

The dynamics of centrosome assembly in  
the early *Drosophila* embryo



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# Abstract

The dynamics of centrosome assembly in the early *Drosophila* embryo

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Centrosomes are important for many cellular processes. They comprise of a pair of centrioles surrounded by an amorphous pericentriolar material (PCM). In *Drosophila*, although more than hundreds of proteins localise to PCM, it is believed that the PCM assembly is governed by a small subset of proteins—Polo, Spd-2 and Cnn forming a network-like underlying scaffold. This scaffold appears to be plastic and adopts different behaviours in many cell types. Therefore, *Drosophila* centrosomes allow us to understand how a simple set of proteins are wired to satisfy biochemical and biophysical needs. In this thesis, I describe my discovery that Cnn scaffold assembly is initiated and timed by a Polo oscillation. Centriolar Ana1 helps recruit Polo, which inhibits Ana1 activity, resulting in a time-delayed negative feedback oscillation. Polo then triggers a Spd-2 oscillation which together build the Cnn scaffold. In the process, I created an automated analysis pipeline to look into the scaffold assembly in different systems efficiently. Moreover, I discovered that in addition to a Cnn solid scaffold centrosomes also possess a liquid TACC scaffold that is organised by Spd-2 and Aurora A. The liquid TACC scaffold can enrich many centrosomal proteins.

## Abbreviations

|                  |                                                                               |
|------------------|-------------------------------------------------------------------------------|
| <b>3D</b>        | 3-dimensional                                                                 |
| <b>3D-SIM</b>    | 3-dimensional structured illumination microscopy                              |
| <b>Ana1</b>      | Anastral spindle 1                                                            |
| <b>Ana2</b>      | Anastral spindle 2                                                            |
| <b>APC/C</b>     | Anaphase-promoting complex/cyclosome                                          |
| <b>Asl</b>       | Asterless                                                                     |
| <b>AU</b>        | Arbitrary units                                                               |
| <b>Aur A</b>     | Aurora A kinase                                                               |
| <b>Bld10</b>     | Basal body protein                                                            |
| <b>BSI</b>       | Back illumination sensor                                                      |
| <b>CaM</b>       | Calmodulin                                                                    |
| <b>CCD</b>       | Charge-coupled device                                                         |
| <b>CCO</b>       | Cdk-cyclin cell-cycle oscillator                                              |
| <b>Cdc25</b>     | Cell division control protein 25                                              |
| <b>Cdc28</b>     | Cell division control protein 28                                              |
| <b>Cdk / CDK</b> | Cyclin-dependent kinase                                                       |
| <b>Cdk5rap2</b>  | Cyclin-dependent kinase 5 regulatory subunit associated protein 2             |
| <b>CDK6</b>      | Cyclin-dependent kinase 6                                                     |
| <b>Cep / CEP</b> | Centrosomal protein                                                           |
| <b>CHC</b>       | Clathrin heavy chain                                                          |
| <b>Chk / CHK</b> | Check point kinase                                                            |
| <b>CM</b>        | Common motif                                                                  |
| <b>Cnn</b>       | Centrosomin                                                                   |
| <b>CnnGROWTH</b> | Cnn growth rate                                                               |
| <b>CnnIN</b>     | Cnn 'IN' rate                                                                 |
| <b>CnnOUT</b>    | Cnn 'OUT' rate                                                                |
| <b>CS</b>        | Centrosome separation                                                         |
| <b>CSV</b>       | Common-separated values                                                       |
| <b>Cyc / CYC</b> | Cyclin                                                                        |
| <b>CPAP</b>      | Centromal P4.1-associated protein                                             |
| <b>CPC</b>       | Chromosomal passenger complex                                                 |
| <b>ch-TOG</b>    | Colonic and hepatic tumour overexpressing gene                                |
| <b>e</b>         | endogenous                                                                    |
| <b>EDTA</b>      | Ethylenediaminetetraacetic acid                                               |
| <b>EGTA</b>      | Ethylene glycol-bis( $\beta$ -aminoethyl ether)-N, N, N', N'-tetraacetic acid |



|                  |                                                                             |
|------------------|-----------------------------------------------------------------------------|
| <b>EM</b>        | Electron microscopy                                                         |
| <b>ESCRT</b>     | Endosomal sorting complexes required for transport                          |
| <b>ET</b>        | Electron cryo-electron tomograph                                            |
| <b>FRAP</b>      | Fluorescent recovery after photobleaching                                   |
| <b>FCS</b>       | Fluorescence correlation spectroscopy                                       |
| <b>G phase</b>   | Gap phase                                                                   |
| <b>GFP</b>       | Green fluorescent protein                                                   |
| <b>IDR</b>       | Intrinsically disordered region                                             |
| <b>Klp</b>       | Kinesin-like protein                                                        |
| <b>LCD</b>       | Low complexity domain                                                       |
| <b>LLPS</b>      | Liquid-liquid phase separation                                              |
| <b>LLSD</b>      | Liquid-like spindle domain                                                  |
| <b>LoG</b>       | Laplacian of Gaussian                                                       |
| <b>M phase</b>   | Mitosis                                                                     |
| <b>Mit.</b>      | Mitosis                                                                     |
| <b>MSPS</b>      | Minispindles                                                                |
| <b>MT</b>        | Microtubule                                                                 |
| <b>MTOC</b>      | Microtubule organising centre                                               |
| <b>Myt / MYT</b> | Membrane-associated tyrosine- and threonine-specific cdc2-inhibitory kinase |
| <b>MZT</b>       | Maternal-to-zygotic transition                                              |
| <b>min</b>       | minute                                                                      |
| <b>mRNA</b>      | Messenger ribonucleic acid                                                  |
| <b>NA</b>        | Numerical aperture                                                          |
| <b>NEB</b>       | Nuclear envelope breakdown                                                  |
| <b>NG</b>        | Neongreen                                                                   |
| <b>NM</b>        | New mother                                                                  |
| <b>nM</b>        | Nanomolar                                                                   |
| <b>nm</b>        | Nanometer                                                                   |
| <b>ns</b>        | Not significant                                                             |
| <b>ODE</b>       | Ordinary differential equation                                              |
| <b>OM</b>        | Old mother                                                                  |
| <b>PAGE</b>      | Polyacrylamide gel electrophoresis                                          |
| <b>PBD</b>       | Polo box domain                                                             |
| <b>PBS</b>       | Phosphate buffered saline                                                   |
| <b>PCM</b>       | Pericentriolar material                                                     |
| <b>Plk / PLK</b> | Polo-like kinase                                                            |
| <b>Plp</b>       | Pericentrin-like protein                                                    |

|                    |                                               |
|--------------------|-----------------------------------------------|
| <b>PPTase</b>      | Phosphatase                                   |
| <b>R</b>           | Receptor                                      |
| <b>RFP</b>         | Red fluorescent protein                       |
| <b>ROI</b>         | Region-of-interest                            |
| <b>S phase</b>     | Synthesis phase                               |
| <b>Sas / SAS</b>   | Spindle assembly abnormal                     |
| <b>SAC</b>         | Spindle assembly checkpoint                   |
| <b>SCF</b>         | Skp, Culin, F-box                             |
| <b>SD</b>          | Standard deviation                            |
| <b>SDS</b>         | Sodium dodecyl sulphate                       |
| <b>SoRA</b>        | Super resolution via optical re-assignment    |
| <b>Spd / SPD</b>   | Spindle defective                             |
| <b>Spd-2GROWTH</b> | Spd-2 growth rate                             |
| <b>Spd-2IN</b>     | Spd-2 'IN' rate                               |
| <b>Spd-2OUT</b>    | Spd-2 'OUT' rate                              |
| <b>STAN</b>        | STIL/Ana2 domain                              |
| <b>STIL</b>        | SCL/TAL1 interrupting locus                   |
| <b>sec</b>         | second                                        |
| <b>TACC</b>        | Transforming acidic coiled-coil               |
| <b>TBS</b>         | Tris-buffered saline                          |
| <b>Thr</b>         | Threonine                                     |
| <b>TIFF</b>        | Tag image file format                         |
| <b>TPX</b>         | Targeting protein for <i>Xenopus</i> Klp2     |
| <b>TPXL</b>        | TPX-like                                      |
| <b>ubq / Ubq</b>   | Ubiquitin                                     |
| <b>V</b>           | Volt                                          |
| <b>wt / WT</b>     | Wildtype                                      |
| <b>XMAP</b>        | <i>Xenopus</i> microtubule associated protein |
| <b>ZYG</b>         | Zygotic defective                             |
| <b>γTuRC</b>       | γ-tubulin ring complex                        |
| <b>μl</b>          | Microliter                                    |
| <b>μM</b>          | Micromolar                                    |
| <b>μm</b>          | Micrometer                                    |

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# 1. Introduction

## 1.1 Thesis overview

Cells are the basic building blocks of life. The inside of a cell is crowded with many different organelles—specialized subcellular structures that perform specific functions. For example, cells typically contain hundreds of mitochondria that produce energy, and hundreds of peroxisomes that make important chemicals. When cells grow and divide—and it is estimated that 330 billion cells divide every day in a typical human (Sender & Milo, 2021)—they must accurately double the amount of each organelle prior to cell division so that the two cells formed after division inherit the right amount of each organelle. It is assumed that cells must have mechanisms that allow them to measure or sense the amount of each organelle (Rafelski & Marshall, 2008), but is not clear how they might do this.

Centrioles are tiny organelles that help organise cells (see **Section 1.3**), and they are potentially an excellent system for studying how cells make the right amount of an organelle. Cells are born with just two centrioles (called mothers) and before the cell divides each mother gives birth to precisely one daughter (that grows off the side of each mother). When the cell divides, each new cell inherits one of these mother/daughter centriole pairs, and these pairs then separate to generate two mothers so the cycle can start again. Unlike many other organelles, centrioles have a well organised barrel-shaped structure, so, if we can understand why mother centrioles give birth to just one daughter, and how the daughter barrel then grows to the same *length* as the mother barrel, we will

understand how cells make precisely the right amount of centriole during each division cycle.

The Raff laboratory recently developed methods to measure the kinetics of daughter centriole growth in the early *Drosophila* embryo (Aydogan et al., 2018). These measurements revealed something unexpected: centrioles grow adaptively. This means that when the nuclei in the embryo divided quickly, the centrioles grew quickly but for a short period; when the nuclei divided slowly, the centrioles grew slowly but for a longer period. As a result, the centrioles always grew to the same size no matter how quickly or slowly the nuclei in the embryos were replicating and dividing. Importantly, Polo-like Kinase (Plk4), a key regulator of centriole assembly, was found to oscillate at the base of the growing daughter centriole, and this oscillation appeared to regulate centriole assembly (Aydogan et al., 2020). This raised the exciting possibility that oscillations in the levels of a key regulator might be one way to regulate organelle biogenesis.

In this thesis, I describe that I use the early *Drosophila* embryo system to extend these studies to investigate how centrioles organise another organelle, the centrosomes. Centrioles form centrosomes when they recruit a complex matrix of proteins—the pericentriolar material (PCM)—around themselves. I show that, like the centrioles, centrosomes also grow to consistent size each time they divide in these embryos and that, remarkably an oscillation in Polo kinase (a relative of Plk4) appears to regulate centrosome growth. I also perform experiments to investigate the fundamental physical

nature of the PCM, and present evidence that centrioles organise two independent PCM scaffolds, one which behaves like a solid, and the other that behaves like a liquid.

## 1.2 The cell cycle

From prokaryotes to eukaryotes, cells divide to produce more cells and pass on their genetic material. In unicellular organism, the decision to divide or not to divide is influenced by several environmental cues, for example the availability of nutrients. To divide, cells need to replicate their genomes and synthesise the appropriate amount of their many cellular components to pass on to their descents. Therefore, the process of cell division needs to be meticulously regulated. The series of events that ultimately lead to the production of two daughter cells is termed “the cell cycle”.

### 1.2.1 The eukaryotic cell cycle

In eukaryotes, the cell cycle can be classified into either interphase or M-phase. Interphase consists of Gap1 (G1), Synthesis (S), and Gap2 (G2) phases while M-phase consists of mitosis and cytokinesis. During G1, cells grow and, if they reach a certain size (often termed the Restriction Point), they commit to dividing and enter S-phase, where the duplication of DNA (and also the centrioles) occurs. During G2, cells grow further and prepare for M-phase when duplicated chromosomes condense, the nuclear envelope breaks down and the duplicated chromosomes are captured and then segregated upon the bipolar mitotic spindle (Prosser & Pelletier, 2017). Finally, the two sets of chromosomes are partitioned into two daughter cells by the constriction of the cell



membrane during cytokinesis (Gegan et al., 2011). Once cells exit mitosis, they can either continue to grow and proliferate, or enter into a quiescent state (G0).

This fundamental organisation of the cell cycle is highly conserved, but precise details can vary enormously depending on the species and cell type (Figure 1.1). For example, some proliferating mammalian cells can take >24 hours to complete a cell cycle, while budding yeast can divide approximately every 90 minutes (Forsburg & Nurse, 1991). In the early syncytial fly embryo (where multiple nuclei divide synchronously within a common cytoplasm) the cycle takes only ~10 minutes and lacks Gap phases (comprising only alternating S and M phases) and cytokinesis (Farrell & O'Farrell, 2014). Despite these variations, the cell cycle is designed to be robust and unidirectional so as to orchestrate innumerable molecular events (Rombouts & Gelens, 2021). This robustness is achieved by a switch-like behaviour as cells pass through each phase of the cycle; this behaviour emerges from the multiple layers of transcriptional, post-translational controls of the major cell cycle regulators (Harashima et al., 2013).

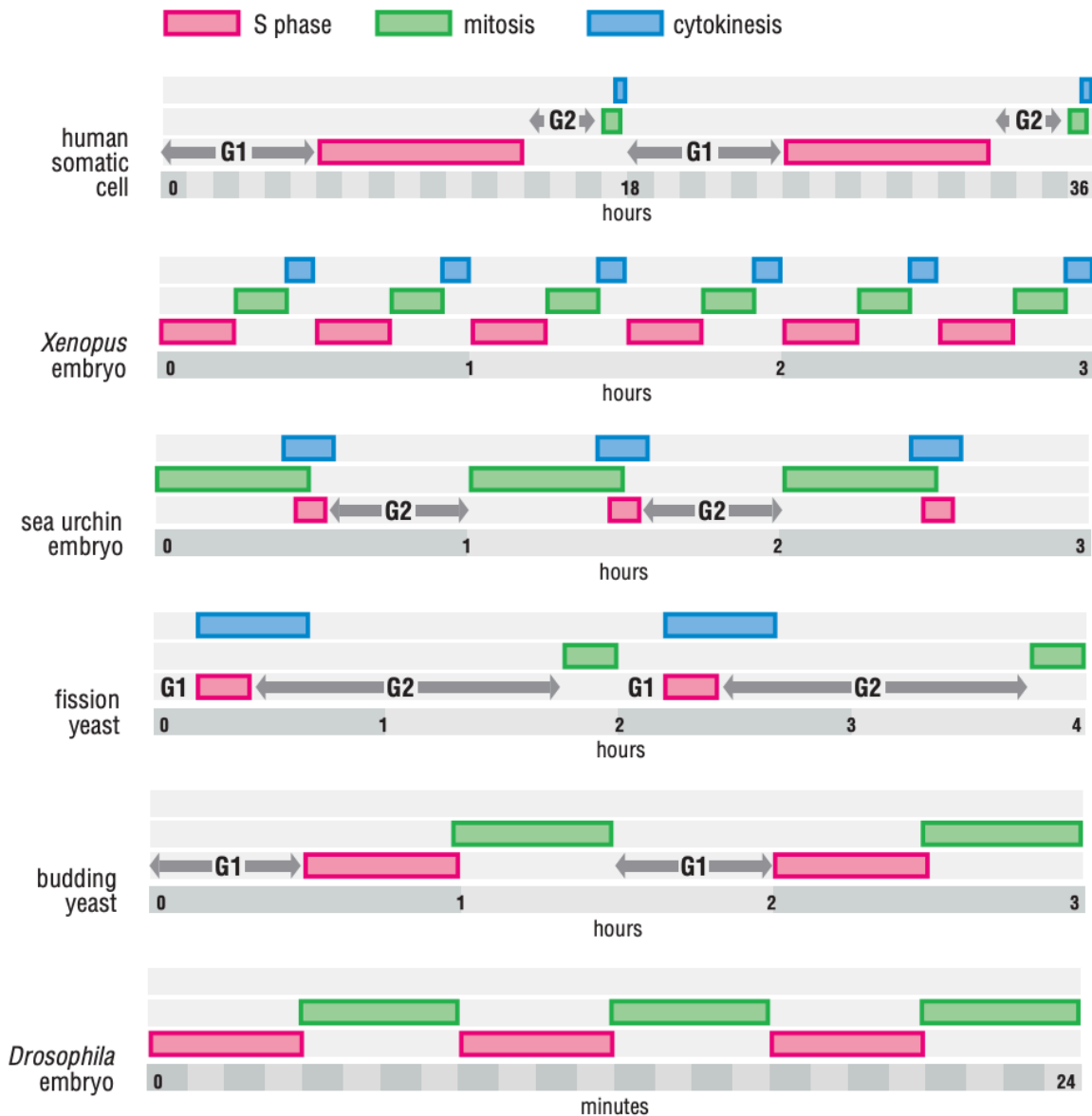


Figure 1.1 Variations of cell cycle structure in different cell types

Adopted from Morgan, 2007

### 1.2.2 Regulation of the eukaryotic cell cycle

Underlying this complexity is the regulation of the main class of protein kinases that regulate cell cycle progression, the Cyclin-Dependent Kinases (Cdks) (Bloom & Cross, 2007). In mammalian systems, several types of Cdks interact with several types of

regulatory cyclin subunits, and these are intertwined into a sophisticated network offering redundancy and robustness to the system (Lindqvist et al., 2009). For example, In human cells, Cyclin D1-Cdk4/6 and Cyclin E-Cdk2 are important to regulate the G1/S transition (Dong et al., 2018; Resnitzky et al., 1994). Cyclin A-Cdk2 promotes S-phase progression, and Cyclin B-Cdk1 promotes the G2/M-phase transition (Hocheegger et al., 2008). In contrast, the budding yeast *S. cerevisiae* has just a single Cdk (*cdc28*) which associates with several different cyclins at different cell cycle phases (Bloom & Cross, 2007). In all systems, there seems to be significant functional redundancy between Cdks and cyclins (Uhlmann et al., 2011). Most remarkably, in the fission yeast *S. pombe* a single Cdk and single cyclin can functionally substitute for the four Cdk-cyclin complexes that normally act during mitosis and the 6 Cdk-cyclin complexes that normally act during meiosis (Coudreuse & Nurse, 2010; Gutiérrez-Escribano & Nurse, 2015).

Cdk activities are regulated by three major mechanisms (Bloom & Cross, 2007; Harashima et al., 2013): (1) Binding to and degradation of Cyclins; (2) Phosphorylation and dephosphorylation of Cdk that may activate or inactivate its activity; (3) Binding to Cdk inhibitors. Based on theoretical considerations, (1) and (2) are sufficient to generate oscillation in Cdk activity that can drive unidirectional and periodic behaviours during cell cycle (Tyson & Novák, 2015). During interphase, the low level of Cyclin B prevents Cdk from premature activation. Low Cdk1 activity is also maintained by Wee1/Myt1-related kinases that phosphorylate Cdk1 on inhibitory sites to prevent premature mitotic entry (Kornbluth et al., 1994; Mueller et al., 1995). As cell cycle progresses, Cyclin B levels gradually increase, which increases the proportion of active Cdk1 in the cell. This active

fraction of Cdk1/Cyclin B inhibits Wee1/Myt1 kinases (Okamoto & Sagata, 2007) and activates the protein phosphatase Cdc25 (Deibler & Kirschner, 2010), which removes the Wee1/Myt1 inhibitory phosphorylation on Cdk1. This combination triggers a burst of Cdk1 activation that drives the irreversible entry into mitosis (Mochida et al., 2016). During mitosis, CDK sets the scene for its own inactivation by activating the Anaphase Promoting Complex/Cyclosome (APC/C), which ultimately induces the proteasome-dependent degradation of Cyclin B and Securin to trigger the exit from mitosis and the separation of the replicated sister chromatids, respectively (reviewed in Primorac & Musacchio, 2013). The spindle assembly checkpoint (SAC) comprises another regulatory network that monitors the status of the kinetochores on the replicated sister chromatids to ensure that cells do not activate the APC/C (and so do not exit mitosis) until all of the sister chromosomes are correctly aligned for segregation on the mitotic spindle (reviewed in Musacchio & Salmon, 2007).

### 1.2.3 The nuclear cycles of the early *Drosophila* syncytial embryo

Cell cycles of *Drosophila* syncytial embryo are modified because the cell cycle progression, which is normally driven by Cyclin accumulation (through a multilayered transcription) and degradation, relies heavily on maternal proteins and mRNAs so that the oscillatory cell cycle dynamics are autonomous in a closed system (Calzone et al., 2007). Deposited with a rich supply of maternal proteins and mRNA, the nuclei in the early *Drosophila* embryos undergo 13 rounds of rapid and nearly-synchronous division that largely do not rely on transcription of the genome (Edgar & Schubiger, 1986). These cycles are termed nuclear cycles because the nuclei in the embryo proliferate without

cytokinesis so that the many nuclei are immersed in a shared cytoplasm. As mentioned briefly above, these nuclear cycles are modified, and comprise only alternating S-phase and M-phase periods with no Gap-phases (Farrell & O'Farrell, 2014) (Figure 1.1). After 9 rounds of nuclear division, most nuclei and their associated centrosomes migrate to the cortex forming a monolayer of syncytial blastoderm (Foe & Alberts, 1983; Raff & Glover, 1989). The nuclei undergo 4 more rounds of nuclear division, with the length of S-phase gradually lengthening from nuclear cycles 11 – 13 (Farrell & O'Farrell, 2014). This is thought to occur because the total amount of DNA in these embryos increases exponentially at each nuclear cycle so that DNA replication factors become limiting, triggering a DNA replication checkpoint cascade comprising Mei-41, Grapes and Wee1 (the *Drosophila* homologues of the vertebrate S-phase checkpoint proteins ATM, Chk1 and Wee1). This activates Wee1 to phosphorylate Cdk1 and so prevent premature entry into mitosis until all the DNA in the embryo is replicated. The duration of the nuclear cycle stretches even further in cycle 14 because a G2-like phase is introduced and cellularisation occurs concomitantly. During cycle 14, cells start to degrade maternal transcripts and initiate zygotic transcription in a process called maternal-to-zygotic transition (MZT). By the end of cycle 14, cells fully compartmentalize the cortical nuclei and the cells no longer divide synchronously (Foe, 1989).

This system is particularly advantageous for live imaging-based quantification studies of centriole and centrosome assembly (Figure 1.2). First, the embryos are very resistant to photodamage, and the centrioles and centrosomes are localised very close to the embryo cortex from nuclear cycle 10-14, making these structures very easy to image at high

resolution (Raff, 2005). Second, the rapid and near-synchronous nuclear cycles allow one to image several hundred centrioles/centrosomes as they synchronously proceed through multiple rounds of division (allowing one to accurately average the behaviour of multiple centrioles/centrosomes) (Aydogan et al., 2018). Finally, *Drosophila* embryos can be easily manipulated genetically, and also by the microinjection of drugs or inhibitory antibodies (Raff & Glover, 1988).

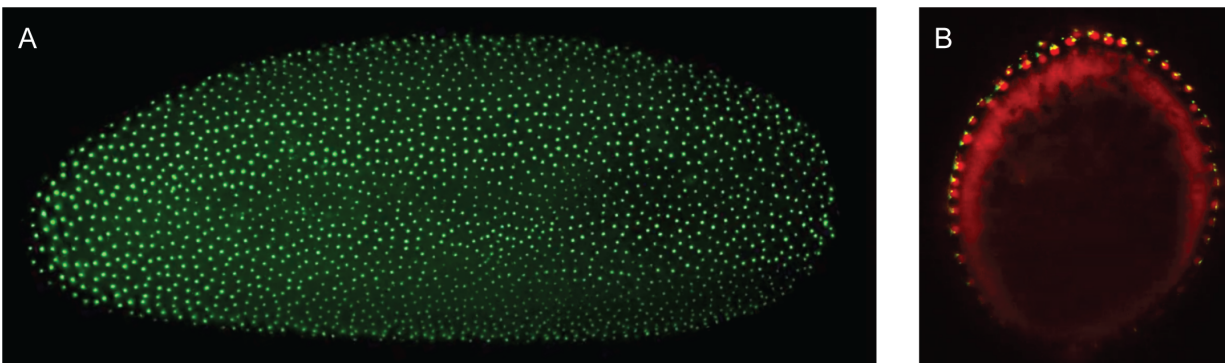


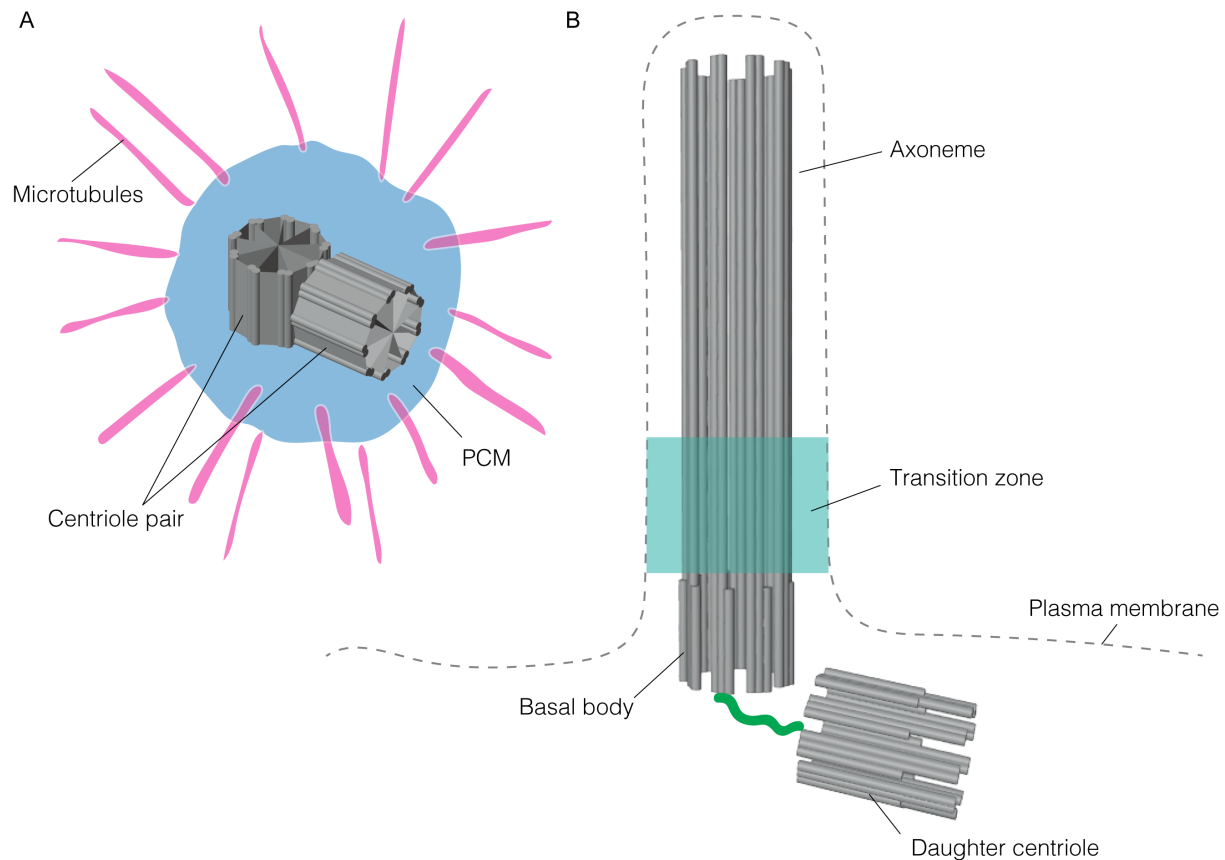
Figure 1.2 Lightsheet images of an early *Drosophila* embryo

(A) A side view of an early *Drosophila* embryo in nuclear cycle 12 labelled with NG-Cnn. (B) A cross-section of an early *Drosophila* embryo in nuclear cycle 12 labelled with NG-Cnn and Histone-RFP.

### 1.3 Centrioles

Centrioles are 9-fold radially symmetric microtubule-based structures that form two important cellular structures: centrosomes and cilia (Figure 1.3). The centrosome consists of a pair of centrioles (an older mother and younger daughter) surrounded by a cloud of pericentriolar material (PCM) that functions as the major microtubule organising centre (MTOC) in most proliferating animal cells (reviewed extensively in Conduit et al., 2015). In most quiescent cells that centrioles migrate to the cell cortex, where the mother centriole forms a basal body that gives rise to the cilium, a structure that generates cell

motility and senses environmental cues (Fry et al., 2014; Nachury, 2014). Centrioles are ancient organelles present in all major eukaryotic groups (Cavalier-Smith, 2002). The last eukaryotic common ancestor is thought to have had centrioles, but centrioles are not found in all present-day eukaryotes (Carvalho-Santos et al., 2011; Cavalier-Smith, 2002). The centriole's role at the base of the cilium is thought to have emerged first, as seen by the co-distribution of centrioles and basal bodies throughout eukaryotes (Azimzadeh, 2014; Marshall, 2009). Centrosomes are thought to have evolved more recently, as they are not always found in organisms that contain centrioles/cilia (Azimzadeh, 2014). Centrosomes and cilia have several important functions in animal cells and mutations in genes encoding several centrosome and cilia proteins have been linked to a variety of human diseases and developmental disorders (Chavali et al., 2014; Nigg & Holland, 2018; Nigg & Raff, 2009).



**Figure 1.3 Centrosomes and cilia**

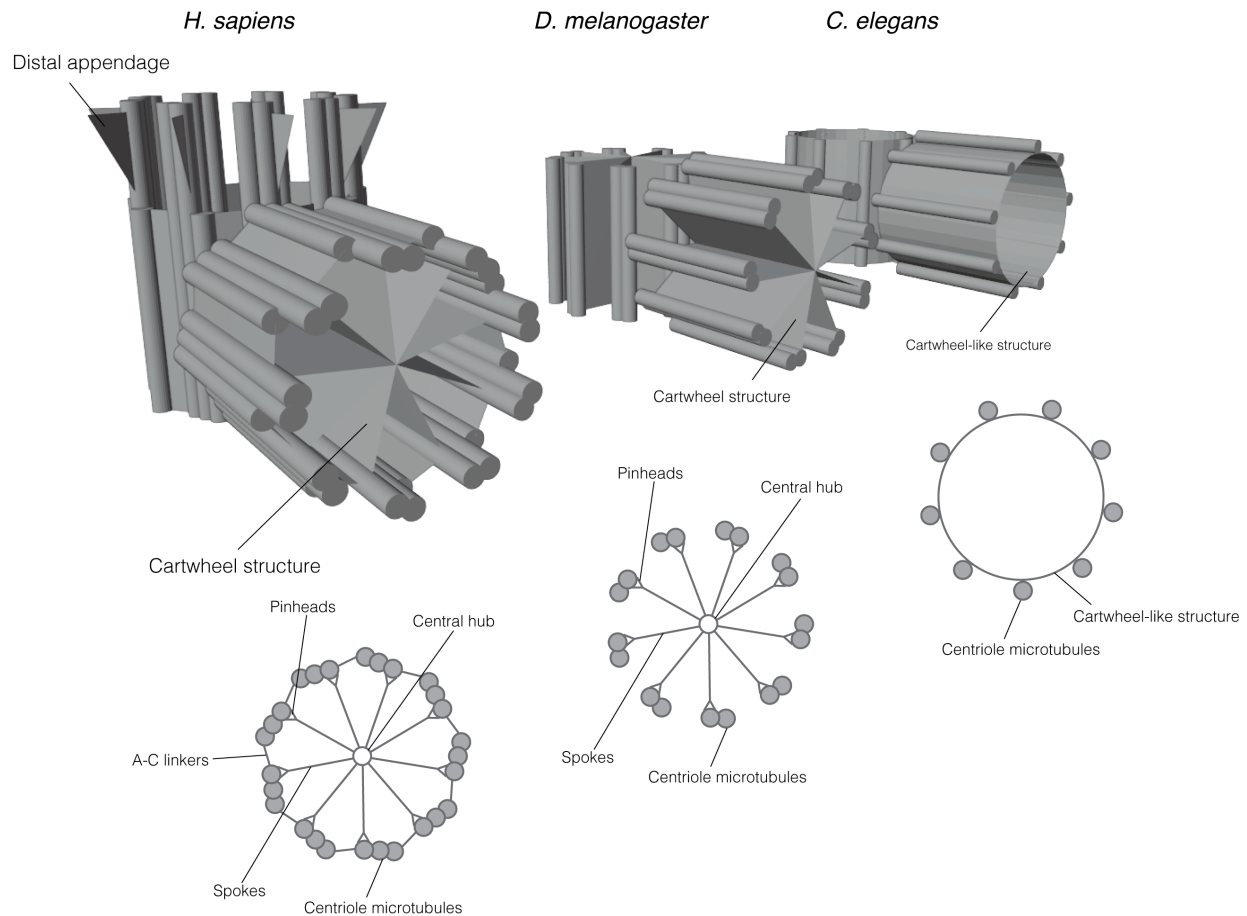
(A) Schematic shows a centrosome consisting of a pair of centrioles (mother and daughter) surrounded by PCM that nucleates microtubules. (B) Schematic shows a cilium that consists of an axoneme and a basal body separated by a transition zone.

### 1.3.1 Centriole structure

The centriole's 9-fold symmetric structure is highly conserved (Carvalho-Santos et al., 2011). At its centre is a cartwheel comprising a central hub from which nine, evenly spaced spokes radiate (Figure 1.4). Elegant cryo-electron tomograph (ET) studies (Guichard et al., 2013) show how the ring-like cartwheel units stack on top of each other and are joined to the outer centriole MT by a "pinhead". The centriole microtubules can be singlets, doublets or triplets, depending on the species and the cell type (Gönczy, 2012; Winey & O'Toole, 2014). In many species, including *D. melanogaster* and *C. elegans*, the



cartwheel is present in both mother and daughter centrioles, whereas in mammalian cells the cartwheel is disassembled as the daughter centriole matures into a mother (see **Section 1.3.2** for details of the centriole duplication cycle). In vertebrate centrioles, the mother centrioles acquire distal and sub-distal appendages that play an important part in docking the mother centriole to the cortex during cilium formation (Ishikawa et al., 2005; Pedersen et al., 2008), and anchoring MTs (Kobayashi & Dynlacht, 2011), respectively—although in species that lack these structures, the mother centrioles can still dock with the cortex to form cilia and can still organise MTs.



**Figure 1.4 The structure of centrioles in vertebrate, *Drosophila* and *C. elegans***

Schematics display simplified 3D structures of a typical centriole pair and cross-sections of daughter centrioles in *H. sapiens*, *D. melanogaster* and *C. elegans* (indicated). The mother centriole is oriented vertically, and the daughter centriole is shown in an orthogonal and engaged configuration. Several features of these centrioles are highlighted.

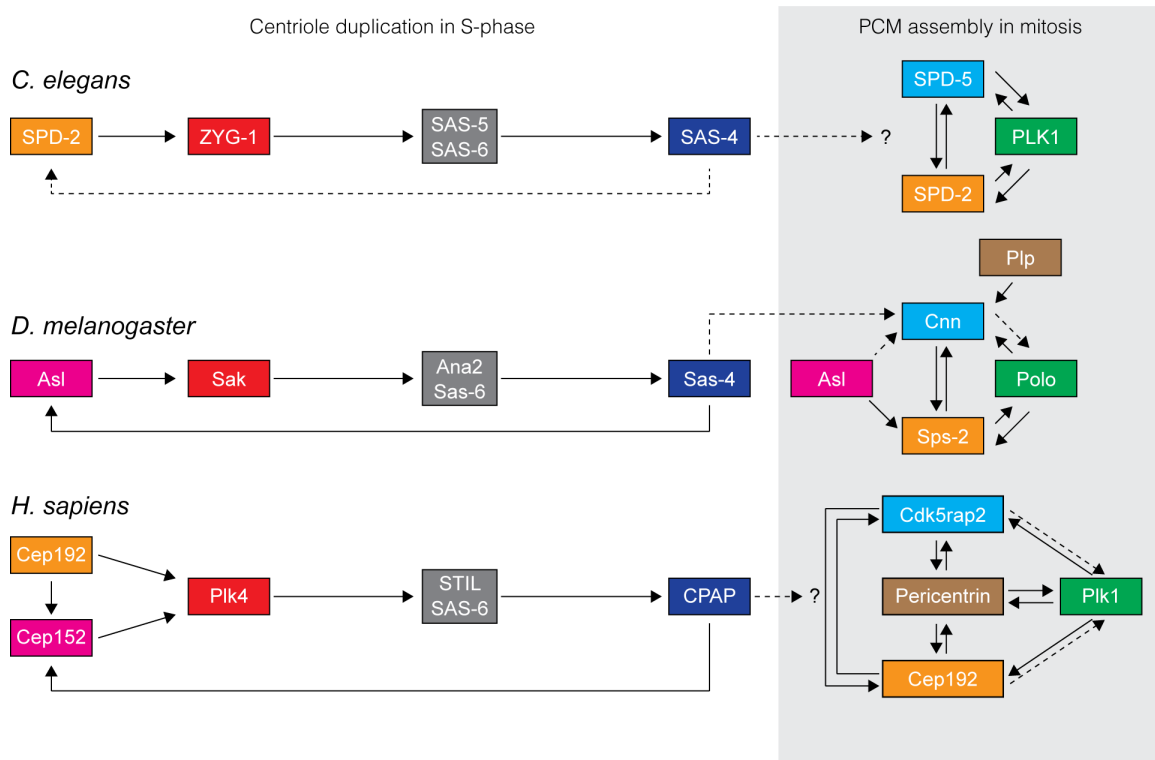
### 1.3.2 Centriole duplication

The cartwheel is the earliest structure formed during the assembly of a new daughter centriole, which in all species examined occurs exclusively during S-phase (so centrioles duplicate during the same time window that the cell's genome is being duplicated) (Gönczy, 2012). Sas-6/SAS6/HsSas-6 (*D. melanogaster*/*C. elegans*/*H. sapiens*) appears to be the main structural component of the cartwheel and it is one of the first proteins to

be recruited to the site of centriole formation (Delattre et al., 2006; Leidel et al., 2005; Pelletier et al., 2006; Rodrigues-Martins et al., 2007). Sas-6 contains an N-terminal globular domain that allows for the oligomerisation of nine Sas-6 dimers to form the central hub, which is linked to an extended coiled-coil domain that forms the emerging spokes (Cottee et al., 2015; Guichard et al., 2017; D. Kitagawa et al., 2011; Van Breugel et al., 2011). Sas-6 can self-assemble into a symmetric, cartwheel-like structures *in vitro* (Guichard et al., 2017; D. Kitagawa et al., 2011; Van Breugel et al., 2011). However, other proteins such as Ana2/STIL and Sas-4/CPAP (see below) are thought to facilitate the 9-fold symmetry *in vivo* (Cottee et al., 2011).

The molecular pathway leading to Sas-6 cartwheel formation and subsequent daughter centriole biogenesis starts when Plk4, a member of the Polo-like kinase family, is recruited to mother centrioles (Bettencourt-Dias et al., 2005; Habedanck et al., 2005) (Figure 1.5). Initially, Plk4 is recruited symmetrically around the entire mother centriole—by SPD-2 in worms, Asl in flies, and a combination of Cep152 and Cep192 (Asl and Spd-2 homologues, respectively) in human (Conduit et al., 2015). In a process that is poorly understood, this ring of Plk4 resolves into a single “dot” on the side of the mother that marks the site of daughter centriole assembly (Ohta et al., 2014; Yamamoto & Kitagawa, 2019). Plk4 can dimerise and trans-auto-phosphorylate its activation loop to become active (Klebba et al., 2015; Lopes et al., 2015), which can then trigger the destruction of the dimer via the phosphorylation of a phospho-degron motif that engages with the Slimb/SCF ubiquitin ligase system, targeting the protein for proteasome-dependent degradation (Cunha-Ferreira et al., 2009; Guderian et al., 2010; Rogers et al., 2009). It is

thought that this degradation may play a part in helping to resolve the initial Plk4 ring into a single dot (Nigg & Holland, 2018). Activated Plk4 can also phosphorylate a conserved STAN domain on Ana2/STIL which allows Ana2/STIL to recruit Sas-6 molecules to build daughter centrioles (Dzhindzhev et al., 2014; Kratz et al., 2015; Moyer et al., 2015; Ohta et al., 2014). Phosphorylated Ana2/STIL also helps to recruit Sas-4/CPAP to further stabilise the cartwheel structure (Gartenmann et al., 2020; Tang et al., 2011) and to promote the interaction between the growing cartwheel and the centriolar microtubules (McLamarrah et al., 2018; Moyer & Holland, 2019; Schmidt et al., 2009; Tang et al., 2009, 2011).



**Figure 1.5 Centriole and mitotic PCM assembly pathways in *C.elegans*, *D. melanogaster*, and *H. sapiens***

Schematics show the difference of centriolar and mitotic PCM assembly pathway in *C. elegans*, *D. melanogaster*, and *H. sapiens*. Solid lines represent confirmed interactions. Dotted lines represent hypothesised interactions. Gray region separates centriole and PCM assembly pathway in S-phase and mitosis, respectively. Adapted and modified from Conduit et al., 2015.

### 1.3.3 Centriole growth control

As mentioned at the start of this thesis, centrioles are an excellent model for studying the regulation of organelle biogenesis. The studies described above reveal that mother centrioles form only a single daughter because the key enzyme initiating centriole biogenesis, Plk4, is ultimately recruited and retained at only a single site on the side of the mother centriole (although the molecular mechanism ensuring that this occurs is unclear). If Plk4 is overexpressed, then mother centrioles can nucleate multiple daughters (Habedanck et al., 2005; Kleylein-Sohn et al., 2007). These studies, however, do not shed any light on how daughter centrioles subsequently grow to the correct length.

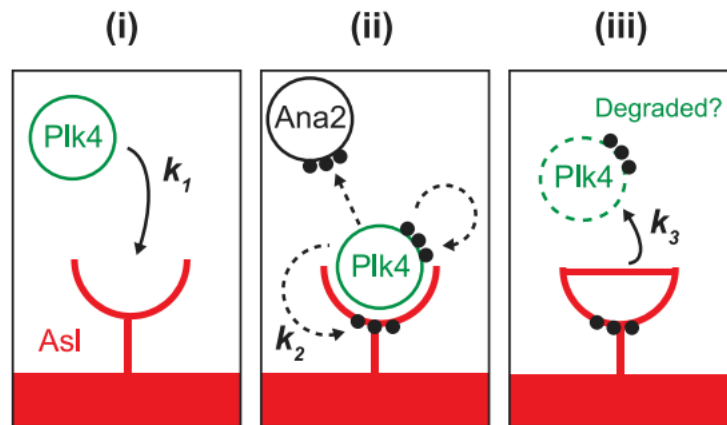
Recent studies in the Raff laboratory used the irreversible incorporation of the cartwheel component Sas-6 into the growing centrioles in living *Drosophila* embryos as a proxy to measure the kinetics of daughter centriole growth (Aydogan et al., 2018, 2020). These studies revealed that centriole growth is adaptive: during nuclear cycle 11 (when S-phase is relatively short), the centrioles grow quickly, but for a short period; during cycles 12-13 (as S-phase gets successively longer), the centrioles grow progressively more slowly, but for a progressively longer period. As a result, the centrioles grow to the same size irrespective of the length of S-phase.

Intriguingly, the levels of Plk4 at the centriole oscillate during S-phase in these early fly embryos (Aydogan et al., 2020) and also in human cultured cells (Yamamoto & Kitagawa, 2019). In fly embryos, this oscillation is also adaptive: during nuclear cycle 11 (when centrioles grow quickly but a short period of time) the oscillation has a high amplitude and

short period; in subsequent cycles (when centrioles grow more slowly, but for longer periods) the Plk4 oscillation amplitude is lower but it has a longer period. It seems that this oscillation can influence the rate and period of centriole growth and that there is a “threshold” level of centriolar Plk4 that is required for centriole growth to occur (allowing the Plk4 oscillation to effectively time the period of centriole growth by setting the length of time that Plk4 levels are above this threshold).

In collaboration with mathematicians, Aydogan, Steinacker et al., developed a simple model to explain how a Plk4 oscillation might be generated at centrioles during S-phase (Figure 1.6). In this model, Plk4 is recruited to centrioles by Asl. This binding activates Plk4, allowing Plk4 to: (1) phosphorylate Ana2 (to drive centriole assembly); (2) phosphorylate itself (perhaps to ultimately drive its destruction); (3) phosphorylate the Asl receptor. It has been shown that Plk4 can phosphorylate Asl at multiple sites in flies, and also its homologue Cep152 in humans, but the significance of these phosphorylation events is unclear (Boese et al., 2018; Hatch et al., 2010). Aydogan, Steinacker et al., postulated that this phosphorylation eventually switches off the ability of Asl to recruit Plk4. These interactions therefore form a classical delayed negative feedback loop in which an activator (Asl) recruits its own inhibitor (Plk4) to generate an oscillation (Novák & Tyson, 2008). Note that this model generates a single oscillation (i.e. rise and fall) in Plk4 levels during S-phase. It was hypothesised, therefore, that a burst of phosphatase activity during mitosis might “reset” the Asl receptors (by dephosphorylating them), so allowing the system to oscillate again during the next nuclear cycle. In this way, the core Cdk/Cyclin

Oscillator (CCO) that drives the nuclear cycles in these embryos would entrain the Plk4 oscillation to the nuclear cycles.



**Figure 1.6 A mathematical model explains Plk4 oscillation**

See text for details. Note that this model considers only Plk4 at the site of daughter centriole assembly—a reasonable assumption as super-resolution imaging showed that in these embryos Plk4 is only very transiently and weakly localized in a ring around the entire mother centriole, while it remains concentrated at the site of daughter assembly throughout the nuclear cycles. Adapted from Aydogan et al., (2020)

### 1.3.4 Centriole maturation

Once fully-grown, the daughter centriole eventually loses its close orthogonal arrangement with its mother (a process termed centriole disengagement) during mitosis (Fry, 2015). This disengagement is required to allow the daughter to then mature into a mother that can itself duplicate and organise PCM (see **Section 1.4.2**) (Novak et al., 2014; W. J. Wang et al., 2011). In fly embryos, disengagement and maturation must occur very quickly, as the newly disengaged daughter centriole must be ready to duplicate and organise PCM within no more than 1-2 minutes—and potentially within just a few seconds

or tens of seconds, as the precise timing of these events is hard to measure and is currently unclear.

In these embryos it seems that the recruitment of Polo to the newly formed centrioles at the start of mitosis is required to initiate centriole maturation. Polo/Plk1 kinases have a conserved Polo Box Domain (PBD) that recruits the kinase to multiple different locations within the cell during mitosis by binding to phosphorylated S-S/T(p) motifs in target proteins. Importantly, mutating the first Ser in this motif to a Thr (creating a T-S/T motif) prevents PBD binding, but often does not prevent phosphorylation of the following S/T residue. *Drosophila* Sas-4 is phosphorylated by Cdk1 on T200 at the start of mitosis to create an S-T(p) motif that is required to recruit Polo to the newly formed daughter centrioles for the first time (Novak et al., 2016). Mutating this motif blocks this recruitment and, although the daughter centrioles still disengage from their mothers, the daughters fail to mature into mothers and so are unable to duplicate or to recruit any PCM. The Polo recruited by Sas-4 seems to initiate the recruitment of Asl to new mother centrioles, which is essential for centriole duplication (Cizmecioglu et al., 2010; Dzhindzhev et al., 2010; Novak et al., 2014, 2016) (Figure 1.5). Independently, it also initiates a cascade of phosphorylation events that create further S-S/T(p) motifs in the centriole protein Ana1/Cep295 and in the centriole/PCM protein Spd-2 that are essential for the subsequent recruitment of the mitotic PCM (Alvarez-Rodrigo et al., 2019, 2020). These later phosphorylation events will be discussed in more detail in **Section 1.4.3**.



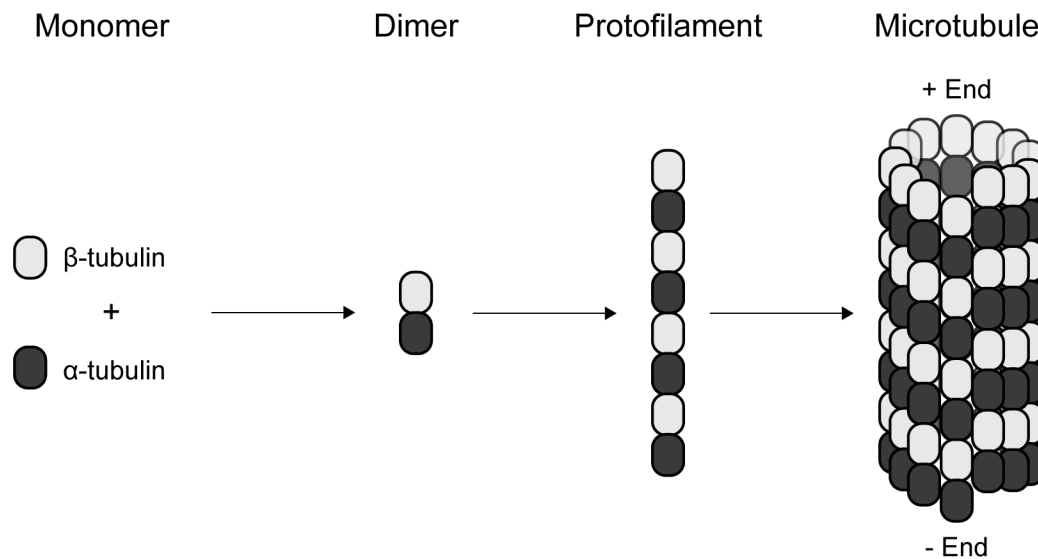
## 1.4 Centrosomes

Centrosomes are formed when centrioles recruit around themselves a matrix of proteins collectively termed the pericentriolar material (PCM) (Conduit et al., 2015). Centrosomes are best known for their role as major microtubule-organising centres (MTOCs) that nucleate and organise microtubules from their PCM (Kuriyama & Borisy, 1981). Centrosomes are also thought to act as important coordination centres within the cell, a place where many signalling and regulatory proteins interact with one another and where decisions are made (Arquint et al., 2014; Doxsey, 2001; Vertii et al., 2016). For example, as cells prepare to enter mitosis, Cdk1/CyclinB activity is first detected at centrosomes or at the centrosome-equivalent in yeast (the spindle pole body), and then spreads throughout the rest of the cell (Basu et al., 2020; Grallert et al., 2013; Jackman et al., 2003); at the end of mitosis, the Anaphase Promoting Complex/Cyclosome appears to first be activated at centrosomes, and then spread into the spindle or the cytoplasm, at least in some systems (Huang & Raff, 1999; Raff et al., 2002; Wakefield et al., 2000).

### 1.4.1 Centrosome functions

During interphase in many cell types, the single centrosome organises an extensive MT array that helps to organise the cell (Figure 1.3A). MTs are composed of  $\alpha/\beta$  dimers of tubulin that usually assemble into a closed MT comprising 13 protofilaments (Kollman et al., 2011) (Figure 1.7). MTs are intrinsically polar polymers, with a slow-growing minus-end and a fast growing plus-end (Fan et al., 1996; Mitchison, 1993), and centrosomes nucleate and organise MTs from their minus ends. In this way, the interphase centrosomal array of MTs is polarised, with the MT minus ends clustered at the centrosome (and so

positioned near the centre of the cell), and their plus-ends spreading outward towards the cell cortex. MT motor proteins can move on MTs either towards their plus-ends (most Kinesin-family proteins) or minus ends (Dynein-family proteins, and some specialized Kinesins), and these motors use the polarised MT array to help organise the interphase cell—for example, moving and position various cargoes and organelles within the cell (Barlan & Gelfand, 2017).

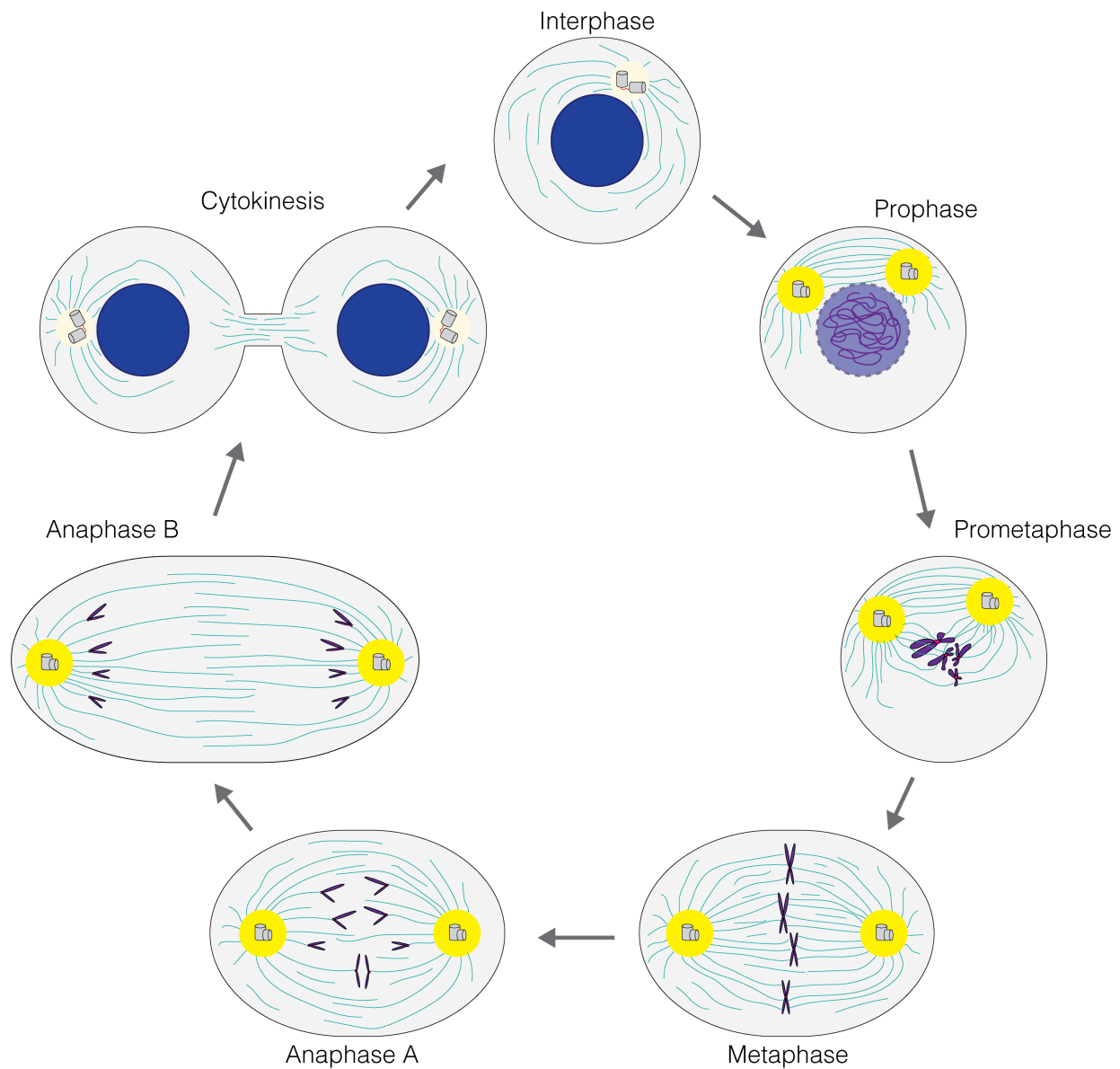


**Figure 1.7 Ultrastructure of microtubules**

Microtubules are hollow and cylindrical structure consisting of repeating units of  $\alpha/\beta$ -tubulin heterodimers arranging longitudinally to form protofilaments. 13 protofilaments associate laterally to form MT lattice in cells. MTs exhibit polarity with  $\alpha/\beta$ -tubulin heterodimers oriented in a head-to-tail fashion within the polymer lattice.

As cells prepare to enter mitosis (Figure 1.8), the two centrosomes (formed around the duplicated centriole pairs generated in S-phase) organise an extensive array of astral MTs. These MTs help to break down the nuclear envelope (Beaudouin et al., 2002) (**prophase**), allowing the MTs to interact with condensed chromosomes (**prometaphase**) and to align them at the centre of the bipolar spindle (**metaphase**). The SAC (see **Section 1.2.2**) holds cells in prometaphase/metaphase until all the duplicated sister chromatids

are properly aligned on the spindle (with one sister attached to one pole, and the other to opposite pole). When this arrangement is achieved, the sister chromatids will separate from one another and move towards their respective spindle poles (**anaphase A**) and the spindle will elongate (**anaphase B**), usually forming a prominent midbody between the separating chromatids. The Chromosomal Passenger Complex (CPC) becomes concentrated at the midbody and at the cell cortex between the separating centrosomes, and this complex orchestrates the remodeling of the cell membrane to pinch the cell into two daughters (**cytokinesis**) (M. Kitagawa & Lee, 2015). The ESCRTIII complex is required for the final stage of cell division that pinches and seals the membrane around the two new daughter cells (**abscission**) (Horváth & Müller-Reichert, 2020; Lafaurie-Janvore et al., 2013). Centrosomal abnormalities are thought to jeopardize these processes and so lead to aneuploidy, a common hallmark of cancer (Chavali et al., 2014; LoMastro & Holland, 2019).



**Figure 1.8 Microtubule organisation during interphase and different phases of mitosis**

See text for detail.

Although these preceding paragraphs emphasise the central role of centrosomes in organising interphase and mitotic cells, it is important to note that in many cell types (e.g. most epithelial and muscle cells), centrosomes are not major organisers of the interphase MT arrays (Bugnard et al., 2005; Efimov et al., 2007; Rivero et al., 2009; Srsen et al., 2009) and that, even in cells where they are, MTs and motor proteins can often self-

organise into functional interphase arrays even in the absence of centrosome (Nédélec et al., 1997). Perhaps surprisingly, centrosomes also appear to be largely dispensable for mitosis. For example, *Drosophila* somatic cells lacking centrosomes can proceed through mitosis relatively normally—although spindle assembly is significantly slowed (Basto et al., 2006). Interestingly, flies that lack centrioles (and therefore lack centrosomes and cilia) can proceed through most of development relatively normally, but they die as adults due to the lack of cilia in their sensory neurons (Basto et al., 2006). In contrast, a lack of centrioles/centrosomes in vertebrate cells seems to activate a p53-dependent checkpoint that triggers either apoptosis or senescence (Bazzi & Anderson, 2014). In the absence of p53, however, these cells can divide relatively normally, indicating that centrosomes are not essential for cell division in most vertebrate cells. It is not clear how vertebrate cells sense a lack of centrioles or centrosomes, why it is important that they do so, or why flies seem to lack this pathway.

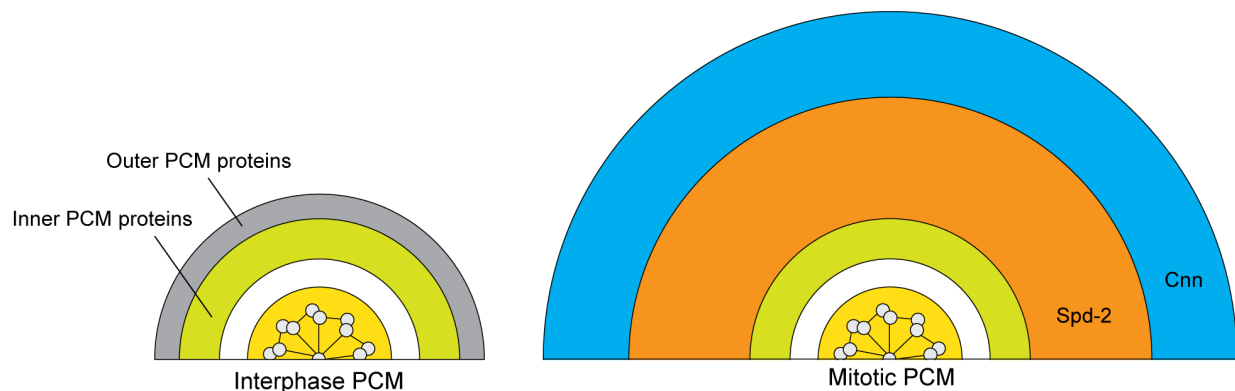
These observations suggest that the ability of centrioles to form cilia represents their most important (and probably ancestral) function, while the ability to form centrosomes may have evolved later, perhaps as a mechanism to ensure that the duplicated centrioles are always accurately segregated into the two daughter cells during cell division. Some cell types, however, seem to have become addicted to their centrosomes for cell division. The rapid nuclear and cell division in the early *Drosophila* and *C. elegans* embryos, for example, are dramatically perturbed in the absence of centrioles/centrosomes (and these cells do not make cilia) (Gönczy et al., 1999, 2000; Kirkham et al., 2003; Stevens et al., 2007; Varmark et al., 2007).

In mice that lack centrioles/centrosomes the developmental defects are much more severe, although this is not due to a failure in cell division but rather due to the activation of a p53-dependent checkpoint that seems to monitor cells for the presence of centrioles (Bazzi & Anderson, 2014; Insolera et al., 2014). Thus, mice without centrioles develop much more normally if p53 is absent although, like flies, they eventually die due to defects associated with the lack of cilia.

#### 1.4.2 Centrosome maturation

The PCM appears to be composed of several hundred proteins (Alves-Cruzeiro et al., 2014). Many of these help the centrosome to nucleate and organise MTs, such as  $\gamma$ -tubulin and its associated proteins that form  $\gamma$ -tubulin ring complex ( $\gamma$ -TuRC) (Liu et al., 2020, 2021; Zheng et al., 1991), while others are important signalling molecules, cell cycle regulators and checkpoint proteins (Arquint et al., 2014; Doxsey, 2001; Vertii et al., 2016). The PCM is organised exclusively by the mother centriole (Conduit et al., 2014b; Izquierdo et al., 2014; Kuriyama & Borisy, 1981), which organises relatively small amounts of PCM during interphase. Super-resolution microscopy studies revealed that the interphase PCM is at least partially organised, with proteins such as Plp/Pericentrin and Asl/Cep152 having one terminus directly attached to the centriole, and the other stretching outwards away from the centriole—an organisation that is conserved in humans and flies (J. Fu & Glover, 2012; Lawo et al., 2012; Mennella et al., 2012; Sonnen et al., 2012). These “inner” centriole proteins are surrounded by a more amorphous “outer” layer of proteins such as  $\gamma$ -tubulin and Cnn/Cdk5rap2. Plp/Pericentrin, seems to play a

particularly important part in organising the interphase PCM (J. Fu & Glover, 2012; Lawo et al., 2012; Mennella et al., 2012; Sonnen et al., 2012) (Figure 1.9).



**Figure 1.9 The organisation of interphase and mitotic PCM in *Drosophila***

Interphase PCM has less amount of protein. Inner and outer PCM proteins are close to the centriole wall. During centrosome maturation, outer PCM proteins such as Spd-2 and Cnn massively expand and form protein matrix that enhances the recruitment of  $\gamma$ -tubulin and nucleation of microtubules.

The PCM expands dramatically as cells prepare to enter mitosis in a process termed centrosome maturation (Palazzo et al., 1999). Classical electron microscopy (EM) studies revealed that the expanded mitotic PCM is generally amorphous (Gould & Borisy, 1977; Kuriyama & Borisy, 1981). Electron tomography (ET) of purified mitotic centrosomes from the early embryos of clams and flies revealed a fibrous matrix surrounding the centriole (Moritz, Braunfeld, Fung, et al., 1995; Schnackenberg et al., 1998) in which  $\gamma$ -TuRCs were embedded (Moritz, Braunfeld, Sedat, et al., 1995). When mixed with tubulin, these purified centrosomes could nucleate MTs, but this was prevented if the centrosomes were washed with high salt, which removed the  $\gamma$ -TuRCs (and many other proteins), but left behind an electron dense “centromatrix” (Moritz et al., 1998; Schnackenberg et al., 1998). If the salt-stripped centrosomes were mixed with cytoplasmic embryo extracts, they regained the ability to bind  $\gamma$ -tubulin and to nucleate MTs; if the extracts were depleted of  $\gamma$ -tubulin, they no longer restored the ability of the centrosomes to nucleate MTs. These

findings support the idea that centrosomes largely nucleate MTs through the  $\gamma$ -TuRCs recruited (Liu et al., 2020; Oakley et al., 1990; Stearns et al., 1991; Stearns & Kirschner, 1994; Zheng et al., 1991). The picture that emerges from these studies is of a fibrous pericentriolar “scaffold” that binds centrosomal proteins such as  $\gamma$ -tubulin from the cytosol that permeates the scaffold, thereby concentrating these proteins within the PCM.

#### 1.4.3 Spd-2, Polo and Cnn cooperate to assemble a mitotic PCM scaffold in *Drosophila*

A genome-wide RNAi screen for genes required for mitotic PCM assembly in *Drosophila* cultured cells identified *polo*, *centrosomin (cnn)* and *Spindle-defective-2 (Spd-2)* as the strongest hits (Dobbelaere et al., 2008), and flies that are mutant for both *cnn* and *Spd-2* can enter mitosis, but centrosomes fail to mature, indicating that Cnn and Spd-2 proteins are strong candidates for being important components of the mitotic PCM scaffold (Conduit et al., 2014b). Most individual centrosome proteins turnover throughout the volume of the mitotic PCM, indicating that they are constantly binding to, and being released from, an underlying scaffold structure (Conduit et al., 2014b). In contrast, Cnn and Spd-2 are only recruited into the PCM in a region close to the surface of the mother centriole, where they assemble into a fibrous-like structure that appears to then flux outwards, away from the mother centriole (Conduit et al., 2014b). In fly embryos, the Cnn scaffold fluxes outwards along the centrosomal MTs, allowing it to extend beyond the Spd-2/Polo scaffold; in fly brain cells, this outward flux of Cnn does not occur, and the Spd-2/Polo and Cnn scaffolds largely spatially overlap (Conduit et al., 2015; Conduit et al., 2014b). Together, these studies suggest that Spd-2 and Cnn are not binding to an

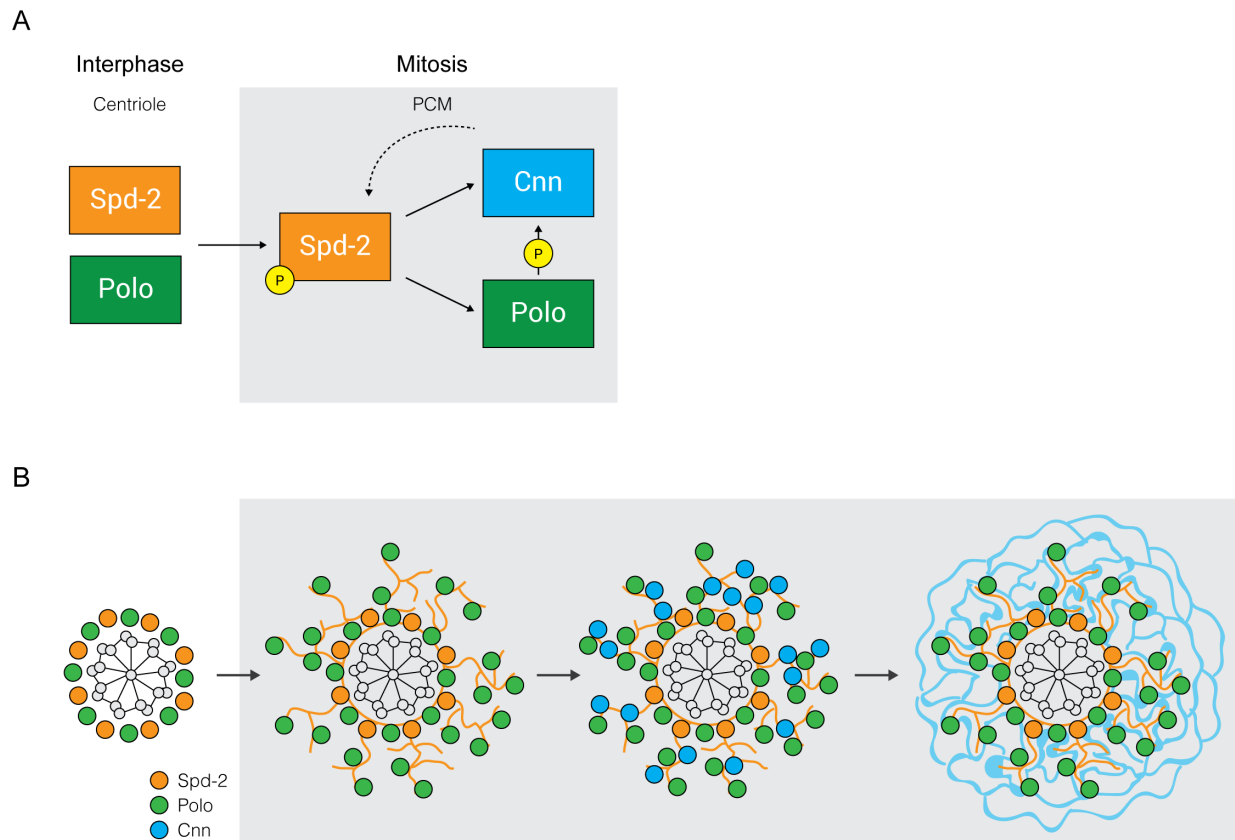


underlying PCM scaffold, but instead that they cooperate with Polo to assemble the underlying scaffold.

The Raff laboratory has proposed a molecular model of mitotic PCM scaffold assembly in *Drosophila* embryos (Figure 1.10). In this model, Spd-2 is constantly being recruited to and released from the mother centrioles. As cells prepare to enter mitosis, the Spd-2 at the centriole becomes phosphorylated, allowing it to assemble into a PCM scaffold. The Spd-2 scaffold fluxes outwards from the mother centriole and recruits other PCM proteins, but it is structurally weak, and so can only accumulate to a moderate extent around the mother centrioles. The Spd-2 scaffold, however, can recruit both Polo—via phosphorylated S-S/T(p) motifs in Spd-2 (Alvarez-Rodrigo et al., 2019)—and Cnn; the Polo phosphorylates Cnn, allowing it to form its own independent scaffold that is structurally stronger than the Spd-2 scaffold (Conduit et al., 2014a; Lucas & Raff, 2007). Importantly, it seems the Cnn scaffold cannot recruit more Spd-2 or Polo itself, but it interacts with and helps to maintain Spd-2 scaffold that originates from the mother centrioles. In this way, the interactions between these proteins forms a positive feedback loop that drives the outward expansion of the mitotic PCM scaffold around the mother centriole: with Spd-2 recruiting Polo and Cnn to form the Cnn scaffold, which then stabilises the Spd-2 scaffold, allowing Spd-2 scaffold to recruit more Polo and Cnn (Figure 1.10).

It has recently been shown that this positive feedback loop relies on Polo that is initially recruited to centrioles by phosphorylated S-S/T motifs in Ana1/Cep295 (Alvarez-Rodrigo

et al., 2020)—a centriole protein that is essential for centriole maturation and so for mitotic PCM assembly (Izquierdo et al., 2014). The centriolar pool of Polo then phosphorylates further S-S/T motifs in Spd-2, which allows the Spd-2 that is fluxing away from the centriole to recruit Polo into the expanding PCM scaffold (Alvarez-Rodrigo et al., 2020).



**Figure 1.10 Molecular model of PCM scaffold assembly**

**(A)** Cartoon depicts the molecular model of PCM scaffold. Importantly, the Cnn scaffold cannot recruit Polo or Spd-2, but it can help to maintain Spd-2 scaffold (indicated by dotted arrow). **(B)** During interphase, centriole recruits Spd-2 (orange circle) and Polo (green circle) which remains inactive. As cells prepare to enter mitosis, Spd-2 is phosphorylated and expands into a Spd-2 scaffold (orange line) which recruits more Polo and Cnn (blue circle). Polo phosphorylates Cnn to form a Cnn scaffold (blue line) which moves further away from Spd-2 scaffold.

#### 1.4.4 The molecular components of the mitotic PCM scaffold have been conserved in evolution

Genetic approaches have identified a very similar set of proteins that are required for mitotic PCM assembly in *C. elegans* (for example Dammermann et al., 2004; Decker et al., 2011; Hamill et al., 2002; Kemp et al., 2004; Pelletier et al., 2004). As in flies, the centriole/centrosome protein SPD-2 recruits PLK1 (the worm homologue of Polo) and a protein called SPD-5. Homologues of SPD-5 have not been identified outside of worm species; but, although Cnn and SPD-5 exhibit little amino acid sequence homology, they are both large proteins with several predicted coiled-coil regions and appear to be functional homologues. As with fly Polo and Cnn, worm PLK1 phosphorylates SPD-5 at multiple sites, which allows SPD-5 to assemble in a scaffold structure that is required for centrosome maturation (Woodruff et al., 2015, 2017; Wueseke et al., 2016). SPD-5 appears to be the major component of the mitotic PCM scaffold in worms, and centrosome maturation is essentially abolished in the absence of either SPD-2, PLK1 or SPD-5.

Vertebrate homologues of Spd-2/SPD-2, Polo/PLK1 and Cnn (Cep192, Plk1 and Cep215/Cdk5Rap2, respectively) all have important roles in centrosome assembly, indicating that this mitotic PCM assembly pathway is likely to have been conserved in evolution (Conduit et al., 2015). In vertebrates, Cep192/Spd-2 not only recruits Plk1 to centrosomes, but also recruits Aurora A, another protein kinase that has a major role in orchestrating mitotic events at the centrosome (Joukov et al., 2010). There appears to be an intricate interplay between Cep192/Spd-2, Aurora A and Plk1 in vertebrates, with Cep192/Spd-2 helping to activate Aurora A, which in turn phosphorylates Cep192/Spd-2 to allow it to recruit Plk1 (Joukov et al., 2010, 2014; Meng et al., 2015). It is not yet clear

if the details of this pathway are conserved in flies or worms, but Aurora A also has an important part in regulating mitotic centrosome assembly in both species (Berdnik & Knoblich, 2002; Hannak et al., 2001). In Chapter 6, I will present evidence that Spd-2 has a central role in harnessing the activity of Polo and Aurora A to assemble a robust mitotic PCM scaffold in *Drosophila* embryos. In vertebrate cells, another large centriole and PCM proteins, Pericentrin, also has an important role in mitotic centrosome assembly (Doxsey et al., 1994; Zimmerman et al., 2004), which is dependent on its phosphorylation by Plk1 (K. Lee & Rhee, 2011). In flies, the Pericentrin-like-protein (Plp) has a role in both interphase (Mennella et al., 2012; Roque et al., 2018) and mitotic (Lerit et al., 2015; Martinez-Campos et al., 2004; Richens et al., 2015) PCM assembly, but its role in mitotic PCM assembly is relatively minor compared to Spd-2 and Cnn. Pericentrin-family proteins appear to interact directly with Cnn-family proteins in both vertebrates and flies (Kim & Rhee, 2014; Lerit et al., 2015; Rose Citron et al., 2018).

#### 1.4.5 The mitotic centrosome and phase separation

Recently, there has been an explosion of interest in the concept that membraneless organelles such as the centrosome can form by the phase separation of their constituent molecules into droplets—more recently termed biomolecular “condensates”—that have gel- or liquid-like properties. Condensate assembly is usually driven by proteins (often in association with nucleic acids) that have intrinsically disordered regions (IDRs) and/or low complexity domains (LCDs), or multiple copies of domains that interact with one another with relatively low affinity (Ditlev et al., 2018; Y. H. Lin et al., 2018). This rapidly expanding

field has been reviewed extensively (Banani et al., 2017; Boeynaems et al., 2018; Eggert, 2018; Shin & Brangwynne, 2017; Wheeler & Hyman, 2018).

The observations described in the previous Section have certainly been interpreted to suggest that Spd-2, Polo and Cnn assemble into a fibrous, solid-like PCM scaffold. Recent studies on the behaviour of the worm SPD-5 protein *in vitro*, however, have suggested that the concept of liquid-liquid phase separation (LLPS) may be very relevant to the assembly of mitotic centrosomes. Woodruff et al. purified full-length recombinant SPD-5 from insect cells and showed that it can self-assemble *in vitro* into large, micron-scale aggregates; importantly, this assembly is catalysed by purified recombinant SPD-2 and PLK1 (Woodruff et al., 2015). These assemblies appear to function as bona fide scaffolds *in vitro*, as they can specifically recruit SPD-2 and PLK1, but not several other non-centrosomal proteins.

Although these SPD-5 scaffolds formed *in vitro* initially appeared to be solid-like, it was subsequently shown that, in the presence of molecular crowding agents, purified SPD-5 can form spherical condensates that look much more like centrosomes (Woodruff et al., 2017). These SPD-5 condensates have transient liquid-like properties, and they can recruit ZYG-9 (the worm homologue of ch-TOG/XMAP215, a protein that promotes MT growth and stability) and TPXL-1 (the worm homologue of TPX-2, a protein that promotes MT stability). Crucially, these condensates can also concentrate tubulin, and so they can nucleate MT asters. Based on these observations, Woodruff et al. suggest that the mitotic PCM is best described as a “selective phase” that concentrates MT regulators and tubulin,

allowing the PCM to organise MT arrays. This interpretation is consistent with an earlier mathematical model that successfully modelled the growth of the centrosomes in early *C. elegans* embryos by considering the centrosome as a liquid-droplet (Zwicker et al., 2014).

Perhaps importantly, the SPD-5 condensates formed *in vitro* are only transiently liquid-like, as they rapidly “mature” into a more gel- or solid-like state (Woodruff et al., 2017). Dynamic studies suggest that both SPD-5 and Cnn molecules exist in a more solid-like state at centrosomes *in vivo*, as they both cannot easily internally re-arrange once incorporated into centrosomes in living fly or worm embryos (Conduit et al., 2010; Conduit et al., 2014a; Laos et al., 2015). This led Woodruff et al. to suggest that SPD-5 might initially form a liquid-like condensate that expands around that mother centriole and then hardens once the centrosome reaches its full size, which might be important to allow the centrosomes to withstand the strong MT-dependent forces that act upon it during mitosis (Woodruff et al., 2017).

While these findings have created considerable excitement in the centrosome field (Rale et al., 2018; Woodruff, 2021), some caution seems advisable, as it is not clear how closely this *in vitro* system accurately mimics MT nucleation from centrosomes *in vivo*. For example, centrosomes normally nucleate MTs by recruiting  $\gamma$ -TuRCs, and these are not present in the SPD-5/ZYG-9/TPXL-1/Tubulin condensates reconstituted by Woodruff et al. On the other hand, worm embryos have a less efficient pathway that allows centrosomes to organise MT arrays independently of  $\gamma$ -tubulin (Hannak et al., 2001; Sampaio et al., 2001)—perhaps these condensates mimic this alternative pathway. In

Chapter 6, I describe experiments that suggest that the centrioles in *Drosophila* embryos may organise both a solid-like Cnn-based PCM scaffold, and a liquid-like scaffold based on the PCM protein TACC.

## 1.5 Centrosome size control

As described at the start of this Chapter, centrioles are an attractive model system for studying how organelle size is controlled (Aydogan et al., 2018, 2020; Marshall, 2009). This is also true of mitotic centrosomes as, although the mitotic PCM is not as clearly structured as centrioles (indeed it is widely regarded as being largely amorphous and unstructured), centrosome size is tightly regulated during the cell cycle (Conduit et al., 2015). Moreover, mitotic centrosomes have an unusual problem to solve, in that cells preparing to enter mitosis have two spatially separated centrosomes that must grow to the same size before the cells enter mitosis: mitotic spindles assembled from centrosomes of unequal size are unbalanced and have problems accurately segregating their chromosomes (Meraldi, 2016; Tan et al., 2015). Moreover, in some cell types, such as the *Drosophila* larval neuroblast, the two centrosomes can initially be very different in size at the start of the centrosome maturation process, yet the centrosomes grow to approximately the same size by the time the mitotic spindle is fully assembled (Conduit & Raff, 2010; Januschke et al., 2011; Rebollo et al., 2007; Rusan & Peifer, 2007). It is not clear how such size equality is achieved, as one might expect that if two centrosomes of unequal size start to mature at the same time, the centrosome that is initially larger would outcompete the smaller centrosome for building blocks due to its larger surface area and volume (Raff, 2019).

#### 1.5.1 Centrosome flux and centrosome size homeostasis

An attractive feature of the Spd-2/Polo/Cnn scaffold model of PCM assembly (**Section 1.4.3**) is that it can potentially explain this conundrum. Spd-2 and Cnn molecules appear to flux through the PCM, only incorporating into the mitotic PCM in the central regions around the mother centriole and only leaving the PCM when they have fluxed outwards to reach the more peripheral regions (Conduit et al., 2010; Conduit et al., 2014b). Thus, there is a rate at which the molecules are incorporated into the PCM in the central regions (**CnnIN** and **Spd-2IN**) and a rate at which they leave the PCM from peripheral regions (**CnnOUT** and **Spd-2OUT**). As the incorporation rates are effectively determined by the mother centrioles (and this may be one reason why it is important that centrioles grow to the same size) CnnIN and Spd-2IN are independent of initial PCM size (i.e. the two centrosomes in a cell will incorporate new Cnn and Spd-2 molecules at their centrioles at the same rate, no matter what the initial size of their PCM). It seems likely, however, that the “off” rate at the periphery will vary depending on PCM size: the larger the PCM, the larger its surface area and volume, and so the higher the probability that an individual molecule will leave from the periphery. If this is correct, then centrosomes will grow to a constant homeostatic size (where the CnnIN and Spd-2IN in the centre is equal to CnnOUT and Spd-2OUT at the periphery), irrespective of their initial size.

#### 1.5.2 A limiting component model for centrosome growth in *C. elegans* embryos

Studies in early *C. elegans* embryos have suggested a very different model of centrosome size control. In these embryos the size of the mitotic centrosomes halves at each



successive cell division, raising the possibility that the developing embryos might contain a constant total amount of a limiting component that is simply redistributed between all of the growing centrosomes in the embryo at each successive division cycle (Decker et al., 2011). Intriguingly, SPD-2 appears to behave as a limiting component in these embryos, and it functions, at least in part, by recruiting PLK1 (the worm homologue of Polo/Plk1) into the PCM. Moreover, centrosome size was shown to set the length of the mitotic spindle in these embryos, potentially because it controls the length scale of a TPXL1 gradient along the spindle MTs (Greenan et al., 2010). These studies provided important insights into how the size of key organelles such as the centrosome and spindle might be regulated so that their size effectively scales with the size of the cell (Decker et al., 2011; Greenan et al., 2010; Hara & Kimura, 2009). In contrast, the Plk4 oscillator model of centriole size control described in **Section 1.3.3**, provides a mechanism that would not be expected to scale with cell size, so ensuring that centrioles grow to a consistent size no matter what the size of the cell.

The “limiting component” model of organelle growth control is potentially an attractive way to explain the growth of organelles whose size scales with cell size—such as spindle length and nuclear volume (Hara & Kimura, 2009; Jorgensen et al., 2007). A limiting cytoplasmic pool of a key building block provides a powerful mechanism for the cell size-dependent regulation of growth without any need for an active mechanism to measure size or modulate growth rates (Goehring & Hyman, 2012); the larger the cell, the more building blocks it will have, and so the larger the organelle will grow. However, there are some problems with the simple model proposed to explain centrosome sizing in *C.*

*elegans* embryos. Perhaps most importantly, it is unclear how such a model could explain how two centrosomes of initially different size could ultimately grow to the same size, as clearly happens in certain cell types (Raff, 2019). Moreover, it is perhaps difficult to envisage how, in large cells like those present in the early *C. elegans* embryos, a limiting component can diffuse rapidly enough within the cell such that effectively every molecule of the component is sequestered at the centrosome during the ~10 minutes period of centrosome growth.

## 1.6 Thesis Summary

In this thesis I first investigate the dynamics of centrosome growth in the syncytial *Drosophila* embryo during nuclear cycles 11-13 (**Chapter 3**). I find that, in contrast to the situation in the early *C. elegans* embryo, centrosomes grow in a homeostatic manner: growing quickly but for a short period in cycle 11, and more slowly but for a longer period during successive cycles—so that the centrosomes grow to a relatively consistent size at each cycle. I provide evidence that an oscillation in the centriolar levels of Plk1 plays an important part in setting the parameters of centrosome growth. I then investigate the mechanisms that ensure the differential growth rate of the centrosomes at successive cycles (**Chapter 4**). I find that the cytosolic levels of the key PCM scaffolding proteins Cnn and Spd-2 decline at successive cycles, potentially explaining, at least in part, why the centrosomes grow more slowly at successive cycles. Finally, I make the surprising observation that TACC, a well-known and conserved centrosomal protein that regulate MT stability, seems to form a liquid-like scaffold around the mother centriole (**Chapter 5**). My data suggests that TACC scaffold is dependent on Spd-2 and Aurora A, indicating

that Spd-2 plays an important part in organising a solid-like Polo/Cnn-dependent scaffold and a liquid-like Aurora A/TACC-dependent scaffold around the mother centrioles. Finally, I discuss the significance of my results, with a focus on how they relate to the mechanisms of organelle size control, and the debate about whether the PCM is built upon a solid-like and/or liquid-like scaffold.

## 2. Material and Methods

### 2.1 Fly Husbandry

#### 2.1.1 Fly handling

Flies were maintained on *Drosophila* culture medium (0.8% agar, 8% cornmeal, 1.8% yeast extract, 1% soya, 8% malt extract, 2.2% molasses, 0.14% nipagen, and 0.625% propionic acid). They were kept in 8 cm x 2.5 cm plastic vials or 0.25-pint plastic bottles. Embryos were collected from apple juice plates (25% apple & raspberry juice, 2.5% sucrose, and 2.25% agar) supplemented with fresh yeast suspension. Standard fly handling techniques were employed.

#### 2.1.2 Drosophila stocks

Oregon R flies were used as the wild type (WT) stock in all experiments. Balancer chromosomes and markers were used as previously described (Lindsley and Zimm, 1992). The complete list of experimental fly lines is listed on Table 2.2. And alleles used to generate these lines are listed on Table 2.1.

Table 2.1 *D. melanogaster* alleles used in this study.

| Allele                | Source                   |
|-----------------------|--------------------------|
| Ubq-MSPS-GFP          | (M. J. Lee et al., 2001) |
| Ubq-GFP-TACC          | (Barros et al., 2005)    |
| stella <sup>592</sup> | (M. J. Lee et al., 2001) |

|                        |                                                                                        |
|------------------------|----------------------------------------------------------------------------------------|
| ncd-γ-tubulin37c-GFP   | (Hallen et al., 2008)                                                                  |
| Ubq-Grip71-GFP         | (Conduit et al., 2014b)                                                                |
| Grip75-GFP (CRISPR)    | (Tovey et al., 2020)                                                                   |
| Grip128-GFP (CRISPR)   | (Tovey et al., 2020)                                                                   |
| Ubq-AuroraA-GFP        | (Lucas & Raff, 2007)                                                                   |
| cycB <sup>2</sup>      | (Jacobs et al., 1998)                                                                  |
| cnn <sup>f04547</sup>  | Exelixis stock no. f04547, Exelixis Stock Centre (Harvard Medical School, Boston, MA). |
| cnn <sup>HK21</sup>    | (Megraw et al., 1999; Vaizel-Ohayon & Schejter, 1999)                                  |
| Ubq-NG-Cnn             | Lisa Gartenmann                                                                        |
| Ubq-Spd-2-GFP          | (Dix & Raff, 2007)                                                                     |
| spd2 <sup>z35711</sup> | (Giansanti et al., 2008)                                                               |
| spd2 <sup>G20143</sup> | (Dix & Raff, 2007)                                                                     |
| Polo-TRAP-GFP          | (Buszczak et al., 2007)                                                                |
| Ubq-Spd-2-RFP          | (Conduit et al., 2014a)                                                                |
| Ubq-Spd-2-mCherry      | (Alvarez-Rodrigo et al., 2019)                                                         |
| Ubq-Cnn                | Saroj Saurya                                                                           |
| Ubq-Spd-2              | Saroj Saurya                                                                           |
| ana1 <sup>mecB</sup>   | (Avidor-Reiss et al., 2004; Blachon et al., 2009)                                      |
| Ubq-Ana1               | Saroj Saurya                                                                           |
| Ubq-Ana1-mCherry       | (Alvarez-Rodrigo et al., 2020)                                                         |
| Ubq-Ana1-S34T-mCherry  | (Alvarez-Rodrigo et al., 2020)                                                         |
| GFP-Cnn (CRISPR)       | Paul Conduit                                                                           |

|                    |                          |
|--------------------|--------------------------|
| Ubq-GFP-Cnn        | (Lucas & Raff, 2007)     |
| Spd-2-NG (CRISPR)  | Saroj Saurya             |
| Ubq-Spd-2-NG       | Lisa Gartenmann          |
| ePlk4-NG           | (Aydogan et al., 2020)   |
| sak <sup>aa</sup>  | (Aydogan et al., 2018)   |
| NG-Sas-6 (CRISPR)  | Thomas Steinacker        |
| NG-Asl (CRISPR)    | Thomas Steinacker        |
| Ubq-Klp10A-GFP     | Saroj Saurya             |
| Ubq-Calmodulin-GFP | Anna Caballe             |
| mNG                | Thomas Steinacker        |
| Ana2-NG (CRISPR)   | Thomas Steinacker        |
| Ubq-RFP-TACC       | (M. J. Lee et al., 2001) |
| Ubq-GFP-TACC-S863L | (Barros et al., 2005)    |
| Ubq-GFP-TACC-S863E | Teresa Barros            |
| aur <sup>1</sup>   | (Glover et al., 1995)    |
| msps <sup>p</sup>  | (Cullen et al., 1999)    |

Table 2.2 *D. melanogaster* strains used in this study.

| Genotype                             | Chapter | Figure                     |
|--------------------------------------|---------|----------------------------|
| Ubq-GFP-TACC ; stella <sup>592</sup> | 3, 5    | 3.2 – 3.5, 3.10, 5.1 – 5.2 |
| Ubq-MSPS-GFP                         | 3       | 3.2 – 3.5, 3.10            |
| ncd-γ-tubulin37c-GFP                 | 3       | 3.2 – 3.5, 3.10            |

|                                                                                                                            |      |                               |
|----------------------------------------------------------------------------------------------------------------------------|------|-------------------------------|
| Ubq-Grip71-GFP                                                                                                             | 3    | 3.2 – 3.5, 3.10               |
| Grip75-GFP (CRISPR)                                                                                                        | 3    | 3.2 – 3.5, 3.10               |
| Grip128-GFP (CRISPR)                                                                                                       | 3    | 3.2 – 3.5, 3.10               |
| Ubq-AuroraA-GFP                                                                                                            | 3    | 3.2 – 3.5, 3.10               |
| Ubq-GFP-TACC ; stella <sup>592</sup> / +                                                                                   | 3    | 3.6                           |
| Ubq-GFP-TACC ; stella <sup>592</sup> / cycB <sup>2</sup>                                                                   | 3    | 3.6                           |
| ncd-γ-tubulin37c-GFP / +                                                                                                   | 3    | 3.6                           |
| ncd-γ-tubulin37c-GFP / cycB <sup>2</sup>                                                                                   | 3    | 3.6                           |
| Ubq-NG-Cnn, cnn <sup>f04547</sup> / cnn <sup>HK21</sup>                                                                    | 3, 4 | 3.7 – 3.10, 4.1 – 4.3, 4.6    |
| Ubq-Spd-2-GFP ; spd2 <sup>z35711</sup> / spd2 <sup>G20143</sup>                                                            | 3    | 3.7 – 3.10                    |
| Polo-TRAP-GFP / +                                                                                                          | 3    | 3.7 – 3.10, 3.16 – 3.18, 3.20 |
| Ubq-GFP-TACC ; stella <sup>592</sup> / Ubq-Spd-2-mCherry                                                                   | 3    | 3.11                          |
| ncd-γ-tubulin37c-GFP / Ubq-Spd-2-mCherry                                                                                   | 3    | 3.12                          |
| cnn <sup>f04547</sup> , Ubq-GFP-Cnn / cnn <sup>HK21</sup> ; Ubq-Spd-2-RFP, spd2 <sup>G20143</sup> / spd2 <sup>z35711</sup> | 3    | 3.13                          |
| Polo-TRAP-GFP / Ubq-Spd-2-mCherry                                                                                          | 3    | 3.14                          |
| cnn <sup>f04547</sup> / + ; Polo-TRAP-GFP / +                                                                              | 3    | 3.16                          |
| Ubq-Cnn / + ; Polo-TRAP-GFP / +                                                                                            | 3    | 3.16                          |
| Polo-TRAP-GFP / spd2 <sup>z35711</sup>                                                                                     | 3    | 3.17                          |
| Polo-TRAP-GFP / Ubq-Spd-2                                                                                                  | 3    | 3.17                          |
| Polo-TRAP-GFP / ana1 <sup>mecB</sup>                                                                                       | 3    | 3.18                          |
| Polo-TRAP-GFP / Ubq-Ana1                                                                                                   | 3    | 3.18                          |
| Ubq-Ana1-mCherry / + ; Polo-TRAP-GFP / +                                                                                   | 3    | 3.21                          |

|                                                                     |      |                |
|---------------------------------------------------------------------|------|----------------|
| Ubq-Ana1-S34T-mCherry / + ; Polo-TRAP-GFP / +                       | 3    | 3.21           |
| Ubq-Ana1-mCherry / Ubq-Spd-2-GFP ; Spd-2 <sup>G20143</sup> / +      | 3    | 3.22           |
| Ubq-Ana1-S34T-mCherry / Ubq-Spd-2-GFP ; Spd-2 <sup>G20143</sup> / + | 3    | 3.22           |
| NG-Cnn (CRISPR)                                                     | 4    | 4.2 – 4.3      |
| Ubq-NG-Cnn, cnn <sup>f04547</sup> / +                               | 4    | 4.3            |
| GFP-Cnn (CRISPR)                                                    | 4, 5 | 4.3, 5.1 – 5.2 |
| cnn <sup>f04547</sup> , Ubq-GFP-Cnn / cnn <sup>f04547</sup>         | 4    | 4.3            |
| cnn <sup>f04547</sup> , Ubq-GFP-Cnn                                 | 4    | 4.3            |
| cnn <sup>f04547</sup> , Ubq-GFP-Cnn ; Ubq-GFP-Cnn                   | 4    | 4.3            |
| NG-Cnn (CRISPR) / cnn <sup>f04547</sup>                             | 4    | 4.3            |
| Spd-2-NG (CRISPR)                                                   | 4    | 4.7 – 4.10     |
| Spd-2-NG (CRISPR) / spd2 <sup>z35711</sup>                          | 4    | 4.9            |
| Ubq-Spd-2-NG / + ;<br>Spd-2-NG (CRISPR) / spd2 <sup>z35711</sup>    | 4    | 4.9            |
| Ubq-Spd-2-NG ; Spd-2-NG (CRISPR)                                    | 4    | 4.9            |
| Ubq-GFP-TACC, Ubq-RFP-Cnn                                           | 5    | 5.1            |
| cnn <sup>HK21</sup> / + ; Ubq-GFP-TACC / +                          | 5    | 5.3            |
| cnn <sup>f04547</sup> / cnn <sup>HK21</sup> ; Ubq-GFP-TACC / +      | 5    | 5.3, 5.7       |
| cnn <sup>HK21</sup> / + ; Ubq-Spd-2-NG / +                          | 5    | 5.4, 5.6       |
| cnn <sup>HK21</sup> / cnn <sup>f04547</sup> ; Ubq-Spd-2-NG / +      | 5    | 5.4, 5.6       |
| cnn <sup>HK21</sup> / + ; Ubq-AuroraA-GFP / +                       | 5    | 5.4, 5.6       |
| cnn <sup>HK21</sup> / cnn <sup>f04547</sup> ; Ubq-AuroraA-GFP / +   | 5    | 5.4, 5.6       |



|                                                                                        |   |          |
|----------------------------------------------------------------------------------------|---|----------|
| cnn <sup>f04547</sup> / + ; ncd-γ-tubulin37c-GFP / +                                   | 5 | 5.4, 5.6 |
| cnn <sup>f04547</sup> / cnn <sup>HK21</sup> ;<br>ncd-γ-tubulin37c-GFP / +              | 5 | 5.4, 5.6 |
| cnn <sup>HK21</sup> / + ; Ubq-MSPS-GFP / +                                             | 5 | 5.4, 5.6 |
| cnn <sup>HK21</sup> / cnn <sup>f04547</sup> ; Ubq-MSPS-GFP / +                         | 5 | 5.4, 5.6 |
| cnn <sup>HK21</sup> / + ; Ubq-Klp10A-GFP / +                                           | 5 | 5.4, 5.6 |
| cnn <sup>HK21</sup> / cnn <sup>f04547</sup> ; Ubq-Klp10A-GFP / +                       | 5 | 5.4, 5.6 |
| cnn <sup>HK21</sup> / + ; Ubq-Calmodulin-GFP / +                                       | 5 | 5.4, 5.6 |
| cnn <sup>HK21</sup> / cnn <sup>f04547</sup> ; Ubq- Calmodulin -GFP / +                 | 5 | 5.4, 5.6 |
| Ubq-Spd-2-RFP, cnn <sup>f04547</sup> / cnn <sup>HK21</sup> ;<br>Ubq-GFP-TACC / +       | 5 | 5.5      |
| Ubq-Spd-2-RFP, cnn <sup>f04547</sup> / cnn <sup>HK21</sup> ; AuroraA-<br>GFP / +       | 5 | 5.6      |
| cnn <sup>HK21</sup> / cnn <sup>f04547</sup> ;<br>Ubq-GFP-TACC / aur <sup>1</sup>       | 5 | 5.7      |
| cnn <sup>HK21</sup> / cnn <sup>f04547</sup> ;<br>Ubq-GFP-TACC / spd2 <sup>z35711</sup> | 5 | 5.7      |
| cnn <sup>HK21</sup> / cnn <sup>f04547</sup> ;<br>Ubq-GFP-TACC / msps <sup>P</sup>      | 5 | 5.7      |
| Ubq-GFP-TACC / Ubq-RFP-TACC                                                            | 5 | 5.8      |
| Ubq-RFP-TACC / + ; Ubq-GFP-TACC-S863L<br>/ +                                           | 5 | 5.8      |
| mNG / Ubq-RFP-TACC                                                                     | 6 | 6.1      |
| Ana2-NG (CRISPR) / + ;<br>Ubq-RFP-TACC / +                                             | 6 | 6.1      |
| Spd-2-NG (CRISPR) / Ubq-RFP-TACC                                                       | 6 | 6.1      |
| NG-Cnn (CRISPR) / + ;<br>Ubq-RFP-TACC / +                                              | 6 | 6.1      |

## 2.2 Biochemistry and Cell Biology

### 2.2.1 Embryo harvesting and fixation for protein expression analysis

0 – 2 hours old embryos were collected and washed three times with washing buffer (1X PBS, 0.05% Triton X-100), dechorionated with 60% bleach for 2.5 minutes. They were then washed three times with washing buffer and moved into a glass vial containing 2 mL of methanol with 3% 0.5M EGTA, and 2 mL heptane. Samples were gently swirled and allowed to sink to the bottom of the vial. Embryos that floated at the methanol-heptane interface and excess heptane were discarded. Samples were washed with methanol with 3% 0.5M EGTA 3 times and transferred to a new tube containing only methanol. They were left at 4°C for 24 hours before usage and put at -20°C for long term storage.

### 2.2.2 SDS-PAGE and Western blotting

The methanol was removed from embryos. Embryos were subsequently washed with PBT washing buffer (1X PBS, 0.2% Triton X-100) for 15 minutes at room temperature. The process was repeated for three times. 20 well-fixed and pre-cellularised embryos were selected under dissection microscope and re-suspended in 20 µl washing buffer. They were then mixed with 20 µl of 2X SDS loading buffer, boiled for 10 minutes, and spun down to remove insoluble impurities.

Samples were then run on a 3-8% NuPAGE acrylamide gel (Cat. EA03785BOX, Life technologies) using NuPAGE tris-acetate SDS running buffer (Cat. LA0041, Life technologies) at 150V. Samples were then transferred from the acrylamide gel to a

nitrocellulose membrane (Cat. 162-0112, Bio-Rad) in a BIO-RAD Mini Trans-Blot system filled with transfer buffer (25 mM Tris-base + 192 mM glycine + methanol 20% + 0.5 ml 20% SDS, pH 7.4). They were run at 100V for 2 hours. The membrane was blocked for 1 hours at room temperature using blocking buffer (TBS, 4% milk powder, 0.1% Tween-20); primary antibody (Table 2.3) was added to the buffer with an additional incubation at 4°C overnight under constant shaking. It was washed in washing buffer (TBS + 0.1% Tween-20) for 10 minutes, repeated 3 times before incubation with secondary antibody (Table 2.4) in blocking buffer for 1 hour. The washing procedure that is same as the one after primary antibody incubation was repeated. The membrane was briefly immersed in diluted HRPO-substrate (SuperSignal West Femto Maximum Sensitivity Substrate, Cat. 34095, Thermo Fischer Scientific) and exposed on X-ray film.

Table 2.3 Primary antibodies used in this thesis

| Protein | Species | Dilution | Source/Reference                   |
|---------|---------|----------|------------------------------------|
| Cnn     | Rabbit  | 1:1000   | Lab stock 37, (Lucas & Raff, 2007) |
| Spd-2   | Rabbit  | 1:500    | Lab stock 57, (Dix & Raff, 2007)   |

Table 2.4 Secondary antibodies used in this thesis

| Species | Reacted to | Conjugation | Dilution | Source/Reference |
|---------|------------|-------------|----------|------------------|
| Swine   | Rabbit     | HRP         | 1:3000   | Dako, P0399      |

### 2.2.3 Live embryo handling and microinjection for live imaging

Embryos were dechorionated by hand and desiccated for 1 minute at 25°C before mounted on coverslip dish (MatTek). The dish was painted with a stripe of glue prepared by dissolving double sided tape (Sellotapes) in heptane. The embryos were covered with

Voltalef grade H10S oil (Arkema) before imaging. For protein encoded RNA injection experiments, embryos were collected at 1-hour interval before desiccation. Embryos were incubated at 25°C for 1 hour to express the fluorescence fusion protein before imaging. For colchicine injection, embryos were collected and aged for 1.5 hours before desiccated and mounted on PerkinElmer Spinning Disk for injection. All microinjection requires slightly longer desiccation time for 5-7 minutes at 25°C.

## 2.3 Microscopy

### 2.3.1 Spinning disk microscope

Images of embryos were acquired at room temperature using a PerkinElmer ERS spinning disk confocal system mounted on a Zeiss Axiovert 200M microscope. A 63X, 1.4NA oil objective was used for all acquisition. The oil objective was covered with an immersion oil (ImmersoIT 518 F, Carl Zeiss) with a refractive index of 1.518 to minimize spherical aberration. The detector used was a charge-coupled device (CCD) camera (Orca ER, Hamamatsu Photonics, 15-bit), with a gain of 200 V. The system was equipped with 405 nm, 488 nm Argon gas lasers, and a diode pumped 561 nm solid-state laser. All red/green fluorescently tagged samples were acquired using UltraVIEW ERS 'Emission Discrimination' setting. The emission filter of these images was set as followed: green emission filter: 520 nm, band pass centre filter and red emission filter: 620 nm band pass centre. 0.5- $\mu$ m thick z-section were used for all images, but the number of sections, time step, laser power, and exposure were dependent on the specific experiment. The software for microscopy setting and manipulation was Volocity (PerkinElmer).

### 2.3.2 Spinning disk super-resolution microscopy

Imaging was based on an Olympus IX83 microscope equipped with a 60X, 1.3NA silicon immersion lens. The super-resolution acquisition was performed using a SoRA disk (Yokogawa), a 3.2X magnification lens (Olympus), and a photometrics BSI camera (95% QE – 6.5µm pixels). Samples were excited with QBIS 488 or 561 nm laser (Coherent) with 525/50 or 617/75 nm emission bandpass filters, respectively. A 'Quad' dichroic of 405/488/561/640 nm was used. At least 6 z-sections with each of 0.125 µm-thickness were used for each image. The images were processed using a super-resolution processing with a standard strength and deconvolved using a constrained iterative deconvolution function with 5 iterations in CellSense software (Olympus).

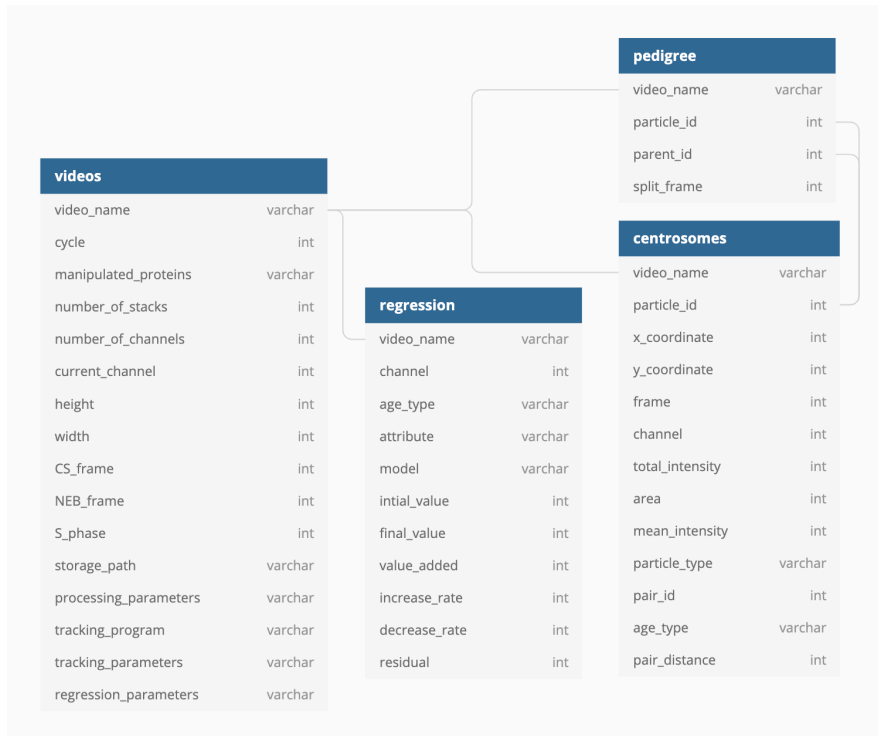
### 2.3.3 Fluorescence recovery after photobleaching (FRAP)

Live embryos were mounted on PerkinElmer ERS spinning disk microscope with settings described. Multiple circular regions of interests (ROI) with each diameter around 3.5 - 4µm were created on different centrosomes. A 488 nm laser with 100% laser power of 20 iterations were used to FRAP each sample. In some samples, different centrosomes were bleached at different timepoints in the same embryos, to compare the behaviour at different times in the nuclear cycle.

## 2.4 Data analysis

### 2.4.1 Data processing and representation

All raw time-series images taken from spinning disk microscope were exported in TIFF format. The photobleaching of fluorophores was corrected by exponential decay correction. The corrected images were z-projected by maximum intensity projection. The background was estimated and corrected by uneven illumination background correction. The details of processing and information of each videos were stored in standard format (“videos” in Figure 2.1). I wrote manual Python scripts to automate all of these processing steps. Single time snapshots from living embryos were also exported in TIFF format. The background was estimated and corrected by uneven illumination background correction (Soille, 2004). The best slices were selected, and maximum intensity projected into a single plane. The background is not subtracted for very dim images and images used for comparing the fold-change between fluorescence signal at centrosomes and the cytoplasm (see Chapter 6). Specific ROIs including dim centrioles were selected by FIJI. All raw data were stored as a hard copy locally and uploaded to OMERO server. Processed data were also stored locally. Python scripts for this step are listed on index 1 of Table 2.6.



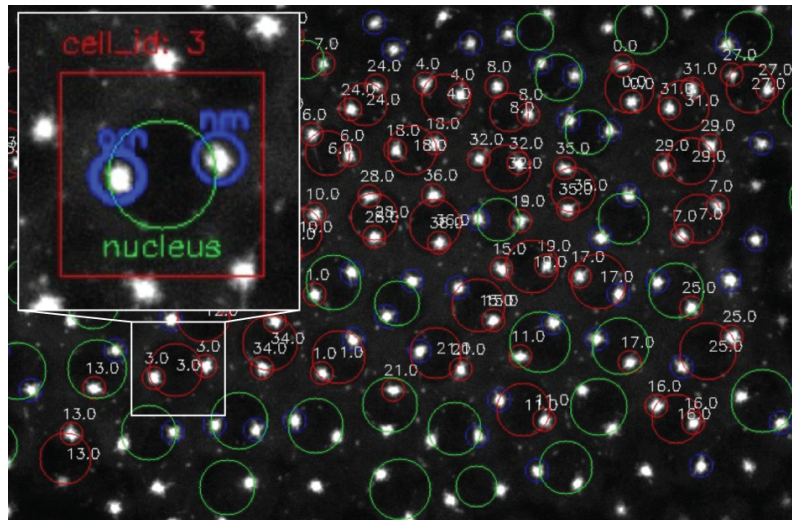
**Figure 2.1 Standard data storage format used in this thesis**

Each column of data represents certain data collected in the pipeline. “Videos” store the information of images and their processing method. “Centrosomes” store the information of each centrosome detected and their features. “Pedigree” stores the information of the relationship between centrosomes. “Regression” stores the data on the mathematical regression used to fit each embryo.

## 2.4.2 Objection Detection and Tracking

Corrected time-series images were tracked by TrackMate (Tinevez et al., 2017). Laplacian of Gaussian (LoG) detector with a protein dependent blob size was used to detect centrosomes. The quality of the detection was subsequently assessed and adjusted to maximize the sensitivity of centrosome detection and to remove irrelevant particles such as flares. The detected particles across different frame were then linked by a LAP tracker (Jaqaman et al., 2008). No gap was allowed within a track. In some instances, for example FRAP and morphological analyses of phase-separated particles (scoring fission and fusion events), centrosomes were manually identified by comparing

successive frames. A tight bounding box was drawn using an open-source software [OpenLabelling](#). I wrote a manual Python script to link annotated particles across frames. The last focal plane of the Z-stack was extracted separately from raw images to allow the manual determination of time point when Nuclear Envelope Breakdown (NEB) occurred. Python scripts for this step are listed on index 2 of Table 2.6.



**Figure 2.2 Centrosome and nucleus detection**

A snapshot from  $\gamma$ -tubulin-GFP labelled live embryo shows the detection of centrosome and nucleus (Inset) Magnification of a linked centrosomes and nucleus unit. Blue circles are used to annotate old mother and new mother of centrosome. Green circle is used to annotate the nucleus. Red square represents a unit of cell in the syncytium.

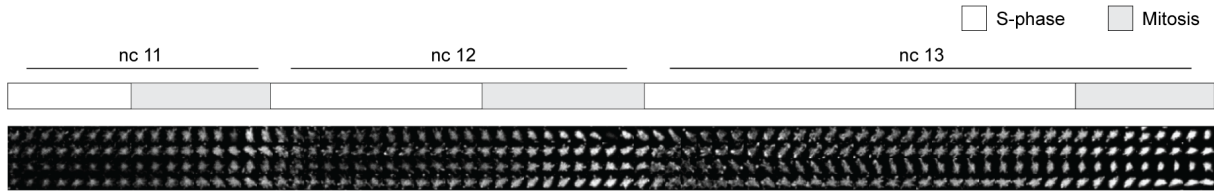
### 2.4.3 Data Extraction

I wrote a custom Python script to extract various parameters of centrosome growth from each embryo. First, I extracted sequential images of centrosomes at each timepoint in an individual track (illustrated for four centrosomes visualized with the centrosomal protein GFP-Cnn in Figure 2.3A). This allowed me to confirm that the tracking had worked well. I then applied an Otsu algorithm (Otsu, 1979) adjusted to the first frame of each embryo to find an appropriate segmentation intensity threshold to maximize the detection of a



contiguous centrosome area, while excluding any flares of PCM proteins that had detached from the main body of the centrosomes (illustrated by the red boundary in Figure 2.3B). This allowed me to calculate the “area”, the sum of pixel intensities within that area (Figure 2.3C), the number of flares (illustrated by yellow boundaries in Figure 2.3D) for each centrosome at each timepoint. Single time snapshots from living embryos were also analysed similarly. The distance between the centroid of each centrosome in single time dual channel snapshots were calculated to show the displacement between phase-separated blobs and centriole. Two centrosomes with the shortest inter-centrosomal distance and within a preset distance threshold were annotated as a pair. The brighter centrosome in a pair was annotated as “Old Mother” while the dimmer one was annotated as “New Mother”. Python scripts for this step are listed on index 2 of Table 2.6.

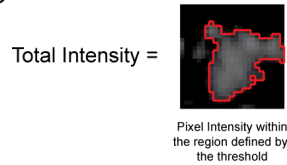
A



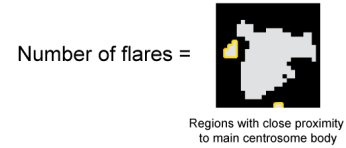
B



C



D

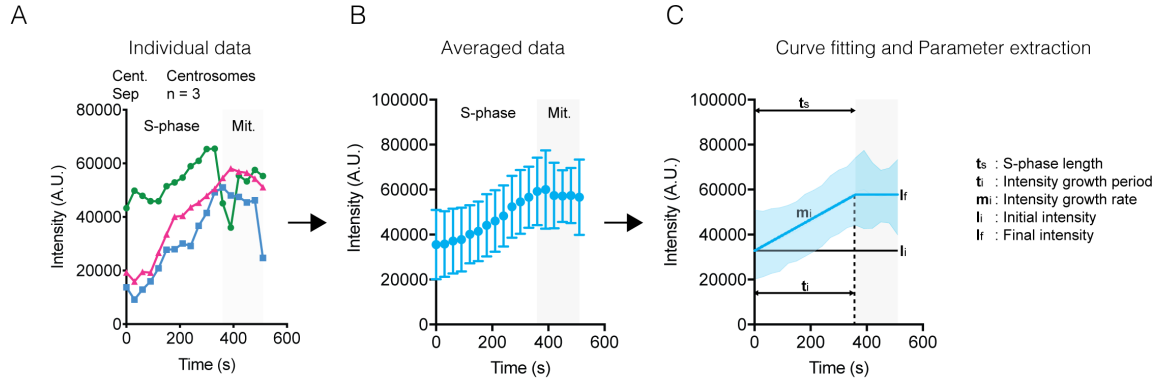


**Figure 2.3 Feature extraction of centrosomes**

(A) Micrographs show successive images of 4 centrosomes (here visualized with GFP-Cnn) tracked continuously over nuclear cycles 11 – 13. I wrote a custom Python script to extract these images so that I could manually inspect all Tracks. Period of S-phase and mitosis are indicated above the Tracks. (B) Micrograph illustrates the output of a custom Python script I wrote to segment individual centrosomes (red boundary) and then calculate their areas. (C) Micrograph illustrates how the area calculated in B was then used to sum all pixel intensities within the area to calculate a "total intensity", representing the "size" of the centrosome (i.e. the total amount of GFP-Cnn at the centrosome at this particular timepoint). (D) Micrograph illustrates how "flares" of GFP-Cnn are present to the left and bottom of the centrosome but are not included, as they are not contiguous with the rest of the centrosome.

#### 2.4.4 Quantification and Curve Fitting for centrosome dynamics

Total intensities and areas of centrosomes were retrieved and averaged for each frame to obtain a "growth curve" for each embryo (illustrated using GFP-Cnn as an example in Figure 2.4A, B). Growth curves were approximated with some pre-defined regression models depending on proteins (Table 2.5) (using Cnn as an example in Figure 2.4C). Multiple growth parameters were extracted from the fitted curves to provide descriptions of behaviours of different proteins. These data were stored as a CSV file in the dataset. The curve fitting and parameter extraction were performed by manual Python script co-written with Felix Zhou. Python scripts for this step are listed on index 3 of Table 2.6.



**Figure 2.4 Quantification of Cnn growth dynamics at cycle 12**

(A) Graph shows the intensity of three centrosomes measured over time during nuclear cycle 12. (Cent. Sep., Time at which centrosome separation was obvious. Mit., Mitosis identified by nuclear envelope breakdown described in method details) Note that the start of S-phase is defined when centrosome separation is obvious. (B) Average intensity of the same centrosomes as described in (A). (C) Regression analysis on intensity over time. (described in main text and method details) Intensity growth parameters extracted from the analysis.

### 2.4.5 Radial Profile Analysis

Some time-series images with proteins that did not exhibit flaring behaviour were studied by their radial distribution around centriole (Conduit et al., 2014b). Data were processed, tracked, and stored with procedures mentioned previously. A certain-sized bound box was cut around each centrosome. The centre of the centrosome was adjusted to the pixel with the highest intensity. The radial profile of a centrosome was obtained by summing and binning all pixel intensities according to their pixel distance away from the centre. The profile of individual centrosome was obtained to extract the pixel intensity at 0-, 3- and 5-pixel away from the centre. The recovery dynamics of the total intensity and radial profiles were regressed using a [one phase decay equation](#) to obtain  $t_{1/2}$  supplied by Prism 9 (Table 2.5). I also calculated the initial recovery rate by differentiating the recovery

dynamics and obtaining the slope of the first timepoint after FRAP. Python scripts for this step are listed on index 2 of Table 2.6.

Table 2.5 Equations used in this thesis

| Manual model                                                                                                                                          |                                                                                                    |
|-------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------|
| For the analysis of dynamics of total intensity of Spd-2, Polo, Aurora A, Grip71, and Grip75, and Grip128                                             |                                                                                                    |
| Equation:                                                                                                                                             | <i>There is no equation to this model</i>                                                          |
| Initial:                                                                                                                                              | Intensity at first frame                                                                           |
| Final:                                                                                                                                                | Maximum intensity                                                                                  |
| Peak time:                                                                                                                                            | Time at peak intensity                                                                             |
| Increase rate:                                                                                                                                        | $(Final - Initial) / Peak\ Time$                                                                   |
| Linear                                                                                                                                                |                                                                                                    |
| For 1) the analysis of the dynamics of dynamics of total intensity of Cnn in cycle 11; 2) the analysis of the slope in dosage-response curve of Spd-2 |                                                                                                    |
| Equation:                                                                                                                                             | $Y = m * X + c$                                                                                    |
| Initial:                                                                                                                                              | $c$                                                                                                |
| Final:                                                                                                                                                | $m * X_{last\_frame} + c$                                                                          |
| Peak time:                                                                                                                                            | $X_{last\_frame}$                                                                                  |
| Increase rate:                                                                                                                                        | $m$                                                                                                |
| Linear increase plateau                                                                                                                               |                                                                                                    |
| For the analysis of dynamics of total intensity of Cnn in cycle 12                                                                                    |                                                                                                    |
| Equation:                                                                                                                                             | $Y = m * X + c \text{ when } X < X_0$ $Y = b \text{ when } X > X_0$ $\text{where } X_0 > 0, b > 0$ |
| Initial:                                                                                                                                              | $c$                                                                                                |
| Final:                                                                                                                                                | $b$                                                                                                |
| Peak time:                                                                                                                                            | $X_0$                                                                                              |
| Increase rate:                                                                                                                                        | $m$                                                                                                |

| Linear increase-decrease                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                |
|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <p>For the analysis of dynamics of total intensity of Cnn in cycle 13, TACC, MSPS, <math>\gamma</math>-tubulin, and area of Cnn</p> <p><i>Equation:</i></p> $Y = m_0 * X + c \text{ when } X < X_0$ $Y = m_1 * X + c \text{ when } X > X_0$ <p>where <math>X_0 &gt; 0, m_0 &gt; 0, m_1 &lt; 0</math></p> <p><i>Initial:</i> c</p> <p><i>Final:</i> <math>m_0 * X_0 + c</math></p> <p><i>Peak time:</i> <math>X_0</math></p> <p><i>Increase rate:</i> <math>m_0</math></p> <p><i>Decrease rate:</i> <math>m_1</math></p> |
| One phase decay                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         |
| <p>For the analysis of recovery profile in FRPA experiments</p> <p><i>Equation:</i></p> $Y = (Y_0 - \text{Plateau}) * \exp(-K * X) + \text{Plateau}$ <p><math>Y_0</math>: Y value when <math>X = 0</math></p> <p><i>Plateau:</i> Y value when <math>X \rightarrow \infty</math></p> <p><math>K</math>: Rate constant</p> <p><math>t_{1/2}</math>: Derived parameter. Computed by <math>\ln(2)/K</math>.</p>                                                                                                             |
| Michaelis-Menton kinetics                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               |
| <p>For the analysis of dosage-response curve of Cnn</p> <p><i>Equation:</i></p> $V = V_{max} * [S] / (K_M + [S])$ <p><math>V</math>: Output variable, typically reaction rate</p> <p><math>V_{max}</math>: V value when <math>[S] \rightarrow \infty</math></p> <p><math>K_M</math>: Michaelis constant</p> <p><math>[S]</math>: Input variable, typically concentration</p>                                                                                                                                            |

#### 2.4.6 Statistical analysis

The details of statistical tests, sample size, and definition of centre and dispersion were included in relevant figure legends. Python scripts for this step are listed on index 4 and 5 of Table 2.6.

Table 2.6 Python programs used in this thesis

|   | Program                                          | Purpose                                                                                                                                                                                                                                                                                                                                                                               |
|---|--------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 1 | <a href="#">io.video_io.py</a>                   | APIs for pre-processing videos ( <a href="#">io.utils.py</a> ) and raw data managements                                                                                                                                                                                                                                                                                               |
| 2 | <a href="#">data_processing.dynamics.py</a>      | APIs for detection ( <a href="#">data_processing.detection.py</a> ), tracking ( <a href="#">data_processing.detection.py</a> ), feature extraction ( <a href="#">data_processing.utils.py</a> ), radial profiling ( <a href="#">data_processing.radial.py</a> ), pedigree analysis ( <a href="#">data_processing.pedigree.py</a> ) and general data management of centrosome dynamics |
| 3 | <a href="#">regression.batch.py</a>              | APIs for curve fitting ( <a href="#">regression.models.py</a> and <a href="#">regression.utils.py</a> ) and parameter extraction                                                                                                                                                                                                                                                      |
| 4 | <a href="#">statistics.hypothesis_testing.py</a> | APIs for testing normality, equality of standard deviation and choosing statistical tests for different data                                                                                                                                                                                                                                                                          |
| 5 | <a href="#">statistics.correlation.py</a>        | APIs for testing correlation and significance                                                                                                                                                                                                                                                                                                                                         |

### 3. A centrosomal Polo/PLK1 oscillation regulates mitotic centrosome size in early *Drosophila* embryos

#### 3.1 Introduction

Centrosomes are important organisers of the cell that are formed when mother centrioles recruit a matrix of pericentriolar material (PCM) around themselves (Conduit et al., 2015). The PCM is thought to contain several hundred proteins, including many proteins that help nucleate and organise microtubules (MTs), allowing the centrosome to function as a dominant MT organising centre (MTOC) in many eukaryotic cells (Arquint et al., 2014; Conduit et al., 2015). Several signalling molecules, cell cycle regulators, and checkpoint proteins are also recruited to the PCM, and the centrosome is thought to function as an important coordination centre in the cell—a place where proteins meet and interact at a relatively high concentration, and where decisions are made (Arquint et al., 2014).

In interphase, many cells organise relatively little PCM, but there is a dramatic increase in PCM recruitment as cells prepare to enter mitosis—a process termed centrosome maturation (Palazzo et al., 1999). It is important that the two centrosomes in the cell grow to the same size, as centrosome size asymmetry can lead to imbalances in spindle forces and so to inefficient chromosome segregation (Meraldi, 2016). How mitotic centrosome growth is regulated to achieve this size homeostasis is largely unknown, but it is clear that the centrioles play an important part in setting centrosome size. Centrioles are essential to initiate efficient mitotic PCM assembly (Basto et al., 2006; Bobinnec et al., 1998; Cabral et al., 2019; Conduit et al., 2010), and in worm embryos, the size of the

centriole is correlated with the ultimate size of the mitotic PCM (Kirkham et al., 2003). Moreover, it has recently been shown that centrioles are continuously required to recruit PCM throughout the centrosome growth period, indicating that the mitotic PCM itself cannot catalyse its own growth without the centrioles (Cabral et al., 2019).

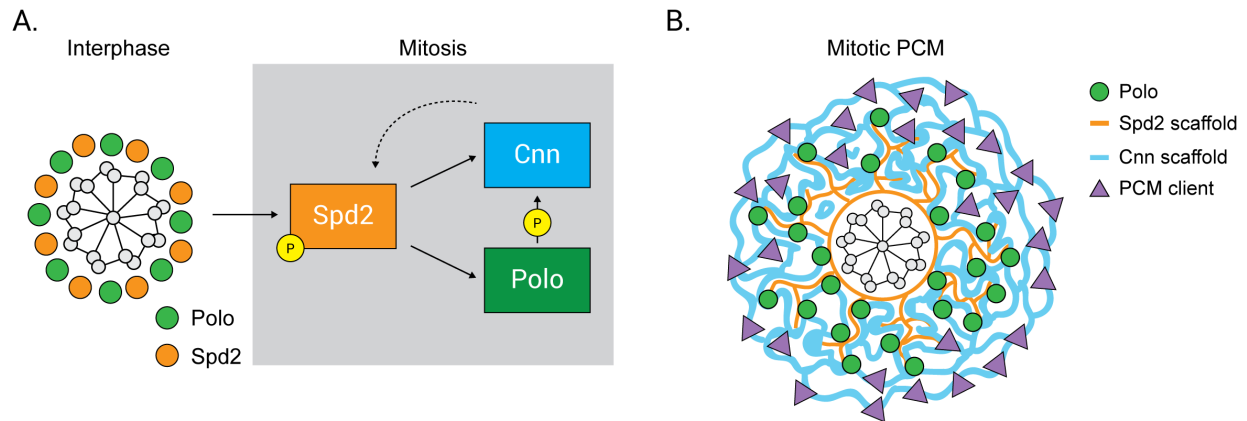
The mitotic protein kinase Polo/PLK1 plays a particularly important part in centrosome maturation (Conduit et al., 2014a; Lane & Nigg, 1996; Novak et al., 2016; Woodruff et al., 2015), and it is recruited to centrosomes by scaffolding proteins that are phosphorylated on S-S/T(P) motifs to create Polo-box-binding sites (Elia et al., 2003). In vertebrates, PLK1 is recruited to centrosomes by PBD-binding motifs in Cep192 (Joukov et al., 2010, 2014; Meng et al., 2015), which is required for centrosome maturation (Gomez-Ferreria et al., 2007; Zhu et al., 2008). In flies and worms, Spd-2/SPD-2 (the fly/worm homologue of Cep192) is concentrated at centrioles and centrosomes and its phosphorylation also helps recruit Polo/PLK1 to the mitotic PCM where it can phosphorylate Cnn/SPD-5 (in flies/worms) to initiate the assembly of a PCM-scaffold (Alvarez-Rodrigo et al., 2019; Decker et al., 2011).

In fly embryos, Spd-2, Polo and Cnn have been proposed to form a positive feedback loop that drives the expansion of the mitotic PCM-scaffold around the mother centriole (Figure 3.1) (Alvarez-Rodrigo et al., 2019; Conduit et al., 2014a). This Spd-2/Polo/Cnn scaffold initiates the recruitment of the many PCM-client proteins, such as  $\gamma$ -tubulin and the  $\gamma$ -Tubulin ring complex ( $\gamma$ -TuRC) (Conduit et al., 2014a). It has previously been shown that in early *C. elegans* embryos centrosome size is set by a limiting pool of SPD-2



(Decker et al., 2011). The total embryonic pool of SPD-2 appears to remain constant throughout the early cell division cycles, so centrosomes halve in size after each round of cell division as the limiting pool of SPD-2 is divided equally amongst the exponentially increasing number of centrosomes. This concept of a “limiting pool” of a key organelle building block is potentially a powerful mechanism with which to regulate organelle size (Goehring & Hyman, 2012). However, it was unclear if this mechanism might be more generally applicable to centrosomes in other cell types.

In this Chapter I developed methods that allowed me to measure the centrosomal levels of various fluorescently-tagged proteins as they are recruited to the centrosomes in preparation for mitosis in living syncytial *Drosophila* embryos. My findings revealed some complicated and unexpected behaviours of the PCM scaffold and client proteins, leading me to propose a very different model of how centrosome size is regulated in these early *Drosophila* embryos.



**Figure 3.1** Schematics show the positive feedback between Spd-2, Polo, and Cnn, and the general structure of PCM

**(A)** Centrioles recruit Spd-2 and Polo in interphase. As cells prepare to enter mitosis, Spd-2 is phosphorylated, allowing it to form a scaffold that can recruit PCM proteins and that fluxes away from the centriole. The Spd-2 scaffold recruits both Polo and Cnn (solid arrows), and Polo phosphorylates Cnn to form an additional Cnn scaffold that can also recruit PCM proteins but that also stabilises the Spd-2 scaffold (indicated by the dotted arrow). These interactions form a positive feedback loop that appears to drive the expansion of the mitotic PCM around the mother centriole (Alvarez-Rodrigo et al., 2019). **(B)** The spatial organisation of the Spd-2/Polo/Cnn scaffold during mitosis. The scaffold can recruit PCM client proteins such as  $\gamma$ -tubulin, MSPS, and TACC (Conduit et al., 2014b), but only the centriole can generate the Spd-2 scaffold and only the Spd-2 scaffold can recruit Polo.

## 3.2 Results

### 3.2.1 Developing a pipeline to measure centrosome growth

At the start of my studies, methods had already been developed in the Raff laboratory to use the Fiji Plugin TrackMate to track individual centriole pairs (labelled with various fluorescently-tagged centriole proteins) as the daughter centrioles grow during S-phase (Aydogan et al., 2018, 2020). From these datasets one can measure the fluorescence intensity of each centriole pair at successive timepoints, and so calculate an average centriole growth curve for each embryo (usually averaging ~30-100 centriole pairs depending on the nuclear cycle). From this data it was often possible to fit a mathematical

regression to describe the protein's behaviour. For example, the fluorescent intensity of the centriole cartwheel protein Sas-6-GFP increases linearly at the start of S-phase (defined as the time of "centrosome separation" (CS)—the time at which the two mother centrioles have separated sufficiently to first be identified as two individual centrioles) and then reaches a plateau a few minutes before the embryos enter mitosis, as judged by Nuclear Envelope Breakdown (NEB). From these mathematical regressions one can derive certain parameters from each embryo, such as the initial and final fluorescence intensity of the centrioles (indicating their initial and final size) and their growth rate and growth period, and so calculate an average value for each of these parameters that describes the kinetics of Sas-6 growth across multiple embryos (Aydogan et al., 2018).

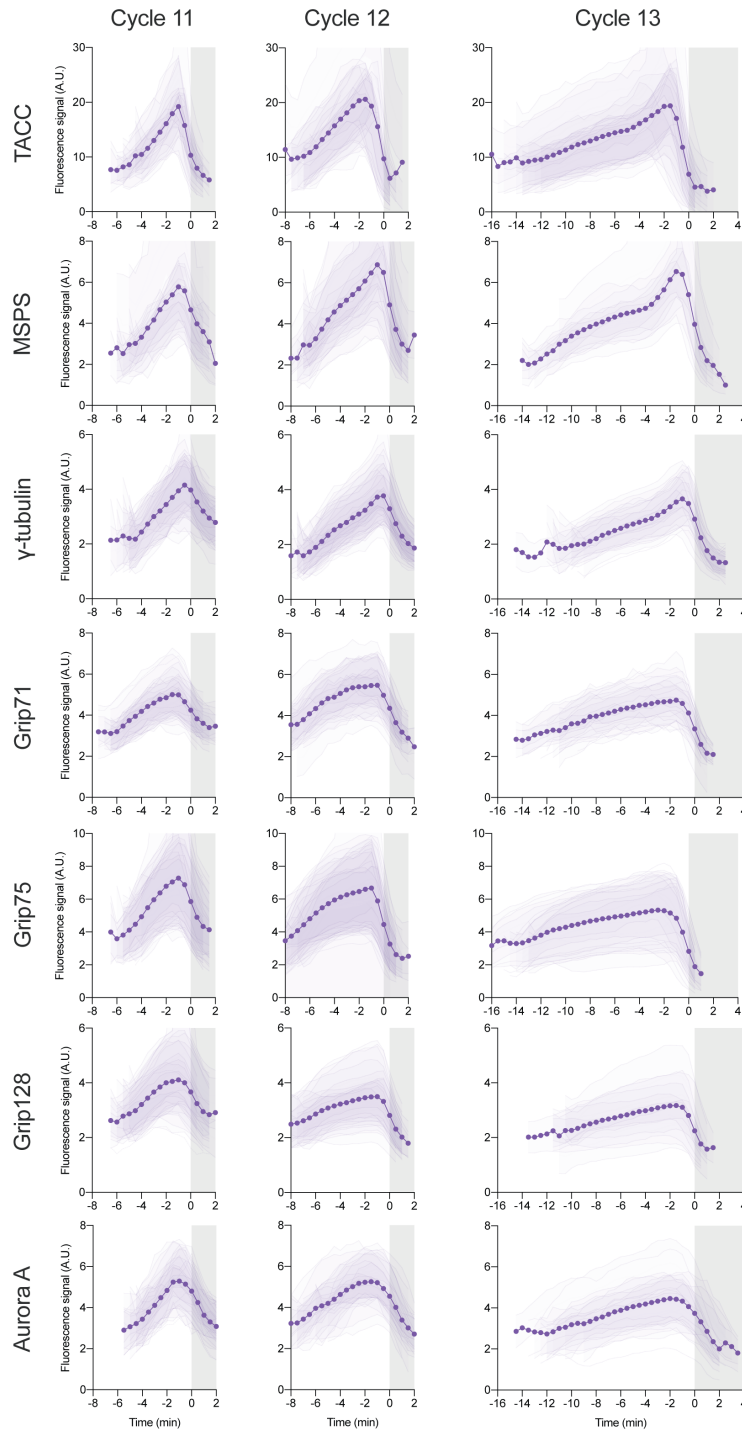
I wanted to apply these methods to describe the behaviour of centrosomes as they grow during S-phase in preparation for mitosis. This is a more complicated problem than tracking centriole growth because centrioles are tiny (~150nm long and wide in fly embryos), and so behave as diffraction-limited "dots" on standard confocal microscope systems (meaning that in practice they all have an identical circular shape). Centrosome proteins incorporate into a scaffold that surrounds the centrioles, and so they are spread over a larger area (~200-1000nm, depending on the protein). They are therefore resolvable on standard microscope systems, and so the shape of each centrosome in an embryo will be different. In addition, some centrosome proteins, such as Cnn and TACC, exhibit extensive "flaring", with large chunks of the proteins breaking away from the main body of the centrosome at the periphery (M. J. Lee et al., 2001; Megraw et al., 1999, 2002). I therefore developed a semi-automated pipeline to analyse centrosome behaviour,

using TrackMate to identify and track the centrosomes in movies of embryos expressing fluorescently-tagged centrosome proteins, and then custom Python codes to segment the centrosomes and calculate their area and fluorescent intensity (Table 2.6). In this Chapter, Importantly, I did not include the discussion of centrosome size (measured by area of centrosome) and protein density (measured by fluorescent intensity divided by area) because they are physically more sophisticated that may involve the regulations of molecular crowding and protein recruitments for example Cnn compacts during mid-S-phase presumably due to intrinsic elasticity between Cnn molecules (data not shown) while it is recruited continuously throughout S-phase. Comparatively, centrosome size that is represented by the fluorescent intensity (a measure of protein abundance) can be more intuitively understood and described by kinetic models using ODEs. This pipeline that allowed me to extract these parameters is summarised **Section 2.4**.

### 3.2.2 The centrosome growth rate and growth period are inversely regulated so that centrosomes grow to a similar size at nuclear cycles 11, 12 and 13

It has previously been shown that in early *C. elegans* embryos centrosome size is set by a limiting pool of the key centrosome assembly protein SPD-2 (Decker et al., 2011). The total embryonic pool of SPD-2 appears to be constant through early development, so centrosomes halve in size after each round of cell division as the limiting pool of SPD-2 is divided amongst the exponentially increasing number of centrosomes. I wanted to test whether centrosomes in early syncytial *Drosophila* embryos behaved in a similar manner, so I first measured centrosome growth by quantifying the centrosomal fluorescence levels of seven PCM-client proteins (GFP-TACC, MSPS-GFP,  $\gamma$ -tubulin-GFP, Grip71-GFP,

Grip75-GFP, Grip128-GFP, and Aurora A-GFP) in living embryos during nuclear cycles 11-13 (Figure 3.2). The centrosomal levels of all seven proteins oscillated during each cell cycle, with their levels increasing through most of S-phase (when the centrosomes in these embryos are growing in preparation for mitosis), and then rapidly decreasing just before the embryos entered mitosis—as judged by NEB. Thus, surprisingly, the centrosomes in these early embryos reach their maximum size (defined by the *amount* of protein recruited to the centrosome) just prior to NEB, and the centrosomes then decrease in size during mitosis.



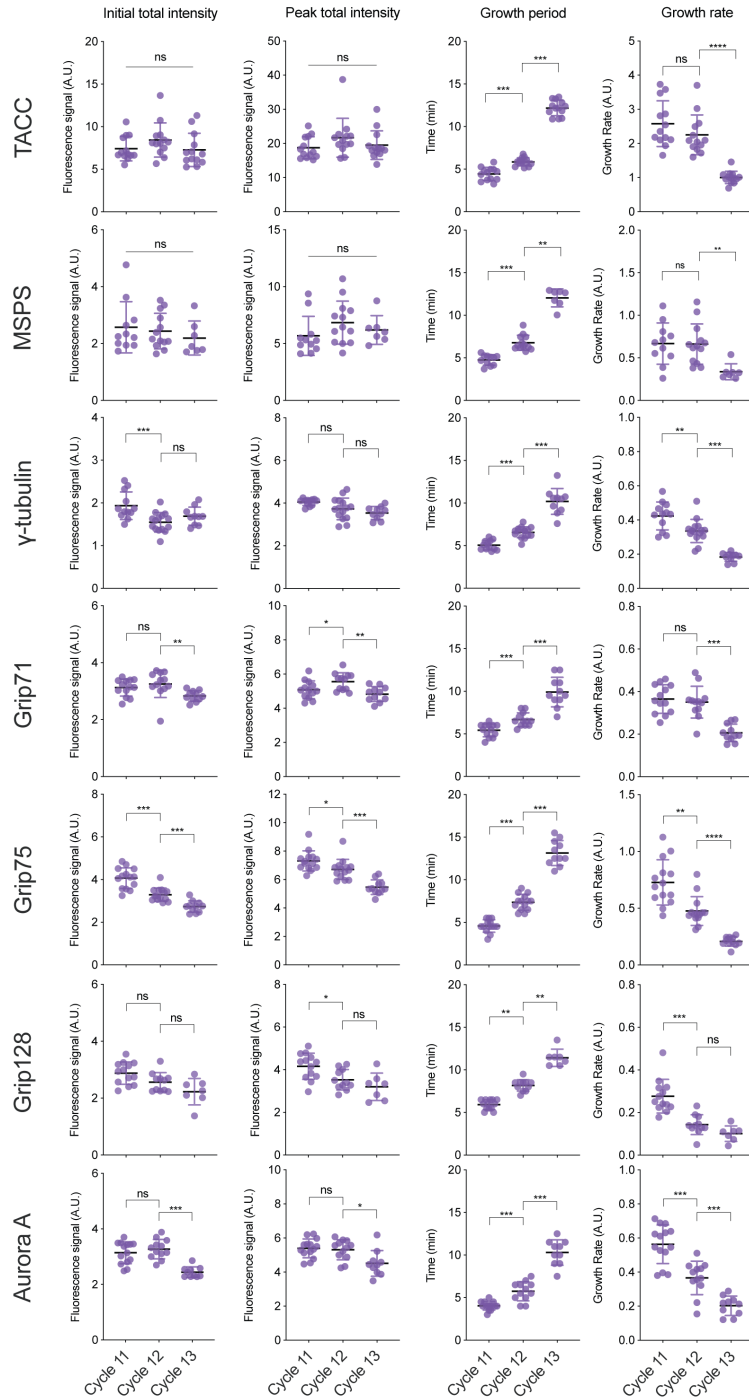
**Figure 3.2 Centrosomal recruitment dynamics of several PCM client proteins.**

Graphs show how the average centrosomal fluorescent intensity – dark lines (Solid purple line represent the average of embryos in each cycle; opaque purple shades represent SD for each individual embryo) – changes over time during nuclear cycles 11, 12, and 13 for several PCM client proteins (as indicated). Because the length of the nuclear cycle varies from embryo to embryo, all individual embryo tracks were aligned to NEB ( $t=0$ ). The white parts of the graphs represent S-phase, and the gray parts represent mitosis. Note how the fluorescent intensity of all proteins peaks and then starts to decline before NEB.

Each PCM client protein exhibited a distinct recruitment profile, but they broadly fell into two classes: (1) TACC, MSPS and  $\gamma$ -tubulin grew at a relatively constant rate during most of S-phase, but the growth rate seemed to increase noticeably towards the end of S-phase (this was most clearly seen during cycle 13, when the S-phase period is the most

extended) before centrosomal levels sharply declined prior to NEB; (2) Grip71, Grip75, Grip128, and Aurora A grew more constantly during S-phase, with no obvious increase in growth rate late in S-phase, and a more gradual transition from growth to shrinkage prior to NEB. Note that Grip71, Grip75, and Grip128 are not considered “core” components of the  $\gamma$ -TuRC (Liu et al., 2020), so it is perhaps not too surprising that they appear to exhibit slightly distinct recruitment kinetics to  $\gamma$ -tubulin.

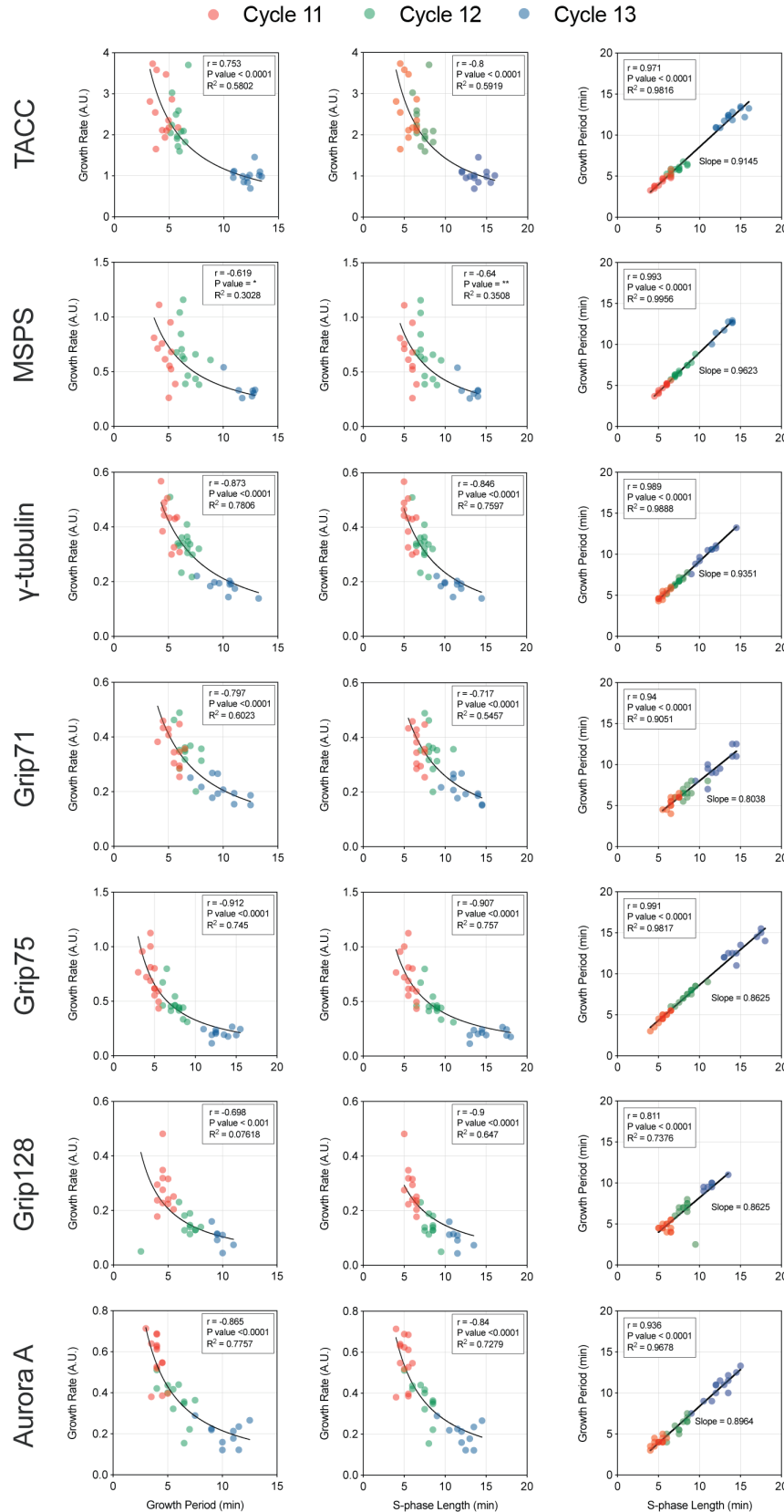
For ease of comparison I decided to fit a simple mathematical regression to each embryo dataset, plotting a straight line between the minimum and maximum fluorescence intensity timepoints (see **Section 2.6.4**), allowing me to calculate an average initial and final centrosome size and an average rate and period of centrosome growth for each PCM Client protein at each successive division cycle. In contrast to the situation in early *C. elegans* embryos, the centrosomes in these embryos were initially of similar sizes at the start of each nuclear cycle, and then grew to approximately the same maximum size by the end of each nuclear cycle (Figure 3.3). While four of the client proteins grew to a slightly smaller size at successive cycles, the size decrease at each cycle was often not statistically significant, and, even over two cycles, was very modest: ~13% ( $\gamma$ -tubulin), ~26% (Grip75), ~24% (Grip128) and ~16% (Aurora A), and nothing like the ~75% decrease that was observed over two cycles in *C. elegans* embryos (Decker et al., 2011).



**Figure 3.3 Growth parameters of PCM client proteins.**

Bar charts show the initial fluorescent intensity, the peak fluorescent intensity, the growth period, and the growth rate of several PCM client proteins (as indicated) during nuclear cycles 11, 12, and 13. Data were extracted from mathematical regression fitted to individual growth curves from embryos in Figure 3.2 and using the equations in Table 2.5. Comparison across nuclear cycles 11, 12, and 13 was performed using either an ordinary one-way ANOVA (Gaussian-distributed and variance-homogeneous), a one-way Welch ANOVA (Gaussian-distributed and variance-unequal), or a Kruskal-Wallis's test (non-Gaussian-distributed). If the comparison was significant, multiple testing was performed. Multiple hypothesis testing P-values were calculated by either Tukey-Kramer's test (Gaussian-distributed and variance-homogeneous), Games-Howell's test (Gaussian-distributed and variance-unequal), or Mann-Whitney's U test (non-Gaussian-distributed) (\*:  $P < 0.05$ , \*\*:  $P < 0.01$ , \*\*\*:  $P < 0.001$ , \*\*\*\*:  $P < 0.0001$ , ns: not significant). Gaussian distribution was tested using D'Agnostino and Pearson's test. The homogeneity of variance was tested using Levene W test.





**Figure 3.4 The growth rate and growth period of the PCM clients are correlated with each other and with S-phase duration**

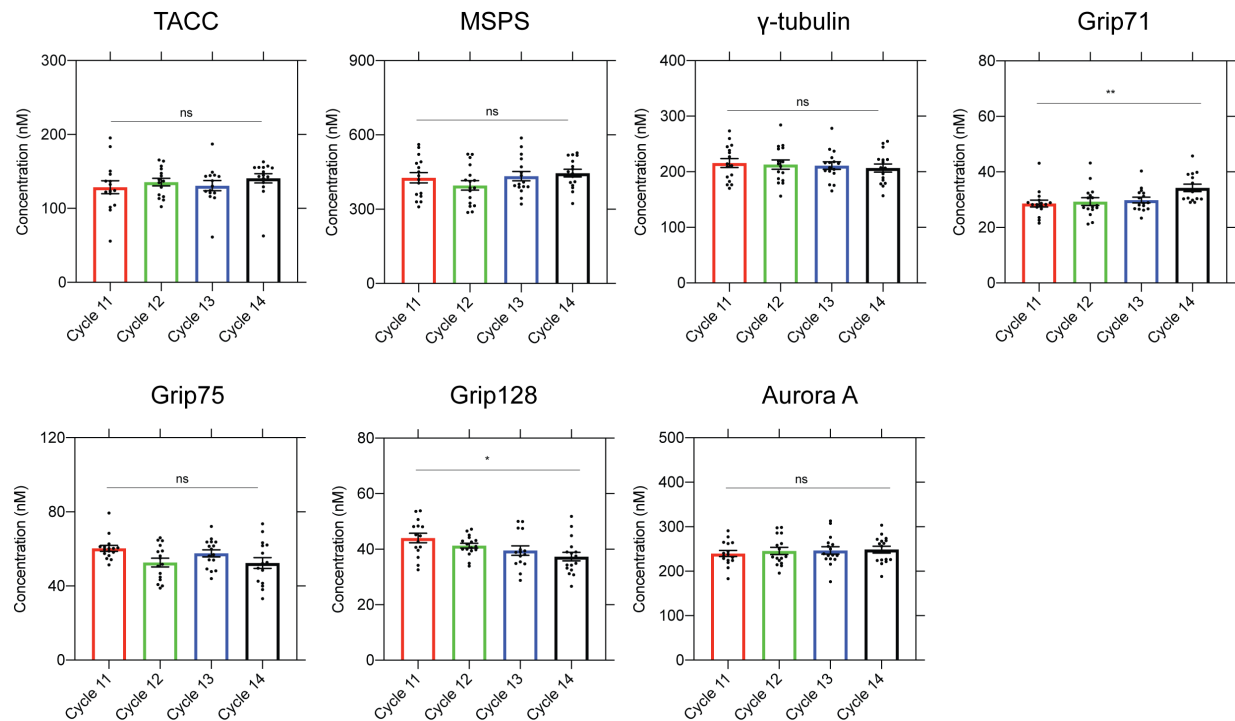
Scatter plots show the correlation between the centrosome growth rate and period (left), growth rate and S-phase length (middle), and growth period and S-phase length (right) for PCM client proteins (as indicated). Lines indicate mathematically regressed fits for inverse-linear (left and middle) and linear (right) correlations. Goodness of fit is indicated by R<sup>2</sup> value. The strength of correlation (r-value) and the statistical significance (P-value) of the correlation were calculated by either Pearson test (bivariate Gaussian-distributed) or Spearman test (bivariate non-Gaussian distributed data). Bivariate Gaussian distribution was calculated by Henze-Zirkler test.

I conclude that in these early fly embryos the centrosomes grow to an approximately similar size during cycles 11, 12 and 13 — even as their numbers double at each successive cycle. Interestingly, I noticed that this size homeostasis seemed to be enforced by an inverse-linear correlation between the centrosome growth rate and growth period for each protein ( $r = -0.61$ - $-0.86$ ;  $p < 0.005$ - $0.0001$ ) (Figure 3.4, left column of graphs), with the growth *period increasing* and the growth *rate decreasing* at successive cycles so that the centrosomes ultimately grew to a similar size (Figure 3.3).

### 3.2.3 The cytoplasmic concentration of several centrosome client proteins remains relatively constant at successive nuclear cycles

I reasoned that this apparent homeostatic relationship between the centrosome growth rate and period might be a passive consequence of the biology of early embryo development. The increase in the growth period at successive cycles could simply be due to the general lengthening of S-phase in the embryo (Farrell & O'Farrell, 2014), while the decrease in the centrosome growth rate could be due to a decrease in the cytosolic concentration of the PCM client proteins—as the exponentially increasing number of centrosomes would be expected to sequester exponentially increasing amounts of centrosomal proteins at each successive cycle. To test this possibility, Thomas Steinacker in the lab used Fluorescence Correlation Spectroscopy (FCS) to measure the cytosolic concentration of all the PCM clients that I had tested. Perhaps surprisingly, the concentration of the clients was essentially constant at the start of cycles 11, 12 and 13 (Figure 3.5, supplied by Thomas Steinacker). This leads to two important conclusions: (1) the slowing of centrosome growth at successive cycles is not a passive process driven

simply by the decrease in the cytosolic concentration of PCM components; (2) There may be specific mechanisms in the embryo that ensure that the cytosolic concentration of PCM clients is kept constant at successive cycles.



**Figure 3.5 The cytosolic concentration of several PCM client proteins remain constant across nuclear cycles 11-14.**

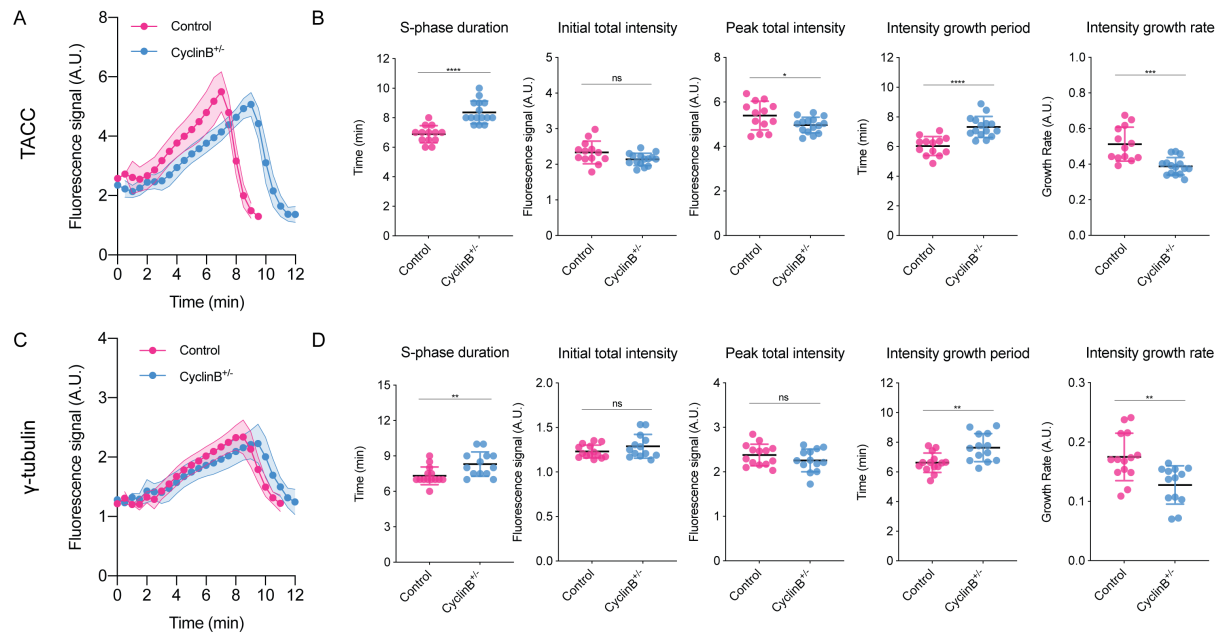
Bar charts compare the cytosolic concentration of several PCM client proteins (as indicated) measured by FCS at the start of nuclear cycles 11 - 14. Each dot represents the average reading from a single embryo. Statistical significance was assessed using ordinary one-way ANOVA in Prism 9. Experiments were performed and graphs were prepared by Thomas Steinacker.

### 3.2.4 The core Cdk/Cyclin Oscillator (CCO) strongly influences the centrosome growth rate and growth period

I wondered whether the CCO, which drives the rapid nuclear divisions in these early embryos (Farrell & O'Farrell, 2014), might influence the centrosome growth period and growth rate to ensure their inverse-linear relationship. As the CCO determines S-phase

length (Farrell & O'Farrell, 2014), I examined whether this parameter correlated with the centrosome growth rate and period. For all of the PCM clients I tested, there was a very strong linear correlation between S-phase length and the centrosome growth period ( $r=0.94-0.99$ ;  $p<0.0001$ ), and a slightly less strong, but still very significant, inverse-linear correlation between S-phase length and the centrosome growth rate ( $r= -0.64-0.88$ ;  $p=0.01-0.0001$ ) (Figure 3.4). Thus, the CCO is likely to directly influence the growth period, and to perhaps more indirectly influence the growth rate, of these PCM clients.

To more directly test this possibility, I examined embryos in which I had halved the genetic dose of *Cyclin B*, which slightly extends S-phase length (presumably because it takes the embryos longer to reach the critical level of Cyclin B/Cdk1 activity that triggers NEB). I examined the growth kinetics of two PCM clients, TACC and  $\gamma$ -tubulin (Figure 3.6A, C). As anticipated by our findings above, halving the dose of *Cyclin B* extended the centrosome growth period, but slowed the centrosome growth rate, so that the centrosomes grew to nearly the same size as in control embryos (Figure 3.6B, D). Importantly, FCS measurements by Thomas Steinacker revealed that the cytoplasmic concentration of both PCM clients was not altered by halving the dose of *Cyclin B* (data not shown). Together, these findings indicate that the CCO influences both the centrosome growth rate and period to help ensure an inverse relationship between these parameters that, in turn, helps ensure that mitotic centrosomes grow to a consistent size.



**Figure 3.6 The growth dynamics of TACC and  $\gamma$ -tubulin are influenced by the CCO**

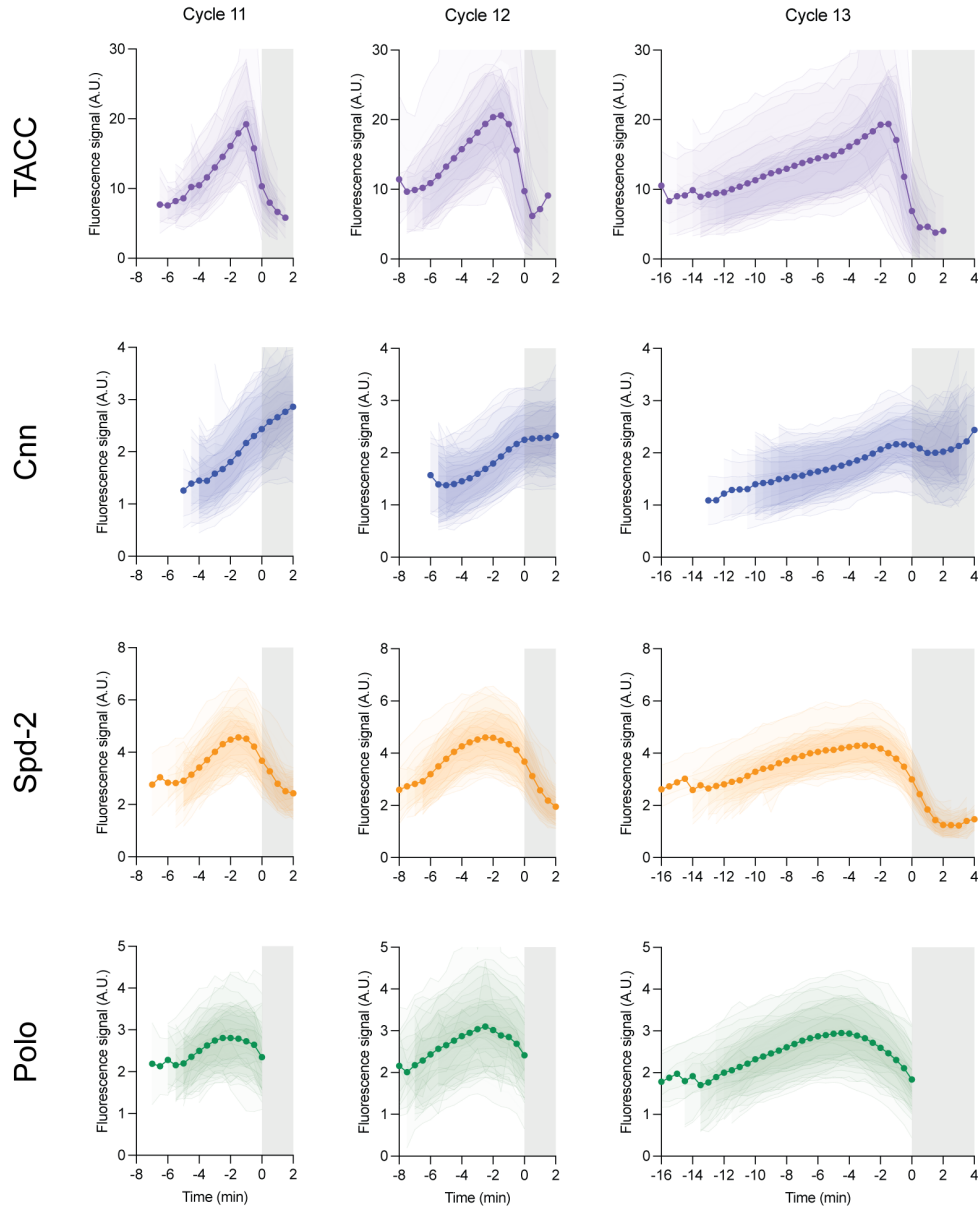
(A, C) Graphs show the average ( $\pm$ SD) centrosomal growth dynamics of (A) GFP-TACC and (C)  $\gamma$ -tubulin-GFP in either wildtype (Control, pink line) or *Cyclin B* half-dose embryos (laid by *CyclinB*<sup>+/-</sup> females, blue line). The average curve from each group was calculated from > 12 embryos. (B, D) Bar charts compare various growth parameters (as indicated) for WT (pink) and Cyclin B half-doses (blue) embryos: these data are derived from the growth curves shown in (A, C). Statistical significance was assessed using an unpaired t-test (\*:  $P < 0.05$ , \*\*:  $P < 0.01$ , \*\*\*:  $P < 0.001$ , \*\*\*\*:  $P < 0.0001$ , ns: not significant).

### 3.2.5 PCM-scaffold proteins exhibit distinct dynamics to the PCM-client proteins

I wondered whether the growth kinetics of the PCM clients might simply reflect the growth kinetics of the underlying PCM scaffold that recruits them. In flies PCM-clients are recruited by a scaffold formed by Spd-2, Polo and Cnn (Conduit et al., 2014a; Conduit et al., 2014b), so I examined the centrosomal dynamics of these proteins during cycles 11-13 (Figure 3.7).

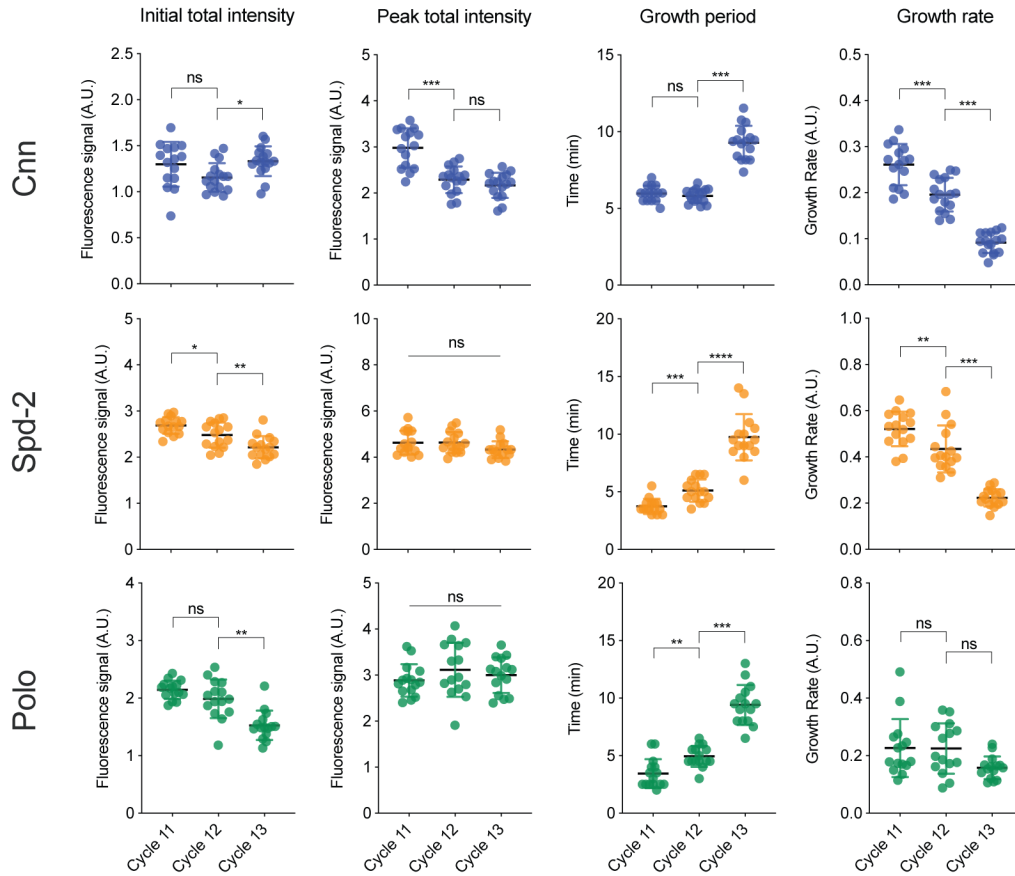
Cnn exhibited growth kinetics that were quite distinct from the PCM-clients. Like the clients, the centrosomal levels of Cnn increased through most of S-phase, but whereas

the centrosomal-levels of the clients peaked and then rapidly started to decline shortly before mitosis, this was not the case for Cnn. During cycle 11, centrosomal Cnn levels often continued to increase even after the embryos had entered mitosis, while in cycles 12 and 13 centrosomal Cnn levels peaked and then largely plateaued at about the time (cycle 12), or a few minutes before (cycle 13), the embryos entered mitosis (Figure 3.7, Figure 3.8). As a result, there was little correlation between S-phase length and the Cnn growth period in cycles 11 and 12, although this correlation was restored in cycle 13 (Figure 3.9). I draw two conclusions from this data: (1) The growth of the Cnn scaffold is not as directly influenced by the CCO as the growth of the client proteins (particularly during cycles 11 and 12); (2) The amount of the PCM-client proteins recruited to centrosomes does not simply follow the amount of the underlying Cnn-scaffold.



**Figure 3.7 Recruitment dynamics of PCM scaffold proteins**

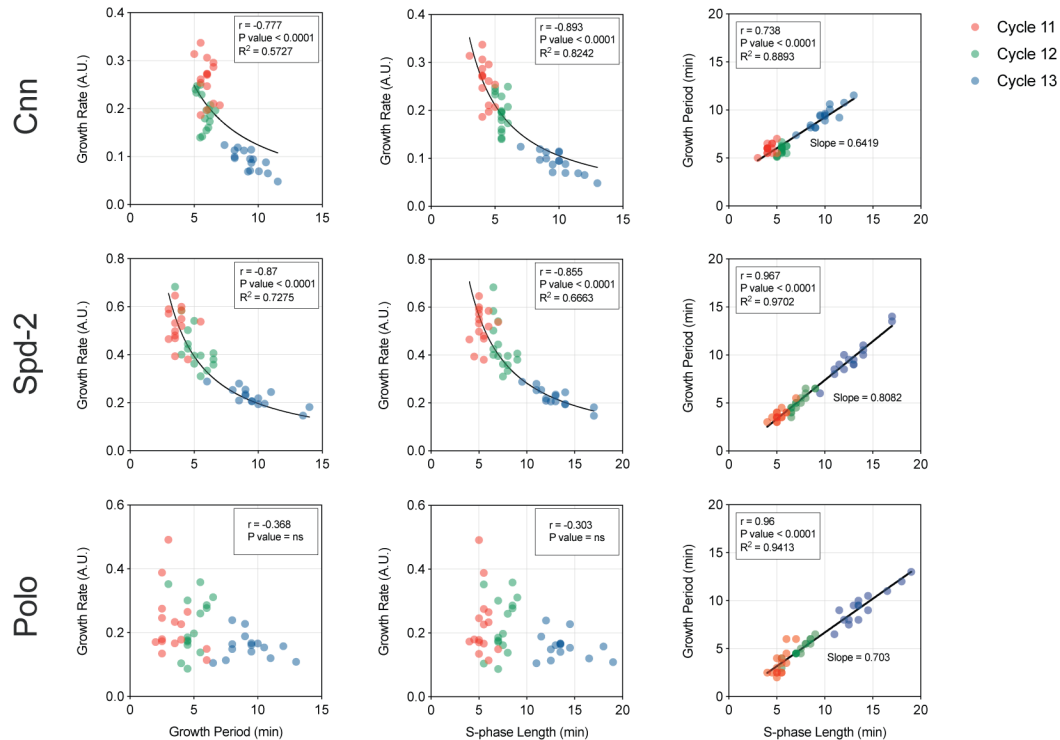
Graphs show how the average centrosomal fluorescent intensity changes over time during nuclear cycles 11, 12, and 13 for GFP-TACC (extracted from Figure 3.2 for comparison), NG-Cnn, Spd-2-GFP, and Polo-GFP (as indicated). All individual tracks were aligned at NEB ( $t=0$ ). The white parts of the graphs represent S-phase, and the gray parts represent mitosis. Note how the centrosomal levels of Cnn can continue to rise, and/or plateau, even as Spd-2 and Polo levels start to decline. Also note that I was unable to measure the centrosomal levels of Polo during mitosis, as Polo strongly localizes to kinetochores at this stage making it impossible to computationally track the centrosomes.



**Figure 3.8 Growth parameters of PCM scaffold proteins**

Bar charts show the initial fluorescent intensity, the peak fluorescent intensity, the growth period, and the growth rate of NG-Cnn, Spd-2-GFP, and Polo-GFP (as indicated) during nuclear cycles 11, 12, and 13. Data were extracted from mathematical regression fitted to individual growth curves from the embryos averaged in Figure 2.4 and using the equations shown in Table 2.5. Comparison across nuclear cycles 11, 12, and 13 was performed using either an ordinary one-way ANOVA (Gaussian-distributed and variance-homogeneous), a one-way Welch ANOVA (Gaussian-distributed and variance-unequal), or a Kruskal-Wallis's test (non-Gaussian-distributed). If the comparison was significant, multiple testing was performed. Multiple hypothesis testing P-values were calculated by either Tukey-Kramer's test (Gaussian-distributed and variance-homogeneous), Games-Howell's test (Gaussian-distributed and variance-unequal), or Mann-Whitney's U test (non-Gaussian-distributed) (\*:  $P < 0.05$ , \*\*:  $P < 0.01$ , \*\*\*:  $P < 0.001$ , \*\*\*\*:  $P < 0.0001$ , ns: not significant). Gaussian distribution was tested using D'Agostino and Pearson's test. The homogeneity of variance was tested using Levene W test.



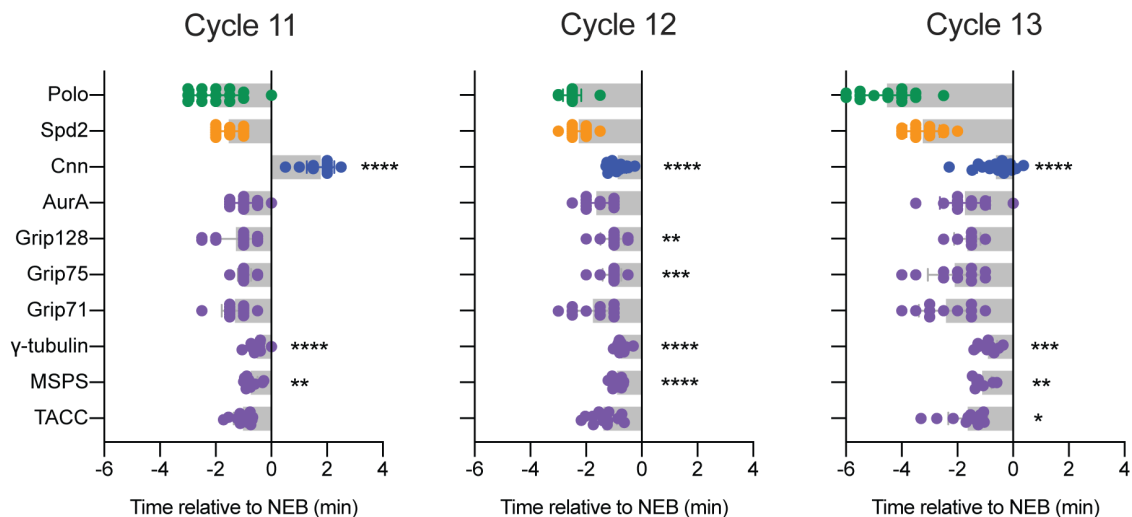


**Figure 3.9 Correlations of PCM scaffold proteins**

Scatter plots show the correlation between the centrosome growth rate and period (left), growth rate and S-phase length (middle), and growth period and S-phase length (right) for NG-Cnn, Spd-2-GFP, and Polo-GFP (as indicated). Lines indicate mathematically regressed fits for inverse-linear (left and middle) and linear (right) correlations. Goodness of fit is indicated by  $R^2$  value. The strength of correlation (r-value) and the statistical significance (P-value) of the correlation were calculated by either Pearson test (bivariate Gaussian-distributed) or Spearman test (bivariate non-Gaussian distributed data). Bivariate Gaussian distribution was calculated by Henze-Zirkler test.

Like the PCM-client proteins, the centrosomal levels of Spd-2-GFP and Polo-GFP oscillated during each nuclear cycle (Figure 3.7), with both proteins peaking prior to NEB and growing to essentially the same size at each cycle (Figure 3.8, Figure 3.9). Surprisingly, I noticed that at every cycle the Polo and Spd-2 centrosomal oscillations peaked before the Cnn scaffold and PCM client centrosomal levels peaked (Figure 3.10). I confirmed this differential timing in embryos co-expressing Spd-2-mCherry with GFP-Cnn or  $\gamma$ -tubulin-37c-GFP (Figure 3.11, Figure 3.12). Thus, perhaps surprisingly, the

centrosomal levels of the Cnn scaffold and the PCM-client proteins can still be increasing during S-phase even as the centrosomal levels of the Spd-2 and Polo scaffold decrease.



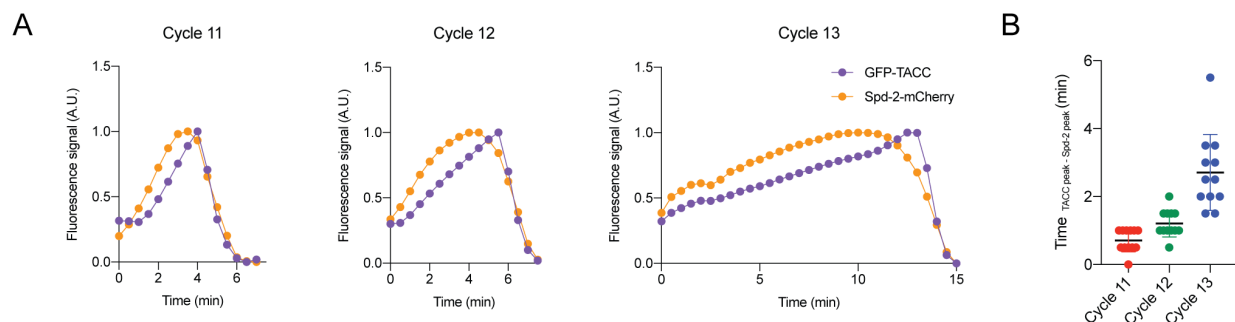
**Figure 3.10 Relative timings of the peaks of individual PCM protein recruitment compared to NEB**

Charts show the average time at which centrosomal protein levels peak relative to NEB during nuclear cycle 11, 12, and 13 for several proteins (as indicated). Data were extracted from the data shown in Figure 3.2 and Figure 3.7. On average, Polo and Spd-2 peak around 2 to 4 min before NEB, while the other proteins peak between 0 and 2 min before NEB (except for Cnn which on average peaks after NEB during cycle 11, and slightly before NEB during cycle 12 and 13). The statistical significance was computed using Kruskal-Wallis's test between any proteins comparing to Spd-2's peaks (\*:  $P < 0.05$ , \*\*:  $P < 0.01$ , \*\*\*:  $P < 0.001$ , \*\*\*\*:  $P < 0.0001$ , comparisons that were not significant are not indicated).

As Spd-2 is a major recruiter of Polo into the PCM (Alvarez-Rodrigo et al., 2019), I expected that the Polo-GFP oscillation would simply follow the Spd-2 oscillation, but the Polo-GFP oscillation actually peaked slightly before the Spd-2-GFP oscillation (Figure 3.10). I confirmed this relative timing in embryos co-expressing Spd-2-RFP and Polo-GFP (Figure 3.14). Thus, the decline in centrosomal Polo levels is not simply driven by the decline in Spd-2 levels. Moreover, whereas Spd-2 dynamics were similar to the PCM client proteins in that the growth period and rate exhibited the typical inverse-linear

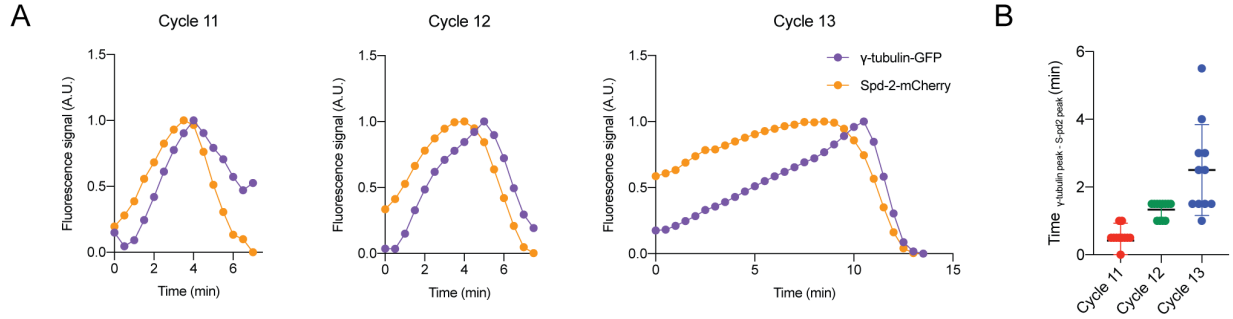
relationship to each other ( $r = -0.87$ ,  $p < 0.0001$ ) and the typical linear and inverse-linear relationship to S-phase length ( $r = 0.97$ ,  $p < 0.0001$ ;  $r = -0.86$ ,  $p < 0.0001$ ), respectively, this was not the case for Polo (Figure 3.9). Although the Polo growth period was still strongly correlated with S-phase length ( $r = 0.96$ ,  $p < 0.0001$ ), there was no significant correlation between the Polo growth rate and S-phase length ( $r = -0.303$ ,  $p = \text{n.s.}$ ); as a result, there was also no significant correlation between the Polo growth rate and period ( $r = -0.37$ ,  $p = \text{n.s.}$ ).

Taken together, these quantitative measurements reveal unexpected complexity in the assembly dynamics of the mitotic centrosomes in these embryos. Most importantly, an oscillation in centrosomal Polo levels precedes oscillations in the centrosomal levels of all the other PCM scaffold and client proteins, and the Polo oscillation appears to be regulated in a manner that is fundamentally distinct from all the other centrosomal proteins, as its growth rate is not correlated with S-phase length.



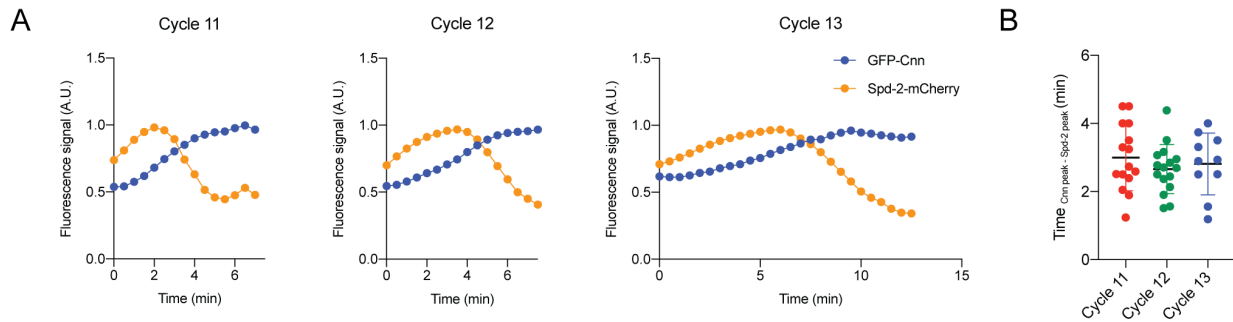
**Figure 3.11 TACC can continue to accumulate at centrosomes even as centrosomal Spd-2 levels decline**

**(A)** Graphs show how the average centrosomal fluorescent intensity changes over time during nuclear cycles 11, 12, and 13 for embryos ( $n = 12$  for each cycle) co-expressing GFP-TACC (purple) and Spd-2-mCherry (orange). The fluorescent intensity was rescaled to between 0 and 1 in each cycle. **(B)** Bar charts compare the time difference between the peak of the Spd-2 oscillation and the peak TACC level of each embryo across nuclear cycles 11, 12, and 13.



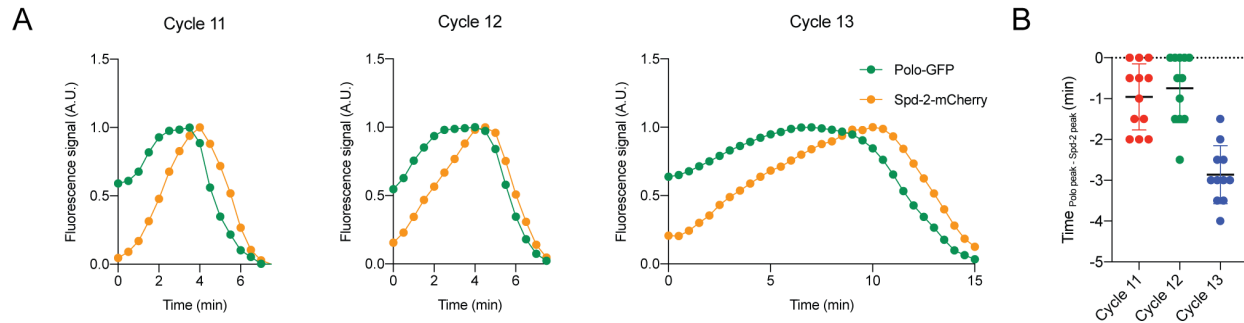
**Figure 3.12  $\gamma$ -tubulin can continue to grow even as centrosomal Spd-2 levels decline**

(A) Graphs show how the average centrosomal fluorescent intensity changes over time during nuclear cycles 11, 12, and 13 for embryos ( $n = 12$  for each cycle) co-expressing  $\gamma$ -tubulin-37c-GFP (purple) and Spd-2-mCherry (orange). The fluorescent intensity was rescaled to between 0 and 1 in each cycle. (B) Bar charts compare the time difference between the peak of the Spd-2 oscillation and the peak  $\gamma$ -tubulin level of each embryo across nuclear cycles 11, 12, and 13.



**Figure 3.13 The Cnn scaffold can continue to grow even as centrosomal Spd-2 levels decline**

(A) Graphs show how the average centrosomal fluorescent intensity changes over time during nuclear cycles 11, 12, and 13 for embryos ( $n = 8 - 12$  for each cycle) co-expressing GFP-Cnn (blue) and Spd-2-mCherry (orange). The fluorescent intensity was rescaled to between 0 and 1 in each cycle. (B) Bar charts compare the time difference between the peak of the Spd-2 oscillation and the peak Cnn level of each embryo across nuclear cycles 11, 12, and 13.



**Figure 3.14 The peak of the Polo oscillation precedes the peak of the Spd-2 oscillation**

(A) Graphs show how the average centrosomal fluorescent intensity changes over time during nuclear cycles 11, 12, and 13 for embryos ( $n = 12$  for each cycle) co-expressing Polo-GFP (green) and Spd-2-mCherry (orange). The fluorescent intensity was rescaled to between 0 and 1 in each cycle. (B) Bar charts compare the time difference between the peak of the Spd-2 oscillation and the peak of the Polo oscillation for each embryo across nuclear cycles 11, 12, and 13.

### 3.2.6 Mathematical modelling reveals that an underlying Polo oscillation can plausibly explain the kinetics of PCM scaffold assembly

The protein kinase Plk4 is related to Polo/Plk1 and is a master regulator of centriole assembly. The Raff lab recently showed that Plk4 is recruited to centrioles in an oscillatory manner, and this oscillation appears to help time and execute daughter centriole assembly (Aydogan et al., 2020). As Polo/PLK1 is an important regulator of mitotic centrosome assembly, I wondered whether the centrosomal Polo oscillation is important for regulating mitotic centrosome assembly. Over the last few years the Raff laboratory has developed a relatively simple molecular model to explain how Spd-2, Polo and Cnn might cooperate to form the PCM scaffold in these embryos (Conduit et al., 2015; Conduit et al., 2014a; Feng et al., 2017) (Figure 3.1). In a collaboration with a mathematician in the laboratory, Zachary Wilmott, I turned to mathematical modelling to test whether this molecular model could plausibly explain how an underlying oscillation in centrosomal Polo

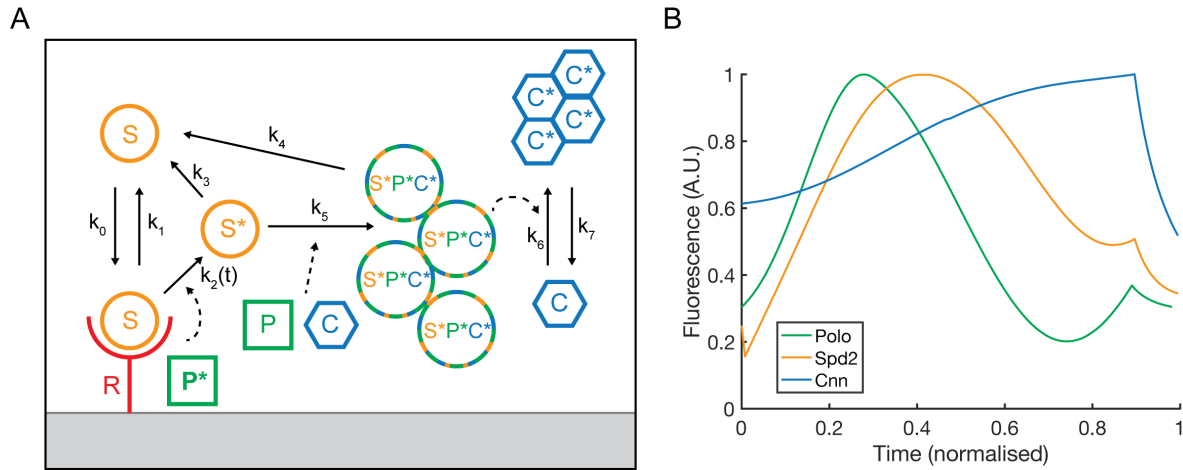
levels might lead to the unexpected kinetics of PCM scaffold assembly that I had observed (Note that this model does not address the recruitment of PCM clients).

In this model (Figure 3.15A) we *assume* that active Polo ( $P^*$ ) is generated at centrioles in an oscillatory manner during S-phase, with levels peaking at approximately mid-S-phase (I explore how this oscillation might be generated in the next Section). We allow a centrosomal Receptor (R) to recruit Spd-2 (S) to the centriole [ $k_0$ ]. The receptor can then either release S back into the cytoplasm [ $k_1$ ], or S can be phosphorylated by  $P^*$  at the centriole to convert it to a form of Spd-2 that can form a scaffold ( $S^*$ ) [ $k_2(t)$ ] (note that this rate constant has a time component as it is proportional to the amount of  $P^*$  at the centriole, which changes over time due to the underlying oscillation we impose).  $S^*$  can be converted back to S [ $k_3$ ] by a phosphatase (that we assume is active in the cytosol at a constant low level), but it can also bind both Polo (P) and Cnn (C) from the cytosol to form a scaffold ( $SPC^*$ ) [ $k_4$ ] that comprises  $S^*$ ,  $P^*$ , and  $C^*$  and this population of  $S^*$  is more resistant to dephosphorylation and so converts back to S relatively slowly [ $k_5$ ]. The stabilised  $SPC^*$  scaffold then effectively acts as a catalyst to phosphorylate more cytoplasmic C and convert it into a form that can assemble into an independent scaffold ( $C^*$ ) [ $k_6$ ]. For simplicity, I show this as the  $SPC^*$  scaffold catalysing the generation of  $C^*$  from C but, in reality, the  $SPC^*$  scaffold is probably constantly releasing its  $C^*$ , which is then replaced by another molecule of C from the cytosol. In this way the  $SPC^*$  scaffold is constantly recruiting P and C to maintain the  $SPC^*$  scaffold, and releasing phosphorylated  $C^*$  to generate the  $C^*$  scaffold. The  $C^*$  scaffold disassembles at the end of mitosis when it is dephosphorylated by a phosphatase [ $k_7(t)$ ]. Interestingly, in initial versions of this

model this last constant had a time component as we allowed the phosphatase to be active in a burst that peaks during late mitosis—a reasonable assumption as the Cnn and SPD-5 scaffolds are rapidly disassembled in late mitosis in flies and worms, respectively, and in worms this is driven by a Phosphatase (Enos et al., 2018). This modelling revealed, however, that this was unnecessary, and the model worked equally well if we allowed C\* to be dephosphorylated by a phosphatase that was active at a constant rate throughout the cycle, but we allowed the rate of Cnn disassembly (**CnnOUT**, see **Chapter 4**) to increase as its size increases—as appears to be the case (Conduit et al., 2010).

Zachary Wilmott expressed these reactions as a series of Ordinary Differential Equations (ODEs) and examined the behaviour of the system. Encouragingly, he found that this relatively simple model can recapitulate several of the key features of scaffold assembly dynamics (Figure 3.15B). Most importantly, an underlying oscillation in *centriolar* Polo levels can drive a subsequent oscillation in Spd-2 levels within the PCM, and these oscillations can drive a relatively steady growth of the Cnn scaffold that can continue to grow and plateau even as the Polo and Spd-2 levels decline (just as we observe in vivo), (Figure 3.13, Figure 3.14).

This mathematical modelling suggests that our molecular model of Spd-2/Polo/Cnn scaffold assembly, when combined with an underlying oscillation in the centriolar levels of active Polo, could plausibly explain several of the unexpected features of PCM scaffold assembly dynamics that I have observed.



**Figure 3.15 Mathematical model of PCM scaffold assembly**

(A) In this model we allow the level of active Polo kinase (P\*) to oscillate at the centriole. Spd-2 (S) is recruited to centrioles ( $k_0$ ) by a Receptor (R) and it can either be released back into the cytosol ( $k_1$ ) or phosphorylated by P\* ( $k_2[t]$ ) to form S\* (so the rate of production of S\* depends on the amount of P\* at the centriole, which varies over time due to the oscillation). S\* is rapidly converted back to S by a Phosphatase ( $k_3$ ), but it can also bind to Polo (P) and Cnn (C), ( $k_4$ ) which activates Polo and allows it to phosphorylate C to convert it to C\*, forming a more stable S\*P\*C\* scaffold (thus allowing more total S\* to accumulate around the centriole). This scaffold can constantly bind new C and convert it to C\* ( $k_6$ ), which is then released, and which can form its own independent C\* scaffold. For simplicity, we depict this as the S\*P\*C\* scaffold catalysing the conversion of cytoplasmic C to scaffold-associated C\*. C\* is converted back to C by a Phosphatase ( $k_7$ ). (B) Graph shows the numerical simulation of dynamics of Cnn (blue) and Spd-2 (orange) driven by Polo (green) oscillation (an artificial sine wave).

### 3.2.7 Ana1 helps to recruit Polo to centrioles in an oscillatory manner

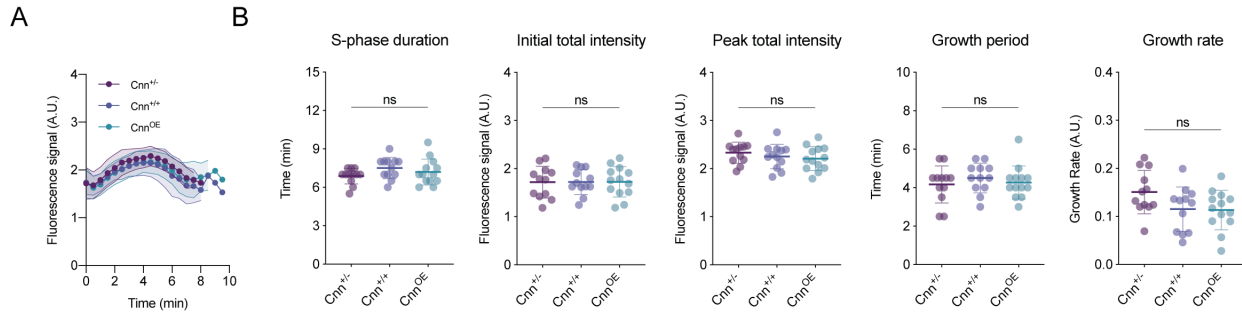
A key feature of this model is that Polo levels at the centriole oscillate during S-phase as the centrosomes grow in preparation for mitosis. This oscillation is very unlikely to simply reflect the overall activity of Polo in the embryo which, like Cdk/Cyclin activity (Deneke et al., 2016), peaks during mitosis (Stefano Di Talia, Duke University (USA), *personal communication*). It has previously been shown in our laboratory that the Polo required to promote mitotic PCM assembly appears to be recruited to centrioles and centrosomes in *Drosophila* embryos by a sequential phosphorylation cascade (Alvarez-Rodrigo et al.,



2019, 2020): the centriole protein Ana1 is initially phosphorylated on multiple S-S/T motifs to allow it to recruit Polo to mother centrioles; this pool of Polo can then phosphorylate Spd-2 on multiple S-S/T motifs, allowing Spd-2 to recruit more Polo and to initiate the positive feedback loop (with Cnn) that drives the expansion of the mitotic PCM (Figure 3.1). I therefore tested whether altering Cnn, Spd-2, or Ana1 levels influenced the Polo oscillation (note that I show that the manipulations I use here to alter the levels of these proteins have the expected effect on their centriole/centrosome levels in **Chapter 4**).

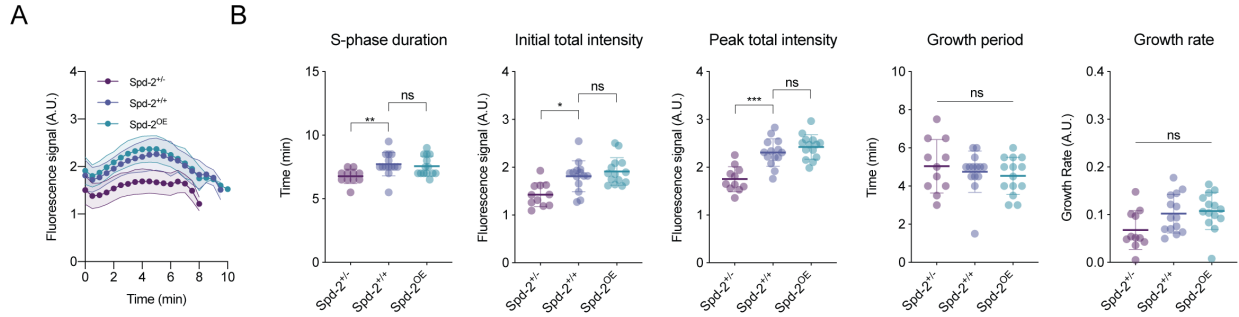
Changing the levels of Cnn had little effect on the Polo oscillation, suggesting that Cnn itself does not recruit Polo into the PCM (Figure 3.16), as predicted in our molecular model. In contrast, halving the genetic dosage of Spd-2 significantly reduced the amplitude of the Polo oscillation, while overexpressing Spd-2 had little effect (Figure 3.17). Neither perturbation altered the period of the Polo oscillation, suggesting that although Spd-2 normally helps recruit Polo to centrosomes, it is not primarily involved in timing Polo recruitment. Interestingly, decreasing or increasing the dosage of Ana1 led to a corresponding decrease or increase in the levels of Polo recruited to centrioles at the start of S-phase but it had little effect on the final peak Polo levels (Figure 3.18). I suspect that this is because at the start of S-phase most of the total centrosomal Polo is being recruited by centriolar Ana1, whereas later in S-phase, when the PCM has expanded, most of the total centrosomal Polo is being recruited to the PCM by Spd-2. Importantly, decreasing Ana1 levels significantly altered the period of the Polo oscillation, even though it had no effect of S-phase length; increasing Ana1 levels had the opposite effect, although this

was modest and not statistically significant (Figure 3.18). This suggested that Ana1 may be involved in setting the period of the Polo oscillation.



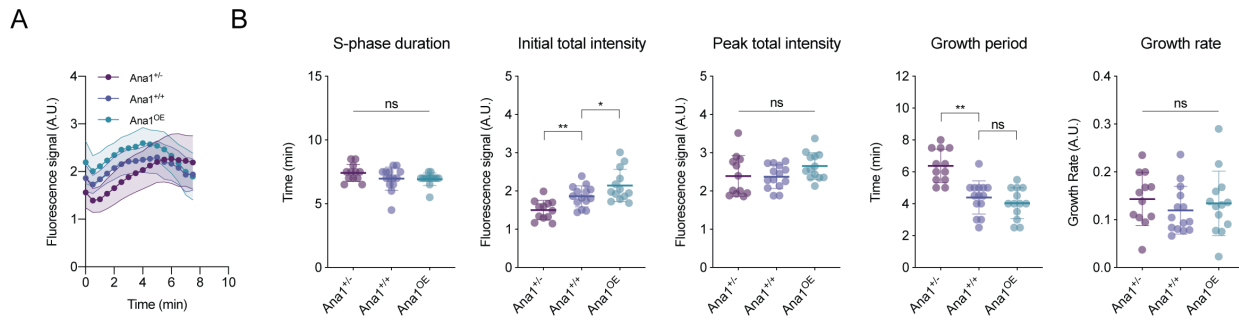
**Figure 3.16 The Polo oscillation is not detectably perturbed by changing Cnn levels**

**(A)** Graphs show how the average centrosomal fluorescent intensity ( $\pm$ SD) of Polo-GFP changes over time during nuclear cycle 12 in embryos where the dosage of Cnn has either been reduced in (Cnn<sup>+/-</sup>), is normal (Cnn<sup>+/+</sup>), or is increased (Cnn<sup>OE</sup>). These perturbations bring about the expected changes in centrosomal Cnn levels (see Chapter 4). The average curve from each group was calculated from > 12 embryos. **(B)** Bar charts compare various growth parameters (as indicated) in these embryos. Statistical significance was assessed by either an ordinary one-way ANOVA (Gaussian-distributed and variance-homogeneous) or a Kruskal Wallis's test (non-Gaussian-distributed), (ns: not significant). Gaussian distribution was tested using D'Agnostino and Pearson's test. The homogeneity of variance was tested using Levene W test.



**Figure 3.17 The period of the Polo oscillation is not changed by changing Spd-2 levels**

**(A)** Graphs show how the average centrosomal fluorescent intensity ( $\pm$ SD) of Polo-GFP changes over time during nuclear cycle 12 in embryos where the dosage of Spd-2 had either been reduced (Spd-2<sup>-/-</sup>), is normal (Spd-2<sup>+/+</sup>), or is increased (Spd-2<sup>OE</sup>). These perturbations bring about the expected changes in centrosomal Spd-2 levels (see Chapter 4). The average curve from each group was calculated from > 12 embryos. **(B)** Bar charts compare various growth parameters (as indicated) in these embryos. Statistical significance was first assessed by either an ordinary one-way ANOVA (Gaussian-distributed and variance-homogeneous) or a Kruskal Wallis's test (non-Gaussian-distributed), (ns: not significant). If the comparison was significant, multiple testing was subsequently performed. Multiple hypothesis testing P-values were calculated by either Tukey-Kramer's test (Gaussian-distributed and variance-homogeneous) or Mann-Whitney's U test (non-Gaussian-distributed) (\*: P<0.05, \*\*: P<0.01, \*\*\*: P<0.001, ns: not significant). Gaussian distribution was tested using D'Agostino and Pearson's test. The homogeneity of variance was tested using Levene W test.



**Figure 3.18 The period of the Polo oscillation is shifted by changing Ana1 levels**

(A) Graphs show how the average centrosomal fluorescent intensity (±SD) of Polo-GFP changes over time during nuclear cycle 12 in embryos where the dosage of Ana1 had either been reduced (Ana1<sup>+/-</sup>), is normal (Ana1<sup>+/+</sup>), or is increased (Ana1<sup>OE</sup>). The average curve from each group was calculated from > 12 embryos. (B) Bar charts compare various growth parameters (as indicated) in these embryos. Statistical significance was first assessed by either an ordinary one-way ANOVA (Gaussian-distributed and variance-homogeneous), a one-way Welch ANOVA (Gaussian-distributed and variance-unequal), or a Kruskal Wallis's test (non-Gaussian-distributed), (ns: not significant). If the comparison was significant, multiple testing was subsequently performed. Multiple hypothesis testing P-values were calculated by either Tukey-Kramer's test (Gaussian-distributed and variance-homogeneous) or Mann-Whitney's U test (non-Gaussian-distributed) (\*: P<0.05, \*\*: P<0.01, ns: not significant). Gaussian distribution was tested using D'Agnostino and Pearson's test. The homogeneity of variance was tested using Levene W test.

### 3.2.8 A mathematical model to explain how an interaction between Ana1 and Polo could generate a centriolar Polo oscillation

Previous studies in the laboratory suggested that the Plk4 centriolar oscillation could be generated by a classical delayed negative feedback loop (Novák & Tyson, 2008), in which an Activator (the Plk4 centriole receptor Asl) activates its own Inhibitor (Plk4—which acts as an inhibitor because the activated Plk4 is proposed to phosphorylate Asl at multiple sites to turn off the ability of Asl to recruit Plk4) (Aydogan et al., 2020). I wondered whether Polo and Ana1 might interact in a similar manner to generate the Polo oscillation. Collaborating with Zachary Wilmott, we generated a model to explain how the Polo/Ana1 interaction might generate a Polo oscillation (Figure 3.19).

We speculate that at the start of the centrosome growth period Ana1 starts to be phosphorylated by a kinase (Kinase in Figure 3.19) on S-S/T motifs to generate A\*, which can recruit Polo to mother centrioles. Recruitment to A\* activates Polo (by an unknown mechanism) to generate P\*, which is then released from A\* (as Polo turns over very rapidly at centrosomes in these embryos, see below), generating a high local concentration of P\* close to the centriole surface. The pool of P\* performs two functions (Figure 3.19): (1) It phosphorylates centriolar Spd-2 on multiple S-S/T motifs to generate S\* (and so initiate the assembly of the mitotic PCM scaffold, as described above); (2) It phosphorylates A\* at additional sites (i.e. not the original S-S/T motifs) to generate A<sup>^</sup>, which can no longer recruit Polo. In this way, the activator (Ana1), activates its own inhibitor (Polo) to generate the Polo oscillation. As is the case with the Plk4 oscillation (Aydogan et al., 2020), we envisage that this system can be reset at each cycle when A<sup>^</sup> is dephosphorylated in mitosis by a PPTase to regenerate dephosphorylated Ana1. An attractive feature of this model is that it can intuitively explain why halving the dose of the Ana1 receptors lengthens the period of the Polo oscillation (as shown in Figure 3.18): less Ana1 receptors leads to the production of less P\* (and I observe that less Polo is concentrated at centrioles in early S-phase when I reduce the dosage of Ana1, Figure 3.18) so A\* is converted to A<sup>^</sup> more slowly, and the period of the oscillation is extended.

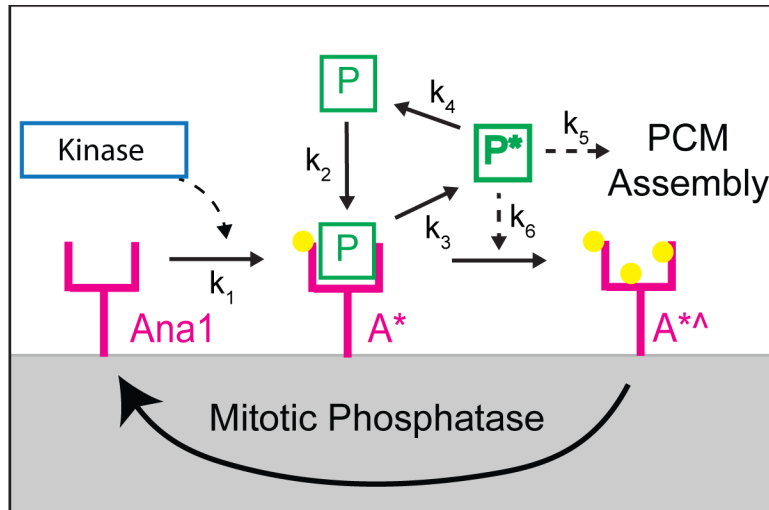


Figure 3.19 A schematic illustration of how Polo might interact with Ana1 to generate a centriole oscillation in active Polo levels

See text for details.

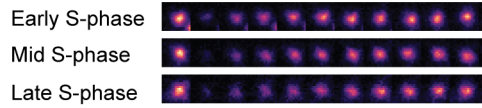
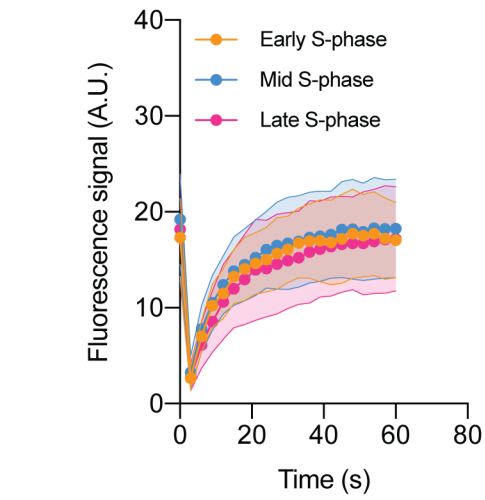
### 3.2.9 Polo rapidly turns over at centrosomes, and the rate of Polo recruitment during S-phase declines at the centriole before it starts to decline in the PCM

A crucial feature of this model is that  $P^*$  is rapidly released from its Ana1 ( $A^*$ ) receptors. If this was not the case, then halving the dose of Ana1 should not alter the period of the Polo oscillation, because once bound  $P^*$  should phosphorylate Ana1 at the same rate no matter what the density of nearby Ana1 receptors. Polo/PLK1 generally turns over rapidly at the sites it is recruited to during mitosis (Conduit et al., 2014b; García-Álvarez et al., 2007; Kishi et al., 2009), and I used fluorescence recovery after photobleaching (FRAP) to show that Polo had a  $t_{1/2}$  of 6.9, 8.2, and 9.1 secs in early-, mid- and late-S-phase, respectively (Figure 3.20A). This confirms that Polo is rapidly turning over, and that any  $P^*$  that is generated at centrosomes is therefore likely to be rapidly releasing from its receptors. These dynamics support the possibility that the scaffolding proteins Ana1 and Spd-2 constantly recruit (and I postulate, activate) Polo to create  $P^*$ , which is then rapidly

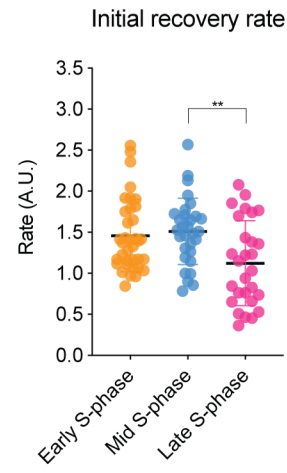
released to generate a high local concentration of P\* at the mother centriole and within the expanding PCM.

If this model is correct, I would expect the rate of Polo recruitment to be relatively low in early S-phase (when it is mainly A\* recruiting Polo, as the Spd-2 PCM scaffold is small), to increase in mid-S-phase (when both A\* and S\* recruit Polo) and to decrease in late S-phase (when both A\* and S\* would be in decline). I did observe this trend in Polo recruitment rates (as measured by FRAP) at different stages of S-phase, but it was not very convincing (Figure 3.20B). This trend might be obscured, however, if the amount of A\* at the centriole and S\* in the PCM do not rise and fall in synchrony. If this was the case, I would predict that the rate of Polo recruitment to the centrioles would decrease before the rate of Polo recruitment to the PCM (as in our model the A\* pathway at the centriole would be switched off before the S\* pathway in the PCM is inactivated by the gradual dephosphorylation of the SPC\* scaffold). To test this possibility, I compared the Polo recruitment rate in early and late S-phase at the central pixels (i.e. at the centrioles, *green pixel* in Figure 3.20C) and in the outer pixels (i.e. in the PCM, the average of the *orange pixels* in Figure 3.20C). Consistent with our model, in late S-phase there was a more dramatic decrease in the rate of Polo recruitment to the central pixels (~44%,  $p < 0.0001$ ) than to the peripheral pixels (~20%,  $p < 0.05$ ) (Figure 3.20D, E).

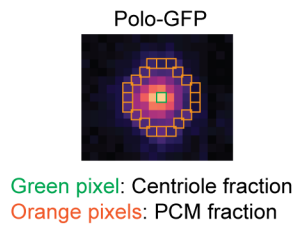
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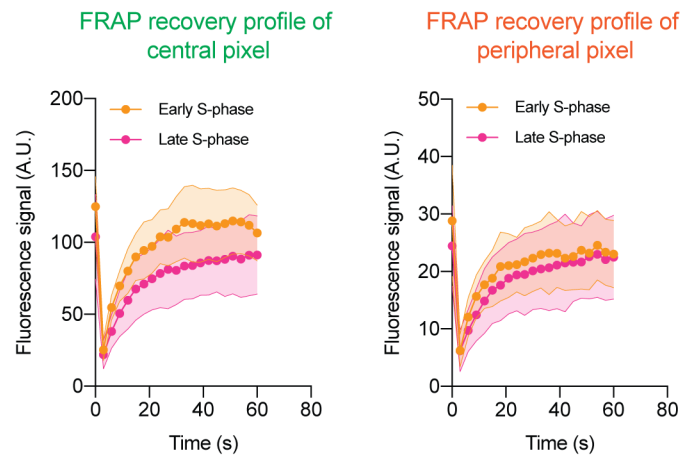
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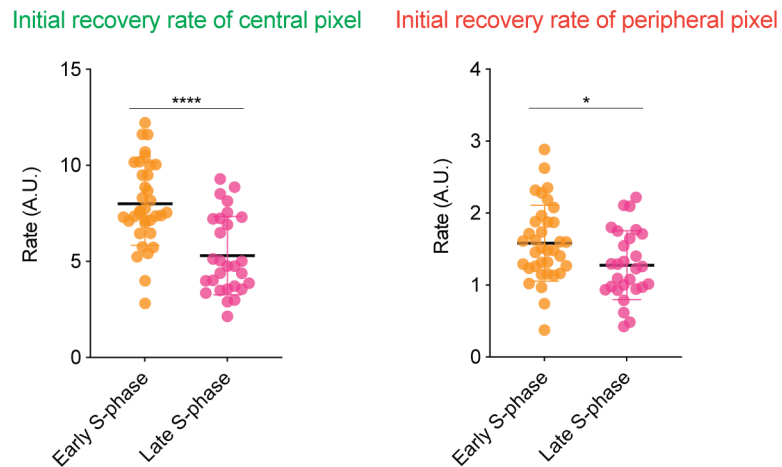
C



D



E





**Figure 3.20 The pattern of Polo recovery during cell cycle as revealed by FRAP**

**(A)** (Upper panel) Graph shows how the average centrosomal fluorescent intensity of Polo-GFP changes over time after FRAP during early-, mid-, and late-S-phase (as indicated) ( $n > 28$  centrosomes for each set). (Lower panel) Micrographs show typical FRAP recovery profiles at single centrosomes (The height of the montages measures  $1.65 \mu\text{m}$ ). **(B)** Bar chart compares the initial recovery rate (the slope of the recovery curve after the first 3 seconds of FRAP) in each embryo. Statistical significance was assessed by a Kruskal Wallis's test (non-Gaussian distributed), (ns: not significant). Normality of data was assessed using a D'Agostino and Pearson's test. **(C)** Micrograph illustrates the central green pixel (the pixel with the highest fluorescent intensity) used to measure the centriole fraction of Polo-GFP, and the peripheral orange pixels (that are 5 pixels away from the centre pixel) used to measure the PCM fraction of Polo-GFP. **(D)** Graphs show the initial recovery rate of the central *green pixel* and the peripheral *orange pixels* during early- and late-S-phase. Statistical significance was assessed using an unpaired t-test (\*:  $P < 0.05$ , \*\*\*\*:  $P < 0.0001$ ).

### 3.2.10 Perturbing the ability of Ana1 to recruit Polo perturbs the Polo oscillations

The Raff laboratory recently generated a form of Ana1 in which all 34 S-S/T motifs were mutated to T-S/T (Ana1-S34T) so that Ana1 could no longer recruit Polo via its PBD (Alvarez-Rodrigo et al., 2020). The Ana1-S34T protein largely rescued the *ana1* mutation, allowing flies to form centrioles that appeared to duplicate and assemble cilia normally, but that could not efficiently recruit Polo and so could not efficiently recruit mitotic PCM. As a result, embryos laid by *ana1* mutant females expressing Ana1-S34T fusion proteins died during early embryonic development—as centrosomes are essential for the rapid rounds of mitosis in the early embryos (Peel et al., 2007; Varmark et al., 2007), but not for cell division in *Drosophila* somatic cells (Basto et al., 2006). Thus, although Ana1 is essential for centriole, mitotic centrosome and cilia assembly, its ability to recruit Polo is only essential for mitotic centrosome assembly.

To examine how Ana1-S34T might influence the Polo-GFP oscillation, I expressed Polo-GFP with WT-Ana1-mCherry or Ana1-S34T-mCherry but in the presence of two copies of the endogenous WT *ana1* gene; I did this because embryos expressing Polo-GFP and Ana1-S34T-mCherry die very early in development if there is no WT Ana1 protein present (Alvarez-Rodrigo et al., 2020). Importantly, after some pilot analysis, I decided to measure the oscillations at old mother (OM) and new mother (NM) centrosomes independently, as they behaved quite differently (Figure 3.21A). I believe that this is because NM centrosomes (organised by the NM centrioles) are recruiting mitotic PCM for the first time, whereas OM centrosomes (organised by the OM centrioles) have already recruited mitotic PCM in previous cycles and, in these rapidly dividing embryos, some of this PCM

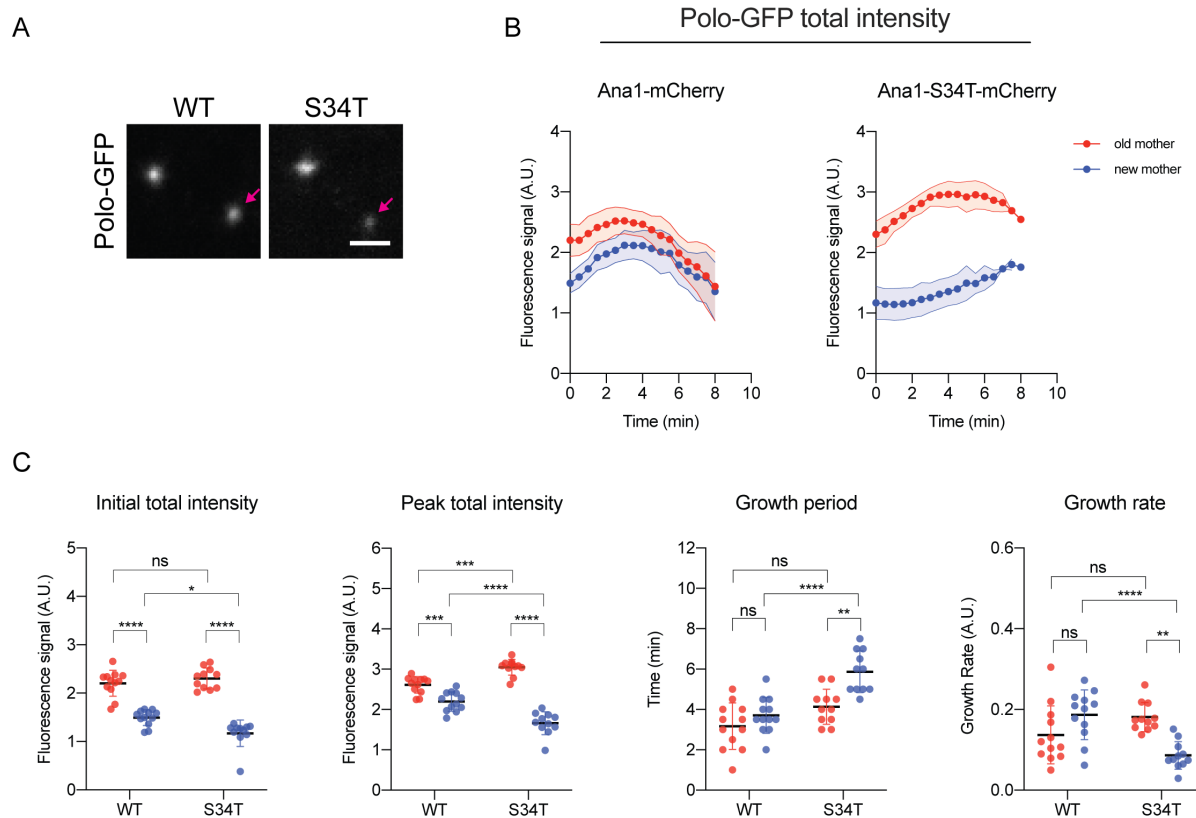
remains associated with the OM centrioles as they leave mitosis and then immediately start to grow again during the next cycle (see below).

In embryos expressing Polo-GFP and WT-Ana1-mCherry, the Polo oscillation looked similar on OM and NM centrosomes (Figure 3.21B), although significantly less Polo was initially present on NMs (Figure 3.21C). As describe above, this is presumably because NMs are recruiting Polo for the first time, whereas OMs retain some PCM (and so some Polo) from the previous cycle. In embryos expressing Polo-GFP and Ana1-S34T-mCherry, the Polo oscillation was relatively normal on OMs, but was dramatically perturbed on NMs (Figure 3.21B), exhibiting a lower amplitude, a longer period, and a reduced growth rate (Figure 3.21C). I draw two conclusions from this data: (1) The ability of Ana1 to recruit Polo to centrioles plays an important part in generating, and defining the parameters of, the Polo oscillation on NM centrosomes; (2) For some reason, OM centrosomes can compensate for the presence of some Ana1-S34T receptors on their centrioles and so organise a relatively normal Polo oscillation.

In our modelling, the Polo oscillation is effectively a readout of the amount of  $P^*$  being generated at the centrosome by  $A^*$  and  $S^*$  (Figure 3.19). I realised that this could potentially explain why OM and NM centrosomes behave so differently in the presence of Ana1-S34T. NM centrosomes are recruiting mitotic PCM for the first time, so they would initially rely only on  $A^*$  to generate  $P^*$ , which would then subsequently generate  $S^*$  (to recruit more  $P^*$ ). In this scenario, reducing the amount of  $A^*$  (by expressing Ana1-S34T, which cannot be converted to  $A^*$ ) would dramatically slow the generation of  $P^*$ —so NM

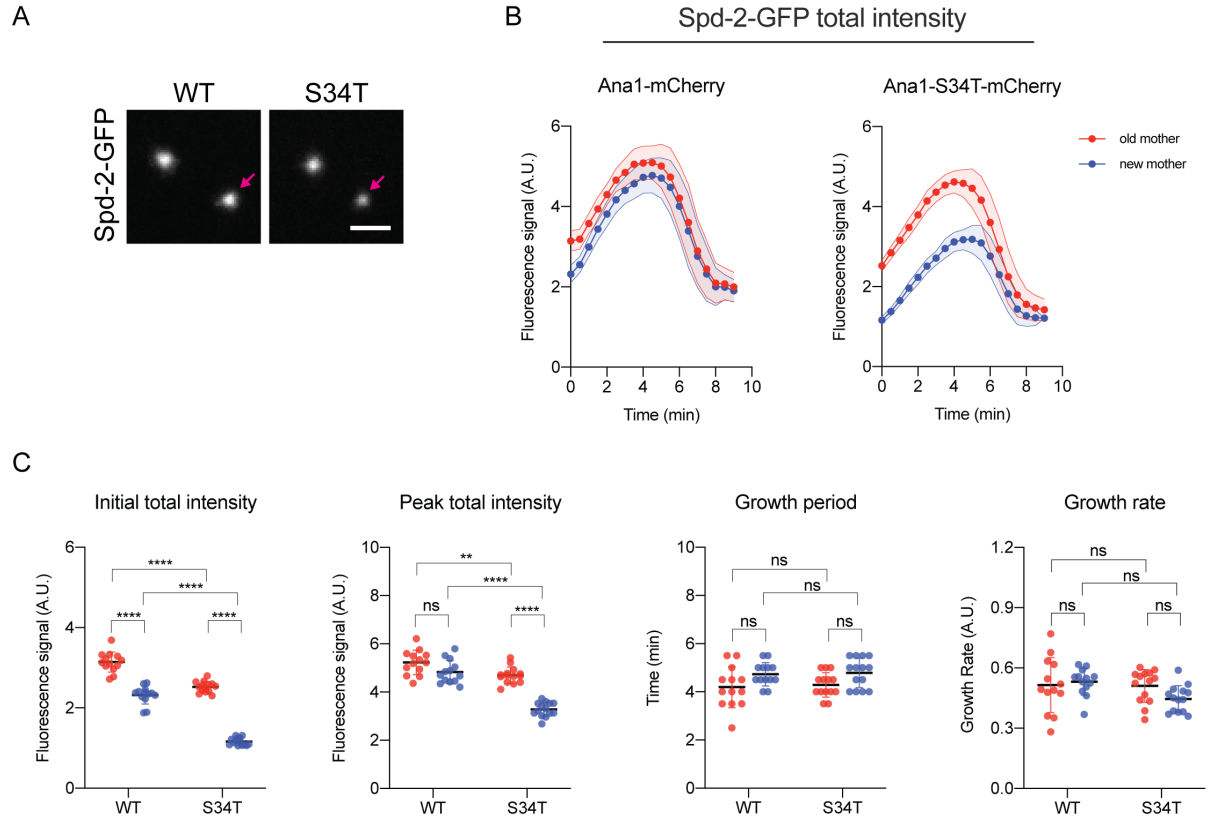
centrioles would recruit Polo more slowly, but for a longer period (as P\* is required to inactivate A\*, Figure 3.19), as I observe (Figure 3.18). At OM centrosomes, however, some Spd-2 (in the form of S\*) might still remain within the PCM organised during the previous cycle so at the start of S-phase, this residual S\* may still be present, and this could generate some P\* at the start of the cycle to partially compensate for the reduction in A\* levels in embryos expressing Ana1-S34T.

To test the plausibility of this idea I examined how expressing either WT Ana1-mCherry or Ana1-S34T-mCherry (as before, always in the presence of the endogenous WT Ana1) influenced the Spd-2-GFP oscillation at OM and NM centrosomes (Figure 3.22B). In embryos expressing WT Ana1-mCherry, NMs recruited slightly less Spd-2 at the start of S-phase than OMs (presumably because the NMs are recruiting Spd-2 for the first time); but the amplitude and period of the Spd-2 oscillation was similar at OMs and NMs (Figure 3.22C). In contrast, in embryos expressing Spd-2-GFP and Ana1-S34T-mCherry, although the OMs organised a relatively normal Spd-2 oscillation, the NMs recruited much less Spd-2 (Figure 3.22C). These observations support my hypothesis that in embryos expressing Ana1-S34T the presence of substantial levels of previously recruited Spd-2 at OMs could help to generate a relatively normal Polo oscillation, but this is not the case at NM centrosomes, where Spd-2 is being recruited for the first time.



**Figure 3.21 Recruitment dynamics of Polo-GFP on old mother and new mother centrosomes in either one copy Ana1-mCherry or Ana1-S34T-mCherry genetic background**

(A) Micrograph shows representative images of centrosomal Polo-GFP in a genetic background of one copy of Ana1-mCherry (WT) or Ana1-S34T-mCherry (S34T) with two copies of endogenous *ana1*. Pink arrows indicate new mother centrosomes (NMs). Scale bar represents 2  $\mu$ m. (B) Graphs show the average centrosomal fluorescent intensity of Polo-GFP on old mother (red) and new mother (blue) in a genetic background of one copy of Ana1-mCherry (left panel) or Ana1-S34T-mCherry (right panel) with two copies of endogenous Ana1. (C) Bar charts compare various growth parameters (as indicated) of Polo-GFP on old mother (red) and new mother (blue) in a genetic background of one copy of Ana1-mCherry (WT) or Ana1-S34T-mCherry (S34T). Statistical significance was first assessed with a two-way ANOVA to confirm the difference of mean. And then multiple comparison was performed using a Šidák's multiple comparisons test (\*\*:  $P < 0.01$ , \*\*\*:  $P < 0.001$ , \*\*\*\*:  $P < 0.0001$ , ns: not significant).



**Figure 3.22 Recruitment dynamics of Spd-2-GFP on old mother and new mother centrosomes in either one copy Ana1-mCherry or Ana1-S34T-mCherry genetic background**

(A) Micrograph shows representative images of centrosomal Spd-2-GFP in a genetic background of one copy of Ana1-mCherry (WT) or Ana1-S34T-mCherry (S34T) with two copies of endogenous *ana1*. Pink arrows indicate new mother centrosomes (NMs). Scale bar represents 2  $\mu$ m. (B) Graphs show the average centrosomal fluorescent intensity of Spd-2-GFP on old mother (red) and new mother (blue) in a genetic background of one copy of Ana1-mCherry (left panel) or Ana1-S34T-mCherry (right panel) with two copies of endogenous Ana1. (C) Bar charts compare various growth parameters (as indicated) of Polo-GFP on old mother (red) and new mother (blue) in a genetic background of one copy of Ana1-mCherry (WT) or Ana1-S34T-mCherry (S34T). Statistical significance was first assessed with a two-way ANOVA to confirm the difference of mean. And then multiple comparison was performed using a Šidák's multiple comparisons test (\*\*:  $P < 0.01$ , \*\*\*\*:  $P < 0.0001$ , ns: not significant).

### 3.3 Discussion

#### 3.3.1 Centrosome size homeostasis

In this Chapter I examined how centrosome size is regulated in early *Drosophila* embryos. I started by analysing the behaviour of several PCM client proteins and found that, perhaps surprisingly, the centrosomes grew to approximately the same size at successive nuclear cycles. This is in contrast to the situation in early *C. elegans* embryos, where centrosomes halve in size at successive cell divisions (Decker et al., 2011). I conclude that early *Drosophila* embryos do not use the elegant “limiting component” mechanism that has been proposed to regulate centrosome size in *C. elegans* embryos (Goehring & Hyman, 2012). It was always difficult to imagine that such a mechanism might operate in *Drosophila* embryos, which are large (~500µm long by 150µm wide) and where the centrosomes increase in number so rapidly (dividing every 10mins) that the rate of diffusion would, intuitively, not allow all the molecules of Spd-2 (the fly homologue of SPD-2, the proposed limiting component in worm embryos) to simply be equally divided up between the centrosomes at each cycle. Also, intuitively, a similar argument would apply to the early *C. elegans* embryo, which is also large (50µm long by 30µm wide) and divides relatively rapidly (every ~15-20mins). The comparison of both systems is outlined in **Error!**

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Table 3.1 Table compares features of *D. melanogaster* and *C. elegans* embryos

|                            | <i>D. melanogaster</i> embryos                                                                                                                                                    | <i>C. elegans</i> embryos                                                                                                                                                       |
|----------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Size of embryos            | ~500 $\mu$ m x ~150 $\mu$ m                                                                                                                                                       | ~50 $\mu$ m x ~30 $\mu$ m                                                                                                                                                       |
| Duration of cell cycle     | ~10 mins for the first 13 nuclear cycles                                                                                                                                          | ~15 – 20 mins for the first cleavage                                                                                                                                            |
| Mode of cell division      | <ul style="list-style-type: none"> <li>- Syncytial nuclear division</li> <li>- Symmetrical cell division</li> </ul>                                                               | <ul style="list-style-type: none"> <li>- Cellular cleavage with cell size approximately halves every cycle before gastrulation</li> <li>- Asymmetrical cell division</li> </ul> |
| Size scaling of centrosome | <ul style="list-style-type: none"> <li>- ~6<math>\mu</math>m<sup>3</sup> in nuclear cycle 11</li> <li>- Volume is stable across nuclear cycle 11 – 13 (Data not shown)</li> </ul> | <ul style="list-style-type: none"> <li>- ~12<math>\mu</math>m<sup>3</sup> in cleavage cycle 1</li> <li>- Volume is halved in each cycle</li> </ul>                              |

The centrosome size homeostasis that I observe in fly embryos seems to be enforced by an inverse-linear relationship between the centrosome growth rate and period: centrosomes grow to approximately the same size at each cycle because they grow rapidly, but for a short time, in cycle 11, and more slowly, but for a longer time, during successive cycles. For the client proteins I tested, it seems clear that this inverse relationship is largely enforced by the Cdk/Cyclin Oscillator (CCO) that times the nuclear cycles in these early embryos. There is a very strong correlation ( $r=0.94-0.99$ , depending on the client) between S-phase length (which is dictated by the CCO) and the growth period of the PCM clients. This strongly suggests that the interaction between the clients and the PCM scaffold is regulated by Cdk/Cyclin activity, potentially via Cdk1 phosphorylation of the PCM-scaffold, individual PCM-client proteins, or both. It seems that a threshold level of Cdk/Cyclin activity triggers an abrupt change in the interaction between the scaffold and client proteins such that client levels within the scaffold start to



drop rapidly when this threshold is reached. My data hints that different PCM clients may be influenced in slightly different ways by the CCO. It has been shown that adjusting the number of Cdk1 phosphorylation sites can change the activation threshold of nuclear export of Whi5, thus timing of Whi5 localization in yeast (Örd et al., 2019). I wondered if centrosomal clients are ordered similarly by the CCO in *Drosophila*. For example, TACC, MSPS and  $\gamma$ -tubulin all exhibited a subtle increase in their rate of binding to the PCM a few minutes before they reached their maximal levels, whereas Grip71, Grip75, Grip128, and Aurora A did not. Clearly more work will be required to better understand how the PCM clients interact with the underlying PCM-scaffold, and how these interactions are regulated by the CCO.

My data also reveals that there is a slightly less strong inverse-linear correlation between S-phase length and the PCM client growth rate ( $r = -0.64$ - $-0.88$ , depending on the client), indicating that the CCO is also likely to influence the centrosome growth rate. This is perhaps most convincingly demonstrated in the experiments where I halved the dose of Cyclin B: this extended S-phase length and the centrosomes grew more slowly but for a longer period to attain their normal size. Thus, simply halving the dose of Cyclin B slows the rate of centrosome growth. One possible explanation for this finding is that PCM clients can only start to bind to the scaffold when they have been phosphorylated to some extent by the CCO, so the gradual slowing of the rise in Cdk/Cyclin activity during cycles 11-13 (Deneke et al., 2016) may reduce the pool of phosphorylated client proteins that can bind to the scaffold (although, as Thomas Steinacker's FCS analysis showed, the *total* pool of PCM client proteins remains relatively constant at successive cycles). As

discussed below, changes in the rate of PCM scaffold assembly at successive cycles are also likely to contribute to the slowing of the PCM client growth rate, and while several parameters of scaffold-growth are influenced by the CCO, at least one (the rate of Polo accumulation) is not. The involvement of multiple mechanisms that influence the PCM client growth rate—not all of which are highly correlated with CCO activity—probably explains why the inverse-linear correlation between CCO activity and the centrosome growth rate is not as strong as the linear correlation between CCO activity and the centrosome growth period.

### 3.3.2 A Polo oscillation appears to govern the assembly of the PCM scaffold

To my surprise, the dynamics of PCM scaffold assembly did not closely mirror the dynamics of PCM client recruitment, and the relationship between the CCO and scaffold assembly was much more complicated. For example, the Cnn scaffold could continue to grow during mitosis in cycle 11, but it stopped growing and plateaued prior to mitosis in cycles 12 and 13 (Figure 3.7), so that, unlike all the other centrosome proteins I analysed, there was no clear correlation between the Cnn growth period and S-phase length during cycles 11 and 12 (Figure 3.9). Moreover, and again in contrast to all the other centrosome proteins I analysed here, there was no significant inverse-linear correlation between the Polo growth rate and S-phase length (Figure 3.9), indicating that the CCO does not strongly influence the rate at which Polo accumulates at centrosomes—and the Polo growth rate remains nearly constant over cycles 11-13, (Figure 3.9).

I was initially very puzzled by these findings, but a potentially important clue came when I noticed that the levels of Polo and Spd-2 peaked and then started to decline at centrosomes before the levels of Cnn, or any of the client proteins, had peaked. Thus, centrosomes can continue to grow (at least when measured by the amount of Cnn or PCM client proteins recruited) even as Polo and Spd-2 levels are in decline. Moreover, the oscillation in Polo levels actually preceded the oscillation in Spd-2 levels, which was surprising because Spd-2 is a major recruiter of Polo into the PCM (Alvarez-Rodrigo et al., 2019). Together, these findings made me wonder whether an oscillation in Polo levels at the centriole might regulate PCM growth just as an oscillation in Plk4 levels regulates daughter centriole growth (Aydogan et al., 2018, 2020). I collaborated with a mathematician (Zachary Wilmott) to express the molecular model of how we think Polo, Spd-2 and Cnn cooperate to assemble the PCM scaffold into a simple mathematical model of ODEs. We found that imposing a pulse of centriolar Polo activity on this simple model could broadly mimic key features of PCM scaffold assembly—inducing a following pulse in Spd-2 recruitment, and the near linear growth of a Cnn scaffold that could continue to grow and plateau even as Polo and Spd-2 levels declined.

Ana1 is a centriole protein that is required to recruit Polo to centrioles to promote centrosome assembly (Alvarez-Rodrigo et al., 2020), and I found that perturbing Ana1 levels strongly influences the Polo oscillation. I developed a mathematical model with Zachary Wilmott to explain how the interaction between Polo and Ana1 could be organised in a classical delayed negative feedback loop to generate a Polo oscillation. I stress that the models presented here are no doubt highly simplified. For example, such

ODE-based models have no spatial element, and it seems certain that a more sophisticated spatial model—taking into account separate interactions at the surface of the centriole and in the bulk of the PCM—will be required to develop these ideas further. Nevertheless, the success of the simple model in crudely recapitulating some of my kinetic observations suggests that our ideas about how Ana1, Polo, Spd-2 and Cnn interact to form a PCM scaffold are plausible, and may ultimately be useful in explaining PCM scaffold assembly.

My studies have revealed unexpected potential mechanistic similarities between the processes that control daughter centriole and mitotic PCM biogenesis. In both cases, we now have evidence that a local (i.e. centriolar) oscillation in the levels of a key regulating enzyme (Plk4 or Polo) can drive the near linear recruitment of a key scaffolding component (Sas-6 or Cnn) for centriole or mitotic centrosome assembly, respectively. Moreover, in both cases, the properties of the oscillation seem to be adaptive and to be influenced by the CCO in a way that ensures that the structures the enzymes build grow to a relatively consistent size. In **Chapter 6** I discuss whether these mechanisms may provide insight into how the biogenesis of other organelles is regulated.

### 3.3.3 Comparing the mechanisms of centrosome assembly in fly and worm embryos

Fly and worm embryos use a very similar set of proteins to build a mitotic PCM scaffold, with Spd-2/SPD-2 initially recruiting Polo/PLK-1 to the centriole, and the Polo/PLK-1 then phosphorylating Cnn/SPD-5 to catalyse the production of a Cnn/SPD-5 scaffold. Given this high degree of conservation (although it should be noted that Cnn and SPD-5 share

very little sequence homology), it is perhaps surprising that the two systems seem to be regulated in very different ways with SPD-2 seeming to act as a limiting component to ensure the centrosomes halve in size at every cell division in the early worm embryo, and Polo and Spd-2 oscillating in an adaptive manner to ensure centrosomes grow to a consistent size in the early fly embryo. Moreover, it is clear that in worm embryos centrosomes reach their maximal size during mitosis, and maintain this size until anaphase-onset, when the mitotic PCM is disassembled (Enos et al., 2018; Mittasch et al., 2020; Woodruff et al., 2015). I was very surprised to find that centrosomes in early fly embryos reach their maximal size prior to NEB, with Spd-2, Polo and all the PCM client proteins already leaving the centrosome before mitosis proper even begins—although the Cnn scaffold largely remains in place until the end of mitosis.

Despite these important differences, I suspect that the fundamental mechanisms that govern mitotic centrosome growth will eventually prove to be similar between the two systems, and that these mechanisms are simply regulated in slightly different ways to adapt to the different biological requirements. For example, it may make sense for centrosomes to reach their maximal size prior to NEB in fly embryos because these embryos have very strong chromatin- and Augmin-pathways of spindle assembly (where the mitotic chromatin and MT-nucleator Augmin can drive the assembly of a mitotic spindle around the chromosomes without centrosomes) (Hayward et al., 2014). Thus, centrosomal MTs might be most important for breaking down the nuclear envelope (Hayward et al., 2014) in fly embryos and, after that point, resources are diverted to the chromatin/Augmin pathways, with the centrosomes simply needing enough MTs to

efficiently capture the MTs organised around the chromatin. In contrast, spindle assembly in worm embryos appears to be almost entirely dependent on centrosomes (Gönczy et al., 1999, 2000; Sönnichsen et al., 2005). I therefore envisage that the centrosome assembly pathways may simply be regulated in different ways to ensure that centrosomes reach the appropriate size at the appropriate time in each system.

It is easy to imagine how the putative Polo oscillation system I have identified here might be adapted to allow centrosomes to reach and maintain their maximal size during mitosis. For example, the putative Polo receptors (of which Ana1 seems to be an example in fly embryos) are simply not turned off until the end of mitosis, rather than being gradually inactivated in the run-up to mitosis, as we envisage in the *Drosophila* embryo (Figure 3.19). Interestingly, a recent study quantified how different PCM proteins were disassembled at the end of mitosis in worm embryos and found that PLK-1 was the first protein to leave the PCM, closely followed by SPD-2, with the other PCM client proteins then following afterwards with SPD-5 being the last (Mittasch et al., 2020). This is the same ordering I observe in fly embryos, although the relative timing, and timing relative to the cell cycle, is different in the two systems. Moreover, in both flies and worms it seems clear that the Cnn/SPD-5 scaffold is disassembled late in mitosis and this disassembly is dependent on a PPTase, which has been identified in worms (Enos et al., 2018).

## 4. Cytoplasmic concentrations modulate centrosome dynamics

### 4.1 Introduction

In the previous chapter, I examined the behaviour of several PCM proteins in *Drosophila* syncytial embryos and discovered that the dynamics of centrosome assembly are more complicated than I had anticipated. Unexpectedly, centrosomes grow to a relatively consistent size at successive nuclear cycles, and they do so by homeostatically adjusting their growth rate and period. I found that the levels of Polo at the centriole oscillate, and mathematical modelling revealed that this oscillation could plausibly explain the assembly kinetics of the PCM scaffold. Importantly, the dynamics of Polo, Spd-2, and Cnn I observed reflect only the overall behaviours of interdependent molecular interaction events. For example, the abundance of protein at a given time in centrosome can be divided in two components: 1) the rates of influx (entering the system) and 2) outflux (leaving the system). The balance of these rates will ultimately determine the overall behaviours of the system. It has been shown previously in the Raff laboratory that Spd-2 and Cnn exhibit a similar centrosomal flux: both proteins are recruited into the PCM close to the centriole and then flux outwards, only leaving the PCM from the more peripheral regions. Thus, the changing size of the Spd-2 and Cnn scaffolds depends on the rate at which these proteins are being recruited into the PCM around the centriole (**Spd-2IN** and **CnnIN**) and the rate at which they leave the PCM at the periphery (**Spd-2OUT** and **CnnOUT**). The rates may also be interdependent on other factors such as the presence of other centrosomal proteins and cytoplasmic concentration of them, which influence the overall dynamics.

In this chapter, I describe my analysis on which, if any, of these parameters are regulated in the embryo to bring about the changes I observe in the centrosome growth rate at successive nuclear cycles.

## 4.2 Results

### 4.2.1 The rate of Cnn incorporation into the PCM (CnnIN) decreases from cycle 11 - 13

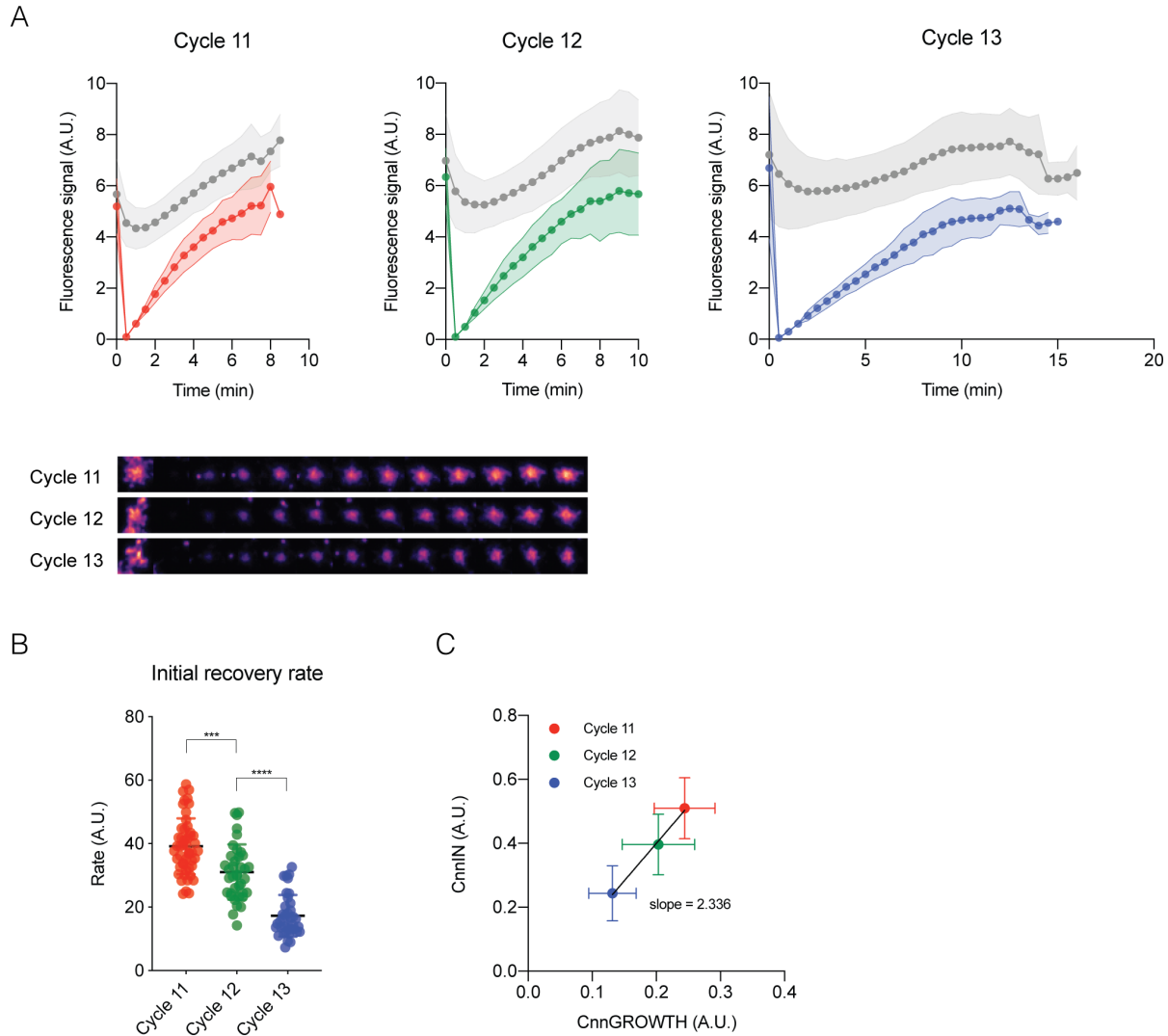
As described above, the rate of growth of the Cnn scaffold is determined by its rate of addition in the central regions (CnnIN) and its rate of loss at the PCM periphery (CnnOUT) (Conduit et al., 2010, 2015). I wanted to test whether the slowing of the Cnn growth rate observed from cycle 11 – 13 (Figure 3.7) was due to a decrease in CnnIN. I performed a FRAP analysis at the start of each nuclear cycle on a NG-Cnn (Neongreen-Cnn) transgene whose expression was driven by the ubiquitin promoter (**Ubq-NG-Cnn**, generated by Lisa Gartenmann) (Figure 4.1A). As observed previously (Conduit et al., 2010; Conduit et al., 2014b), Cnn levels first recovered in the central region of the centrosome, and the recovering fluorescence then appeared to spread outwards. I measured the initial NG-Cnn recovery rate during the first 30 seconds after FRAP (so a reasonable estimate of CnnIN) and found that it slowed significantly at successive cycles (Figure 4.1B).

Importantly, Cnn levels at the centrosome are increasing in early S-phase (see Figure 3.7), so this fluorescence recovery might represent turnover of the existing Cnn pool, addition of new Cnn molecules, or both. To assess this, I calculated the ratio of the initial recovery rate (CnnIN, measured from the colored lines in Figure 4.1A) to the total growth



rate (CnnGROWTH, measured from the gray lines in Figure 4.1A) at each cycle. This ratio was 2.09, 1.95 and 1.85 at cycles 11, 12 and 13, respectively, indicating that the photobleached centrosomes acquire fluorescence intensity at approximately twice the rate of the unbleached centrosomes (Figure 4.1C). While this might suggest that almost half of the Cnn in the central region is turning over, previous studies have strongly indicated that Cnn is only incorporated into the PCM (CnnIN) around the centrioles and only leaves the PCM (CnnOUT) at the periphery (Conduit et al., 2010; Conduit et al., 2014b). I therefore speculate that CnnIN is larger than CnnGROWTH because a significant fraction of the *total* Cnn molecules in the PCM are actually leaving the PCM from the periphery even when the centrosomes are growing (i.e. CnnOUT is not zero), but this is not the case for new Cnn molecules that have only just incorporated into the PCM—as these molecules have not had time to migrate outwards towards the periphery and leave the PCM. Thus, in my FRAP experiments I am measuring only new molecules adding into the central regions of the PCM (so this is a reasonably accurate measurement for CnnIN, as CnnOUT for these molecules is near zero), while in my centrosome growth experiments I am measuring the behaviour of all the centrosomal Cnn molecules so CnnGROWTH (CnnIN - CnnOUT) is less than CnnIN because CnnOUT is not zero for the *total* population of centrosomal Cnn molecules. In support of this interpretation, although the growth rate at the bleached centrosomes was initially much faster than at the unbleached centrosomes, the two rates were very similar by the end of S-phase; I suspect this is because the newly added fluorescent Cnn molecules have had time to spread throughout the PCM and so their off rate (CnnOUT) is now similar to that of the *total* Cnn molecules.

These studies therefore suggest that the reduction I observe in the rate of FRAP recovery at cycles 11 – 13 is largely due to a reduction in CnnIN, and this reduction presumably at least partly explains why the Cnn scaffold grows more slowly at successive cycles.

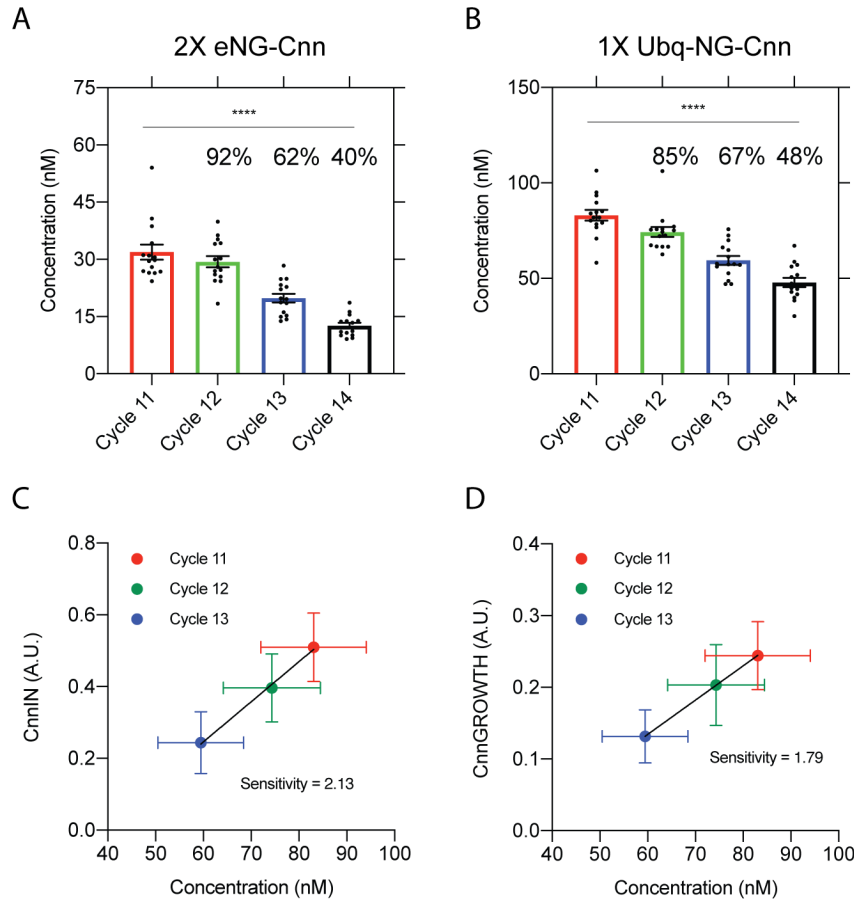


**Figure 4.1 CnnIN and growth rate of Cnn decreases during nuclear cycles 11 – 13**

**(A)** Graphs show the dynamics ( $\pm$ SD) of recovery of 1X Ubq-NG-Cnn (which is expressed at similar levels to the endogenous protein, see below) after Fluorescent Recovery After Photobleaching (FRAP) at early S-phase of cycle 11 – 13. Each colored line is an average of  $\sim$ 35 - 50 centrosomes. Gray lines illustrate the averaged centrosomal intensity ( $\pm$ SD) of unbleached centrosomes. Micrographs show examples of photobleached centrosomes tracked every 30 second in nuclear cycle 11, 12, and 13. **(B)** Bar chart compares the average initial recovery rate ( $\pm$ SD) of centrosomal Ubq-NG-Cnn (CnnIN) in nuclear cycle 11, 12, and 13. Statistical significance was assessed using a Dunnett's T3 multiple comparison test (\*\*\*:  $P < 0.001$ , \*\*\*\*:  $P < 0.0001$ ) **(C)** Graph shows the ratio of CnnIN ( $\pm$ SD) and CnnGROWTH ( $\pm$ SD) of NG-Cnn extracted from the data in (A). Slope was extracted from a linear regression model supplied by Prism 9.

#### 4.2.2 The cytosolic concentration of Cnn decreases from cycle 11 – 13

In the previous Chapter, Thomas Steinacker used FCS to show that the gradual slowing of the growth rate of the PCM client proteins was not due to any decrease in their cytosolic concentration at successive cycles (Figure 3.5). To test if this was the case for Cnn, Thomas performed a similar analysis using a CRISPR eNG-Cnn knock-in at the endogenous *cnn* locus (**endogenous NG-Cnn**, generated by Saroj Saurya) and also a ubiquitin promoter-driven NG-Cnn (**Ubq-NG-Cnn**) (Figure 4.2B). Although NG-Cnn was expressed at ~2.5X higher levels from the ubiquitin transgene, the cytosolic concentration of Cnn dropped significantly in both lines at successive nuclear cycles, and by approximately the same percentage (Figure 4.2A, B). Moreover, when we plotted the *concentration* of NG-Cnn at each cycle against *either* CnnIN or CnnGROWTH (Figure 4.2C, D), I again found a linear relationship, with a sensitivity of 2.1 or 1.7, respectively (note that for this analysis I used data from the Ubq-NG-Cnn line, which is expressed at levels most similar to the endogenous Cnn protein). This indicates the growth rate is relatively sensitive to the cytosolic concentration of Cnn (with every 1% change in Cnn concentration leading to a ~2% change in the growth rate). These data suggest the fall in the cytosolic Cnn concentration at the start of S-phase (~10-15% and ~30% from cycles 11-13 and 12-13, respectively) could entirely explain the drop in the growth rate (~20% and ~45% from cycles 11-13 and 12-13, respectively) given the sensitivity of the system (where a 1% change in concentration equated to a 2% change in growth rate).



**Figure 4.2 The initial recovery rate determines the rate of Cnn incorporation during nuclear cycles 11 – 13**

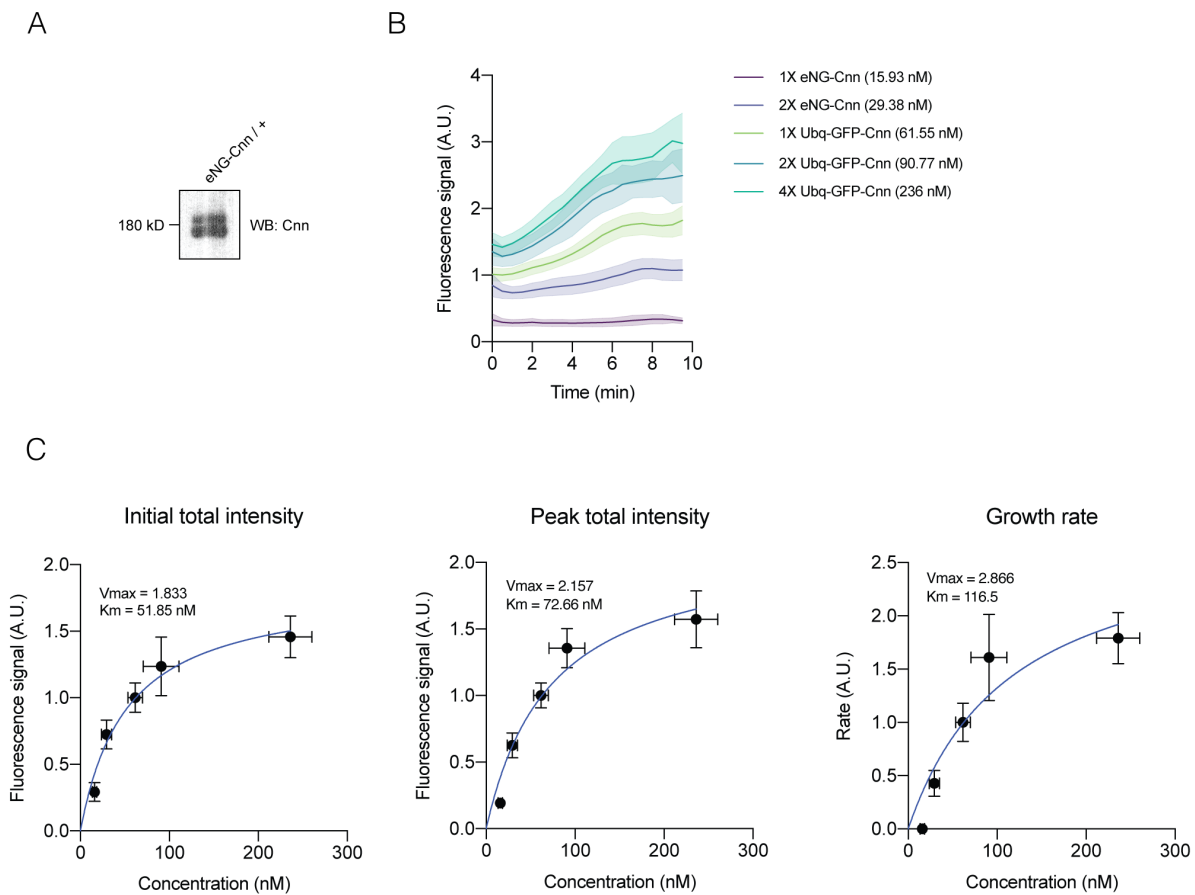
(A, B) Bar chart compares the cytosolic concentration of (A) CRISPR NG-Cnn or (B) Ubq-NG-Cnn at the start of nuclear cycles 11 - 14. Statistical significance was assessed using an ordinary one-way ANOVA in Prism 9. Experiments were performed and graphs were prepared by Thomas Steinacker. (C, D) Graph shows the correlation of cytoplasmic concentrations of NG-Cnn against (C) Spd-2IN or (D) Spd-2GROWTH during cycles 11 - 13. Sensitivity was calculated by a mid-point method ( $\delta Y/Y_0 / \delta X/X_0 \rightarrow \delta Y / \delta X * X_0 / Y_0$  where  $X_0$  and  $Y_0$  are centroid along Y-axis and X-axis): slope \* average of concentrations / average of parameters in y-axis. Slope was extracted from a linear regression model supplied by Prism 9.

To test this plausibility of this idea I wanted to test whether changing the cytosolic concentration of Cnn could influence the Cnn growth rate within a single cycle. I varied the cytoplasmic concentration of Cnn and measured the dynamics of Cnn recruitment during nuclear cycle 12. At the same time, Thomas Steinacker used FCS to measure the

cytosolic concentration of Cnn at the start of nuclear cycle 12 under each condition. Together we tested the following constructs (either knocked-in at the endogenous *cnn* locus or expressed in a *cnn* null mutant background, with the cytosolic concentration of each protein measured by Thomas given in brackets): **1X eNG-Cnn** (the CRISPR NG knock-in at the endogenous *cnn* locus) ( $\sim 16nM$ ); **2X eNG-Cnn** ( $\sim 29nM$ ); **1X Ubq-GFP-Cnn** (a fusion of GFP to a full length Cnn cDNA, driven by the ubiquitin promoter) ( $\sim 61nM$ ); **2X Ubq-GFP-Cnn** ( $\sim 91nM$ ); **4X Ubq-GFP-Cnn** ( $\sim 236nM$ ). To accurately compare the centrosomal fluorescence of NG-Cnn and GFP-Cnn I applied a “scaling” factor that I calculated by comparing the centrosomal fluorescence levels of 1X Ubq-NG-Cnn and 1X Ubq-GFP-Cnn, which Thomas Steinacker showed were expressed at very similar levels (data not shown). I also performed a western blot that revealed that the endogenous Cnn is expressed at a higher level than the eNG-Cnn knock-in (Figure 4.3A). Thus, we estimate that endogenous Cnn is normally present in embryos at  $\sim 75-85nM$  in cycle 11 and at  $\sim 50-60nM$  in cycle 13.

Cnn dynamics showed a graded response to the alteration of its cytoplasmic concentration, and all the growth parameters (initial intensity, peak intensity, and growth rate) grew steadily as the Cnn concentration increased from  $\sim 15nM$  to  $\sim 90nM$  but appeared to have largely saturated by  $\sim 200nM$  (Figure 4.3A, B). Thus, Cnn scaffold assembly exhibits typical Michaelis-Menton kinetics. This is in line with our thinking that the centriole produces S\* (a form of Spd-2 that can bind both Polo and Cnn) that effectively acts as an enzyme to catalyse the production of the Cnn scaffold (C\*) from cytosolic Cnn (C) (Figure 4.4A). The cytosolic concentration of Cnn appears to be  $\sim 50-$

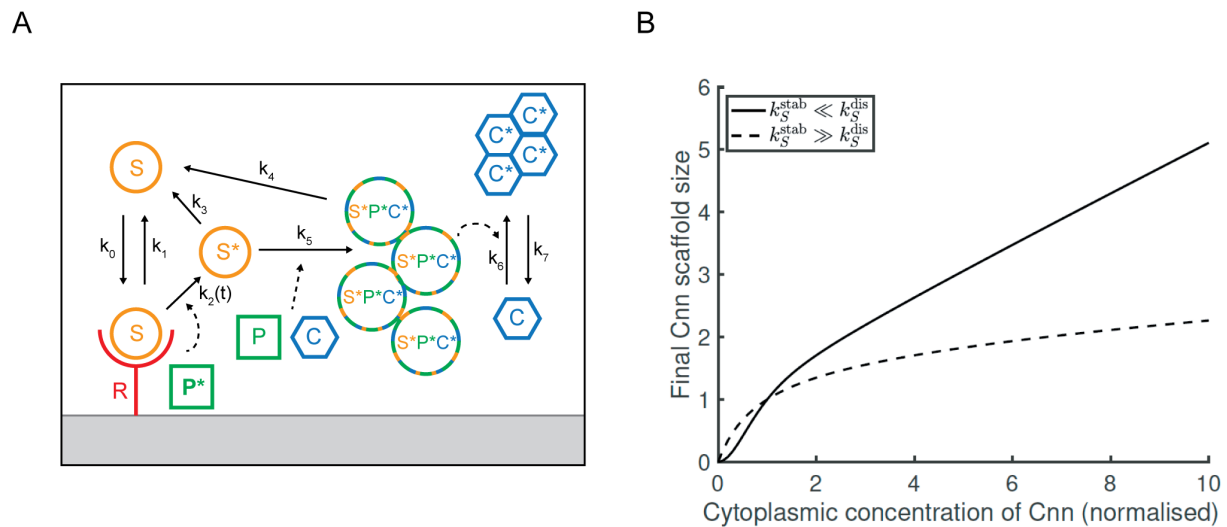
85nM during cycles 11 – 13 (see above), so within the range where small changes in the cytosolic concentration of Cnn can significantly change the Cnn scaffold growth parameters.



**Figure 4.3 Cnn dynamics are concentration dependent but saturate at high concentration**

**(A)** Western blot compares embryonic expression levels of endogenous Cnn (lower band) to that of a CRISPR NG-Cnn knock-in (upper band) at the endogenous *cnn* locus. Embryos were collected from heterozygous females with one copy of the endogenous allele and one copy of the knock-in allele. **(B)** Graph shows the dynamics ( $\pm$ SD) of NG-Cnn or GFP-Cnn at different concentrations (as indicated). Each set has > 10 embryos. **(C)** Graphs show the response of initial intensity, peak intensity, and growth rate (CnnGROWTH) to concentration (as indicated). Dots are measured data from dynamics and FCS ( $\pm$ SD). Blue lines are a Michaelis-Menton Kinetics fitted model regressed in Prism 9.

Zachary Wilmott used the mathematical model of PCM scaffold assembly we had developed in **Chapter 3** to examine the effect of changing the concentration of Cnn (Figure 4.4A). He found that under conditions where Cnn binds to and stabilises  $S^*$  faster than  $S^*$  normally decays (i.e.  $k_5^{\text{stab}}(k_5) \gg k_5^{\text{dis}}(k_3)$ ) the system saturates at higher Cnn concentrations (Figure 4.4B), because Cnn eventually stabilises essentially all the  $S^*$  that is produced—so the rate of  $S^*$  production becomes limiting. In contrast, if Cnn binds to and stabilises  $S^*$  more slowly than  $S^*$  normally decays (i.e.  $k_5 \ll k_3$ ), then the system does not saturate and the Cnn scaffold can continue to grow linearly over a large concentration range—because  $S^*$  production does not become limiting. Thus, if our model is correct, we would predict that Cnn bind to  $S^*$  efficiently, which seems biologically plausible.



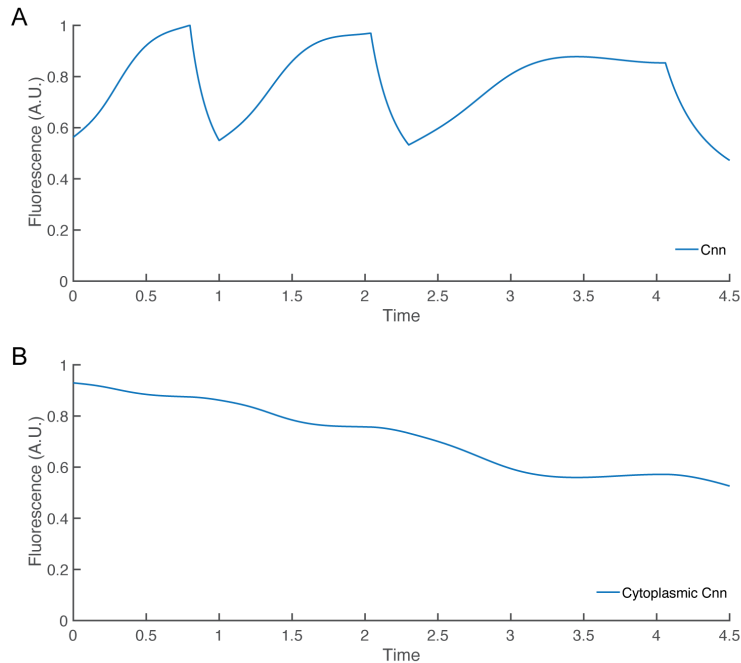
**Figure 4.4 Mathematical modelling shows a dose saturation relationship of final scaffold size**

(A) Mathematical model described in Figure 3.15. (B) Final Cnn scaffold size as a function of the cytoplasmic concentration of Cnn for the cases in which  $k_5^{\text{stab}}(k_5) \gg k_5^{\text{dis}}(k_3)$  (dotted line) and  $k_5^{\text{stab}}(k_5) \ll k_5^{\text{dis}}(k_3)$  (solid line). This graph was prepared by Zachary Wilmott.



#### 4.2.3 Centrosomes could potentially sequester Cnn to cause a drop in its cytosolic concentration

These observations demonstrate that the reduction in the cytosolic concentration of Cnn that we observe between cycles 11 – 13 could largely explain the reduction in the Cnn growth rate. But they do not explain *why* cytosolic Cnn levels drop at successive cycles. An attractive possibility is that the exponentially increasing number of centrosomes sequester more Cnn at each cycle. To test the plausibility of this idea, Zachary Wilmott ran simulations of our PCM scaffold assembly model in which he kept the total amount of Cnn molecules within the embryo constant and allowed centrosome numbers to increase exponentially over an equivalent of nuclear cycles 11 – 13. In this model, the concentration of Cnn in the cytoplasm dropped by successively larger amounts during each successive S-phase (Figure 4.5A, B).



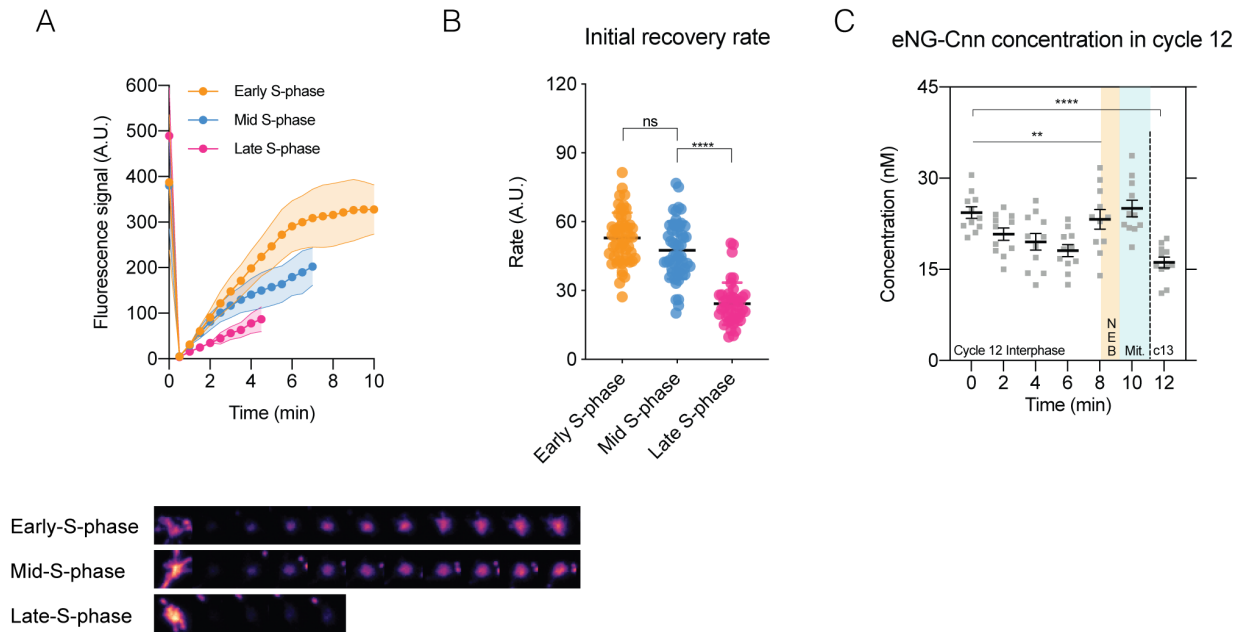
**Figure 4.5 Mathematical model predicts Cnn concentration drop in cycles 11, 12, and 13**

Graphs show numerical simulations (based on model described in Chapter and illustrated in Figure 4.4A) of the growth of the Cnn scaffold (**A**) and the corresponding changes in the cytosolic concentration of Cnn (**B**) over nuclear cycles 11-13. Note that the periodic drop in Cnn levels at the end of each cycle (A) occurs because in our model the existing Cnn scaffold is redistributed from the single parental centrosome to the two daughter centrosomes.

If this model is correct, then it would predict that the cytosolic concentration of Cnn should steadily fall during each round of S-phase (as the Cnn starts to become sequestered at the growing centrosomes), and there should be a concomitant drop in CnnIN. I used FRAP to measure the initial recovery rate (i.e. CnnIN) in early-, mid- and late-S-phase of cycle 12 and found that CnnIN decreased as S-phase progressed, as predicted (Figure 4.6A, B). Moreover, Thomas Steinacker showed using FCS that the cytosolic concentration of Cnn also fell *during* S-phase of cycle 12 (Figure 4.6C). The fall in the cytosolic Cnn concentration during S-phase (~25%) could potentially explain the more dramatic drop in the growth rate (~55%), given the sensitivity of the system calculated above (where a 1% change in concentration equated to a 2% change in growth rate).

Given that the levels of Polo and Spd-2 peak in mid-S-phase and then start to decline, it seems likely that the reduction in Polo/Spd-2 levels in late-S-phase (~20% drop compared to the peak in mid-S-phase, Figure 3.7) will also contribute to the reduction in CnnIN as S-phase progresses.

Intriguingly, the FCS data revealed that cytosolic Cnn levels start to rise again in mitosis, and then dropped suddenly at the start of the next S-phase. This is puzzling, as Cnn levels at the centrosome generally plateau during mitosis in cycle 12, suggesting that Cnn molecules, on average, are not being released from the centrosome back into the cytosol during mitosis. These observations suggest that Cnn protein is being synthesized in these embryos (explaining why cytosolic levels increase during mitosis) and that our idea that cytosolic Cnn levels drop at each cycle because more Cnn is sequestered at centrosomes is, at best, only a partial explanation, as it cannot explain the dramatic drop in cytosolic Cnn levels that Thomas observes at the end of mitosis.



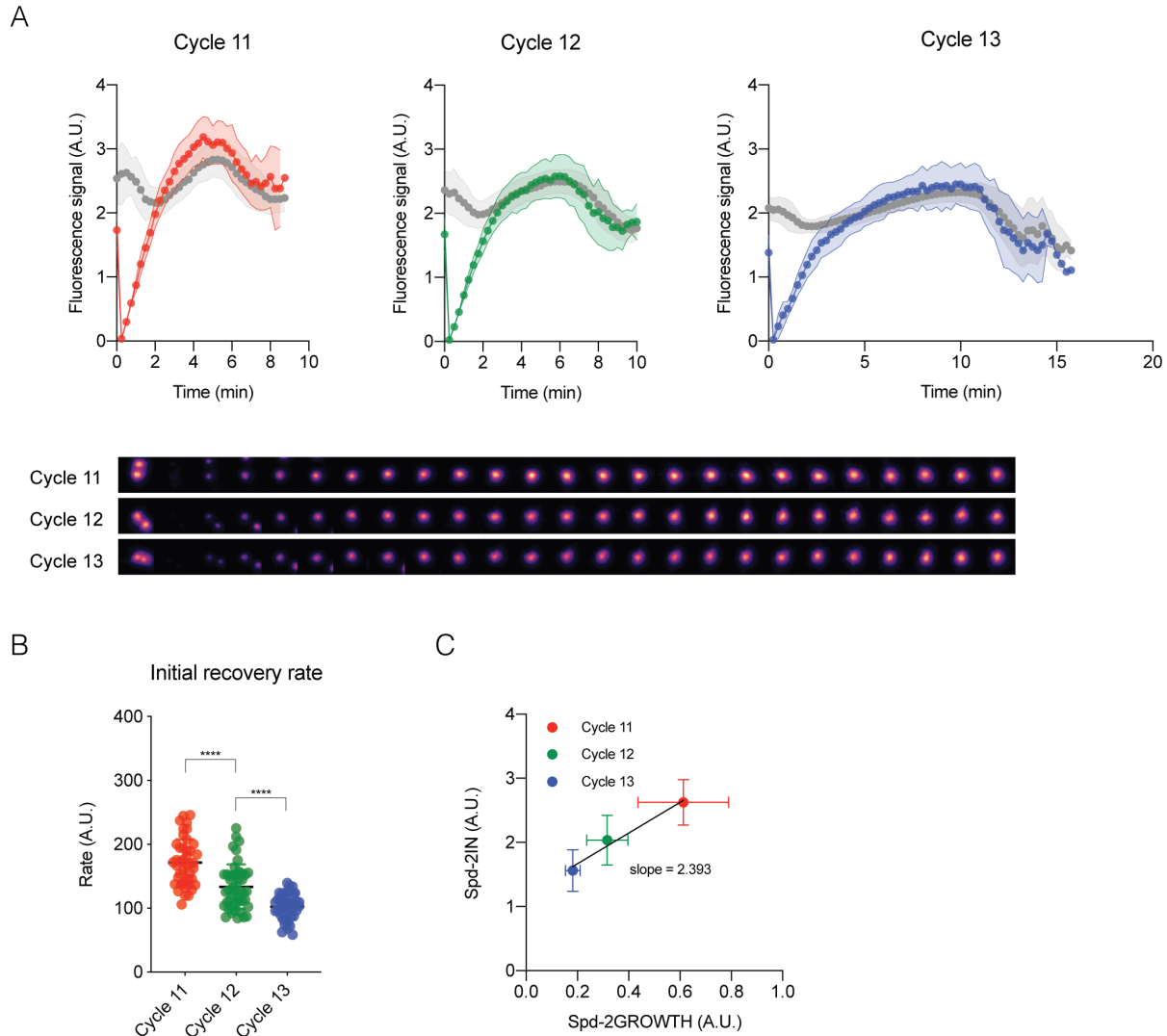
**Figure 4.6 Dynamics of CnnIN and Cnn concentration during cycle 12**

(A) Graphs show the dynamics ( $\pm$ SD) of recovery of centrosomal NG-Cnn fluorescent intensity ( $n = \sim 40$  centrosomes for each set) after FRAP in early S-phase (orange line), mid S-phase (blue line), and late S-phase / early mitosis (pink line). Micrographs shows examples of individual photobleached centrosomes tracked every 30 second. (B) Bar chart compares the initial recovery rate (CnnIN) of early S-phase, mid S-phase, and late S-phase / early mitosis. Each dot is a centrosome. Statistical significance was first assessed by a Kruskal Wallis's test (non-Gaussian distributed). If it was significant, multiple testing using Dunn's test was performed (\*\*\*:  $P < 0.001$ , ns: not significant). (C) Graph shows the changes of concentration of NG-Cnn during cycle 12. Statistical significance was determined by a RM one-way ANOVA with a Geisser-Greenhouse correction. Orange region shows an approximate time window of nuclear envelope breakdown (NEB). Cyan region represents an approximate duration of mitosis (Mit.). Experiments were performed and graph was prepared by Thomas Steinacker.

#### 4.2.4 The rate of Spd-2 incorporation into the PCM (Spd-2IN) decreases from cycle 11 – 13

Although Cnn and Spd-2 exhibit very different growth kinetics at centrosomes, both proteins grow at a slower rate at successive nuclear cycles (Figure 3.8). I wondered whether, like Cnn, the rate of Spd-2 incorporation into the PCM (Spd-2IN) slowed at successive cycles and whether this could potentially be explained by a decrease in the cytosolic concentration of Spd-2.

I used FRAP to examine if Spd-2IN changed at successive cycles. I found that, like CnnIN, Spd-2IN decreased at each cycle (Figure 4.7A). Interestingly, the ratio of the initial recovery rate (Spd-2IN, measured from the colored lines in Figure 4.7A) to the total growth rate (Spd-2GROWTH, measured from the gray lines in Figure 4.7A) at each cycle was 4.29, 6.43, and 8.58 in cycles 11-13, respectively (Figure 4.7C). Thus, the photobleached centrosomes initially re-acquire fluorescence intensity at much higher rate than the unbleached centrosomes, and this difference is more pronounced at later cycles. As was the case for Cnn, I suspect that this difference is, at least in part, explained by the rate of Spd-2OUT being a significant factor that slows the overall growth rate (Spd-2GROWTH, which equals  $\text{Spd-2IN} - \text{Spd-2OUT}$ ) but does not influence newly incorporated Spd-2 molecules, which have not had time to flux outwards to the more peripheral regions. In support of this interpretation one can see that at the start of S-phase the total Spd-2 at the centrosomes is actually in decline in these graphs, even though Spd-2IN is high—suggesting that Spd-2OUT is higher than Spd-2IN at the end of mitosis/early S-phase (Figure 4.7A). Moreover, as was the case with Cnn, although the growth rate at the bleached centrosomes was initially much faster than at the unbleached centrosomes, the two rates were very similar by the end of S-phase, potentially because the newly added fluorescent Spd-2 molecules have by then had time to spread throughout the PCM and so their off rate (Spd-2OUT) is now similar to that of the total Spd-2 molecules.



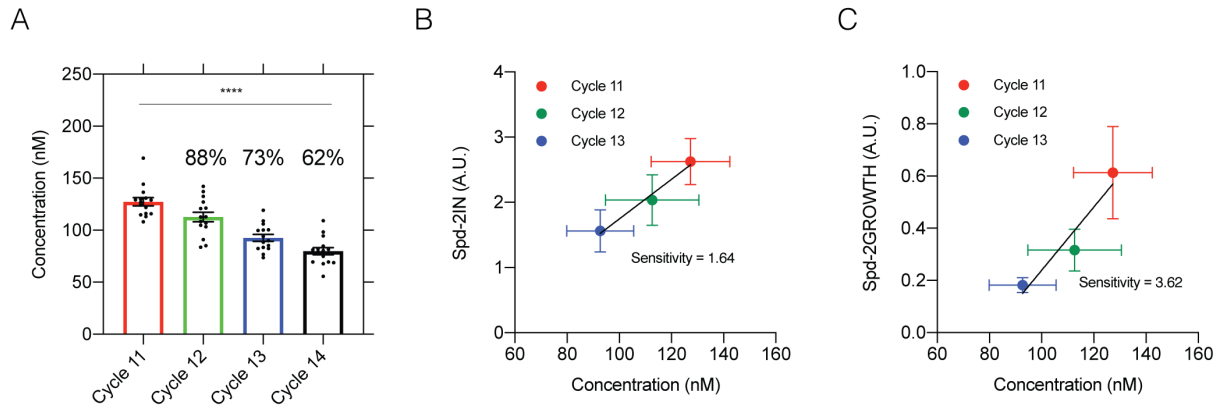
**Figure 4.7 Spd-2IN and growth rate of Spd-2 decreases during nuclear cycles 11 – 13**

(A) Graphs show the dynamics ( $\pm$ SD) of recovery of CRISPR knock-in Spd-2-NG (which is expressed at similar levels to the endogenous protein, see below) after Fluorescent Recovery After Photobleaching (FRAP) at early S-phase of cycle 11 – 13. Each colored line is an average of  $\sim 35$  - 50 centrosomes. Gray lines illustrate the averaged centrosomal intensity ( $\pm$ SD) of unbleached centrosomes. Micrographs show examples of photobleached centrosomes tracked every 30 second in nuclear cycle 11, 12, and 13. (B) Bar chart compares the average initial recovery rate ( $\pm$ SD) of centrosomal Spd-2-NG (Spd-2IN) in nuclear cycle 11, 12, and 13. Statistical significance was assessed using a Dunnett's T3 multiple comparison test (\*\*\*:  $P < 0.001$ , \*\*\*\*:  $P < 0.0001$ ) (C) Graph shows the ratio of Spd-2IN ( $\pm$ SD) and Spd-2GROWTH ( $\pm$ SD) of Spd-2-NG extracted from the data in (A). Slope was extracted from a linear regression model supplied by Prism 9.

#### 4.2.5 The cytosolic concentration of Spd-2 decreases from cycle 11 – 13 and strongly influences Spd-2IN

In **Section 4.2.2** I collaborated with Thomas Steinacker to show that the cytosolic concentration of Cnn gradually decreased from nuclear cycles 11 – 13, and we concluded that this was likely to play a significant part in driving the corresponding decreases in CnnIN. To test if this was also the cases for Spd-2, Thomas Steinacker used FCS to analyse an endogenous CRISPR Spd-2-NG knock-in line generated by Saroj Saurya and found that the cytosolic concentration of Spd-2 at the start of S-phase dropped significantly at successive nuclear cycles (Figure 4.8A). Moreover, when we plotted the concentration of Spd-2 at each cycle against either Spd-2IN or Spd-2GROWTH, we found a near-linear relationship with a sensitivity of 1.64 or 3.62 (Figure 4.8B, C). Thus, a 1% change in Spd-2 concentration leads to a ~1.6% change in Spd-2IN and a ~3.6% change in Spd-2GROWTH. The larger effect on Spd-2GROWTH may be due to the Polo/Spd-2/Cnn positive feedback loop, as increasing levels of Spd-2 might not only *increase* Spd-2IN but may also *decrease* Spd-2OUT (as more Spd-2 is retained within the PCM), so amplifying the effect of the change in concentration on Spd-2GROWTH.

These data suggest that the fall in the cytosolic Spd-2 concentration at the start of S-phase (~12% and ~17% from cycles 11-12 and 12-13, respectively) could entirely explain the drop in the growth rate (~24% and ~23% from cycles 11-12 and 12-13, respectively) given the sensitivity of the system (where a 1% change in concentration equated to a ~3.6% change in growth rate).



**Figure 4.8 The initial recovery rate determines the rate of Spd-2 incorporation during nuclear cycles 11 – 13**

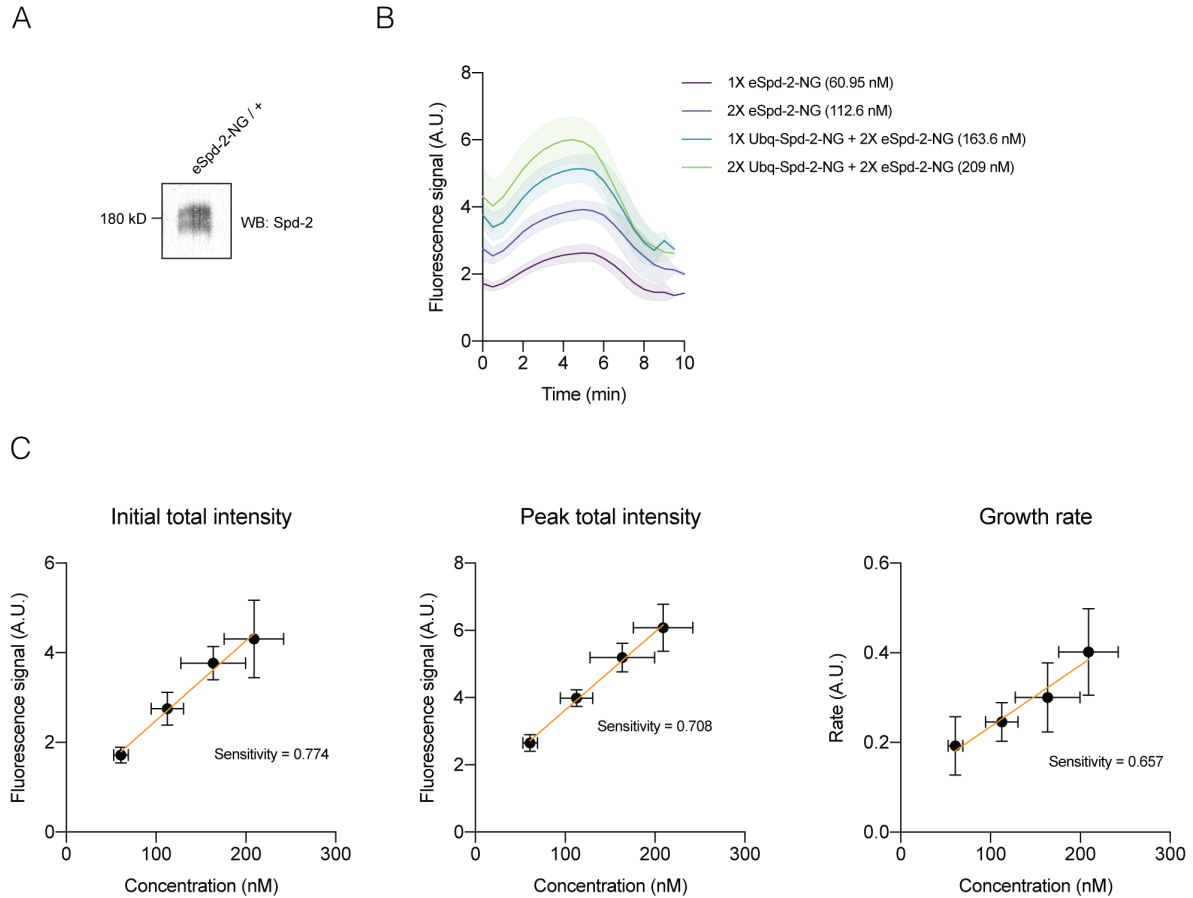
(A) Bar chart compares the cytosolic concentration of CRISPR Spd-2-NG knock-in at the start of nuclear cycles 11 - 14. Statistical significance was assessed using an ordinary one-way ANOVA in Prism 9. Experiments were performed and graphs were prepared by Thomas Steinacker. (B, C) Graph shows the correlation of cytoplasmic concentrations of Spd-2-NG against (B) Spd-2IN or (C) Spd-2GROWTH during cycles 11 - 13. Sensitivity was calculated by a mid-point method ( $\delta Y/Y_0 / \delta X/X_0 \rightarrow \delta Y / \delta X * X_0 / Y_0$  where  $X_0$  and  $Y_0$  are centroid along Y-axis and X-axis): slope \* average of concentrations / average of parameters in y-axis. Slope was extracted from a linear regression model supplied by Prism 9.

To directly test whether changing the cytosolic concentration of Spd-2 could influence the Spd-2GRWOTH within a single cycle, I varied the cytoplasmic concentration of Spd-2 and measured the dynamics of Spd-2 recruitment during nuclear cycle 12. At the same time, Thomas Steinacker used FCS to measure the cytosolic concentration of Spd-2 at the start of nuclear cycle 12 under each condition. Together we tested the following constructs that were all expressed in the absence of any endogenous untagged Spd-2 (the cytosolic concentration of each protein measured by Thomas is shown in brackets): **1X eSpd-2-NG** (the CRISPR knock-in at the endogenous *Spd-2* locus) ( $\sim 61nM$ ); **2X eSpd-2-NG** ( $\sim 113nM$ ); **1X Ubq-Spd-2-NG**; **2X eSpd-2-NG** ( $\sim 164nM$ ); **2X Ubq-Spd-2-NG**; **2X eSpd-2-NG** ( $\sim 209nM$ ). I also performed a western blot that revealed that the endogenous Spd-2 is expressed at a similar level to the eSpd-2-NG knock-in (Figure 4.9A).



Spd-2 dynamics showed a graded response to the alteration of its cytoplasmic concentration, and all the growth parameters (initial intensity, peak intensity, and growth rate) increased nearly linearly as the Spd-2 concentration increased from ~60nM to ~209nM (Figure 4.9B, C). Unlike our similar experiments with Cnn (Figure 4.3), these parameters did not appear to saturate at the highest Spd-2 concentration, but I note that the normal Spd-2 concentration at the start of cycle 12 is ~112nM and so we effectively only examined conditions where we had approximately halved or doubled the normal concentration of Spd-2. In contrast, for our analysis of Cnn, we were able to explore a broader range of conditions, and we only observed clear saturation when we had increased the normal concentration in cycle 12 (~40-60nM) by ~3-4X (~236nM). Thus I suspect the Spd-2 growth parameters would eventually saturate if we could push the cytosolic concentration of Spd-2 high enough.

From these experiments I conclude that the cytosolic concentration of Spd-2 during cycles 11 – 13 is ~90-130nM, so well within the range where small changes in the cytosolic concentration of Spd-2 can significantly alter the Spd-2 scaffold growth parameters.

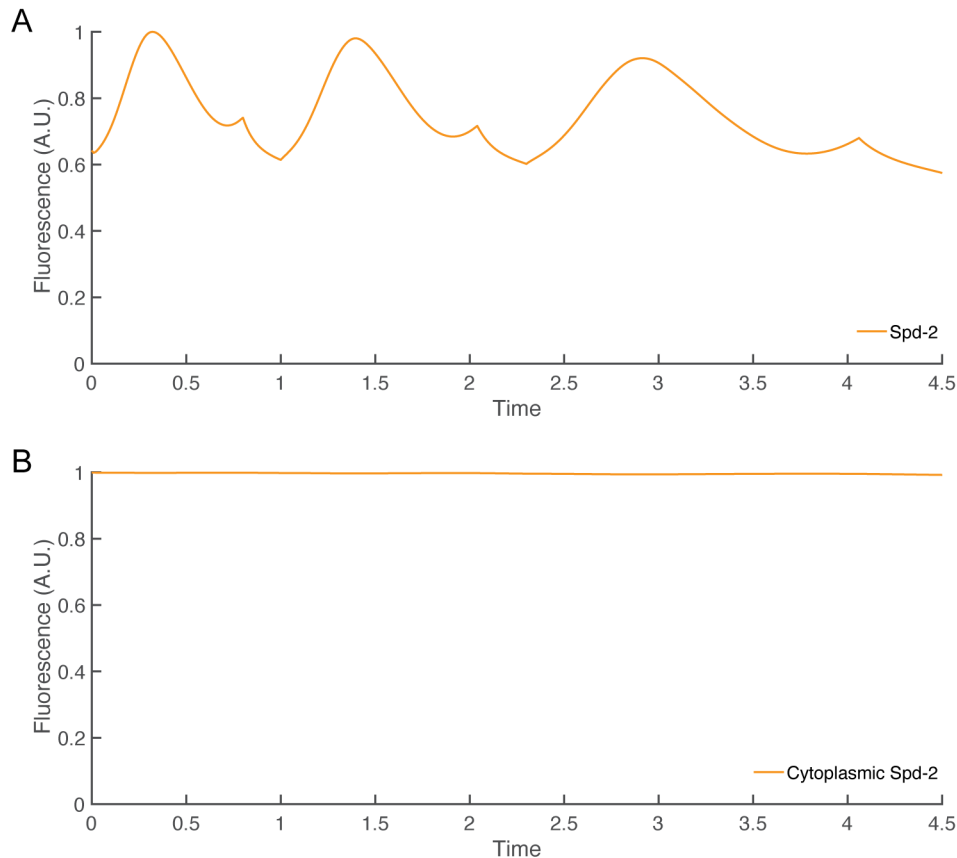


**Figure 4.9 Spd-2 dynamics are concentration dependent**

**(A)** Western blot compares embryonic expression levels of endogenous Spd-2 (lower band) to that of a CRISPR Spd-2-NG knock-in (upper band) at the endogenous *Spd-2* locus. Embryos were collected from heterozygous females with one copy of the endogenous allele and one copy of the knock-in allele. **(B)** Graph shows the dynamics ( $\pm$ SD) of Spd-2-NG at different concentrations (as indicated). Each set has > 10 embryos. **(C)** Graphs show the response of initial intensity, peak intensity, and growth rate (Spd-2GROWTH) to concentration (as indicated). Dots are measured data from dynamics and FCS ( $\pm$ SD). Blue lines are a linear regressed model regressed in Prism 9. Sensitivity was calculated by a mid-point method ( $\delta Y/Y_0 / \delta X/X_0 \rightarrow \delta Y / \delta X * X_0 / Y_0$  where  $X_0$  and  $Y_0$  are centroid along Y-axis and X-axis): slope \* average of concentrations / average of parameters in y-axis. Slope was extracted from a linear regression model supplied by Prism 9.

#### 4.2.6 The sequestration of Spd-2 at centrosomes is unlikely to be the primary cause of the drop in its cytosolic concentration at successive cycles

These observations demonstrate that the reduction in the cytosolic concentration of Spd-2 that we observe between cycles 11 – 13 could help to explain the concomitant reduction in the Spd-2 growth rate. To examine whether this concentration-drop could be caused by the exponentially increasing number of centrosomes sequestering more Spd-2 at successive cycles, Zachary Wilmott ran simulation of our PCM scaffold assembly model in which he kept the total amount of Spd-2 molecules within the embryo constant and allowed centrosome numbers to increase exponentially over an equivalent of nuclear cycles 11 – 13. Perhaps surprisingly, and in contrast to our previous modelling of Cnn (Figure 4.5), the concentration of Spd-2 in the cytosol did not drop at successive nuclear cycles (Figure 4.10B). An inspection of the model revealed that the two proteins behave differently in this respect because Spd-2 is present in the embryo at a higher concentration than Cnn, and because significantly less Spd-2 is recruited to centrosomes than Cnn at each cycle.

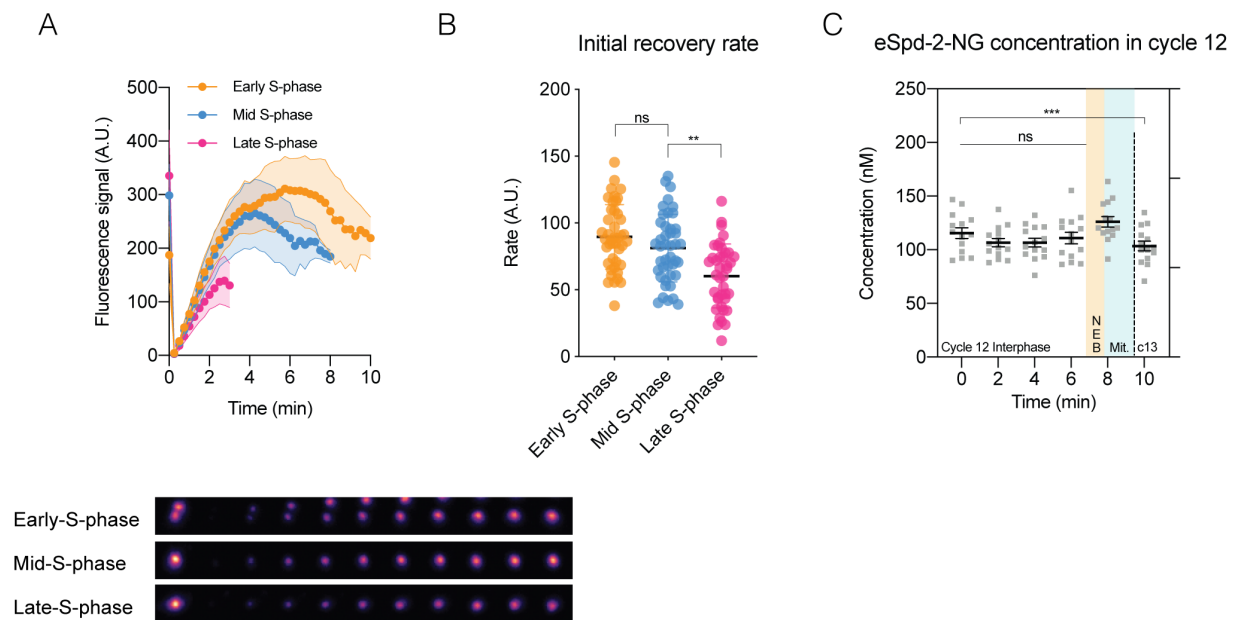


**Figure 4.10 Mathematical model predicts Spd-2 concentration stays constant in cycles 11, 12, and 13**

Graphs show numerical simulations (based on model described in Chapter and illustrated in Figure 4.4A) of the growth of the Spd-2 scaffold **(A)** and the corresponding changes in the cytosolic concentration of Spd-2 **(B)** over nuclear cycles 11-13. Note that the levels of Spd-2 do not drop at the end of each cycle.

In light of these findings, I wondered whether Spd-2 would behave differently to Cnn in terms of how its incorporation rate (Spd-2IN) and cytosolic concentration would vary during nuclear cycle 12. I used FRAP to measure Spd-2IN in early-, mid- and late-S-phase of cycle 12 and found that, as for CnnIN, this rate decreased as S-phase proceeded (Figure 4.11A, B). In contrast to Cnn, however, Thomas Steinacker showed using FCS that the cytosolic concentration of Spd-2 did not decrease during S-phase of cycle 12, although, like Cnn, cytosolic Spd-2 levels started to rise as embryos entered mitosis, and

then dropped sharply as the embryos entered the next round of S-phase (Figure 4.11C). I draw two important conclusions from these data: (1) Spd-2-IN decreases *during* cycle 12, even though the cytoplasmic concentration of Spd-2 does not significantly decrease; (2) The sequestration of Spd-2 at centrosomes cannot explain the drop in the cytosolic levels of Spd-2 at successive nuclear cycles.



**Figure 4.11 Dynamics of Spd-2IN and Spd-2 concentration during cycle 12**

(A) Graphs show the average recovery of centrosomal intensity ( $\pm$ SD) of Spd-2-NG after FRAP in early S-phase (orange line), mid S-phase (blue line), and late S-phase / early mitosis (pink line). Each set consists of > 30 centrosomes. Micrographs show examples of photobleached centrosomes tracked every 30 second. (B) Bar chart compares the initial recovery rate (Spd-2IN) of early S-phase, mid S-phase, and late S-phase / early mitosis. Each dot is a centrosome. Statistical significance was first assessed by a Kruskal Wallis's test (non-Gaussian distributed). If it was significant, multiple testing using Dunn's test was performed (\*\*\*:  $P < 0.001$ , ns: not significant). (C) Graph shows the changes of concentration of Spd-2-NG during cycle 12. Statistical significance was determined by a RM one-way ANOVA with a Geisser-Greenhouse correction. Orange region shows an approximate time window of nuclear envelope breakdown (NEB). Cyan region represents an approximate duration of mitosis (Mit.). Experiments were performed and graphs were prepared by Thomas Steinacker.

### 4.3 Discussion

In this chapter, I have used FRAP to show that the rate at which Spd-2 and Cnn are recruited into the PCM (Spd-2IN and CnnIN) decreases at successive nuclear cycles. If we assume that Spd-2 and Cnn do not turnover within the PCM (i.e. they only flux through it, incorporating at the centriole and then leaving at the periphery) then my measurements of the recruitment rates and growth rates (Spd-2GROWTH and CnnGROWTH) allow us to infer the off-rates (as  $GROWTH = IN - OUT$ ), potentially allowing us to calculate all three parameters and so derive a full description of how each parameter changes within and between each nuclear cycles. Such an analysis, for example, would allow us to infer that for Spd-2 and Cnn, both the IN and OUT rates decrease at each successive cycle (inferred by calculating the OUT rate by subtracting the measured GROWTH rate from the measured IN rate), but that the IN rates decrease slightly faster, so the overall GROWTH rate decreases. I remain cautious in interpreting my data in this way as these calculations assume there is absolutely no turnover of Spd-2 and Cnn molecules within the PCM. Although much of my data—and previously published data (Conduit et al., 2010; Conduit et al., 2014b)—is consistent with this possibility, it cannot be ruled out that there is some turnover and that this may vary depending on the conditions.

Joao Monterio in the laboratory has been following the behaviour of single molecules of Spd-2 and Cnn within the PCM and his preliminary data suggests that the vast majority of molecules bind near the centriole and flux outwards, but a significant fraction can leave the PCM before they reach the periphery. It is probably more accurate, therefore, to say that Spd-2 and Cnn molecules bind near the centriole and then have an increasing

probability of leaving the PCM as they flux outwards. When these single molecule data have been properly assessed it may be possible to calculate firm rates for these growth parameters with more confidence. Nevertheless, my data indicate that the reduction in Spd-2IN and CnnIN are likely to play a major part in reducing the overall growth rate of these scaffolding proteins at successive nuclear cycles.

Why do Spd-2IN and CnnIN decrease at successive nuclear cycles? Thomas Steinacker showed that the cytosolic concentration of both proteins decreases at the start of S-phase at successive cycles, and our data together suggests that this decrease is likely to be sufficient to explain the decrease in Spd-2IN and CnnIN. We found that, at near-physiological concentrations, Spd-2IN and CnnIN are sensitive to the cytosolic concentration of protein (with a 1% change in cytosolic concentration giving a ~1.5-3.6% change in the IN rate, depending on the protein and nuclear cycle). At this sensitivity, the reduction in protein concentration at each cycle is sufficient (and often more than sufficient) to explain the reduction in the overall growth rate. Again, I remain cautious about interpreting this data to derive absolute numbers. My data suggests that both the IN and OUT rates can change for each protein at each cycle, and our positive feedback model would intuitively suggest that any changes to the IN rate may also influence the OUT rate—perhaps explaining why both these parameters seem to decrease at successive cycles. In addition, it seems likely that several other mechanisms could influence the IN rate at each cycle; for example, the rate at which Ana1 is converted to A\* by a protein kinase, (Figure 3.19).

Interestingly, Thomas and I also collaborated to show that Spd-2IN and CnnIN both decreased as S-phase progressed within a single nuclear cycle; for Cnn this was correlated with a decrease in its cytosolic concentration, but this was not the case for Spd-2. Thus, for Spd-2 at least, other factors can influence Spd-2IN as S-phase progresses. An obvious possibility is that the conversion of Ana1 to A\* and then to A<sup>Δ</sup>, which we propose to generate the Polo oscillation (Figure 3.19), leads to the decline in Spd-2IN as S-phase progresses.

Together, these studies highlight the potential importance of regulating the cytosolic concentration of centrosome proteins to ensure their appropriate assembly kinetics. For all the PCM scaffold and client proteins the rate of their assembly decreases from nuclear cycle 11-13. For the client proteins and the scaffold protein Polo, their cytosolic concentration remains constant throughout these cycles, while the concentration of the scaffold proteins Spd-2 and Cnn reduces at each cycle. Our data and mathematical modelling suggest that for Cnn this reduction occurs, at least in part, because increasing amounts of Cnn are sequestered at the exponentially increasing number of centrosomes generated at each cycle. I note that this is potentially an elegant mechanism that would allow embryos to effectively “count” the number of centrosomes they have by effectively monitoring the amount of Cnn in the cytoplasm to set their growth rate accordingly. In this scenario, the rate of Cnn scaffold assembly would naturally slow as centrosome numbers increase.



My data also suggests, however, that this mechanism cannot on its own explain how the cytosolic concentration of Cnn is regulated in the embryo. During nuclear cycle 12, for example, cytosolic Cnn levels are initially depleted as centrosomes assemble (as expected), but then rise as the embryos enter mitosis (even though Cnn levels at the centrosome remain constant) before falling rapidly as the embryo enters the next cycle. Moreover, the cytosolic concentration of Spd-2 exhibits a similar behaviour: levels drop slightly in early S-phase and then rise as embryos enter mitosis (although these trends were not statistically significant) before levels fall significantly as the embryos enter the next cycle. (Note that our modelling did not predict that cytosolic levels of Spd-2 would change during S-phase because Spd-2 is expressed at higher levels, and less total Spd-2 is sequestered at centrosomes, compared to Cnn). We conclude that both Spd-2 and Cnn are being synthesized in the cytosol (at least in late S-phase, and potentially throughout S-phase) and that a fraction of the cytosolic protein is then rapidly degraded towards the end of mitosis. We currently do not know how this potential degradation might be regulated, but an intriguing possibility is that the Spd-2 and Cnn molecules that have bound to centrosomes and then been released as they flux through the PCM are somehow marked for subsequent degradation at the end of mitosis. This would effectively be a variation of the “centrosome counting” mechanism, as the amount of Spd-2 and Cnn degraded at every cycle would again be proportional to the number of centrosomes present in the embryo at that cycle.

## 5. Mitotic centrosomes are organised by co-existing solid- and liquid-like scaffolds

### 5.1 Introduction

Although the pathway of mitotic PCM assembly has been extensively studied, particularly in flies and worms, it remains unclear whether the PCM scaffold is best described as a solid-like or liquid-like structure (Raff, 2019; Woodruff, 2021). During the past few years there has been an explosion of interest in the idea that non-membrane bound organelles like the centrosome might be formed by liquid-liquid phase separation, and there is now compelling evidence that structures such as P-granules, stress-granules and various processing bodies in the cytoplasm and nucleus can be thought of as biomolecular condensates that often have liquid-like properties (Banani et al., 2017).

In worm embryos, the main PCM scaffolding protein SPD-5 (the functional homologue of Cnn in flies) can form liquid-like condensates *in vitro* that can concentrate several other PCM proteins and nucleate MTs, prompting speculation that the centrosome in worm embryos functions primarily as a selective condensate that nucleates MTs by concentrating tubulin (Woodruff et al., 2017). However, the evidence that centrosomal SPD-5 is liquid-like *in vivo* is thin (Woodruff et al., 2017), and FRAP studies indicate that SPD-5 molecules within the PCM undergo very little internal rearrangement, consistent with a more solid-like scaffold (Laos et al., 2015). Moreover, in flies, the Cnn scaffold also appears to be solid-like *in vivo* (Conduit et al., 2014a; Feng et al., 2017), and two small fragments of the Cnn molecule can self-assemble into very large solid-like macromolecular structures *in vitro*, particularly if the fragments have been phosphorylated

by recombinant Plk1 (Feng et al., 2017). Recently, Pericentrin (PCNT), a key centrosome scaffolding protein in mammalian cells, was also reported to form liquid-like condensates at centrosomes (Jiang et al., 2020). Thus, it remains controversial whether the mitotic PCM scaffold is most usefully described as solid- or liquid-like.

During the course of my studies, I noticed that the PCM protein TACC seemed to have some liquid like properties, with small flares of the protein breaking off from the edges of the PCM and forming round droplets in the cytoplasm—as described previously (Lee et al., 2001). I did not think too much about this until a paper was published showing that in mouse oocyte spindles TACC3 organises a liquid-like spindle domain (LLSD) that depends on the phosphorylation of TACC3 by Aurora A and that promotes acentrosomal spindle formation (So et al., 2019). Our group has also shown that Aurora A-dependent phosphorylation of TACC occurs in flies, and that this is important for TACC function at centrosomes (Barros et al., 2005). Although So et al., concluded that the LLSD structure was a specific feature of acentrosomal meiotic spindles (So et al., 2019), I wondered whether Aurora A and TACC might be working together to generate a similar structure at centrosomes in fly embryos.

In this Chapter, I present evidence that fly centrosomes indeed organise a liquid-like TACC scaffold that can recruit several centrosomal proteins. Like the LLSD in mouse oocyte spindles, the assembly of the TACC scaffold is promoted by Aurora A and MSPS. I show that Spd-2 is also important for TACC scaffold assembly, raising the exciting possibility that Spd-2 might recruit Polo and Cnn to organise a solid-like scaffold (as

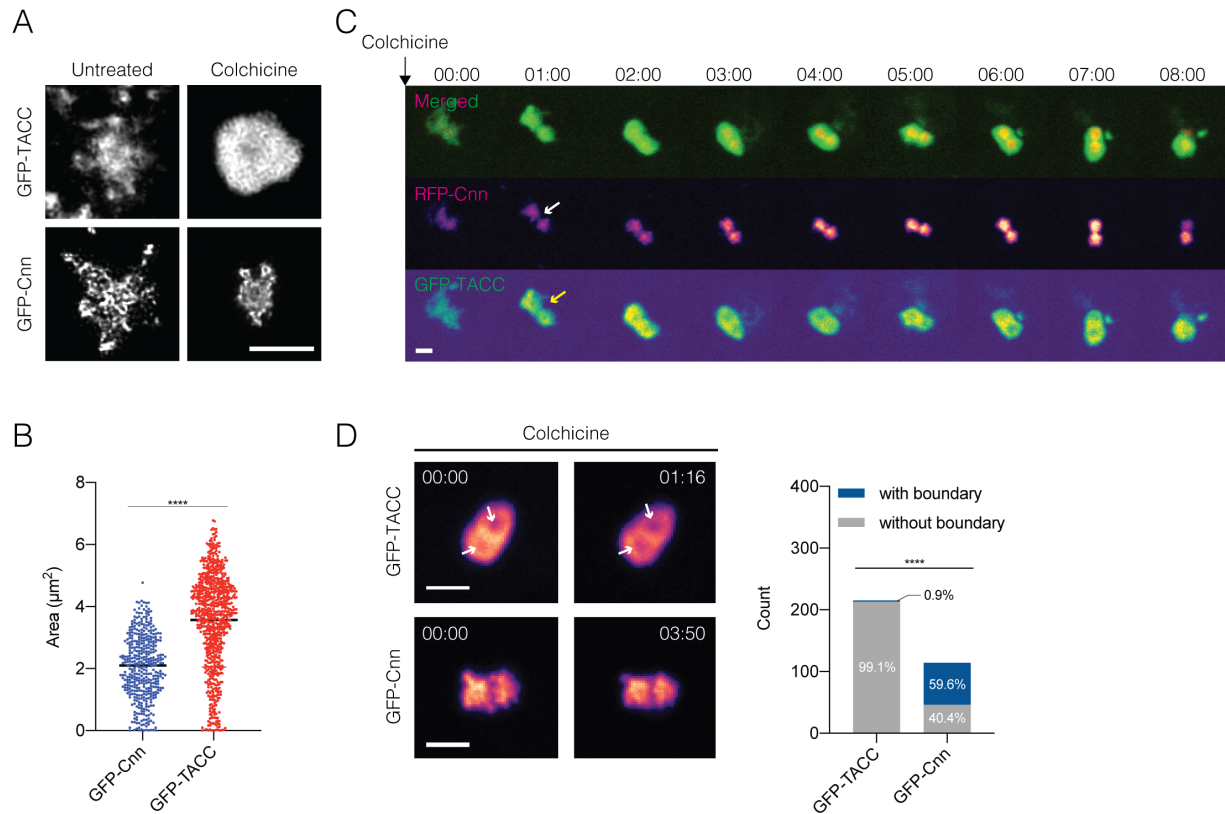
shown in **Chapters 3 and 4**), and also recruit Aurora A and TACC to organise a liquid-like scaffold.

## 5.2 Results

### 5.2.1 Centrosomal Cnn and TACC appear to behave as solid- and liquid-like scaffold, respectively

I wanted to more rigorously test my impression that the TACC protein exhibits liquid-like behaviour at centrosomes. I first imaged fly embryos expressing either GFP-TACC or GFP-Cnn using a SoRA super-resolution spinning disc system. As shown previously, the GFP-Cnn scaffold forms discrete extensions that spread outwards from the mother centriole along the centrosomal MTs (Figure 5.2A). In contrast, although GFP-TACC extended around the centriole in a similar manner, the TACC “structure” was much more diffuse and amorphous (Figure 5.2A). I injected colchicine into these embryos to remove the centrosomal MTs. The GFP-Cnn scaffold collapsed back into a smaller structure that appeared polygonal with distinct puncta, but the GFP-TACC structure remained relatively large—significantly larger than the GFP-Cnn structure (Figure 5.2B) and it was more diffuse and had a smoother surface than the GFP-Cnn scaffold (Figure 5.2A). Due to surface tension, liquid condensates can internally rearrange to adopt a spherical morphology to minimize their contact with the surrounding solvent, so these observations are consistent with the possibility that the Cnn scaffold and TACC structure are solid-like and liquid-like, respectively.

I next co-expressed GFP-TACC and RFP-Cnn in the same embryos so that I could compare their behaviour more directly. I first used spinning disc confocal microscope to image these embryos immediately after colchicine injection into embryos where the centrosomes were just starting to separate in early S-phase (Figure 5.2C). The two centrosomes failed to separate properly (due to the depolymerisation of the MTs) but the Cnn scaffold usually remained organised in two clearly distinct domains, while the larger TACC structures usually fused to form one contiguous domain (Figure 5.2C, D). Interestingly, GFP-TACC appeared to be largely excluded from the two more central GFP-Cnn scaffold regions (arrows, Figure 5.2C, D).

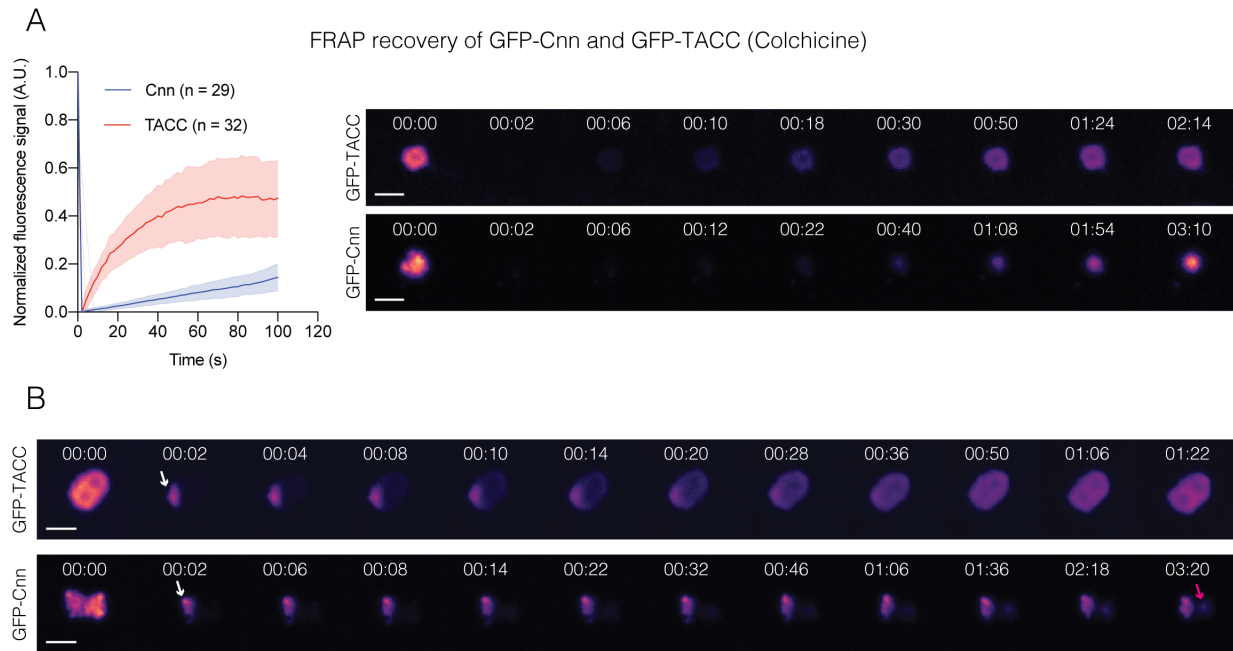


**Figure 5.1 Centrosomal Cnn and TACC exhibits distinct behaviours**

(A) Super resolution images show GFP-TACC and GFP-Cnn in WT or colchicine-treated embryos. (B) Bar chart compares the average area occupied by GFP-Cnn or GFP-TACC in centrosomes. Statistical significance was assessed using a Mann-Whitney's test (\*\*\*\*:  $P < 0.0001$ ). (C) Standard resolution time-lapse images of a centrosome labeled with GFP-TACC and RFP-Cnn after colchicine injection. (D) Images show and graphs quantify the absence or presence of a boundary between the centrosomal fluorescence of either GFP-TACC or GFP-Cnn. White arrows indicate regions excluded by Cnn scaffold. Statistical significance was determined by a Fischer's exact test. (A, C, D) Scale bars represent 2  $\mu\text{m}$ ; time (min:sec) is indicated in each time-lapse image.

To compare how the Cnn or TACC molecules were being recruited to their respective structures, I bleached colchicine treated centrosome pairs and monitored the pattern of GFP-Cnn or GFP-TACC fluorescence recovery over time (Figure 5.2A). As shown previously in non-colchicine treated embryos (Conduit et al., 2010), GFP-TACC fluorescence appeared to recover evenly throughout the TACC structure, whereas GFP-Cnn preferentially recovered in the central regions (around the centriole) and then spread

outwards. GFP-TACC recovered more quickly than GFP-Cnn, consistent with previous reports that CnnON is low during mitosis (Conduit et al., 2014a) (these colchicine-treated embryos enter mitosis and then arrest there due to activation of the spindle-assembly checkpoint). To test whether the Cnn or TACC molecules were capable of internally rearranging within these structures, I *partially* bleached colchicine treated centrosome pairs and monitored the pattern of GFP-Cnn or GFP-TACC fluorescence recovery over time (Figure 5.2B). The unbleached region of the GFP-Cnn scaffold (white arrow, Figure 5.2B) largely retained its fluorescence over the time-course of the experiment (~3mins). I could detect no diffusion of the unbleached molecules into the bleached area, and fluorescence seemed to recover only around the central area of the centrosomes (most easily visualized at the centrosome that was completely bleached—red arrow, Figure 5.2B). In contrast, GFP-TACC molecules in the unbleached region (white arrow, Figure 5.2B) appeared to rapidly diffuse into the nearby unbleached areas of the TACC structure, and I could detect no specific incorporation of new TACC molecules around the centrosomes. These findings are consistent with the possibility that the Cnn-scaffold is solid-like (as its constituent molecules do not easily internally rearrange) while the TACC structure is liquid-like (as its constituent molecules appear to readily internally rearrange).



**Figure 5.2 FRAP revealed centrosomal Cnn and TACC behaves as solid and liquid, respectively**

(A) Image illustrates and graph quantifies the centrosomal recovery of GFP-TACC and GFP-Cnn in colchicine-treated embryos (B) Images illustrate the partial bleaching of GFP-TACC and GFP-Cnn in colchicine treated embryos. White arrows highlight the non-bleached region, red arrow highlights the recovery of GFP-Cnn around the bleached centrosomes (A, B) Scale bars represent 2  $\mu$ m; time (min:sec) is indicated in each time-lapse image.

### 5.2.2 The TACC liquid-like structure can assemble independently of the Cnn scaffold

As the TACC structure assembles around the central Cnn scaffold I tested whether the Cnn scaffold is required for its assembly. I imaged embryos that expressed GFP-TACC in a *cnn*<sup>-/-</sup> genetic background and found that GFP-TACC was still strongly concentrated around centrosomes but it was more dispersed than normal (



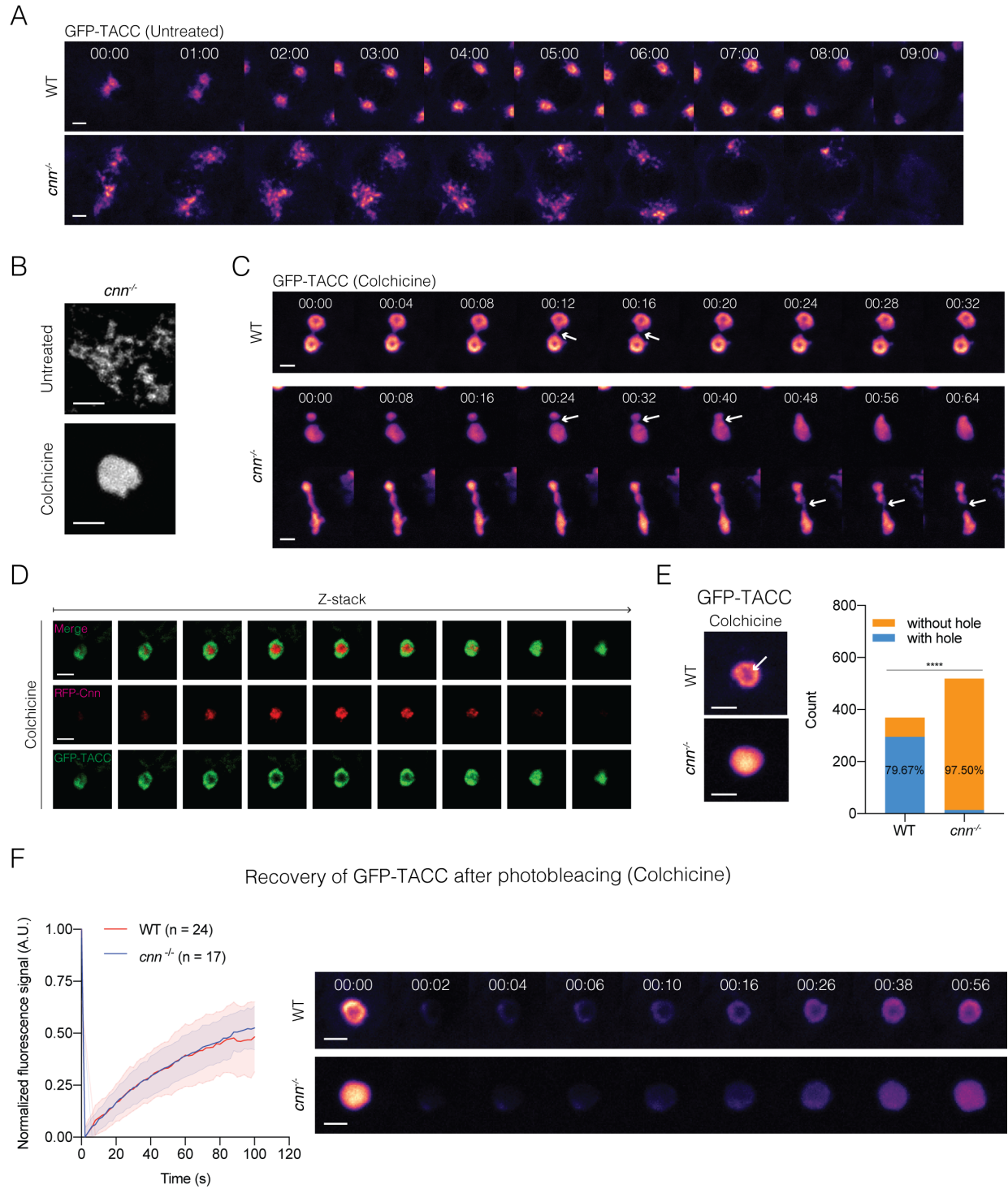
Figure 5.3A). Time lapse movies suggested that GFP-TACC was still being recruited to centrosomes, but was then rapidly dispersed outwards along the centrosomal MTs. When I injected colchicine into these embryos, the scattered “clouds” of GFP-TACC stopped spreading outwards and gradually coalesced into large rounded blobs (

Figure 5.3B) that usually formed around the centrioles (see below). During this period, I observed several clear examples of GFP-TACC blobs fusing and fissioning (

Figure 5.3C). These TACC structures appeared more homogeneous in the absence of Cnn, and GFP-TACC was no longer excluded from the central region (where the Cnn scaffold is normally present) (

Figure 5.3D, E). When I used FRAP to analyse the rate of TACC recruitment to these structure in the presence or absence of Cnn I found that it was indistinguishable (

Figure 5.3F) I conclude that centrosomes can still recruit and organise a liquid-like TACC structure even in the absence of Cnn.

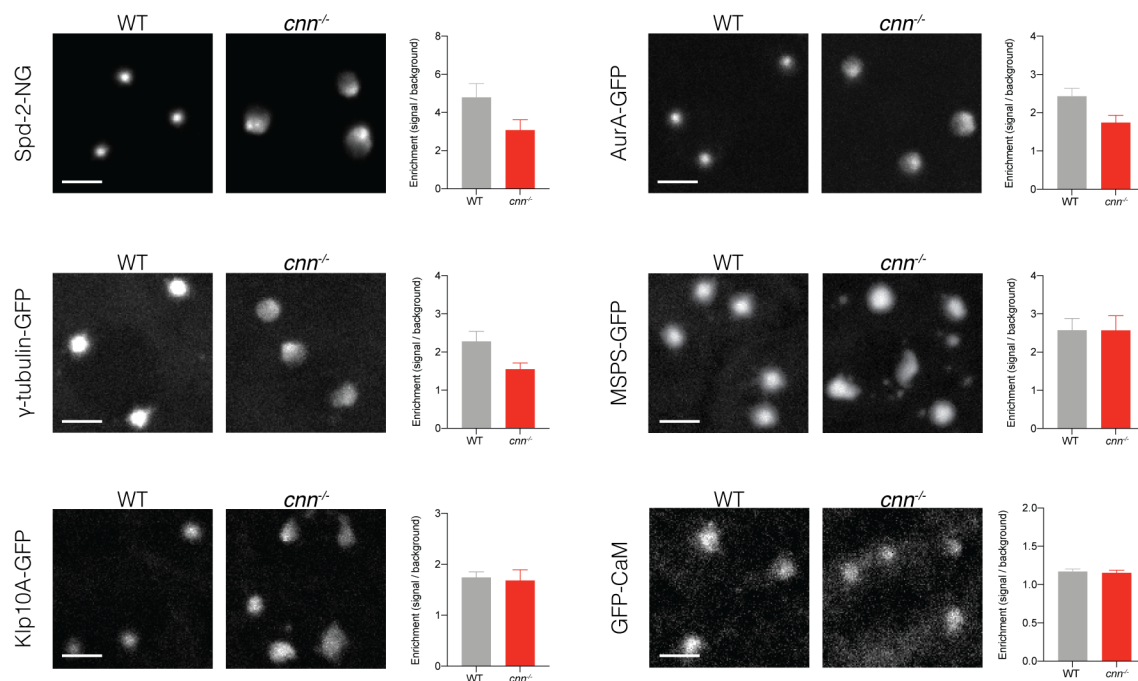


**Figure 5.3 TACC liquid scaffold formation is independent of Cnn solid scaffold**

**(A)** Images shows an example “clouds” of GFP-TACC expressing in a *cnn*<sup>-/-</sup> genetic background. **(B)** Micrograph compares super resolution images of GFP-TACC expressing in a *cnn*<sup>-/-</sup> genetic background in WT or colchicine treated embryos. Scale bar represents 2 μm. **(C)** Images illustrate GFP-TACC fusing or fissioning in a WT and *cnn*<sup>-/-</sup> genetic background. Arrows indicate fission and fusion events. Scale bar represents 2 μm. **(D)** Micrograph illustrates super resolution images of Z-stacks of a centrosome labeled with GFP-TACC and RFP-Cnn after colchicine injection. **(E)** Images show and graph quantifies the absence or presence of a hole between the centrosomal fluorescence of GFP-TACC in either WT or *cnn*<sup>-/-</sup> genetic background. Statistical significance was determined by a Fischer’s exact test. **(F)** Images illustrate and graph quantifies the centrosomal recovery of GFP-TACC in a WT and *cnn*<sup>-/-</sup> genetic background treated with colchicine. **(A-F)** Scale bars represent 2 μm; time (min:sec) is indicated in each time-lapse image.

### 5.2.3 The TACC scaffold is a selective condensate that forms around centrosomes and concentrates several centrosomal proteins

I wondered if the TACC structure could function as a “scaffold” that can recruit centrosome “client” proteins. To test this, I examined the distribution of several centrosomal proteins in colchicine treated embryos at either normal centrosomes or at TACC structures formed in *cnn*<sup>-/-</sup> embryos (to rule out any recruitment of the client proteins to these structures by the Cnn scaffold). I found that the centrosomal proteins Spd-2-NG, AuroraA-GFP,  $\gamma$ -tubulin-37c-GFP, MSPS-GFP, Klp10A-GFP and GFP-Calmodulin (GFP-CaM) were all enriched at centrosomes and also in the TACC structures, demonstrating that the TACC structure can function as a scaffold to recruit centrosomal proteins even in the absence of Cnn (Figure 5.4).



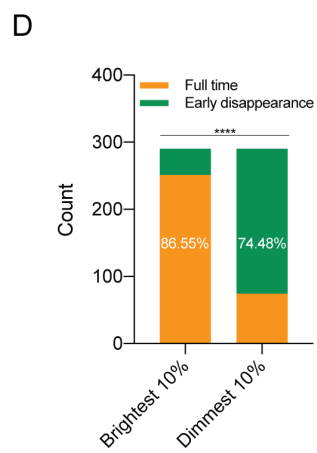
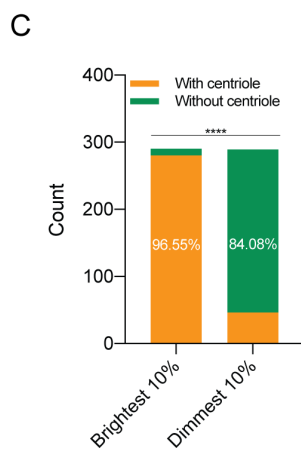
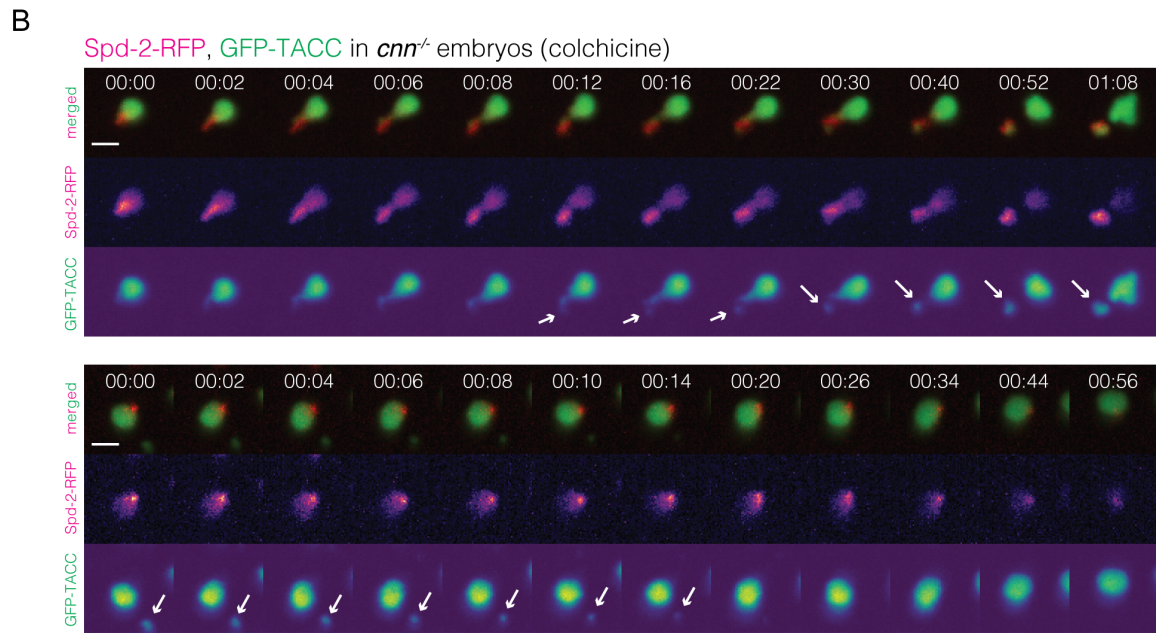
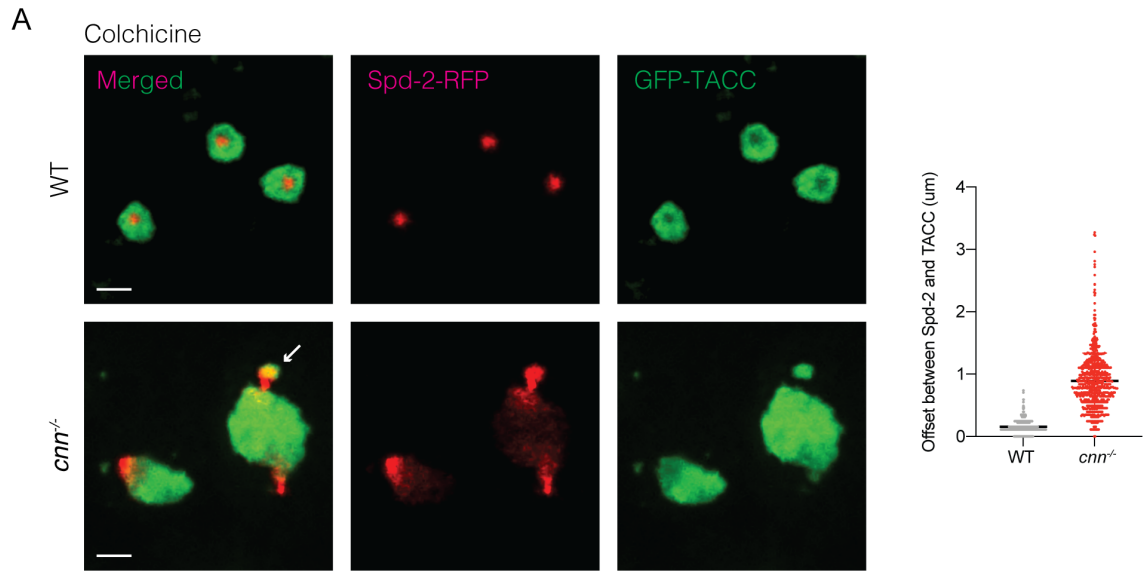
**Figure 5.4 TACC scaffold can concentrate several centrosomal proteins independently of Cnn scaffold**

Images show and graphs quantifies the localisation of several centrosomal proteins (indicated) in TACC scaffold. Enrichment ratio was calculated by the dividing mean intensity within the blobs by the mean intensity surrounding the blob (>1 means a positive enrichment to the blob). Scale bar represents 4  $\mu$ m.



Although the TACC structure can form in the absence of Cnn, it was striking that the majority of the TACC structures were still associated with centrioles. The centrioles were usually offset towards the edge of the PCM in these structure—as reported previously (Lucas & Raff, 2007) (Figure 5.5A). This centriole “offset” was easiest to quantify and visualize in colchicine-treated *cnn*<sup>-/-</sup> embryos co-expressing Spd-2-RFP and GFP-TACC, and in some rare cases the centrioles appeared to slightly detach from the edges of the TACC scaffold (arrows, Figure 5.5A). This centriole mispositioning is thought to occur because the PCM scaffold is weakened in the absence of Cnn, so the centriole cannot maintain its position at the centre of the scaffold (Lucas & Raff, 2007)—a theory that is consistent with my new data suggesting that the TACC scaffold is liquid-like.

In the rare instances where I was able to make a movie of a centriole detaching from a TACC scaffold, the detached centriole recruited more TACC scaffold, while the centriole-less scaffold gradually appeared to lose Spd-2 and TACC, suggesting that the centrioles are required for TACC scaffold assembly (Figure 5.5B). As these movies were rare, I analysed all the TACC scaffolds that formed in *cnn*<sup>-/-</sup> embryos shortly after colchicine injection on the basis of their brightness (Figure 5.5C). I found that >95% of the brightest TACC scaffolds, but only ~16% of the dimmest TACC scaffolds, were associated with a centriole (Figure 5.5D). Moreover, in movies of these embryos, ~85% of the brightest scaffolds (that largely contained centrioles) remained visible for the duration of the movie (usually 3-5 mins), while ~74% of the dimmest scaffolds (that usually lacked centriole) dissipated over this time course (Figure 5.5E, F). Taken together, these data strongly suggest that centrioles can generate a liquid-like TACC scaffold.

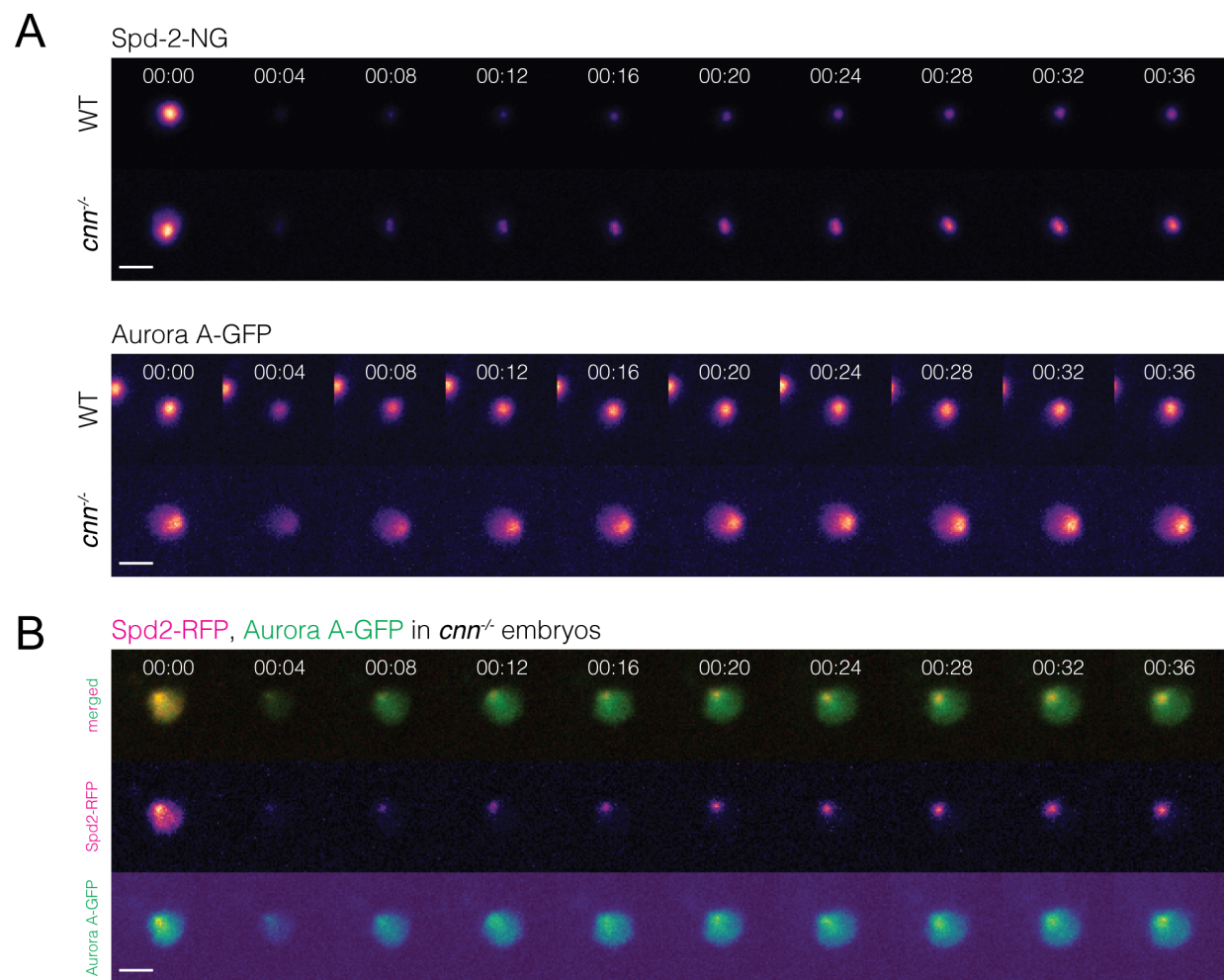


**Figure 5.5 TACC liquid-like scaffold formation depends on centrioles**

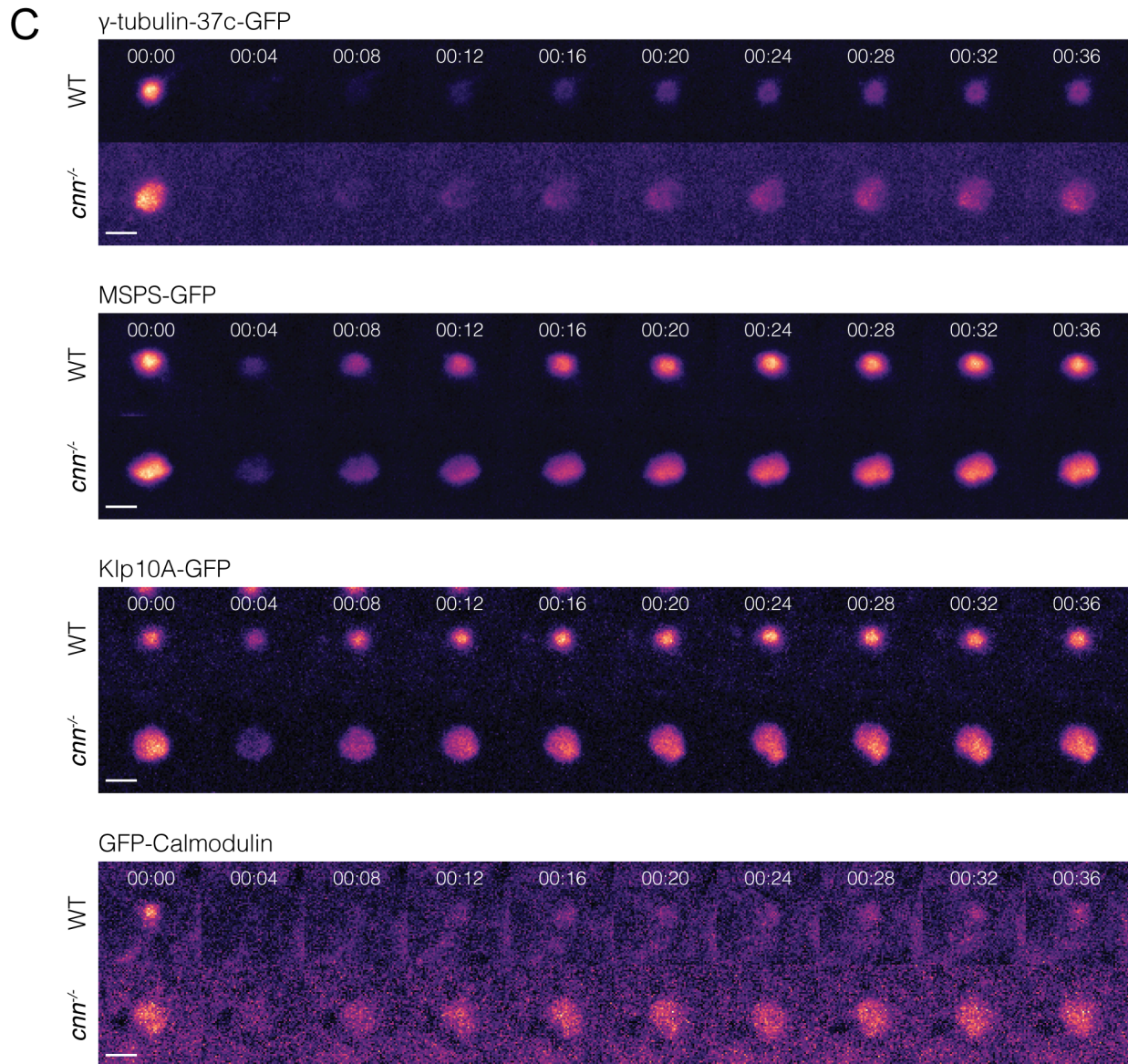
**(A)** Images compare embryos expressing Spd-2-RFP and GFP-TACC in a WT and *cnn*<sup>-/-</sup> genetic background (arrow indicates a loosely attached GFP-TACC blob). Graph quantifies the offset between GFP-TACC liquid blobs and the closest Spd-2-RFP labeled centrioles. **(B)** Time-lapse images of centrosomes labeled with Spd-2-RFP and GFP-TACC after colchicine injection. Arrows indicate associated or detached GFP-TACC liquid blobs. Time (min:sec) is indicated in each time-lapse image. **(A, B)** Scale bars represent 2  $\mu$ m. **(C)** Chart compares the proportion of centriole-bearing blobs in the brightest 10% and dimmest 10% blobs. Statistical significance was determined by Fischer exact test. **(D)** Chart compares the proportion of liquid blobs which lasted for the whole imaging duration in the brightest 10% and dimmest 10% blobs. Statistical significance was determined by a Fischer's exact test.

#### 5.2.4 Spd-2, Aurora A and MSPS are required for the assembly of the TACC scaffold

From the Spd-2 distribution in the TACC scaffold, it looked as though Spd-2 might initially be recruited to the centrioles and then spread outwards into the TACC scaffold (arrowheads, Figure 5.5A). I confirmed that this was the case using FRAP (Figure 5.6A). Strikingly, I noticed that Aurora A exhibited a similar behaviour, preferentially incorporating into the TACC scaffold in the immediate vicinity of the centriole (although, unlike Spd-2, it also seemed to incorporate into the bulk TACC scaffold directly from the cytoplasm) (Figure 5.6B). In contrast, the PCM proteins tubulin-37c-GFP, MSPS-GFP, Klp10A-GFP and GFP-Calmodulin all incorporated evenly throughout the TACC scaffold (Figure 5.6C).







**Figure 5.6 Centrosomal proteins display different pattern of recovery in TACC liquid-like scaffold**

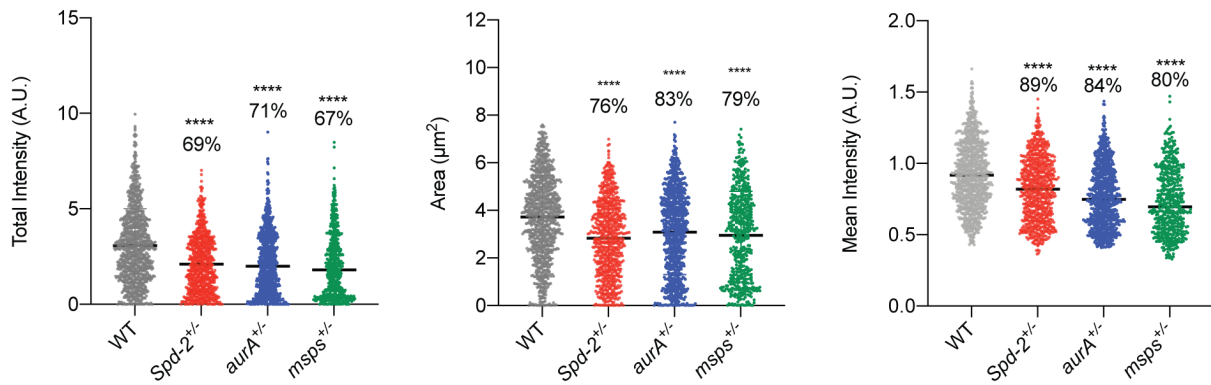
(A) Time-lapsed images show the centrosomal recovery of Spd-2-NG and Aurora A-GFP after photobleaching in WT or *cnn*<sup>-/-</sup> embryos. (B) Time-lapsed images show the centrosomal recovery of Aurora A-GFP and Spd-2-RFP co-expressed in *cnn*<sup>-/-</sup> embryos photobleaching. (C) Time-lapsed images show the centrosomal recovery of several centrosomal proteins (indicated) after photobleaching in WT or *cnn*<sup>-/-</sup> embryos. (A-C) Scale bars represent 1  $\mu$ m; time point (min:sec) is indicated in each time-lapse image. Time points were sampled linearly in these images.

It has previously been shown that Spd-2 plays an important part in recruiting Aurora A into the mitotic PCM in *Drosophila* (Conduit et al., 2014b), *Xenopus* (Joukov et al., 2010,

2014) and human cells (Tsou & Stearns, 2006). Moreover, Aurora A is required to form the TACC3 LLSD in mouse oocytes (So et al., 2019), and Aurora A also phosphorylates TACC to promote TACC function at the centrosome (Barros et al., 2005; Kinoshita et al., 2005). Taken together with my new experiments, these observations raised the exciting possibility that Spd-2 plays a central role in driving the assembly of *two* PCM scaffolds around the centriole: first, a Polo/Cnn solid-like scaffold; second, an Aurora A/TACC liquid-like scaffold.

In support of this possibility, I examined the ability of centrioles to organise a TACC scaffold (again, in colchicine treated embryos in the absence of Cnn) in embryos where I also halved the genetic dosage of Spd-2 or Aurora A. Both perturbations significantly reduced the total amount of TACC scaffold assembled around the centriole, and also the *density* of these TACC scaffolds (i.e. total fluorescence intensity/size of scaffold) (Figure 5.7A, B). In mouse oocytes, the ability to form the LLSD is dependent on TACC and Aurora A, and also on the MT stabilising protein ch-TOG (MSPS in flies) and Clathrin Heavy Chain (CHC), both of which have been shown to bind to TACC proteins in several systems and are important for TACC function at centrosomes (Barros et al., 2005; Hood et al., 2013; Kinoshita et al., 2005; M. J. Lee et al., 2001; Thakur et al., 2014). I found that halving the genetic dosage of *mmps* also reduced the total amount and density of the TACC scaffolds (Figure 5.7C). I am currently performing the genetic crosses to allow me to perform the same analysis for CHC. Taken together, these results strongly suggest that the centrosomal TACC scaffold I have characterised here has similar molecular

composition and dependencies as the LLSD scaffold characterised in mouse oocyte spindles (that lack centrosomes) (So et al., 2019).



**Figure 5.7 *Spd-2*, *Aurora A* and *MSPS* are important for the TACC liquid-like scaffold formation**

Bar charts show the total intensity (left), area (middle), and mean intensity (right) of centrosomes in WT or *Spd-2*<sup>+/-</sup> (A), *aurora A*<sup>+/-</sup> (B) and *msps*<sup>+/-</sup> (C) in *cnm*<sup>-/-</sup> embryos injected with colchicine. Statistical significance was assessed by a Mann-Whitney test (\*\*\*\*: P<0.001). Each set consists of ~500 – 1000 centrosomes.

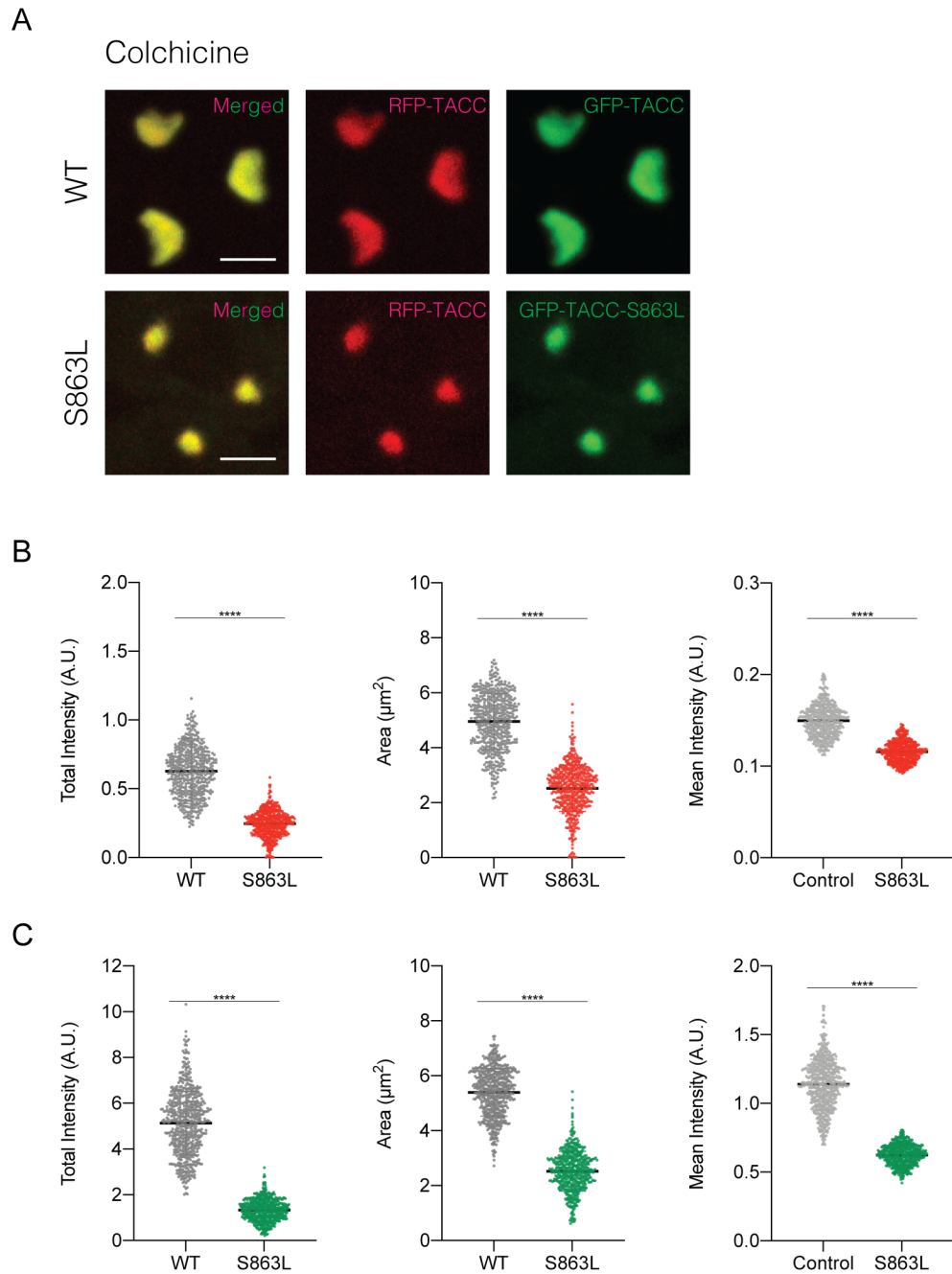
It was shown previously in flies, frogs and humans that Aurora A phosphorylates TACC at a conserved Ser residue (S863 in flies). It was originally postulated that this allowed TACC to recruit the MT stabilising protein MSPS/XMAP215 to centrosomes, thus explaining why TACC is required to stabilise centrosomal MTs (Barros et al., 2005; Gergely et al., 2003; Kinoshita et al., 2005). It was subsequently shown, however, that TACC proteins also interact with CHC and that this interaction is also important for several aspects of centrosome and mitotic spindle function (Burgess et al., 2018; W. Fu et al., 2010; Hood et al., 2013; C. H. Lin et al., 2010). It is now thought that Aurora A phosphorylates TACC primarily to promote its interaction with CHC, rather than with MSPS/ch-TOG/XMAP215, and that these proteins form a variety of complexes relevant for several aspects of mitotic spindle assembly (Barros et al., 2005; Burgess et al., 2018;



W. Fu et al., 2010; Gergely et al., 2003; Hood et al., 2013; M. J. Lee et al., 2001; C. H. Lin et al., 2010). As TACC, Aurora A, MSPS and CHC are all required for the assembly of the LLSD in mouse oocytes, I wondered whether the phosphorylation of TACC on S863 by Aurora A might initiate TACC scaffold assembly by allowing TACC to interact with CHC—with non-phosphorylated TACC potentially continuing to interact with proteins such as MSPS to perform other functions at the centrosome/spindle independently of TACC scaffold assembly. In support of this possibility, embryos completely lacking TACC protein do not develop at all because they fail in pronuclear fusion, while embryos expressing only a form of TACC that cannot be phosphorylated on S863L (GFP-TACC-S863L, which can still be recruited to centrosomes, but at significantly lower levels than normal) can proceed through early development, but mostly die at late syncytial stages due to centrosome/spindle defects (Barros et al., 2005)—indicating that S863 phosphorylation is not essential for all TACC functions.

I am currently generating the fly stocks to allow me to test this possibility; in particular to examine whether GFP-TACC-S863L is capable of forming a liquid-like scaffold. This analysis may be complicated, as I believe the mutant TACC will still be recruited to centrosomes by the Cnn scaffold—explaining why GFP-TACC-S863L is still recruited to centrosomes (Barros et al., 2005)—so it will be necessary to perform this analysis in the absence of endogenous Cnn and endogenous TACC, and in the presence of TACC-S863L and of suitable markers to allow me to follow the behaviour of any TACC scaffold that assembles. In the meantime, I have expressed GFP-TACC-S863L in WT embryos expressing WT RFP-TACC (Figure 5.8). Interestingly, the mutant protein seems to have

a dominant effect, and both the GFP-TACC-S863L and RFP-TACC proteins are concentrated in a much smaller area than I observe in embryos only expressing RFP-TACC. This is consistent with the possibility that the mutant protein can still be recruited to the central Cnn scaffold (and it also prevents the WT protein from doing so). Clearly, however, more experiments will be required to test these interesting possibilities.



**Figure 5.8 S863L mutation perturbs localisation TACC**

(A) Images compare embryos co-expressing RFP-TACC with either GFP-TACC (WT) or GFP-TACC-S863L (S863L). Scale bar represents 4  $\mu\text{m}$ . (B, C) Bar charts show the total intensity (left), area (middle), and mean intensity (right) of centrosomes in red channel (B) or green channel (C) in these embryos.

### 5.3 Discussion

The physical nature of the mitotic centrosome has recently been a topic of much debate in particular as to whether centrosomes should join the ever-increasing list of non-membrane bound structures and organelles that appear to be formed by liquid-liquid phase separation (Boeynaems et al., 2018; Eggert, 2018; Shin & Brangwynne, 2017; Wheeler & Hyman, 2018). The results presented in this Chapter raise the exciting possibility that mitotic centrosomes in fly embryos are formed by *both* a solid-like Cnn scaffold and a liquid-like TACC scaffold. Moreover, the centriole/centrosome protein Spd-2 appears to catalyse the production of both scaffolds: recruiting Polo to phosphorylate Cnn and drive Cnn scaffold assembly, and Aurora A to phosphorylate TACC and drive TACC scaffold assembly. Crucially, Spd-2 has the ability to flux outwards from the centriole and it appears to be captured within the Cnn and TACC scaffolds, allowing the scaffolds to expand outwards around the mother centrioles. Currently, the central role of Spd-2 in catalysing the assembly of the TACC scaffold remains somewhat speculative, but I note that Spd-2 is essential for recruiting Aurora A to mitotic centrosomes in fly brain cells (Conduit et al., 2014b), and in vertebrate cells it is well established that Cep192 (the homologue of Spd-2) recruits both Polo and Aurora A to centrosomes, where each one helps to activate the other (Joukov et al., 2010, 2014). Thus, it is a real possibility that Spd-2 functions as a crucial nexus in the run up to mitosis that allows centrioles to coordinate the assembly of both a solid-like and a liquid-like PCM scaffold.

This work was performed towards the end of my studies, so further experiments are required to test this hypothesis. For example, I have shown that the assembly of the TACC

scaffolds is not dependent on the Cnn scaffold but I also need to test whether the assembly of the Cnn scaffold is independent of the TACC scaffold. This is not trivial, as embryos lacking TACC fail in pronuclear fusion and so do not develop at all (meaning that I cannot directly examine the Cnn scaffold at centrosomes in the absence of TACC). As assembly of the liquid-like spindle domain (LLSD) in mouse oocytes is dependent on both TACC and Aurora A, I speculate that the assembly of the TACC scaffold at fly centrosomes will depend on the ability of Aurora A to phosphorylate TACC at S863 (and my preliminary experiments are consistent with this possibility). If this is the case, the TACC-S863L mutant will allow me to study centrosome behaviour in the absence of the TACC scaffold—as fly embryos expressing only TACC-S863L do develop, although they usually die prior to cellularisation. If my hypothesis is correct, then the TACC-S863L still recruited to centrosomes (presumably by the Cnn scaffold), provides enough function to allow the embryos to develop (perhaps because TACC-S863L can still recruit MSPS to centrosomes, as this binding is not dependent on phosphorylation by Aurora A), but the centrosomes in these embryos should completely lack the TACC scaffold. I am currently generating the stocks that should allow me to test whether TACC-S863L is unable to form a scaffold.

My results indicate the molecules required for TACC scaffold assembly at centrosomes are similar to the molecules required for LLSD assembly in mouse meiotic spindles. TACC, Aurora A, CHC and ch-TOG (MSPS in flies) are all required for this process in the mouse system, and the same seems to be true in the fly system (although I have not yet tested the requirement for CHC). At a first glance, this may seem surprising, as So et al

concluded that the LLSD structure was a specific feature of acentrosomal meiotic spindles, and they could detect no similar structure when they generated acentrosomal spindles in mitotic cells (they did this by depleting centrioles from somatic cells using the Plk4 inhibitor centrinone) (So et al., 2019). I believe, however, that my results are entirely consistent with these findings, as the TACC scaffold in fly embryos is not generated by the mitotic spindle but is generated by the centrosomes. Thus, I would expect that the acentrosomal spindles generated in mouse somatic cells would not be able to assemble a TACC scaffold/LLSD because they lack centrosomes. Indeed, in the mouse meiotic spindle, several centriole proteins (including Cep192) are concentrated at the poles of the acentriolar meiotic spindle. It will be important to test if the assembly of the LLSD in mouse meiotic spindles is dependent on Cep192. I suspect that the centrosomal TACC scaffold and the meiotic spindle LLSD will turn out to be similar structures that function to promote efficient centrosome and/or spindle assembly.

Is the ability to organise both a solid-like and liquid-like scaffold a property common to mitotic centrosomes in other species? I believe this is likely to be the case. As discussed above, Cep192/Spd-2 proteins appear to play a very important (and possibly an essential) part in mitotic PCM assembly in many higher eukaryotic cells (Decker et al., 2011; Dix & Raff, 2007; Giansanti et al., 2008; Gomez-Ferreria et al., 2007; Woodruff et al., 2015), and there is evidence from several systems that this family of proteins are important for the recruitment of both Polo/Plk1 and Aurora A to mitotic centrosomes. In contrast, in several systems neither TACC-family proteins (Gergely et al., 2000, 2003) nor Cnn/Cdk5Rap2-family proteins (Barr et al., 2010; Megraw et al., 2001) appear to be

essential for mitotic centrosome assembly. A possible explanation this finding is that these protein families can generate independent scaffolds that can partially support mitotic centrosome assembly in the absence of the other scaffold, while Cep192/Spd-2 proteins are required for the assembly of both scaffolds. Interestingly, in *C. elegans* embryos, mitotic PCM assembly appears to be completely dependent on both SPD-2 and SPD-5 (the worm homologue of Cnn) (Decker et al., 2011; Woodruff et al., 2015, 2017). These embryos express a TACC homologue (TAC-1) that has an important role in spindle assembly, but TAC-1 lacks the N-terminal domain present in other TACC proteins that is required for LLPS (Bellanger & Gönczy, 2003; Le Bot et al., 2003; Srayko et al., 2003). Thus, I would predict that these embryos cannot form a TAC-1 liquid-like scaffold, potentially explaining why SPD-5 is essential in this system.

Importantly, it has been shown in fly brain cells that some mitotic PCM scaffold can still assemble in the absence of Spd-2, as long as Cnn is present (Conduit et al., 2014b). This presumably reflects the ability of Cnn to form a scaffold independently of Spd-2 if it is phosphorylated by Polo at a sufficient rate. Spd-2 normally catalyses Cnn scaffold assembly by bringing Cnn and active Polo together at a high concentration, and I speculate that it does the same for the Aurora A/TACC scaffold. Clearly there are proteins other than Spd-2 that can recruit Polo to centrioles (Alvarez-Rodrigo et al., 2019, 2020) and this is likely to also be the case for Aurora A. Thus, Spd-2/Cep192 proteins might normally ensure robust and efficient assembly of both the Cnn and TACC scaffolds, but centrioles are likely to have alternative pathways of Polo and Aurora A recruitment that can organise residual levels of mitotic PCM even in the absence of Spd-2/Cep192. The

strength of these alternative pathways may vary depending on the cell type, potentially explaining why, for example, fly brain cells can organise significant amounts of mitotic PCM in the absence of Spd-2, but fly embryos cannot (Conduit et al., 2014b).

In summary, my studies suggest that Spd-2 normally plays an important part in catalysing the assembly of the mitotic centrosome by promoting the assembly of a solid-like Polo/Cnn scaffold and a liquid-like Aurora A/TACC scaffold. These pathways may represent a fundamental logic that underpins the biogenesis of all mitotic centrosomes. The precise molecular details of mitotic centrosome assembly, however, are likely to vary between species and cell types. For example, centrosome assembly may rely to different extents on the different scaffolds, may engage a variety of alternative mechanisms to recruit and activate the key scaffolding proteins, and may even use alternative molecules—such as Pericentrin (Jiang et al., 2020)—to form additional scaffolds. In **Chapter 6** I discuss why it might be an advantage for mitotic centrosomes to organise both a solid-like and liquid-like scaffold.



## 6. Discussion

In this thesis I have made two observations that may have wider implications for understanding centrosome biogenesis, and perhaps organelle biogenesis more generally. First, I present evidence that a centriolar oscillation in Polo levels plays an important part in determining the kinetics of centrosome growth. Second, I find that centrosomes appear to organise both a solid-like and liquid-like scaffold. In this final Chapter, I briefly discuss why these findings are potentially important.

### 6.1 The potential role of oscillations in regulating organelle biogenesis

As discussed in Chapter 1, cells are densely packed with many different organelles and the biogenesis of these organelles appears to be tightly regulated so that cells make the right amount of each organelle at the right time and in the right place (Cole, 2016; van Bergeijk et al., 2016). It is largely unclear, however, how cells regulate and coordinate the biogenesis of their many organelles. The Raff laboratory recently made the surprising observation that in *Drosophila* embryos, centriole biogenesis appears to be regulated by a localised oscillation in the centriolar levels of Plk4 (Aydogan et al., 2020). When the amplitude of the Plk4 oscillation is high, its period is short, and centrioles grow quickly but for a short period of time; when its amplitude is low, its period is longer, and centrioles grow more slowly and for a longer period of time. As a result, centrioles grow to a consistent size (Aydogan et al., 2018, 2020). My data suggests that a similar mechanism operates to ensure that mitotic centrosomes grow to a consistent size in *Drosophila* embryos. Polo/Plk1 is an important regulator of mitotic centrosome biogenesis, and I

found that its centriolar levels oscillate as the centrosomes grow in preparation for mitosis, and this oscillation appears to play an important part in regulating centrosome growth.

It is currently unclear how the Plk4 and Polo centriolar oscillations are generated, but mathematical modelling indicates that a plausible explanation is that these kinases are activated by binding to their centriolar receptors. This activation allows the kinases to promote the assembly of key organelle-scaffolding structures (Sas-6 in the case of centrioles, Spd-2 and Cnn in the case of centrosomes), but also to phosphorylate their receptors in a way that eventually inhibits further binding. This is a classical “delayed negative feedback” oscillating system, in which an activator (the centriolar Plk4 and Polo receptors) activates its own inhibitor (Plk4 and Polo) (Novák & Tyson, 2008). Although these models are attractive, the experimental support for them is relatively weak. Most importantly, although there is good evidence that Plk4 and Polo can phosphorylate candidate centriolar receptors such as Asl (Boese et al., 2018) and Ana1 (Alvarez-Rodrigo et al., 2020), respectively, the significance of these phosphorylations is unclear, and there is certainly no evidence that their phosphorylation inhibits binding to their respective kinases. It is crucial that this is tested more rigorously, either by reconstituting these interactions in vitro, or identifying the relevant phosphorylation sites and testing their importance in vivo.

Intuitively, one can see how this type of oscillating system could be an effective way of ensuring that an organelle grows to a consistent size, even as cellular conditions vary. By linking the growth rate of the organelle to the rate at which the signal to grow is inactivated,

the organelle could maintain a relatively constant size no matter how active the kinase. Moreover, a powerful advantage of oscillating systems is their ability to be entrained by other oscillating systems, such as the cell cycle oscillator or the circadian clock (Lu & Cross, 2010; Matsuo et al., 2003; Yang et al., 2010). It seems to be a universal aspect of centriole and centrosome biology, for example, that centrioles duplicate in S-phase and that they acquire a dramatically expanded PCM in preparation for mitosis. In the *Drosophila* embryo, the Plk4 and Polo oscillations are entrained by the basic Cdk/Cyclin oscillator (CCO) to ensure that they occur at the right time, and the spatially restricted activation of the kinases (to either a single site on the surface of the mother centriole for Plk4, or the entire mother centriole surface for Polo) ensures that they occur in the right place.

In the case of the Plk4 oscillation, it has been shown that the oscillation can occur even when the CCO is perturbed, supporting the idea that the system is inherently oscillatory (Aydogan et al., 2020). In the modelling of the system, this is because the system essentially oscillates between two states: the centriolar Plk4 receptor Asl molecules are either bound to Plk4 and being phosphorylated (to become inactive), or they are not binding Plk4 and being dephosphorylated (to reset the system for another round of Plk4 binding). The CCO entrains the system by ensuring that all the Asl receptors are coordinately “reset” during mitosis by a burst of phosphatase activity, so that they all enter S-phase in an unphosphorylated state, ready to bind and activate Plk4. In the absence of a robust CCO, individual Asl receptors continue to oscillate between these states, but they are no longer coordinated, so the centrioles in the embryo duplicate stochastically

and more slowly. It will be important to test whether the Polo oscillation that promotes centrosome assembly also has the ability to oscillate independently of a robust CCO.

Is it plausible that similar oscillatory systems could regulate the biogenesis of other organelles—perhaps even membrane-bound organelles such as mitochondria and peroxisomes? While this might initially seem far-fetched, Mustafa Aydogan (a former student in the Raff laboratory) searched available datasets (Y. Wang et al., 2018) to find that Plk4 protein levels exhibited an oscillation that was entrained to the circadian clock in mouse livers (Aydogan et al., 2020). These liver cells are essentially quiescent, so the centrioles are not duplicating, and the cells are not dividing. The significance of this oscillation, if any, is unclear, and it may simply reflect that the Plk4/Asl system is inherently oscillatory, and so therefore likely to become entrained to any other dominant oscillating system (the circadian clock in the case of the quiescent liver cells). Intriguingly, however, ~8% of the ~8000 proteins analysed in these experiments also exhibited an oscillatory behaviour that was entrained to the circadian clock, although this point was not commented on by the authors (Y. Wang et al., 2018). This set of oscillating proteins included several key enzymes involved in organelle biogenesis (Mustafa Aydogan and Jordan Raff, personal communication). Again, the significance of these oscillations, if any, is unknown, but these findings may indicate that many proteins in the cell could be part of systems that can exhibit oscillatory behaviour, and so have the potential to be entrained by other oscillators. Moreover, the biogenesis of several membrane bound organelles, such as peroxisomes and lipid droplets, are now thought to occur at specialised membrane contact sites, where key biogenesis proteins are concentrated (Cohen et al.,

2018; Jackson, 2019). This may provide a mechanism to spatially restrict the key activities that drive the biogenesis of membrane-bound organelles, in the same way that the centrioles provide a platform to spatially organise the Plk4 and Polo oscillations.

In summary, it seems possible that spatially restricted oscillations in key regulatory activities might be a fundamental aspect of organelle growth control, allowing cells to regulate and coordinate the growth of their many different organelles. Clearly there is much work to be done to test this interesting hypothesis.

## 6.2 The physical nature of the mitotic centrosome

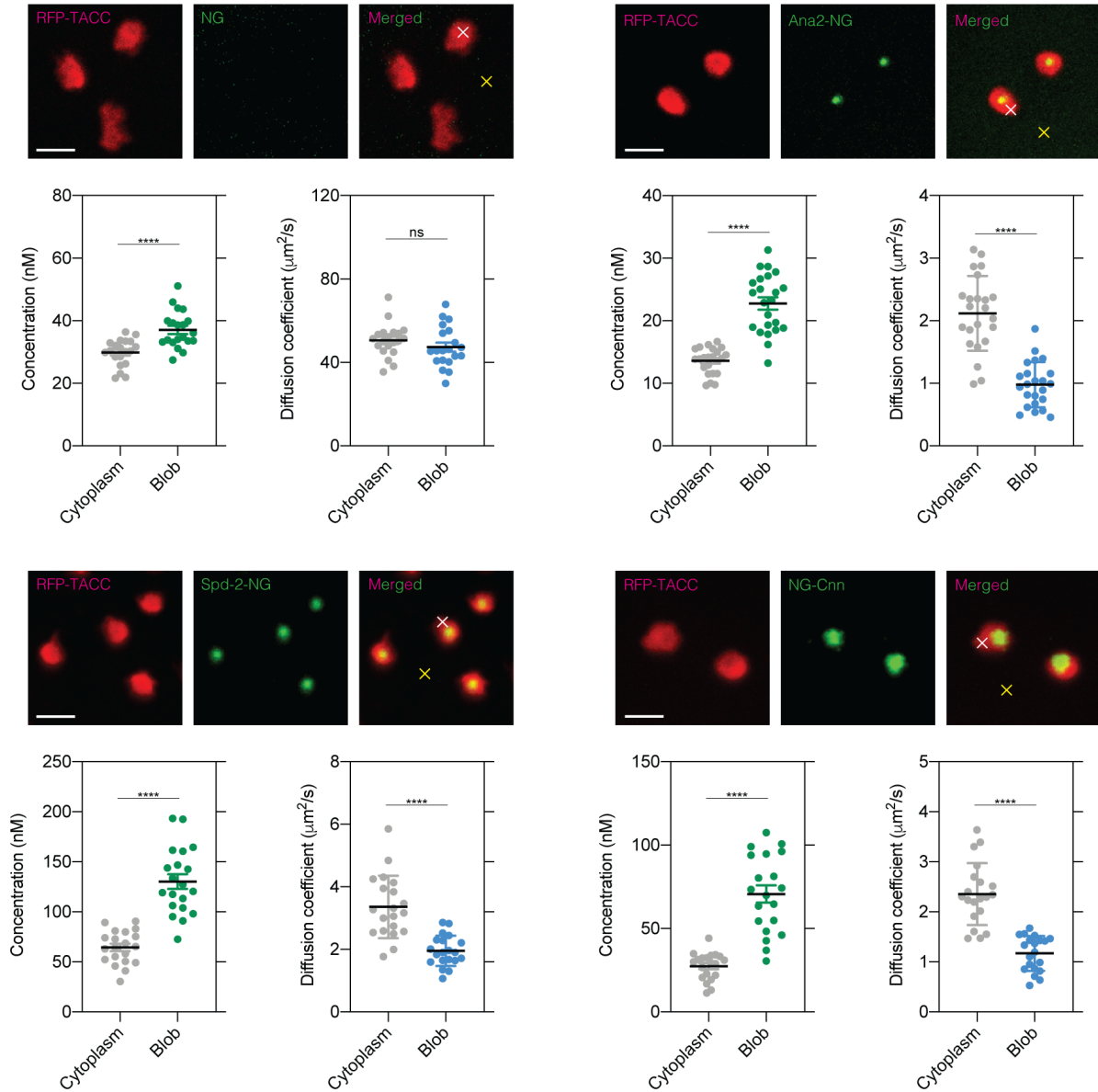
Why would the centrosomes in the early *Drosophila* embryo organise both a solid-like and liquid-like scaffold? A solid-like scaffold would intuitively provide more structural strength to the PCM, potentially allowing the mitotic centrosome to withstand the large forces that push and pull on the many MTs that the centrosomes nucleate and organise. There is good evidence that the Cnn scaffold does provide structural strength to the PCM, as almost all PCM components (including TACC and Spd-2) are present at much lower levels than normal in the absence of Cnn, and these proteins can be seen to be rapidly dissipating back into the cytoplasm as they are pulled away from the centrosome along the centrosomal MTs (Lucas & Raff, 2007). Moreover, in colchicine injected embryos the centrioles can efficiently maintain their position within the centre of the mitotic PCM if Cnn is present, but not when it is absent, even though essentially all PCM proteins can still be recruited to the PCM. I would now speculate that this displacement of the centrioles occurs because although the centrioles can still organise a TACC scaffold (explaining

why the PCM can still recruit many centrosome proteins), this scaffold is liquid-like and structurally weaker, so the centrioles cannot maintain their central position. As postulated by Lucas and Raff (2007), this process may be analogous to when beads (or bacteria such as *Listeria* or *Shigella*) recruit an actin scaffold at their surface that fluxes outwards: if the actin scaffold is strong, the beads/bacteria can maintain their central position within the scaffold; if the scaffold is weak, the beads/bacteria “break symmetry” and move to the edge of the scaffold (Van Oudenaarden & Theriot, 1999). This can lead to the movement (“rocketing”) of the beads/bacteria, similar to the centriole “rocketing” observed in embryos in the absence of Cnn (Lucas & Raff, 2007).

These observations can explain why it might be important for centrosomes to organise a scaffold with solid-like properties. Why might it be an advantage for centrosomes to also organise a scaffold with liquid-like properties? Several hundred proteins are probably recruited to the mitotic PCM, and the centrosome is often viewed as an important coordination centre—a place where lots of proteins can interact with one another at a high concentration, integrating information and changing their behaviour appropriately (Arquint et al., 2014). *A priori*, it would make sense if this structure were liquid-like, allowing selected molecules (“clients”) to quickly diffuse and interact with each other within the scaffold at higher concentrations than occur in the cytosol (Banani et al., 2017). I suspect that the TACC scaffold might function as a “net” that is assembled around the centrioles to “capture” centrosome proteins. This would not only allow these molecules to efficiently interact with one another but would also provide a high local concentration of building blocks to drive the assembly of the centrosome upon the Cnn scaffold.

I am currently collaborating with Thomas Steinacker to test this idea. I am generating stocks expressing RFP-TACC and endogenously tagged CRISPR NG-fusions to various candidate proteins and Thomas is using FCS to examine the concentration and diffusion rate of the NG-fusions within the outer region of the TACC scaffold (that extends beyond the Cnn scaffold). Our preliminary experiments are encouraging. Neon Green (NG) expressed on its own is very slightly concentrated by the TACC scaffold (~25% enrichment compared to the nearby cytoplasm), but its diffusion rate is essentially unchanged, indicating that NG is largely passively diffusing through the scaffold (Figure 6.1). In contrast, both Spd-2-NG and NG-Cnn are enriched by ~100-150% in the TACC scaffold region, and their diffusion drops significantly, indicating that they are likely to be interacting with the TACC scaffold and/or other proteins enriched in the scaffold (Figure 6.1). Intriguingly, we found that the core centriole building block Ana2-NG is concentrated by ~66% in the TACC scaffold, and its diffusion rate also drops significantly. This raises the possibility that the TACC scaffold may also interact with centriole building blocks to increase their local concentration for centriole assembly.

A



**Figure 6.1 Centrosomal proteins are specifically concentrated in TACC liquid blob in native condition**

Spinning disk confocal images show mNG, Ana2-NG, Spd-2-NG, and NG-Cnn co-expressed RFP-TACC. Yellow crosses indicate FCS measurement in cytoplasm. White crosses indicate FCS measurement in TACC liquid. White scale bars measure 4  $\mu\text{m}$ . Bar charts compare the concentrations or diffusion coefficients of these proteins (as indicated) in cytoplasm and in liquid blobs. Significance was determined by a paired t-test (\*\*\*\*:  $P < 0.0001$ ).



Taken together, these preliminary findings offer some support for my hypothesis that mitotic centrosomes assemble a liquid-like scaffold, at least in part, to allow them to efficiently capture and sequester centrosome building blocks from the surrounding cytoplasm. I am hoping that I will be able to test whether this is the only function of the TACC scaffold by examining the behaviour of centrosomes in the presence of only TACC-S863L. If, as I suspect, this protein can perform many of TACC's normal functions (such as interacting with MSPS) but it cannot form scaffold (as it cannot be phosphorylated by Aurora A), then I may be able to test whether the embryos expressing this protein die only because they cannot assemble centrosomes (or centrioles) quickly enough (because they can no longer capture building blocks in the expanded TACC scaffold net), or whether these centrosomes lack some other essential functions that are normally provided by the liquid-like TACC scaffold.

### 6.3 Concluding Remarks

The studies described in this thesis illustrate the power of the early *Drosophila* embryo system to provide quantitative descriptions of cell biological processes, and how these descriptions can reveal potentially important new biological insights. There is intense interest in the regulation of organelle biogenesis and in the potential role of liquid-liquid phase separation in this process. I believe that many further insights in these areas can be gained by continuing to dig deeper into the fundamental mechanisms that regulate centriole and centrosome assembly in this beautiful system.

## 7. References

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