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Identification and Characterization of New Players Involved in the Centriole Maintenance Program

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If you want to go fast, go alone. If you want to go far, go together.

Abstract

Organelle inheritance and its persistence is essential not only upon cell division, but also differentiation, fertilization and disease. Amongst those, a structure that always raised much interest is the centrosome. The centrosome is the primary microtubule-organizing centre (MTOC) in animal cells, regulating cell shape and polarity in interphase, and spindle pole organization during mitosis. The centrosome is formed by two orthogonally arranged centrioles that are surrounded by a matrix of proteins called the pericentriolar material (PCM), which is essential for microtubule (MT) nucleation. Control of centriole number and function is very important, and dysfunction of these organelles is associated with a variety of human diseases. Despite being resistant to MT depolymerization and instability, centrosomes are eliminated from the oocytes from most metazoan species and can be inactivated in differentiated cells, suggesting that centrosomes are not intrinsically stable.

It is not completely understood how centrosomes are eliminated or inactivated. However, a recent study in *Drosophila melanogaster* proved the existence of a centriole maintenance program. This program ensures centriole stability through PCM maintenance, which protects the centrosome, and is mediated by Polo kinase. To allow centriole elimination in the oocyte, this program is "shut down". Perturbing this program prevents centriole loss and leads to female sterility.

In order to identify new players involved in the centriole maintenance program, we performed a RNAi screen in *Drosophila* cultured cells. We chose candidate proteins that were described as components of the different modules of the centrosome and depleted them in S-phase arrested cells. We showed that while removal of some of the modules of the centrosome strongly destabilize the centrosome, others do not have a major effect in centrosome destabilization.

Out of all the candidate proteins tested, the centriolar wall protein Ana1 showed the strongest effect on centrosome destabilization. We show that depletion of Ana1 in *Drosophila* oogenesis impairs PCM accumulation mediated by Polo, suggesting that this protein has a key role in the centriole maintenance program.

Together, these results allowed us to identify the principal structural features that contribute to centrosome stability and also brought us new insights into the centriole maintenance program. However, further studies are needed to understand how this program is regulated which is important not only for successful sexual reproduction, but also for centriole life span and its impact on different tissues in homeostasis and disease, shaping the cytoskeleton.

Keywords: Centrosome, *Drosophila melanogaster*, Centrosome Stability, RNAi screen, Ana1.

Summary (Portuguese)

O centrossoma é, em células animais, o principal centro organizador de microtúbulos (COMT). Em interfase regula a mobilidade, adesão e polaridade celulares, enquanto que em mitose participa na formação e organização do fuso mitótico.

Cada centrossoma é composto por dois centríolos. Os centríolos são estruturas cilíndricas formadas por nove microtúbulos (organizados em singletos, dupletos ou tripletos) dispostos de forma radial em redor de uma estrutura central (a *cartwheel*). Um dos centríolos do par é denominado de centríolo-mãe, enquanto que o outro, ortogonalmente disposto em relação ao primeiro é denominado de centríolo-filho. Um centríolo tem aproximadamente 200nm de largura e pode possuir entre 200 a 500nm de comprimento, dependendo do tipo de célula. Em conjunto ambos os centríolos recrutam e participam na organização de uma matriz proteica densa - o material pericentriolar – que os rodeia e é responsável pela nucleação de microtúbulos. Finalmente, em fases específicas de diferenciação, os centríolos associam-se à membrana celular para formar a corpo basal, a partir do qual crescerá o cílio ou flagelo da célula.

A simetria radial dos centríolos é extremamente conservada nos diferentes ramos da árvore filogenética, porém a estrutura centriolar pode variar ligeiramente de espécie para espécie. Em Humanos os centríolos são compostos por nove tripletos de microtúbulos, ao passo que os centríolos em *Drosophila melanogaster* são formados por nove dupletos de microtúbulos. Em algumas espécies os centríolos-mãe possuem apêndices distais e sub-distais que permitem a ligação do centríolo à membrana celular e a ancoragem de microtúbulos, respetivamente.

O número de centríolos presente nas células é altamente controlado, e a sua duplicação é regulada tanto temporalmente - em coordenação com a replicação do DNA - como espacialmente - apenas um centríolo-filho se forma junto a cada centríolo-mãe por cada novo ciclo celular. Dependendo da fase do ciclo celular em que se encontra, a maioria das células tem um (fase G1) a dois centrossomas (fase S, G2 e M) (dois a quatro centríolos). Alterações no número de centríolos estão associadas a diversas doenças como o cancro, microcefalia ou ciliopatias.

Diferentes estudos demonstram que os centrossomas são resistentes a tratamentos de despolimerização de microtúbulos. Consequentemente, estes organelos são descritos como estruturas altamente estáveis; no entanto, várias observações têm demonstrado que os centrossomas não são estruturas intrinsecamente estáveis como inicialmente se pensava. Em diversos sistemas celulares estes organelos podem ser eliminados ou inativados, como na gametogénese feminina ou em células diferenciadas, respetivamente.

A eliminação de centrossomas durante a oogénese é comum na maioria dos metazoários, equinodermes e molúsculos. Porém, enquanto que no caso dos metazoários a eliminação dos centrossomas ocorre antes das divisões meióticas, em equinodermes e molúsculos estas estruturas só desaparecem durante as divisões meióticas. A eliminação dos centrossomas durante a gametogénese feminina implica que o embrião dependa do centríolo fornecido pelo esperma para que ocorram as primeiras divisões celulares no embrião, após a fertilização.

A eliminação de centrossomas na oogénese é um processo biológico muito importante e o conhecimento dos mecanismos que o regulam é ainda muito escaço. Um estudo publicado recentemente pelo nosso grupo desvendou um mecanismo de regulação da eliminação de centrossomas durante a oogénese em *Drosophila*. Em *Drosophila* a oogénese inicia-se no germário com a divisão assimétrica de uma célula estaminal que dá origem a uma nova célula estaminal e um cistoblasto. O cistoblasto sofre quatro divisões consecutivas com citocinese incompleta formando um grande cisto composto por dezasseis células em que apenas uma dará origem ao oócito, sendo que as restantes são denominadas de *nurse cells.* Nesta fase, os centrossomas das *nurse cells* agrupam-se e migram para o oócito, formando um grande COMT que se desintegra ao longo da oogénese, e que portanto, não participa na formação

do fuso meiótico. No referido estudo observou-se que durante a oogénese a cinase Polo (Polo like kinase em humanos) é deslocalizada do centrosoma. Sendo a Polo responsável pelo recrutamento do material pericentriolar, também este é perdido durante o processo. Consistentemente, a remoção da Polo dos centríolos do oócito, leva à perda prematura de centrossomas durante a oogénese, ao passo que a expressão ectópica desta proteína com a sua localização forçada nos centríolos do oócito, leva à retenção de centrossomas até à fase de divisão meiótica, com acumulação de proteínas do material perientriolar. Esta retenção anormal de centrossomas nos óocitos levou à falha do desenvolvimento embrionário após a fertilização.

Os resultados obtidos neste estudo e reproduzidos com sucesso em células de *Drosophila,* sugerem que as proteínas que formam o material pericentriolar, auxiliadas pela *Polo,* funcionam como um escudo protetor à volta dos centríolos impedindo a sua destabilização, o que pressupõe a existência de um mecanismo de manutenção da estabilidade dos centríolos. Em situações em que os centrossomas são eliminados, como nos oócitos, este mecanismo de estabilização é desativado: a *Polo* e as proteínas do material pericentriolar deixam de ser acumuladas no centrossoma, levando à sua destabilização e consequente eliminação. De forma semelhante ao que acontece na oogénese em *Drosophila,* em células diferenciadas - como o músculo esquelético, neurónios ou células epiteliais - a inativação dos centrossomas também parece ser realizada através da remoção das proteínas do material percientriolar, sugerindo que o mecanismo de estabilidade de centrossomas não é exclusivo para a oogénese, mas sim universal. Paralelamente, o mecanismo que permite a estabilização dos centrossomas mediado pelas proteínas do material pericentriolar e pela Polo permanece pouco claro.

As proteínas do material pericentriolar poderão funcionar como um escudo que impede: (1) o acesso de proteínas que possam destabilizar os centríolos; ou (2) a saída de proteínas que contribuem para a estabilidade dos centríolos.

De forma a identificar proteínas envolvidas no mecanismo de estabilização dos centríolos foi realizado um rastreio (*screen*) por RNA de interferência (RNAi) em células de *Drosophila melanogaster* bloqueadas na fase S durante oito dias. O bloqueio nas células em fase S impede o funcionamento do ciclo de duplicação de centríolos permitindo desacoplar estabilidade e biogénese centrossomal. Desta forma, o numero de centríolos é mantido constante, o que permite tirar vantagem para analisar o efeito (d)estabilizador das diferentes proteínas testadas.

As células foram transfectadas com RNAi's específicos para diferentes proteínas (proteínas candidatas) que foram descritas como fazendo parte da estrutura do centrossoma. Foram depletadas proteínas do material pericentriolar, proteínas centriolares como as da *cartwheel*, da parede centriolar e da zona distal do centríolo (*cap*) e ainda a *Polo like kinase 4* (Plk4), a principal reguladora da biogénese dos centrossomas. O efeito que a remoção destas proteínas provocou na estabilidade dos centrossomas foi analisado através do número de centríolos por célula utilizando ensaios de imunofluorescência, no qual os centrossomas foram detetados utilizando anticorpos específicos para diferentes proteínas de diferentes partes/módulos do centrossoma. Os resultados obtidos foram comparados com um controlo (*mCherry* RNAi) e sempre que o número de centríolos era reduzido comparativamente ao controlo foi considerado que a proteína era importante para a estabilização do centrossoma.

Os nossos estudos sugerem que os módulos que contribuem significativamente para a estabilidade dos centrosomas incluem proteínas do material pericentriolar, da parede centriolar e da *cartwheel*. Por outro lado, as proteínas da *cap* e Plk4 não parecem apresentar um papel relevante na estabilidade do centrossoma. De todas as proteínas candidatas testadas, a proteína da parede centriolar Ana1 revelou um dos fenótipos de perda de centríolos mais forte. Assim, prosseguimos com o estudo desta proteína *in vivo*. Simultaneamente, expressámos ectopicamente a Polo e depletámos a Ana1 nos centrossomas de óocitos de *Drosophila.* Observámos uma redução na manutenção dos centríolos em estádios avançados da oogénese sugerindo que a Ana1 apresenta um papel extremamente importante na estabilidade dos mesmos.

Em conjunto, este estudo demonstra a importância das proteínas do material pericentriolar, da *cartwheel* e da parede centriolar para a manutenção e estabilização do centrossoma. Foi ainda possível identificar uma proteína que poderá ter um papel preponderante no mecanismo de estabilização dos centríolos. Contudo, é necessário investigar qual o papel dos diferentes módulos do centrossoma (o material pericentriolar, a *cartwheel* e a parede centriolar) na sua estabilização e compreender se estes têm funções específicas ou redundantes. É igualmente importante perceber qual o papel da Ana1 no mecanismo de estabilização dos centríolos, assim como a sua relação com a *Polo* e com as proteínas do material pericentriolar. Apenas o conhecimento detalhado do mecanismo que confere estabilidade aos centrossomas nos permitirá atuar sobre este mecanismo quando desregulado em doenças como o cancro.

Palavras-chave: Centrossoma, *Drosophila melanogaster*, Estabilidade Centrossomal, RNAi *screen*, *Ana1*.

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List of abbreviations

% - percentage
µg – micrograms
µL – microliters
µm – micrometers
aMTOC – acentrosomal Microtubule Organizing Centre
Ana – Anastral
Aph - Aphidicolin
Asl – Asterless
Bld – Bald 10
BSA – Bovine Serum Albumin
CDK1 - Cyclin-Dependent Kinase 1
CDK5RAP2 – Cycling Dependent Kinase 5 Regulatory Subunit Associated Protein 2
cDNA – complementary DNA
Cep – Centrosomal protein
Cnn - Centrosomin
CP – Centrosomal protein
CPAP – Centrosomal protein 4.1-associated protein
ddH_2O – Double-Distilled water
DMEL – Drosophila melanogaster cultured cells
DNA – Deoxyribonucleic acid
D-Plp – Drosophila pericentrin-like protein
dsRNA - double-strand RNA
Eg5 – Kinesin-5
EM – Electron Microscopy
FBS – Fetal Bovine Serum
GFP – Green Fluorescent Protein
GPCR – G-Protein Coupled Receptor
HU – Hydroxyurea
Klp – Kinesin like protein
MAP – Microtubule Associated Protein
MCPH - Autosomal Recessive Primary Microcephaly
$MgCl_2$ – Magnesium Chloride
$MgSO_4$ – Magnesium Sulphate
mL – milliliter.
mM – millimolar
mRNA – messenger RNA
MT – Microtubule
MTOC – Microtubule Organizing Centre
NA – Numerical aperture
Nap – Nucleosome assembly protein
Nek – NIMA Related kinase
PACT - Pericentrin-AKAP-450 Centrosomal Targeting
PCM – Pericentriolar Material
PCNT – Pericentrin
PCR – Polymerase Chain Reaction
PFA – Paraformaldehyde
Plk – Polo-like Kinase
PPI γ - Protein Phosphatase γ

PSG - Penicilin-Strepnomicin-L-Glutamine
Px – pixels
RNA – Ribonucleic acid
RNAi - RNA interference
Roi - region of interest

RT – Room Temperature
RT-PCR – Reverse-Transcriptase PCR
SAC - Spindle Assembly Checkpoint
SAK – Snk/Plk-akin kinase
Sas – Spindle assembly
SIM – Structured Illuminated Microscopy
siRNA – Silencing RNA
Spd2 – Spindle defective 2
STIL - SCL/TAL -1-interrupting locus protein
UAS - Upstream Activating Sequence
γ-TuRC - γ-Tubulin Ring Complexes

1. Introduction

1.1. Cytoskeleton

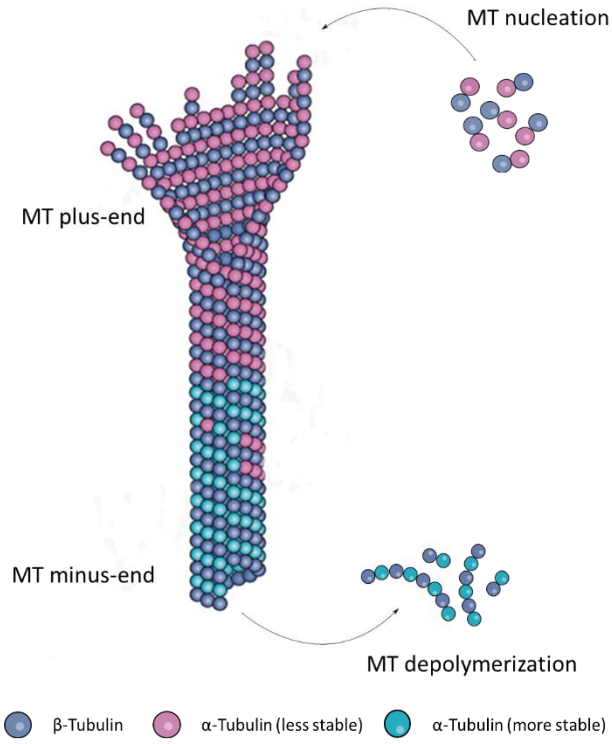
1.1.1. Function and key components

Cells depend on the cytoskeleton to allow not only spatial organization and transport of its contents, but also to physically and biochemically connect them to the external environment. Despite being frequently denominated as "the skeleton of the cell", the cytoskeleton is a dynamic and adaptive structure whose component polymers and regulatory proteins are in constant flux[1]. Upon externally applied forces and chemical signals, the cytoskeleton can reorganize its components and generate directed forces that stimulate changes in cell shape and enable its movement[1]. In Eukaryotic cells, the cytoskeleton is formed by three distinct classes of protein filaments: microtubules (MTs), actin filaments and intermediate filaments. As my thesis work is related with the centrosome, an organelle built by MTs, I will focus on this class of protein filaments.

1.1.2. Microtubules

MTs are the stiffest of the three protein filaments mentioned above. MTs have been implicated in a wide range of cellular processes. These structures have been implicated in the regulation of intracellular transport, chromosome separation during cell division, cell polarity and morphogenesis of cilia and flagella[2].

MTs are composed of α- and β-tubulin heterodimers that associate to form hollow tubes called protofilaments (Figure 1.1). The protofilaments are polarized structures, they harbour two distinct ends: the plus end, at which β-tubulin is exposed; and the minus end at which α-tubulin is exposed. *In vitro*, with purified tubulin solutions, both, MT plus and minus ends, are able to grow, however at very different rates. While minus ends grow (nucleate) slowly and disassemble (depolymerize) occasionally, the plus ends nucleate faster and disassemble frequently; suggesting that MT minus ends are more stable than its plus ends[2].



Nucleation and disassembly of MTs is controlled by a large number of proteins known as microtubule-associated proteins (MAPs) which can promote or suppress the dynamic behaviour at both ends. Particularly, MT nucleation at the plus ends is mostly dependent on γ-tubulin ring complexes (γ-TuRC). Localization and activity of the γ-TuRC can be regulated by several factors (such as CDK5RAP2/Cep215 in humans, Cnn in *Drosophila*) leading to its recruitment to microtubule organizing centres (MTOCs), such as the centrosome or membrane organelles (nucleus and golgi apparatus)[2,3].

Figure 1.1: Microtubules are dynamic structures composed of α- and β-tubulin. MTs are polarized structures which harbour two distinct ends. MTs' plus-ends nucleate fast and depolymerize slowly while MTs' minus ends nucleate slowly and depolymerize faster. As more α- and β-tubulin monomers are added to the MT's plus-end, α-tubulin monomers get more stable and, as a result, the MT gets stiffer. α-tubulin is represented in pink (less stable) and light blue (more stable); β-tubulin is represented in purple (Adapted from Akhmanova et al., 2015)[2].

1.2. Centrosomes

1.2.1. Centrosomes: the major microtubule organizing-centre in animal cells

In animal cells, the major site of MT nucleation and anchoring is the centrosome. Centrosomes were first described in 1887 by Theodor Boveri who wrote that: *"the centrosome represents the dynamic centre of the cell; its division creates the centres of the forming daughter cells, around which all other cellular components arrange themselves symmetrically."; "The centrosome is the true division organ of the cell, it mediates the nuclear and cellular division*"[4]. Indeed, just as Boveri predicted, centrosomes play a key role in the formation of the spindle poles during mitosis, ensuring correct chromosome segregation. Moreover, these organelles can persist during interphase near the nucleus as (MTOCs) regulating not only cell mobility, adhesion and polarity, but also positioning of MT-associated organelles[5,6].

Since Boveri's observations using light microscopy, scientists focused on studying this structure in detail. The explosion of the study of biological structures by Electron Microscopy (EM) in the 1950's showed that centrosomes are formed by two cylinder-shaped structures arranged orthogonally called centrioles (Figure 1.2A). Centriole structure is highly conserved in different branches of the phylogenetic tree. Their nine-fold symmetry is highly conserved, nevertheless, they can show structural differences among organisms or cell type[7]. The canonical centriole has approximately 200 nm in diameter, however, its length can vary between 200 and 500 nm[5,8]. These structures are surrounded by an electron-dense and protein-rich matrix, the pericentriolar material (PCM) that confers MT nucleating and anchoring capacity to the centrosome[8].

Centrioles are assembled around a cartwheel structure: a central tubule with nine radially-arranged spokes, each one linked to a single MT (singlet). In addition to the simple singlet structure, MTs can be organized in doublets (pairs) or triplets (Figure 1.2B). The two centrioles that form the centrosome can be differentiated according to the time they were formed. The oldest centriole is called the mother centriole while the youngest, formed in the following cell cycle, is called the daughter centriole[9]. The mother centriole can have distal and/or sub-distal appendages, important for docking to the membrane and MT anchoring, respectively (Figure 1.2A)[9,10]. Association of the centriole to the plasma membrane is important to form cilia (for more details see chapter 1.2.4).

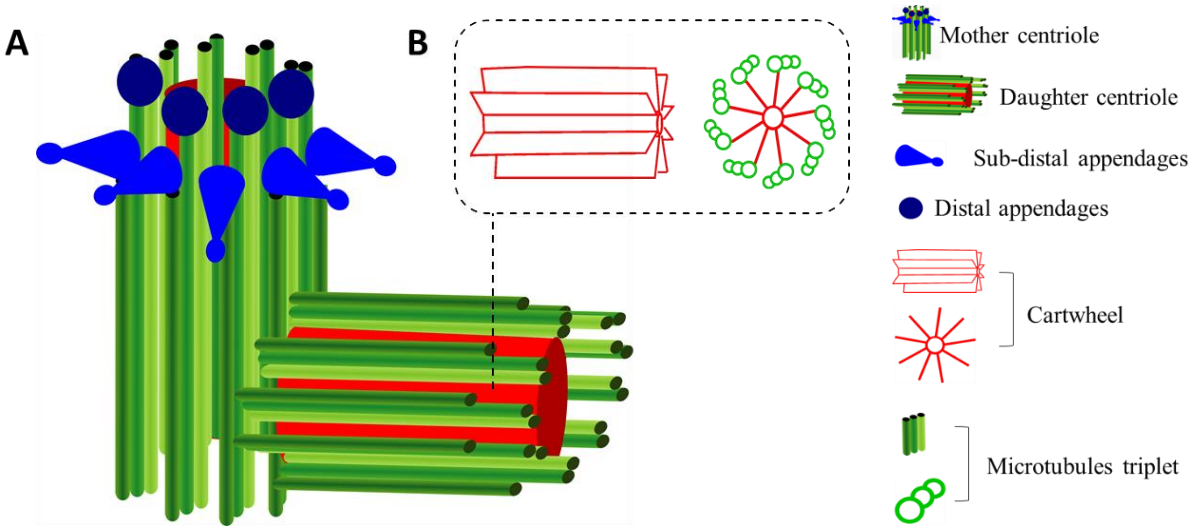


Figure 1.2: A schematic illustration of the centriole structure. (A) In most vertebrate cells new centrioles (also called daughter centrioles) are arranged in an orthogonal fashion close to the oldest centriole (the mother centriole). Daughter centrioles are assembled around a central cartwheel. The mother centriole usually loses its cartwheel and its often decorated with distal (dark blue) and sub-distal (blue) appendages. **(B)** The cartwheel structure is composed by a central tubule with nine-radially arranged spokes. The cartwheel sets the nine-fold symmetric arrangement of the MTs which can be organized in singlets, doublets or triplets.

1.2.2. Centrosome components and its structure

Centrosomes were described more than one century ago but research on these structures only took off recently with the onset of new technologies. Centrosome components have been identified through the years using different techniques such as mass-spectrometry analysis of purified human centrosomes[11,12] and RNAi screens in *C. elegans*[13], *D. melanogaster*[14,15,16] and human cells[17]. Some of the identified proteins have been validated using localization, depletion by RNAi and mutagenesis studies[5,9].

All these studies allowed the identification and characterization of proteins that are involved in centriole duplication: Polo-like kinase 4 (Plk4), CPAP, Sas6, Cep295 or STIL[14,15,18]; proteins required for both centriole duplication and maturation/PCM recruitment: Cep152, Cep135, CP110 and Cep97[16]; and proteins involved in centrosome maturation/PCM recruitment: Cep215, Polo-like Kinase 1 (Plk1), Cep192, Pericentrin (PCNT) and y-tubulin[16].

Super resolution techniques, such as Structured Illumination Microscopy (SIM) allowed to understand how these proteins were organized within the centrosome. Studies in *Drosophila* identified five different zones at the centrosome. The centriolar core can be divided in two different zones: zone I which contains the cartwheel proteins STIL/Ana2 (humans/*Drosophila*) and Sas6 (Figure 1.3 in red); and zone II which holds proteins associated with the MTs that form the centriolar wall (Figure 1.3 in green). Inner PCM proteins are accommodated in the zone III of the centrosome (Figure 1.3 in light orange), while outer PCM proteins can be found at zone IV (Figure 1.3 in dark orange). Finally, zone V contains proteins that are known to be at the distal end of the centriole, also denominated cap (Figure 1.3 in blue)[19]. Table 1.1 shows the arrangement of the main known centrosomal proteins and their known functions. Centrosomes' organization is likely to be conserved as many of the proteins, like Cep135/Bld10, CP110, Cep152/Spd2, Cep192/Asl, γ-tubulin, Cep215/Cnn, CPAP/Sas4 and PCNT/D-Plp, were shown to organize similarly in human centrosomes[20,21] (Table 1.1).

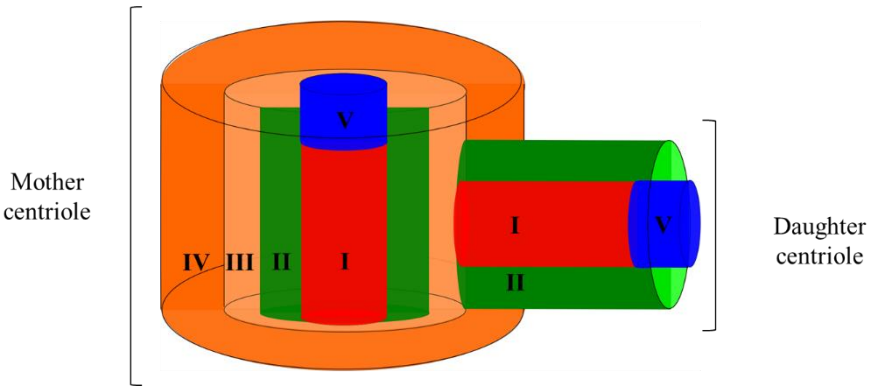


Figure 1.3: Schematic illustration of the *Drosophila* centrosome showing its organization in different zones. Zone I, depicted in red, shows the cartwheel; zone II, in green, represents the centriolar wall; zone III, in light orange, illustrates the inner PCM while zone IV, depicted in dark oranges, shows the outer PCM. Zone V is represented in blue showing the cap. Adapted from Fu et al., 2012.[19]

Table 1.1: Spatial organization of the principal known proteins that form the centrosome. Adapted from Lattao *et al*. 2017)[6]. * Indicates the proteins that show a conserved localization pattern in Drosophila and human cells[20,21].

Module	Zone	Proteins		Function
		Human	*Drosophila*	
Cartwheel	I	SAS6	Sas6*	Centriole duplication
		STIL	Ana2	
Centriolar wall	I/II	Cep135	Bld10*	Centriole elongation
		Cep295	Ana1*	
	II	Cep152	Spd2*	Centriole maturation
		PLK1	Polo	
		α-tubulin	α-tubulin	
	II/III	Cep192	Asl*	
		CPAP	Sas4*	Centriole biogenesis
PCM	III	Plk4	SAK*	
		PCNT	D-PLP*	Centriole maturation
	III/IV	Cep215	Cnn*	
		γ-tubulin	γ-tubulin*	
	IV	Cep152	Spd2*	
		Aurora A	Aurora A	
Cap	V	CP110	CP110*	Centriole elongation
		Cep97	Cep97	

SIM allowed not only to understand the spatially arrangement of proteins in the centrosome, but also to elucidate the architecture of the PCM. The PCM was originally considered to be a disorganized and amorphous pool of proteins, however, it has been shown that PCM proteins occupy specific places around the centriole forming a rigid structure[22,23]. PCM organization can be divided in two layers of proteins. The first layer comprises the D-Plp/PCNT and Asl/Cep152 and connects the centriole core to

the remaining PCM proteins: Spd2/Cep192, Cnn/Cep215 and y-tubulin, which form the second layer (Figure 1.4) [8,22].

1.2.3. Centriole duplication cycle

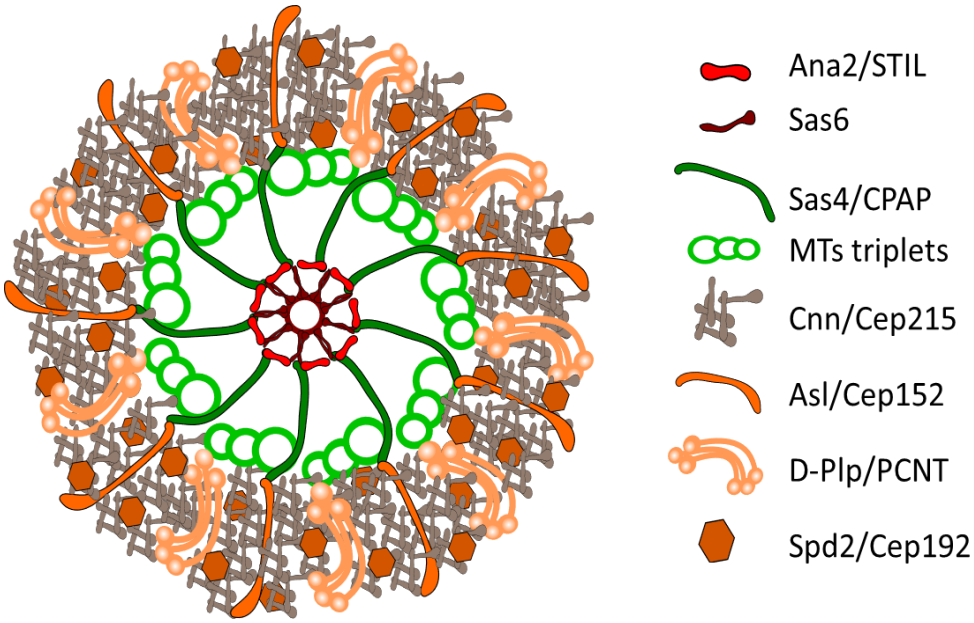


Figure 1.4: Schematic representation of the architectural elements of the PCM at the centrosome. It is possible to distinguish two different layers of organization: (1) the PCM fibres which correspond to the elongated coiled-coil proteins Pericentrin/D-Plp and Cep152/Asl; and (2) a PCM matrix comprising Cep152/Cnn, Cep192/Spd2 and γ-Tubulin (not shown). Adapted from Mennella et al., 2014[8].

The number of centrioles is highly regulated in cycling cells. To ensure the presence of the correct number of centrioles, cells depend on a tightly regulated centriole duplication cycle. This cycle is spatially and temporally coordinated: only one centriole forms near each pre-existing centriole and its formation can only occur once per cell cycle. Centriole duplication is coordinated with the DNA replication cycle. Interestingly, it has been shown that some of the proteins that regulate the DNA duplication cycle are also involved in the centriole duplication cycle. From these proteins, we can highlight three major mitotic kinases: Polo-like kinase 1 (PLK1; Polo in Drosophila), cyclin-dependent kinase 1 (CDK1) and Aurora A, which phosphorylate several components of the centrosome and mitotic apparatus, and also regulate each other[9,24–28].

Newly born cells inherit one centrosome formed by a pair of orthogonally engaged centrioles (Figure 1.5A). The orthogonal arrangement of the centrioles is lost as cells exit mitosis (disengagement) and enter in G1 phase, in a process dependent on PLK1 and the protease separase, which together licence centrioles for duplication in S-phase[29]. However, the two centrioles remain connected through their proximal ends by a linker containing C-Nap1 and rootletin. At this stage Cep152/Asl recruits Plk4/SAK to each centriole[30] (Figure1.5B). By the end of G1, beginning of S phase, Plk4/SAK triggers procentriole formation (daughter centriole) at the proximal end of the each one of the existing centrioles, by recruiting the cartwheel proteins SAS6 and STIL/Ana2 (Figure 1.5C). After the establishment of the procentriole's cartwheel, centriolar MTs start to be added to the structure and centriolar wall proteins like CPAP/Sas4, Cep120 are recruited promoting procentriole elongation (Figure 1.5D). The daughter centriole elongates trough S and G2 phases. Elongation is counteracted by the recruitment of cap proteins like CP110 and Cep97 allowing the procentriole to acquire only an equivalent size to its mother centriole (Figure 1.5E)[30]. Proteins like Cep135/Bld10 or Cep295/Ana1 have also been described as important for procentriole elongation[31,32,33].

At the transition of G2 phase to mitosis each centrosome starts to accumulate PCM in a process called centrosome maturation, which is dependent on PLK1 and Aurora A activation. At this stage Cep152/Asl and CPAP/Sas4 recruit Cep192/Spd2, Cep215/Cnn and γ-tubulin to the centrosome. In human cells, at the exit of mitosis, the mother centriole loses the cartwheel and acquires appendages[34]. Before mitotic entry, more PCM proteins are added to the centrosomes in a process regulated by PLK1/Polo[35]. Phosphorylation of this protein by Aurora A, promotes the recruitment of PCM proteins like Cep192/Spd2, Cep215/Cnn and PCNT/D-Plp[36]. These proteins increase MT nucleation and promoting te formation of the mitotic spindle (Figure 1.5F)[8].

After acquiring PCM components, centrosomes start to separate to ensure spindle bipolarity in mitosis, a process mainly regulated by CDK1. CDK1 phosphorylates and inactivates protein phosphatase γ (PPI γ) which allows Nek2A activation and, therefore, phosphorylation of Nap1 and rootletin, breaking the link that connected the two mother centrioles. After centrosome disjunction, CDK1 phosphorylates Aurora A leading to the activation of the centrosome-associated PLK1/Polo. PLK1/Polo phosphorylates Nek9 and Nek6, promoting phosphorylation of a kinesin-5 subfamily protein (Eg5). This kinesin, which can also be directly phosphorylated by CDK1, promotes the binding of the centrosome to MTs facilitating its movement to the spindle poles (Figure 1.5G)[30,36].

The two centrosomes will form the poles of the bipolar mitotic spindle. As cells usually divide halfway between the two spindle poles, each daughter cell will inherit a single centrosome, each one containing a mother-daughter pair of centrioles[9,30,36].

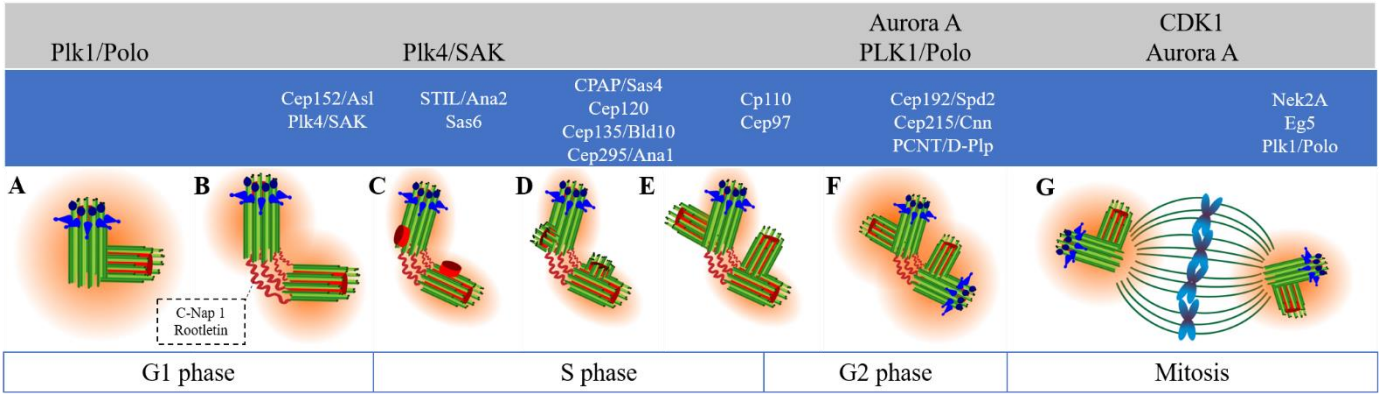


Figure 1.5: The centrosome duplication cycle is coupled with the DNA replication cycle. (A) Newly born cells inherit one centrosome formed by a pair of orthogonally engaged centrioles**. (B)** Centriole assembly is triggered, at the end of G1 phase, by PlK4/Sak and Cep152/Asl. **(C)** In S phase STIL/Ana2 and Sas6 are recruited to each of the centrioles allowing cartwheel assembly. **(D)** Centriole elongation starts right after cartwheel formation and CPAP/Sas4, Cep120, Cep135/Bld10 and Cep295/Ana1 are recruited to the centrosome**. (E)** Between S and G2 phases CP110 and Cep97 are recruited to the daughter centriole to ensure correct centriole length. **(F)** Daughter centrioles start to acquire PCM proteins, such as, Cep192/Spd2, Cep215/Cnn and PCNT/D-Plp. **(G)** Upon mitotic entry, centrosomes disengage to allow the formation of the bipolar spindle and ensure correct chromosome segregation. The major regulators in each phase are shown in grey, while the principal proteins involved in each phase are shown in blue.

1.2.4. The dual life of centrioles: when centrioles become basal bodies

Interestingly, centrioles are not only components of the centrosome; these structures can also function as basal bodies to form cilia. When cells exit the cell cycle, the centrosome can then migrate from the cytoplasm to the apical plasma membrane. There, the distal end of the mother centriole (assisted by its appendages) associates with the ciliary vesicles and docks to the membrane forming the basal body. The basal body acts as a template to nucleate the axoneme which can give rise to a cilium or a flagella[6,10,37].

Cilia can be classified as motile, such as the sperm flagella, or immotile, such as primary cilia which exist in most cells[38]. Most of the motile cilia are formed by nine MT doublets with a central MT pair, and have dynein arms connecting the neighbouring MTs of the axoneme. On the other hand, immotile cilia do not possess dynein arms and are either formed by nine MT doublets or a central MT pair[38] (Figure 1.6).

Primary cilia are approximately 5-10µm in length and extend to the extracellular environment which allows these structures to play important roles as signalling centres. It has been described that primary cilia are able to detect mechanical signalling, such as fluid movement across the cell surface and chemical signals like the concentration of G-protein coupled receptors (GPCRs)[39]. Also, these structures have been proposed to have a role in Hedgehog and Wnt signalling pathways[38]. Moreover, it was observed that primary cilia are present in retinal photoreceptors and are packed with phototransducing pigments, such as rhodopsin, suggesting that primary cilia are also able to detect light (Figure 1.6)[39].

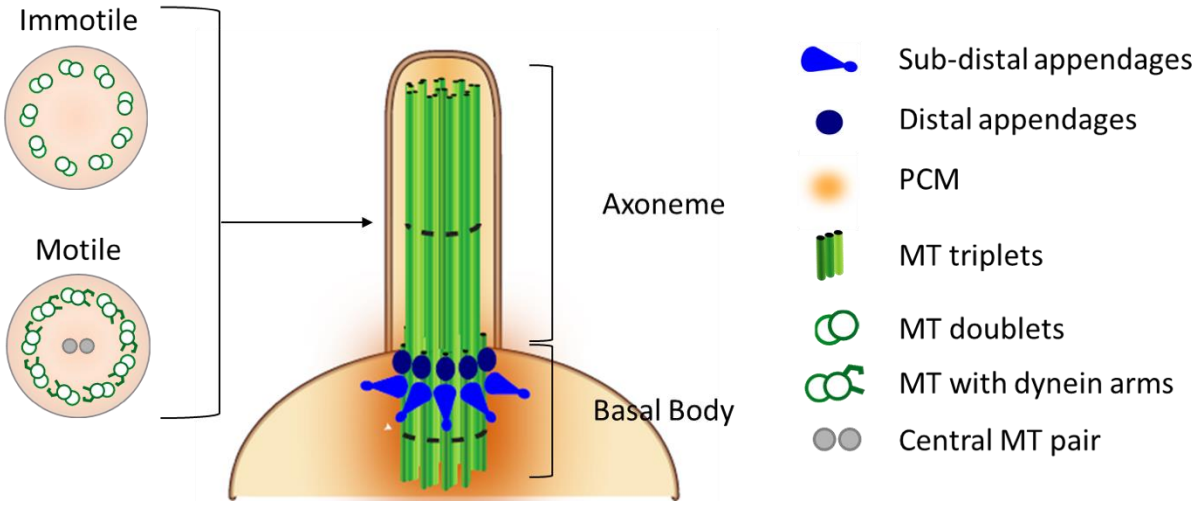


Figure 1.6: The cilia structure. Centrosomes can migrate and tether to the plasma membrane forming the basal body. The basal body serves as a template for the growth of the axoneme which is the skeleton of the cilia or flagella. Motile and immotile cilia are composed by MTs doublets arranged in a nine-fold symmetry. However, while most of the motile cilia possess a central MT doublet and depend on dynein arms to connect the MTs, immotile cilia can either have the central MT doublet or not and do not possess dynein arms.

1.2.5. Centrosomes, centrioles and basal bodies in human disease

Centrosome number and assembly in cells is tightly controlled. Most cells either have one or two centrosomes (two to four centrioles) according to its cell cycle phase. However, not only numerical but also structural centrosome aberrations have been implicated in several human diseases like cancer, microcephaly and ciliopathies.

Centrosome aberrations and cancer

Cancer cells frequently show centrosomes that have defects. Numerical abnormalities, such as centrosome amplification, are perhaps the best characterised centrosomal defects in cancer[38]. It has been proposed that supernumerary centrosomes are a consequence of overduplication (loss of cell cycle control) or division failure[40]. However, cells can cope with excessive numbers of centrosomes either by clustering these into two MTOCs during mitosis[38,41], which allows cells to undergo a bipolar division. These mechanisms allow cells with supernumerary centrosomes to survive, however, these cells frequently show chromosome instability and segregation errors. Cells with abnormally high numbers of centrosomes, that are able to undergo mitosis, frequently show chromosome attachment errors which

are not sensed by the spindle assembly checkpoint (SAC). Therefore, these cells can show lagging chromosomes leading to loss of a fragment of a chromosome or whole-chromosome aneuploidy, promoting tumorigenesis[42,43]. For instance, a population that has lost a fragment or a chromosome that contained a tumour suppressor gene can have a proliferative advantage[38,44]. Although it is still unclear whether centrosome defects are a consequence of mitotic abnormalities or if they actively contribute to tumorigenesis in human tumorigenesis, in mice it was shown that centrosome deregulation is sufficient to drive development of spontaneous tumors[45].

Structural abnormalities were also observed at centrosomes in cancer cells. These defects are probably connected with an altered expression of centrosomal proteins that can either lead to abnormally enlarged or reduced centrosomes and consequent enhanced or reduced MT nucleation[38,41].

Centrosomes and diseases of brain development

Mutation of MT-regulation and centrosome proteins have both been linked to dwarfism and neurodevelopment disorders like: disorders of neural migration, disorders of growth where the brain is disproportionately affected and, primary microcephalies, where the brain alone is affected and significantly reduced in size[38]. Interestingly, 10 out of 13 genes implicated in autosomal recessive primary microcephaly (MCPH) code for proteins that were found to localize to the centrosomes and/or thr the mitotic spindle poles[41]. Vertebrate neurogenesis starts with an expansion phase where asymmetrical divisions will give rise to neural precursors. The neural precursors will then differentiate to form neurons or basal progenitors. It has been proposed that aberrant centrosomes can deregulate the asymmetric division essential for the formation of neural precursors in the beginning of neurogenesis. As a consequence, there is a decrease in the pool of neural precursors that is thought to be the cause of MCPH[38]. It was also observed that centrosome amplification leads to abnormal cell division, followed by aneuploidy and p53 dependent cell cycle arrest which results in the generation of a microcephaly brain (a brain with a small size)[46].

Ciliopathies

Pathologies caused by defects in cilia show a wide diversity of symptoms and affect different organs, including the kidney, retina, liver, brain or bones. These are mostly caused by mutations in proteins that can be present at the primary cilia and/or at the centrosome. Interestingly, these diseases are generally recessive, meaning that mutations at both alleles of the same gene are required to give rise to a ciliopathy[41]. These mutations can affect ciliary signalling in different ways: through changes in the cilia structure; at the level of the sensory of signalling molecules; or in the capacity of transducing the signal to its targeting molecules[38].

1.2.6. Centrosomes fate in different cell types

Centrosomes have been described as very stable structures. It was observed that these organelles were resistant to MT depolymerization treatments, such as cold treatment[47]. Also, experiments in *Xenopus* embryos, showed that, during mitosis, MTs grow faster. This faster growth rate results in MT instability which can lead to their release from the centrosome. However, centrosomes did not seem to be affected, suggesting that MT instability does not affect centrosome stability[48]. Moreover, recent work from Balestra *et al.* shows that in *C. elegans* the two paternally centrioles marked by GFP::Sas-4 could be detected in all embryos up to the ~350-cell stage, suggesting that they are stable structures which persist through embryonic development[49].

Despite the existing evidence towards centrosomes being considered highly stable structures, several observations suggest that centrosomes are not intrinsically stable as originally thought. In metazoans, echinoderms and molluscs these organelles are eliminated during oogenesis. While in metazoans centriole elimination occurs before meiotic stages[50,51], in echinoderms and molluscs, centrosomes are observed until meiotic stages, being eliminated during the meiotic divisions[52]. As a result of centrosome elimination during female gametogenesis, it is widely accepted that centrosomal

inheritance depends on the centrosome provided by the male gamete upon fertilization[51]. Centrosomes are also known to be inactivated, and in some cases even lost, in cells undergoing differentiation[3].

It is not completely understood how centrosomes are eliminated or inactivated, nevertheless, a recent study suggests that this might be under the regulation of a universal maintenance program[53].

Centrosomes during gametogenesis

Given the technical difficulties in obtaining enough material for analysis of mammalian oocytes, most of the knowledge in this area comes from studies in invertebrates and lower vertebrates, such as *D. melanogaster*. In *Drosophila*, oogenesis begins at the tip of the germarium, with the asymmetric division of a stem cell giving rise to a new stem cell and a cystoblast. The cystoblast undergoes four consecutive divisions with incomplete cytokinesis forming a large cyst composed of sixteen interconnect cells. One of these cells becomes the oocyte while the remaining cells are called nurse cells[54]. The oocyte will further progress into meiosis, while the nurse cells produce massive amounts of mRNA and proteins that will be deposited into the oocyte and sustain the initial stages of embryonic development[55].

Early in oogenesis, when the four mitotic divisions are complete, the centrioles of the 15 nurse cells cluster and migrate to the oocyte where they can nucleate MTs. The clustered centrosomes will act as a large MTOC in the oocyte, contributing for the transport of mRNA and proteins from the surrounding nurse cells to the oocyte[51,55,56].

The MTOC in the oocyte is active (with MT nucleation capacity) from stages 2 to 6[54] but can be detected up until stage 9 by EM[57]. In mid stages (stages 7/8), the MTOC starts to lose MT nucleation capacity, coincident with the loss of PCM components (Pimenta-Marques et al.,2016). In the late stages, of oogenesis (from 9 to 12 stage), the MTOC disperse into the cytoplasm and disappear beyond detection before meiosis occurs[51] (Figure 1.7).

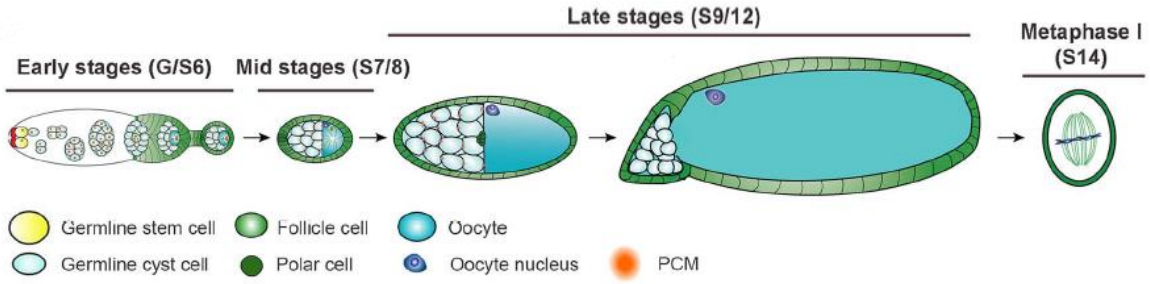


Figure 1.7: ***Drosophila melanogaster's*** **oogenesis.** After asymmetric division of a stem cell, the cystoblast formed divides four times giving rise to a cyst of 16 interconnected cells. Only one of the 16 cells becomes the oocyte while the others are called nurse cells. All centrioles from the nurse cells migrate to the oocyte. Centrioles have been observed as late as stage 12/13, but not in meiosis (I and II). Adapted from Pimenta-Marques et al., 2016[53].

The male contributes with a centriole, at the base of the flagella, which can organize a functional centrosome by recruiting the PCM and centriolar proteins present in the cytoplasm of the oocyte.In the subsequent embryonic divisions, this centrosome is able to enter the canonical centriole duplication cycle, as described before[51].

Interestingly, during spermatogenesis, centrosomes can be partially or fully degenerate according to the species. Degeneration occurs in three stages: 1) loss of MT nucleating capacity by loss of PCM proteins like γ-tubulin; 2) loss of centrosomal proteins (either centriolar and/or PCM proteins, depending on the species); and 3) disintegration of centrioles[51,56].

The mechanisms underlying centrosome elimination and degeneration during gametogenesis are poorly understood. However, a recent study in *D. melanogaster* has shed some light on centrosome elimination during oogenesis. It was observed that during oogenesis the PCM and Polo kinase (PLK1 orthologue) are downregulated at the MTOC. Moreover, depletion of Polo in oogenesis leads to

accelerated centrosome loss. Consistently, depletion of either Polo or PCM components in *Drosophila* cultured cells leads to a reduction in the number of centrosomes per cell. By tethering Polo to the oocyte's centriole, it was possible to observe retention of these structures until the meiotic division stages, with accumulation of PCM proteins. As expected, the presence of centrosomes in such late stages leads to aborted embryonic development (Figure 1.8)[53].

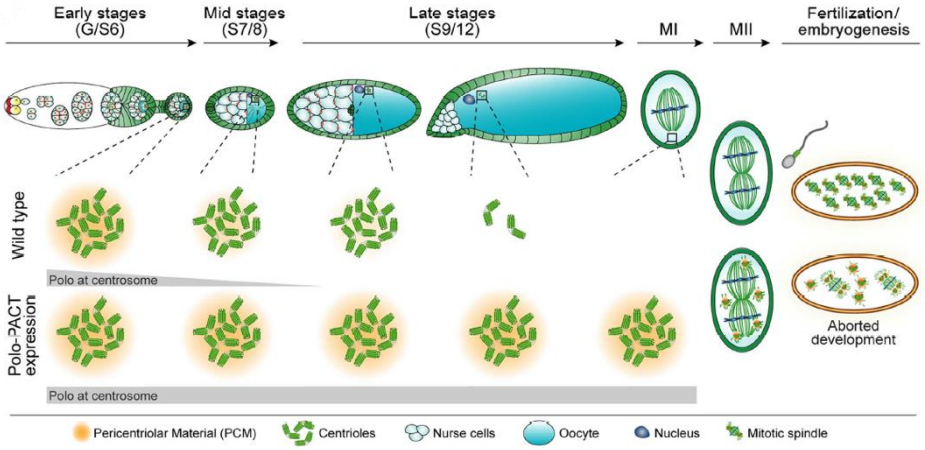


Figure 1.8: Polo is downregulated during oogenesis and tethering this protein to the centrosome allows PCM accumulation and centrosome maintenance until meiotic division. In a wild-type fly, during oogenesis, Polo and PCM components are down-regulated at the oocytes. Centrosomes are not stable and are eliminated, leading to their absence in meiotic division - the centriolar maintenance program is **down-regulated**. The centriole is then provided by the sperm upon fertilization ensuring successful embryonic development. By ectopically expressing and forcing the localization of Polo on centrioles, PCM is maintained at the centrioles, which confers them stability. Consequently, centrosomes are kept until meiosis and upon fertilization, the high number of centrosomes leads to abnormal mitotic divisions with consequent failure of zygotic development – the centriolar maintenance program is **up-regulated**. Adapted from Pimenta-Marques et al., 2016. [53]

Not only Polo, but also Spd2 (a PCM protein) is downregulated in *Drosophila* oogenesis at the transcriptional level[58], which may contribute for its downregulation at the MTOC. Interestingly, this mechanism may be conserved in other species that eliminate centrosomes during oogenesis. In *Xenopus* the PCM protein γ-tubuling[59] and Polo are downregulated during oogenesis[60] which suggests that regulation of the PCM by Polo for centrosome elimination might be part of a universal program.

Similarly to what happens during *Drosophila* oogenesis, differentiated cells, such as skeletal muscle, neurons and epithelial cells, also remove PCM from their centrosomes to inactivate them[59], which I discuss below.

Muscle

During muscle differentiation, myoblasts fuse to give rise to syncytial myotubes that arrange MTs in a longitudinal fashion. The MT minus ends, that normally are associated with the centrosome, are mostly linked to the Golgi apparatus[61] and to the nuclei in myotubes[62,63]. Also, it was observed that PCM proteins like pericentrin, ninein and γ-tubulin are delocalized from the centrosome but are present at the Golgi and the nuclei[61,64]. This suggests that either PCM proteins are being relocated from the centrosome to the nuclei/Golgi; or PCM proteins are being degraded from the centrosomes and newly formed PCM proteins are being recruited to the nuclei/Golgi, where they promote MT nucleation.

Neurons

Similarly to what was observed during muscle differentiation, studies show that during neuronal differentiation the centrosomes are also inactivated. When axons start to form in rat hippocampal neurons, the PCM protein γ-tubulin is localized at the centrosome in all neurons. As neurons keep

differentiate, γ-tubulin is detected in 97% of the neurons, however its intensity its reduced by 52%. In mature neurons, centrosomal γ-tubulin could only be detected in 42% of the neurons and showed an intensity reduction by 81%. The observed reduction of centrosomal γ-tubulin seems to be related with the specific loss of Nedd1, which is part of the γ-TuRC, suggesting that loss of this protein leads to centrosome inactivation[3,65].

Epithelial cells

Studies in simple epithelia, like *C. elegans* intestinal epithelium and *Drosophila* trachea epithelium, have shown that the centrosomal MTOC is inactivated and MTs start to nucleate from acentrosomal MTOCs (aMTOC) at the apical surface of the cell is coincident with γ-tubulin removal from the centrosome[3]. The mechanisms that induces γ-tubulin release from the centrosome in *C. elegans* are not fully understood, nevertheless, this delocalization seems to be related with a decrease in CDK activity[66].

In stratified epithelia, like mammalian epithelia, it was recently suggested a two-step mechanism that controls centrosome inactivation. Muroyama et al. showed that, upon cell-cycle exit, a decrease in CDK is sufficient to promote degradation of Need1, which leads to delocalization of the γ-TuRC from the centrosome. It was also proposed that, as cells continue to differentiate, additional centrosomal proteins like Cep215, which recruits the γ-TuRC and is an active MT nucleator, is also lost from the centrosome. Therefore, the authors suggest that centrosome inactivation is a process regulated by distinct protein sub-complexes that are delocalized from the centrosome with different kinetics[3,67].

1.2.7. Centrosomes are not intrinsically stable

Centrosome elimination and inactivation studies suggests the existence of a mechanism that can be turned on or off depending on the cell type or cell cycle stage: a centriole maintenance program. When the centriole maintenance program is activated, cell cycle regulators like CDK and PLK1/Polo are also activated promoting PCM maintenance/accumulation at the centrosome and preventing centrosome elimination/inactivation. However, how Polo and the PCM are protecting the centrosome is still unknown.

Differentiation induces MT cytoskeleton reorganization in many cell types and species. MT reorganization is generally characterized by the formation of an aMTOC. This suggests that inactivation and possible elimination of centrosomes in cells is an important step during development. On the other hand, it has been shown that centrosome amplification can trigger cell invasion in cancer[42]. It is therefore important to understand how this maintenance program works during development; this would provide ideas on how to manipulate centrosomes in disease.

1.3. The fruit fly, *Drosophila melanogaster*

1.3.1. *Drosophila melanogaster* as a model organism

The fruit fly, *Drosophila melanogaster*, was introduced as an experimental organism at the beginning of the 20th century and ever since it has contributed to multiple areas of biology. *Drosophila* was the second multicellular organism (after *C. elegans*) to have its genome completely sequenced[68]. Its genome encodes more than 14 000 genes on only four chromosome pairs. The first pair corresponds to the sex chromosomes while the remaining three pairs are autosomes, (2L, 2R, 3L, 3R and 4) that are extremely well-annotated (*www.ensembl.org* or *www.flybase.org*). By comparing the fly genome with the ones of mammals, there is approximately 40% identity between homologs, either at the nucleotide level or protein sequence level. However, this percentage can increase to 80/90% or higher when

comparing conserved functional domains[69]. Comparing *Drosophila* with humans, over 70% of the proteins involved in human disease exist in the fly.

The information about *Drosophila's* genome combined with its short life cycle, which allows for the rapid generation of a large progeny makes the fruit fly a powerful model for comparative genomic studies. In *Drosophila*, the development of the embryo to an adult requires on average 9-11 days. However, this generation time can change according to the temperature: if the flies are at 25ºC, the cycle takes 9 to 11 days, if the flies are at 18ºC this process can take up to 19 days[70].

Upon fertilization, embryogenesis is completed in approximately 24h hours. Fertilization is followed by the three larval stages: first, second and third instar. The two first instar last on average one day whereas the third instar requires approximately two days. At the end of the third instar stage, larval development is complete and the animals metamorphose and form a pupa. The flies remain in the pupal case for four to five days, before emerging as an adult. Adult flies become sexually mature after eight to twelve hours after eclosion[70].

Nowadays, there are large collections of *Drosophila* stocks, such as, stocks with "foreign pieces of DNA" (P-elements) inserted that can potentially disrupt gene function; chemically and radiation-induced mutants and transgenic fly mutants that harbour specific siRNA to examine the effects of tissue specific gene knockout. Many of these stocks are maintained due to the presence of balancer chromosomes that assist with the chromosomal mapping of traits and the propagation of recessive (often lethal) mutations[71]. Moreover, with the development of the GAL4/UAS system, it became possible to target gene expression not only spatially (specific tissues) but also temporally (specific developmental stages). To obtained this system Brand and Perrimon cloned the yeast transcription factor GAL4 into a P-element vector and showed that one could place a given promoter upstream of GAL4 or integrate the GAL4 element into the genome to use endogenous transcriptional enhancers to express GAL4. The expression of this driver is accompanied with another p-element vector which contains an UAS (upstream activating sequence), pUAST. UAS sequences are connected to a general promoter and a cloning site to allow for the insertion of any gene of interested. Therefore, expression of the gene of interest is controlled by the presence of the UAS element that depends on the expression of the GAL4 protein[70,72]. This system allows for temporal and spatial control of gene expression.

The characteristics of *Drosophila* as well as the resources available makes the fruit fly a great model organism that allows correlation of genetics and phenotype.

1.3.2.*Drosophila* cultured cells and RNAi screens

Besides the advantages of studying cellular processes in the organism itself, *Drosophila* cell culture has become a great tool due its simplicity and minimal maintenance costs. The availability of the fully sequence genome allows the possibility of producing specific double-strand RNA (dsRNA) that can be directly added to the culture medium leading to the degradation of specific mRNAs with few complications of off-target effects. Thus, RNA interference (RNAi) screens have been the main approach to study novel molecules and elucidate mechanisms of cell growth, signalling, regulation of the cell cycle progression and mitosis[73].

1.3.3. Centrosomes in *Drosophila*

Different studies have shown that centriolar core proteins, necessary to define the centriole nine-fold symmetry, recruitment and tethering of centriolar MTs, as well as know regulators of centriolar biogenesis, seem to be conserved among eukaryotes[74,75].

The presence of the majority of the human centrosomal components and regulators in *D. melanogaster* suggests that the main mechanisms involved in centriole biogenesis are conserved among

these two species. For this reason, *D. melanogaster* is one of the best model organisms to study centrosomes.

1.4. Objectives

Previous work suggests the existence of a centriole maintenance program that is "shut down" to allow centriole elimination in oogenesis. In this program, Polo (PLK1 ortholog) is down-regulated and delocalized from centrosomes, leading to loss of PCM and centriole destabilization, however, the mechanism that allows centriolar stabilization trough Polo kinase and the PCM remains unclear.

We hypothesize that the PCM might be conferring stability to the centrosome by functioning as a shield that either prevents accessibility of proteins that destabilize the centriolar structure, or that prevents the loss of stabilizing proteins that form the centriolar structure.

As so, the objective of the work described in this thesis was to identify centrosomal proteins that might have a role in the stabilization/destabilization of the centrosome itself. In order to identify these proteins, I performed a candidate screen in *Drosophila* cultured cells, taking advantage of the "8 Day Centriole Stability Assay" that was previously developed in our lab. This assay allows to uncouple centrosome maintenance from centrosome biogenesis once centriole number is kept constant.

Identifying new players involved in centrosome stabilization will not only allow to link the stabilizing structural features to the maintenance program, but also open new venues to understand not only how does this program is regulated in development but also how it is deregulated in disease.

2. Materials and methods

2.1. Cell Culture

D. melanogaster cells (DMEL) were cultured in T75 flasks with Express 5 SFM medium (Gibco, USA) supplemented with 1x Penicilin-Strepnomicin-L-Glutamine (PSG) (Gibco, USA) at 25ºC. When at least 85-90% of the flask's surface was covered by a cell monolayer (85-90% confluence), cells were split to a new flask with fresh medium.

2.2. Immunostaining optimization

To increase the power of the present screen, several *Drosophila* antibodies for centrosomal proteins were tested (Table 2.1). Tests were performed in DMEL asynchronous cells. $0{,}7x10^6$ Cells were added to a 24-well plate containing glass coverslips previously washed in a 70% ethanol solution. Cells were allowed to adhere for approximately 1 hour before fixation and staining.

Table 2.1: List of primary antibodies tested. Rb1 and Rb2 correspond to different animals in which the antibodies were raised.

Primary Antibodies	Supplier/Reference
Ana1 Rb1	Metabion (generated in our lab)
Ana1 Rb2	
Ana2 Rb1	
Ana2 Rb2	
CP110 Rb1	
CP110 Rb2	
Centrin Rb1	
Centrin Rb2	
Centrobin Rb3	
Sas4 Rb1	
Sas4 Rb2	

2.2.1. Treatment of coverslips

After washing the coverslips with the 70% ethanol solution, coverslips were added to a 24-well plate. Concanavalin A was added to each coverslip, and washed afterwards. After 30 minutes, cells were added to the coverslips.

2.2.2. Fixation methods

When the cells were adherent to the coverslips, the medium was removed from the wells.

PFA fixation: Cell fixation was performed with 400µL of a fixative solution containing paraformaldehyde (PFA) (Table 2.2) during 10 minutes at room temperature (RT).

Table 2.2: Paraformaldehyde fixative solution.

Solutions	Final concentration
PIPES pH 6,8	60 mM
HEPES pH 7,0	30 mM
EGTA pH 6,8	10 mM
$MgSO_4$	4 mM
ddH_2O	-
Paraformaldehyde	4%

Methanol fixation: Cell fixation was performed with 400µL of cold methanol (-20ºC) during 10 minutes at -20ºC.

2.2.3. Blocking conditions

Two different blocking agents were used to block the cells: PBSTB or PBS+FBS.

Block with PBSTB: 1mL of PBSTB (PBS with 1% BSA and 0,1% of Triton-X) was added to the cells for 1 hour at RT.

Block with PBS+FBS: 1mL of PBS with 10% FBS was added to the cells for 30 minutes at RT.

The tested primary antibodies were diluted in the same solution used for cell blocking. Cells were stained not only with the primary antibodies listed in Table 2.1, but also with chicken anti-D-Plp (1:2000). The chicken anti-D-Plp antibody is highly used[18] and specifically recognises the PCM protein D-Plp at the centrosome. Therefore, colocalization of this antibody with the tested antibodies will suggest that these are also specifically recognizing the centrosome. The primary antibodies were thus validated using colocalization with this antibody.

Cells were incubated with the primary antibodies over night at 4ºC in a dark and humid container to avoid evaporation. Cells were washed with PBS and PBSTB and incubated with the secondary antibodies anti-rabbit Alexa 488 (1: 1 000) and anti-chicken Cy5 (1:1 000) and simultaneously stained for DNA with DAPI (1:500) in blocking solution for 2 hours at RT in the dark. Finally, cells were washed with PBS and coverslips were mounted using DAKO mounting medium (Agilent).

2.3. Generation of dsRNA

Synthesis of *mCherry*, Ana1, Ana2, Asl, Bld10, CP110, Cep97, Cnn, D-Plp, Plk4, Sas4, Sas6 and Spd2 dsRNA were produced as described before[73] using the T7 RiboMAX™ Express RNAi System Kit (Promega) and the primers listed on Table 2.3.

Table 2.3: List of primers used for dsRNA synthesis and RT-PCR. The sequence highlighted in blue corresponds to the promoter sequence of the T7 RNA polymerase that allows dsRNA production.

Name	CG Number	Primers	
		Forward (5'-3')	Reverse (3'-5')
mCherry	-	TAATACGACTCACTATAGGGATGGTGAGCAAGGG	TAATACGACTCACTATAGGGGTTGACGTTGTAGG
Ana1	CG6631	TAATACGACTCACTATAGGGAGAATGGCTCTGCAGCTAACAGTAA	TAATACGACTCACTATAGGGAGATTGACCAAAACATGCTCACGCC
Ana2	CG8262	TAATACGACTCACTATAGGGAGAATGTTTGTTCCCGAAACGGAGGA	TAATACGACTCACTATAGGGAGATGCTGGGAGCGGTGCGAGGA
Asl	CG2919	TAATACGACTCACTATAGGGAGATTATGGTGAATGCCTTCGAC	TAATACGACTCACTATAGGGAGAACTAGCTCAGCCTGCATGATG
Bld10	CG17081	TAATACGACTCACTATAGGGAGAACCACCACAACGACCAAA	TAATACGACTCACTATAGGGAGAGATCCTTTCCCTTCTTCTT
CP110	CG14617	TAATACGACTCACTATAGGGAGAAAGAAGCGCGAGGTGCAGCT	TAATACGACTCACTATAGGGAGAATGCGATTATGCCGCCTTGG
Cep97	CG3980	TAATACGACTCACTATAGGGAGATGTTAAGTCTTCCACCATCGC	TAATACGACTCACTATAGGGAGAGATATGCTACTTACGAAGGCCC
Cnn	CG4832	TAATACGACTCACTATAGGGAGAACCTCCAGGCGGCGGCAACT	TAATACGACTCACTATAGGGAGATGGCTCGAGCGGCATCCTT
D-Plp	CG33957	TAATACGACTCACTATAGGGAGAGGAGCGCCTAAAGAACAGTG	TAATACGACTCACTATAGGGAGACTGATCGAGCTGTTTGTGGA
Plk4	CG7186	TAATACGACTCACTATAGGGAGAATACGGGAGGAATTTAAGCAAGTC	TAATACGACTCACTATAGGGAGATTATAACGCGTCGGAAGCAGTCT
Sas4	CG10061	TAATACGACTCACTATAGGGAGATCTCGCGGCGCTTAGTCGTT	TAATACGACTCACTATAGGGAGAGGCGCAGGATTGGGAGGTG
Sas6	CG15524	TAATACGACTCACTATAGGGAGATGTAGTGCGCATGCTGAAGGAC	TAATACGACTCACTATAGGGAGAGCTGCGCTGCTCGTTTATTTTG
Spd2	CG17286	TAATACGACTCACTATAGGGAGATCGCGTTCCAGCCAAGCAAAGA	TAATACGACTCACTATAGGGAGAATCCCCCACCTCCGTTAAGACTCAG
eIF4a	CG9075	TAATACGACTCACTATAGGGAGAAATGAGATACCTCAGGATGGCCC	TAATACGACTCACTATAGGGAGAACGTTAGTGCCGCCAATGCA

2.4. 8 Day Centriole Stability Assay

DMEL cells were counted and $15x10^6$ cells were added to a 6-well plate with Express 5 SFM medium (Gibco, USA) supplemented with 1x L-Glutamine (Gibco, USA). Cells were allowed to adhere for 1 hour before transfection.

To perform transfection, after removal of the medium, 40µg of each of the dsRNA produced (diluted in 1mL of Express 5 SFM medium (Gibco, USA) supplemented with 1x L-Glutamine (Gibco, USA) was added to each well. One hour after transfection cells were arrested in S-phase by cotreatment with 10µM aphidicolin (Aph) (Sigma) and 1.5mM hydroxyurea (HU) (Sigma) diluted in Express 5 SFM medium (Gibco, USA) supplemented with 1x PSG (Gibco, USA) was used. Cells were maintained at 25ºC (Day 0, Figure 2.1).

At the 4th day of the treatment, the old medium was removed and cells were subject to a second round of dsRNA transfection. dsRNA was diluted in 1mL of Express 5 SFM medium (Gibco, USA) supplemented with 1x L-Glutamine (Gico, USA) and Aph and HU as described before. After one hour, 3 mL of Express 5 SFM medium (Gibco, USA) supplemented with 1xPSG, Aph and HU was added to each well and cells were maintained at 25ºC (Day 4, Figure 2.1).

Cells were harvested for immunostaining and RT-PCR on the 8th day of the assay (Day 8, Figure 2.1).

Each experience included two different controls: a negative and a positive control. As a negative control, I added 40µg of *mCherry* dsRNA to cells. As *mCherry* is a fluorescent construct, not produced by the cell machinery, it is very likely to not interfere with any cell processes and should not alter the number of centrosomes in cells. As a positive control, I added 10µg of each Asl, Cnn, D-Plp and Spd2 dsRNA – "All PCM RNAi". Asl, Cnn, D-Plp and Spd2 are important components of the PCM and the combined depletion of these four proteins is sufficient to lead to centriole loss in arrested DMEL cultured cells[53].

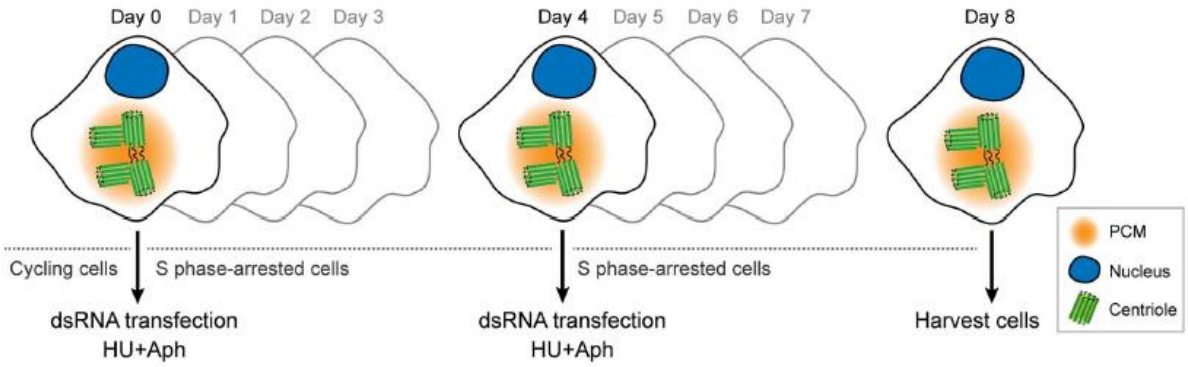


Figure 2.1: Schematic representation of the "8 day centriole stability assay". Cells were arrested with Aph and HU at the beginning of the assay. Cells were subjected to two rounds of dsRNA transfection. The first transfection was performed at day zero of the assay and the second at day four. Cells were harvested for immunostaining and RT-PCR at day eight.

2.5. Immunostaining of DMEL cells

DMEL cells were plated onto glass coverslips previously washed in a 70% ethanol solution and allowed to adhere for 1 hour. After removal of the medium, cells were fixed using a fixative solution containing PFA (Table 2.2) for 10 minutes at RT. After fixation cells were washed with PBS, permeabilized and blocked with PBSTB (as described in chapter 2.2.3 – Block with PBSTB) during 1 hour at RT.

Cells were incubated with the primary antibodies (Table 2.4) diluted in PBSTB over night at 4ºC in a dark and humid container to avoid evaporation. After washing with PBSTB, cells were

incubated with the secondary antibodies (Table 2.5) diluted in PBSTB and stained with DAPI (1:500) for 2 hours at RT in the dark. Cells were washed with PBS and coverslips were mounted using DAKO mounting medium (Agilent).

To stain the cells with the Rabbit anti-Sas4 antibody conjugated with Alexa 568, cells were washed with PBSTB and PBS and blocked with PBS containing 10% Rabbit serum (Metabion) for 30 minutes at RT. After blocking, cells were incubated with the antibody diluted in PBS with 10% rabbit serum for 1 hour at RT in the dark.

Table 2.4: List of primary antibodies used for immunostaining of DMEL cells.

Primary antibodies	Dilution	Supplier/Reference
Chicken anti-D-PLP	1:2000	Kindly provided by David Glover[18]
Rabbit anti-CP110	1:10 000	Metabion
Rabbit anti-Sas4 conjugated with Alexa 568	1:500	Metabion
Rabbit anti-Bld10	1:5000	Kindly provided by Tim Megraw[31]
Rat anti-Ana1	1:500	Kindly provided by Jordan Raff[32]
Guinea Pig anti-Ana1	1:500	Kindly provided by Jordan Raff[76]

Table 2.5: List of secondary antibodies used for the immunostaining of DMEL cells.

Secondary Antibodies	Dilution	Supplier/Reference
Anti-Chicken Cy5	1:1000/1:500	Bethyl Laboratories
Anti-Rabbit Alexa-488	1:1000	Molecular Probes
Anti-Rabbit Alexa-555	1:500	Molecular Probes
Anti-Rat Dylight A488	1:500	Bethyl Laboratories
Anti-Guinea Pig HRP	1:500	-

2.6. Imaging and centriole scoring in DMEL cells

Cell imaging was performed on a commercial Nikon High Content Screening microscope, based on Kikon Ti equipped with a Andor Zyla 4.2 sCMOS camera, using a 100X 1.49 NA objective, DAPI+GFP fluorescence filter sets and controlled with the Nikon Elements software. Images were acquired as Z-series (0,2 µm apart; 51 slices). Acquired images were deconvoluted using the AutoQuantX software and the analysis and centriole scoring software was written in ImageJ (NHI, USA).

2.6.1. Centriole identification

All images were deconvolved and assembled as maximal intensity projections. Automated identification of centrioles involved 2 different steps: 1) Individual cell identification; and 2) Individual centriole identification.

First, cells were identified based on the background provided by the anti-Sas4 antibody staining using the Otsu's threshold method. The threshold allowed the use of the "Analyse Particles" plugin of

ImageJ which defines a region of interest (*roi)* for each cell identified. The resulting *rois* were used to create several images of individualized cells. In order to identify the centrioles within the cells, a second threshold was applied in the individualized cell images. This threshold was based on the individual anti-D-Plp, anti-CP110, anti-Sas4, anti-Bld10 or anti-Ana1 antibodies signals using the Yen's threshold method. Again, the threshold allowed to run the "Analyse Particles" plugin and thus identification and counting of centrioles per cell using each marker. All the centrioles identified using this method were manually curated to ensure correct centriole identification. The script used to identify and count the centrioles can be found in annex (chapter 7.1).

To avoid any kind of bias, identification and counting of centrioles in the different experimental conditions was performed blindly. At least 100 cells per sample in three independent experiments were analysed. Statistical analysis was performed using a one-way ANOVA test. Finally, all images were processed in ImageJ (NHI, USA) and Adobe Photoshop (Adobe Systems, USA).

2.6.2. Quantification of the Ana1 signal in DMEL cells

All images were deconvolved and assembled as sum intensity projections. The *rois* used to identify each cell during centriole counting were applied in the sum intensity projections and also to obtain final images of the individualized cells. Centrioles in each cell were identified using the *rois* obtained for the counting of centrioles marked by Ana1. Raw integrated intensities were measured from at least100 cells per sample in three independent experiments. To avoid any kind of bias, intensity measurements of the different experimental conditions was performed blindly. Statistical analysis was performed using a Mann-Whitney t-test. The script used to identify and measure the intensity signal of Ana1 marked centrioles can be found in annex (chapter 7.2).

2.7. RT-PCR

At the 8th day of the "8 Day Centriole Stability Assay", cells from each of the samples were harvested to perform a Reverse-Transcriptase PCR. Total mRNA was extracted from these cells using the RNeasy Mini Kit (Qiagen) according to the manufacturer instructions. mRNA was quantified and 1µg of each sample was used to synthesize cDNA using the Transcriptior First Strand cDNA Synthesis kit (Roche), using the Random Hexamer primers. The cDNA produced was used as a template for PCR to confirm the knock-down of the mRNA of the gene-of-interested. The PCR was carried out using the primers used for dsRNA production (Table 2.3).

2.8. In vivo studies in *Drosophila melanogaster*

2.8.1. Fly work and genetics

All flies were reared according to standard procedures and maintained at 25ºC.

In order to deplete Ana1 in females ectopically expressing GFP-Polo-PACT in the germline, transgenic fly line stocks bearing a GFP-Polo-PACT construct[53] were crossed with a fly bearing an RNAi construct for Ana1. Knock down for Ana1 was performed by using the UAS RNAi line P[TRiP.HMJ23356]attP40 (BDSC#61867). As a control, a UAS RNAi line for mCherry (P{VALIUM20-mCherry}attP2; BDSC#35785) was also combined with GFP-Polo-PACT expressing females. Both RNAi fly lines come from the *Drosophila* transgene RNAi project (TriP)[77].The transgene constructs were expressed in the female germ-line by using the maternal driver V32-Gal4 (kindly provided by Daniel St Johnston). This driver induces expression around stages 3/4 of oogenesis, already after the initial four consecutive mitosis at the germarium (Figure 2.2).

As a control for efficient depletion of Ana1 by the RNAi line, Ana1 RNAi was driven by a maternal Gal4 (Nanos-Gal4) which induces expression of the construct since the germ-line stem cells. In this case, the UAS-Ana1-RNAi construct will be expressed before the initial four mitosis at the germarium. Since Ana1 is required for centriole duplication[21,32,78], we expect that if the RNAi line is working, centriole duplication should be affected early in development (Figure 2.2).

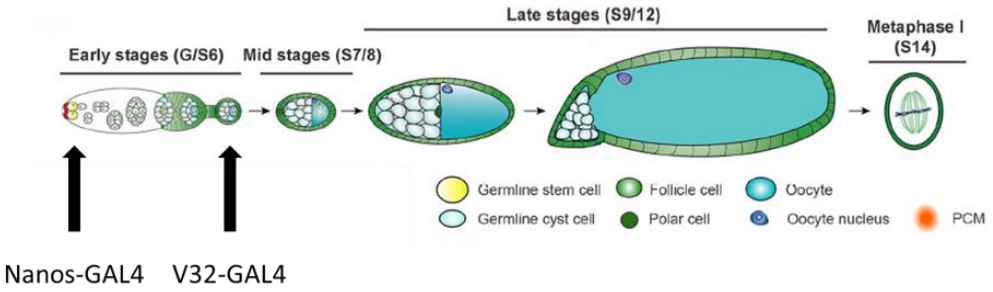


Figure 2.2: Schematic representation of the phases in *Drosophila* oogenesis where the drivers used are expressed. Nanos-Gal4 driver is induced in the early stages of oogenesis, before the four consecutive mitosis in the germarium. V32-Gal4 driver is induced in the end of the early stages of oogenesis after the four consecutive mitosis in the germarium.

All crosses and expression profiles are depicted in figure 2.3.

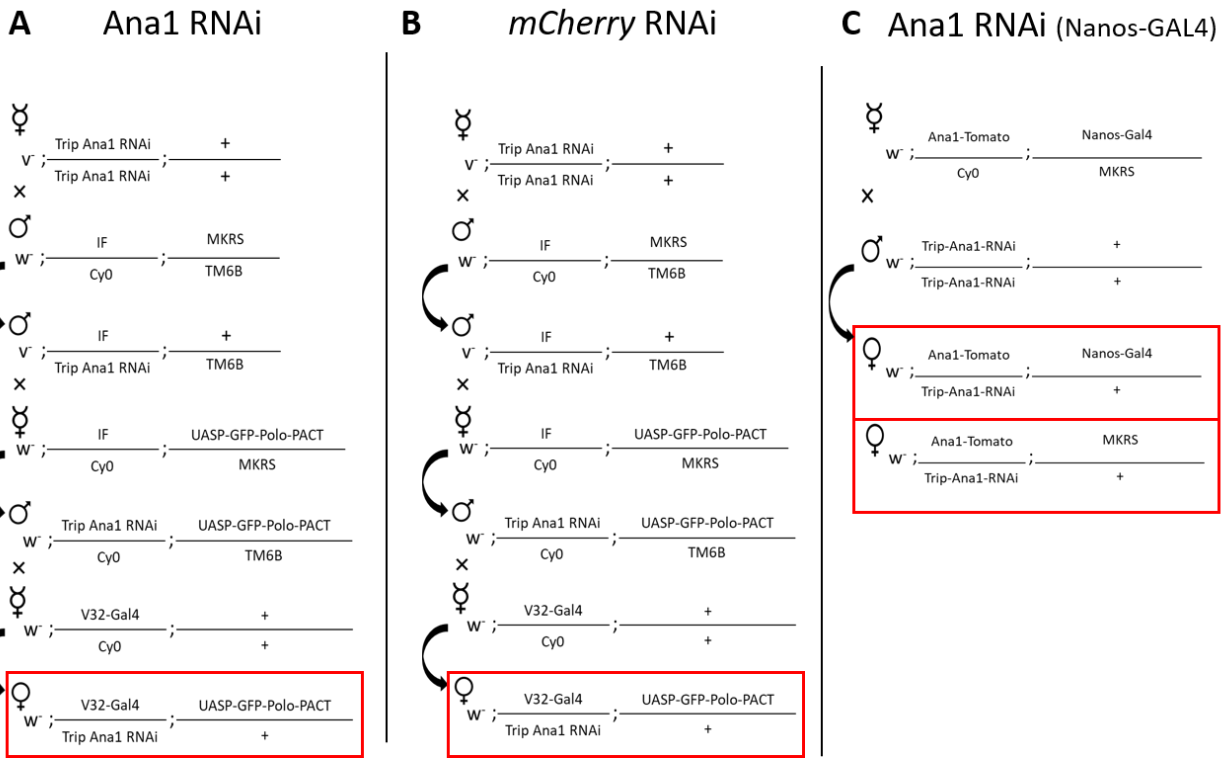


Figure 2.3: Schematic representation of the crosses performed to obtain the flies to perform in vivo studies in *Drosophila melanogaster*. (A) Depletion of Ana1 in the *Drosophila* female germline (after the 4 consecutive mitosis at the germarium) upon overexpression of the construct GFP-Polo-PACT. **(B)** Negative control: depletion of *mCherry* in the *Drosophila* female germline (after the 4 consecutive mitosis at the germarium) upon overexpression of the construct GFP-Polo-PACT. **(C)** Control for Ana1 RNAi line: depletion of Ana1 in the *Drosophila* female germline (before the consecutive mitosis at the gemarium). The flies highlighted in red represent the genotype of the flies in which the experiment was carried.

2.8.2. Dissection of flies and- immunostaining of ovaries

To perform ovary staining, 3-5 day-old females, were fed with yeast for two days. For ovary dissection, females were anesthetized with ether and further transferred to BRB80 buffer (80 mM Pipes pH 6.8, 1 mM $MgCl_2$, 1mM EGTA) at RT, supplemented with 1× protease inhibitors (Roche). Their ovaries were extracted with pre-cleaned forceps. Individualized ovaries were then incubated for one hour at RT in BRB80 buffer supplemented with 1× protease inhibitors (Roche) and 1% Triton X-100 without agitation. Ovaries were fixed with cold methanol (-20ºC) for 15 minutes. Three wash steps with PBT (1× PBS supplemented with 0.1% of Tween) at RT for 15 minutes were performed and ovaries were left to permeabilize overnight in PBT at 4ºC. In the following day, ovaries were blocked for 1 hour at RT with PBT containing 2% of BSA (Gibco). Primary antibodies (Table 2.6) were incubated overnight at 4ºC in PBT with 1% of BSA. After 3 wash steps, secondary antibodies (Table 2.7) were incubated in PBT with 1% of BSA for 2 hours at RT. Ovaries were then washed in PBS and DNA was stained using Vectashield mounting medium containing DAPI (Vector Laboratories) mounted onto coverslips in the same mounting medium.

Table 2.6: List of primary antibodies used for immunostaining of *Drosophila* ovaries.

Primary Antibodies	Dilution	Supplier/Reference
Rabbit anti-CP110	1:5000	Metabion
Mouse anti-γ-Tubulin; clone GTU88	1:50	Sigma

Table 2.7: List of secondary antibodies used for the immunostaining of Drosophila ovaries.

Primary Antibodies	Dilution	Supplier/Reference
Anti-rabbit Alexa 555	1:250	Jackson Immunoresearch Laboratories
Anti-mouse Alexa 647	1:250	Jackson Immunoresearch Laboratories

2.8.3. Imaging, analysis and quantification

Ovaries were imaged as Z-series (0,29 µm apart) on a Leica TCS SP5 upright confocal laser scanning microscope. Images were acquired with the same exposure for each protein at stage 10 of oogenesis. Images were processed as maximum-intensity projections and assembled into panels using ImageJ (NHI, USA) and Adobe Photoshop CS (Adobe Systems, USA) programs.

To quantify the signals of the ectopic expression of GFP-Polo-PACT and from the immunostaining for CP110 and γ-Tubulin, I first defined the centrosomal region. Centrosomal region was determined by the co-localization of the GFP signal from GFP-Polo-PACT with the Alexa-555 signal of the CP110 antibody staining in the maximum-intensity projections. To remove the cytoplasmic background staining before quantification, the intensity of three different regions with 10 px of diameter was measured, again in maximum-intensity projections. The mean of the intensities measured was subtracted from the signal of the original image stacks and the centriolar signal (for GFP-Polo-PACT, CP110 or γ-Tubulin) was measured in sum-intensity projections. Intensity measurements were performed using ImageJ software (NHI).

3. Results

3.1. Screen optimization

Previous work in *Drosophila* shows that the PCM confers stability to the centrosome[53]. How the PCM stabilizes the centrosome is not known; however, we hypothesize that it can act as a shield preventing either accessibility of proteins that destabilize the centrosome, or loss of centriolar proteins that stabilize the centrosomal structure.

To identify proteins that play a role in stabilization/maintenance of the centrosomal structure, I performed a candidate screen in DMEL cells and analysed the number of centrioles per cell by an immunostaining assay. In these immunostainings different parts/modules of the centrosomes were analysed to understand which parts of the structure were possibly affected (for details see Materials and Methods, chapters 2.4 and 2.5).

3.1.1. Immunostaining

To understand what is happening to the centrosome structure it is important to use tools that allow the study of the different parts of the centrosome. Therefore, I started by testing several primary antibodies previously produced by the host lab. These were developed with the purpose of recognizing several *Drosophila* centriolar proteins (Table 2.1 – Materials and Methods). I tested these antibodies using different dilutions and different staining conditions, such as: coverslip treatment, cell blocking conditions and cell fixation methods. All the tested conditions are described in Materials and Methods (chapter 2.2) and the results are summarized in table 3.1.

Table 3.1: Optimization of different primary antibodies generated to recognize several centrosome proteins. Rb1 and Rb2 – Different rabbits in which the antibodies were generated**. CoA -** coverslips treated with Concanavalin A; **MeOH** - Cells fixed with methanol; **PFA** – cells fixed with a fixative solution containing paraformaldehyde; **y** – obvious co-localization between D-PLP and the tested antibody; **n** – no obvious co-localization between D-PLP and the tested antibody; **US** – Presence of unspecific signal.

Test #	Primary Antibody	Dilution	Coverslip treatment	Fixation	Blocking	Immunostaining
1	CP110 Rb1	1:500	CoA	MeOH	PBS+FBS(10%)	y; US
	CP110 Rb2					y; US
	Centrobin Rb3					n
	Sas4 Rb1					y
	Sas4 Rb2					y
	Ana1 Rb1	1:100				n
	Ana1 Rb2					n
	Ana2 Rb1					n
	Ana2 Rb2					n
	Centrin Rb1					n
	Centrin Rb2					n
2	CP110 Rb1	1:1000	CoA	MeOH	PBS+FBS(10%)	y; US
	CP110 Rb2					y; US
	Ana1 Rb1	1:500				n
	Ana1 Rb2					n
	Ana2 Rb1					n
	Ana2 Rb2					n
	Centrin Rb1					n
	Centrin Rb2					n

3	CP110 Rb1	1:2 000	CoA	MeOH	PBS+FBS(10%)	y; US
		1:5 000				y; US
		1:10 000				y; US
4	CP110 Rb1	1:5 000	-	PFA	PBSTB	y
		1:10 000				y
	Sas4 Rb2	1:500				y

All antibodies were tested in cycling *Drosophila* cultured cells. To validate that the tested antibodies are targeted to the centrosome, cells were co-stained with a well described antibody that recognizes the PCM protein D-Plp with a robust signal[18]. From all the tested antibodies, anti-CP110 (1:10000; Rb1) and anti-Sas4 (1:500; Rb2) showed a clean immunostaining with a strong signal, obvious co-localization with anti-D-Plp and little unspecific signal, when cells were fixed with PFA and blocked with PBSTB (indicated in grey in table 3.1 and Figure 3.1). Centriolar proteins CP110 and Sas4 are described below (chapter 3.2.1).

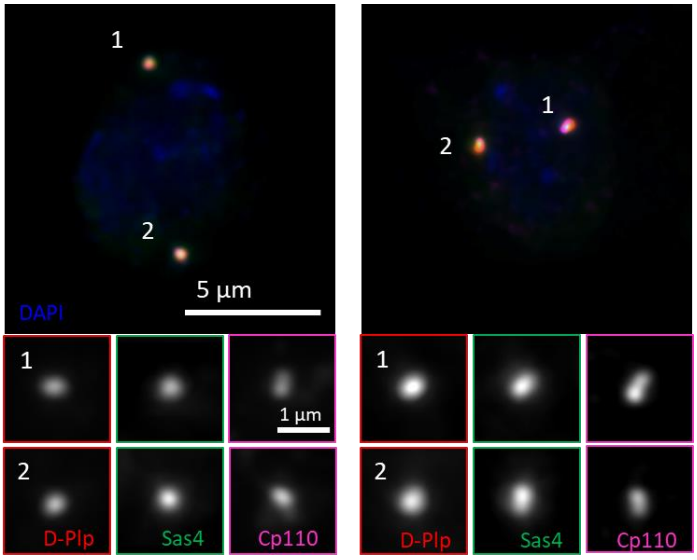


Figure 3.1: CP110 and Sas4 antibodies show a clear immunostaining signal and co-localization with D-Plp. Cells were stained for D-Plp (red), Sas4 (green), CP110 (magenta) and DNA (blue). Immunostaining was performed as described in Materials and Methods chapter 2.5. All images were acquired with the same exposure conditions. Scale bar, 5µm. Enlargements of the indicated areas (numbers) are shown. Scale bar, 1 µm.

To further validate that the chosen antibodies are specifically identifying the centrosomal proteins they were raised against, I depleted CP110 and Sas4 in arrested DMEL cells (as described in Materials and Methods chapter 2.4). After harvesting and fixing the cells, they were simultaneously immunostained with anti-CP110, anti-Sas4 and anti-D-Plp using the previously described conditions and centriole number was scored. Both CP110- and Sas4-depleted cells showed a decrease in the number of centrioles marked with either CP110 (Figure 3.2A) or Sas4 (Figure 3.2B), respectively, comparing with the negative control (*mCherry* RNAi).

These results suggest that the antibodies are specific for CP110 and Sas4, functioning as reliable markers for the centriole, and therefore were used in the following assays. One should note that all the other antibodies used to score centrioles had been previously optimized[21].

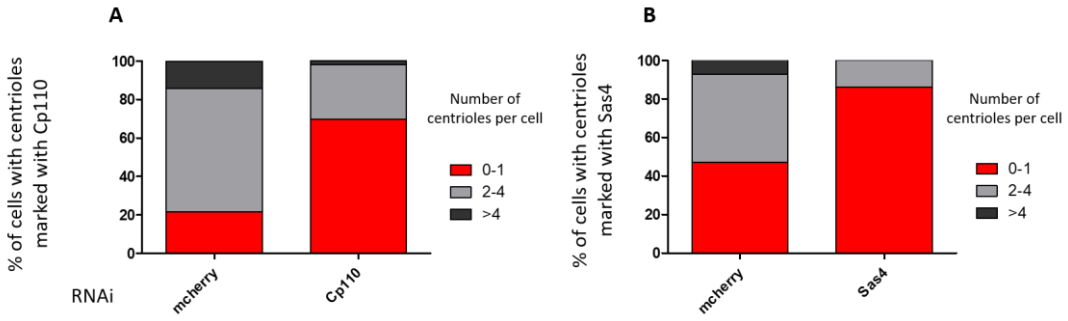


Figure 3.2: CP110 and Sas4 antibodies are specific. Histograms show that depletion of Cp110 or Sas4 in Drosophila cultured cells leads to an increase in the percentage of cells with 0-1 centrioles (red) either marked with CP110 (A) or Sas4 (B). Consistently, there is a decrease in the percentage of cells with 2-4 centrioles (grey) and more than 4 centrioles (black) marked with both antibodies. One experience was performed. More than 100 cells were counted per condition.

3.1.2. Centriole scoring

To investigate the influence of removing candidate proteins from the centrosome structure I developed an automatic process (Macro) for centriole counting in ImageJ (NHI, USA). This tool allowed me to count several assays (each one with different conditions and different immunostainings) faster and in an unbiased way. The script for the automatic count can be found in annexe (chapter 7.1).

To validate the built Macro, I compared both automatic and manually centriole scoring (figure 3.3). DMEL cultured cells were arrested and were depleted for four major PCM proteins (Asl, Cnn, D-Plp and Spd2 – "All PCM"), which was previously shown to lead to a decrease in centriole number[53]. As a control, cells were transfected with *mCherry* dsRNA. Centrioles were scored using three different markers: CP110, Sas4 and D-Plp. The obtained results are shown in Figure 3.3.

The data shows that the number of centrioles counted by the developed Macro are not significantly different from the number of centrioles I counted manually, even when using several different markers. This shows that the Macro does not introduce errors in centriole scoring. As the developed Macro is a reliable tool for centriole scoring in *Drosophila* cultured cells, all further experiments were scored by using this automatic process.

3.2. Candidate screen for identification of protein involved in centrosome stability

3.2.1. Candidate Selection

In order to identify proteins that contribute to centrosome stability I conducted an RNAi screen by depleting candidate proteins that are described as being part of the different modules that form the centrosomal structure: the cap, the cartwheel and the centriolar wall. The candidate proteins will only

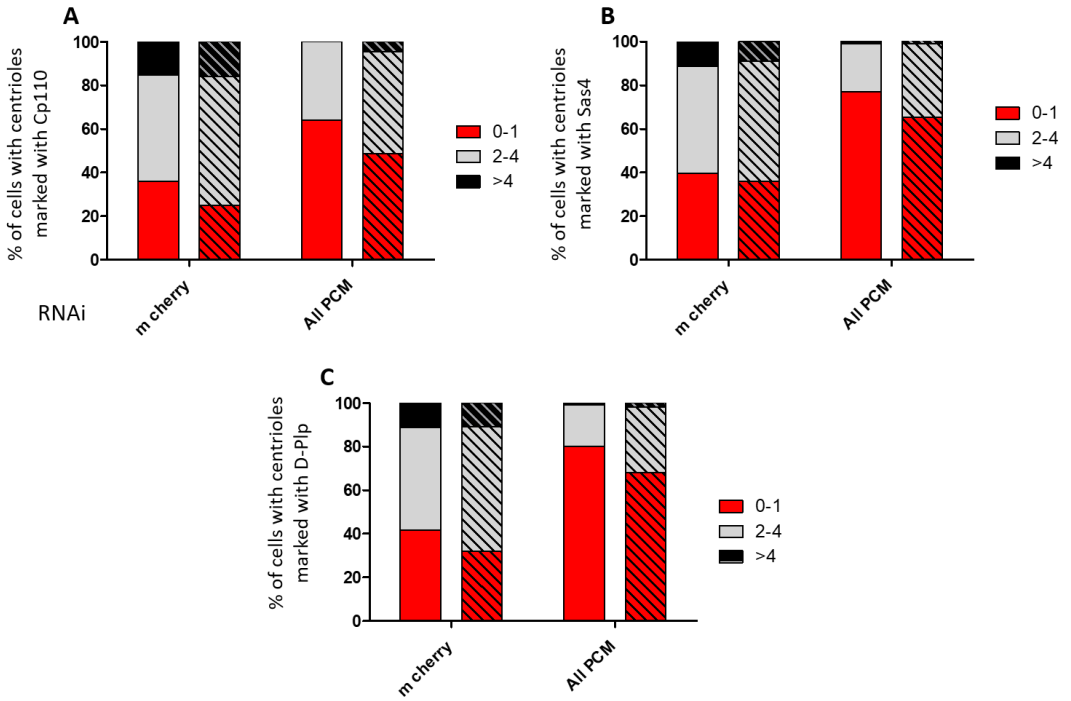


Figure 3.3: Automatic centriole counting does not introduce errors in centriole scoring. Centrioles were counted manually and automatically using three different markers: CP110 **(A);** Sas4 **(B)** and D-Plp **(C).** The histograms represent the percentages of cells with 0-1(red), 2-4 (grey) and >4 (black) centrioles, marked with the respective markers. The plain histograms show the results obtained with the manual counting while the striped histograms represent the results obtained using the automatic process. Only one experience was performed, and more than 100 cells were counted per condition.

be briefly described, and, for the matter of simplicity, I will divide the centrosome in zones as previously described and characterized by SIM (see Introduction, chapter 1.2.2).

Upon duplication, the cartwheel proteins Sas6 and Ana2, are the first ones to be recruited to the mother centriole, to allow procentriole formation and lie at the very core of the centriole (zone I). Sas6 is a conserved coiled-coil protein that is able to homo-oligomerize *in vitro* to form ring like structures that resemble the cartwheel structure[79,80]. After cartwheel formation, Ana 2 is phosphorylated by Plk4 and recruited to the mother centriole, allowing centriole duplication[81].

After cartwheel assembly, centriolar wall proteins, like Bld10, Ana1 and Sas4, start to accumulate at the centriole. Bld10 and Ana1 have been described as centriolar core proteins. Using SIM, it was possible to observe that Ana1 forms a small ring around the little punctate formed by Bld10 which allowed to divide the centriolar core in two different zones: zone I, occupied by Bld10, and zone II which is occupied by Ana1[19,33]. Both proteins were shown to interact Fu et al., 2015) and participate in centriole duplication[33,40], centriole maturation[33,78] and centriole elongation[31,32,40]. Interestingly, Bld10 is able to bind and stabilize centriolar MTs[82].

Sas4 is the main component of zone II and provides the connection between the cartwheel, by interacting with Ana2, and the centriolar MTs that form the centriolar wall[83,84]. This protein has been described, not only as important for centriole duplication and elongation[85,86] but also for centriole maturation, particularly, for PCM recruitment to the newly formed centriole[87].

Cap proteins, like CP110 and Cep97, are the next module to be recruited to the centrosome. CP110 and Cep97 are present at the distal end of both centrioles, which corresponds to zone V of the centrosome[19]. CP110 is a coiled-coil protein necessary for centriole duplication[40,88] and control of centriole length[86,89]. This protein is recruited to the centrosome by Cep97 and loss of both proteins promotes centriole overelongation[90,91]. Depletion of CP110 in *Drosophila* cultured cells leads to shortening of the centrioles due to exposure to the microtubule depolymerizing protein Klp10A[92].

I also tested Plk4 which is a master regulator of centriole duplication. Depletion of this kinase in cycling *Drosophila* cultured cells leads to centrosome loss and disorganized mitotic spindle[18], on the other hand its overexpression leads to centrosome amplification[6,93,94]. Plk4 has been described as part of the centrosome structure, being localized in zone III of the centrosome[19] and interacting with Ana2[81] and Asl[95].

We hypothesised that, besides being important for centrosome biogenesis and maturation, proteins which form different parts of the centrosome might also play different roles in maintenance of its structure. Table 3.2 summarizes the main known functions for each of the candidates as well as their known interactors. Figure 3.4 shows the spatial organization of the different proteins at the centrosome.

Table 3.2: Chosen candidate proteins for the RNAi screen.

Protein	Module	Function	Known Interactors
Ana2	Cartwheel	Centriole duplication	Plk4; Sas6; Sas4
Sas6		Centriole assembly and duplication; Cartwheel assembly	Ana2; Bld10
Bld10	Centriolar wall	Centriole stability, duplication and elongation	Ana1; SAS6
Ana1		Centriole duplication and elongation	Asl; Bld10
Sas4		Centriole duplication, elongation and maturation	Asl; Cnn; D-Plp; Ana2
Cp110	Cap	Centriole duplication and elongation	Cep97
Cep97		Centriole elongation	Cp110
Plk4	Biogenesis Regulator	Centriole assembly and duplication	Sas4; Sas6; Asl

3.1.1. 8 Day centriole stability assay

All candidate proteins were tested for a role in centrosome stability by depletion in DMEL cultured cells using the "8 Day Stability Assay" described in chapter 2.4. In this assay, cells are arrested in S phase (using Aph and HU) preventing centriole duplication, which allows to uncouple centrosome biogenesis from maintenance, and, as the cells do not duplicate their centrosomes, their number is kept

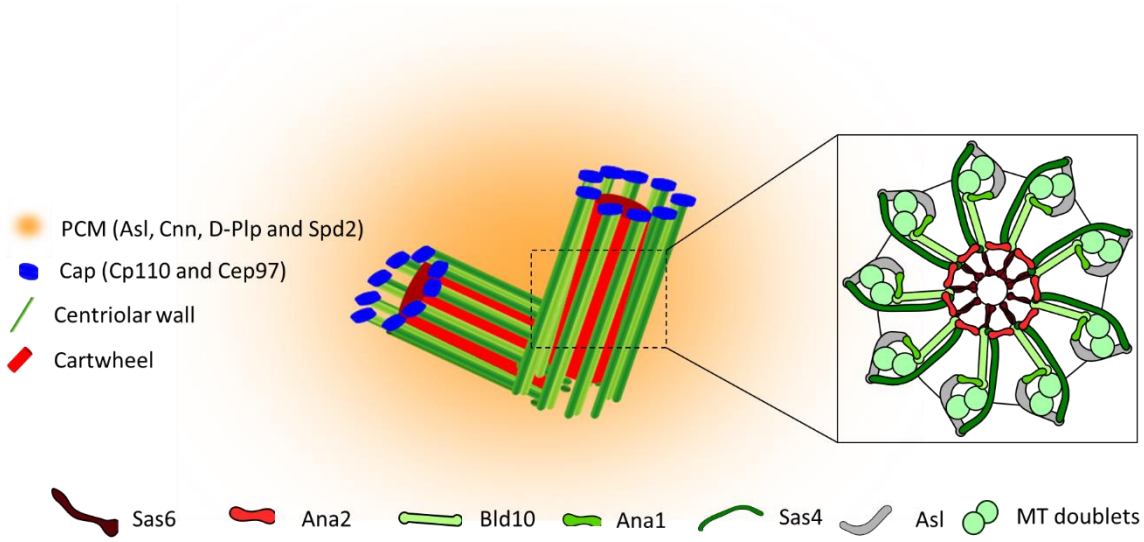


Figure 3.4: Schematic representation of the Drosophila centrosome and its main components.

constant. We can use the constant number of centrosomes in our advantage to understand if depletion of the candidate proteins affects or not centrosome stability. After mitosis, each daughter cell should have inherited a centrosome with two centrioles which start to disengage during G1 phase. At the end of G1/early S phase, procentriole formation starts in each of the centriole. As cells were blocked in S phase, each cell should have either two to four centrioles.

If the candidate protein is important for centriole stabilization we expect a decrease in the number of centrioles upon depletion (increase in the percentage of cells with zero to one centrioles, an abnormally low number); on the other hand, if the protein does not contribute to centriole stabilization we ecpect the number of centrioles to not change significantly.

The depletion phenotype of each candidate protein was compared with a negative control, which should not change the number of centrioles in cells. I also used a positive control which drastically alters centriole numbers to make sure that the experience was correctly performed. As a negative control cells were transfected with RNAi against *mCherry*, which is not encoded in the *Drosophila* genome. As a positive control, cells were simultaneously depleted for four major PCM proteins (Asl, Cnn, D-PLP and Spd2 – "All PCM"), which is known to induce centriole loss in arrested DMEL cultured cells[53].

Three independent experiments that included all the candidates and the controls were performed and at the 8th day cells were harvested for immunostaining and RT-PCR (Materials and Methods, (chapters 2.5 and 2.6, respectively).

3.2.2. Confirmation of mRNA depletion

RNAi targets the endogenous messenger RNA for degradation, not allowing it to be expressed. I performed an RT-PCR to confirm that the candidate RNAs were effectively depleted in DMEL cultured cells (Materials and Methods, chapter 2.6). As a readout for the amount of mRNA in the different RNAi conditions, I used eIF4a elongation factor, a house keeping gene, as a loading control. As expected, there were no differences in the mRNA levels of the eIF4a between the negative control (*mCherry* RNAi) and the depletion of the different candidate proteins (Figure 3.5). In contrast, I observed a decrease in the mRNA levels of the different candidate proteins in comparison with the respective control (Figure 3.5). The reduction in the intensity of the bands was variable among the different candidates, however, all of them show a reduction in their respective mRNA levels, which suggests that the protein levels of each candidate protein should be reduced upon RNAi treatment, in comparison with the control (*mCherry* RNAi) (Figure 3.5).

Unfortunately, it was not possible to confirm CP110 and Sas4 depletion using RT-PCR. However, both proteins were depleted and its presence at the centrosome was analysed by immunostaining. In both cases, I observed a reduction in the number of centrioles marked with either CP110 and Sas4, suggesting that these proteins were significantly depleted from the centrosome (Figures 3.10C and 3.9C, respectively).

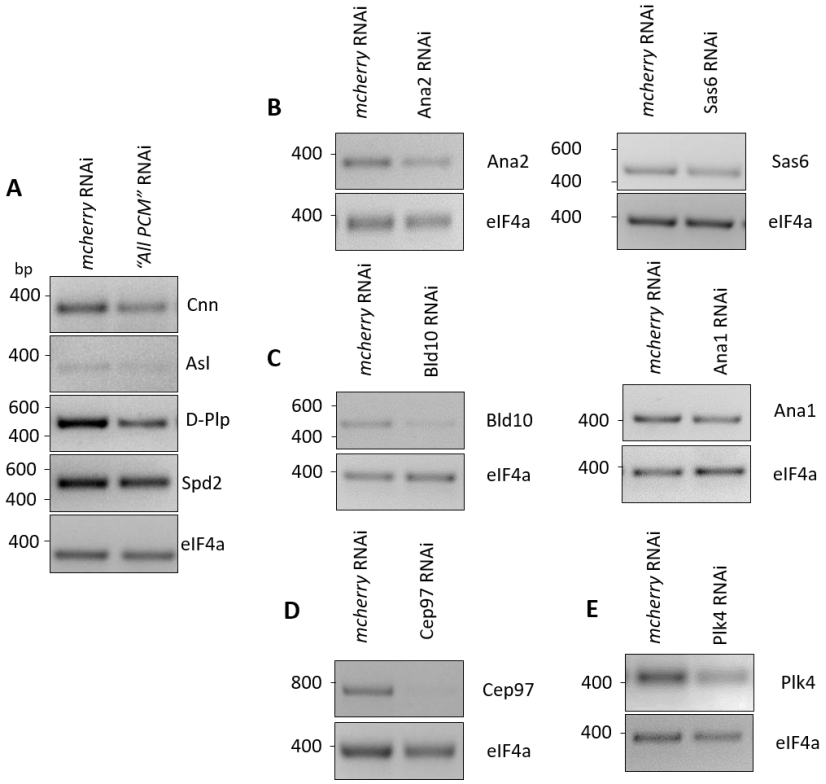


Figure 3.5: Centrosomal RNAs were depleted to different extents in DMEL cells. Comparison of the intensity of the bands amplified from the cDNA obtained from the different RNAi experimental samples (right samples) and the negative control (left samples), shows a decrease in the intensity levels of most bands, suggesting depletion of all mRNA of the candidate proteins.

3.2.3. Stability phenotypes upon depletion of the different centrosome proteins

In order to understand how the different candidate proteins contribute for centriole stability, I scored centriole number using 5 different centrosomal markers: D-Plp, Sas4, Bld10, Ana1 and CP110, which label the different structures. Two different sets of immunostainings were performed to allow analyses of the centrosomes with the different markers. I performed the first immunostaining with CP110, Sas4 and D-Plp, and the second immunostaining using Bld10, Ana1 and D-Plp. D-Plp antibody is well characterized[18] and shows a robust signal, therefore, it was used in both immunostainings aterial and Methods, chapter 2.5).C and

D-Plp localizes in the zone III of te centriole and was therefore used as a PCM marker. Bld10 and Ana1 are centriolar wall proteins, while Sas4 lies between the centriolar wall and the PCM. Accordingly, these proteins were used as centriolar wall markers. Finally, CP110 is a cap protein present at the distal tip of the centriole and was used a cap marker. The use of all these markers will provide insights on how the candidates may be affecting the

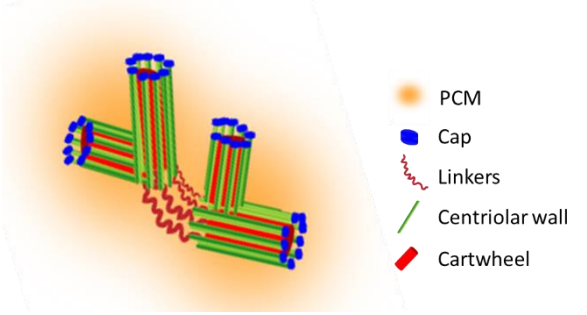


Figure 3.6: The centrosome is formed by several different modules: the cartwheel, the centriolar wall, the cap and the PCM.

different modules that form the centriole: the **PCM** (D-PLP), the **centriolar wall** (Bld10, Ana1 and Sas4) and the **Cap** (CP110) (Figure 3.6).

Removal of the PCM

Previous work showed that removal of PCM proteins leads to centriole destabilization[53]. To better understand the importance of the PCM on centriole stability I depleted four different PCM proteins ("All PCM RNAi) and analysed the number of centrioles marked with either D-Plp, Sas4 and CP110 (Figure 3.7A) or D-Plp, Bld10 and Ana1 (Figure 3.7B).

As I am arresting DMEL cultured cells in S phase, centriole duplication is prevented and the number of centrioles is kept constant. Therefore, these cells should have between two to four centrioles. However, *Drosophila* cultured cells can cope with large number of centrioles per cell, and therefore it is expected that approximately 30% of the cells will have an abnormal centriole number[14]. As a small part of the population shows more than four centrioles per cell we chose to look to the distribution of the number of centrioles per cell in all counted cells to evaluate centriole destabilization.

It is possible to observe that removal of PCM proteins leads to a shift in the dispersion of the population towards lower centriole numbers (decrease in the mean, median, first and third quartiles) using either PCM (orange), centriole (green) or cap (blue) markers, which suggests a decrease in the number of centrioles per cell (Figure 3.7C). Note that, although in the RT-PCR the intensity of the D-Plp band was not strikingly different from the control (*mCherry* RNAi) (Figure 3.5), there is a decrease in the number of centrioles per cell marked with D-Plp suggesting that we are effectively depleting D-Plp from the centrosome.

As expected, the observed results suggest that centrioles are being destabilized and, therefore, disassembled. In contrast with all the other centrosome markers, Ana1 does not seem to be lost from the centrioles at the same rate. Not only the mean number of centrioles marked with Ana1 is higher than the mean number observed using all the other markers (Figure 3.7C, green), but also, it is possible to observe the presence of dots positive for the staining with this marker when all the other markers are no longer present (Figure 3.7B). It is very likely that Ana1 is a stable protein at the centrosome and, upon centrosome destabilization, one of the last proteins to be lost from the structure. Perhaps the Ana1 dots observed correspond to centriole remnants, however, I will further tackle this hypothesis in the future.

As it was expected, my data suggests that depletion of PCM proteins from the centrosome destabilizes all the modules that form the centrosome: PCM (orange), the centriolar wall (green) and the cap (blue).

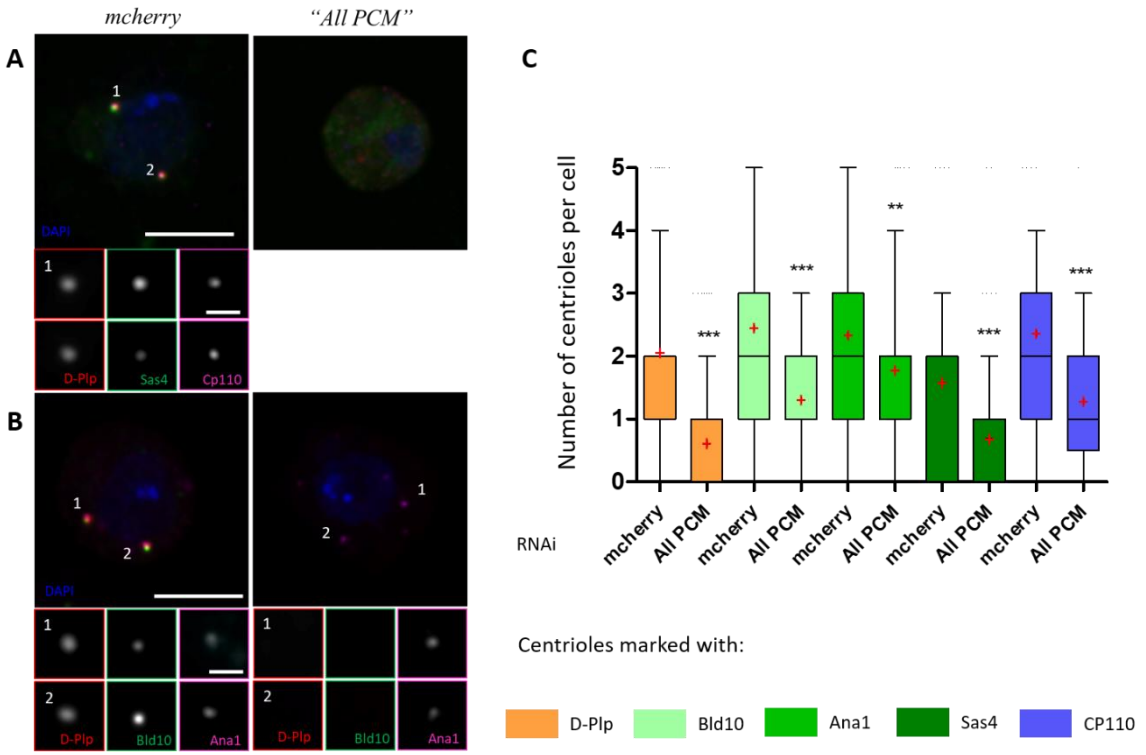


Figure 3.7: Removal of PCM proteins leads to destabilization of all the modules that form the centrosome. (**A** and **B**) Representative images of the immunostaining of "All PCM"- depleted cells and control cells. **(A)** Centrioles were identified using antibodies to detect D-Plp (red), Sas4 (green) and CP110 (magenta) and **(B)** antibodies to detect D-Plp (red), Bld10 (green) and Ana1 (magenta). In both cases D-Plp was used as a PCM marker (red) and DAPI (blue) was used to stain the DNA. All images were acquired with same exposure. Scale bar, 5µm. Enlargements of the indicated areas (numbers) are shown. Scale bar, 1 µm. **(C)** Plot representing the distribution of the number of centrioles per cell. The box limits represent the first and third quartiles and the band inside the box corresponds to the median of the distribution. The whiskers in the box represent the 10% and 90% percentile. The red cross represents the mean of distribution (n≥100 cells per condition in each experiment) **P<0.01; ***P<0.001 (analysis of variance using a One-Way ANOVA).

Removal of the Cartwheel

I analysed the cartwheel role in centrosome stability by counting centrioles marked with D-Plp, Sas4 and CP110 (Figure 3.8A) or D-Plp, Bld10 and Ana1 (Figure 3.8B) in Ana2- or Sas6-depleted cells. Similarly to what was observed upon depletion of PCM proteins, depletion of the cartwheel proteins Ana2 and Sas6 leads to a decrease in the number of centrioles per cell using either PCM (orange), centriolar (green) or cap (blue) markers (Figure 3.8C).

Interestingly, Ana2 depleted cells (highlighted by red in Figure 3.8C) show a lower mean number of centrioles per cell when compared with Sas6 depleted cells, except when centrioles are marked with CP110 (blue). In Ana2-depleted cells the mean number of centrioles per cell is slightly higher than the mean observed for Sas6-depleted cells. This suggests that although Ana2 removal form the centrosome leads to a strong destabilization of the PCM and centriolar wall proteins, it does not destabilize the cap at the same extent. Interestingly, from all the markers tested, removal of Ana2 had the strongest effect in the centriolar wall protein Sas4. Again, Ana1 seemed to be the least affected protein upon depletion of both Ana2 or Sas6.

Altogether these results suggest that removal of cartwheel proteins affects the centrosome structure by destabilizing the PCM (orange) and the centriolar wall (green), but not the cap (blue). Furthermore, within the cartwheel, Ana2 is likely to have a more important role in centrosome stability in comparison with Sas6.

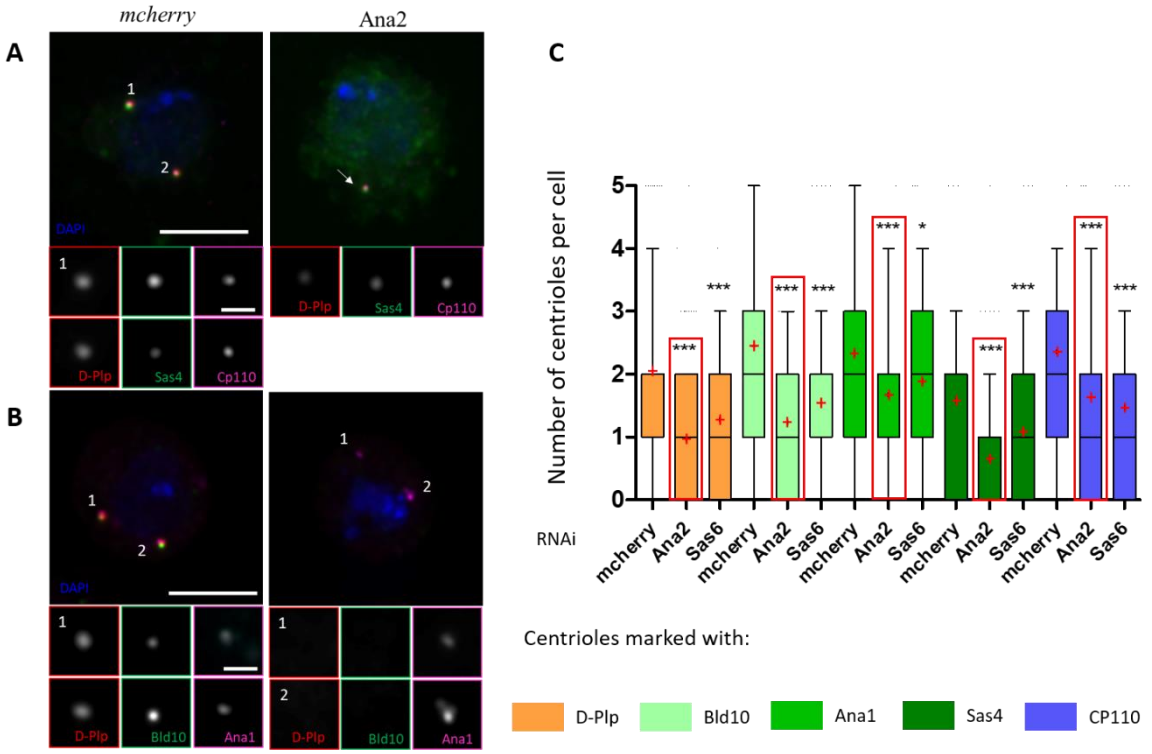


Figure 3.8: Removal of cartwheel proteins Ana2 and Sas6 leads to destabilization of the PCM and the centriolar wall but not the cap modules. (**A** and **B**) Representative images of the immunostaining of Ana2-depleted cells and the respective control (*mCherry* RNAi). Centrioles were identified by using antibodies to detect D-Plp (red), Sas4 (green) and CP110 (magenta) and **(B)** antibodies to detect D-Plp (red), Bld10 (green) and Ana1 (magenta). In both cases D-Plp was used as a PCM marker (red) and DAPI (blue) was used to stain the DNA. All images were acquired with same exposure. Scale bar, 5µm. Enlargements of the indicated areas (numbers) are shown. Scale bar, 1 µm. **(C)** Plot representing the distribution of the number of centrioles per cell. The box limits represent the first and third quartiles and the band inside the box corresponds to the median of the distribution. The whiskers in the box represent the 10% and 90% percentile. The red cross represents the mean of distribution. (n≥100 cells per condition in each experiment). *P<0.1; ***P < 0.001; (analysis of variance using a One-Way ANOVA).

Removal of the Centriolar Wall

To understand if centriolar wall proteins play a role in centrosome stability I depleted three different centriolar wall proteins: Ana1, Bld10 and Sas4. The number of centrioles was accessed by immunostaining of treated cells with D-Plp, Sas4 and CP110 antibodies (Figure 3.9A) or D-Plp, Bld10 and Ana1 antibodies (Figure 3.9B). Depletion of these proteins led to a reduction in the number of centrioles per cell with all the markers used (Figure 3.9C). Despite affecting all the modules that form the centrosome, removal of centriolar wall proteins does not lead to an accentuated reduction in the number of centrioles marked with CP110 in comparison with all the other markers, suggesting that the cap is not affected in the same extension as the PCM or the centriolar wall.

Bld10-depleted cells show the lowest number of centrioles per cell marked with Bld10 (Figure 3.9C in light green), suggesting that Bld10 was effectively depleted from the centrosome. Similarly, Ana1- and Sas4-depleted cells show the lowest number of centrioles marked with Ana1 and Sas4,

respectively, which suggests that also Ana1 and Sas4 are being effectively depleted (Figure 3.9C in green and dark, respectively).

Depletion of Ana1 (highlighted by red in Figure 3.9C) was the condition that led to accentuated loss of centrioles marked with either D-Plp, Sas4, Bld10 or CP110 when compared with the remaining centriolar wall proteins tested (Bld10 and Sas4). This suggests that the depletion of this protein severely affects centrosome structure. Interestingly, even upon Ana1 depletion itself we were able to observe cells that presented centrioles marked with Ana1 (Figure 3.9B).

Our data suggest that depletion of centriolar wall proteins leads to destabilization of the PCM, the centriolar wall and the cap. Furthermore, from all the centriolar wall proteins tested, Ana1 seems to have an important role on centrosome stability.

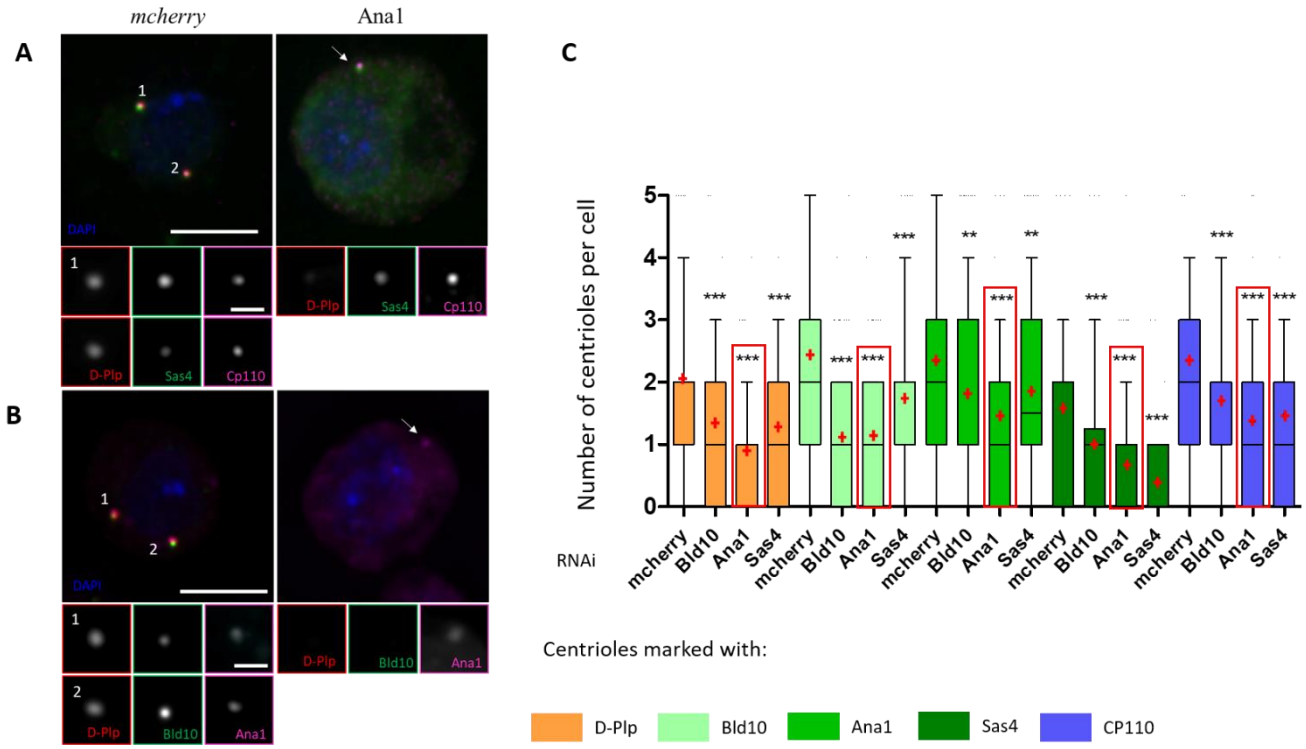


Figure 3.9: Removal of centriolar wall proteins leads to destabilization of all the modules that form the centrosome. (**A** and **B**) Representative images of the immunostaining of Ana1-depleted cells and control (*mCherry* RNAi). Centrioles were identified by using antibodies to detect D-Plp (red), Sas4 (green) and CP110 (magenta) and **(B)** antibodies to detect D-Plp (red), Bld10 (green) and Ana1 (magenta). In both cases D-Plp was used as a PCM marker (red) and DAPI (blue) was used to stain the DNA. All images were acquired with same exposure. Scale bar, 5µm. Enlargements of the indicated areas (numbers) are shown. Scale bar, 1 µm. **(C)** Plot representing the distribution of the number of centrioles per cell. The box limits represent the first and third quartiles and the band inside the box represents the median of the distribution. The whiskers in the box represent the 10% and 90% percentile. The red cross represents the mean of distribution (n≥100 cells per condition in each condition). **P < 0.01, ***P < 0.001; (analysis of variance using a One-Way ANOVA).

Removal of the cap

To test the importance of the cap on centrosome stability I depleted two cap proteins: CP110 and Cep97. Centrioles were analysed by immunostaining with D-Plp, Sas4 and CP110 antibodies (Figure 3.10A) or D-Plp, Bld10 and Ana1 antibodies (Figure 3.10B).

Depletion of CP110 led to a significant reduction in the number of centrioles marked with either Bld10 (light green) or Sas4 (dark green) or CP110 (blue) (highlighted in red in Figure 3.10C). The accentuated reduction in the number of centrioles marked with CP110 upon depletion of CP110 itself suggests that CP110 was efficiently depleted (Figure 3.10C in blue). Nevertheless, depletion of this protein only produced a small reduction in the number of centrioles per cell marked with D-Plp (orange) and no changes were observed between the control and CP110 depleted cells when centrioles were marked with Ana1 (green) (Figure 3.10C). These results suggest that CP110 depletion leads to

centrosome destabilization. However, one should note that the centrosomes in these cells were not as strongly destabilized as the ones observed previously: neither the mean nor the variability outside the upper and lower quartiles drastically decreased in comparison with the control (*mCherry* RNAi). Moreover, we observed a large number of centrioles in CP110-depleted cells marked with D-Plp, Sas4, Bld10 and Ana1 (Figure 3.10A and B).

On the other hand, removal of Cep97 only induced a decrease in the number of centrioles per cell when centrioles were marked with either Sas4 (dark green) or CP110 (blue) (Figure 3.10C). As Cep97 is a cap protein known to interact with CP110, it is expected that depletion of these protein leads to a reduction in the number of centrioles marked with CP110, which, in addition confirms that this protein was efficiently depleted. On the other hand, reduction in the number of centrioles marked with Sas4 upon Cep97 depletion was not expected and a direct link between Cep97 and the centriolar wall protein Sas4 has not been shown.

Collectively, as removal of cap proteins leads to a mild reduction in the number of centrioles per cell, our results suggest that depletion of cap proteins does not seem to strongly destabilize the centrosome structure.

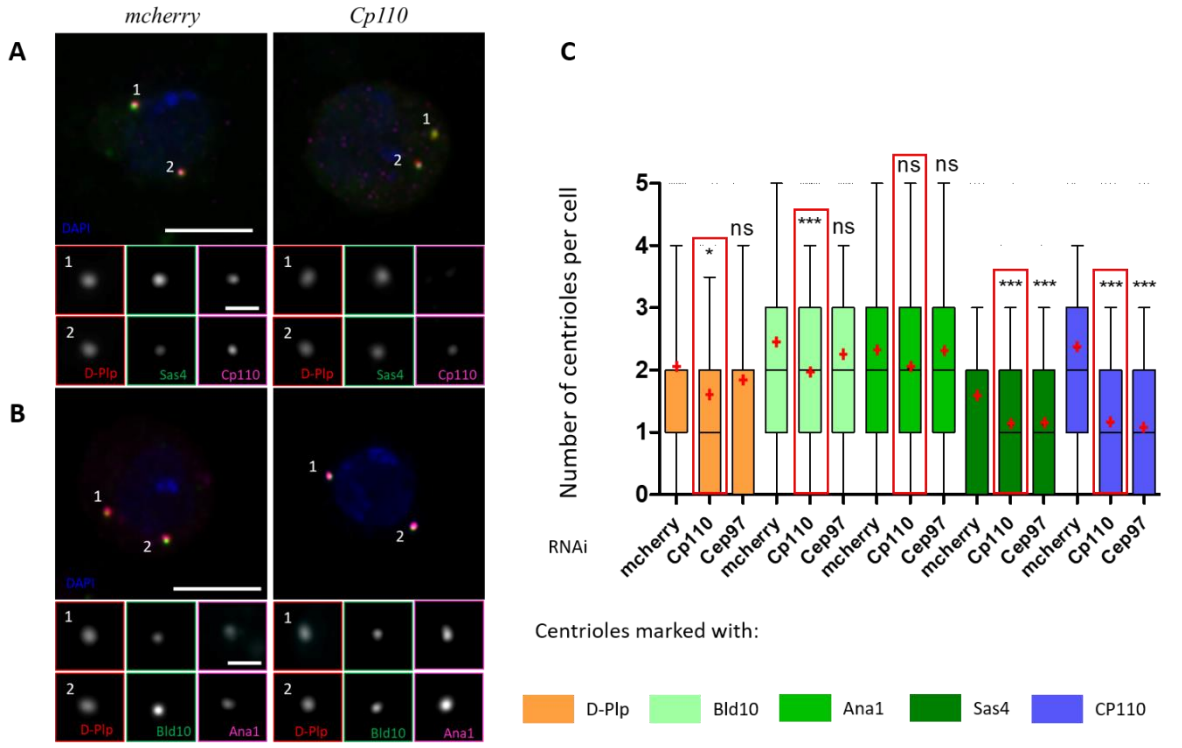


Figure 3.10: Removal of cap proteins does not cause destabilization of the centrosome. (**A** and **B**) Representative images of the immunostaining CP110-depleted cells and the control cells (*mCherry* RNAi). Centrioles were identified by using antibodies to detect **(A)** D-Plp (red), Sas4 (green) and CP110 (magenta) and **(B)** antibodies to detect D-Plp (red), Bld10 (green), Ana1 (magenta) and D-PLP. In both cases D-Plp was used as a PCM marker (red) and DAPI (blue) was used to stain the DNA. All images were acquired with same exposure. Scale bar, 5µm. Enlargements of the indicated areas (numbers) are shown. Scale bar, 1 µm. **(C)** Plots show the distribution of the number of centrioles per cell. The box limits represent the first and third quartiles and the band inside the box represents the median of the distribution. The whiskers in the box represent the 10% and 90% percentile. The red cross represents the mean of distribution. (n≥100 cells per condition in each experiment). *P<0.1; ***P < 0.001; ns, not significant (analysis of variance using a One-Way ANOVA)

Removal of Plk4

In order to understand the importance of Plk4, the master regulator of centriole biogenesis, I depleted Plk4 and analysed the presence of centrioles using D-Plp, Sas4 and CP110 (Figure 3.11A) or D-Plp, Bld10 and Ana1 (Figure 3.11B) as markers. Depletion of Plk4 led to a decrease in the number of centrioles per cell when D-Plp (orange), Bld10 (light green), Sas4 (dark green) and CP110 (blue)

were used as markers. Again, no significant changes regarding the number of centrioles were observed when centrioles were marked with Ana1 (green) (Figure 3.11C).

Although depletion of Plk4 leads to a reduction in the number of centrioles, this reduction was not as strong as the ones observed upon depletion of PCM, cartwheel or centriolar wall proteins. Similarly to what we observed upon cap depletion, Plk4-depleted cells do not show an accentuated decrease neither in the mean nor in the variability outside the upper and lower quartiles in comparison with the control (*mCherry* RNAi), except when centrioles where marked with Sas4. The reduction in the number of centrioles marked with Sas4 was expected as it has been shown Sas4 and Plk4 are interactors (for details see discussion).

Overall, our results suggest that Plk4 does not play a major role in centrosome destabilization.

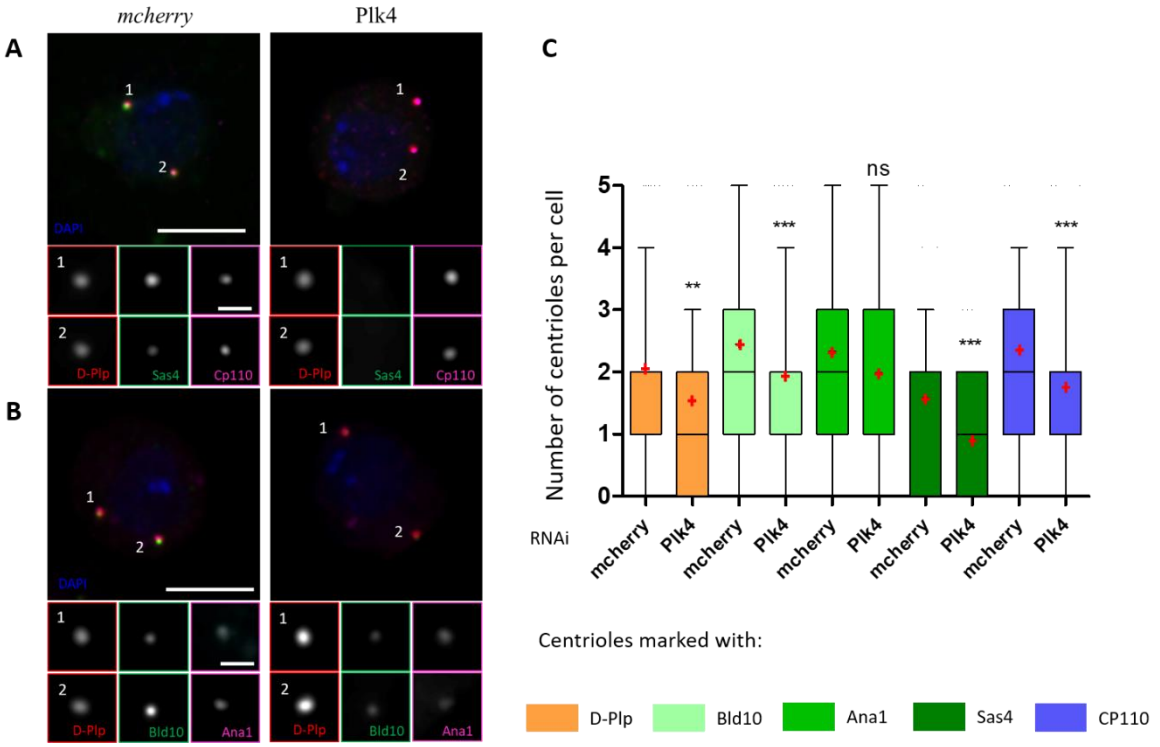


Figure 3.11: Removal of Plk4 does not cause destabilization of the centrosome. (**A** and **B**) Representative images of the immunostaining Plk4-depleted cells and control cells (*mCherry* RNAi). Centrioles were identified using **(A)** antibodies to detect D-Plp (red), Sas4 (green) and CP110 (magenta) and **(B)** antibodies to detect D-Plp (red), Bld10 (green) and Ana1 (magenta). In both cases DAPI (blue) was used to stain the DNA. All images were acquired with same exposure. Scale bar, 5µm. Enlargements of the indicated areas (numbers) are shown. Scale bar, 1 µm. **(C)** Plot represents the distribution of the number of centrioles per cell. The box limits represent the first and third quartiles and the band inside the box represents the median of the distribution. The whiskers in the box represent the 10% and 90% percentile. The red cross represents the mean of distribution. (n≥100 cells per condition in each experiment). **P < 0.01, ***P < 0.001; ns, not significant (analysis of variance using a One-Way ANOVA).

The present screen in *Drosophila* cultured cells shows that not only PCM (as it was previously reported) but also cartwheel and centriolar wall proteins are important modules of the centrosome that contribute for its stability.

3.3. Two different pools of Ana1 are present at the centrosome

From all the candidate proteins tested, depletion of Ana1 was one of the candidates that showed a higher increase in the percentage of cells with abnormally low numbers of centrioles (zero to one centrioles). Despite the strong phenotype shown upon depletion of Ana1 (Figure 3.9C), we observed that upon depletion of any of the candidate proteins it was possible to observe a large number of dots

positive for Ana1 staining. These results led us to hypothesis that: (1) the antibody does not recognize specifically Ana1; or (2) Ana1 is highly stable at the centrosome, which makes its complete depletion difficult. Therefore, this might be one of the last proteins to be lost from the centrosome upon centrosome disassembly.

To show that Ana1 is being effectively depleted from the centrosome we could have performed a western blot. In this case, we would expect to observe a strong band in the control (*mCherry* RNAi) and a faint band in Ana1-depleted cells. However, even if we were able to detect a decrease in the band correspondent to Ana1 in Ana1-depleted cells using the western blot this would exclude the possibility that the antibody is recognizing any other protein besides Ana1 in the immunostaining. Therefore, to understand if Ana1 antibody used is specifically recognizing Ana1 at the centrosome, we chose to stain our cells using a different Ana1 antibody (anti-Ana1 raised in guinea-pig, Figure 3.12A). Moreover, to confirm that Ana1 is being sufficiently depleted from the centrosome, we compared Ana1 signal in the control (*mCherry* RNAi) and in Ana1-depleted cells (Figure 3.12B).

The staining using Ana1 antibody raised in guinea-pig was performed in Ana1-depleted cells, "All PCM"-depleted cells and control cells (*mCherry* RNAi) (Figure 3.12A). "All PCM" depletion leads to a strong destabilization of the centrosome and consequent reduction in the number of centrioles per cell. Therefore, we expect that if Ana1 antibody raised in guinea-pig is recognizing any other protein besides Ana1, the number of centrioles in these cells will be larger than the number observed in the samples that were stained with the Ana1 antibody raised in rat.

Comparison of the number of centrioles marked with Ana1 antibody raised in guinea-pig (plain histograms – Figure 3.12A) and Ana1 antibody raised in rat (striped histograms – Figure 3.12A) showed no significant difference. These results not only show that the antibody used recognizes specifically this protein, but also suggest that either Ana1 is not being sufficiently depleted or it is very stable at the centrosome. To further investigate Ana1 depletion from the centrosome I quantified the signal of Ana1 in control cells (*mcherry* RNAi) and in Ana1-depleted cells. Although there is no significant decrease in the number of centrioles marked with Ana1, there is a statistically significant reduction in the intensity of Ana1 signal at the centriole in Ana1-depleted cells (Figure 3.12B). This shows that Ana1 is being depleted at some extent from the centrosome.

Altogether, these results show that Ana1 is reduced at the centrosome and that the antibody used to analyse this protein is specific. Therefore, it is likely that there are two distinct pools of Ana1 at the centrosome: one stable, which we cannot deplete by RNAi; and one dynamic that is possibly being removed. It is likely that the RNAi treatment is depleting the dynamic pool of Ana1 but not the stable pool, explaining the presence of this protein at the centrosome upon its depletion. Interestingly, the depletion of the dynamic pool of Ana1 is enough to cause a big reduction in the number of centrioles marked with either PCM, centriolar wall or cap markers suggesting that this protein has an important role in centrosome stability. Probably the structures that remain are centrosome remnants instead of proper centrioles, but this will have to be investigated using ultrastructural approaches, such as EM.

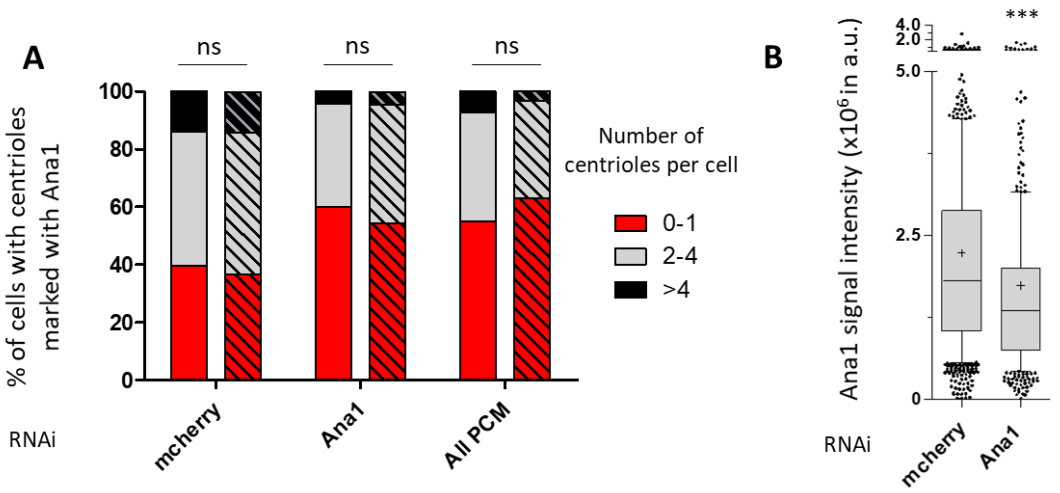


Figure 3.12: Ana1 is reduced at the centrosome and Ana1 antibodies are specific. (A) Ana1 antibodies specifically recognizes Ana1. Percentage of cells with 0-1 (red), 2-4 (grey) or >4 (black) centrioles marked with Ana1 antibody. The plain histograms represent he Ana1 staining using the anti-Ana1 antibody raised in guinea pig while the striped histograms represent the staining using the anti-Ana1 antibody raised in rat. For each condition more than 100 cells were counted; ns, not significant (analysis of variance using an ANOVA). **(B)** Ana1 is being depleted from the centrosome. Quantification of the Ana1total integrated intensity levels at the centrioles. Box plots show the dispersion of the population. The box limits represent the first and third quartiles and the band inside the box the median of the distribution. The cross shows the mean of the distribution. The whiskers in the box represent the 10% and 90% percentile. The data was analysed from three independent experiments (n≥100 cells per conditions). ***P<0.0001 (unpaired Mann-Whitney test).

3.4. *In vivo* studies in *Drosophila melanogaster*

The candidate screen in *Drosophila* cultured cells shows that both cartwheel and centriolar wall proteins contribute for centriole stabilization. Ana1- and Ana2- depleted cells both show an accentuated increase in the percentage of cells with an abnormally low number of centrioles per cell, suggesting that these proteins have a key role in centriole stabilization.

In human cells, newly formed centrioles in Cep295-depeted cells (Ana1 human orthologue) do not acquire PCM, compromising centrosome maturation and leading to their disassembly at the end of the next cell cycle[78]. Interestingly, Ana1 has been shown to interact with Polo by a yeast-two hybrid assay[96]. Taking into account that Polo plays a key role in centrosome stabilization by PCM maintenance at the centrioles[53], we hypothesize that Polo allows PCM maintenance at the centriole in a process mediated by Ana1.

Taking advantage of the tools available in the lab, I investigated whether Ana1 is important for centriole stability *in vivo*, and if this role is through Polo and PCM maintenance at the centrioles. To test this hypothesis, I over-expressed the GFP-Polo-PACT construct in the female *Drosophila's* germ line. Expression of this construct allows ectopic tethering of Polo at the centrosome and prevents PCM loss. As a consequence, centrosomes are abnormally kept until meiotic stages of oogenesis[53]. We expect that, if Ana1 has a role in keeping PCM at the centrosome trough Polo, then centriole maintenance is likely to be affected in this background if Ana1 is depleted.

Ana1 was specifically depleted at the female germline by driving the expression of an UAS-RNAi against Ana1, using the maternal V32-Gal4 driver in a UASp-GFP-Polo-PACT background. For this experiment I performed two different controls. As a negative control was induced the expression of an UAS-RNAi against *mCherry*, again, using the maternal V32-Gal4 driver in a UASp-GFP-Polo-PACT background. As a control for the efficient depletion of Ana1 by the RNAi line, Ana1 RNAi was driven by a Nanos-GAL4, which induces expression of the RNAi line in the germarium before the four consecutive mitosis, and therefore affecting centriole duplication in these cycles.

Centrioles were identified by GFP-Polo-PACT, which localizes at the centrosome and two different antibodies: a rabbit anti-CP110 and a rat anti-γTubulin (as described in Materials and Methods). As I wanted to access if there was a reduction in the maintenance of centrioles at the late stages of oogenesis, we choose to analyse stage 10 oocytes (late stage). The intensity of the signal of each one of the markers was measured and the results are shown in Figure 3.14.

As expected, depletion of Ana1 before the four consecutive mitosis at the germarium (Ana1 RNAi driven by a Nanos-Gal4) leads to disappearance of the Ana1 MTOC signal in mid stages of oogenesis (stage 7/8) in comparison with flies without the inducing the expression of the RNAi line (Figures 2.3 and 3.13).

The levels of all the markers used to identify the centrioles were significantly decreased upon Ana1 depletion, in contrast to the *mCherry* RNAi control (Figure 3.14B). This results strongly suggest the presence of less centrioles at the oocyte[53]. Thus, it is likely that Ana1 regulates PCM acquisition at the centrosome trough Polo.

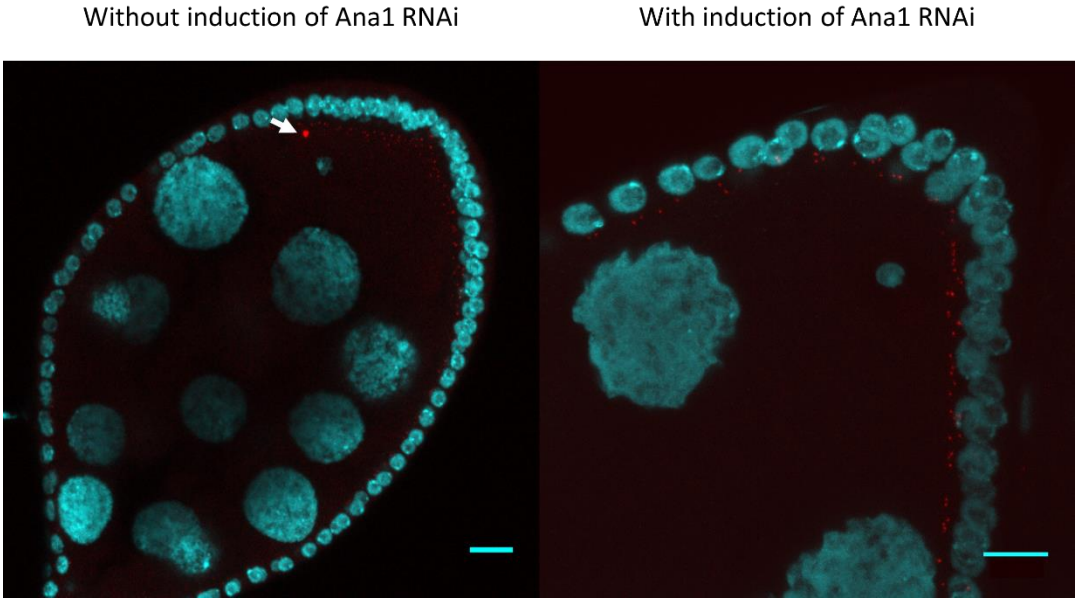


Figure 3.13: Ana1 is being efficiently depleted by the RNAi line. Induction of depletion of Ana1 using a Nanos-Gal4 leads to disappearance of the Ana1 MTOC signal whereas flies where the expression of the RNAi line was not induced show Ana1 at the MTOC.

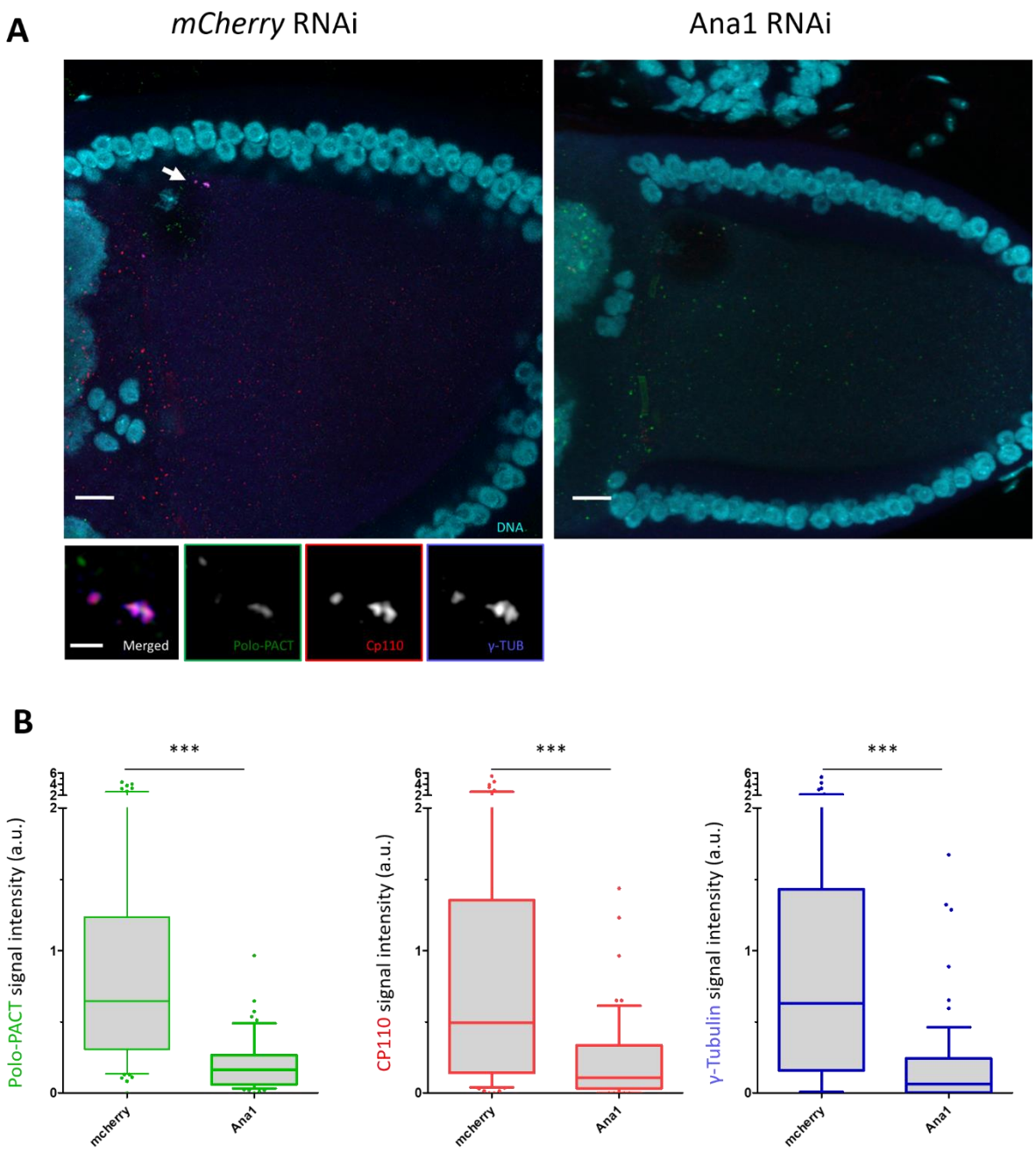


Figure 3.14: **Down-regulation of Ana1 when Polo is tethered to the centrosome leads to loss of centrioles in the late stages of *Drosophila* oogenesis.** Ana1RNAi or *mCherry* RNAi and GFP-Polo-PACT were simultaneously expressed in the germline of the fruit-fly. (**A**) Representative images of a stage 10 oocyte in a control situation (*mCherry* RNAi) and upon Ana1 depletion (Ana1 RNAi). GFP-Polo-PACT (green) expression under a maternal promoter and immunostaining for CP110 (red; using a rabbit anti-CP110 antibody) were used as centriolar markers. PCM was detected by immunostaining for γ-Tubulin (blue). All images were acquired with same exposure. Scale bar, 10 µm. Enlargements of the indicated areas (arrows) are shown. Scale bars, 5 µm. (**B**) Quantification of the total integrated intensity of co-localized GFP-Polo-PACT, CP110 and γ-Tubulin as a proxy for centriole numbers in stage 10 oocytes. Box plots show the distribution of the measured intensities for GFP-Polo-PACT (green), CP110 (red) and γ-Tubulin (blue) signals from n=27 oocytes for *mCherry*-RNAi (control) and n=27 for Ana1-RNAi. The box limits represent the first and third quartiles and the band inside the box the median of the distribution. The whiskers in the box represent the 10% and 90% percentile. The plots show three technical replicates. ***P<0.001 (unpaired Mann-Whitney test).

4. Discussion

To identify new players involved in the centriole maintenance program I performed a screen in *Drosophila* cultured cells arrested in S phase. In this screen, to test how different centrosome components affect centrosome stability I depleted several proteins known to be part of the centrosome structure: PCM, cartwheel, centriolar wall and cap proteins, as well as Plk4, the major regulator of centriole biogenesis. I analysed the effect on centrosome stability by scoring the number of centrioles per cell using markers to identify the different modules of the centrosome.

Depletion of these proteins showed a reduction in the number of centrioles however, not to the same extent. This allowed the distinction of two groups of candidate proteins: (1) proteins that show a **strong** effect in centriole destabilization and (2) proteins that show a **mild** effect in centriole destabilization.

Removal of PCM, cartwheel or centriolar wall proteins showed a strong effect in centriole destabilization. Depletion of these proteins led to a decrease in the number of centrioles per cell and consistently showed destabilization of all the modules studied (PCM, centriolar wall and cap).

Depletion of cap proteins and Plk4 (the principal centriole biogenesis regulator) showed a mild effect in centriole destabilization. Despite leading to a reduction in the number of centrioles per cell, neither the mean number of centrioles nor the variability outside the upper and lower quartiles changed significantly comparing with the control. This suggests that depletion of these proteins did not consistently show destabilization of all the modules of the centrosome.

The PCM confers MT nucleating capacity to the centrosome[8]. To study the importance of this module on centrosome stabilization I simultaneously depleted four important ,proteins known to be part of the PCM: Asl, Cnn, D-Plp and Spd2[19,20,22]. My observations show that removal of the PCM proteins shows a strong effect in centrosome stabilization (Figure 3.7). PCM's role in centriole biogenesis has been acknowledged. Asl, Cnn, D-Plp and Spd2 have an important role in centrosome maturation[16,97], in a process regulated by Polo[76,98,99]. Recently it was shown that loss of PCM leads to centriole elimination in DMEL cells and thus centriole destabilization[53]. My results confirm these observations and, in addition, show that PCM removal from the centrosome destabilizes the cap. This suggests that PCM has a key role in keeping centriole integrity, however, how the PCM is protecting the centrosome remains unclear.

To understand the importance of the cartwheel in centrosome stabilization I depleted Ana2 and Sas6, which interact to form the cartwheel at the core of the centriole (Figure 3.4)[81,68]. Depletion of these proteins led to a strong effect in centrosome destabilization (Figure 3.8), suggesting that removal of the cartwheel from centrioles can lead to centrosome destabilization.

Both these proteins have important roles during centriole duplication[,83,93,100-101] and recently it has been proposed that these can also contribute to centriole stabilization, as inhibition of cartwheel removal from the centrosome in human cells seems to stabilize the centrosome[78]. These results are in agreement with my observations and suggest that cartwheel might have a role in centriole stabilization.

To test the role of centriolar wall proteins in centrosome stabilization I depleted 3 different centriolar wall proteins: Bld10, Ana1 and Sas4. Depletion of each one of these proteins also showed a strong effect in centrosome destabilization, with Ana1 being the candidate showing the most accentuated centriole loss (Figure 3.9). These proteins are known to be involved in centriole elongation during centriole biogenesis[,21,31,32,102] and are able to interact with centriolar MTs[19-21, 82,103]. It was also shown that in *Drosophila* and *C. elegans*, depletion of Sas4 leads to a reduction in the levels of PCM in newly formed centrioles[87,104]. A similar phenotype has been observed in daughter centrioles of Cep295-depleted human cells (Ana1's orthologue in humans), which cannot acquire PCM components[78]. This led us to hypothesize that the phenotype that we observed upon removal of centriolar wall proteins might be due to destabilization of the centriolar MTs and/or to an impairment in PCM maintenance which can

lead to different degrees of centrosome destabilization. We propose that the different effects in centrosome destabilization observed upon depletion of the three centriolar wall proteins tested may be related to the interactions established by these proteins at the centrosome. Both Sas4 and Ana1 are known to connect the cartwheel to the PCM. On one hand, Sas4 not only interacts with the cartwheel protein Ana2[83], but also with several PCM proteins, such as: Asl[105], Cnn and D-Plp[106]. On the other hand, Ana1 binds to the centriolar wall protein Bld10[32,107], which might be how it is connected to the cartwheel, and interacts with the PCM protein Asl[32,107], which might act as the link to the PCM (see Figure 3.4).

From all the centriolar wall proteins tested, Ana1 showed the strongest phenotype. Therefore, we hypothesize that Ana1 has a leading role in centriole stabilization in comparison with the link set by Sas4. Ana1 depletion leads to removal of the centriolar wall protein Bld10 (see Figure 3.9) and, probably, triggers delocalization of the PCM protein Asl. Removal of these proteins would lead not only to a substantial destabilization of the centriolar MTs, but also to an impairment in PCM maintenance. Sas4 depletion, on the other hand, despite promoting PCM destabilization, does not promote loss of the centriolar protein Bld10, suggesting that centriolar MTs are not being affected at the same extent. Therefore, we suggest that Ana1 has a role in keeping not only PCM but also the centriolar MTs stable at the centrosome.

Altogether, the results suggest that **accentuated** loss of centrioles is related with destabilization of either the PCM and/or the cartwheel. Moreover, depletion of centriolar wall proteins suggests that these are important in stabilising the centriolar MTs.

In order to study the role of cap proteins in centriole stabilization, I depleted two different cap proteins: CP110 and Cep97. It has been reported that these two proteins interact with each other and that Cep97 is able to stabilize CP110[90]. Both are recruited to the distal part of the centriole during centriole elongation and, not only control centriole length, but also inhibit the ciliogenesis program[86,90,108].

In the present screen, depletion of CP110 resulted in a significant reduction in the number of centrioles. In contrast, removal of Cep97 does not show the same effect (Figure 3.10C). Interestingly, for both proteins, we observed a reduction in the number of centrioles marked with either the cap protein CP110 or the centriolar wall protein Sas4 (Figure 3.10C). As CP110 and Cep97 are known interactors, it was expected that removal of either of these proteins would lead to CP110 delocalization from the centrosome. However, the reduction in the number of centrioles marked with Sas4 was unexpected. Sas4 lies on the interface of centriolar wall and the PCM[19,20], and a recent study using a yeast-two-hybrid assay shows that Sas4 can directly interact with CP110[96]. This data suggests that CP110 might be important for Sas4 stabilization at the centrosome, which would also explain the decrease of centrioles marked with Sas4 in Cep97-depleted cells (Figure 3.10C). Despite leading to PCM destabilization, Sas4 removal mediated by CP110, does not seem to be sufficient to trigger destabilization of the centriolar wall.

Interestingly, a study in *Drosophila* cultured cells showed that in the absence of CP110, centrioles are shortened, but, co-depletion of the cap protein CP110 and a MT-depolymerizing protein (Klp10A) causes centriole elongation[92]. These results indicate that cap proteins might serve as a barrier against MT-depolymerizing proteins. However, as centrioles were slightly destabilized upon cap proteins removal, our results suggest that either this destabilization is not induced by "MT-depolymerize proteins" activity or, if it is, MT depolymerase proteins acting upon centriolar MTs are not sufficient to cause major effects in the centrosome structure, at least, at this specific cell cycle stage. We hypothesize that depletion of cap proteins leads to destabilization/removal of Sas4 and, consequently, compromising PCM maintenance at the centrosome.

We finally asked whether the major regulator of centriole duplication, Plk4, could play a role in centrosome stability. Despite the significant role of this protein in centriole biogenesis, removal of Plk4 showed a reduction in the number of centrioles per cell, however, this reduction was not as accentuated

as the one observed upon removal of PCM, cartwheel or centriolar wall proteins. The strongest reduction in the number of centrioles per cell was observed when these were marked with Sas4 (figure 3.11C). These results suggest that this protein does not have a major role in centrosome stabilization, however it can promote an accentuated reduction of Sas4 levels at the centrosome. Sas4 loss from the centrosome upon Plk4 removal is consistent with the studies that show that Plk4 can phosphorylate Sas4[102], through an interaction mediated by the PCM protein Asl[109]. As it was mentioned before, Sas4 lies between the centriolar wall and the PCM[19,20], so it is possible that removal of Plk4 leads to the destabilization of the Sas4-Asl-Plk4 complex. Destabilization of this complex might compromise PCM integrity at the centrosome and consequently lead to its destabilization, explaining the reduction of centrioles per cell observed upon depletion of Plk4 (Figure 3.11C).

Interestingly, it seems that the **mild** effect in centriole destabilization, either induced by removal of cap proteins or Plk4, can be correlated with removal and/or destabilization of Sas4 at the centrosome. As Sas4 interacts with several PCM proteins (Asl, Cnn and D-Plp)[105,106], destabilization of this protein might lead to inefficient PCM maintenance at the centrosome. However, one should note that Sas4 removal from the centrosome is not enough to trigger an accentuated centrosome destabilization, which is consistent with previous data that shows that only by removing simultaneously several PCM proteins we are able to significantly affect the centrosome[53].

Together, the results obtained in this study show that removal of either the PCM, cartwheel or centriolar wall proteins is sufficient to lead to a strong destabilization of the centrosome. Both cartwheel and PCM have already been implicated in centrosome stabilization[78]. While depletion of single PCM components do not destabilize the centrosome, simultaneous removal of different PCM proteins in *Drosophila* cultured cells leads to a decrease in the number of centrioles, showing that PCM has a key role in centrosome stabilization[53]. Also, *Drosophila* cultured cells at interphase lack MT-nucleating capacity and show low levels of the PCM protein γ-Tubulin at the centrosome. However, the centrosomes are kept[110]. In contrast with human centrosomes, *Drosophila* centrosomes do not lose the cartwheel during centriole maturation. Therefore, one might think that the low levels of PCM proteins in *Drosophila* interphase centrosomes might be counterbalanced by the presence of the cartwheel, which suggests that the cartwheel can also contribute to centrosome stabilization.

Nevertheless, only recently a direct link between cartwheel and PCM was established. Daughter centrioles of Cep295-depleted human cells (Ana1's orthologue in humans) are not able to accumulate PCM. The daughter cell that inherits this centriole (a non-competent-MTOC) cannot undergo normal centriole duplication in the next cell cycle and *de novo* centrioles are formed. These *de novo* centrioles are lost before mitosis in every cell cycle. However, by inhibiting cartwheel loss in these centrioles (by inhibiting Polo's activity), *de novo* centrioles became stable in most post mitotic cells[78], supporting the idea that not only PCM but also the cartwheel contribute to centrosome stabilization. Interestingly, inhibition of post-translation modification in the centriolar MTs is sufficient to destabilize the centriolar MTs, leading to PCM dispersal and consequent disappearance of centrosomes[111].

We therefore suggest that the PCM and the cartwheel are the two principal modules that contributes to centrosome stabilization through maintenance/stabilization of the centriole wall. It is possible that cartwheel and the PCM protect the centriolar wall which is a key element that allows maintenance of the centrosome structure. In the future, it will be important to determine whether the PCM and the cartwheel have redundant or distinct functions in centrosome stability.

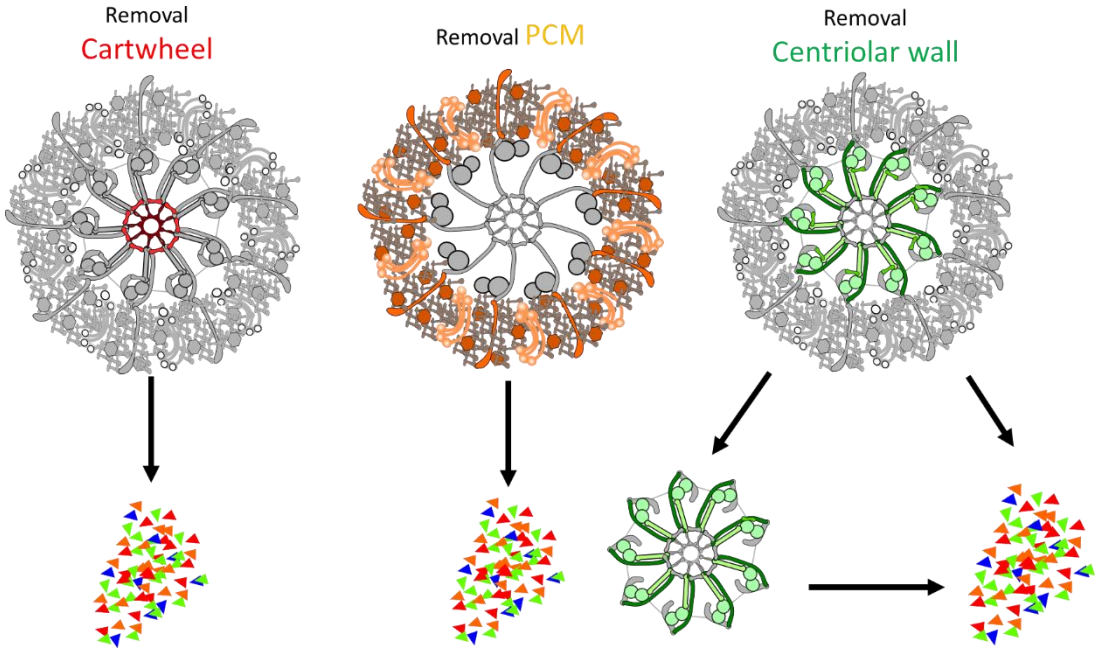


Figure 4.1: PCM and the cartwheel protect the centriolar wall. We propose that PCM and the cartwheel are the main modules that confer stability to the centrosome. Upon removal of either one of these, centrosomes are destabilized and therefore disassembled. On the other hand, centriolar wall proteins, as they are the key elements that allow maintenance of the centrosome structure, its removal might destabilize the PCM and/or the cartwheel, leading to its loss form the centrosome and consequent disassembling.

Notably, out of the centrosome components tested in this study, two specific proteins stood out: the centriolar wall protein Ana1 and the cartwheel protein Ana2. Both proteins were recently shown to interact with Polo by a yeast-two-hybrid assay[96]. Since Polo stabilizes the centrosome through PCM maintenance[53] we, therefore, hypothesize that Ana1 and Ana2 might also be stabilizing the centrosome through PCM.

In fact, just as it was shown for Cep295 (human orthologue of Ana1) in human cells[78], removal of Polo leads to an impairment in PCM acquisition in newly formed centrioles[35]. Polo has a key role in centriole elimination during *Drosophila*'s oogenesis, this protein is down-regulated during oogenesis, however, its ectopic expression at the centrosome leads to PCM accumulation and prevents centrosome loss[53]. Therefore, we asked whether Ana1 might also play a role in centriole elimination in *Drosophil*a's oogenesis.

To answer this question, I specifically depleted this protein at the *Drosophila*'s female germline in the presence of a GFP-Polo-PACT construct that allows maintenance of centrioles through PCM accumulation until late stages of oogenesis. Upon Ana1 depletion, it was observed a decrease in the signal intensity of centriole (GFP-Polo-PACT and CP110) and PCM (γ-Tubulin) markers (Figure 3.14) which suggests that Ana1 removal has a significant effect in destabilizing the centrosome, even in the presence of Polo at the centrosome.

Our data strongly suggests that Ana1 role in centrosome stabilization is very likely to be through PCM. Ana1 is a centriolar wall protein, thus, removing this protein would destabilize the ultimate structure that allows maintenance of the centrosome arrangement and lead to loss of PCM, which is consistent with our hypothesis. It has been shown that Ana1 and Polo can interact[96], however how these two proteins are contributing to centrosome stabilization warrants further investigation.

5. **Conclusion and future perspectives**

Centrosomes were considered stable structures for a long time, however, recent studies suggest that stability is not an intrinsic property of centrosomes. These organelles can be eliminated during oogenesis or inactivated in differentiated cells types and, either elimination or inactivation, seems to be related with PCM loss from the centrosome.

The importance of PCM maintenance for centrosome stability led us to hypothesize that the PCM might be protecting the centrosome by functioning as a shield that prevents either accessibility of proteins that destabilize the centriolar structure, or loss of stabilizing proteins that form the centriolar structure.

Here we identified the PCM, the cartwheel and the centriolar wall as important modules that assure centrosome stabilization. We hypothesize that the PCM and the cartwheel work (either together or individually) to protect the centriolar wall, which is the key component that allows maintenance of the centrosome structure. Ultrastructural analysis of centrioles would allow us to understand how the centrosomes being disassembled upon destabilization. Moreover, further studies are needed to understand if cartwheel and PCM have redundant roles in centrosome stability.

Out of all the candidate proteins tested, the centriolar wall protein Ana1 showed the strongest effect in centrosome stabilization. Depletion of this protein in *Drosophila*'s oogenesis suggest that this protein plays a key role in the centriole maintenance program. Ana1 removal from the centrosome seems to impair PCM accumulation mediated by Polo, however, further studies are needed to understand whether Ana1 acts upstream or downstream of Polo, or, if both work together to allow PCM maintenance at the centrosome.

This study not only shows the importance of PCM and cartwheel to centriole maintenance but also discloses a new key protein in the centriole maintenance program. We propose that this is a universal mechanism that can be turn on and off (allowing the presence or elimination/inactivation of centrosomes) according with cell type or cell cycle stage. It is therefore important to understand how this mechanism works and its regulated in development to be able to tackle it when it is deregulated in diseases.

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7. Annex

7.1. Macro used to identify and count centrioles

```
selectImage("MAX_C2-07.tif");
run("Duplicate...", "title=MAX_C2-07_mask");
run("Median...");
setAutoThreshold("Otsu dark stack");
run("Maximum...");
run("Median...");
run("Convert to Mask");
run("Watershed");
run("Analyze Particles...", "size=1-Infinity display exclude clear add");
roiManager("Save ");


selectWindow("MAX_C4.tif");
run("Select None");
selectWindow("MAX_C4.tif");
run("Duplicate...", "title=Subtract C4");
run("Subtract...", );
rois=roiManager("count");
for(i=0;i<rois;i++) {
        selectImage("Subtract");
        roiManager("select", i);
        run("Duplicate...", "title=C4_07_cell"+i+1);
        };
run("Images to Stack", "method=[Copy (center)] name=MAX_stack_C4 title=C4_07_cell use");
run("Save");
selectWindow("MAX_stack_C4.tif");
run("Duplicate...", "title=measure duplicate");
setOption("BlackBackground", false);
run("Convert to Mask", "method=Yen background=Dark calculate");
run("Analyze Particles...", "size=1-Infinity show=[Count Masks] display exclude clear add
stack");roiManager("Save");
run("Select None");
selectWindow("MAX_C2-07.tif");
run("Duplicate...", "title=Subtract C2");
run("Subtract...");
roiManager("deselect");
roiManager("delete");
roiManager("Open", //Mask//);rois=roiManager("count");
for(i=0;i<rois;i++) {
        selectImage("Subtract");
        roiManager("select", i);
        run("Duplicate...", "title=C2_07_cell"+i+1);
        };

run("Images to Stack", "method=[Copy (center)] name=MAX_stack_C2_07 title=C2_07_cell use");
run("Save");
selectWindow("MAX_stack_C2_07.tif");
run("Duplicate...", "title=measure duplicate");
```

```
setOption("BlackBackground", false);
run("Convert to Mask", "method=Yen background=Dark calculate");
run("Analyze Particles...", "size=1-Infinity show=[Count Masks] display exclude clear add stack");
roiManager("Save");


run("Select None");
selectWindow("MAX_C3-07.tif");
run("Duplicate...", "title=Subtract C3");
run("Subtract...");
roiManager("deselect");
roiManager("delete");
roiManager("Open", //Mask//);
rois=roiManager("count");
for(i=0;i<rois;i++) {
        selectImage("Subtract");
        roiManager("select", i);
        run("Duplicate...", "title=C3_07_cell"+i+1);
        };
run("Images to Stack", "method=[Copy (center)] name=MAX_stack_C3_07 title=C3_07_cell use");
run("Save");
selectWindow("MAX_stack_C3_07.tif");
run("Duplicate...", "title=measure duplicate");
setOption("BlackBackground", false);
run("Convert to Mask", "method=Yen background=Dark calculate");
run("Analyze Particles...", "size=1-Infinity show=[Count Masks] display exclude clear add stack");
roiManager("Save ");
```

7.2. Macro used to quantify Ana1's signal at the centrosome

```
open("SUM_C2.tif");
selectWindow("SUM_C2.tif");
run("Duplicate...", "title=Mask");
roiManager("Open", “Mask");
rois=roiManager("count");
for(i=0;i<rois;i++) {
        selectImage("Mask");
        roiManager("select", i);
        run("Duplicate...", "title=C2_05_cell"+i+1);
        };
run("Images to Stack", "method=[Copy (center)] name=SUM_stack_C2_05 title=C2_05_cell use");
run("Save");
roiManager("Deselect");
roiManager("Delete");
roiManager("Open", "Ana1 rois");
roiManager("Measure");
saveAs("Measurements_C2_05");
run("Close All");
```