foundation that institutes the centriole's nine-fold symmetry (Keller et al., 2014; Kitagawa et al., 2011; Leidel et al., 2005; Nakazawa et al., 2007; van Breugel et al., 2011). SAS-6 is a critical component in this structure since SAS-6 binding to the CEP135 (Bld10 in Drosophila) facilitates the homodimerization and oligomerization of the SAS-6 and establishes the nine-fold symmetry (Cottee et al., 2015; Guichard et al., 2017; Kitagawa et al., 2011; van Breugel et al., 2011; van Breugel et al., 2014). The SAS-6 molecules are added to the cartwheel for centriole elongation with a rate of growth set by PLK4 activity.

Following the cartwheel assembly, the procentrioles get elongated throughout the S and G2 phases into their full length. The final length they reach varies according to the cell type and species (Figure 1.2). In vertebrates, the centriole MT triplets extend approximately 300 nm. Compared to the cytosolic MTs, the growth rate of centriole MTs are much slower; however, they are much more stable (Kochanski & Borisy, 1990). Recent studies have shown that specialized proteins, known as microtubule-associated proteins (MAPs), regulate the centrioles' low but steady growth (Sharma et al., 2016; Zheng et al., 2016). One of the essential players in the centriole length determination is CPAP which associates directly with centriole tubules through its N terminus. CPAP controls the growth and stabilization of the elongating centriole (Sharma et al., 2016; Zheng et al., 2016). Other proteins take a positive role during centriole elongation with unknown mechanisms, such as POC1(Keller et al., 2009), hPOC5 (Azimzadeh et al., 2009), Asterless (CEP152 in humans) (Galletta et al., 2016), CEP295 (Anal in Drosophila) (Chang et al., 2016; Saurya et al., 2016), Centrobin (Gudi et al., 2015) and SPICE1 (Comartin et al., 2013). The distal end of the centriole is capped by CP110 and CEP97, which restricts centriole elongation in mammalian cells (Franz et al., 2013; Schmidt et al., 2009; Spektor et al., 2007).

With the dissolution of the S-M linker and acquiring the PCM, they disengage from the parental centrioles, a phenomenon called a centriole-to-centrosome conversion (Bettencourt-Dias & Glover, 2007). The centriole maturation is completed with the acquisition of appendages to both distal and subdistal parts of the older centriole. The duplicated centrosomes are separated during the G2/M transition and form the bipolar spindle with the loss of the "G1-G2 tether" by phosphorylation (Mardin & Schiebel, 2012). The subdistal appendage assembly is initiated by the recruitment of ODF2, followed by the recruitment of other proteins that are not characterized thoroughly (Huang et al., 2017; Ishikawa et al., 2005). hNinein and CEP170 proteins are also a part of subdistal appendages which bind to the MTs. Other proteins localize near the subdistal appendages, such as dynein–dynactin complex, EB1, and Kif3a (Huang et al., 2017), anchoring the MTs to the mother centrioles. The assembly of the distal appendages is initiated by the recruitment of the OFD1 and C2cd3. Their recruitment is followed by CEP83 recruitment, and CEP83 also continues recruiting other distal appendage components such as Cep89, SCLT1, FBF1, and Cep164 (Tanos et al., 2013) (Thauvin-Robinet et al., 2014) (Figure 1.2).

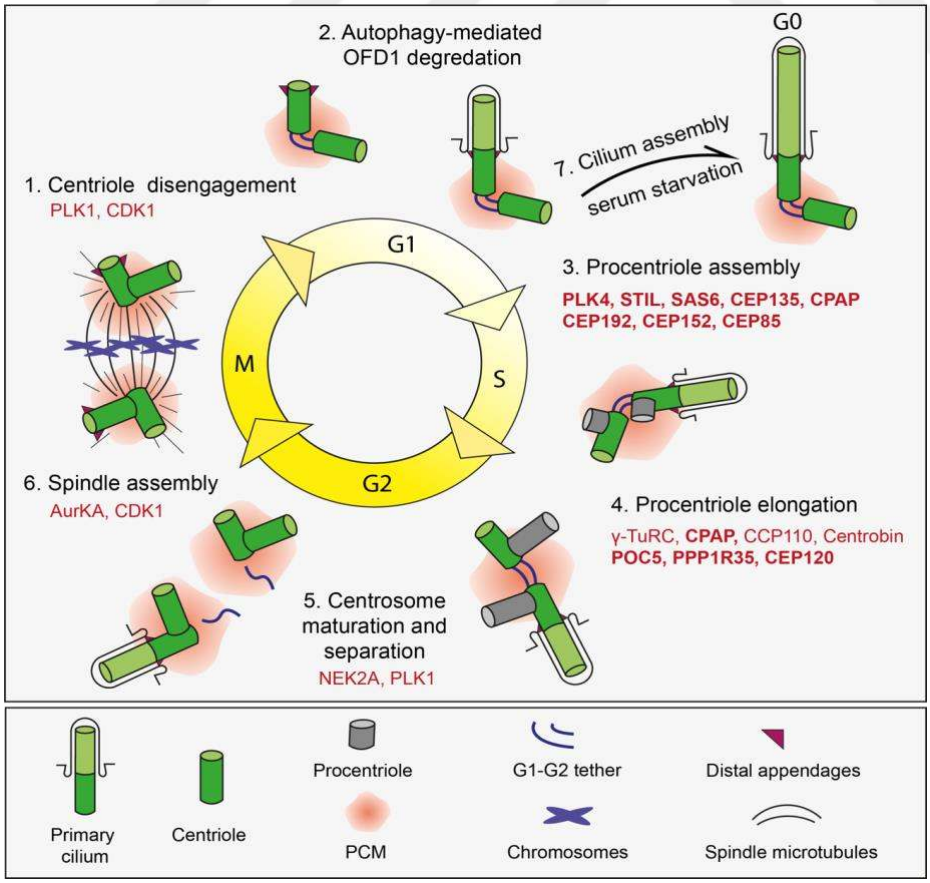

Figure 1.2: The regulation during the biogenesis of the centriole and cilium during the cell cycle. The formation of centrioles and cilia is a multi-step, highly regulated process closely correlated with the cell cycle. Most animal cells in the G1 phase contain a single centrosome of two centrioles joined at their proximal ends by the G1-G2 tether. The two centrioles only replicate once during the G1 and S phases, forming one procentriole next to each existing parental centriole. The sequential recruitment and activity of a conserved group of proteins, including the kinase PLK4, the scaffold STIL, and the component of the cartwheel SASS6, as well as regulators of these proteins, in the centriolar compartment, controls this process. Procentrioles develop during the S and G2 stages after centriole duplication begins. The G1-G2 tether dissolves in late G2, separating the two centrosomes. Fully elongated centrioles lose their cartwheel and recruit additional PCM material in a process known as centriole-to-centrosome conversion to become ready for bipolar spindle assembly. The bipolar spindle, which evenly distributes a pair of centrioles and genetic material to daughter cells, is put together by centrosomes during mitosis. During mitosis, distal appendages momentarily disassemble. The centriole pairs disengage and lose their orthogonal configuration after the conclusion of mitosis. (Adapted from (Arslanhan et al., 2020).

The events during the centriole duplication led to the centrioles being different in age and structure. The old centriole that facilitates the assembly of the procentriole is called the “mother” centriole, whereas the newly formed younger centriole is called the “daughter” centriole. The mother centriole is the centriole that acquires the distal and subdistal appendages during the centriole maturation. Different appendages participate in diverse cellular events. The distal appendages help the mature centrosome to be docked to the plasma membrane along with the formation of the basal body of the primary cilium during ciliogenesis. On the other hand, the subdistal appendages act as an anchor for a subset of cytoplasmic microtubules (Gupta & Kitagawa, 2018; Remo et al., 2020).

For a complete centrosome formation, PCM recruitment is needed. Initially, PCM had been considered an amorphous matrix of proteins; however, recent studies revealed that PCM is an organized structure (Baumann, 2012). The proximal end of the centrioles is responsible for the recruitment of the PCM, which shows microtubule-organizing center activity with the nucleation and anchoring of the MTs. PCM components are usually structured as concentric rings around the centrioles in interphase cells, with few

exceptions (Holland et al., 2010). PCM expands and recruits more components during mitosis that accumulate in a cloud-like matrix. The mitotic PCM is less structured than the interphase PCM (Fry et al., 2017).

1.2. Primary Cilium

Centrosomes have different functional roles in distinct cell cycle stages. As mentioned, centrosomes nucleate the microtubule cytoskeleton in interphase cells, forming the spindle poles during mitosis. However, in the quiescent cells, the mature centriole of the centrosome docks to the plasma membrane and forms the non-motile microtubule-based structure called the primary cilium (Mirvis et al., 2018; Pedersen et al., 2012)

The primary cilium has several functionally and structurally different regions with different roles (Figure 1.3). The most proximal part of the primary cilium is the ciliary gate which consists of transition fibers and a transition zone. The distal appendages of the mother centriole, which correspond to transition fibers, help with the docking process of the centrosome to the plasma membrane. The docked centrosome is referred to as the basal body, along with the transition fibers from the transition zone. The cilium content is regulated via the transition zone, and it functions as a diffusion barrier that controls the entry and exit of the ciliary proteins (Garcia-Gonzalo et al., 2011; Garcia-Gonzalo & Reiter, 2017). The outer doublets start forming from the transition zone, surpassing the primary cilium (Molla-Herman et al., 2010). The axonemal microtubules that elongate with the plasma membrane forms the ciliary axoneme. The ciliary axoneme provides the primary cilium with rigidity and flexibility. In addition, it also serves as a track for the trafficking of the ciliary proteins (Nachury, 2018; Nakayama & Katoh, 2018). The ciliary cargo is transported bi-directionally through the primary cilium with the intraflagellar transport (IFT) complexes. These complexes are essential for the assembly and the maintenance of the cilium; they traffic structural cargoes such as tubulin dimers and other types of proteins such as ciliary membrane receptors, which play a role in signaling (Lechtreck, 2015). Despite the IFT, different kinds of conserved proteins take a role in protein trafficking, such as the Bardet-Biedl syndrome (BBS) proteins (Ye et al., 2018). The axonemal MTs do not encompass the entire cilium; the nine radially arranged microtubule doublets turn into MT singlets towards the distal end of the cilium. The ciliary tip is the part between the distal end of axonemal MTs that turns into the singlets

and the ciliary membrane (Pedersen et al., 2012; Soares et al., 2019). The ciliary tip is an essential compartment of the primary cilium since it is critical in many processes such as IFT remodeling, cilium length regulation, and Hedgehog signaling (Soares et al., 2019).

Primary cilium acts as the cellular antenna since it is crucial for the signaling initiated by external stimuli (Berbari et al., 2009; Singla & Reiter, 2006). The structural and functional abnormalities in primary cilium cause a broad range of diseases known as ciliopathies.

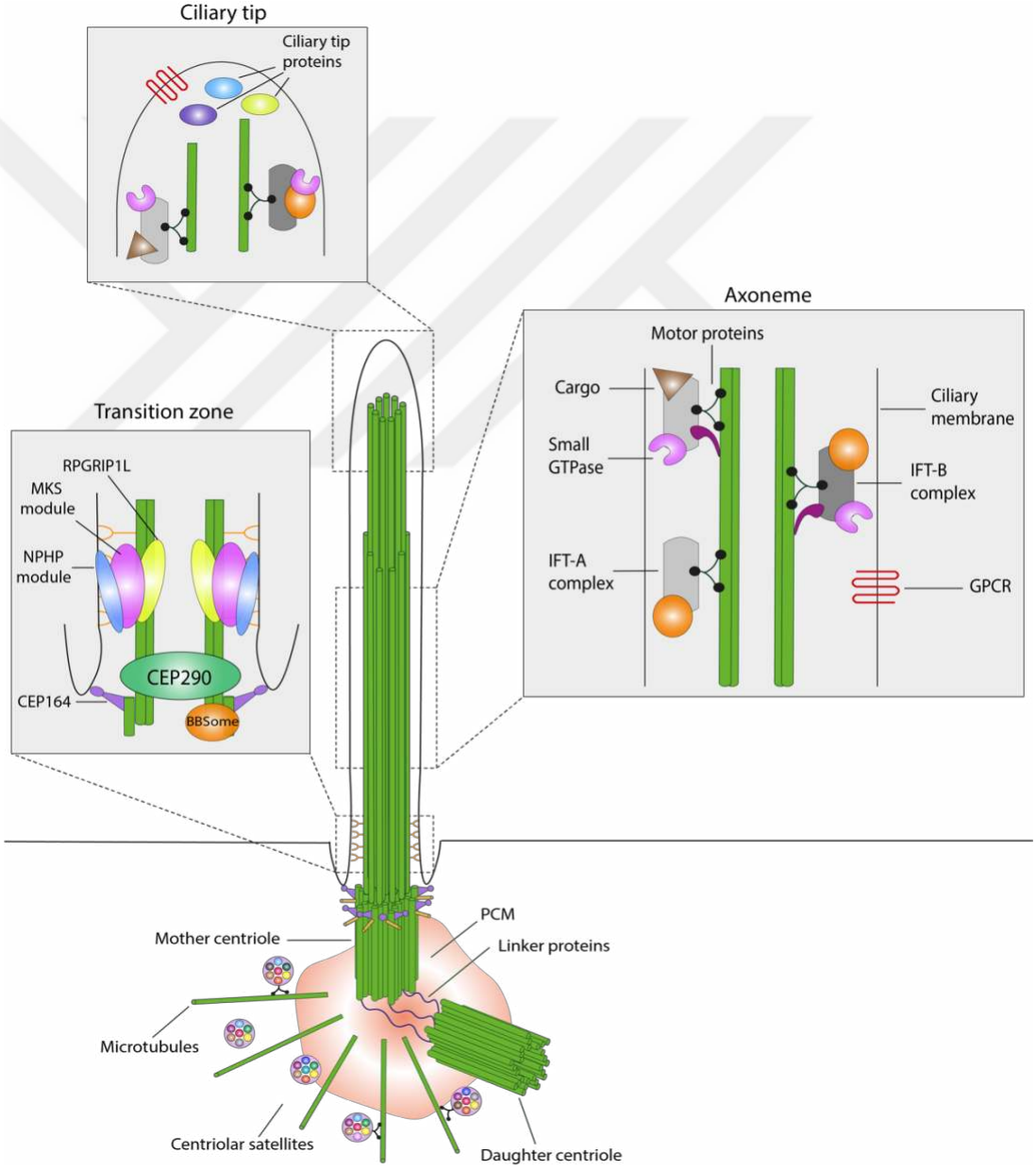

Figure 1.3: The structure of the primary cilium and its specialized sub-compartments. After docking the mother centriole to the plasma membrane, the primary cilium is assembled in quiescent cells. The primary cilium comprises three distinct parts: ciliary gate, ciliary axoneme, and ciliary tip. The ciliary gate is composed of transition fibers and the transition zone. The distal appendages of the mother centriole, such as Cep164 and Cep290, helps with the formation of the transition zone. The elongated axonemal microtubules form the ciliary axoneme. The ciliary axoneme is important for IFT and helps with the structure of the primary cilium. At last, the most proximal part of the cilia is the ciliary tip which is a vital part of the primary cilium (Adapted from (Arslanhan et al., 2020).

1.3. Centriolar Satellites

The third component of the centrosome/cilium complex is centriolar satellite They are less understood in terms of structure, function, and regulation (Arslanhan et al., 2020). Centriolar satellites are electron-dense membrane-less granules 70-100 nm in size clustered around the centrosome and cilia (Kubo et al., 1999; Odabasi et al., 2020; Prosser & Pelletier, 2020). These structures were first observed in electron microscopy studies (Balczon et al., 1994; Kubo et al., 1999) (Figure 1.4). There are variations in the subcellular localization of the centriolar satellites in various cell types and tissues, which indicates that there might be cell and tissue-specific functions of the centriolar satellites. The studies on non-centrosomal MTOCs show that the major MTOC dictates the localization of the centriolar satellites (Kubo & Tsukita, 2003; Sanchez & Feldman, 2017; Srsen et al., 2006; Vladar & Stearns, 2007).

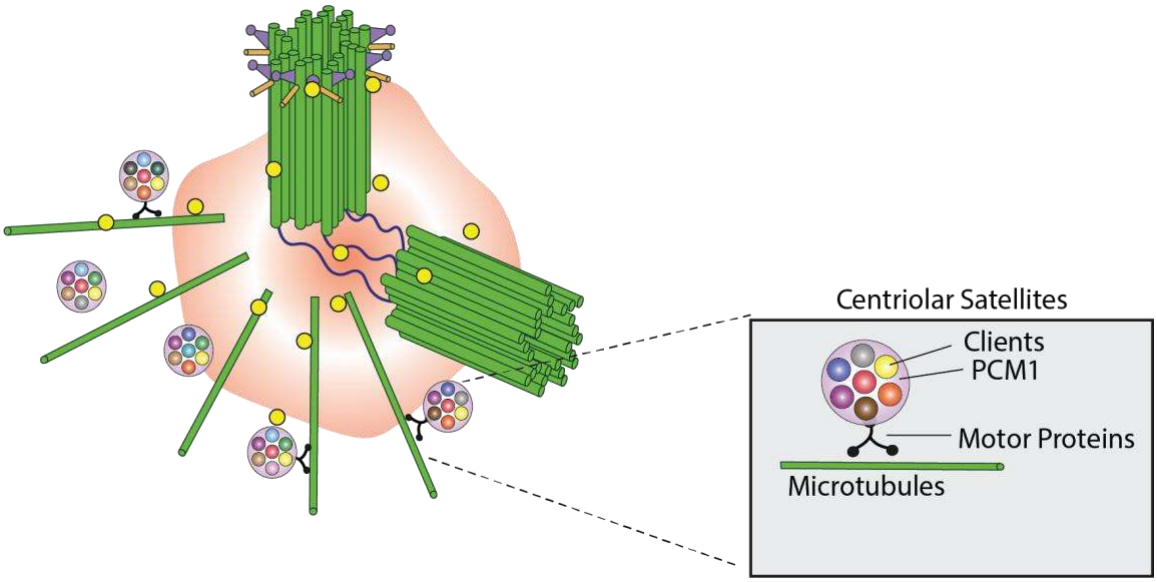

Figure 1.4: The structure and localization of centriolar satellites. The centriolar satellites are membrane-less electron-dense granules that are localized around the centrosome. PCM1 is the main scaffolding protein of centriolar satellites, which maintains satellite integrity. There is a variety of centriolar satellite clients, and each granule is heterogeneous. They are doing motor-directed movement controlled by motor proteins and microtubules (Adapted from (Arslanhan et al., 2020).

Centriolar satellites are dynamic structures, and this behavior is important for their cellular localization and protein targeting functions. The initial studies, both *in vivo* and in cells on the centriolar satellites, show that they have a motor and microtubule-dependent retrograde and anterograde motility (Kubo et al., 1999). On the other hand, recent studies on the dynamic behavior of the satellites show that along with their motor-dependent motility, most of the satellite granules also display diffusive motility (Conkar et al., 2019). Their dynamic behavior is not only limited to motility; centriolar satellites also undergo fission and fusion events like liquid-like behavior (Banani et al., 2017; Conkar et al., 2019).

Centriolar satellites are macromolecular protein complex assemblies. The first centriolar satellite protein identified was PCM1 (Pericentriolar Material 1) in human cells using human autoimmune antiserum (Balczon et al., 1994). With the identification of PCM1, molecular insights on centriolar satellites started to emerge. PCM1 is the main scaffolding protein of centriolar satellites that is important for assembling and maintaining these structures (Kubo et al., 1999; Odabasi et al., 2019; Wang et al., 2016).

Recent studies that took advantage of high-throughput proteomics and mass-spectrometry revealed the satellite proteome in different mammalian cell types. The centriolar satellite proteome is highly enriched in centrosomal and ciliary proteins, which is another crucial line of evidence of a regulatory and functional relationship between the centriolar satellites and the mammalian centrosome/cilium complex (Firat-Karalar et al., 2014; Gheiratmand et al., 2019; Gupta et al., 2015; Quarantotti et al., 2019). Dissecting the satellite composition, dynamics, and function showed that centrosomal and ciliary protein abundance regulation mediates the role of the centriolar satellites (Odabasi et al., 2020; Prosser & Pelletier, 2020). There are three proposed action mechanisms for regulating protein abundance by satellites 1) bidirectional movement toward and away from the centrosome and cilium by active transport, 2) to limit the centrosomal and ciliary proteins sequestration of them in centriolar satellites, and 3) proteostatic regulation by the centriolar satellites (Arslanhan et al., 2020).

Compared to the centrosome and primary cilia, the centriolar satellite cell dynamics also change. However, their behavior is much more different than the centrosome and cilia. Centriolar satellites lack precise number control, show liquid-like behavior, and have compositional and structural heterogeneity (Arslanhan et al., 2020; Kubo & Tsukita, 2003; Odabasi et al., 2020). While centriolar satellites are usually clustered around the centrosome and cilia in most cell types, their distribution changes during cell division. In mitosis, centriolar satellites first cluster around the spindle poles in metaphase and then dissolve where they are redistributed among the cytoplasm. They condensate back to their initial state upon mitotic exit. The mitotic dissolution of the centriolar satellites is regulated by the kinase activity of the dual-specificity kinase DYRK3. During mitosis, the DYRK3 levels and activity increase However, upon the mitotic exit, the APC/C-mediated ubiquitination leads to the degradation of the DYRK3, which results in the re-assembly of the centriolar satellites, and they re-cluster around the centrosome. Their dissolution by the DYRK3 is another line of evidence that they show liquid-like behavior since it is a mitotic dissolvase identified for dissolution of membrane-less granules such as stress granules (Arslanhan et al., 2020; Odabasi et al., 2020; Rai et al., 2018). The reason and the importance of the mitotic dissolution of centriolar satellites are unknown; however, it might be related to the centriolar satellite functions during mitosis (Arslanhan et al., 2020).

The functions of the centriolar satellites have been concluded from the phenotypic changes in their residents' acute or chronic depletion and cellular processes associated with the residents (Hori & Toda, 2017). Upon serum induced cilium assembly or release of key ciliogenesis factors upon environmental stress the centriolar satellite composition have identified to be changed. Proteins such as AZI1/Cep131 and Cep290 is released from the satellites to promote ciliogenesis by the activation of the p38 MAPK pathway (Tollenaere et al., 2015; Villumsen et al., 2013). The autophagy dependent degradation of the centriolar satellite pool of the OFD1 also have a role in the promotion of the ciliogenesis (Tang et al., 2013). There are also enzymes that have been identified in the centriolar satellite proteome. For example MIB1, E3 ubiquitin ligase, has shown to be sequestered by the centriolar satellites to prevent the degradation of KIAA0586/Talpid3 for ciliogenesis and GABARAP for formation of autophagosome (Joachim & Tooze, 2018; Wang et al., 2016).

Other than the mammalian cells, satellite remodeling have been also shown in the differentiation processes for the multiciliated epithelial cells (Kubo & Tsukita, 2003; Sorokin, 1968; Vladar & Stearns, 2007). In the multiciliated cells, during the centriole amplification stage in there is duplication of new centrioles from the centrioles that are already formed and deuterosomes. The fibrous granule formation in the close proximity of these new centrioles is regulated by centriolar satellites. With the further differentiation to form motile cilia, the fibrous granules are changing both in terms of size and abundance; only a small pool left in the basal bodies which can be seen in the apical surface of these mature multiciliated epithelial cells (Zhao et al., 2021).

Collective results of these studies indicate that centriolar satellites have a variety of roles in different cellular processes such as centriole duplication, cilium assembly/disassembly, microtubule nucleation, microtubule organization, progression during mitosis, autophagy, neurogenesis, stress response, and nuclear alignment (Aydin et al., 2020; Hori & Toda, 2017; Odabasi et al., 2019; Prosser & Pelletier, 2020; Wang et al., 2016).

2. Liquid-Liquid Phase Separation in Cell Physiology

Organisms have the same building blocks in biology, from prokaryotes to mammals. However, unlike the assembly of manufactured machines, which is composed of static parts attached, in biological systems, dynamic molecule interactions of

macromolecules such as proteins and RNA are the driving force for the assembly of these structures. Even though the biological systems can be considered a dynamic "wet soup," there is coherent motion and communication in these complex three-dimensional structures. There are different organizational levels within the organisms. The highest form is the organism, and cells give rise to various tissues, organs, and systems. The cells also have distinct parts known as organelles, a mesoscale organization in these biological systems with specific roles in many cellular processes. Cell compartmentalization is critical for important biological events. Understanding the achievement of this compartmentalization is essential to uncovering cellular dynamics.

2.1. Cell Compartmentalization and Membrane-bound organelles

Membrane-bound organelles are considered characteristic of eukaryotic cells. They are a mesoscale organization that compartmentalizes the cell to increase the reaction rate by sequestering and storing the molecules (Nunnari & Walter, 1996). They are well-defined compartments with specific cellular roles that are membrane-bound and vesicle-like. All the organelles are considered special functional units enclosed by a lipid bilayer membrane. Even though they are considered organelles, centrosomes and ribosomes were the only exceptions to organelles not surrounded by a membrane. The membrane-bound organelles are usually micrometer large, easily detectable with microscopy, and can be isolated using techniques such as cell fractionation. Organelle identity can be defined using the markers which mark different proteins such as luminal, transmembrane, and peripheral (Mellman & Warren, 2000).

2.2. Cell Compartmentalization and Membrane-less Organelles/Compartments

Along with the membrane-bound organelles, there are also other micron-large assemblies that contain different types of biomolecules that are not enclosed by a membrane. These structures are classified as membrane-less organelles. Like the membrane-bound organelles, these structures are important for cellular compartmentalization and have specific roles in different cellular processes. The centrosomes and ribosomes are the early evidence of membrane-less organelles; however, another type of cellular compartment not enclosed by a bilayer membrane was found later.

In addition to centrosomes and ribosomes, the first membrane-less compartment discovered was P granules (also known as C.elegans germ granule), which were identified during studies on *C.elegans* zygote) (Brangwynne et al., 2009). P granules are considered a mix of RNA or RNA-binding proteins (RBPs) that accumulate on the posterior side of the one cell C.elegans zygote before cell division (Brangwynne et al., 2009; Voronina et al., 2011). Other than P granules, different types of membrane-less compartments have been identified over the years. Nucleolus (Boisvert et al., 2007), Cajal bodies (Morris, 2008), paraspeckles (Fox et al., 2018), speckles (Spector & Lamond, 2011), and stress granules (van Leeuwen & Rabouille, 2019) are some of the examples for these multi-component assemblies. Just like the membrane-bound organelles, there are specific markers that are used for the identification of membrane-less-compartments.

2.3. Formation of the Membrane-less Compartments: Liquid-liquid phase separation in biology

The specific proteins, nucleic acids, and other molecules are in a restricted space in the context of the membrane-bound organelles. These molecules are already sequestered within a lipid bilayer which helps them perform their functions. Also, leakage from these compartments has serious consequences such as apoptosis (Ow et al., 2008). On the other hand, membrane-less compartments are maintained in the surrounding environment without a boundary and can perform a dynamic exchange of materials. With or without a membrane, all of these structures control complex biochemical and biological reactions in time and space; however, the organization of the membrane-less organelles is more intriguing. The formation and maintenance of membrane-less compartments is an emerging concept for cellular organization. These dynamic cellular assemblies can still form and function for specific biological processes without needing an enclosing membrane have been a trending concept among these scientists and thought to be facilitated by a phenomenon known as the liquid-liquid phase separation (LLPS).

The matter has a lot of different states; however, in most basic terms of physics, it is usually defined as gas, liquid, or solid forms. These states have different degrees of orders of the molecules that they possess (Hyman et al., 2014). By observation, one can separate liquids from solids since they are more rigid in shape and contain a higher degree of order. The molecules in the solid are caged, meaning they are kept in a specific area

for a long time. On the other hand, liquids are more flexible in order, and they can rearrange easily. The states of order in water can be an excellent example to illustrate the differences between solids and liquids. When the water freezes, it forms ice, a more rigid, stable, and not easily deformed structure that is not dynamic. The form of the ice will maintain its shape. As far as liquid water is concerned, the state is more dynamic and retains its container form. The rapid motion of the liquids allows the components to be easily mixed; a chemical reaction can occur anywhere in the liquid. This is also a good explanation in terms of biology since biological reactions tend to take place in liquids (Chong & Forman-Kay, 2016; Hyman et al., 2014).

The cytoplasm of the cell is long thought of as liquid; upon the disruption of the lipid bilayer of the cell, the cytoplasm will flow out. The lipid membrane is the structure that gives the cytoplasm its form, like the liquid matter inside a container. All of these compartments, whether membrane-bound or not, have physical characteristics and states of matter. Since the cytoplasm is liquid-like, all of these compartments have been acknowledged as solid-like compartments. In terms of membrane-less compartments, one cannot think of them as rigid solid-like structures; they do possess liquid-like properties. Liquid-liquid phase separation, which defines these membrane-less compartments, is defined as the following; the stable co-existence of two distinct liquid phases, which was a homogenous solution of macromolecules in the first place (Brangwynne et al., 2009; Hyman et al., 2014). Two miscible fluids will mix because the mixed state will have a higher entropy which is thermodynamically favorable, like coffee and milk. However, fluids can also demix, like the oil droplets in the water, as in the case of liquid-liquid phase separation. The entropy in these cases is not driving these fluids to be in the mixed state because of the physical interactions between the different molecules. The energy reduction caused by the favor of having neighbors molecules and disfavor of not like neighbor ones oppose the mixing by the system's entropy.

However, it is important to note that in the case of LLPS, there are not only simple liquids but also complex liquids that are worth mentioning. Many different kinds of liquids show similar properties. Since it has the proper viscoelasticity, the dough inside a bowl will also take the shape of its container (Hyman et al., 2014). In the biological context, one can give the example of the actomyosin cytoskeleton in terms of viscoelasticity. Upon force, the actomyosin cytoskeleton will initially respond with an elastic behavior; however, if the force is continued to be applied, its shape will change.

Overall the actomyosin gel acts like a liquid in more extended time periods and acts as a solid in shorter periods, meaning that; it is a complex biological fluid (Humphrey et al., 2002; Janmey et al., 1994; MacKintosh et al., 1995; Shin et al., 2004). Some liquid crystals can be an example of complex liquids, such as a meiotic spindle (Gatlin et al., 2010; Inoue, 2008; Itabashi et al., 2009; Shimamoto et al., 2011).

In describing the actomyosin cytoskeleton, the term gel was mentioned. A gel is usually defined as a polymeric structure that contains a crosslinked network. Different from chemical gels, biological gels are more similar to physical gels since weaker bonds hold them than covalent bonds. However, it is essential to note that even though there are few of them, there are examples in biology, such as fibrin gels, that are physically crosslinked (Munster et al., 2013). Due to the lifetime of the crosslink, actomyosin gels can go between liquid-like and solid-like behavior (Fritzsche et al., 2013). Hydrogels can be another example of gels in biology, and they have a high water content with crosslinked components. Hydrogels can also be distinguished both as chemical and physical gels. Nuclear pore complexes and RNA-Binding Proteins are examples of hydrogels (Frey & Gorlich, 2007; Han et al., 2012; Kato et al., 2012; Kwon et al., 2013; Peppas et al., 2000; Schwartz et al., 2013).

Membrane-less compartments formed by the liquid-liquid phase separation have similar properties in terms of structures. These liquid-like compartments, in vivo and in vitro, have spherical shapes; even though they perform dynamic exchange with the surrounding cytoplasm, they can fuse to form bigger assemblies and deform under shear stress. The P-granules, the first membrane-less compartment to be identified, have these properties, which led to the conclusion that P granules are liquid droplets that LLPS forms. The studies of Hyman and co-workers with the fluorescently labeled constitutive P-granule protein indeed showed that these structures were spherical, showed dynamic behavior like fusion, deformation under shear stress, and had fast internal rearrangement by active material exchange (Brangwynne et al., 2009).

One might ask about the novelty of LLPS since it is a known matter in the physics concept. The idea of proteins going under the phase transitions is not novel since protein crystallographers have been observing different states of proteins during the crystallization process. Recent studies done in vitro show that oligomeric peptides and biological macromolecules such as RNA can go into LLPS in vitro by different stimuli such as pH, temperature, crowding agents, and increasing concentrations (Dumetz et al.,

2008; Gomes & Shorter, 2019; Jain & Vale, 2017; Van Treeck et al., 2018; Wang et al., 2017).

2.4. Understanding Molecular Language that causes Phase Separation

2.4.1 Multivalency

When a protein becomes highly packed due to its density, it can lead to a jammed state in which the molecules within a protein cannot rearrange anymore. Even though this kind of crowded state was found in silico studies, it was also shown that liquid states could be achieved. If the system does not go into a highly dense-packed state and stays in a concentration range, other components can also be attached loosely by different interactions (Fusco & Charbonneau, 2013). These interactions between components can be defined as valency, interaction strength, and the range of the interaction. To achieve a liquid state, all of these parameters should be considered. An extended range of interactions, moderate valency, and binding energy is favorable in the liquid state (Asherie et al., 1996). In terms of biology, these parameters are considered in different ways. The multiple protein binding sites in a protein sequence would define the valency. The multivalency of the Interaction partners of a protein is an important consideration since it is one of the critical drivers of liquid-liquid phase separation. In vitro studies of weak multivalent interactions between signaling proteins showed that this can lead to the formation of liquid droplets. The engineered Nck and N-WASP proteins formed liquid droplets in vitro, containing one hundredfold higher protein concentration from the surrounding medium. In addition, the studies have shown that the model proteins which contained the tandem repeats of the ligand, the SH3 domain, or its interaction partner, the proline-rich motif (PRM), were also able to phase separate. When the number of repeats increased, which also increased the interaction strength, the liquid droplets became gelified. This also shows how these interactions lead to a highly packed state where a saturation within a droplet is reached. In this case, the specific multivalent protein-protein interactions cause phase separation (Li et al., 2012). It is also important to highlight that the multivalency does not only rise from protein-protein binding but also other macromolecular interactions such as protein-RNA interactions.

2.4.2 Intrinsically Disordered Proteins and Intrinsically Disordered Regions/Domains

The concept of multivalency described previously can arise from the protein-protein interactions in the ordered domains. However, looking at the different protein domains, the intrinsically-disordered domains and their interactions are as important as the ordered domains. The stretches of sequences with low complexity within the protein are named intrinsically disordered regions (IDR). A protein itself can also be referred to as an intrinsically disordered protein (IDP) despite stretches of disordered regions. These sequences can be defined within a protein since they are typically enriched in terms of polar and charged amino acids such as glycine (G), serine (S), glutamine (Q), proline (P), glutamic acid (E), lysine (K), and arginine I, and they often show conformational heterogeneity in the three-dimensional structure (Castello et al., 2012; Kato et al., 2012; Tompa, 2012; Uversky, 2017; Vucetic et al., 2003). The hydrophobic amino acid residues which lead to higher-order folding in a protein are usually missing in the IDRs however, these sequences can also contain some interspersed amino acids with aromatic residues such as tyrosine (Y) and phenylalanine (F); helping with the patterning of the polypeptide backbone to form specific interactions (Clifford P. Brangwynne, 2015). The precipitation of the components of the RNP granules by the biotinylated isoxazole showed that having an IDR or/and RNA-recognition motifs (RRMs) caused successful precipitation. The importance of disordered regions in determining the phase separation was demonstrated in this case (Kato et al., 2012). The germ granule protein, an RNA helicase that contains IDRs in both the N and C terminus, known as DDX4, is one of the most studied examples in terms of phase separation and the importance of the IDRs. The N-terminal IDR of the protein DDX4 was sufficient to form into liquid droplets both in vitro and in vivo studies (Nott et al., 2015). Again a closely related RNA helicase to DDX4, known as LAF-1, forms similar kinds of liquid droplets (Elbaum-Garfinkle et al., 2015). Both IDRs in these RNA helicases contain a mix of positively and negatively charged amino acids, meaning they are polyampholytic. Not just the presence of the charges but also the charge distribution and pattern along the protein sequence also promote the phase separation of the protein (Nott et al., 2015; Pak et al., 2016). The charge pattern's effect is still not yet uncovered; however, simulations suggest they might help with the conformational properties. The simulations also show that the LAF-1 IDR domains cause significant fluctuations in the conformation, allowing low protein density and high permeability to these liquid droplets (Das & Pappu, 2013; Das et al., 2015; Holehouse et al., 2017; Wei

et al., 2017). Indeed, many intracellular RNP condensates exhibit low density (Feric & Brangwynne, 2013; Handwerger et al., 2005). These large fluctuations in conformational changes also affect the intermolecular interactions that dictate biophysical properties in these liquid droplets. The systematic evaluations for the protein sequence and their effect on the biophysical behavior are still ongoing (Quiroz & Chilkoti, 2015).

Even though the homotypical interactions among the different IDRs have been highlighted, heterotypical interactions such as RNA/protein interactions are also essential to consider. In soft matter physics, it is known that oppositely charged polymers can form complexes and then go into phase separation in a process called complex coacervation if sufficient concentrations are achieved. The IDR of the nephrin (NICD) with the net charge of -21 causes nephrin to form complexes with positively charged proteins and go into phase separation in vitro to neutralize its charge (Pak et al., 2016).

2.4.3. RNA- and DNA- binding domains

A particular class of proteins known as RNA-binding proteins (RBPs) has biologically relevant phase behaviors. The assembly of these RBPs depends on the LLPS of the protein and RNA. The RNA-binding proteins contain multiple different domains that lead to multivalent interactions, such as IDR and RRMs. These domains synergistically lead to liquid-liquid phase separation. Even though many RBPs can go into phase separation *in vitro* without additional RNA because of the IDRs, they lack additional regulatory levels that arise from the RNA Recognition Motifs (RRMs), RGG, and oligomerization domains (Guo et al., 2018; Hofweber et al., 2018; Monahan et al., 2017; Qamar et al., 2018; J. Wang et al., 2018; Yoshizawa et al., 2018).

However, in many cases, phase separation is promoted by the presence of the RNA. An example independent from the IDRs is that polypyrimidine tract-binding protein (PTB) only goes under phase separation if the RNA is present (Molliex et al., 2015). N-terminal of the hnRNAP1, which contains two RRMs, also has similar behavior to the PTB (Li et al., 2012). Along with the phase separation, binding to RNA has other effects on the protein. RNA also impacts the protein interaction strength, which can be quantified through dissociation constant or closely related second virial coefficient. The studies conducted with the P granule protein LAF-1 showed that polyadenylate (polyA) RNAs with different sequence lengths have other impacts on the physical properties of the granule droplets. While 50 nucleotide polyA RNA does not affect the saturation of

the concentration on LAF-1, it affects the internal viscosity and leads to a decrease; on the other hand, longer polyA RNA such as 3000 nucleotide has an opposite effect by increasing the droplet viscosity (Elbaum-Garfinkle et al., 2015; Wei et al., 2017). The droplet viscosity effect of the longer polyA RNAs in these droplets also suggests that RNA might have a role in entanglement.

2.4.4. Oligomerization domains

Oligomerization domains are important for assembling large protein subunits and increasing protein valency. In the presence of a protein's high local concentration, these domains can promote phase separation (Shin et al., 2017). A nuclear RBP, TPD-43, has N terminal oligomerization domains, and it was shown that these domains have a role in the polymerization of the proteins. The phosphomimetic mutant within this domain also reduces the phase separation of TPD-43 (A. Wang et al., 2018). Also, if these domains have responsiveness to environmental cues, they can promote phase separation upon specific signals.

2.5. Understanding the regulators and modulators of the phase separation

Many molecular components of a protein can drive phase separation. Other than these drivers, there are other modulators and regulators for the phase separation. The phase separation is a biochemical response by a cell, so it has to be regulated via different processes to respond to extracellular and environmental cues.

2.5.1 Post-translational modifications (PTMs)

Studies for LLPS have revealed that phase separation is mainly driven by different cellular interactions by specific sequences within a protein. As discussed previously, these sequences include low complexity domains, including IDRs, multivalent protein interaction domains, variety of motifs. These sequences within a protein are often post-translational modified; therefore, post-translational modifications (PTMs) are also important for regulating the phase transition. (Bah & Forman-Kay, 2016; Chong & Forman-Kay, 2016; Rhoads et al., 2018; Tompa et al., 2014; Xie et al., 2007)

The PTMs affect the interactions of the specific amino acids both positively and negatively since they alter the aminoacids' properties such as sterocity, charge, or bulkiness. The altered amino acid interactions can have an effect on the phase separation of the specific proteins due to the weakening or strengthing of multivalent interactions between the macromolecules. On the other hand, they can also have different effects on phase separation because they can recruit or exclude specific macromolecules from condensate or into a condensate. By this means, the assembly or disassembly of a particular membrane-less organelle can affect the PTMs in terms of their composition or the material state.

Upon the environmental signals, the macromolecule interactions can be regulated by the PTMs, which can facilitate or antagonize the phase separation in different means of macromolecule interactions (Bah & Forman-Kay, 2016; Bhowmick et al., 2015). The PML protein, the scaffolding protein for the PML bodies in the nucleus, another membrane-less organelle that forms upon LLPS, can be an example. The SUMOylation of the PML protein increases the valency of the protein and leads to the recruitment of other proteins such as Daxx into these membrane-less condensates (Banani et al., 2016; Shen et al., 2006; Zhong et al., 2000). This is an example of a PTM to facilitate phase separation since adding a small-ubiquitin-like modifier is needed to assemble these condensates.

There are also examples to show the antagonizing effect of the PTMs on the condensates. Dissolution of many membrane-less organelles during mitosis is regulated by the protein DYRK3, which phosphorylates these granules (Carpenter et al., 2018; Rai et al., 2018). Similarly, the methylation of the arginine group in the RNA-Binding Proteins such as Ddx4 and FUS leads to the dissolution of the condensates. (Hofweber et al., 2018; Nott et al., 2015; Qamar et al., 2018; Ryan et al., 2018). These are only a few examples of the effect of the PTMs on LLPS and many other ways of modulation.

2.5.2 The Seeding/Nucleation of the Membrane-less Organelles

Phase separation can occur when a protein reaches a certain threshold similar to a switch-like mechanism. Concentration dependency is a known phenomenon for phase separation, and many proteins have a critical concentration that they need to reach to form condensates (Boeynaems et al., 2018). A seeding or nucleating mechanism can help the

cells to promote the phase-separation by enabling certain macromolecules to reach the necessary critical concentration.

RNA

Along with the RBPs, RNA is also important in phase separation and has a vital role in forming membrane-less organelles. Many membrane-less organelles rely on RNAs both for function and formation. In terms of function, P-bodies have a role in mRNA decay, stress granules stores RNA, and there is rRNA synthesis in the nucleoli (Boeynaems et al., 2018; Gomes & Shorter, 2019). RNA is one of the major nucleators in phase separation and has a role in the formation. In the formation of P-granules in *C.elegans,* RNA induces the phase separation of the MEG-3 scaffolding protein, and the translationally stalled mRNAs during stress response are the nucleators for the stress granules. (Gomes & Shorter, 2019; Smith et al., 2016; Van Treeck et al., 2018). Other than mRNA, other RNAs play a role in forming condensates, such as non-coding (nc) RNAs. There are many ncRNAs whose function is unknown; however, they might be important for phase separation, like the Men ϵ/β ncRNA that forms paraspeckles (Mao et al., 2011; Palazzo & Lee, 2015; Shevtsov & Dundr, 2011).

Poly(ADP-ribose)

Other than RNA, RNA-like molecules such as poly (ADP-ribose) (PAR) are utilized for the seeding mechanisms in LLPs. PAR is an ADP-ribose monomer-based polymer with a known function of seeding several stress-based membrane-less organelles, and it is both a PTM and an RNA-like scaffold (Schreiber et al., 2006). Some proteins, such as FUS and TDP-43, are examples that form condensates with the assistance of PAR (Altmeyer et al., 2015; McGurk et al., 2018). Stress granules also contain PAR (Leung et al., 2011).

Polyphosphates

The importance of the charge-charge interactions and the seeds such as RNA have often been highlighted for the LLPS. So, it is important to acknowledge other polyanions such as polyphosphates and their role in seeding these condensates. The polyphosphates' role in seeding amyloids has already been elucidated (Cremers et al.,

2016). Further studies should be conducted to understand the polyphosphate roles in LLPS.

Proline cis-trans isomerization

Looking into the RBPs, the importance of the prion-like domains (PrLD) was emphasized. There are many proline residues in these sequences. The side-chain geometry of the proline amino acids can introduce kinks. Given that PrLD can form aggregates, proline might have a role in preventing abnormal aggregation caused by PrLDs by acting as a fluidizer. So, proline and proline isomerization have a role in mediating phase transitions; for example, stress granules show colocalization with peptidyl-prolyl cis-trans isomerases (Xiang et al., 2015).

2.6. Biological consequences of phase separation

2.6.1. Organization and Cellular Compartmentalization

Thoroughly the LLPS cellular compartmentalization by membrane-less organelles and condensates is enabled. The spatiotemporally organized compartments can rearrange biomolecules within the compartment or the surrounding environment. As an implication of these spatiotemporal organizations, an example from the Drosophila polytene cells can be given. The stresses such as heat shock to these cells cause transcription sites where heat-shock protein (Hsp) genes are actively transcribed, and proteins such as RNA polymerase II are recruited to these sites, which means that there is a phase-separated compartment that is organized for transcription (Ashburner & Bonner, 1979; Bonner, 1981; Harlen & Churchman, 2017; Zobeck et al., 2010). Another example of the organizational aspect of LLPS can be the super-enhancers. The super-enhancers are clustered elements of enhancers, DNA, and coactivator proteins. For the formation of these, the PrLDs of transcription factors have a role. The PrLDs can form dynamic centers that stabilize DNA and recruit proteins that have a role in transcription, such as RNA polymerase II (Boulay et al., 2017; Cho et al., 2018; Chong et al., 2018; Hnisz et al., 2017; Kwon et al., 2013; Pott & Lieb, 2015; Sabari et al., 2018; Shorter, 2017). Depending on the clients and their interaction strengths, it is important to highlight that not all the membrane-less organelles show complete liquid behavior, but they can also be in a gel-like state (Boeynaems et al., 2018; Gomes & Shorter, 2019). In terms of

organization, the LLPS ciliary base can be an example of a gel-like state that controls the ciliary content and its regulation (Endicott & Brueckner, 2018; Kee et al., 2012).

2.6.2. Tuning reactions

Since the phase separation increases the concentration of biomolecules compared to the surrounding environment, a specific microenvironment is created within these condensates that may have a role in tuning biological reactions (Li et al., 2018). The impacts of the reactants for a reaction have a role in reaction rates meaning that phase separation might have a role in both tuning the biochemical reactions and increasing their rates. Cajal bodies can be an in vivo example of increasing reaction rates. The U4/U6·U5 tri-snRNP complex is assembled within the Cajal bodies, and the formation of this complex is 11 times higher within the Cajal bodies compared to the nucleoplasm surrounding these Nuclear RNP granules (Novotny et al., 2011). For tuning reactions in Cajal bodies, the di-snRNP complex cannot leave the nuclear bodies, whereas only fully formed The U4/U6·U5 tri-snRNP complex can. This means the Cajal bodies act as a regulatory filter where only a particular molecule enters or stays in a condensate. The U4/U6·U5 tri-snRNP complex, a reactant for the biochemical reaction, is selectively gathered in a defined compartment (Schaffert et al., 2004).

2.6.3. Cellular fitness

Sensing and responding to stress cells utilize LLPS as a tunable reaction since stress factors might affect and trigger protein misfolding (Boeynaems et al., 2018; Chuang et al., 2018; Gomes & Shorter, 2019). The triggered condensate formation with the environmental stress has a cryoprotective role since the formation of these reversible structures has a role in protein and RNA stores. With the stress release, these compartments dissolve, releasing their clients, and cells can have a rapid recovery for survival. These stress-induced aggregates were thought of as accumulations of misfolded or aggregated proteins; however, with the findings in stress response and LLPS, it is shown that these condensates are an adaptive strategy for the cells (Wallace et al., 2015). Studies in yeast and mammalian cells showed that the translation installment caused by stress leads to stress granule formation, which is later dissolved by heat-shock proteins such as Hsp110, Hsp70, and Hsp40 (Cherkasov et al., 2013). The stress granules also

protect cells from senescence since the accumulation of promoters such as PAI-1, an established senescence promoter, also accumulates within these granules (Omer et al., 2018). This shows the importance of LLPS to help with cellular fitness, primarily upon stress-based environmental cues.

3. Auxin Inducible Degron

The clustered regularly interspaced short-palindromic repeats (CRISPR) have been utilized in various ways in CRISPR-associated (Cas) based technologies. CRISPR-Cas9 gene-knockouts in human cell line generation has revolutionized our understanding of cellular function. CRISPR-Cas9-based gene knock-outs perturb the constitutively active genes, meaning that the knock-out of the essential genes that have a role in cellular viability will lead to lethality. To study these genes, conditional knock-out or deletions should be performed at shorter time points so that they would not cause apoptosis (Blomen et al., 2015; Cong et al., 2013; Hart et al., 2015; Mali et al., 2013; Wang et al., 2015).

The conditional depletion of the gene of interest can be achieved by fusing a destabilizing domain, referred to as the degron, to the gene of interest. There have been examples of conditional depletions such as heat-inducible degron technologies in the budding yeast to study DNA replication. These technologies are adventogous as they do not provide time for an adaptation like in the case of CRISPR/Cas9 knock-out. Thus, the direct effect of the depletion of a protein can be studied. The stability of the degron fused proteins is controlled by several mechanisms such as heat activation or the small molecules (Banaszynski et al., 2006; Bonger et al., 2011; Chung et al., 2015; Dohmen et al., 1994; Iwamoto et al., 2010; Kanemaki et al., 2003; Neklesa et al., 2011).

Auxin-inducible degron (AID) technology is a conditional depletion technology that utilizes a small molecule called auxin, a plant phytohormone. It achieves conditional depletion in non-plant cells such as mammalian cells by transplanting a plant-specific degradation pathway controlled by the auxin. The E3 ubiquitin ligases, the final enzymes in the PiP pathway, control and achieve the enzyme-substrate specificity (Nishimura et al., 2009). They recognize the specific substrate that will lead to the proteasome for degradation. Different E3 ligases, such as the SCF (Skp1-Cullin-F-box) ubiquitin ligases, have a role in this pathway. By expressing the plant-specific F-box protein names TIR1, a part of the SCF complex, and the proteins fused with AID degron, one can achieve

conditional depletion upon auxin addition in non-plant cells. SCF complex formed by the TIR1 protein specifically recognizes the proteins with the AID tag with the auxin addition. Since these proteins are derived from the *Arabidopsis thaliana,* other pathways are not interrupted (Nishimura et al., 2009).

Upon the other degron Technologies, AID enables protein depletion in relatively shorter periods, approximately 10-20 minutes. In addition, AID is a reversible degron, which means that upon removing the auxin-containing medium and changing it, the protein assembly is established again. Even though it is an essential technology, it is still limited in use since it is hard to establish endogenous gene tagging by CRISPR/Cas9. Along with the gRNA's, which are also provided in CRISPR/Cas9-based knock-out one, need to use a donor plasmid that contains homology arms designed according to the site of gRNA break site for the protein of interest with the AID tag (Holland et al., 2012; Kanemaki et al., 2003; Kanke et al., 2011; Lambrus et al., 2015; Natsume et al., 2016; Nishimura et al., 2009; Zhang et al., 2015).

CHAPTER 2: RESULTS

2.1. PCM1 is an IDP, and the sequence has been conserved through evolution

PCM1, the scaffolding protein of the centriolar satellites, regulates the satellite integrity and dynamics in the cells. Due to its highly dynamic structure and ability to form membrane-less spherical granules, they may go under liquid-liquid phase separation (LLPS) to construct these condensates (Gomes & Shorter, 2019). Since intrinsically disordered regions are known to be one of the main drivers of LLPS, I utilized different *in silico* tools to see if PCM1has any IDRs. For this purpose, we used three different IDR prediction algorithms PONDR (VSL2), PONDR (VL3-BA), and IUPred3. All of these sites score each amino acid in a given protein sequence from 0 to 1, 1 being the score for the most disordered. The bioinformatic analyses revealed that PCM1 is a highly disordered protein containing many IDRs since many sequences score above 0.5 (Figure 2.1A). Being a highly disordered protein and having numerous IDRs makes PCM1 an excellent candidate to go under LLPS.

If the PCM1 granule condensation is essential in cellular processes, the sequence-specific features of PCM1 might be evolutionary conserved. To gain the initial insight into how the disordered regions and domain composition of PCM1 were changing during the evolution, we analyzed the set of PCM1 orthologs with Michal Malszycki . We have acquired the sequences for PCM1 orthologs from OMA and NCBI. Many of the sequences were from vertebrates. All the sequences that we could find in vertebrates, and were runned in MobiDB disorder prediction and aligned according to their length. Even though the phylogenetic analysis shows that PCM1 architecture to ordered / disordered regions is relatively conserved with minor variations, no size or disorder variation could be explained by protein phylogeny. The interpretation likely results from different qualities of the genome assemblies between species (Figure 2.1B).

Additionally, the protein seems to evolve according to the species' evolution. It is conserved mainly within vertebrates and dramatically different outside the clade, yet the overall order/disorder structure is maintained. Furthermore, we investigated the presence of known domains such as the coiled-coiled domains and PCM1 C_domain, which have been reported in the literature, across these sequences. We observed that they all have a

PCM1_C domain at the C-terminus and multiple coils throughout the sequence (Figure 2.1B).

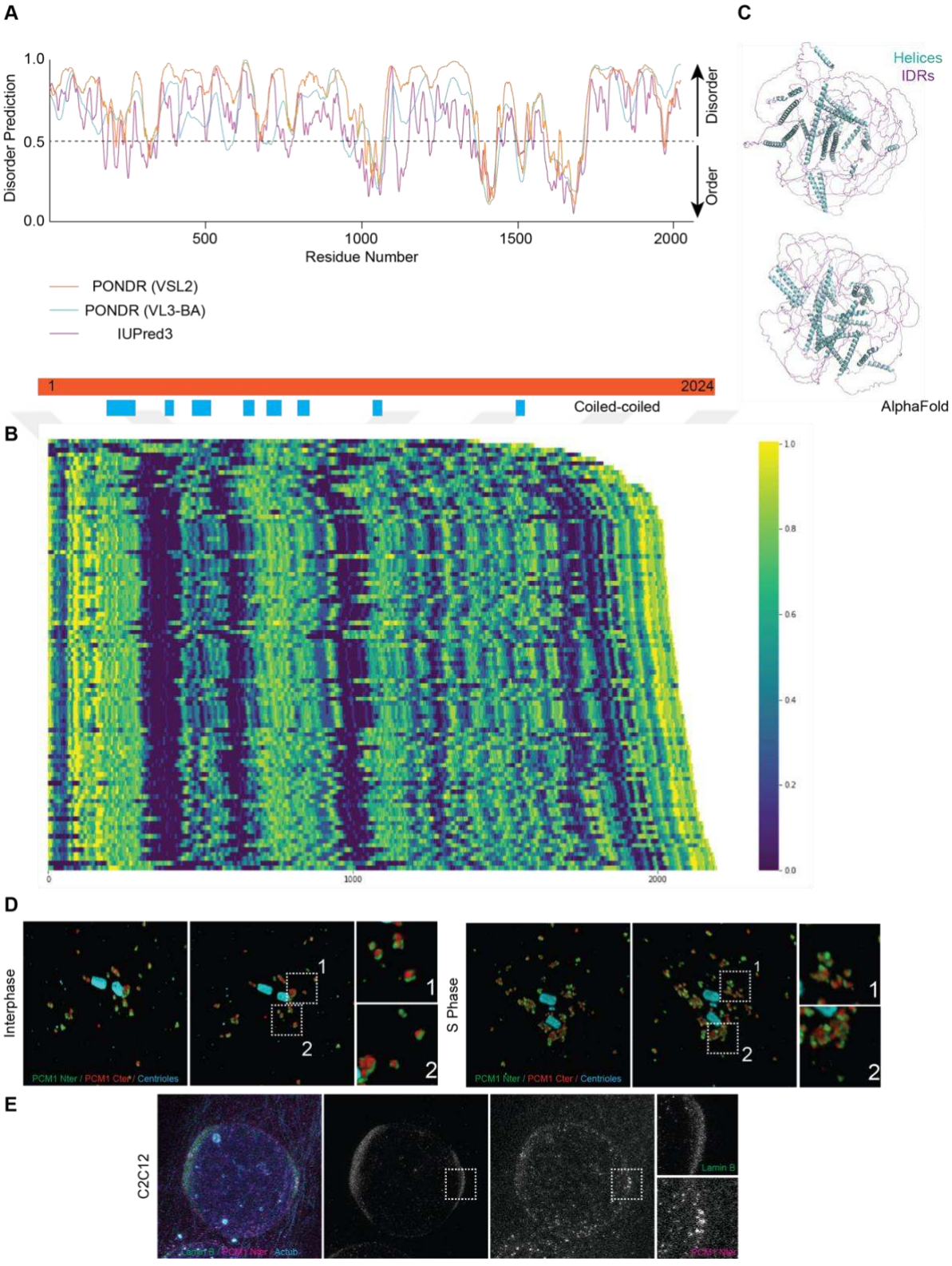

Figure 2.1: The PCM1is an IDP, conserved among the protein orthologs, and the granular shape is also conserved upon the changes in the MTOCs. **A)** The bioinformatic analysis is conducted to find IDR inside PCM1 protein. The 0 being most ordered and 1 being most disordered, the amino acid hits that give a 0.5-1 score are considered as disordered. Most residues inside the PCM1 are above 0.5, showing that the protein is highly disordered. **B)** The bioinformatic analysis was conducted among the protein orthologs from the NCBI and OMA databases. Each line represents a different ortholog. Each amino acid is coded from blue (0) to yellow (1) to show the disorder prediction again. Since the protein orthologs are aligned, each column gives an amino acid in the same position in every individual protein ortholog. Preservation of color in each column dictates the amino acid conservation. **C)** Alphafold prediction of PCM1 3D structure in 2 different angles. While strings show IDRs (in purple), the helixes show structured regions (in blue). **D)** The U-ExM of PCM1 granules was conducted in U2OS cells. The granules are stained with two antibodies, one recognizing the PCM1 N-terminal (shown in green) and one recognizing the PCM1 C-terminal (shown in red). The centrioles are stained with Actub (shown in cyan). The granules are clustered around the centrosome, and it can be resolved that each granule is different in shape and size. Also, the two different terminals of the PCM1 protein do not colocalize into a perfect sphere. **E)**The U-ExM is done to the differentiated C2C12 cells where the MTOC localizes to the nuclear envelope. LaminB (shown in green) labels the outer nuclear envelope. The PCM1 (shown in magenta) localizes outside the outer nuclear envelope and does not show a localization pattern like LaminB. The granules are still conserved in ncMTOCs.

The protein sequence conservation and the IDR predictions needed further assessment in order to understand more about the architectural properties of the PCM1. Even though it has ordered domains like the coiled-coiled domains, the IDRs have an important role in the protein folding (Gomes & Shorter, 2019; Hyman et al., 2014; Li et al., 2012). The “structure-function” paradigm in proteins states that the structure of a protein is dictates the function, however proteins such as PCM1 which are characterized as IDPs challenge this paradigm (Anfinsen & Scheraga, 1975). The lack of folding into a precise 3D structure gives the IDPs a high dynamicity and conformational heterogeneity in terms of ensemble. The composition of the IDR sequences are mostly polar and charged residues instead of hydrophobic and bulky residues, therefore IDPs are mostly characterized as polyelectrolytes (polymers with multiple same charge residues) or

polyampholytes (polymers with both negative and positive charges) (Mao et al., 2010; Radivojac et al., 2007). Even though there is lack of hydrophobic residues which enables the ”hydrophobic collapse”, there are still some IDPs that can adopt compact confirmations by the means of “electrostatic collapse” (Choi et al., 2019; Corti et al., 2019; Das & Pappu, 2013; Mao et al., 2010). Even though PCM1 is an IDP, in order to understand if it has a 3D structure that might be caused by these kind of electrostatical interactions we applied the alpha fold protein structure analysis (Figure 2.1C). The *in silico* protein structure prediction shows that IDR domains are exposed in terms of structure. Therefore, PCM1 is an IDP and it does not have a defined structure dictated by the ordered domains such as coiled-coiled domains or the charged residues that might cause “electrostatic collapse”.

Since the IDRs also provides with heterogeneity in protein confirmation and PCM1 does not have a defined 3D structure, I wanted to investigate the heterogeneity between each granule further. There is a known satellite heterogeneity regarding interaction partners and granule sizes. Depending on binding, different binding partners would affect the PCM1 structure, leading to granule size variation. Therefore in order to elucidate more on the heterogeneity in terms of confirmation we applied expansion-microscopy.

Since PCM1 localizes to the MTOC, the localization pattern varies according to the cell and tissue type. The PCM1 granules accumulate around the centrosome in mammalian cells. However, it is also known that differentiation in cells such as cardiomyocytes and myocytes localizes to the nuclear envelope since the MTOC changes. The redistribution of the PCM1 granules upon MTOC changes might alter the granule integrity. For resolving the protein confirmation, we used U2OS cells where PCM1 accumulates around the centrosome and differentiated myoblasts where PCM1 accumulates to the nuclear envelope. By applying expansion microscopy, we could examine the form of the PCM1 granules with higher resolution. Staining the PCM1 granules with antibodies that recognize the PCM1 N-ter and PCM1 C-ter showed that the granules behave differently in U2OS cells (Figure 2.1 D). The heterogeneity between the granules in size and folding was more apparent in this case which also aligns with our findings of PCM1 being a highly disordered protein. Since IDPs do not have a defined structure, there might be other regulators of protein shape. For example, the protein

structure can be shaped by the binding partners. Other than the heterogeneity and plasticity seen in the granules around the centrosome; we continued to investigate the protein behavior around the nuclear envelope. Our expansion data in ncMTOCs in differentiated C2C12 cells showed that the granules remain intact and do not fuse even though they come close (Figure 2.1E).

Overall, our findings show that PCM1 is an intrinsically disordered protein with conserved ordered/disordered regions within PCM1, PCM1_C domain, and coiled-coiled domains, suggesting that the protein might be under selective pressure to maintain its molecular functions. Also, PCM1 does not have a defined 3D structure, and there is a conserved granular protein confirmation which might be arise from different binding partners.

2.2. Endogenous PCM1 has a stable core and does not dissolve upon 1,6 hexanediol treatment

As PCM1 shows the features of LLPS, we sought to understand the biophysical properties of the centriolar satellite scaffolding protein PCM1. Since the stress granules are the most common structures that show the LLPS features, we used them as a positive control during our studies. We treated U2OS WT and U2OS PCM1 KO cells with sodium arsenate to induce stress granule assembly. Both lines were responsive to stress and formed stress granules. Interestingly, in wild-type U2OS cells, the endogenous PCM1 granules were dispersed/depleted, indicating that the PCM1 might be responsive to cytotoxic stress. However, we did not further investigate this since this is not in the scope of our research. To eliminate the effect of stress granule assembly on PCM1 granules and understand the PCM1 granule dynamics, we performed separate imaging of PCM1 granules in stress-induced and stress-uninduced cells. (Figure 2.2 A,B)

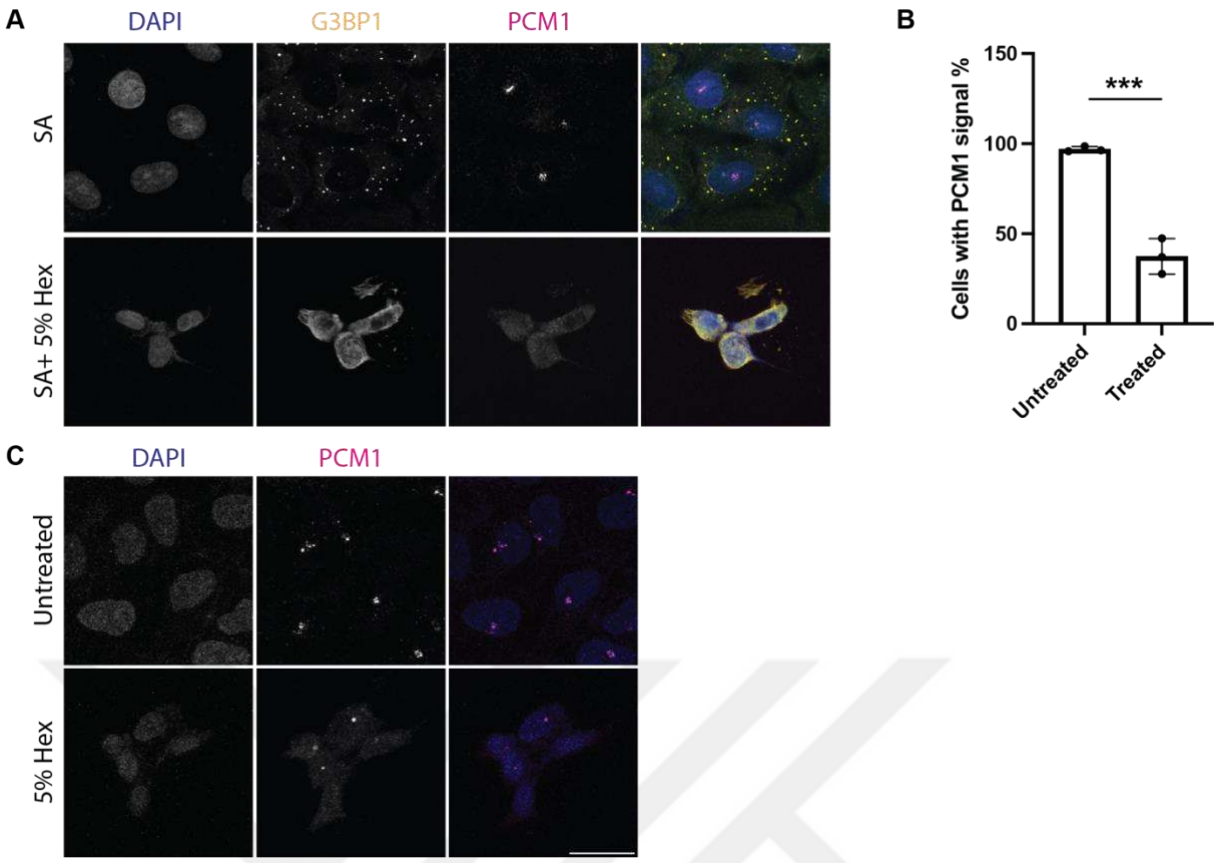

Figure 2.2: The endogenous PCM1 does not dissolve upon 1,6 hexanediol treatment. **A)** The G3BP1 is the main scaffolding protein of the stress granules. Upon 5% 1,6-hexanediol treatment stress granules dissolve, thus they are used as the positive control. In line with the literature the stress granule dissolvement is seen however in stress induced case some cells responds to sodium arsenate by the dispersal of PCM1 granules, so the satellite integrity was not assessed in stress induced conditions. **B)** The quantification for the cells that have lost the PCM1 signal upon the sodium arsenate treatment. C) The PCM1 granules are still intact after the treatment with 5% 1,6- hexanediol in the no stress induced case. This shows that PCM1 has is in gel-like or solid-like stage. Scale bar 5 um.

To start biochemical characterization of PCM1 granules, we used aliphatic alcohol 1,6-hexanediol, which has been shown to dissolve liquid-like cellular assemblies such as stress granules (Jain et al., 2016). In line with the previous literature, treatment with a 3% 1,6-hexanediol containing medium led to intact stress granules, whereas stress granules were completely dissolved in a 5% 1,6- hexanediol containing medium in U2OS WT cells (Jain et al., 2016). In the case of endogenous PCM1 granules, we found that PCM1 granules were still intact and present in each treatment. (Figure 2.2C) Even though

the dynamic behavior of the endogenous PCM1 granules shows liquid-like behavior, they were still intact after the 1,6- hexanediol treatment. We are limited in terms of counting the granules since they are smaller in size, they overlap, and it is hard to distinguish one condensate; the population state of each granule was not concluded quantitatively. Due to the limitations in counting individual centriolar satellites granules, there might be a small population of granules that might be in liquid-like state which dissolved upon 1,6-hexanediol treatment that we could not distinguish. There might be three possibilities in this case. PCM1 granules might act as liquid or solid in different cell cycle stages (1), possess different behavior with age and maturation (2) or be heterogeneous in terms of state indicating that some behave as a liquid and some act as solid (3). To assess their heterogeneity in terms of each condensate, the granules should be counted in an unbiased way. However, since the PCM1 condensates are still intact after the treatment with 5% 1,6-hexanediol, unlike the stress granules, this shows that; the overall satellite population is likely in a gel-like or solid-like state with a subpopulation of granules that are in liquid-like state.

Many membrane-less granules such as nucleolus, Cajal bodies, and P-bodies are enriched in RNA and referred to as the ribonucleoprotein (RNP) granules (Spector & Lamond, 2011). The formation of RNP granules can rely on multiple interactions, and these granules often contain RNA binding domains, prion-related low complexity domains, or IDR. These assemblies have been defined with a stable core and a more dilute shell, combining liquid and solid phases due to different reaction rates with their various macromolecular partners (Gomes & Shorter, 2019). Since the structures with stable cores cannot be disrupted with 1,6- hexanediol treatment due to their solid states, we hypothesized that PCM1 might have a stable RNA core. To test this hypothesis, we treated the cells with RNAse A to deplete the global RNA and observe the behavior of the satellite granules. We treated the U2OS cells with either RNAse A enzyme and fixed the cell 15 minutes after treatment. The cells are stained with the PCM1 antibody to see the centriolar satellite behavior. Our results show that the endogenous PCM1 granules were dissolved/dispersed/not maintained upon the treatment like the control stained with

stress granule marker G3BP1 (Figure 2.3). Our findings suggest that PCM1 might have a stable RNA core and can exist in liquid or solid-like states like the stress granules.

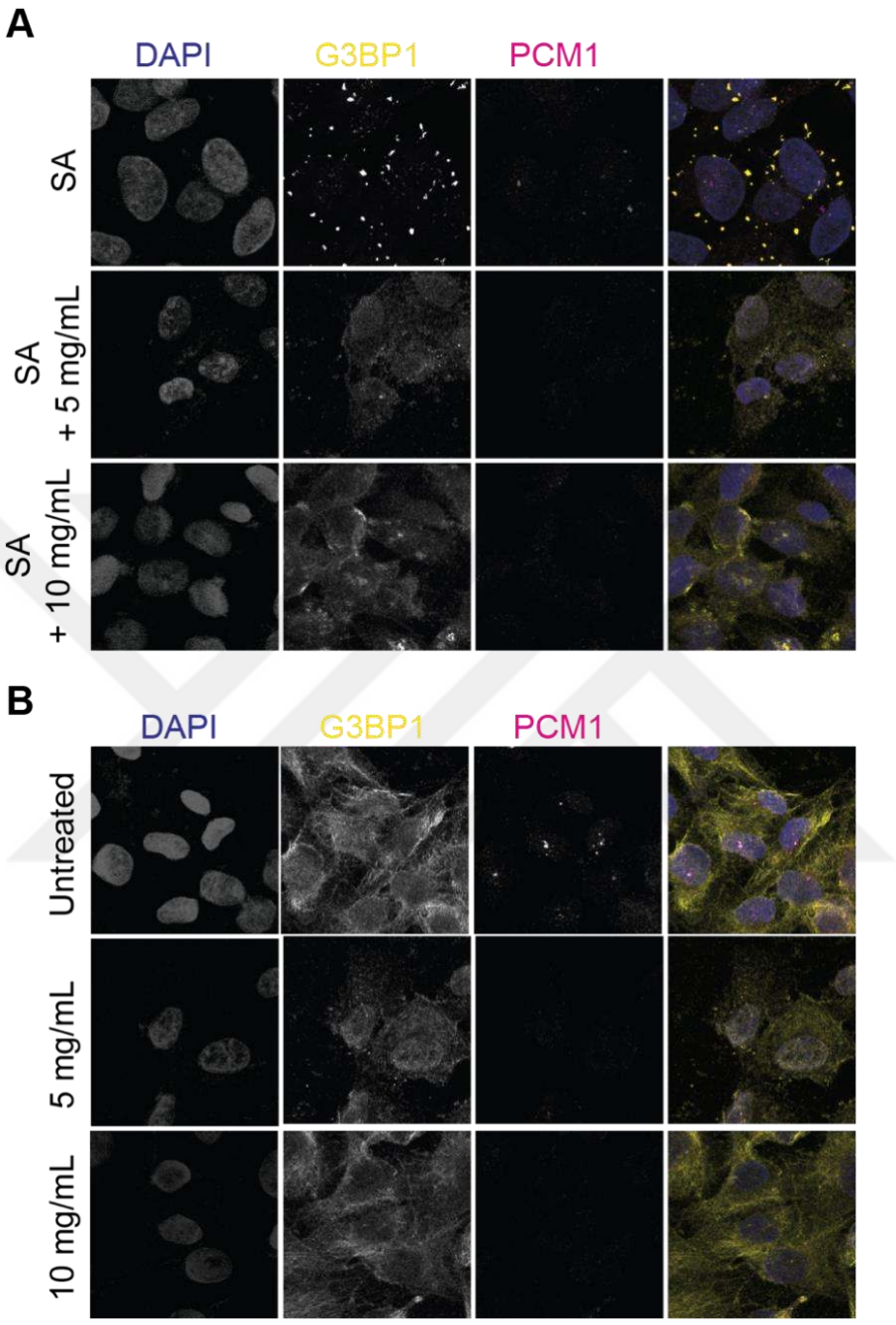

Figure 2.3: The RNAse A treatment to perform global RNA depletion. **A)** G3BP1 is the main scaffolding protein of the stress granules which have been shown to dissolve upon RNAse A treatment. By inducing stress granules upon sodium arsenate treatment, they have been utilized as the positive control. In line with the literature the stress granules have dissolved with RNAse A treatment. **B)** The RNAse A depletion is performed in stress uninduced cells to assess PCM1 behavior. With the global RNA depletion the

PCM1 granules lost their integrity which shows that RNA presence is needed for the satellite integrity. Scale bar 5 um.

2.3. Different domains of PCM1 can drive the granule assembly.

To further understand the granule assembly of PCM1 and the possible domains that might lead to protein oligomerization, we have cloned the different fragments of PCM1 into the N-LAP backbone. PCM1 is a big protein that is composed of 2024 amino acids. Therefore looking at the different fragment behaviors would give us more insight into the granule assembly. We sought to comprehend the fragment localizations and how they act. The fragments were designed according to the literature and previous studies of PCM1. All the eight fragments were categorized into three different groups: condensate forming (1), cytoplasmic (2), granular, and cytoplasmic (3) (Figure 2.4). To assess if endogenous PCM1 has a role in fragment localization through dimerization, we performed the experiments in wild-type and PCM1 KO cells. The protein localization of each fragment was concluded if the determined localization was prominent in the overall transfected cell population. According to the pronounced localization, the following things were observed. Among eight fragments, three showed precise granule formation, which was physiologically larger than the centriolar satellite granules. Four other five had cytoplasmic localization, whereas one of the fragments was bound to the microtubules.

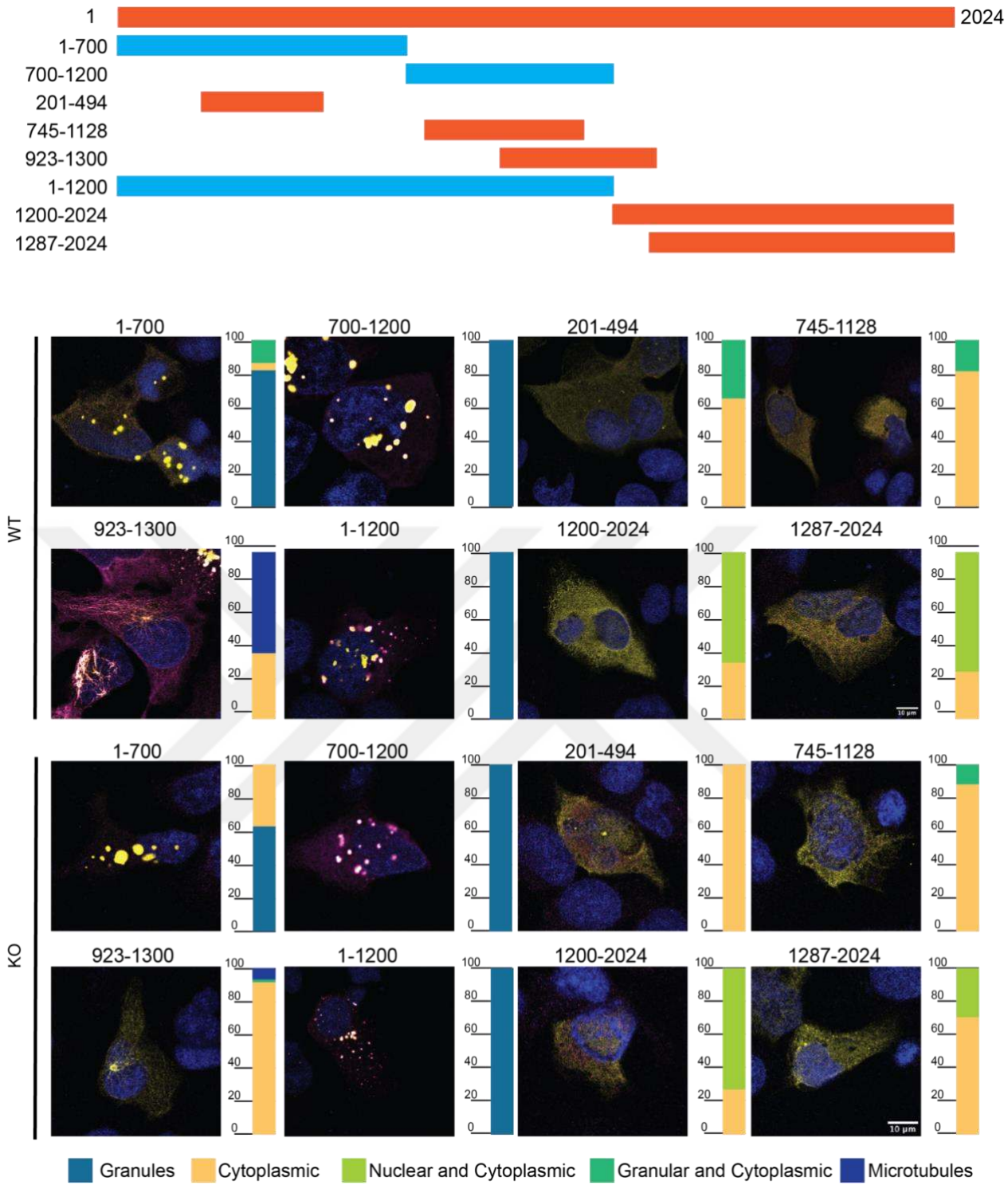

Figure 2.4: This study's schematic representation of the PCM1 fragments. The PCM1 FL comprised 2024 a.a.. Each fragment and the amino acids they encompass compared to the PCM1 FL are shown at the bottom. The fragments shown in blue show the fragments that can form granules. The other fragments (shown in orange, same as the PCM1 FL) show the fragments that do not form granules and have different localization patterns. The upper panel of fragment localizations belongs to the U2OS WT cells. Each

fragment localization IF showed individually, and the column on the right side of the images gives the percentage graph for each different kind of localization. The lower panel is the fragment localization in U2OS PCM1 -/- cells. Each image belongs to different fragments, and the columns on the right side of the images give the percentage graph for each different kind of localization. Scale bar 10 um.

The three prominent condensate-forming fragments consisted of amino acid sequences from the N-terminal part of the PCM1. 1-2100 a.a. and 2100-3600 a.a. fragments being the truncated ones, another sequence 1-3600 a.a. was the most considerable fragment that contains a large portion of the N-terminal. However, two other N terminal fragments: 201-494 a.a. and 745-1128 a.a. that encompass a smaller region of PCM1 N-terminal, were mostly cytoplasmic and did not show a distinct condensate formation like the others. In some cells, along with the cytoplasmic localization, smaller granules were seen; however, the condensates were not prominent in terms of localization and size, as mentioned. The granule forming fragments 1-2100 a.a., 2100-3600 a.a, and 1-3600 a.a. from now on will be referred to as N-ter Small Fragment (N-ter S), Middle-ter Fragment (M-ter), and N-ter Fragment (N-ter), by order. Localization analysis of the fragments showed that three of the fragments encompass more extensive sequences from the N-terminal part of the PCM1 compared to others that are sufficient to form condensates. The other smaller fragments from the N-terminal are not forming granules as efficiently as others, whereas only one sequence being 923-1300 a.a., binds to microtubules. In terms of other fragments, the C-terminal part of the PCM1 behaves cytoplasmic. To conclude, all eight fragments bear the same both in the presence and absence of the endogenous PCM1; the N-terminal region of the protein drives granule assembly, whereas the C-ter acts cytoplasmic (Figure 2.4).

To understand the behavior of the granules formed by each PCM1 fragment whole-cell intensity of the fragments was quantified along with the overexpression of the full-length PCM1. All N-ter or M-ter expressing cells showed granule formation. The whole-cell intensity calculations for these fragments revealed that they form granules regardless of their protein concentration both in WT and PCM1 KO cells (Figure 2.5A, 2.5B). This also showed that endogenous PCM1 did not affect the formation of the granules by the N-ter (1-200 a.a.) and M-ter (700-1200 a.a.) fragments. In the case of NS-

ter and PCM1-FL cells showed two different behaviors. They either formed granules or had cytoplasmic expression (Figure 2.5A, 2.5C).

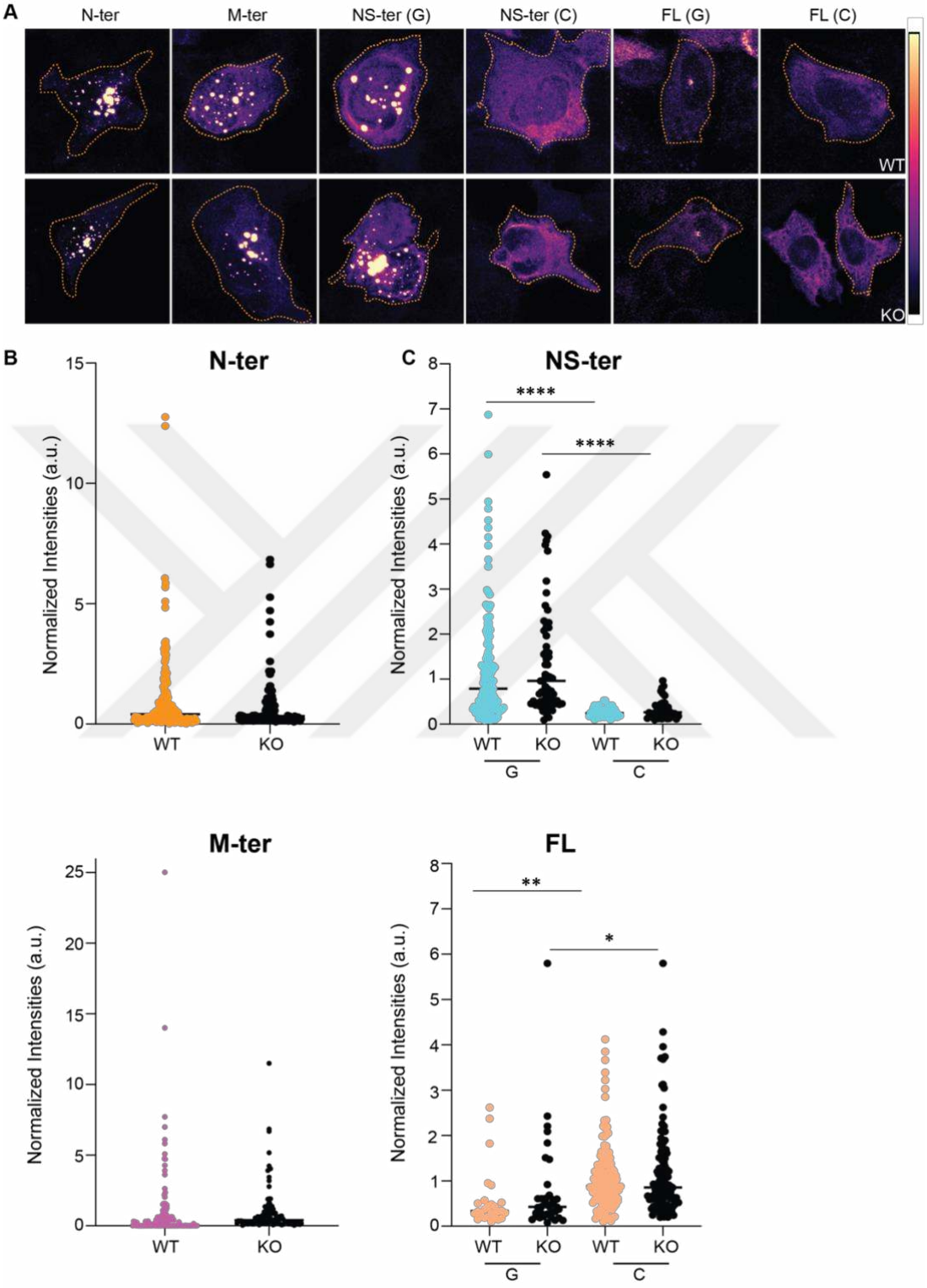

Figure 2.5: The whole cell intensity quantification for each granule forming fragment and the PCM1 FL for assessing the critical concentration and the fragment behavior. **A)** The PCM1 granule forming fragments and the PCM1 FL behavior both in the U2OS WT and the U2OS PCM1 -/- cells are shown in LUT. The white color

represents the pixels with the most intensity; the black color shows the pixels with minimal intensity. The intensities are shown in terms of a gradient that goes from white color to black color. For each cell, the total cellular intensity was measured by drawing the cellular borders represented in orange dashed lines. The N-ter and M-ter only formed granules, whereas the NS-ter and the FL showed either granular or cytoplasmic localization. **B)** Each data set was normalized within itself. The average of each data set was taken, and the total cellular intensities were normalized according to each. The N-ter and M-ter fragments only showed granular localization, and the normalized concentrations within the U2OS WT and U2OS PCM1 -/- cells did not show any significance in the critical concentration needed for the granule formation. C) The NS-ter and FL showed two different kinds of behaviors, one being granular and the other one being cytoplasmic. For this purpose, each data point was normalized according to the cumulative average of granular and cytoplasmic intensities. The NS-ter fragments granular and cytoplasmic expression had high significance both in U2OS WT and U2OS PCM1 -/- cells. In both cases, the mean protein expression level to form granules was higher than the expression level needed for the cytoplasmic localization. For FL, there was a significant difference between the granular and cytoplasmic protein expression levels in both U2OS WT and U2OS PCM1 -/- cells. However, unlike the NS-ter mean protein expression level needed for the cytoplasmic expression was higher. ****P < 0.001 ***P < 0.001, **P < 0.01, *P < 0.05.

For the NS-ter (1-700 a.a.), both WT and KO cells showed mainly granule expression. In WT cells, the transfected population had 74.7% granule formation, whereas in KO cells, the transfected population had 64.7% granule formation. The mean intensities for the granule formation and the cytoplasmic expression had a similar threshold for both WT and KO cells meaning that endogenous PCM1 does not affect granule formation or cytoplasmic expression. The granule expressing cells have a higher mean intensity than the cytoplasmic expressions cells ones in both WT and PCM1 KO cells; and there is significant difference in terms of protein concentration that determines the protein behaviour (Figure 2.5C). NS-ter (1-700 a.a.) needs a higher protein concentration in order to form granules and upon passing a certain threshold it only forms granules. Overall NS-ter (1-700 a.a.) acts mostly granular and protein concentration needed for the formation of the granules is significantly higher compared to the cytoplasmic expression. The plot of normalized intensities for individual cells showed

that cells might be acting differently in similar protein concentrations. (Figure 2.6B) Even though the critical concentration is passed by these cells there might be still other mechanisms that might regulate the granule formation. The granules might have been started seeding at that time point but might require more time to be assembled. Therefore the heterogeneity between the granule formation or the cytoplasmic expression in individual cell level at the same protein concentration needs to be assessed further in time dependent manner.

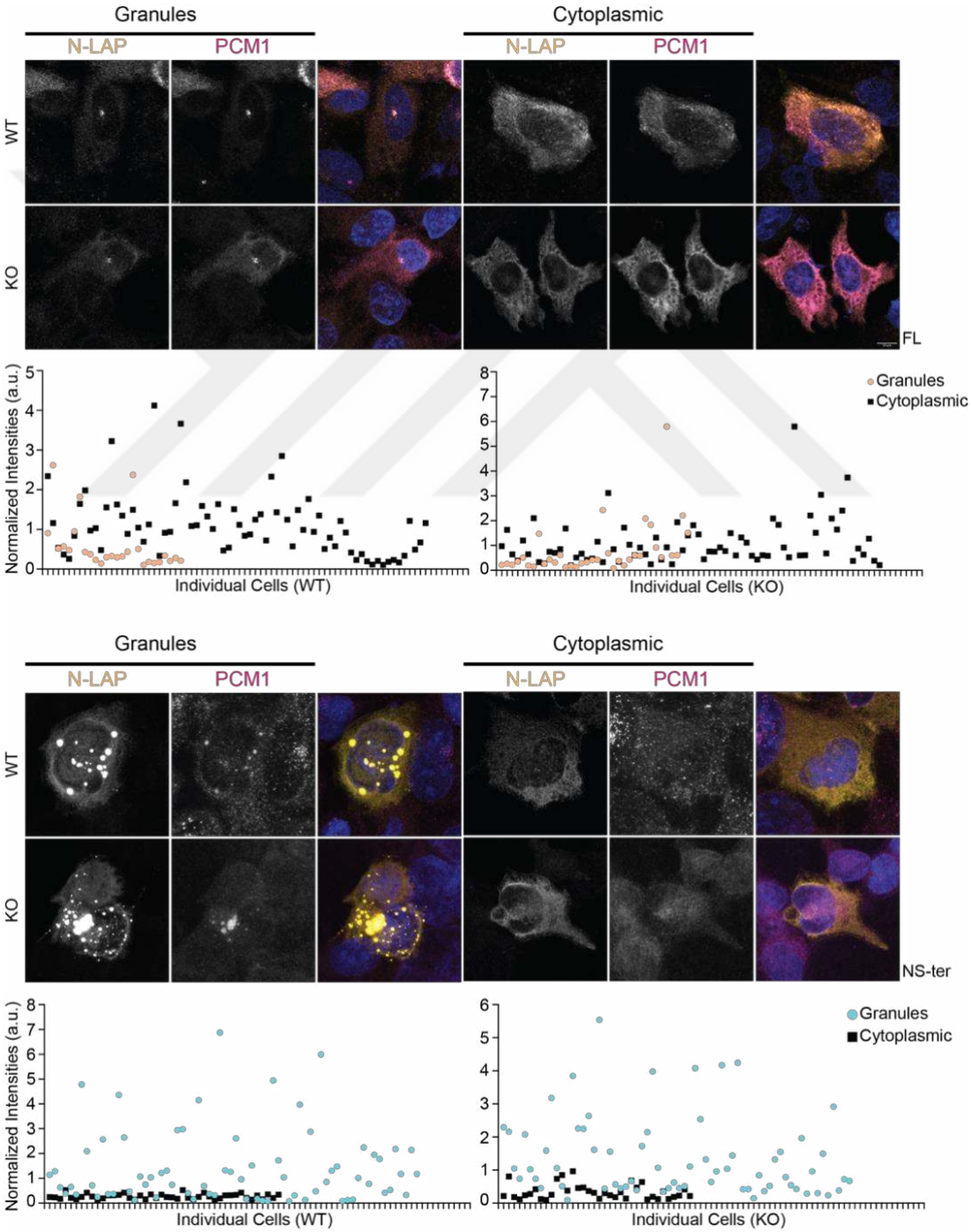

Figure 2.6: Comparison of the individual cell's normalized protein expression levels for PCM1 FL and NS-ter. Since PCM1 FL and NS-ter have two different expressions, granular and cytoplasmic, each normalized intensity for the cells was compared. Each data point corresponds to the cell mean protein expression level, and the shape dictates whether the localization was granular (show in circles; for FL, the color code is orange, for NS-ter, the color code is blue) or cytoplasmic (shown in squares). Scale bar 10 um.

Opposite to the NS-ter, in the PCM1-FL case, the transfected population mostly had cytoplasmic expression. In WT cells, 24% population showed granule formation, whereas, in KO cells, 33% of the cells had granule formation. Since the overexpression of the PCM1-FL leads to cytoplasmic expression and the KO cells have more granule expression, it was hypothesized that there is a specific critical protein concentration for the formation of PCM1-FL granules. After crossing this threshold, the PCM1 mostly acts as cytoplasmic. The cells required a higher protein concentration for the cytoplasmic expression and whole-cell quantifications shows that granule formation and cytoplasmic expression had difference in terms of concentration even though it is not highly significant (Figure 2.5C). This shows that after reaching a certain protein threshold the granule integrity of the centriolar satellites are lost. This aligns with the previous literature since the PCM1 granules are dissolved during mitosis, when the protein expression level is higher compared to non-mitotic cells. Along these lines, in WT cells the significant difference in terms of normalized intensities were higher compared to the PCM1 KO cells and PCM1 KO cells have a higher percentage of granule formation. This also shows that endogenous PCM1 might have an additive effect that leads to increased protein concentration and granules dispersal. Therefore endogenous PCM1 have a certain protein threshold that is needed for the granule formation and surpassing this threshold leads to the cytoplasmic expression. Comparison of the protein levels in individual cell level for PCM1-FL also shows that similar to the NS-ter (1-700 a.a.) , there are some cells that acts differently in similar protein levels (Figure 2.6A). Even though there is small subpopulation of cells that behaves differently, the population behaviour is prominently cytoplasmic.

The behavioral difference in all granule forming fragments and the PCM1-FL again highlights the possible role of C-terminal of the PCM1 in terms of granule

formation and the regulation of the centriolar satellites. The different behaviour for granule formation upon higher protein concentrations in NS-ter (1-700 a.a.) and PCM1 FL also shows that even though the N-terminal is the driver for the granule formation, the C-terminal might also have an effect on the regulation of the protein expression by different cellular pathways.

Our data shows that N-ter and M-ter is forming granules regardless of the protein concentration. The NS-ter is mostly forming granules and needs higher protein concentrations for the formation of these granules. The PCM1-FL needs a specific concentration for the granule formation and overexpression of the protein leads to dispersal of these granules.

2.4. PCM1 N-ter S, M-ter, and Ns-ter condensates are solid-like assemblies, and PCM1 C-ter regulates satellite granules.

Even though the endogenous PCM1 did not show dissolution upon treatment with 1,6-hexanediol, it is known that the centriolar satellites are dispersed during mitosis, and there are no visible granules. So even though they act as solid-like structures, their dissolution in mitosis shows their LLPS behavior. To understand the granule assembly and disassembly dynamics, the granule forming fragments (1-700 a.a. NS-ter, 1-1200 a.a. N-ter, 700-1200 a.a. M-ter) were again tested with biochemical assays to know if they undergo phase separation. Understanding the behavior of the granule-forming fragments might provide an insight into the behavior of the endogenous PCM1. After the 1,6-hexanediol treatment, all fragments had remaining granules, and the transfected population did not show a granule dissolution (Figure 2.7). Even though there are still granules that are intact, there might be a subpopulation of granules that have been dissolved. This indicates that each of the granule-forming agents have an assembly processes and also goes into a gel-like or solid-like state over time.

The granules might become gelated over time and insensitive to hexanediol. Since centriolar satellites dissolve during mitosis and the DYRK3 phosphorylation effect has been shown in the literature, these gel or solid-like condensates formed by PCM1 might require another mechanism such as post-translational modification. Since the N-terminal fragments that have been utilized create larger condensates than the endogenous PCM1

granules, PCM1 C-ter might be indispensable for granule assembly/disassembly and size control of these condensates.

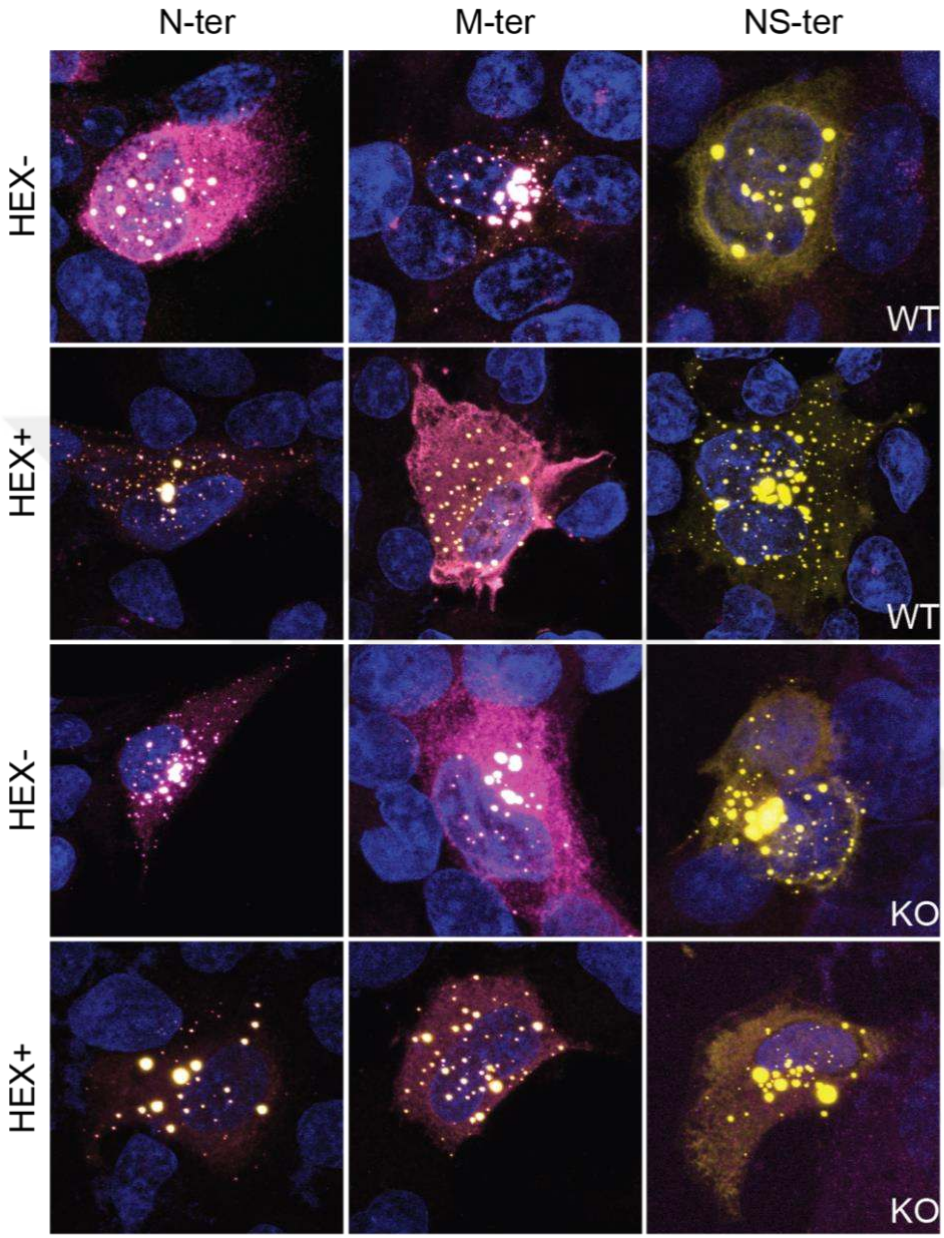

Figure 2.7: The assessment of the biophysical properties of the PCM1 fragments that form granules. The endogenous PCM1 granules treatment with 1,6-hexanediol showed that they are in a gel-like state. The same experiment was performed to assess the phase separation properties of the PCM1 fragments, N-ter, M-ter, and NS-ter(1-700 a.a. NS-ter, 1-1200 a.a. N-ter, 700-1200 a.a. M-ter). The granules for each fragment did not dissolve e upon 1,6-hexanediol treatment, indicating that these fragments are also in a gel-like state.

2.5. PCM1 N-ter S, M-ter, and N-ter have different dynamic behavior and mobility.

To better understand the state of the granule-forming fragments, (1-700 a.a. NS-ter, 1-1200 a.a. N-ter, 700-1200 a.a. M-ter) live cell imaging was performed to assess their dynamic behavior (Figure 2.8). Even though granules were intact after the 1,6-hexanediol treatment, (1) there might be still some granules that have been dissolved (2) the assembly and liquid-to-gel like transition would require time course. For this purpose, we utilized U2OS PCM1 -/- cells to solely understand the granule dynamics without the presence of endogenous PCM1 and performed live cell imaging to assess fragment behaviour. Since granules might become gelated over time, we used transient transfection and imaged positive cells instead of establishing a stable cell line.

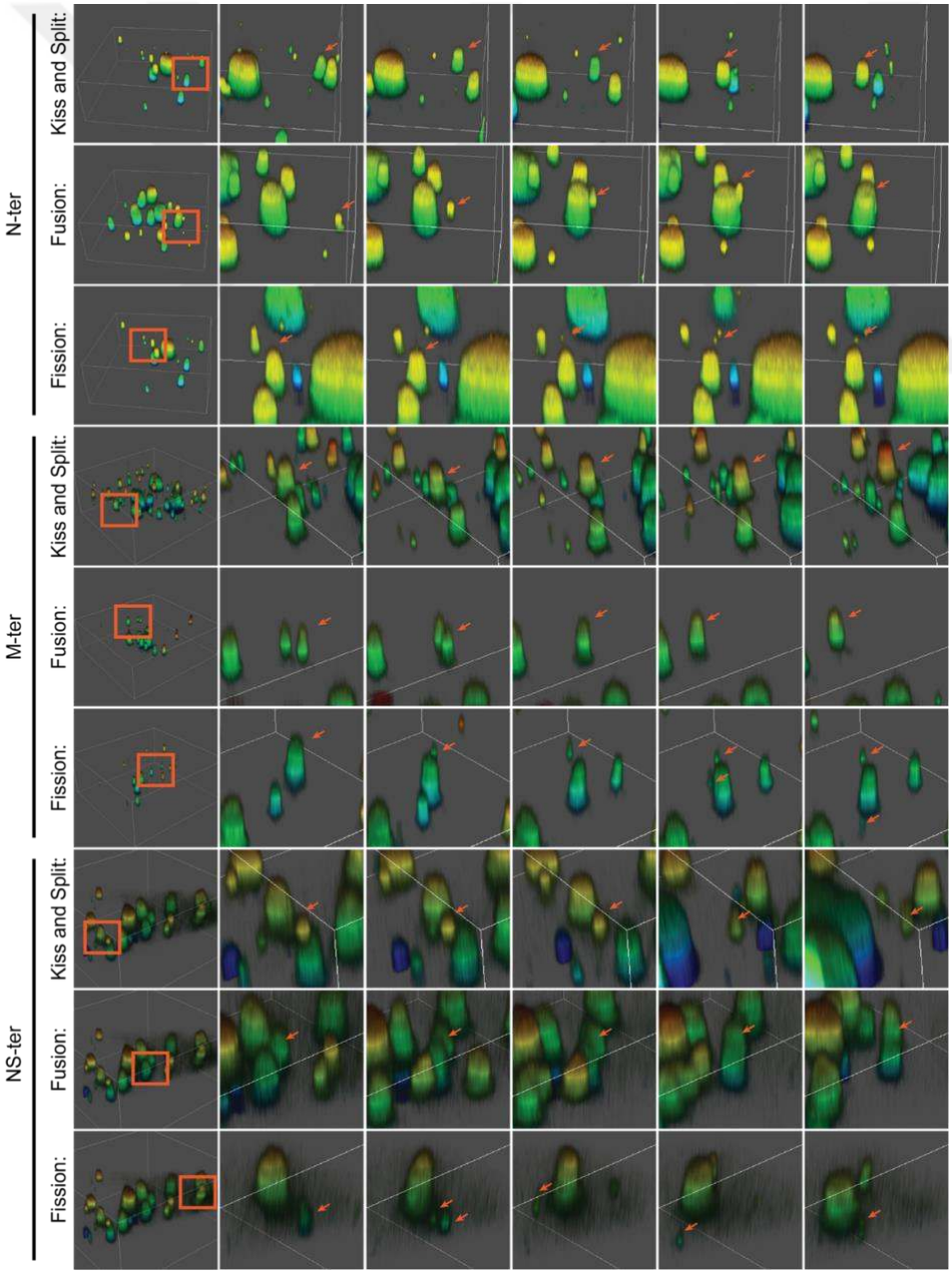

Figure 2.8: The granule forming PCM1 fragments have dynamic behaviour (1-700 a.a. NS-ter, 1-1200 a.a. N-ter, 700-1200 a.a. M-ter). All three PCM1 fragments that form granules have dynamic motility and goes under the kiss and split, fusion and fission events frequently which was able to captured by live-cell imaging. The granules of each fragment are insensitive to 1,6-hexanediol treatment yet they are still mobile indicating the liquid-to-gel transition or intermediate steps for assembly .

Live cell imaging of all the fragments showed that they all have dynamic behavior and show a different kind of motility. In all fragments, it was observed that they do kiss and split, fusion, and fission events (Figure 2.8). This indicates that these entities are 1,6-hexanediol resistant but still have mobile behavior. The granules that shows dynamic behaviour might be an intermediate assembly that is still in liquid-like state. Binding event of different proteins might take different times ranging from seconds to hours. Therefore dynamicity that is captured during live cell imaging's might be reflecting the subpopulation of the liquid-like granules that is still did not complete their assembly and go under a liquid-to-gel like transition.

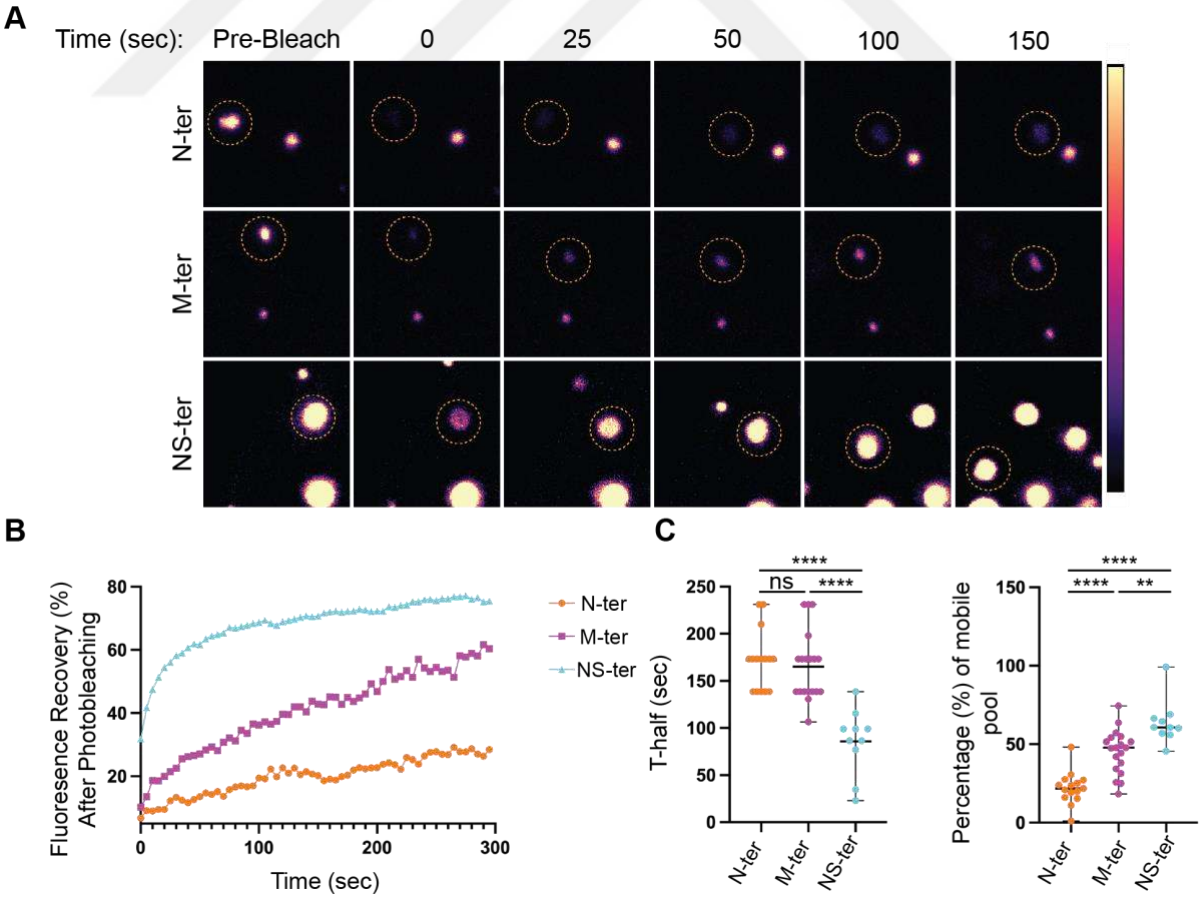

Figure 2.9: The Fluorescence Recovery After Photobleaching experiment of PCM1 fragments that forms granules. **A)** The recovery of the individual granules after photo-bleaching in different time points. Each granules intensity is represented with the LUT images. The intensity scale goes from black to white. The black color represents the lowest intensity whereas the white color represent the maximal intensity that can be seen in a pixel. **B)** The representative experimental recovery curves for each PCM1 fragment chosen from the two technical replicates. **C)** The t-half and mobile pool graphs for each fragment. Among all fragments NS-ter have the lowest t-half and the largest mobile pool. ****P < 0.001 ***P < 0.001, **P < 0.01, *P < 0.05.

To further assess their dynamic behavior, FRAP was performed to see if these granules (1-700 a.a. NS-ter, 1-1200 a.a. N-ter, 700-1200 a.a. M-ter) have active internal/external material exchange with their surrounding environment (Figure 2.9). The N-ter fragment had a minor mobile pool compared to the other two. It showed approximately 22.15% recovery after frapping with and half-life of about 171.9 seconds (Figure 2.9 C). Even though they had a similar half-life, the half-life of the M-ter being 165.2 seconds, the M-ter was more dynamic than the N-ter and showed approximately 45.24% recovery after photo-bleaching. Of all the granule-forming fragments, Ns-ter was the one that showed the highest recovery rate and the shorter half-life. It showed approximately 63.94% recovery with a half-life of nearly 85.93 seconds (Figure 2.9 C) There was no significant difference between the t-half of N-ter and M-ter, whereas the differences between these fragments were NS-ter were highly significant. For the mobile pools, the recovery rates for M-ter and NS-ter were faster and highly significant compared to the N-ter. The mobile pools for M-ter and NS-ter showed significance again; however, it was less significant.

The biochemical assays and the dynamic behavior assessment showed that these granules are heterogenous in terms their phase state, some being liquid-like some being gel-like. They are dynamic in terms of behavior, which might be correlating with the intermediate states of granule assembly or the gelation processes.

2.6. De Novo PCM1 biogenesis assay reveals the disassembly dynamics of PCM1 granules.

Auxin-inducible degron is a degron technology that uses CRISPR-Cas9-based knock-in cell line generation and allows rapid endogenous protein depletion in mammalian cells by using plant hormone auxin. To elucidate the in vivo LLPS properties better in the centriolar satellite granules we applied auxin-inducible degron technology. With the rapid depletion of endogenous protein *in vivo* we aimed to seek the disassembly dynamics. Therefore if there would be a correlation between the behaviour during the disassembly of these granules with the dynamic behaviors that have been reported for membraneless condensates, we would have achieve a more directed diagnostic approach.

For this purpose, the C-terminal of the PCM1 was endogenously tagged with mAID_mClover, on the 9x Myc OsTIR DLD-1 parental cell line. The plasmids and gRNAs were designed by the Gergely Lab and cell line was established in our lab. Genomic DNA PCR validated the biallelic tagged cell lines (Figure 2.10A) For the disassembly dynamics, live cell imaging was performed before and after auxin addition to the system. During disassembly, it was observed that bigger condensates were broken into smaller condensates over time until no condensate was left that could be detected microscopically (Figure 2.10B) .

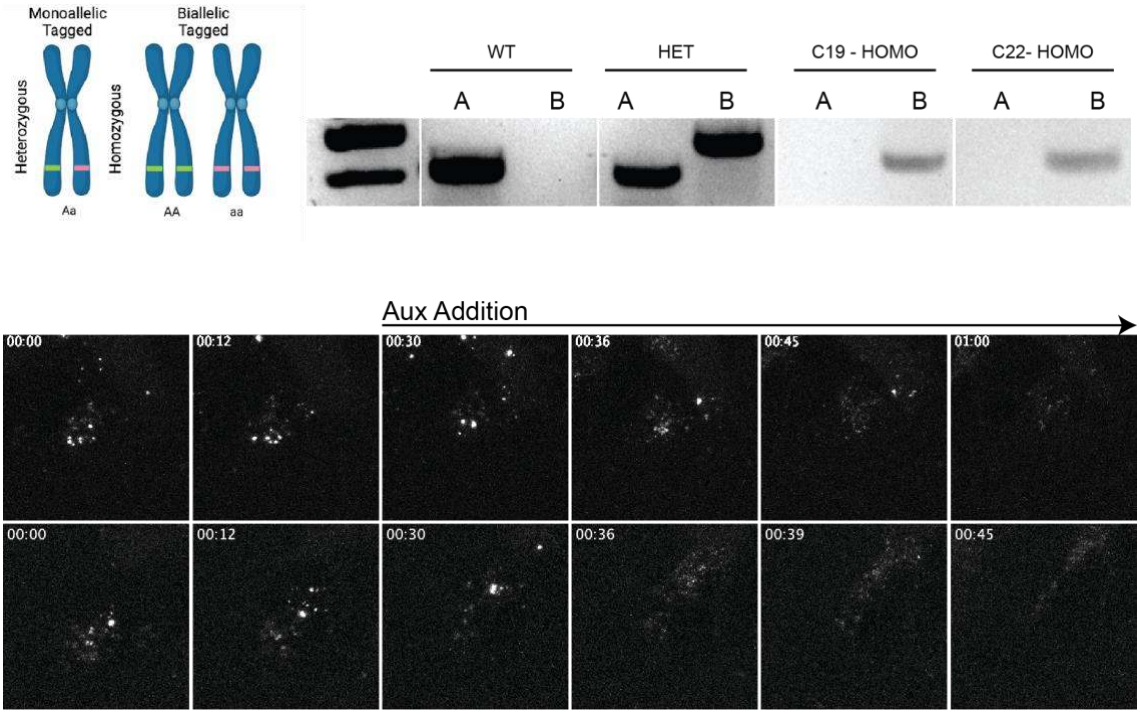

Figure 2.10: The 9x Myc OsTIR1 DLD-1 parental cell line development and validation to perform auxin based degron technology for rapid protein depletion. **A)** The DLD1 PCM1 mAID_mClover genomic DNA PCR to determine the biallelic tagged colonies. **B)** The auxin depletion experiments on the PCM1. After auxin addition in 30th minute the PCM1 granules dissolved individually.

CHAPTER 3: MATERIALS AND METHODS

3.1. Cell Culture and Transfection

U2OS WT, HEK293T U2OS PCM1 -/- cells (Gurkaslar et al., 2020) were grown in the DMEM (PAN Biotech, Cat#P04-03590) supplemented with 10% fetal bovine serum (FBS; PAN Biotech, Cat#P30-3306). 9x Myc OsTIR parental DLD-1 cells (gift from Andrew Holland) were grown in the DMEM supplemented with 10% FBS and %2 L-glutamine (Capricorn). C2C12 cells were grown in a growth medium supplemented with 20% FBS. For the C2C12 differentiation, the medium was used containing %5 Horse Serum (HS) and 2% insulin. The cells were differentiated for five days with medium changes every 12 hours. All cells were cultured at 37 °C and 5% CO_2

For transient transfections, U2OS WT, U2OS PCM1 -/-, and HEK293T cells, were transfected with plasmids using PEI, and samples were allowed for 24 hours.

For the generation of PCM1, C-ter tagged mAID_mClover knock-in line 9x Myc osTIR DLD-1 parental cell line was transfected in 6 well plates with donor plasmid (HA-PCM1-Cter-mAID-mClover-HygR) (gift from Fanni Gergely) and gRNA (gift from Fanni Gergely) by using FuGene according to manufacturer's instructions. On day one, cells were expanded to a 10 cm plate. On day two, selection with hygromycin was started, and the cells were selected with 700 µg/mL Hygromycin B (Gold Bio, Cat# H207/1) for 2-3 rounds. Single-cell dilution was performed, and colonies were screened.

3.2. Cell Line Validation

To isolate the genomic DNA from the DLD-1 cell lines ONE-4-All Genomic DNA Mini-Preps kit (BioBasic) was used with the protocol provided with the kit. Two primer sets performed the genomic DNA PCR, one for detecting the endogenous allele and one for the modified locus. For the endogenous PCM1 the PCM1_fwd: TTTTCAGAAACGGTGGGAGC and PCM1_rev: CTTCTCTGAGCAGCCTAAAGT were used. For the modified locus PCM1_fwd: TTTTCAGAAACGGTGGGAGC and mAID_rev: ACCATCACGTTCTTCCGGTA primers were used. The genomic DNA PCR products were run on 3% agarose gel and visualized with E-Box, Gel Documentation Imaging.

3.3 Cloning

PCM1 (NM_006197), full-length cDNA, was amplified from pEGFP-N1-PCM1. All the PCM1 fragments used in this study were amplified with PCR from PCM1 full-length cDNA and cloned into pDONR221 using Invitrogen Gateway Cloning. pMN444

pEF5-FRT-LAP DEST plasmid (gift from M. Nachury) was used for the Gateway recombination reactions. All plasmids were verified by DNA sequencing. For the PCM1 C-ter knock-in the PCM1_exon39_fwd: CACCGTGAAGACATCTCATATACTC and PCM1_exon39_rev: AAACGAGTATATGAGATGTCTTCAC gRNA's were designed. The oligo cloning performed was cloned into pX330 backbone with BbsI restriction sites into pX330 backbone.

3.4. Cell Treatment

For the 1,6-hexanediol (ChemCruz, Lot#B2519) experiments, the chemical was dissolved in the medium at 5% concentration and given to the cells for 5 minutes. The cells were fixed after the treatment.

For RNAse A (Neofroxx, Cat#1263) treatment, the cells were washed with 1xPBS three times, 1x Cytoskeletal Buffer (CB Buffer) buffer (10 mM PIPES, three mM MgCl2, 100 mM NaCl, 300 mM sucrose, pH 6.9) for three times and incubated at room temperature for 5 minutes with 0.5% Tween-20. The coverslips were washed with 1x PBS once more and treated with 5 mg/mL RNAse A for 15 minutes at 37°Cdegrees. The samples were fixed with 100% cold methanol at -20°C for 15 minutes and stained for immunofluorescence.

For auxin depletion experiments Indole-3-acetic acid sodium salt (Sigma-Aldrich, Cat# I5148-2G) was used. The auxin was added to the media of the cells with 500 uM final concentration.

3.5.Immunofluorescence and Expansion Microscopy

For imaging, cells grown on the coverslip were washed three times with 1X PBS, fixed in ice-cold methanol for 15 minutes at -20°C, and rewashed three times with 1X PBS for rehydration. The cells were blocked with PBS-BT at RT for 1 hour. Primary antibody incubation was performed at RT for 1 hour, followed by a three-time 1x PBS wash. The cells were incubated with secondary antibodies and DAPI (Thermo Scientific, Cat# D1306) at 1:5000 for one hour at room temperature. Coverslips were mounted with Mowiol.

U-ExM was performed as previously described (Gambarotto et al., 2019). Briefly, U2OS WT cells and C2C12 cells were seeded to coverslips. The C2C12 cells were expanded after five days of differentiation. The seeded coverslips were treated with 1.4%

formaldehyde (FA, 36.5– 38%, F8775, Sigma-Aldrich) / 2% acrylamide (AA, 40%, A4058, Sigma-Aldrich) (2X FA / AA) solution in 1X PBS for 5 hour at 37°C. Later on, for gelation, the monomer solution was supplemented with tetramethylethylenediamine (TEMED, 17919, Thermo Scientific) and ammonium persulfate (APS, 17874, Thermo Scientific) was prepared, and the coverslips were incubated for 1 hour at 37°C. After gelation, denaturation was performed with denaturation buffer for one and a half hours at 95C. The denatured gels were stained with primary antibodies at 37°C for 3 hours, followed by a 3x10 minute wash by 1X PBS with 0.1% Triton-X at RT. Secondary antibody incubation was done for two and a half hours at 37°C, followed by the same wash steps. The gel expansion was done in 3 rounds in 150 mL dH2O.

3.6. FRAP module and Analysis

The FRAP module of the Leica SP8 confocal microscope was used for the FRAP experiments. The cells were kept at 37°C with 5% CO2 during imaging while incubated at 10%FBS in DMEM. For bleaching, the PCM1 fragment condensates a z-stack of 6 µm with 1 µm step size and 9.00 zoom was used; for the bleaching, 20 iterations were done in 488 Argon laser with 100% laser power. The maximal projections of each file were taken in Leica LAS X software, and the files were analyzed in ImageJ. The quantifications were done according to the equations in (Sprague et al., 2004) .

3.7. Live Cell Imaging

For the live cell imaging to assess the granule dynamics of PCM1 fragments, Leica SP8 confocal microscope was used in the TC-S module. The cells were incubated at 10% FBS in DMEM and kept at 37°C with 5% CO2. The images were taken at 3 minute per frame frequency for auxin depletion with 1 µm step size , 9 µm stack size and in 2.00 zoom in 512x512 pixel format. Mark and find experiment were defined for specific position acquirements with 63x HC PL APO CS2 63x 1.4 NA oil lens.

3.8.Antibodies

The anti-PCM1 N-ter (Rb), anti- PCM1 C-ter (Rat) (recognizes 700-1200 a.a.) and anti-GFP (Rb) antibody was generated as previously described (Firat-Karalar et al., 2014). The antibodies were used in 1:2000 concentration for immunofluorescence. The other primary antibodies used in this study is G3BP1 (Rb) (Proteintech, Cat#13057-2-

AP) 1:500, Actub (IgG2b) (Sigma, 6-11B-1) 1:1000, rabbit anti PCM1 (Proteintech, 19856-1-AP) at 1:500, LaminB (Rb) (Proteintech) 1:500.

3.9. Statistical tests

For the statistical significance Student's t-test were applied by using the Prism Software (GraphPad Software, La Jolla, CA). The error bars shows the SD. The asterisk placeholder's for the P-values reflected by following key: ****P < 0.001 ***P < 0.001, **P < 0.01, *P < 0.05.

CHAPTER 4: DISCUSSION

The biochemical characterization of the PCM1, the main scaffolding protein of centriolar satellites, revealed that the protein is evolutionarily conserved within the vertebrate taxa with minor changes and has many IDRs. The protein confirmation does not have a definitive 3D structure caused by electrostatic interactions in IDR domains, meaning that other factors might have a role in granule formation for centriolar satellites. The protein conformation of the PCM1 does not change upon differences in protein localization, it acts as granular around the centrosome. Similarly, it still has granular behavior in the ncMTOCs even though it localizes around the nuclear lamina; it strengthens the hypothesis that the 3D confirmation relies on other factors such as the other binding partners. The assumption was that the IDRs of this protein could lead to liquid-liquid phase separation that allows compartmentalization of different centriolar satellite proteins in time and space. Our findings have shown that endogenous PCM1 has a mainly gel-like or solid-like state with the possibility of having a subpopulation with liquid-like properties. Since the centriolar satellites are much smaller in size and more prominent in the number of granules compared to the other membrane-less organelles reported in the literature, such as stress granules, Cajal Bodies, and P granules; the size and number of the granule per cell were not quantified. There might be younger granules that have not solidified over time which might be dissolved upon 1,6-hexanediol addition. Global RNA depletion experiment's revealed that RNA presence helps with the PCM1 integrity; therefore, these granules might have a stable RNA core that helps with the granule formation, such as stress granules.

Three different fragments of PCM1 formed granules, which shows that the domains in the N-terminal of the protein have a role in the granule formation. Since the fragments in the C-terminal of the protein act cytoplasmic, this data also focuses on the importance of the N-terminal for granule formation. These fragments, which form granules, are referred to as N-ter (1-1200 a.a.), M-ter (700-1200 a.a.), and NS-ter (1-700 a.a.) and have different behaviors. They act the same as the endogenous PCM1 and are maintained after 1,6-hexanediol treatment. Like the endogenous PCM1, these fragments might have a subpopulation that acts liquid-like, which might be gelified over time. Therefore, it will be essential to note that the maturation might affect these granules in their phase state.

N-ter and M-ter form granules in WT and PCM1 -/- cells despite the protein concentration. For this case, endogenous PCM1 does not have an additive effect on the granule formation. However, for the NS-ter and the PCM1-FL, two different localization patterns have been observed, both granule formation and cytoplasmic expression. The NS-ter forms granules upon reaching a certain protein concentration, and the threshold needed for granule formation significantly differs from cytoplasmic expression. It does not have a chance in WT or PCM1 -/- cells.

On the other hand, PCM1-FL has the opposite behavior. The protein concentration needed for the cytoplasmic expression is higher, indicating that low protein expression levels are required for granule formation. Also, endogenous PCM1 has an additive effect on this phenomenon since it will increase the present protein concentration. It is also important to highlight that the granules formed by these three fragments are much bigger than the endogenous PCM1. This might be because of the lack of the C-terminal in these fragments. This shows that the very C-terminal end of the PCM1 might have a role in the size and number regulation of these granules. Since NS-ter and PCM1-FL showed different behaviors upon the increasing protein concentration, it also indicates that the C-terminal of the protein might also have a possible role in the cellular processes that helps with the regulation of protein expression levels. Endogenous PCM1 must be held at a critical concentration to preserve granular integrity. Overall, these results suggest heterogeneity in the architecture of these granules, along with the heterogeneity in their phase properties.

The live cell imaging of these granules showed that they have dynamic behavior and do fusion, fission, and kis&split events. Since these granules are insensitive to 1,6-hexanediol treatment, indicating that these assemblies are gel-like or solid-like, there might be several explanations for their dynamicity. First, the mobile granules might be intermediate assembly products still in a liquid state. Second, the liquid-to-gel transition might take longer; we do not know these granules' possible maturation and the aging process. Finally, there might be heterogeneity in the phase state of the granules; a subpopulation of the granules might be liquid-like, whereas others have gel or solid-like behavior. Therefore even though there is no granule disassembly upon hexanediol treatment, their dynamicity can be explained further by other reasoning.

Similarly, FRAP experiments revealed that these granules have lower recovery rates than the other membrane-less organelles. This also aligns with the dynamic behavior since most of the population indicated that these assemblies are gel-like. It would be expected to see low recovery rates and mobile pool; since the internal/external exchange, and granule formation would also take a period. Also, it is essential to note that seeing recovery shows that they are not aggregates but condensates. Yet, their physiological importance to the cells was not in the scope of this research.

The *de novo* PCM1 biogenesis essay done by the AID technology has revealed the dynamics of the granule disassembly. Since the endogenous PCM1 granules have the same dynamic behavior as the other membrane-less organelles, the disassembly process would also give a better insight into the phase separation properties of the centriolar satellites. The overexpression of the PCM1-FL has shown that the protein in high concentration shows cytoplasmic expression tendency, whereas granule formation is only seen in lower expression cases. With the decreased protein concentration over time, more prominent granules have broken into smaller ones until no granule that can be seen microscopically was left. In line with these results, the granules are preserved with the decreasing concentration of the protein. Multistep action mechanisms have been proposed for the disassembly of other membrane-less organelles such as stress granules. Similar to what we have observed in the PCM1, more prominent stress granules also disassemble into smaller ones. The findings in stress granules indicate that this multistep action process might be caused by structural instability caused by RNA titration (Wheeler et al., 2016) . The studies in PCM1 -/- cells (Odabasi et al., 2019) and PCM1 depletion phenotypes show that the satellite granules are dispersed when the PCM1 is no longer present. During disassembly with the titration of PCM1, we have also seen that the satellite granules are not structurally intact anymore. This aligns with the literature and again provides the importance of the PCM1 for satellite architecture and integrity. Overall, the evolutionary analysis, overexpression phenotype, and disassembly dynamics show significant importance of the PCM1 to form centriolar satellite granules in the cells.

For a better assessment of the centriolar satellites, some questions still need to be assessed. We have shown that these granules are sensitive to RNA depletion, so using techniques such as FISH, the RNA presence in these granules should be sought. Also, by purifying the endogenous PCM1 granules *ex vivo* , possible mRNA candidates can be

found with transcriptomic analysis. Further, the presence of these mRNA candidates can be assessed by more precise techniques such as smFISH. An inducible line can be created to understand the maturation and gelatification over time. By this means, we would also see the behavior of endogenous PCM1 and the granule forming PCM1 fragments over a period and better understand the liquid-to-gel-like transition. This line would also help determine the critical protein concentration more quantitively. Lastly, *in vitro*, PCM1 condensate assays should be done with purified PCM1-FL to see the physiological properties. With the purified protein, we would also have more details about the possible drivers of the granule formation.

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