Elucidating the pathway of centrosome formation

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Abstract

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Centrosomes are cellular organelles present in most animal cells, and are formed of two main components: the centrioles and the pericentriolar material (PCM). Centrosomes perform a variety of functions: they are the main microtubule organising centre in the cell, and are important localisation hubs for kinases involved in regulating the cell cycle. Hundreds of proteins are thought to localise to centrosomes, but work in the last decade has narrowed down this list to a handful of proteins that are thought to be essential for centrosome structure and function in *Drosophila*. Asl, Ana2, DSas-4, DSas-6 and Sak have been identified as essential components for centriole duplication, while Cnn and DSpd-2 are thought to be important in establishing the PCM. However, the relative position of these 7 components in the pathway of centrosome assembly in *Drosophila* embryos remains elusive, as a genetics analysis of this process is hampered by the absence of centrioles in most mutant embryos for these proteins.

In this thesis I elucidate the pathway of centrosome assembly in *Drosophila* by using SAPs (DSas-6/Ana2 particles that form in *Drosophila* unfertilised eggs upon moderate expression of DSas-6 and Ana2) as proxy models of centrosomes. I show SAPs are very similar to centrosomes in composition and dynamics but unlike centrosomes are able to form even in the absence of some of the essential centriolar components. SAP analysis in the absence of each of the main centrosome components reveals that: Sak is not required for the recruitment of downstream components; DSas-4 is necessary for Ana2 and DSas-6 to interact; Asl is the most upstream component of the PCM recruitment pathway, followed by DSpd-2; it is likely that there is an additional PCM recruitment pathway. I then take advantage of some of these results to examine how centrosome formation is potentiated after egg activation. My work allows me to propose an improved description of the pathway of centrosome formation in *Drosophila*.

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Abbreviations

γ-TuRC	γ-tubulin ring complex
γ-TuSC	γ-tubulin small complex
3D	three-dimensions
3D-SIM	3D structured-illumination microscopy
aa	amino acid
Ana	anastral
Asl	asterless
bp	base pair
BSA	bovine serum albumin
Cep	centrosomal protein
Cnn	centrosomin
CP	centrosomal protein
C-terminal/terminus	carboxyl-terminal/terminus
co-IP	co- immunoprecipitation
DNA	deoxyribonucleic acid
D-PLP	*Drosophila* pericentrin-like protein
EM	electron microscopy
EMS	ethyl methanesulfonate
FRAP	fluorescence recovery after photobleahing
GFP	green fluorescent protein
Hr	hour
M	molar
Min	minutes
mM	millimolar
MT	microtubules
MTOC	microtubule organising centre
N-terminal/terminus	amino-terminal/terminus
PBS	phosphate buffered saline
PCM	pericentriolar material
Plk	Polo-like kinase
r	Spearman correlation coefficient

RFP	red fluorescent proteins
RNAi	ribonucleic acid interference
ROI	region of interest
RT	room temperature
s	seconds
SAP	DSas-6/Ana2 particle
STIL	SCL/TAL1 interrupting locus
SPD/Spd	spindle defective
TACC	transforming acidic coiled-coil
UAS	upstream activating sequence
Ubq	ubiquitin
WT	wild-type
Y2H	yeast-two-hybrid
ZYG	zygotic defective

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Chapter 1: Introduction

Centrioles are cellular structures characterised by a beautiful, intricate structure, and are important components of two cellular organelles: the centrosome and the cilium. The last common eukaryotic ancestor possessed a centriole (Carvalho-Santos et al., 2010; Hodges et al., 2010), suggesting an evolutionary essential role for this structure. There are several different theories for the early, conserved, role of the centriole. One possibility is that the earliest role of the centriole was in forming the cilium, which may have allowed cells to sense and interact with their environment more efficiently. Another possibility is that motile cilia may have been involved in moving nutrients between cells localised in close proximity, therefore performing an essential role in the establishment of multicellularity.

Centrosomes, containing both centrioles and a surrounding pericentriolar material (PCM), are not present in all eukaryotic cells, having been lost in certain lineages (Marshall, 2009; Azimzadeh et al., 2012). However, they provide extra efficiency in the cellular processes they are involved in, and are essential in specific tissues. The dual role of centrioles in centrosome and cilia make them very important structures to study, with relevance to many human diseases.

1.1 Centrioles, Centrosomes and Cilia

1.1.1 Centriole structure

The centriole is a cylinder-shaped, microtubule (MT) based structure that plays a central role in the formation of the centrosome, cilium and flagellum. The length and diameter of the centriole can vary widely, depending on the species, cell type and even cell cycle stage. For example, centrioles in *Drosophila* embryos are 180-200nm long, while in testis they can be as long as 900nm (Gonzalez et al., 1998). The diameter of the centriole normally ranges from 100 to 250nm.

Centrioles are typically nine-fold symmetric structures, containing 9 MT sets arranged in a circle (Riparbelli et al., 2009). The number of MTs in these 9 sets is dependent on the species and sometimes even on the specific region of the centriole concerned. Vertebrate centrioles usually have 9 sets of MT triplets (Fig.1.1A). The nine MT triplets are organised in a circular arrangement, with an anti-clockwise twist to each triplet. Each triplet contains one A-tubule (inside), B-tubules (middle) and C-tubules (outside). MTs are filamentous structures formed of dimers of alpha and beta tubulin that organise into long protofilaments. These protofilaments then organise in a circular fashion to form the tube-like MT structure (Fig.1.1B). Of the 3 MTs in the centriolar triplets, only the A-microtubule is formed of 13 filaments,

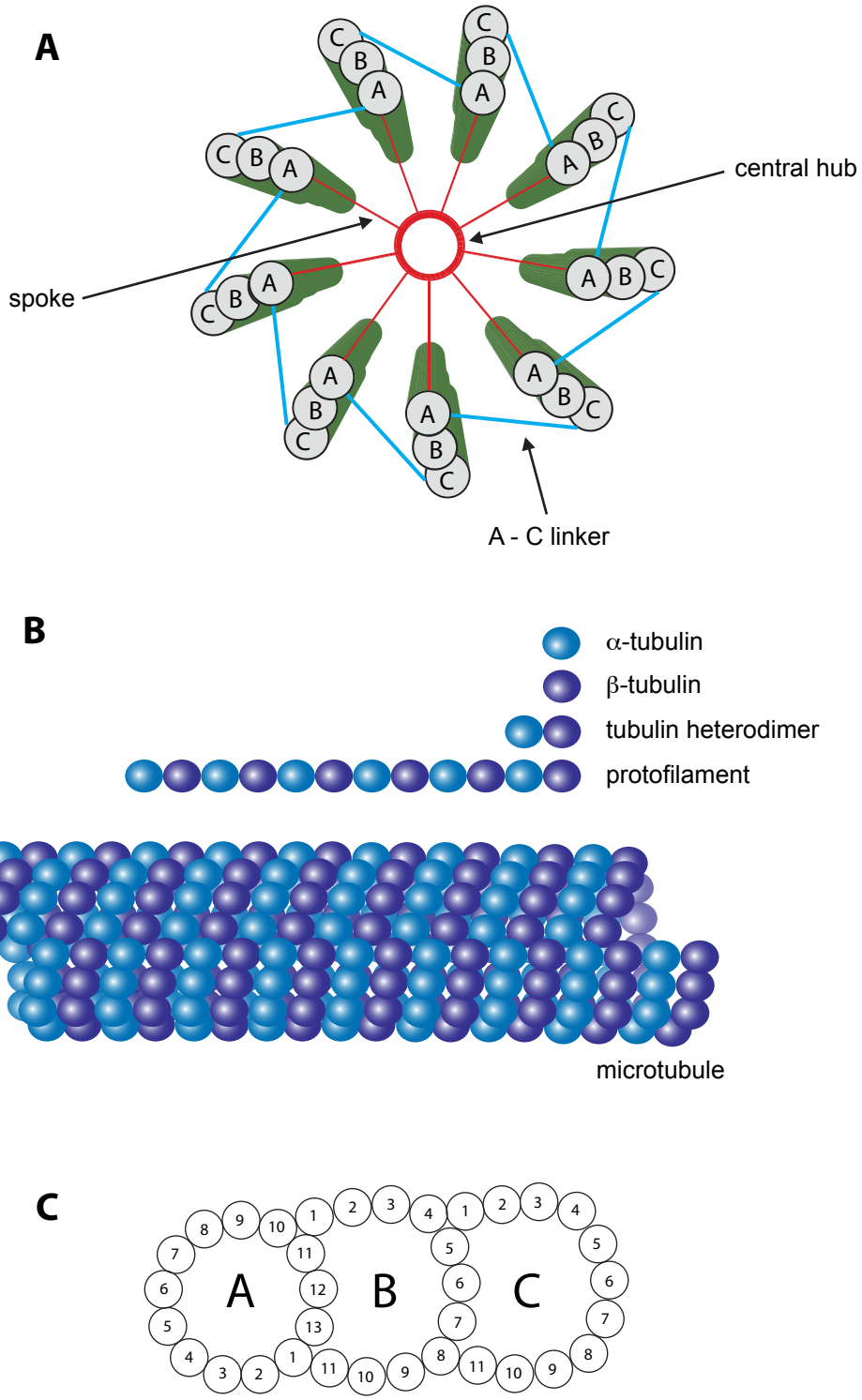


Figure 1.1. Centriolar microtubule organisation
(A) Cross section through a typical vertebrate centriole showing the cartwheel (red). Each cartwheel spoke is connected to a MT triplet (green). MT triplets are connected to each other via A-C linkers (blue). Adapted from Gonczy, 2012. **(B)** Schematic representation of α-tubulin, β-tubulin, tubulin heterodimers, protofilaments and microtubules (formed by the side association of 13 protofilaments into a tube). Adapted from Conduit, 2010. **(C)** Schematic representation of a centriolar MT triplet, with its A, B and C tubules. Note how only the A tubule is formed by 13 protofilaments, while the B and C tubules are incomplete. Adapted from Linck and Stephens, 2007.

while the B- and C- microtubules form incomplete tubules (Fig.1.1C). MT triplets are connected to one another via a linker that connects the A tubule of one triplet to the C tubule of a nearby triplet (Fig.1.1A). Centriolar MTs are very stable structures, and there is little exchange with the cytoplasmic pool of tubulin (Kochanski and Borisy, 1990).

One of the most fascinating features of centrioles is their ability to duplicate once, and only once, per cell cycle. This process occurs during S phase, seemingly in synchrony with DNA replication. Centriole duplication appears to be a template-driven process. A pre-existing centriole, known as the mother centriole, is able to recruit precursor components to its outer wall, and hence template the formation of a daughter centriole in an orthogonal orientation. This process is now quite well understood, and the molecular players in this process are described in detail in section 1.2. A structural component of the centriole that is thought to be very important in the early formation of the daughter centriole is the central cartwheel.

The central cartwheel is composed of a central hub, from which nine-spokes emanate (Fig.1.1A). Each spoke connects, most likely indirectly, to the A-tubule of a MT triplet. It is therefore not surprising that the cartwheel is thought to be the structure that establishes the characteristic nine-fold symmetry of centrioles (Kitagawa et al., 2011c; van Breugel et al., 2011). In agreement with such a role, in *Chlamydomonas* the cartwheel is the earliest recognisable daughter centriole structure formed on the wall of the mother centriole during centriole duplication (Matsuura et al., 2004). Consistent with

this observation, in vertebrate centrioles the cartwheel is only present at the base of the daughter, but not mother, centriole (Guichard et al., 2010) (Fig.1.2C). It should be noted that there are a few exceptions to this arrangement. For example, the short centrioles observed in the *Drosophila* embryo show cartwheel structures along the whole length of both mother and daughter centrioles (Gonzalez et al., 1998) (Fig.1.2B). *C. elegans* centrioles are at the other extreme of the spectrum, as they do not appear to contain cartwheels. *C. elegans* centrioles possess only a central tube, to which nine MT singlets are thought to attach directly (Fig.1.2A). Therefore, when centriole duplication takes place in *C. elegans,* the first structure to be detected on the wall of the mother centriole is not a cartwheel, but rather a short tube (Pelletier et al., 2006).

Recent crystallographic work describing the structure of the cartwheel component SAS-6/DSas-6/hSAS-6 (Kitagawa et al., 2011c; van Breugel et al., 2011) suggests that this essential cartwheel component assembles into rings with nine-spokes, as discussed in more detail in section 1.2.1. This SAS-6/DSas-6/hSAS-6 ring with nine-spokes seems to be the minimal unit of the cartwheel. Recent cryo-EM tomography of the very long basal bodies of *Trychonympha* (Guichard et al., 2012; Guichard et al., 2013) sheds light on the organisation of these rings into a complete cartwheel. This work suggests that 2 rings come together to form a dimer, via interactions of their spokes, as shown in Fig.1.3A and B. The dimers of rings seem to then be stacked on top of each other to form a long cartwheel. This organisation would suggest a mechanism to regulate the length of the cartwheel by

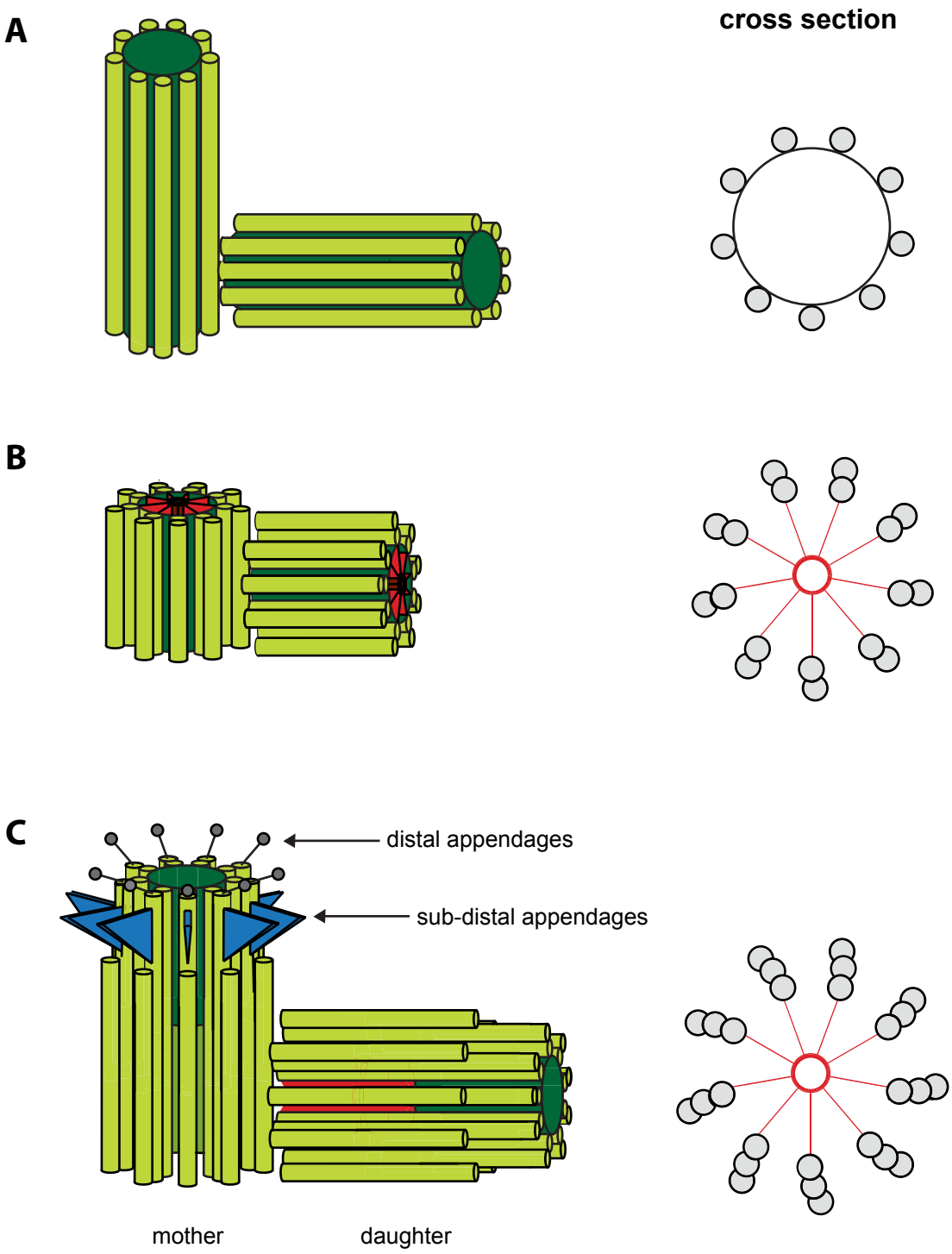


Figure 1.2 The structure of centrioles in different species
Panels show a schematic view (left) or a cross sectional view (right) of typical centrioles found in *C. elegans* **(A)**, *D. melanogaster* **(B)** and vertebrates **(C)**: MTs (green), central cartwheel (red) and appendages (blue/gray). Note how no cartwheel is detectable and only MT singlets are present in *C. elegans* (A). In *Drosophila*, MT doublets are usually present (although this varies in different cell types) and the central cartwheel extends through the length of both mother and daughter centrioles (B). In typical vertebrate centrioles the proximal end of the centriole is composed of MT triplets and the distal end of MT doublets, and only the daughter centriole has a detectable cartwheel, which is usually restricted to the proximal end. The mother centriole usually also has distal and sub-distal appendages associated with its distal end. Adapted from Conduit, 2010 and Nigg and Raff, 2009.

controlling the number of cartwheel dimer rings added to the structure (Fig.1.3C). Interestingly, a recent paper suggests that the central tube of the *C. elegans* centriole might actually have a spiral arrangement (Hilbert et al., 2013). However, as the worm centriole has an atypical structure (Fig.1.2A), it is possible that this spiral arrangement is *C. elegans*-specific.

The vertebrate centriole is also characterised by the presence of distal and sub-distal appendages. These seem to be only associated with the mother centriole, and to be variable in thickness and number depending on the centriole (Paintrand et al., 1992; Sonnen et al., 2012). Several proteins have been reported to localise to the distal appendages: Cep164 (Graser et al., 2007a; Sillibourne et al., 2011), Cep98, Cep83, SCLT1, FBF1 (Tanos et al., 2013) and Cep123 (Sillibourne et al., 2011). The distal appendages are thought to be required for the anchoring of centrioles to the plasma membrane to allow the formation of the cilium, by recruiting vesicles to the centriole (Reiter et al., 2012; Schmidt et al., 2012). Concurrently with a role in ciliogenesis, the depletion or absence of distal appendage proteins leads to cilium formation defects (Ishikawa et al., 2005; Graser et al., 2007a; Tanos et al., 2013).

Several proteins have also been reported to localise to the sub-distal appendages: Ninein (Mogensen et al., 2000), Cep170 (Guarguaglini et al., 2005), ε-tubulin (Chang et al., 2003) and Odf2 (Nakagawa et al., 2001; Ishikawa et al., 2005). Sub-distal appendages have been implicated in

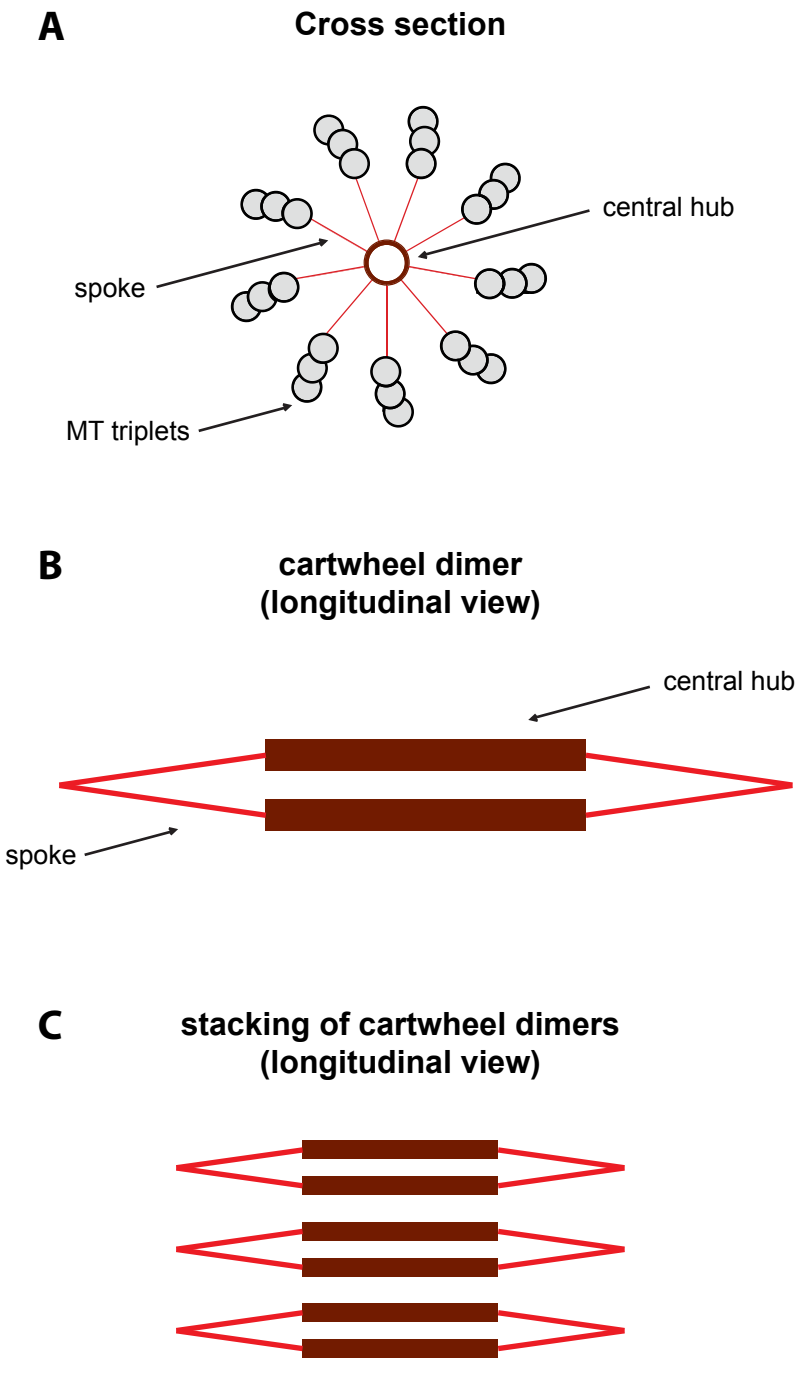


Figure 1.3 The structure of the centriole central cartwheel
(A) Cross section through a cartwheel, showing the central hub and the radiating nine spokes that are attached to the MT triplets. **(B)** In a longitudinal view, the cartwheel minimal unit is a dimer composed of two central hub rings, each with nine spokes emanating outwards that come together at the periphery **(C)** These cartwheel dimers are stacked on top of each other to form the carthweel that extends along the inner volume of the centriole. Adapted from Guichard et al., 2012 and Guichard et al., 2013 .

the anchoring, but not nucleation, of MTs to the vertebrate centriole (Delgehyr et al., 2005). Interestingly, although homologues of some of the vertebrate appendage proteins are found in *Drosophila*, in this organism centrioles do not seem to possess appendages (Carvalho-Santos et al., 2010; Hodges et al., 2010).

While daughter centrioles do not possess appendages, several proteins have, however, been shown to localise exclusively to daughter centrioles. Some of these asymmetrically localised proteins include the poly (ADP-ribose) polymerase PARP-3 (Augustin et al., 2003), the interphase PCM recruitment factor centrobin (Zou et al., 2005; Januschke et al., 2011; Januschke et al., 2013; Jeffery et al., 2013), Cep120 (Mahjoub et al., 2010) and the kinesin Kif16a (Torres et al., 2011).

1.1.2 The Centrosome

1.1.2.1 Centrosome organisation

In many cells, centrioles assemble centrosomes, the major MT organising centres. Centrosomes are formed by two main components. The first of these components is the centriole, described in detail in the previous section. A centrosome will normally contain one (in G1 phase) or two (from S-phase onwards) centrioles, depending on whether centriole duplication has occurred. The centrioles at the centrosome are surrounded by an

electron-dense matrix named the pericentriolar material (PCM). MT nucleation takes place in the PCM (Kuriyama and Borisy, 1981). A schematic representation of a centrosome is shown in Fig.1.4.

1.1.2.2 Functions of the centrosome

One of the most obvious functions of the centrosome is to nucleate MTs, and indeed centrosomes are generally considered to be the major MT organising centres (MTOC) in many cells. The MT organising role of the centrosome is most obvious during mitosis, when two centrosomes, one at each pole, are important players in the formation of the mitotic spindle. Centrosomes are also important regulatory centres in the cell cycle (Doxsey et al., 2005a; Doxsey et al., 2005b). Centrosomes are thought to form “hubs” at which the behaviour of a variety of proteins, such as cell cycle regulators and checkpoint proteins, are integrated (Ma and Poon, 2011). For example, Cyclin B1-Cdk1, is initially activated at centrosomes (Jackman et al., 2003), and cyclin B destruction appears to be initiated there at the end of mitosis (Wakefield et al., 2000). The centrosome is also thought to play other specific roles in specialised cell types. For example, centrosome repositioning within T cells guarantees the transport of lytic granules to the point of contact with target cells (Stinchcombe et al., 2006), while in certain stem progenitor cells the centrosome is thought to be important for proper spindle positioning during asymmetric cell division

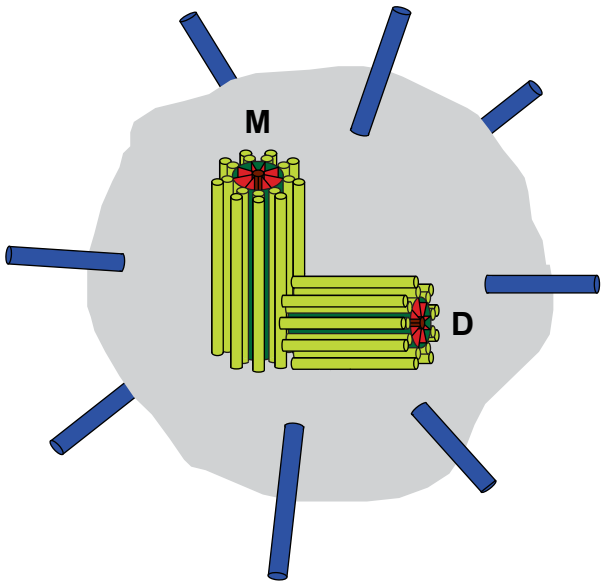


Figure 1.4 The centrosome
The centrosome is usually formed by a pair of centrioles. Centriolar MTs (green), cartwheel (red). The daughter centriole is arranged orthogonally in relation to the mother centriole. The centrioles organise the pericentriolar material (PCM- in grey). The PCM is where MT (blue) nucleation takes place at the centrosome. Note that the centriolar MTs (green) and MTs nucleated from the PCM (blue) are not drawn to the same scale.

(Basto et al., 2006; Gonczy, 2008; Knoblich, 2010; Homem and Knoblich, 2012). Despite playing an important role in these functions, centrosomes are not essential components of every cell. Some organisms, such as higher plants, funghi, red algae and planaria, lack centrosomes completely (Marshall, 2009; Azimzadeh et al., 2012). In other organisms, the requirement for centrosomes is very much cell specific. In many organisms female meiosis is organised in the absence of centrosomes, and in mice acentrosomal cell division takes place in the early stages of embryogenesis (Calarco-Gillam et al., 1983; Schuh and Ellenberg, 2007; Courtois et al., 2012). In flies, centrosomes seem to not be necessary in most cells, with the exception of the very early embryo (Basto et al., 2006; Stevens et al., 2007). However, in those cells where centrosomes seem to be largely dispensable, centrosomes are required to make certain processes, such as spindle formation, more efficient (Basto et al., 2006). For example, while flies lacking the spindle checkpoint are viable, when the centrosome is also lacking there is a high incidence of polyploidy, resulting in death by the larval stage (Buffin et al., 2007). The centrosome is also essential for spindle formation when other MT nucleation pathways, such as the augmin pathway, are absent (Wainman et al., 2009). It is possible that centrosomes evolved to make these processes more robust. An alternative hypothesis is that centrosomes evolved mainly to allow the efficient segregation of centrioles upon cell division, as centrioles are critical for the formation of cilia, which are essential (discussed in section 1.1.3).

1.1.2.3 The centrosome duplication cycle

As centrosomes play a role in establishing the mitotic spindle, it is not surprising that centrosome duplication and maturation is tightly linked to the cell cycle. Over the years several papers have described this process in detail in the context of the cell cycle, by taking advantage of electron microscopy techniques (Robbins et al., 1968; Vorobjev and Chentsov Yu, 1982; Paintrand et al., 1992; Chretien et al., 1997). A schematic representation of this process is shown in Fig.1.5.

In the beginning of G1 each cell inherits 1 centrosome containing 2 centrioles (a mother and a daughter) in an orthogonal arrangement. During G1 the two centrioles lose this 90° organisation in a process known as centriole disengagement (Piel et al., 2000). The kinase Plk1 and, to a lesser extent, the protein separase, have been implicated in this process (Tsou and Stearns, 2006; Tsou et al., 2009). Separase is the enzyme responsible for cleaving cohesin, the ring-like protein complex that maintains sister chromatid cohesion during mitosis (Nasmyth, 2011). Recent work suggests that the connection between mother and daughter centriole might also be maintained by cohesin, suggesting a mechanism to synchronise sister chromatid separation and centriole disengagement, although this remains controversial (Schockel et al., 2011). After disengagement, the two centrioles are spatially separated, but remain attached via a linker until later in the cell cycle.

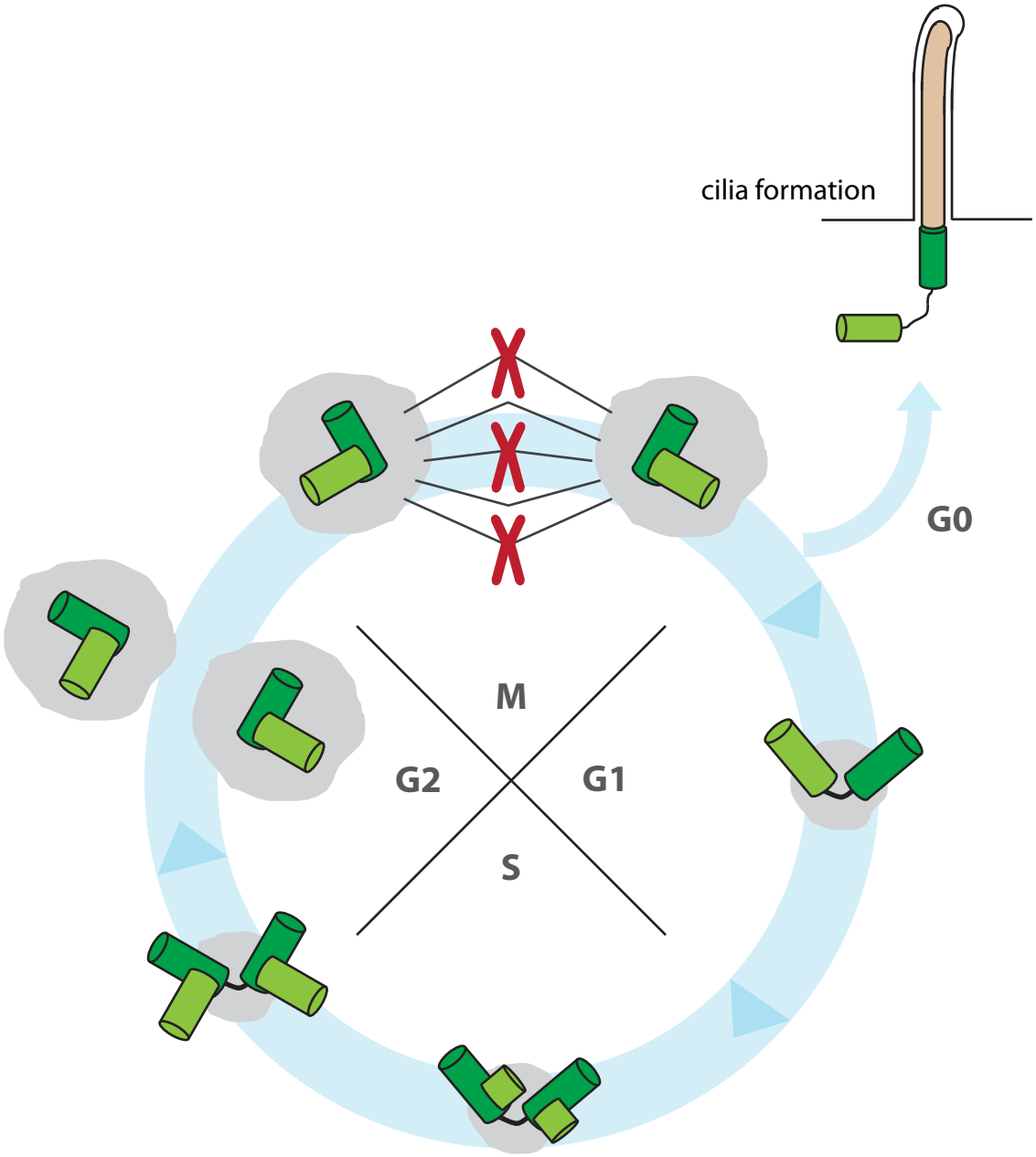


Figure 1.5 The centrosome duplication cycle
A schematic view of the centriole duplication cycle. **G1.** Each cell receives 1 centrosome containing 2 centrioles, which are disengaged but remain attached by a linker structure. **S phase.** Each centriole templates the formation of a procentriole. **G2.** Procentrioles elongate and the 2 centriole pairs separate, forming 2 separate centrosomes. PCM maturation is usually initiated in late G2/M. **M.** The 2 centrosomes move to opposite sides of the cell and nucleate MTs, forming the mitotic spindle. Each centrosome will be segregated to a different daughter cell. Some cell types can become quiescent following mitosis (**G0**) and in some cases the older centriole will become a basal body and form a cilia. Centrioles are shown in green: mother centriole (dark green), daughter centriole (light green). Chromosomes (red), axoneme of the cilium (beige).

During S phase, and seemingly in coordination with DNA replication, both the mother and daughter centrioles inherited from the previous cycle act as mothers, and each templates the formation of a new daughter procentriole at their proximal end. The newly formed procentrioles then elongate during G2. Also during G2, centrosome disjunction takes place. This is the process by which the two now mother centrioles loose the linker that has kept them physically connected since disengagement. The linker between the two inherited centrosomes is thought to be composed of proteins such as Rootletin and C-Nap1 (Mayor et al., 2000; Mayor et al., 2002; Bahe et al., 2005; Yang et al., 2006). During G2, the kinase Nek2 phosphorylates Rootletin and C-Nap1 in order to promote centrosome separation (Faragher and Fry, 2003; Mardin et al., 2010). The kinase Plk1 has also been implicated in this process, upstream of Nek2 (Mardin et al., 2011).

By the end of G2, the cell possesses two centrosomes, each containing two centrioles in an orthogonal organisation. During the G2 to mitosis transition the PCM also increases considerably in size, a process known as centrosome maturation. This increase in PCM size correlates with an increase in MT nucleation capability. During mitosis the two centrosomes move to opposite poles of the cell and nucleate MTs extensively, contributing to the formation of the mitotic spindle. At the end of mitosis each centrosome is segregated to a different daughter cell, the PCM reduces in size, and the cycle begins again (Nigg and Stearns, 2011).

In some cells, mitosis is followed by a state of quiescence (G0). In some quiescent cells, the centriole can template the formation of the cilium, which is described in more detail in section 1.1.3.

1.1.2.4 *de novo* centriole formation

During canonical centriole duplication, new centriole formation occurs only during S phase, and is limited to once per cell cycle. Although the centriole duplication cycle would suggest that centriole formation absolutely requires the presence of a template mother centriole, it is clear that some cells can form centrioles *de novo*. This process occurs normally during mouse embryogenesis. During the first cell divisions of the mouse embryo, cell division is performed via an acentrosomal pathway, but by the 64 cell stage centrioles have been generated *de novo* (Gueth-Hallonet et al., 1993; Courtois and Hiiragi, 2012). The flatworm planaria also uses the *de novo* pathway to generate centrioles in cells that will form cilia, as its precursor cells do not contain centrioles (Azimzadeh et al., 2012). Moreover, when centrioles in human cells were ablated by laser or removed by microsurgery, new centrioles were shown to form *de novo* in the cytoplasm (Khodjakov et al., 2002; La Terra et al., 2005; Uetake et al., 2007). The genetic removal of centrioles in *Chlamydomonas* also induces this *de novo* formation (Marshall et al., 2001). In unfertilised *Drosophila* eggs, which do not possess any centrioles, *de novo* centriole formation can be induced by the overexpression of key centriole formation factors (Rodrigues-Martins et al.,

2007b; Dzhindzhev et al., 2010). These observations suggest that centriole formation can take place in the absence of a template, but that *de novo* formation is normally inhibited by the presence of an existent centriole, presumably to prevent an aberrant number of centrioles forming. It should be noted that a specialised type of *de novo* centriole formation takes place in some multicilliated cells, involving the formation of dense clusters of material called deuterosomes, around which many procentrioles simultaneously form (Dawe et al., 2007). This process is poorly understood.

1.1.3 Cilia

Cilia are cellular structures that are present in most animal cells (Davis et al., 2006). The cilium is present in the plasma membrane and is formed by a central MT-based structure- the axoneme, which is covered by a specialised membrane (Bisgrove and Yost, 2006). Most cells possess a single non-motile primary cilium (Fig.1.6A). The primary cilium is characterised by a 9+0 organisation, i.e. 9 MT doublets organised in a radial fashion (similar to the centriole) containing no MT doublet in the centre (Praetorius and Spring, 2005). Specific vertebrate cells, such as those that form epithelial tissues, can possess hundreds of a second type of cilium, known as motile cilium (Fig.1.6A). Motile cilia are characterised by a 9+2 organisation, i.e. 9 MT doublets organised radially, with an additional MT doublet in the centre (Fisch and Dupuis-Williams, 2011). While most vertebrate cells possess cilia (Davis et al., 2006), invertebrates only form cilia in specific cell types.

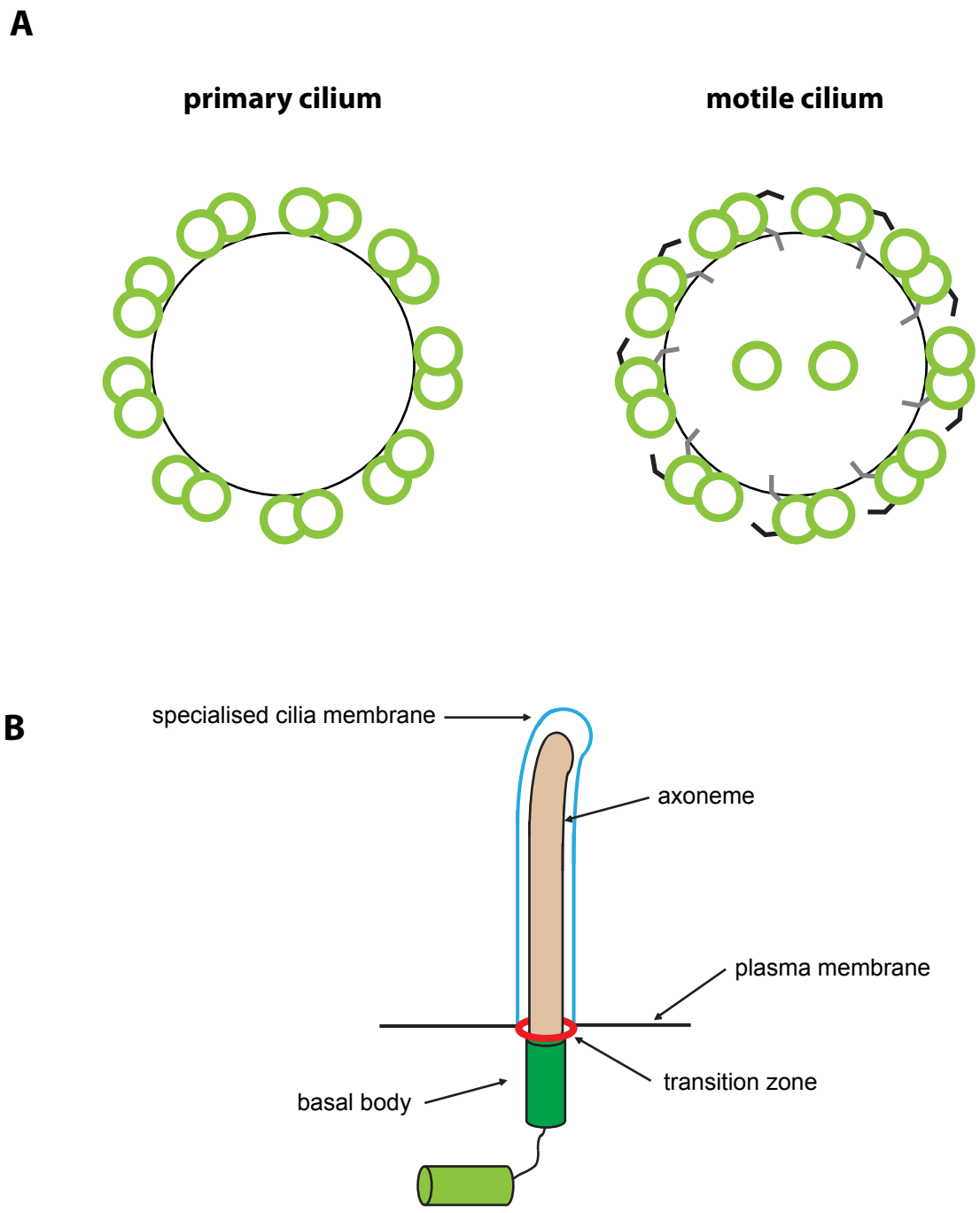


Figure 1.6 The structure of the cilium
(A) A cross-sectional view through the axoneme of a typical primary cilium (left), and a typical motile cilium (right). The primary cilium is composed of 9 MT doublets (green), the so called “9+0” arrangement, while the motile cilium has two additional central MTs, a “9+2” arrangement, and characteristic dynein arms (in black and in grey) associated with the outer MTs. **(B)** Schematic representation of a cilium at the plasma membrane; note how the cilium is surrounded by a specialised membrane (light blue). The axoneme of the cilium (beige) is formed by the extension of MT doublets (not shown) from the basal body (dark green). The area where the basal body transitions into the axoneme is known as the transition zone (red). The basal body is formed from the oldest centriole in the cell, and remains attached by a linker to the younger centriole (light green). Adapted from Dawe et al., 2007.

Drosophila, for example, only possesses cilia in certain types of sensory neurons and sperm (Kernan, 2007). As a consequence, flies lacking centrioles are uncoordinated and infertile, but otherwise viable (Basto et al., 2006).

Cilia are formed when a centriole pair moves to the plasma membrane, and the oldest (mother) centriole becomes anchored to the membrane (Fig.1.6B). This anchored centriole forms the basal body. The extension of the basal body from its distal end leads to the formation of the axoneme and hence the cilium, a process known as ciliogenesis (Bisgrove and Yost, 2006). Centriolar proteins CP110 and Cep97 are binding partners, and are thought to be involved in preventing this process in cycling cells (Spektor et al., 2007; Schmidt et al., 2009). Cep290 is another protein implicated in this process, and is thought to establish the transition zone, i.e. the zone that connects the basal body to the axoneme (Craige et al., 2010). The transition zone is of particular importance because it is the region that controls the entrance of components from the cytoplasm to the ciliary compartment. The ciliary compartment is the region between the axoneme and the ciliary membrane. Components from the cytoplasm are required to enter this region to build and maintain ciliary structure, and maintain the characteristic properties of the specialised ciliary membrane (Kobayashi and Dynlacht, 2011; Reiter et al., 2012).

Cilia are thought to perform a variety of functions. The function of motile cilia has been appreciated for some time, as these cilia are responsible, for

example, for the clearance of mucus from tracheal and bronchial epithelial tissue (Afzelius, 1976). The role of the primary cilium, however, was for a long time a mystery. Specialised functions, such as the establishment of left-right symmetry in the embryo (Afzelius, 1976; Supp et al., 1997) have long been reported, but a general role to justify its existence in most vertebrate cells has long been elusive. It is now thought that the major function of primary cilia is signalling, more specifically playing an essential role in hedgehog signalling (Huangfu et al., 2003). Hedgehog signalling is involved in a plethora of developmental processes, from limb development to stem cell maintenance (Jiang and Hui, 2008). Cilia also play an important, but not essential, role in Wnt/PCP signalling (Simons et al., 2005). Interestingly, the role of cilia in cellular signalling does not seem to be conserved across evolution. For example, *Drosophila* and planaria use hedgehog signalling without involving cilia (Ingham et al., 2011).

1.1.4 Centrosomes, cilia and human disease

As described in previous sections, centrosomes and cilia play many important roles in the cell. Perhaps not surprisingly their dysfunction has been associated with many human diseases.

Centrosome dysfunction was first suggested to be associated with tumorigenesis by Boveri in 1914 (Boveri, 2008), and is now known that a variety of tumours are characterised by excessive numbers of centrosomes

(Lingle et al., 2002; Pihan et al., 2003). Aneuploidy is a common feature of cancers. Based on his work in embryos with multipolar spindes, Boveri suggested that when too many centrosomes are present in a cell, multipolar spindles can form, and this can cause chromosome missegregation. More recent work in *Drosophila* cells shows that when supernumerary centrosomes are present they tend to cluster into two poles, and so cell division is usually bipolar (Sibon et al., 2000; Basto et al., 2008). However, Boveri was right in associating supernumerary centrosomes with aneuploidy. Although cells with too many centrosomes divide in a bipolar fashion, they often undergo an intermediate multipolar stage. During this stage, the attachment of a single kinetochore to both spindle poles can take place (merotellic attachments), and this does indeed lead to elevated levels of chromosome instability (Ganem et al., 2009).

An alternative mechanism by which supernumerary centrosomes can potentially induce tumorigenesis is by interfering with asymmetric cell division. This hypothesis is supported by the observation that excessive centrosome numbers interfere with asymmetric cell division in *Drosophila* neuroblasts, and that these misregulated neuroblasts can induce the formation of tumours when injected in the abdomen of flies (Basto et al., 2008). This could also be a potential mechanism to induce the formation of cancer stem cells from existing stem cell populations.

While a compelling genetic link between centrosomes and human cancer has remained elusive, a surprising role for centrosome dysfunction in human

disease has recently been identified. Several genes that encode centriole and centrosome proteins have been shown to be mutated in patients with primary autosomal recessive microcephaly, a condition characterised by small brain size at birth (Bond et al., 2005; Gul et al., 2006; Hassan et al., 2007; Kumar et al., 2009; Darvish et al., 2010; Guernsey et al., 2010; Kalay et al., 2011; McIntyre et al., 2012; Pagnamenta et al., 2012). However, the mechanistic connection between this phenotype and centrosome dysfunction is uncertain. An attractive possibility is that, as in flies (Basto et al., 2008), centrosome dysfunction may interfere with the asymmetric division of neuronal progenitor cells, therefore leading to reduced brain size (Wang et al., 2009). Other cellular mechanisms linked to centrosomes, such as cilia and/or DNA damage repair, may also be involved in this process (Hsu and White, 1998; Takada et al., 2003; Griffith et al., 2008; Barr et al., 2010). Indeed, other human diseases that include microcephaly but have other additional phenotypes, such as Seckel syndrome and Majewski osteodysplastic primordial dwarfism, have been associated with centrosome problems and have also been linked to DNA damage (Griffith et al., 2008; Ogi et al., 2012; Mokrani-Benhelli et al., 2013).

Ciliopathies is the name given to a varied group of conditions associated with cilia defects. Some of the pathologies in this broad definition include diseases such as Primary Ciliary Diskinesia (PCD), Bardet-Biedl Syndrome (BBS) and polycystic kidney disease (PKD). Considering the developmental role of cilia, and their presence in all human cells, it is not surprising that ciliopathies are characterised by a broad range of phenotypes. These

include infertility, *situs inversus*, mental retardation, obesity, polydactyly, diabetes and kidney defects (Bisgrove and Yost, 2006; Nigg and Raff, 2009). Interestingly, there seems to be a link between cilia function and obesity, as mice mutated in certain ciliary components become obese (Coleman and Eicher, 1990; Sun et al., 2012; Mukhopadhyay and Jackson, 2013). How ciliary defects lead to this plethora of phenotypes is still unclear.

Cilia defects have also been associated with cancer. Reduced presence of cilia has been observed in a variety of cancers, and this is most likely to involve the misregulation of Wnt and Hedgehog signalling (Seeley et al., 2009; Wong et al., 2009; Yuan et al., 2010; Egeberg et al., 2012; Basten et al., 2013), although recently the presence of extra cilia was found to be associated with reduced sonic hedghehog signalling (Mahjoub and Stearns, 2012). Future work in lower organisms, where these signalling pathways are simplified, may help clarify this potential link to tumorigenesis.

1.2 Centriole biogenesis

Proteomics studies of partially purified centrosomes have suggested that the centrosome is likely composed of more than 200 unique proteins (Andersen et al., 2003; Bettencourt-Dias and Glover, 2007; Muller et al., 2010; Jakobsen et al., 2011; Jakobsen et al., 2013). Similar analysis on the components of the centriole and basal body have also revealed many components specific to these structures (Li et al., 2004; Keller et al., 2005;

Kilburn et al., 2007). Dozens of proteins are thought to make the intricate structure of the centriole, and work in the last few years has identified which of these are important in centriole duplication.

Work in *C. elegans* has been crucial in this area, as its embryos are an excellent system with which to identify components required for centriole duplication. While the *C. elegans* egg lacks centrioles (as is the case in other organisms), the sperm brings upon fertilisation a centrosome that contains two centrioles. These two centrioles separate and duplicate, each forming a centrosome. The two resulting centrosomes (each containing two centrioles) will allow the completion of the first cell division. When RNAi against candidate centriole duplication factors is applied in this system, this first cell division still successfully occurs. However, if the depleted protein is important for centriole duplication, the original incoming centrioles will not have duplicated, and as such the second cell division does not take place. This system therefore provides an easy assay in which to assess the contribution of candidate proteins in centriole duplication. Using this set-up, five *C. elegans* proteins have been identified as essential components in centriole duplication: SPD-2, ZYG-1, SAS-6, SAS-5 and SAS-4 (Delattre et al., 2006; Pelletier et al., 2006).

An RNAi screen in fly cells identified several additional components potentially required for centriole duplication in this organism (Dobbelaere et al., 2008). Interestingly, the careful analysis of the role of these components has also narrowed down the list to five essential components. While four of

these components seem to be direct homologues (or at least functional homologues) of the proteins identified in *C. elegans*: Sak/Plk4/Zyg-1, DSas-6/SAS-6, Ana2/SAS-5, DSas-4/SAS-4 (Bettencourt-Dias et al., 2005; Basto et al., 2006; Peel et al., 2007; Rodrigues-Martins et al., 2007a; Rodrigues-Martins et al., 2007b; Basto et al., 2008; Dobbelaere et al., 2008; Dzhindzhev et al., 2010; Stevens et al., 2010a) in flies the homologue of SPD-2, DSpd-2, seems to not be important in duplication (Dix and Raff, 2007; Giansanti et al., 2008), while a new player- Asterless (Asl) has also been identified (Blachon et al., 2008; Dobbelaere et al., 2008; Dzhindzhev et al., 2010). In vertebrates, most of the components are conserved, and the homologues of both DSpd-2 and Asl are thought to play a role in duplication (Zhu et al., 2008; Blachon et al., 2009; Cizmecioglu et al., 2010; Hatch et al., 2010; Sonnen et al., 2013). In the next section I discuss in detail our current knowledge on the five key centriole duplication factors in flies.

1.2.1 Essential centriole duplication proteins

1.2.1.1 Zyg-1/Sak/Plk4

Sak/Plk4 is a kinase of the Polo-like kinase (Plk) family. Plk proteins are characterised by an N-terminus serine/threonine kinase domain and a C-terminus region of homology, the Polo-box (Lowery et al., 2005). While other Plks are characterised by two polo boxes, Sak/Plk4 was until recently thought to have only one Polo-box domain (Fode et al., 1994; Leung et al.,

2002). Sak/Plk4 contains, however, an additional conserved region termed the cryptic polo box. Recently, the crystalisation of this region of *Drosophila* Sak/Plk4 revealed that the cryptic polo-box actually consists of 2 polo-box-like regions, therefore establishing Sak/Plk4 as a Plk protein containing not one but three polo boxes (Slevin et al., 2012).

Sak/Plk4 was first identified as an important player in cell division in both mouse (Fode et al., 1994) and in *C. elegans* (O'Connell et al., 1998), and has been shown to localise to the centriole in a variety of systems (O'Connell et al., 2001; Bettencourt-Dias et al., 2005; Habedanck et al., 2005; Fu and Glover, 2012). The levels of Sak/Plk4 at the centrosome vary with the cell cycle, being reduced in G1 and S phase and peaking in mitosis (Rogers et al., 2009; Sillibourne et al., 2010; Peel et al., 2012).

Complementary work performed with vertebrate Sak/Plk4, as well as with its *Drosophila* (Sak/Plk4) and *C. elegans* (Zyg-1) homologues has established Sak/Plk4 as an essential centriole duplication factor. Work in a mutant of Zyg-1 showed that this protein is essential for the assembly of daughter centrioles (O'Connell et al., 2001). A reduction in centriole numbers was also observed in somatic cells of a hypomorphic mutation of the *Drosophila* Sak/Plk4 (Bettencourt-Dias et al., 2005), as well as by RNAi approaches in *Drosophila* and human cultured cells (Bettencourt-Dias et al., 2005; Habedanck et al., 2005; Dobbelaere et al., 2008; Dzhindzhev et al., 2010). In addition, the overexpression of Sak/Plk4 induces centrosome amplification and centriole overduplication (Habedanck et al., 2005;

Kleylein-Sohn et al., 2007; Peel et al., 2007; Rodrigues-Martins et al., 2007b; Basto et al., 2008).

While similar observations have been reported for other essential centriolar components (see below), Sak/Plk4 has a striking property. Upon overexpression, Sak/Plk4 is the only centriolar protein (with the exception of Asl, see below) that is able induce the formation of *de novo* centrioles in the absence of a template centriole (Rodrigues-Martins et al., 2007a). This observation suggests that Sak/Plk4 is likely to be not only essential for centriole duplication, but indeed one of the most upstream components in this process. A role for Sak/Plk4 in the first steps of centriole formation is supported by additional observations. The analysis of the pathway of centriole assembly in *C. elegans* has shown that Zyg-1 is one of the first centriole proteins to be recruited to the newly formed centriole (Delattre et al., 2006; Pelletier et al., 2006). The pattern of localisation of Sak/Plk4 is also supportive of an early role in centriole duplication. ImmunoEM analysis of human centrioles has shown that Sak/Plk4 localises to the interface between the parental and daughter centriole, suggesting a role in nucleating the formation of the procentriole (Kleylein-Sohn et al., 2007). In addition, recent 3D structured-illumination microscopy (3D-SIM) analysis has shown that Sak/Plk4 is observed as a single dot in the wall of the mother centriole, at the base of the procentriole, and it localises to the site of procentriole formation before any other centriole components can be detected there (Sonnen et al., 2012).

Sak/Plk4 is a kinase, but the identification of the phosphorylation target of Sak/Plk4 has remained elusive. Serine 123 of SAS-6 in *C. elegans* has been identified as a phosphorylation target of Zyg-1 (Kitagawa et al., 2009). This modification was shown to be required for the maintenance of SAS-6 at the centrosome, and in its absence centriole formation is defective. However, this phosphorylated serine is localised in a non-conserved region of SAS-6, and recent studies conclusively demonstrate that this site is not essential for centriole duplication in *C. elegans* (Lettman et al., 2013).

Interestingly, a role for Sak/Plk4 kinase activity has been suggested with regards to its degradation. Sak/Plk4 has been shown to dimerise (Leung et al., 2002; Slevin et al., 2012). Dimerization is thought to promote autophosphorylation in trans, and a variety of phosphosites in Sak/Plk4 have been identified (Guderian et al., 2010; Holland et al., 2010; Sillibourne et al., 2010). Sak/Plk4 autophosphorylation has been shown to promote Sak/Plk4 instability (Holland et al., 2010), most likely mediated by the activity of the proteasome. Proteosome inhibition leads to increased levels of Sak/Plk4 (Holland et al., 2010; Sillibourne et al., 2010; Peel et al., 2012). Sak/Plk4 has been shown to be ubiquitylated (Fode et al., 1994) and ubiquitin ligase complexes in different organisms have been implicated in Sak/Plk4 degradation (Cunha-Ferreira et al., 2009; Rogers et al., 2009; Guderian et al., 2010; Peel et al., 2012). Interestingly, recently published work in *Drosophila* cell culture shows that PP2A phosphatase dephosphorylates Sak/Plk4, suggesting a mechanism for fine tuning of Sak/Plk4 levels (Brownlee et al., 2011).

The regulation of Sak/Plk4 levels is of vital importance. Proteosome inhibition, PP2A phosphatase overactivity and defects in Sak/Plk4 phosphorylation can all induce centrosome amplification (Cunha-Ferreira et al., 2009; Guderian et al., 2010; Sillibourne et al., 2010; Holland et al., 2012). The most obvious implication of the importance of regulating Sak/Plk4 levels is its health consequences when this regulation goes awry: Sak/Plk4 misregulation has been implicated in tumorigenesis (Hudson et al., 2001; Macmillan et al., 2001; Ko et al., 2005; Basto et al., 2008). Although it is currently unclear whether Sak/Plk4 kinase activity is limited to regulating its own stability these observations suggest that this particular role is of vital importance to limit centriole duplication.

1.2.1.2 SAS-6/DSas-6/hSAS-6

SAS-6 was first identified in an RNAi screen in *C. elegans* for genes involved in cell division and spindle assembly (Dammermann et al., 2004; Leidel et al., 2005). It is now known that SAS-6 is a component of the centriole (Leidel et al., 2005; Peel et al., 2007; Rodrigues-Martins et al., 2007a; Yabe et al., 2007; Dammermann et al., 2008; Sonnen et al., 2012). Homologues have been identified in a variety of organisms, in particularly via an evolutionary conserved N-terminal region, the PISA (present in sas-6) motif (Leidel et al., 2005).

SAS-6 and its homologues are essential centriole duplication factors. The absence of SAS-6 leads to centriole formation defects in a variety of systems (Dammermann et al., 2004; Leidel et al., 2005; Kleylein-Sohn et al., 2007; Peel et al., 2007; Rodrigues-Martins et al., 2007a; Strnad et al., 2007; Vladar and Stearns, 2007; Yabe et al., 2007). In addition, overexpression of SAS-6 is able to induce *de novo* formation of MTOCs in *Drosophila* eggs (Peel et al., 2007; Rodrigues-Martins et al., 2007a) and mild centriole overduplication in *Drosophila* embryos (Peel et al., 2007). SAS-6 seems to play an early role in the process of formation of the procentriole. The overexpression of SAS-6 in human cells is able to induce the formation of multiple procentrioles in pre-existing centrioles (Strnad et al., 2007). Sak/Plk4 overexpression is unable to induce the formation of multiple procentrioles in the absence of SAS-6 (Kleylein-Sohn et al., 2007).

SAS-6 is thought to act downstream of Sak/Plk4 in centriole assembly. SAS-6 is only recruited to centrioles after Zyg-1 (Delattre et al., 2006; Pelletier et al., 2006; Sonnen et al., 2012), and Sak/Plk4 can still localise to centrioles in the absence of SAS-6 (Kleylein-Sohn et al., 2007). In addition, recent temporal analysis of the recruitment of different centriolar proteins to human centrioles by super-resolution microscopy has shown that although SAS-6 is indeed one of the earlier components to be recruited to the site of procentriole formation, its recruitment is preceded by Sak/Plk4 (Sonnen et al., 2012). SAS-6 appears to play a structural role in the formation of the centriole. Careful analysis of SAS-6 localisation by ImmunoEM has shown that it localises to the lumen of the centriole (Kilburn et al., 2007; Culver et

al., 2009; Gopalakrishnan et al., 2010). In the absence of SAS-6, centriole duplication is blocked and several structural defects can be observed in centrioles, basal bodies and axonemes. These include cartwheel defects, and defects in the attachment of MT doublet/triplets (Nakazawa et al., 2007; Rodrigues-Martins et al., 2007a; Culver et al., 2009). A structural role in the formation of the central tube/cartwheel of the centriole as also been suggested due to the observation that co-expression of SAS-6 and Ana2 in *Drosophila* testes induces the formation of elongated tube-like structures that exhibit a striking resemblance to the central cartwheel (Stevens et al., 2010b).

Two recent papers describe in molecular detail the X-ray crystallographic structure of SAS-6 from *Chlamydomonads reinhardtii* and zebrafish (Kitagawa et al., 2011c; van Breugel et al., 2011). These structures reveal that although SAS-6 sequence conservation seems to be mainly restricted to the PISA motif, the different SAS-6 homologues are similar with regards to their quaternary structure. Excitingly, these structures immediately suggest how SAS-6 might function in the formation of the centriolar cartwheel.

The crystal structure of SAS-6 shows that one SAS-6 protein is able to interact with another SAS-6 protein via their central coiled-coil regions, forming parallel homodimers. These homodimers can then interact with other dimers via an interaction mediated by the N-terminal globular domains of SAS-6 (Fig.1.7). Specifically, a conserved loop in the N-terminus of

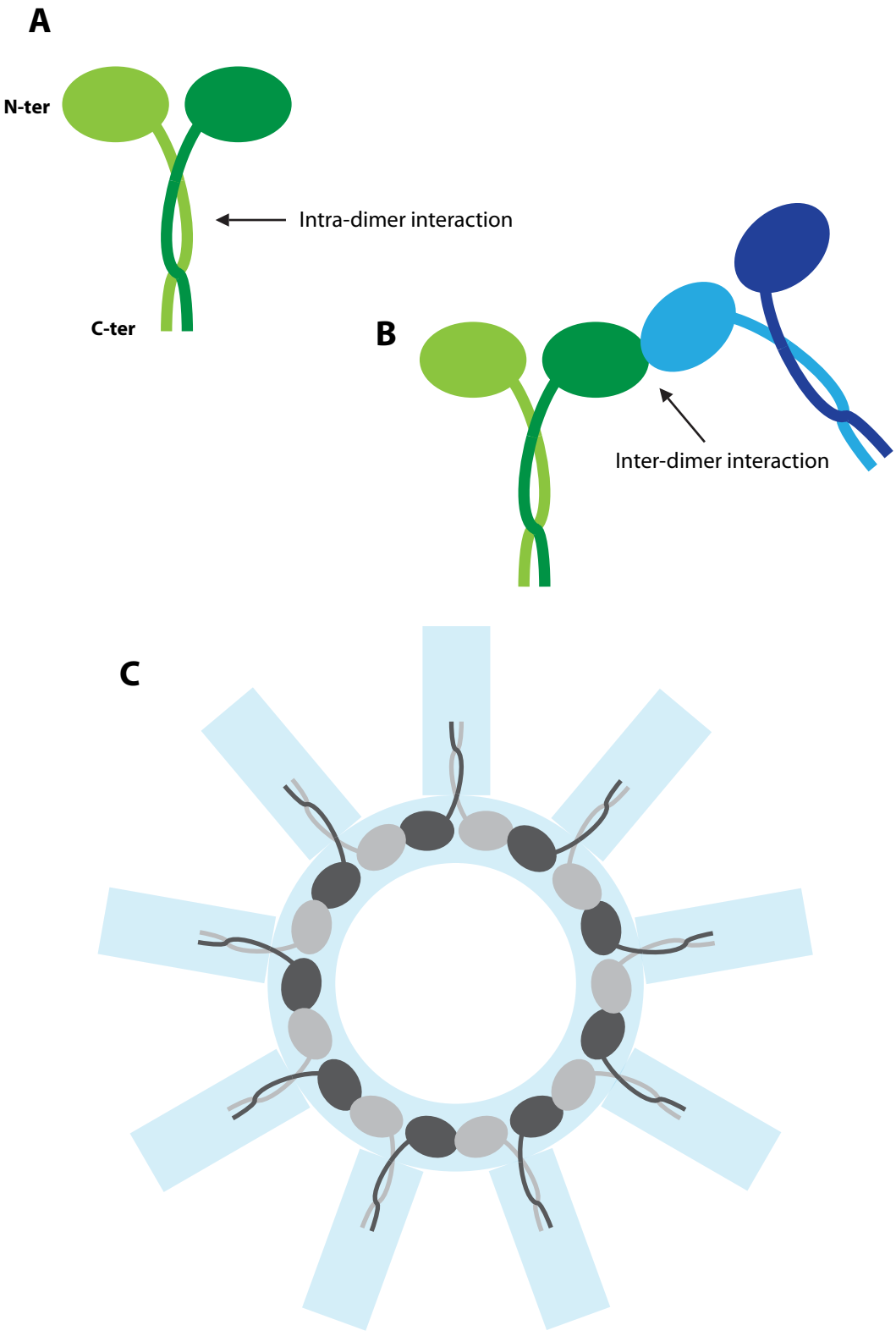


Figure 1.7 The crystal structure of SAS-6 reveals its structural role at the cartwheel
(A) Homodimers of SAS-6 are formed by a "coiled-coil" interaction (in a parallel orientation) between two monomers. **(B)** Inter-dimer interaction is possible via the globular N-terminal domains of two SAS-6 molecules from different homodimers. **(C)** Possible oligomerisation arrangement of SAS-6 homodimers to generate a 9-fold symmetric ring. A potential cartwheel with 9 spokes is shown in light blue. Note how the ring of SAS-6 homodimers could form the central ring of the cartwheel. Note also that these models do not represent the full length SAS-6 (part of the C-terminal coiled-coil domain is lacking as its structure has not been solved yet). Adapted from Cottee et al., 2011.

SAS-6 in one of the dimers fits into a hydrophobic pocket on the N-terminal domain of the other dimer. Both these intra and inter dimer interactions were shown to be of relevance *in vivo*: SAS-6 constructs disrupting either interaction are unable to rescue the centriolar phenotypes caused by the absence of SAS-6 (Kitagawa et al., 2011c; van Breugel et al., 2011). The potential relevance of this oligomerisation capability is obvious: when SAS-6 is allowed to interact *in vitro* at sufficiently high concentrations, it can spontaneously assemble into rings (Kitagawa et al., 2011c; van Breugel et al., 2011). Computer modelling has shown that for SAS-6 dimers to associate to form rings, conformational adjustments are necessary to the existing crystal structures of SAS-6. Interestingly, the ring conformation requiring the least adjustment corresponds to an 8 or 9-fold symmetric ring, with the expected diameter for a cartwheel (van Breugel et al., 2011). SAS-6 is therefore likely to be the main component of the central cartwheel: the inter-dimer interactions establishing the central ring, and the intra-dimer coiled-coils forming at least part of the spokes that emanate outwards towards the centriolar MTs. Other centriolar proteins, however, are likely to be involved in setting the dimensions of the ring, conferring structural integrity to this arrangement and completing the outer spokes. Some of these proteins, such as SAS-5/Ana2/STIL and Bld10/Cep135, are discussed in more detail below.

1.2.1.3 SAS-5/Ana2/STIL

SAS-5 was first identified in a screen in *C. elegans* for genes affecting cell division (Delattre et al., 2004). SAS-5 depletion is associated with problems in centriole duplication and spindle formation (Dammermann et al., 2004; Delattre et al., 2004). It was shown to localise to the centriole, specifically to the lumen (Dammermann et al., 2004; Delattre et al., 2004). SAS-5 is thought to be recruited to the forming procentriole downstream of Zyg-1 (Delattre et al., 2004; Delattre et al., 2006; Pelletier et al., 2006). Interestingly, SAS-5 and SAS-6 seem to be recruited to the procentriole concurrently (Delattre et al., 2006; Pelletier et al., 2006), and to be co-dependent for their localisation (Leidel et al., 2005). SAS-5 and SAS-6 were shown to physically interact by yeast-2-hybrid (Y2H) analysis (Leidel et al., 2005), and the molecular detail of this interaction has been mapped to the central coiled coil region of SAS-6 by crystalisation and mutagenesis analysis (Qiao et al., 2012). Recent work has also shown that SAS-5 localisation to the centriole is dependent on its dephosphorylation by PP2A phosphatase (Kitagawa et al., 2011a; Song et al., 2011).

Due to low sequence homology (Delattre et al., 2004), the identification of homologues of SAS-5 in other organisms has proved difficult. Despite very low sequence similarity, Ana2 and STIL are now considered the *Drosophila* and vertebrate functional orthologues of SAS-5 respectively. STIL is a much larger protein than Ana2, but these two proteins share two regions of sequence conservation: the STAN motif, corresponding to a 90 amino acid

region (Stevens et al., 2010a) and the TIM motif, localised in the extreme C-terminus (Arquint et al., 2012). The TIM motif is of particular interest because it has been identified as a region mutated in patients with microcephaly (Kumar et al., 2009).

Ana2 was first identified in an RNAi screen in *Drosophila* cells for genes required for mitotic spindle assembly (Goshima et al., 2007). Ana2 localises to centrioles (Goshima et al., 2007; Stevens et al., 2010a; Wang et al., 2011), and its depletion in cell culture leads to centriole duplication defects (Dobbelaere et al., 2008). A mutant of Ana2 has recently been reported, but its role has only been examined in neuroblast spindle orientation (Wang et al., 2011). However, homozygous mutant flies are uncoordinated (my own observation), a characteristic phenotype displayed by flies mutated for core centriolar components (Martinez-Campos et al., 2004; Basto et al., 2006; Peel et al., 2007; Blachon et al., 2008), due to the lack of functional cilia (Baker et al., 2004). In addition, overexpression of GFP-Ana2 in spermatocytes induces centriole overduplication (Stevens et al., 2010a). Like SAS-5, Ana2 directly interacts with DSas-6, as shown by Y2H (Stevens et al., 2010a), and co-immunoprecipitation of these two proteins has been reported (Tang et al., 2011). Interestingly, and supportive of a functional relevance to the interaction between Ana2 and DSas-6, the relatively moderate co-overexpression of GFP-DSas-6 and GFP-Ana2 generates D**S**as-6/**A**na2 **p**articles (SAPs) that function as MTOCs in unfertilised *Drosophila* eggs (Stevens et al., 2010a). These SAPs were used as a proxy

model of the centrosome in much of the work in this thesis, and their characteristics are described in detail in subsequent chapters.

Before its role in centriole duplication had been established, vertebrate STIL had been implicated in spindle formation (Pfaff et al., 2007) and in defining left-right asymmetry in mice (Izraeli et al., 1999). STIL is now known to be mutated in a subset of microcephaly patients (Kumar et al., 2009) and is upregulated in certain types of cancer (Erez et al., 2004). Like SAS-5 and Ana2, STIL localises to centrioles (Arquint et al., 2012; Sonnen et al., 2012; Vulprecht et al., 2012) and has been reported to localise specifically to the base of the procentriole (Tang et al., 2011). Depletion of STIL leads to a decrease in centriole numbers, mitotic spindle defects and reduced proliferation in culture cells (Kitagawa et al., 2011b; Tang et al., 2011; Arquint et al., 2012; Vulprecht et al., 2012). In addition, overexpression of STIL can lead to centriole overduplication (Tang et al., 2011; Arquint et al., 2012; Vulprecht et al., 2012). A crucial role in centriole duplication is also supported by the observation that Sak/Plk4-induced centriole overduplication is dependent on the presence of STIL (Vulprecht et al., 2012). The exact nature of the interaction between STIL and hSAS-6, however, is unclear. STIL and hSAS-6 seem to play a role in each other's localisation at the centriole, but their recruitment is not co-dependent, unlike SAS-5 and SAS-6 in *C. elegans* (Tang et al., 2011; Arquint et al., 2012; Vulprecht et al., 2012). In addition, and although STIL and hSAS-6 seem to interact, this interaction does not seem to be direct (Tang et al., 2011; Arquint et al., 2012).

In conclusion, SAS-5 and its functional orthologues Ana2 and STIL are thought to be important centriole duplication factors, acting downstream of Zyg-1/Sak/Plk4, but upstream of other important centriole factors (see below). The exact function of SAS-5/Ana2/STIL, however, remains elusive. In particular, it is unclear whether the direct interaction between SAS-5 and SAS-6 is evolutionarily conserved.

1.2.1.4 SAS-4/DSas-4/CPAP

SAS-4 was first identified in an RNAi screen in *C. elegans* for proteins involved in cell division (Kirkham et al., 2003). Homologues of SAS-4 have been identified in *Drosophila* (DSas-4) and in humans (CPAP). Mutations in CPAP cause microcephaly in humans (Bond et al., 2005; Gul et al., 2006; McIntyre et al., 2012).

SAS-4/DSas-4/CPAP localises to centrioles and mutation or depletion of SAS-4/DSas-4/CPAP is associated with defects in centriole duplication and spindle formation (Hung et al., 2000; Kirkham et al., 2003; Leidel and Gonczy, 2003; Basto et al., 2006; Cho et al., 2006; Peel et al., 2007; Dobbelaere et al., 2008; Kohlmaier et al., 2009; Kitagawa et al., 2011b; Fu and Glover, 2012). In addition, Sak/Plk4-induced centriole overduplication is dependent on the presence of CPAP (Tang et al., 2009). Unlike other core centriole components, however, DSas-4/CPAP overexpression does not seem to induce centriole overduplication (Peel et al., 2007; Schmidt et al.,

2009), and overexpression of DSas-4 in unfertilised *Drosophila* eggs induces the formation of MTOCs that do not contain centrioles (Peel et al., 2007; Dzhindzhev et al., 2010). Considering this fact, it is perhaps not surprising that SAS-4/DSas-4/CPAP is thought to be recruited to centrioles downstream of factors such as SAS-6/DSas-6/hSAS-6 and SAS-5/Ana2/STIL (Delattre et al., 2006; Pelletier et al., 2006; Kohlmaier et al., 2009; Tang et al., 2011).

Early work on centriole formation in worms showed that in the absence of SAS-4, MT singlets cannot be added to the central tube of the forming centriole (Pelletier et al., 2006). A specific region of CPAP, known as PN2-3, can bind to MT dimers (Hung et al., 2004), and CPAP can inhibit MT polymerisation and depolymerise preformed MTs (Hung et al., 2004; Hsu et al., 2008). However, CPAP overexpression is able to induce the formation of MT-based centriole extensions (Kohlmaier et al., 2009; Schmidt et al., 2009; Tang et al., 2009; Kitagawa et al., 2011b). This suggests that CPAP might play an important role in the formation and elongation of centriolar MTs, although future work is required to clarify the seemingly contradictory observation that CPAP has a MT destabilising function. It has also been suggested that CPAP's role in centriole elongation is dependent on its phosphorylation by Plk2 (Chang et al., 2010), and phosphorylation might also be required for CPAP self-assembly (Zhao et al., 2010).

SAS-4 has been reported to localise weakly to the PCM (Dammermann et al., 2008), where its levels are thought to correlate with γ-tubulin levels

(Kirkham et al., 2003). This has led to the suggestion that SAS-4 proteins might be involved directly in PCM recruitment. A recent paper has suggested that in the cytoplasm DSas-4 forms complexes with other PCM components via its N-terminus, and these complexes are then tethered to centrioles via DSas-4's C-terminus (Gopalakrishnan et al., 2011). This hypothesis, however, is not consistent with other published work. Maternally contributed centrioles (which contain some stably incorporated DSas-4) in *dsas-4* mutant *Drosophila* spermatocytes are still able to organise robust levels of PCM (Riparbelli and Callaini, 2011). In addition, the injection of anti-DSas-4 antibodies in *Drosophila* syncytial embryos does not interfere with the recruitment of PCM, although it does block centriole duplication (Conduit et al., 2010). Future work is necessary to clarify the nature of DSas-4's role in PCM recruitment.

1.2.1.5 Asl/Cep152

Asterless (Asl) was first identified in a *Drosophila* screen for mutants affecting male sterility (Bonaccorsi et al., 1998). A vertebrate homologue has been identified, Cep152 (Andersen et al., 2003; Blachon et al., 2008). Cep152 is one of the genes mutated in autosomal primary microcephaly (Guernsey et al., 2010; Kalay et al., 2011). There is no known homologue of Asl in *C. elegans*.

The first identified mutants of *asl*, in particular the most studied *asl*^{1} allele (a hypomorph), were characterised by defects in PCM recruitment and spindle formation in most tissues, while still displaying fully formed centrioles (Bonaccorsi et al., 1998; Giansanti et al., 2001; Varmark et al., 2007; Blachon et al., 2008). Also supportive of Asl's role in PCM recruitment was the observation that injection of anti-Asl antibodies in *Drosophila* syncytial embryos severely inhibits Cnn recruitment to the centriole (Conduit et al., 2010). More recently a stronger allele of *asl* has been identified- *asl*^{mecD} (Blachon et al., 2008). This allele produces a short, unstable protein, leading to defects in centriole duplication and consequently defects in cilia formation (Blachon et al., 2008). RNAi of Asl in fly culture cells and in vertebrate cells also leads to limited centriole duplication, supporting a role for Asl in centriole duplication (Dobbelaere et al., 2008; Cizmecioglu et al., 2010; Dzhindzhev et al., 2010; Hatch et al., 2010). It seems therefore that Asl might have a dual role at the centriole: being involved in both centriole formation and in PCM recruitment. Consistent with this dual role, Asl/Cep152 localises to the outer wall of the centriole where it can potentially interact with the PCM (Blachon et al., 2008; Cizmecioglu et al., 2010; Fu and Glover, 2012; Sonnen et al., 2012). Whether Asl does indeed have a dual role remains a controversial point.

Asl/Cep152 overexpression is able to induce centriole overduplication (Dzhindzhev et al., 2010), and it is required for Sak/Plk4-induced centriole overduplication (Cizmecioglu et al., 2010; Dzhindzhev et al., 2010; Hatch et al., 2010). Moreover, Asl is the only duplication factor (in addition to

Sak/Plk4), that is able to induce the *de novo* formation of *bona fide* centrioles (as opposed to amorphous MTOCs) in *Drosophila* unfertilised eggs (Dzhindzhev et al., 2010). A clue to the role of Asl/Cep152 in centriole duplication came from studies showing that the N-terminus of Asl/Cep152 is able to directly interact with the cryptic polo-box region of Sak/Plk4 (Cizmecioglu et al., 2010; Dzhindzhev et al., 2010; Hatch et al., 2010). The depletion of Sak/Plk4 does not interfere with the localisation of Asl/Cep152 (Cizmecioglu et al., 2010; Dzhindzhev et al., 2010; Hatch et al., 2010), while in the absence of Asl, Sak/Plk4 localisation is perturbed in fly culture cells (Dzhindzhev et al., 2010) and in some cases in human cells (Cizmecioglu et al., 2010). While a lack of Sak/Plk4 localisation in the absence of Cep152 in vertebrate cells has not always been reproducible (Dzhindzhev et al., 2010; Hatch et al., 2010) these data suggest that Asl/Cep152 might be involved in the recruitment of Sak/Plk4 to the centriole. If this is the case then Asl/Cep152 might be responsible for recruiting Sak/Plk4 to the site of procentriole formation. The fact that Asl/Cep152 and Sak/Plk4 have a very similar evolutionary distribution is supportive of a conserved role for this interaction (Hatch et al., 2010).

Interestingly, the C-terminal region of Asl/Cep152 has been shown to mediate its direct interaction with DSas-4/CPAP (Cizmecioglu et al., 2010; Dzhindzhev et al., 2010). In fly culture, depletion of Asl does not interfere with the localisation of DSas-4 (Dzhindzhev et al., 2010). This led to the suggestion that DSas-4 recruits Asl to the centriole (Dzhindzhev et al., 2010). It is unclear, however, whether this is a conserved role of DSas-

4/CPAP, as the depletion of Cep152 in vertebrate cells leads to the lack of CPAP recruitment to the centrosome (Cizmecioglu et al., 2010; Dzhindzhev et al., 2010).

1.2.2 Other important centriole components

The five main centriole duplication players have been characterised in detail and, although many aspects of their function remain to be elucidated, their central role in centriole assembly is well established. However, the centriole is a complex structure formed of dozens of proteins, many of which are still largely uncharacterised. Nevertheless, there is strong evidence that some of these proteins have an important influence in centriole assembly (Goshima et al., 2007; Dobbelaere et al., 2008). Two such proteins are Bld10/Cep135 and CP110.

Bld10/Cep135 is one of the few components, in addition to hSAS-6, known to localise to the cartwheel (Matsuura et al., 2004). The absence of Bld10/Cep135 leads to the absence of basal bodies in *Chlamydomonas* (Hiraki et al., 2007) and *Paramecium* (Jerka-Dziadosz et al., 2010), and in humans Bld10/Cep135 seems to be essential for the formation of the cartwheel (Lin et al., 2013). In *Drosophila*, Bld10/Cep135 is not essential for cartwheel assembly but it does seem to play a structural role (Roque et al., 2012). EM analysis of centrioles formed in the absence of Bld10/Cep135 in these organisms suggests a role specifically in maintaining the stability of

the cartwheel, and on the interaction between the cartwheel and the centriolar MTs. More recently, *in vitro* work shows that Bld10/Cep135 is able to bind MTs, and might be involved in connecting the spoke component of hSAS-6 to the MT triplets. In addition, Bld10/Cep135 can interact with CPAP, and this interaction might be important for CPAP-mediated centriole elongation (Lin et al., 2013).

As previously mentioned, CP110 has been implicated in cilia formation and has been shown to interact with other centriolar proteins in this process, namely Cep97 and Cep290 (Spektor et al., 2007; Tsang et al., 2008; Cao et al., 2012). At the centrosome CP110 has been implicated in centriole duplication (Chen et al., 2002; Dobbelaere et al., 2008) and in centrosome cohesion (Chen et al., 2002). A more specific role for CP110 has been identified in controlling centriole length. CP110 is thought to play an antagonistic role to that of CPAP, as CP110 depletion leads to the formation of elongated centrioles (Schmidt et al., 2009; Korzeniewski et al., 2010). More recently it was shown that CP110's role in controlling centriole MT length requires the interaction with kinesins Kif24 in human cells (Kobayashi et al., 2011) and Klp10A in *Drosophila* (Delgehyr et al., 2012), although the exact nature of this interaction in different species still requires clarification. CP110 levels at the centrosome appear to be tightly regulated by ubiquitylation, and this appears to be important for proper cell division and maintenance of genome stability (D'Angiolella et al., 2010; Li et al., 2013).

Table 1.1- Main *Drosophila* centriole duplication and PCM recruitment factors and respective homologues

***Drosophila* gene**	***C. elegans* homologue**	**Vertebrate homologue**	**Function in *Drosophila***
Sak	*ZYG-1*	*Plk4*	Centriole duplication
DSas-6	*SAS-6*	*hSAS-6*	Centriole duplication (cartwheel)
Ana2	*SAS-5*	*STIL*	Centriole duplication
DSas-4	*SAS-4*	*CPAP*	Centriole duplication and MT nucleation
Asl	-	*Cep152*	Centriole duplication and PCM recruitment
DSpd-2	*SPD-2*	*Cep192*	PCM recruitment
Cnn	*SPD-5*	*CDK5RAP2*	PCM recruitment

1.3 PCM structure and recruitment

The PCM is an important component of the centrosome, and is responsible for many of the functions of the centrosome, including MT nucleation (Kuriyama and Borisy, 1981). It is interesting to note that a recent paper showed that an artificial increase of the size of the PCM could induce centriole overduplication, implicating that the PCM may play a role in centriole duplication (Loncarek et al., 2008). Unlike centrioles, the PCM does not have a well-defined structure, and when examined by EM it is observed as an electron-dense, amorphous 'cloud'. Some immunofluorescence studies have suggested that the PCM is organised in a lattice (Dictenberg et al., 1998), while EM studies of salt stripped centrosomes have shown the presence of a 'centromatrix', providing a scaffold for MT nucleation at the centrosome (Schnackenberg et al., 1998). The nature and composition of this underlying 'matrix' is unclear.

The centrosome is thought to be composed of dozens of proteins (Andersen et al., 2003; Bettencourt-Dias and Glover, 2007; Muller et al., 2010; Jakobsen et al., 2011; Jakobsen et al., 2013), many of which are components of the PCM. The detailed distribution of some of these components was recently described using super-resolution microscopy techniques (Fu and Glover, 2012; Lawo et al., 2012; Mennella et al., 2012; Sonnen et al., 2012). This work, focusing mainly on PCM organisation during interphase, shows that different proteins have different distributions within the PCM. Some PCM components such as DSpd-2/Cep192 are also

part of the centriole, while others, like Cnn, are present exclusively in the PCM fraction. The interphase PCM is also characterised by the presence of reduced amounts of certain PCM proteins, such as Cnn and DPLP in fly cells (Mennella et al., 2012). During G2/mitosis transition, the PCM increases dramatically in size, composition and MT nucleating ability, and appears less organised. This process is known as centrosome maturation, and recent work from the lab suggests that Cnn and DSpd-2 are crucial for this process in *Drosophila* (Paul Conduit, personal communication).

1.3.1 Microtubule nucleation

One of the most important functions of the PCM is to nucleate MTs. γ-tubulin is a component of the PCM, and has long been known to play an important role in microtubule nucleation (Stearns et al., 1991; Zheng et al., 1991). Biochemical work in *Xenopus* and in *Drosophila* (Zheng et al., 1995; Oegema et al., 1999) has shown that γ-tubulin forms at least two types of complexes. A γ-tubulin small complex (γ-TuSC) is composed of two γ-tubulin proteins, and one copy each of two other proteins called Dgrip84 and Dgrip91 in *Drosophila* and GCP2 and GCP3 in mammals (Fig.1.8A). A γ-tubulin ring complex (γ-TuRC) is formed by multiple γ-TuSC subunits, as well as several additional proteins (Fig.1.8B): Dgrip75, Dgrip128, Dgrip163 and Dgp71WD in flies (called GCP4, GCP5, GCP6 and NEDD1 in mammals, respectively). All of these components share a GRIP motif, with the exception of Dgp71WD/NEDD1. More recently, three new proteins:

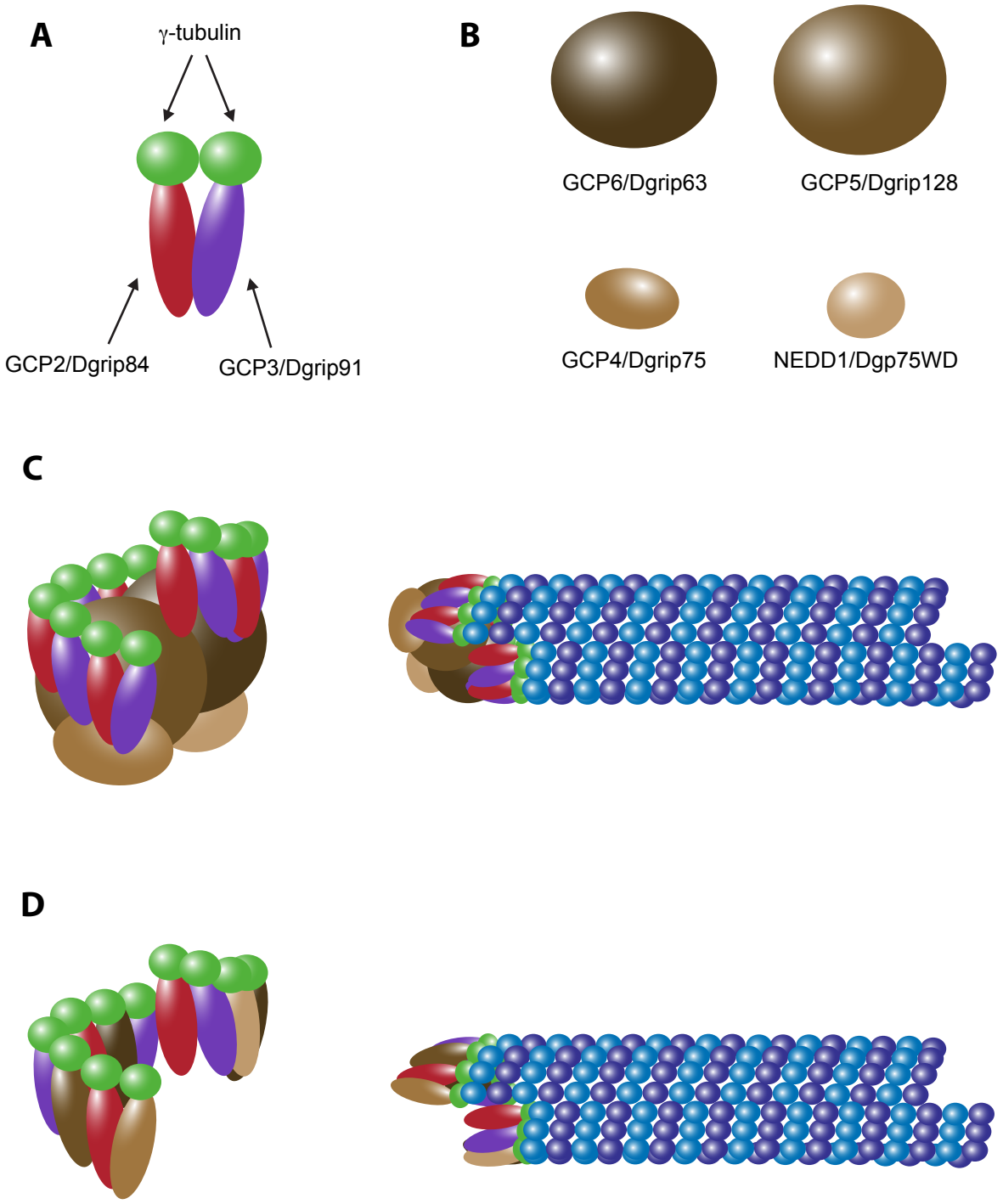


Figure 1.8 Microtubule nucleation from γ-tubulin complexes
(A) Schematic view of the γ-tubulin small complex (γ-TuSC), formed by two γ-tubulin molecules and two additional components, GCP2/Dgrip84 and GCP3/Dgrip91. **(B)** Several additional proteins are thought to bind together with the γ-TuSC to form the γ-tubulin ring complex (γ-TuRC). **(C and D)** Two alternative models for how the γ-TuRC components assemble to template MT nucleation. **(C)** The most established model suggests that γ-TuRC components form a cap to which γ-TuSCs bind in a helical fashion to nucleate MT formation. **(D)** Recently published data suggests that γ-TuSC-like complexes, containing hybrid combinations of the GCP2-6 proteins associate to form a helical template for MT nucleation. Adapted from Kollman et al, 2011.

MOZART1, MOZART2A and MOZART2B have been identified as new, conserved, components of the γ-TuRC (Hutchins et al., 2010).

The most established model of how the γ-TuSC and γ-TuRC complexes mediate MT nucleation is shown in Fig.1.8C. In this model, the additional components of the γ-TuRC complex form a cap, to which several γ-TuSC complexes bind in a helical fashion. As γ-tubulin is a homologue of α-tubulin and β-tubulin, the γ-tubulins in the γ-TuSCs are thought to bind to α/β-tubulin dimers and so promote MT nucleation (Kollman et al., 2011). An alternative model suggests that γ-tubulin might be able to associate longitudinally to form protofilaments that can then nucleate the attachment of α/β-tubulin dimers and hence nucleate MTs (Erickson and Stoffler, 1996). This last model is particularly attractive because it provides an explanation for how MT nucleation might occur in organisms that do not contain γ-TuRC components, such as the yeast *Saccharomyces cerevisiae* (Kollman et al., 2011), or how it still takes place in *Drosophila* when γ-TuRC components are eliminated (Verollet et al., 2006).

More recent structural studies have suggested that an updated model might be required (Fig.1.8D). Crystallisation and EM studies of γ-TuSC complexes have shown that these complexes can assemble into helical structures that can initiate MT formation in the absence of any additional components (Kollman et al., 2010). Moreover, the recent solving of the structure of GCP4 has shown that the additional γ-TuRC components are structurally very similar to the γ-TuSC components Dgrip84/GCP2 and Dgrip91/GCP3

(Guillet et al., 2011). This suggests that MTs could be nucleated by several different γ-TuSC-like complexes that can contain hybrid combinations of any of the GCP2-6 components (Kollman et al., 2011).

1.3.2 Essential PCM recruitment components

The PCM is composed of dozens of proteins, several of which have been implicated in mitotic PCM recruitment. Remarkably, recent work in our lab indicates that just two proteins, DSpd-2 and Cnn, are essential for initiating the recruitment of all the other mitotic PCM components in flies (Paul Conduit, personal communication). Here I discuss in detail our current knowledge of these two proteins and their homologues.

1.3.2.1 SPD-2/DSpd-2/Cep192

SPD-2 was first identified in a screen for cell division mutants in *C .elegans* (O'Connell et al., 1998). DSpd-2 and Cep192 are homologues in *Drosophila* and vertebrates, respectively (Pelletier et al., 2004). All three proteins share a 200 amino acid region of homology (named the 'SPD-2 domain'), in addition to several predicted coiled coil regions (Pelletier et al., 2004; Dix and Raff, 2007; Gomez-Ferreria et al., 2007; Giansanti et al., 2008; Zhu et al., 2008). However, the different homologues seem to play different roles in centrosome function in different organisms.

All three homologues are components of the centrosome, and localise to both the centriole and the PCM. SPD-2/DSpd-2/Cep192 localises mainly to the centriole during interphase and expands considerably into the PCM at the onset of mitosis, during PCM maturation (Kemp et al., 2004; Pelletier et al., 2004; Dix and Raff, 2007; Gomez-Ferreria et al., 2007; Giansanti et al., 2008; Zhu et al., 2008; Fu and Glover, 2012; Sonnen et al., 2012).

A role for all three homologues in PCM recruitment has been established. In all three cases depletion or absence of this protein leads to defects in mitotic spindle formation and decreased recruitment of other PCM components, such as γ-tubulin (Kemp et al., 2004; Pelletier et al., 2004; Dix and Raff, 2007; Gomez-Ferreria et al., 2007; Giansanti et al., 2008; Zhu et al., 2008). While the role of DSpd-2 in PCM recruitment in *Drosophila* has been established, being involved in the recruitment of PCM factors such as Cnn and γ-tubulin to the PCM in both larval brain cells and in spermatocytes, some inconsistency has been observed in the literature with regards to the extent of the this role in different tissues. Work on a *dspd-2* mutant generated by a P-element insertion has argued that this effect is stronger in spermatocytes than in brain cells (Dix and Raff, 2007). Work published on an EMS mutant of *dspd-2*, however, suggests that DSpd-2 plays an equally important role in the recruitment of the PCM in both tissues (Giansanti et al., 2008). It should be noted, however, that the EMS mutant of *dspd-2* seems to be a stronger allele, as the flies have been reported to be uncoordinated, although no defect in centriole duplication has been observed. Due to failure in pronuclear fusion in *dspd-2* mutant embryos, the extent of the role of

DSpd-2 in PCM recruitment in embryos is still unknown (Dix and Raff, 2007). Pronuclear fusion is the process by which the paternal and maternal DNA fuses after fertilisation, and seems to be dependent on a functional PCM in *Drosophila*.

While the role of SPD-2/DSpd-2/Cep192 in PCM recruitment seems to be conserved across evolution, this is not the case with regards to a role in centriole duplication. In *Drosophila*, there is currently no evidence linking DSpd-2 to centriole duplication (Dix and Raff, 2007; Giansanti et al., 2008). In *C. elegans*, on the other hand, SPD-2 appears to be the most upstream component required for the formation of a new procentriole: upon depletion of SPD-2 by RNAi the kinase Zyg-1 cannot be recruited to the place of procentriole formation (Delattre et al., 2006; Pelletier et al., 2006) and centriole duplication is blocked (Kemp et al., 2004; Pelletier et al., 2004). Thus, SPD-2 is involved in both PCM recruitment and centriole duplication in *C. elegans.*

The role of Cep192 in centriole duplication has been a mystery for some time. Work on the role of Cep192 in human cells has confirmed, as is the case in other organisms, that Cep192 has a role in PCM recruitment (Gomez-Ferreria et al., 2007; Zhu et al., 2008). This seems to also be the case in *Xenopus*, where Cep192 has been shown to directly interact with another important PCM component, Aurora-A, and to be responsible for its recruitment to the centrosome (Joukov et al., 2010). The role of Cep192 in centriole duplication is, however, still unclear as two studies in HeLa cells

came to contradictory conclusions on this point (Gomez-Ferreria et al., 2007; Zhu et al., 2008). A recent study, however, examines a previously un-studied isoform of Cep192, shedding light on some of the contradictory results previously observed. This study demonstrates that this isoform of Cep192 is able to bind Plk4, and that this interaction is important for centriole duplication (Sonnen et al., 2013). It is worth noting, however, that recent genome analyses have shown that SPD-2 proteins are present in most organisms that make centrosomes, but are absent in many species that make centrioles but not centrosomes (Azimzadeh et al., 2012). This strongly suggests that evolutionarily the main role of SPD-2 proteins is in PCM recruitment.

1.3.2.2 SPD-5/Cnn/CDK5RAP2

Centrosomin (Cnn) is a *Drosophila* protein that localises to the centrosome. Cnn mainly localises to the outer layer of the centriole in interphase and, as is the case with other PCM components, expands in localisation during PCM maturation at the onset of mitosis (Conduit et al., 2010; Fu and Glover, 2012; Mennella et al., 2012). Cnn is thought to be one of the main players in PCM recruitment during mitosis: in its absence the centrosomal localisation of several PCM components, such as γ-tubulin, CP190 and Aurora-A is reduced (Megraw et al., 1999; Vaizel-Ohayon and Schejter, 1999; Lucas and Raff, 2007; Dobbelaere et al., 2008; Conduit et al., 2010). Interestingly Cnn has been shown to have an unusual dynamic behaviour, being

incorporated close to the centriole and then moving outwards, and this behaviour is most likely associated with a function in establishing the organisation of the PCM (Conduit et al., 2010).

The recruitment of γ-tubulin to the PCM by Cnn is thought to take place via conserved sequence named Cnn Motif 1, or CM1 domain (Zhang and Megraw, 2007). This region is conserved from yeast to humans, and seems to be important for many functions of Cnn. The CM1 domain is required, for example, to promote successful centrosome separation (Zhang and Megraw, 2007). Importantly, the CM1 domain has also been shown to interact with the γ-TuRC complex (Terada et al., 2003). It is therefore not surprising that functional Cnn is required for proper spindle formation and MT nucleation, both from the centrosome and from the cytoplasm (Megraw et al., 1999; Vaizel-Ohayon and Schejter, 1999; Riparbelli and Callaini, 2005; Mahoney et al., 2006; Moutinho-Pereira et al., 2009). Recruitment of some PCM factors, however, seems to be performed by other regions of the protein (Terada et al., 2003; Zhang and Megraw, 2007).

Due to defects in PCM recruitment and spindle formation, embryos laid by *cnn* mutant flies die before cellularisation (Megraw et al., 1999; Vaizel-Ohayon and Schejter, 1999). *cnn* mutant flies are, however, viable and coordinated, suggesting that Cnn does not play a role in centriole duplication (Megraw et al., 2001). Associated with its role in PCM recruitment, Cnn is also thought to be important for other functions in addition to MT nucleation. For example, a role has been suggested for Cnn

in establishing the proper connection between the centriole and the PCM, as in the absence of Cnn the centrioles are seen to 'rocket' around the cytoplasm (Lucas and Raff, 2007). Ten different isoforms of Cnn have been identified (Eisman et al., 2009), and these are thought to have tissue-specific roles.

The human homologue of Cnn is CDK5RAP2. Mutations in the CDK5RAP2 gene have been associated with microcephaly (Bond et al., 2005; Hassan et al., 2007; Darvish et al., 2010; Pagnamenta et al., 2012). Consistent with this, lack of CDK5RAP2 has been associated with defects in mouse neurogenesis (Buchman et al., 2010). CDK5RAP2 seems to play a similar role to Cnn. CDK5RAP22 has a similar localisation at the centrosome, showing reduced levels during interphase, and increasing in centrosome localisation during mitosis (Fong et al., 2008; Zhang et al., 2009). It is not required for centriole duplication, although a role for centriole cohesion and engagement has been described (Graser et al., 2007b; Barrera et al., 2010). As is the case with Cnn, CDK5RAP2's main role is in PCM recruitment, being responsible for the localisation of several PCM components and for MT nucleation and dynamics (Fong et al., 2008; Zhang et al., 2009; Barrera et al., 2010; Choi et al., 2010; Lee and Rhee, 2010). CDK5RAP2 also contains the conserved N-terminus CM1 domain. However, the role of this region may not be conserved in all vertebrates. While the CM1 domain has been shown in human cells to be involved in γ-TuRC recruitment and MT nucleation (Fong et al., 2008), in avian cells this does not seem to be the case, as it seems to be required for the localisation of AKAP350 but not γ-

tubulin (Barr et al., 2010). Interestingly, work on a less conserved region in the C-terminus, the Cnn Motif 2, or CM2 domain, suggests that CDK5RAP2 might also be a Calmodulin-binding protein (Wang et al., 2010).

Cnn/CDK5RAP2 seems to play a conserved, essential role in PCM recruitment at the centrosome across evolution. Interestingly, a homologue has not been identified so far in *C. elegans*, but SPD-5 is a potential functional homologue. Like Cnn and human CDK5RAP2, SPD-5 is required for γ-tubulin recruitment, MT nucleation and mitotic spindle formation (Hamill et al., 2002). In addition its localisation seems to be co-dependent with that of SPD-2 (Kemp et al., 2004; Pelletier et al., 2004; Srayko et al., 2005; O'Toole et al., 2012). This co-dependency has some parallels in *Drosophila*, where interference with DSpd-2 function is known to strongly delocalise Cnn from the centrosome (Conduit et al., 2010).

1.3.3 Other important PCM components

In addition to SPD-2/DSpd-2/Cep192 and SPD-5/Cnn/CDK5RAP2, several other proteins have been implicated as being important for PCM recruitment (Dobbelaere et al., 2008; Mahen and Venkitaraman, 2012).

Several kinases are thought to play important roles in establishing the PCM, in addition to other roles in the context of cell cycle progression. The kinase Polo was first discovered in *Drosophila* (Sunkel and Glover, 1988) and since

then 5 different Polo-like kinases (Plk) have been identified in humans (Bruinsma et al., 2012). This family of proteins is generally characterised by the presence of two polo box regions, which together form the polo box domain, necessary for the phosphorylation of specific substrates. Different Plks have different roles in the cell cycle, and proteins in this family have been implicated in different functions at the centrosome. For example, I described above in detail the crucial role of Plk4 (Sak) in centriole duplication, and Polo was identified in a RNAi screen in *Drosophila* cells as the strongest hit with regards to PCM recruitment, and shown to be involved in Cnn phosphorylation (Dobbelaere et al., 2008). Plk1 is another protein in this family, and in addition to roles in mitotic entry, checkpoint recovery and cytokinesis, it has also been strongly implicated in PCM recruitment. For example, when anti-Plk1 antibodies are injected in human cells, the recruitment of other PCM components, such as γ-tubulin, is reduced (Lane and Nigg, 1996; McInnes et al., 2006). Plk1 is a kinase, and certain PCM components, such as pericentrin/D-PLP (described in more detail below) are known targets of Plk1, and this phosphorylation is required for the maturation of the PCM (Lee and Rhee, 2011). Interestingly, and as mentioned in section 1.1.2.3, Plk1 is also required for centrosome separation, as the phosphorylation of linker proteins by Nek2A kinase is dependent on Plk1 activity, as well as centriole disengagement (Mardin et al., 2011; Tsou et al., 2009).

Another kinase involved in PCM maturation is Aurora-A. Aurora kinases are Serine/Threonine kinases with a variety of roles in mitosis. Aurora was first

identified in *Drosophila* in a screen for genes involved in the centrosome cycle (Glover et al., 1995). There are three types of Aurora: Aurora-A, Aurora-B and Aurora-C (Cowley et al., 2009; Carmena et al., 2012). While mammals possess all three types, *Drosophila* and *C. elegans* only have Aurora-A and Aurora-B (Lukasiewicz and Lingle, 2009). All three Auroras are implicated in different roles in mitosis, but only Aurora-A is thought to play a role at the centrosome. Aurora-A localises to the mitotic spindle poles, and has been implicated in centrosome separation. Aurora-A seems to be play an important role in centrosome maturation, as work in *C. elegans* and *Drosophila* shows that in the absence of this protein several PCM components fail to localise to the centrosome during mitosis (Hannak et al., 2001; Berdnik and Knoblich, 2002; Giet et al., 2002; Bettencourt-Dias et al., 2004). Interestingly, Aurora-A localisation at the centrosome is dependent on Plk1 (De Luca et al., 2006).

There are also other proteins that have been implicated in PCM recruitment in some organisms that may not show a conserved role. One of these proteins is Pericentrin. Pericentrin is the probable vertebrate homologue of Spc110, the yeast protein thought to anchor the yeast-equivalent of the γ-TuSC to the spindle pole body (Knop and Schiebel, 1997; Knop and Schiebel, 1998). There are two main isoforms of pericentrin: Pericentrin A and Pericentrin B, although other isoforms also exist (Flory and Davis, 2003; Miyoshi et al., 2006a). Pericentrin B has been mainly implicated in ciliogenesis (Li et al., 2001; Jurczyk et al., 2004; Miyoshi et al., 2006b; Miyoshi et al., 2009), while Pericentrin A seems to play a role in PCM

recruitment. Pericentrin A has been shown to interact and form a PCM lattice with γ-tubulin (Dictenberg et al., 1998), and this interaction seems to depend on an interaction between Pericentrin A and dynein (Purohit et al., 1999; Young et al., 2000). It also probably interacts with certain γ-TuRC components (Zimmerman et al., 2004). In addition, RNAi depletion of Pericentrin A leads to a partial loss of γ-tubulin during mitosis, although interphase recruitment of γ-tubulin still takes place (Zimmerman et al., 2004). The *Drosophila* homologue of pericentrin, D-PLP (*Drosophila* pericentrin-like protein), has been implicated in PCM recruitment, as it seems to be able to interact with components of the γ-TuRC and restore MT nucleating capacity to salt stripped centrosomes. D-PLP is crucial for organising Cnn and DSpd-2 in interphase, but plays only a minor role in mitosis (Martinez-Campos et al., 2004). Interestingly, flies that lack D-PLP are uncoordinated, suggesting that D-PLP might be an important player in ciliogenesis, and hence more similar to Pericentrin B.

Another protein whose role in PCM maturation and MT nucleation at the centrosome does not seem to be conserved is NEDD1/Dgp71WD. Dgp71WD was first identified in *Drosophila*, and was the first γ-TuRC component identified that does not contain a grip motif, the conserved characteristic sequence of other γ-TuRC components (Gunawardane et al., 2003). The vertebrate homologue of Dgp71WD is NEDD1 (Gunawardane et al., 2003; Luders et al., 2006). NEDD1 is required for γ-TuRC targeting to the centrosome in vertebrates (Haren et al., 2006; Luders et al., 2006; Manning et al., 2010). In *Drosophila*, however, RNAi depletion of Dgp71WD

in cell culture, as well as analysis of Dgp71WD mutant larval brains, only partially impairs the recruitment of γ-tubulin and PCM components to the centrosome (Verollet et al., 2006; Dobbelaere et al., 2008; Reschen et al., 2012). It seems, however, that Dgp71WD plays a role in MT nucleation in female meiosis and other MT nucleation pathways, probably via the Augmin pathway (Verollet et al., 2006; Reschen et al., 2012).

1.4 Thesis outline

In this thesis I attempted to clarify the relative position of the 5 main centriole duplication factors (Ana2, Asl, DSas-4, DSas-6, Sak) and the two main PCM recruitment factors (Cnn and DSpd-2) in the pathway of centrosome assembly in *Drosophila*. To achieve this I have used DSas-6/Ana2 particles (SAPs), centrosome-like structures that form in unfertilised *Drosophila* eggs by moderate overexpression of DSas-6 and Ana2.

Aims and objectives:

- To characterise SAPs in detail: composition, dynamics, and behaviour in the context of the developing embryo (Chapter 3)
- To assess the pathway of PCM assembly by examining SAP and *de novo* centrosome composition in *cnn*, *dspd-2* and *asl*^{1} mutant backgrounds (Chapter 4)
- To assess the pathway of centriole formation by examining SAP composition in *asl*, *dsas-4* and *sak* mutant backgrounds in *in vitro* activated eggs (Chapter 5)
- To use the knowledge acquired from the centriole pathway analysis to address how centrosome formation is potentiated on the transition from oocyte to egg (Chapter 6)

Chapter 2: Materials and Methods

2.1 Fly Husbandry

2.1.1 Fly handling

Flies were kept at 25 °C or 18 °C on *Drosophila* culture medium (0.77% agar, 6.9% maize, 0.8% soya, 1.4% yeast, 6.9% malt, 1.9% molasses, 0.5% propionic acid, 0.03% orthophosphoric acid, 0.3% nipagin) in 9.5 cm x 2.5 cm vials and 177 ml bottles (Dutscher Scientific). Embryos and unfertilised eggs were collected in fruit juice places (25% cranberry juice, 2.5% sucrose and 2.25% agar). Plates were supplemented with fresh yeast. Unless otherwise stated, all embryo/egg collections correspond to 0-3 hrs. Standard fly techniques were used (Roberts, 1986).

For analysis of centriolar mutants in SAPs, mutant pupae were selected and transferred to a petri dish lined with moist paper. Once female flies of the correct genotype eclosed, they were manually fed for 2-3 days with a 1% glucose solution to promote stage 14 oocyte accumulation. Both pupae and uncoordinated flies were maintained at 25 °C.

For stage 14 oocyte imaging, female flies were placed in vials containing large quantities of dry yeast for 2-3 days at 25 °C to promote stage 14 oocyte accumulation.

2.1.2 *Drosophila* stocks

Standard WT lab stocks were used as required. Other fly stocks used are shown in Table 2.1. Transgenic lines containing GFP-DSas-6 and GFP-Ana2 constructs in different chromosomes were used in different SAP experiments as indicated in Table 2.1. The same transgenic lines were used for the respective WT controls.

Table 2.1- Fly stocks used in this thesis

Stock nr	Name	Source	Thesis section
T925	Ubq-GFP-DSas-6#89	(Peel et al., 2007)	3.2.1; 3.2.3; 3.2.5; 3.2.6; 4.2.3; 5.2.1; 5.2.2; 5.2.3; 5.2.4; 6.2.1
T091	Ubq-GFP-Ana2#3F	(Stevens et al., 2010a)	3.2.1; 3.2.4; 3.2.5; 3.2.6; 4.2.3; 4.2.4; 5.2.1; 5.2.4; 6.2.2; 6.2.3; 6.2.5
T1027	Ubq-GFP-Ana2#2, Ubq-RFP-Cnn#80	Raff Lab stock	3.2.3; 6.2.1

T928	Ubq-GFP-Sas6#102	(Peel et al., 2007)	3.2.4; 3.2.6; 4.2.1; 5.2.1; 6.2.2; 6.2.3; 6.2.5
T123	Jupiter-mCherry	Gift from D. St.Johnston	3.2.5
7498-1-M	Ubq-DSas-6#1M	Saroj Saurya	3.2.6
7498-2-2M	Ubq-Ana2#2M	Saroj Saurya	3.2.6
7498-2-1M	Ubq-Ana2#1M	Saroj Saurya	3.2.6
9205-1-4M	UAS-DSas-6	Saroj Saurya	3.2.6
9205-2-3M	UAS-Ana2	Saroj Saurya	3.2.6
T888	pUbqRFPMA8#50	(Lee et al., 2001)	3.2.3
T036	Ubq-RFP-DSas-6#1	(Peel et al., 2007)	3.2.6
U877	pUASp-Sak	(Rodrigues-Martins et al., 2007b)	All chapters concerning *de novo* centrosome formation
	V32 driver	(Peel et al., 2007)	Chapters 4,5 and 6
	V37 driver	(Peel et al., 2007)	3.2.4
F206	cnn^{hk21}	(Megraw et al., 1999; Vaizel-Ohayon and Schejter, 1999)	4.2.1

F372	*cnn*^{f04547}	(Lucas and Raff, 2007)	4.2.1
T094	Ubq-GFP-Ana2#2M	(Stevens et al., 2010a)	4.2.1
F427	*spd2*$^{z3-5711}$	(Giansanti et al., 2008)	4.2.2; 4.2.3
D324	Df(3L)st-j7	(Giansanti et al., 2008)	4.2.2; 4.2.3
L492	*asl*^{1}	(Bonaccorsi et al., 1998)	4.2.4; 4.2.5
D327	Df(3R)ED5177	(Varmark et al., 2007)	4.2.4; 4.2.5; 5.2.2
T1025	Ubq-GFP-Ana2#2, Jupiter-mCherry	Raff Lab stock	5.2.1; 6.2.1
L502	*asl*^{mecD}	(Blachon et al., 2008)	5.2.2
	asl^{B46}	Zsofia Novak, Alan Wainman	5.2.2
T098	Ubq-Ana2-GFP#1M	(Stevens et al., 2010a)	5.2.2; 5.2.3
L521	*dsas-4*^{S2214}	(Basto et al., 2006)	5.2.3
	Sak^{Aa74}	Janina Baumbach, Alan Wainman	5.2.4
T1019	Ubq-GFP-Ana2#2, DSas-4-RFP	Raff Lab stock	6.2.1

T1056	Ubq-GFP-DSas-6#102, DSas-4-RFP	Raff Lab stock	6.2.1
	cort$^{[qw55]}$	(Page and Orr-Weaver, 1996)	6.2.2; 6.2.5
	Df(2L)BSC9	Bloomington stock centre	6.2.2; 6.2.5
	sra^{KO}	(Takeo et al., 2006)	6.2.2; 6.2.3; 6.2.5
	sra^{A426}	(Horner et al., 2006)	6.2.2; 6.2.3; 6.2.5
T975	UAS-GFP-DSas-6#13	Raff lab stock	6.2.6
U836	UAS-Ana2-GFP#6M	(Stevens et al., 2010a)	6.2.6
T914	UAS-DSas-4-GFP	Raff lab stock	6.2.6

2.2 Antibodies used in this thesis

All primary antibodies used and respective concentrations are shown in Table 2.2. For stainnings, Alexa 405, 488, 568 and 647 secondary antibodies from Molecular Probes, Jackson Laboratories were used at 1:1000 dilution. GFP booster antibody (ChromoTek) was also used at 1:500 dilution for the experiment in section 4.2.4.

Table 2.2 Primary antibodies used in this thesis

Protein	Species	Source	Concentration
α-tubulin	Mouse	Sigma (DM1α)	1 μg/ml (1:1000)
γ-tubulin	Mouse	Sigma (GTU-88)	1 μg/ml (1:1000)
Actin	Mouse	Sigma	1.5 mg/ml (1:1000)
Ana1	Guinea Pig	Raff lab stock	serum (1:500)
Ana2	Rabbit	Raff lab stock	0.32 mg/ml (1:500)
Asl	Guinea Pig	Raff lab stock	serum (1:500)
Asl	Rabbit	Raff lab stock	1.46 μg/ml (1:500)
Cnn	Sheep	Raff lab stock	serum (1:500)
Cnn	Rabbit	Raff lab stock	1.6 μg/ml (1:1000)
Dgp71WD	Rabbit	(Verollet et al., 2006)	serum (1:500)
D-PLP	Rabbit	(Martinez-Campos et al., 2004)	0.3 mg/ml (1:500)
DSas-4	Rabbit	(Basto et al., 2006)	0.7 mg/ml (1:500)

DSas-6	Rabbit	(Peel et al., 2007)	0.12 mg/ml (1:500)
DSpd-2	Rabbit	(Dix and Raff, 2007)	0.6 μg/ml (1:500)
GFP	Mouse	Roche	serum (1:500)

2.3 Biochemistry

2.3.1 Centrosome/SAP sucrose gradients

Centrosomes and SAPs were separated from the cytoplasm using a modified version of a previously described protocol for the isolation of centrosomes (Lehmann et al., 2006). All steps were performed in a cold room. Embryo extracts for centrosome isolation were prepared by homogenising an overnight collection of embryos in Buffer 1 (0.2 g : 1 ml ratio). Egg extracts for SAP isolation were prepared by homogenising an overnight collection of eggs expressing GFP-DSas-6 and GFP-Ana2 in Buffer 1 (0.14 g : 1 ml ratio). Buffer 1 is composed of 1% protease inhibitor cocktail (1 mM benzamiding-HCl, 0.5 mM Phenanthroline, 0.01 mg/ml aprotinin, 0.01 mg/ml leupeptin and 0.003 mg/ml pepstatinA), 1% phenylmethanesulfonylfluoride, 1% Dithiothreitol (DTT), 1% Phosphatase Inhibitor Cocktail 2 (Sigma), 1% Phosphatase Inhibitor Cocktail 3 (Sigma), 80 mM of K- piperazine-N,N′-bis(2-ethanesulfonic acid) (PIPES, pH 6.8), 1 mM $MgCl_2$, 1 mM ethylene glycol tetraacetic acid (EGTA), 1 mM guanosine triphosphate (GTP), 100 mM KCl and 14% sucrose. The extracts were then

filtered twice in miracloth (Merk Milipore). The filtered extracts were then centrifuged for 10 min at 1500 x g. Supernatant was centrifuged a second time for 10 min at 1500 x g. 0.30% Triton was added, as well as a 70% sucrose solution in BRB80 (80 mM K-PIPES, 1 mM of $MgCl_2$, 1 mM EGTA and 1 mM GTP) to a final concentration of 50% sucrose. The embryo extract containing 50% sucrose was then layered on top of a sucrose cushion consisting of a 0.5 ml layer of 55% sucrose in BRB80 on top of a 1.5 ml layer of 70% sucrose in BRB80. Tubes were then spun in a Beckman SW 41 Ti Rotor at 27,000 rpm for 1 hr 30min, causing the centrosomes/SAPs in the extract to move into the 70% sucrose layer. Five 0.5 ml cytoplasmic fractions were taken from the top of the tube and fifteen 150 μl "centrosome" fractions were collected from the 70% sucrose layer by piercing the bottom of the tube with a 19 G needle and collecting drops. Samples were flash frozen in liquid nitrogen and stored at -20 °C or -80 °C. Samples selected for western blotting were added to an equal volume of Laemmli sample buffer (0.25 mM Tris.HCl pH 6.8, 8% SDS, 20% β-mercaptoethanol, 40% glycerol, 0.08% bromophenol blue). The solution was then mixed and heated at 95 °C for 10 min. Usually 10 μl was loaded in a single well of a polyacrylamide gel.

2.3.2 Phosphatase treatments

To examine Cnn and DSpd-2 phosphorylation in centrosomes and SAPs, single fractions from sucrose gradients obtained as described in 2.3.1 were

diluted 1:10. Diluted fractions were then incubated with 1 μl of PMSF, 1 μl of DTT, 10 units of alkaline phosphatase (Roche) and 10 μl of phosphatase buffer. Phosphatase inhibitor cocktail 2 and 3 (Sigma) were added as necessary. Solutions were incubated at 37 °C for 4 hrs.

2.3.3 Preparation of other samples for Western blotting

Stage 14 oocytes were dissected in Schneider's solution (Sigma). Oocytes were then transferred with the assistance of forceps into sample buffer, in a ratio of 1 oocyte / μl of 2 x sample buffer. Unless otherwise indicated, eggs and embryo samples used for Western blotting were collected and fixed in methanol/heptane as described in section 2.3.1. Eggs and embryos were re-hydrated by 3 x 15 min washes in PBT, before being transferred to sample buffer, to a ratio of 1 egg / 2 μl of 1 x sample buffer. Oocytes/eggs/embryos were homogenised using a sterile plastic pestle, and then boiled at 95 °C for 10 min. Samples were then frozen in liquid nitrogen and stored at -20 °C or -80 °C. In Fig.6.7A, 5 μl of stage 14 oocyte solution was loaded per lane, and 10 μl of egg/embryo solution was loaded per lane. In Fig.6.8 and 6.9, 10 μl of solution was loaded for all sample types. In Fig.6.7B and C and Fig.6.10, eggs and embryos were prepared at the same ratio of sample buffer as is described for stage 14 oocytes above. 10 μl of stage 14 oocyte solution and 5 μl or eggs and embryos were loaded per lane in this case.

2.3.4 Electrophoresis and Western blotting

3-8% pre-cast NuPAGE polyacrylamide gels (Invitrogen) were used to run samples. Proteins were then transferred overnight at 50 mA onto a nitrocellulose membrane (Amersham) in Western blot buffer (20% methanol, 0.29% glycine, 0.58% Tris, 0.04% SDS). A quick assessment of transfer efficiency was performed by staining the membrane with Ponceau solution (Sigma: P3504). The membrane was then washed in distilled water, as necessary, and blocked in milk solution (PBS, 4% milk powder, 0.1% Tween-20) for 1 hr at RT, while gently shaken. Incubation with primary antibody was performed at RT for 1 hr. The membrane was then washed 3 times in TBS + 0.1% tween-20 (TBST). Incubation for 2 hr with the appropriate secondary antibody (horseradish peroxidase conjugated antibodies, Amersham, 1:3,000 dilution) was then performed. Membrane was then washed 3 times with TBST, followed by a wash of 10 min with gentle agitation. These washing procedures were repeated 3 times. The membranes were developed by enhanced chemiluminescence according to the manufacturer's instructions (Pierce) and exposed to X-ray film.

2.4 Cell Biology

2.4.1 Egg and embryo fixation

Laid embryos and eggs were treated identically, unless otherwise stated. Laid embryos/eggs were dechorionated for 2 min in 60% bleach and washed twice in PBS + 0.2% Triton (PBT). Embryos/eggs were washed into glass vials using heptane, and methanol + 3% 0.5M EGTA was then added. The vials were then shaken for 30s and embryos/eggs were left to sink to the denser methanol/EGTA solution at the bottom. Embryos/eggs were washed and stored in methanol at 4 °C.

In order to image GFP-Ana2 in *de novo* formed centrosomes in an asl^{1}/asl^{df} background I used the following fixation method. Eggs were dechorionated and washed as above, and washed using heptane into glass vials containing 1:10 dilution of buffer (1 M PIPES, 20 mM EGTA, 10 mM MgSO4) in 16% microfiltered formaldehyde. Eggs were swirled in the interface for 5 min. the formaldehyde solution was removed and replaced by methanol. This set up was then shaken. Eggs sinking to the bottom of the vial were then washed and stored in methanol at 4 °C.

2.4.2 *in vitro* activation of stage 14 oocytes and fixation of activated eggs

Stage 14 oocytes from virgin females aged at 25 °C were dissected in PIPES-modified activation buffer (Horner and Wolfner, 2008a), and then incubated in an hourglass (previously washed in distilled water) with the same buffer for 1 hour, replacing the buffer every 5 minutes. Oocytes were then incubated in 60% fresh bleach for 4 min in order to destroy unactivated oocytes and to remove the chorion from activated oocytes. Activated oocytes (i.e. activated eggs) were then washed twice in PBT. All PBT was removed, and activated eggs were then incubated for 5 min in the interphase formed of 2 ml of heptane and 2 ml of methanol/EGTA solution. Methanol/EGTA was then removed and replaced by 4% formaldehyde for 20 min. Formaldehyde was removed and activated eggs were then washed twice with PBS. Activated eggs were then transferred to tape with a pipette. Heptane was added to the oocytes in order to separate the glue from the tape and surround the activated eggs. Forceps were then used to 'roll' the activated eggs on the glue in order to remove the vitelline membrane. Activated eggs were then transferred to an eppendorf using forceps and stored in methanol at 4 °C.

Note that for early optimisation experiments (section 5.2.1) activation was performed for 20 min (4 x 5 min) incubation in activation buffer. For *in vitro* activation of *de novo* formed centrosomes 2 or 4 hour incubations in activation buffer were performed, with buffer changes every 15 min.

2.4.3 Staining of laid and activated eggs/embryos

Laid embryos/eggs and activated eggs were stained identically, unless otherwise stated. Samples were washed 3 x 15 min in PBT and blocked for 2 x 30 min in PBT + 5% BSA + 0.02% azide. Samples were then incubated with primary antibody overnight at 4 °C. The following day samples were washed 3 x 20 min in PBT and then incubated for 2 hrs with secondary antibody at RT. Samples were then washed for 3 x 15 min in PBT, and the mounted in mounting medium (85% glycerol, 2.5% n-propylgallate). For DNA staining, instead of 3 washes in PBT, one wash in PBT was performed, followed by 15 min incubation in Hoechst solution, followed by a last wash in PBT.

2.5 Microscopy and Image Analysis

2.5.1 Imaging of fixed eggs/embryos

All immunostaining images were acquired on an Olympus FV1000 scanning confocal system on an inverted IX81 microscope using a 60X 1.35 NA UPlanSApo Oil objective. 0.5 µm thick confocal sections covering the entire depth of the SAP and centrosome fluorescence were collected. 0.4 µm thick confocal sections were used for *de novo* formed centrosomes.

2.5.2 Quantification of SAP fluorescence

For SAP fluorescence quantification experiments, all eggs containing SAPs, as marked by the presence of GFP, were imaged. Therefore, eggs were imaged 'blind' for the antibody staining in the other channels. As a consequence, each data set included a proportion of eggs containing SAPs that do not behave like centrosomes. However, this proportion should be the same in the control and experimental group, and hence not bias the analysis.

In order to quantify the level of fluorescent signal in individual SAPs, the following protocol was developed. For each staining experiment, a representative SAP of the control sample was used to determine the correct imaging settings. These settings were then used for both control and experimental samples. For all quantification experiments, in each egg an area below the surface of the egg that included the full volume of several SAPs was imaged, generally corresponding to a depth of 9 μm. Analysis of images was performed using Volocity (Perkin-Elmer). The 'find spots' function in the GFP channel was used to find SAPs in the image. The intensity threshold for each image was adjusted to just above the level in which random spots were detected in the background. Spots were then dilated by 6 iterations in order to create 3D objects that included each individual SAP. 3D objects were formed by 739 pixels and had a volume of approximately 15.7 μm^3. Any objects touching the edges of the images were excluded, as well as objects that included more than one SAP or that

overlapped areas of unusually high background fluorescence. The total fluorescence signal within each object was then quantified for the several channels, using the 'measurements' function in Volocity. The 3D objects were then manually moved to areas that did not include SAPs and the background fluorescence averaged across these background 3D objects to determine the average background fluorescence. Average background values for each channel were then subtracted from SAP fluorescent values. Any SAPs in which a negative value was obtained for the GFP channel were excluded. Unless otherwise stated n=1 in an experiment corresponds to the average SAP fluorescence within one egg.

For quantification of fluorescence in *de novo* centrosomes formed by the overexpression of SAK a similar quantification protocol was used, but spots were dilated by 5 iterations in order to create 3D objects that included each individual centrosome. 3D objects for quantification of *de novo* centrosomes were formed by 389 pixels and had a volume of 6.6 μm^3.

For graphic representation, the data was normalised by dividing all values by the respective WT average value. This allowed different antibody stainings for the same experiment to be shown in the same graph.

2.5.3 Statistical analysis

Graphs and statistical analysis of data was performed using GraphPad Prism 6.0 statistical package. All samples were tested for normality (Kolmogorov-Smirnov test) and for equality of variances. If either or both of these criteria were not fulfilled, non-parametric tests were used. Student t-test (parametric) or Mann Whitney (non-parametric) test were used. Correlations were performed using Spearman correlation test.

2.5.4 Imaging of live stage 14 oocytes and laid eggs

Eggs were manually dechorionated before live imaging, unless otherwise stated. For stage 14 oocyte imaging, virgin females were fattened in vials containing large amounts of dry yeast for 2-3 days, and then dissected in Schneider's solution (Sigma). Eggs and oocytes were then glued to glass bottom microwell dishes (MatTek Corporation) and covered with Voltalef oil (Atofina). For imaging of stage 14 oocytes during activation, the oocytes were covered in activation buffer rather than Voltalef oil.

Live images in sections 6.2.1 (stage 14 oocyte imaging during activation and Jupiter-mCherry imaging) were acquired on a spinning disc confocal system (Andor Technology) using an inverted microscope (Eclipse TE2000-U; Nikon) equipped with an electron-multiplying charge-coupled device camera (iXON DU-897E-#BV-500; Andor Technology) using a 60x NA 1.4 oil Plan

Apo VC objective (Nikon). z stacks were projected, dynamic range was adjusted, and .avi files were generated using IQ (Andor Technology).

Live images of SAPs with RFP-Cnn and RFP-DSas-4 in section 6.2.1 were acquired using an Olympus FV1000 scanning confocal system on an inverted IX81 microscope using a 60X 1.35 NA UPlanSApo Oil objective. 0.5 µm thick confocal sections covering the entire depth of the SAP and centrosome fluorescence were collected.

Live images in section 3.2.3 were acquired using a Perkin Elmer ERS Spinning Disk confocal system (running ERS software) mounted on a Zeiss Axiovert microscope and using a 63X, 1.4 NA Oil objective. 0.5 µm thick confocal sections covering the entire depth of the SAPs were taken every 10 seconds.

2.5.5 FRAP analysis of SAPs

FRAP analysis was performed in laid eggs prepared for live imaging using a Perkin Elmer ERS Spinning Disk confocal system, as described in 2.4. The RFP signal was bleached using a focused 568 nm laser.

To calculate the fluorescence profile of each SAP at each time-point, a protocol similar to that developed for FRAP analysis in centrosomes (Paul Conduit, personal communication) was used. Fluorescence quantification

was performed using Fiji analysis software. Fluorescence was measured along a 6 μm line drawn through the center of each SAP such that the peak fluorescence was in the middle of the line. The angle of the line was chosen based on the area of the PCM that remained most constant in shape throughout the movie. Fluorescence along each line was then measured and the average background fluorescence subtracted for each time point. This quantification was performed in n=16 SAPs for RFP-Cnn analysis and n=20 SAPs for RFP-DTACC. For each time point, the values directly opposite each other on the left and on the right of the line were averaged and these values were then plotted as mirrored images to give a final, symmetrical fluorescence curve for each time point. In order to compare the shape of the curves, when indicated curves were normalized so that the peak value at each time point was 1.

2.5.6 SAP/centrosome injection into developing embryos

SAPs were obtained from a 0-3 hr collection of eggs (donors) expressing Ubq-GFP-DSas-6 and Ubq-Ana2-GFP. Eggs were manually dechorionated and lined up on a glued slide. Donors were inspected to confirm the presence of the SAPs, and a glass needle was then used to extract the cytoplasm (containing the SAPs). Some of this cytoplasm (~2% of egg volume) was then injected into 0-1 hour old Jupiter-mCherry-expressing embryos (recipients), that had been manually dechorionated and glued to a

glass slide. Recipient embryos were then imaged in a Perkin Elmer ERS Spinning Disk confocal system as described above.

2.5.7 Image manipulation and presentation

All images shown are maximum intensity projections unless otherwise stated. Contrast and brightness were adjusted for easier observation using Fiji analysis software. All control and experimental images were adjusted in a similar way.

Chapter 3: SAPs as models for centrosomes

3.1 Summary

In order to elucidate the pathway of centrosome assembly in the *Drosophila* egg using a classic genetics approach, it is necessary to look at the composition of centrosomes in the absence of the most important players in this pathway. However, this type of analysis is often not possible. Strong mutations in some of the key centriolar proteins, such as Asl and DSas-4, lead to a complete absence of centrioles. In addition, mutations in critical PCM recruitment factors, such as DSpd-2, lead to defects in pronuclear fusion upon fertilisation of the embryo.

Previously published work has shown that the mild overexpression (ubiquitin promoter) of one copy of GFP-DSas-6 and one copy of GFP-Ana2 leads to the formation of centriole-like structures in unfertilised *Drosophila* eggs (Stevens et al., 2010a). We call these structures **SAPs** for D**S**as-6/**A**na2 **P**articles. Although the SAPs contained DSas-4 and organised MT asters, EM analysis shows that they do not contain centrioles, and a rather amorphous electron dense area is present at their core (Fig.3.1). This is in contrast to the well-ordered SAP structure observed in spermatocytes upon expression of GFP-DSas-6 and GFP-Ana2 at the same levels, which consist of extended inner cartwheel-like structures (Stevens et al., 2010b). This led me to hypothesize that SAPs might be able to form in eggs even in the

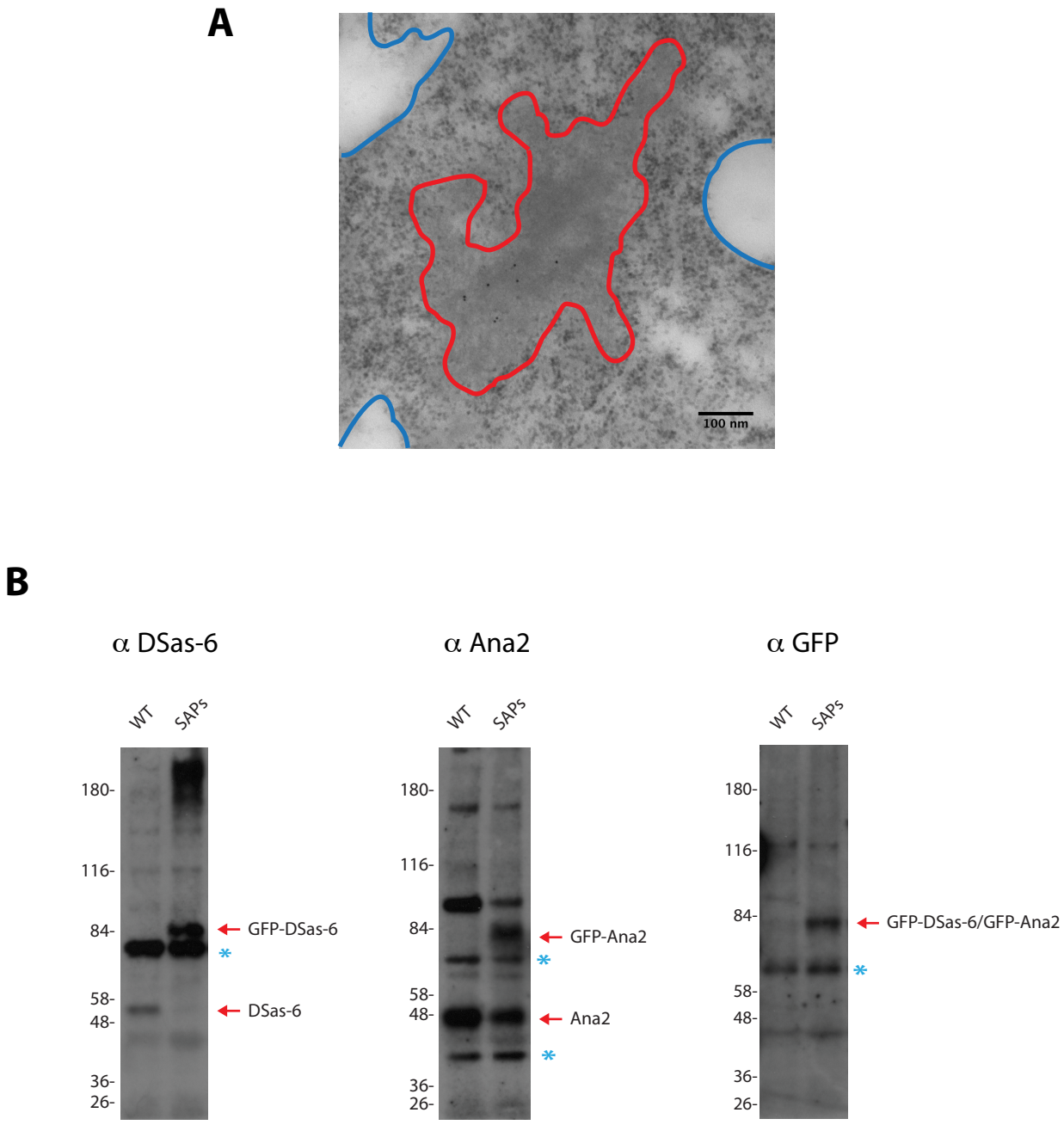


Figure 3.1 SAPs in eggs do not have a highly organised structure
(A) Panel shows a SAP (identified by immuno-gold against Cnn) formed in an unfertilised egg by overexpression of GFP-DSas-6 and GFP-Ana2, examined by electron tomography (EM). Area within the red line corresponds to a SAP, while the blue lines correspond to the edges of yolk granules present in the egg. This image was kindly provided by Helio Roque. Scale bar = 100 nm. **(B)** Western blot of WT eggs and eggs expressing GFP-DSas-6 and GFP-Ana2 (labelled SAPs) blotted with antibodies indicated above of each blot. Asterisks indicate prominent non-specific bands. Note how in eggs expressing the transgenes there is an apparent downregulation of endogenous DSas-6 and Ana2. The mechanism for this is unclear.

absence of proteins that are essential for centriole assembly, such as DSas-4 and Asl. In addition, as they do not rely on a previous centriole template to form, they may also be able to form in mutants that fail in pronuclear fusion (such as DSpd-2 and weak hypomorphs of Asl). I will show in Chapters 4 and 5 that this is indeed the case. In this chapter I describe the detailed characterisation of the SAPs in eggs. I look at their composition and dynamics, and conclude that SAPs closely resemble centrosomes in all criteria analysed, and hence may be considered a good model with which to examine the process of centrosome assembly.

3.2 Results

3.2.1 SAPs have a similar composition to centrosomes

Previously published work shows that SAPs in eggs contain the centriolar component DSas4, the PCM components Cnn and D-PLP, and organise robust MT asters (Stevens et al., 2010a). To confirm these results, and to determine whether any other centrosomal proteins were present in the SAPs, I examined the localisation of several centriolar and centrosomal proteins in the SAPs using antibodies (unless otherwise stated). I compared the localisation of centriolar components (Asl, DSas-4, Ana1) and PCM components (D-PLP, DSpd-2, γ-tubulin, Dgp71WD, RFP-DTACC and Cnn) in SAPs (eggs) and centrosomes (embryos). As shown in Fig.3.2A, all tested proteins localised to the SAPs, and they all showed a qualitatively similar localisation to that observed for each protein in centrosomes: Asl, DSas-4, Ana1 localised as a small dot at the centre at the SAP; DSpd-2, γ-tubulin and Dgp71WD had a slightly larger area of localisation; while RFP-DTACC and Cnn exhibited a much more extended localisation. In addition, as shown in Fig.3.2B, I found that the levels of DSas-4 at the SAPs were correlated with the levels of Cnn ($r=0.7362$, $p<0.0001$). This is reminiscent of work done in *C. elegans* centrosomes, where the levels of SAS-4 were seen to correlate with the levels of γ-tubulin (Kirkham et al., 2003). I also confirmed that SAPs are able to organise MT asters, by staining for α-tubulin (Fig.3.2A). SAPs are therefore composed of all tested centrosomal

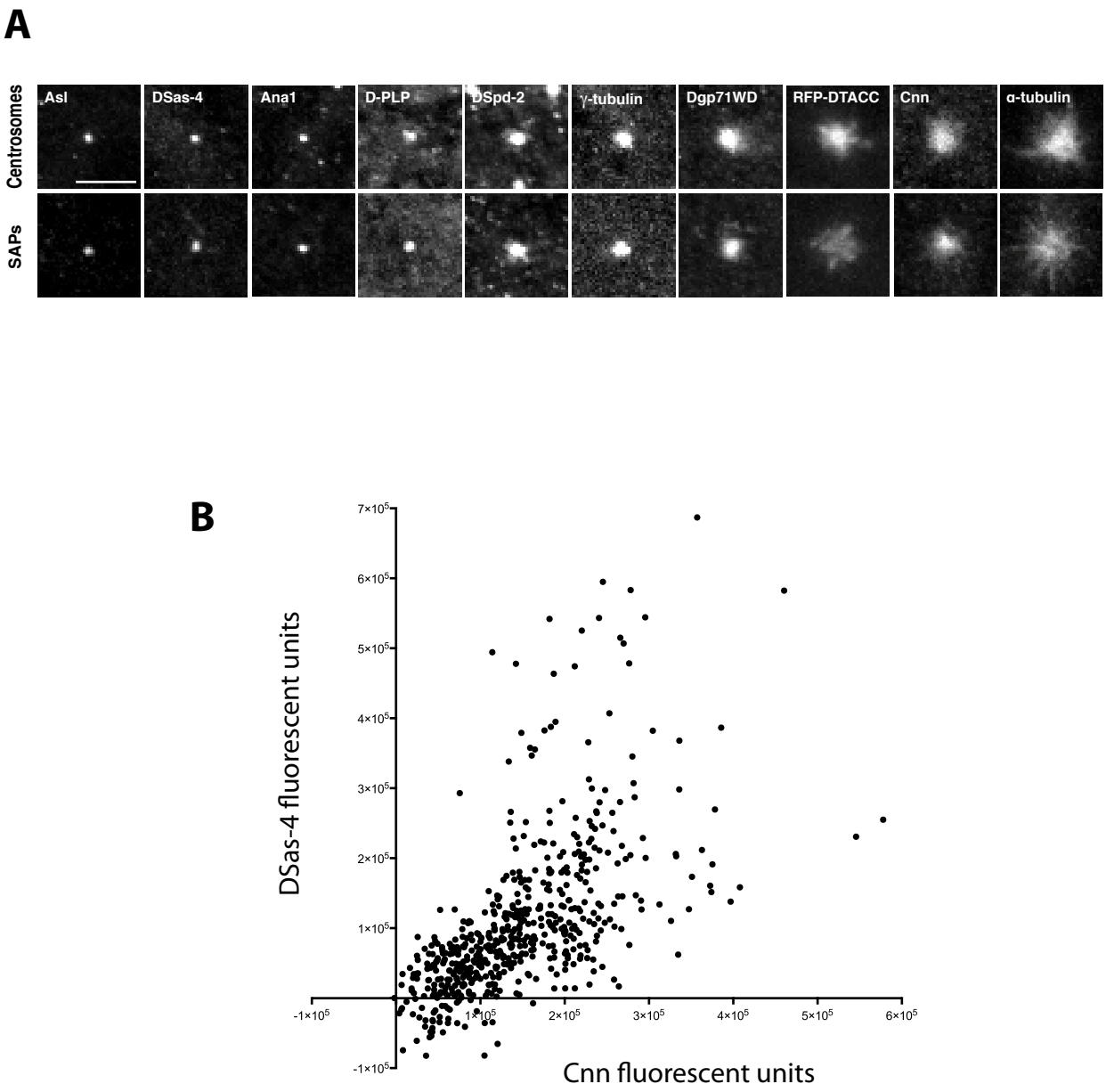


Figure 3.2 All tested centrosomal components are present in SAPs
(A) Images show localisation of several centriolar and PCM components in centrosomes (upper panels) and SAPs (lower panels). Note how the localisation of all the tested proteins is similar in SAPs and in centrosomes. Scale bar=5μm. **(B)** Correlation analysis of DSas-4 and Cnn levels in SAPs ($r=0.7362$, $p<0.0001$). Each dot corresponds to a single SAP. n= 553 SAPs from 20 eggs.

proteins, and these show a similar distribution to that observed at centrosomes.

3.2.2 PCM proteins are post-translationally modified in SAPs in the same way as centrosomes

Both published work and work from our lab suggests that certain PCM proteins might be phosphorylated. Cnn phosphorylation is mentioned several times in the literature. An early paper by Li and Kaufman in 1996 showed that Cnn is present in a higher band in centrosome IPs from embryos, and that this high band shifts down upon phosphatase treatment (Li and Kaufman, 1996). Work in cultured S2 cells that have been enriched for mitotic cells by synchronisation with colchicine, also reported the presence of a higher molecular weight phosphorylated band, whose presence was dependent on Polo kinase (Dobbelaere et al., 2008). Previous work in our lab has also shown that Cnn is present as a higher molecular weight band at the centrosome in larval neuroblasts (Conduit, 2010). DSpd-2 was identified as a candidate in a screen for potential proteins undergoing changes in phosphorylation state during the stage 14 oocyte to egg transition (Krauchunas et al., 2012).

Importantly, recent work in embryos in our lab has shown that Cnn and DSpd-2 are post-translationally modified specifically at the centrosome (Paul Conduit, personal communication). When centrosomes were separated from

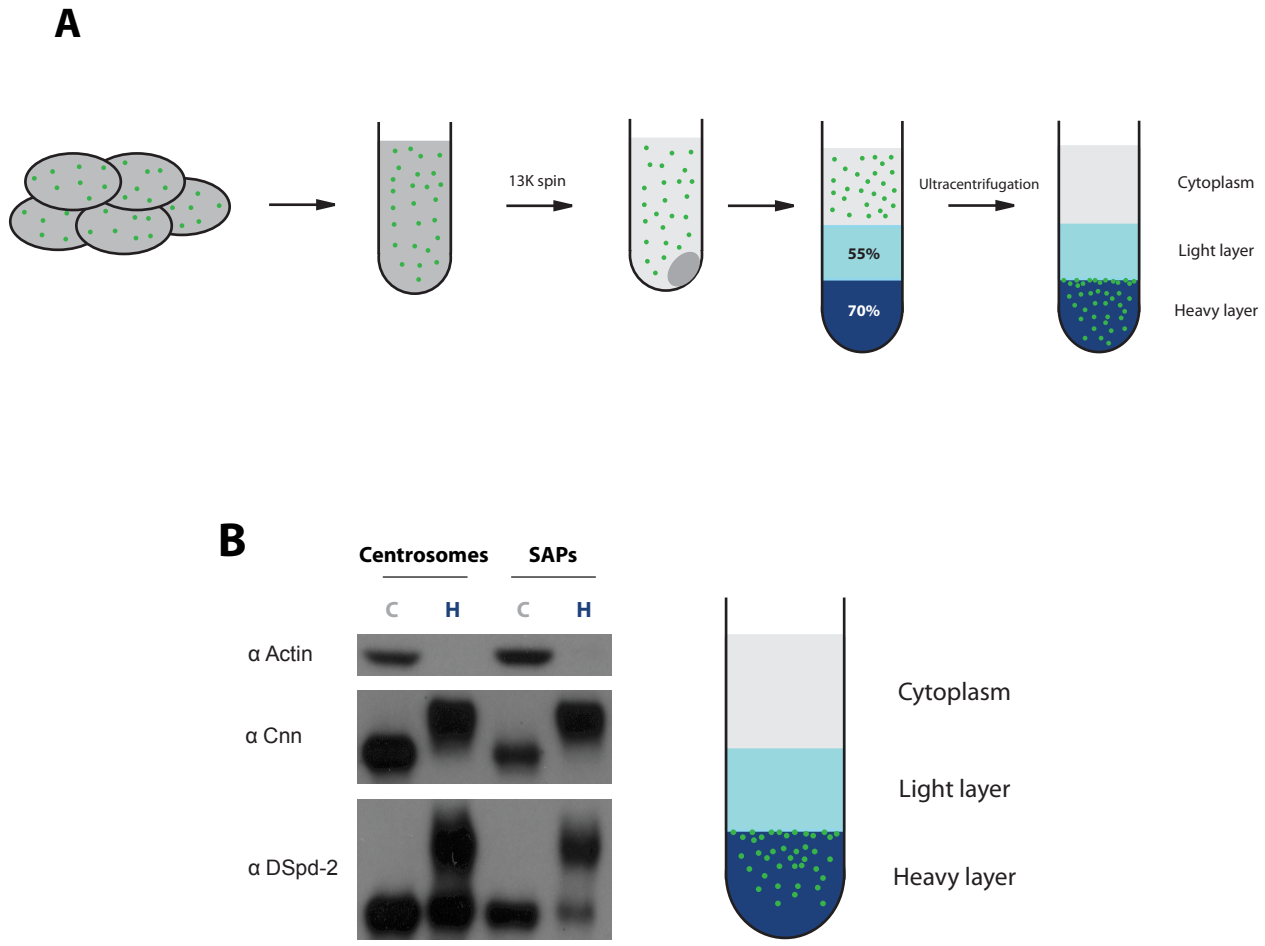


Figure 3.3 PCM proteins are post-translationally modified in SAPs and in centrosomes

(A) Schematic representation of the protocol for SAP/centrosome isolation by sucrose gradient centrifugation. An embryo extract (in the case of centrosomes) or an egg extract (in the case of SAPs) is cleared of larger debris by low speed centrifugation and the supernatant is then loaded on a sucrose cushion and subjected to high speed ultracentrifugation. The heavy centrosomes/SAPs move through the light sucrose layer (light blue) and become trapped in the heavy sucrose layer (dark blue), while the lighter cytoplasmic proteins remain in the cytoplasmic layer at the top of the tube (grey). **(B)** A western blot of samples from cytoplasmic and heavy (centrosomes/SAPs) fractions, probed for actin, Cnn and DSpd-2, as indicated. Note how both in centrosomes and SAPs, Cnn is present as a higher MW band when compared with Cnn in the cytoplasm. DSpd-2 is present in centrosomes and SAPs as a double band, while it is exclusively in the faster migrating form at the cytoplasm. As expected, actin is only present in the cytoplasmic fraction.

the cytoplasm using a sucrose gradient, it was clear that Cnn is present as one band in the cytoplasmic fractions, and as a higher weight band in the centrosomal fractions (Fig.3.3B). Probing these fractions with antibodies for DSpd-2 also showed interesting differences: while DSpd-2 is a single band in the cytoplasmic fractions, a significant proportion of the protein in the centrosomal fractions was shifted to a higher molecular weight band. In both cases this shift was due to phosphorylation, as shown by treatment with phosphatases and phosphatase inhibitors (Fig.3.4). I therefore wondered if the Cnn and DSpd-2 that localised to the SAPs might also be post-translationally modified in the same way as occurs at centrosomes.

To test this possibility I isolated SAPs from the cytoplasm in eggs using a sucrose gradient protocol similar to the one used to isolate centrosomes (Fig.3.3A). As a control, I also isolated centrosomes from embryos using the same protocol. SAP/centrosome and cytoplasmic samples were then examined by western blotting (Fig.3.3B). I confirmed that the sucrose gradient had been successful by probing the membrane for actin, a cytoplasmic component. As expected, actin was present only in the cytoplasmic fraction, but not in the centrosome/SAP (heavy) fractions. I then examined the behaviour of the PCM components Cnn and DSpd-2. As previously described (Paul Conduit, personal communication and Fig.3.3B) Cnn was present in cytoplasmic fractions as a single band, and this band shifted to a higher form in the centrosomic fractions. Remarkably, a similar shift was observed in the SAP fraction, suggesting that Cnn is being post-translationally modified both at the centrosomes and at SAPs in a similar

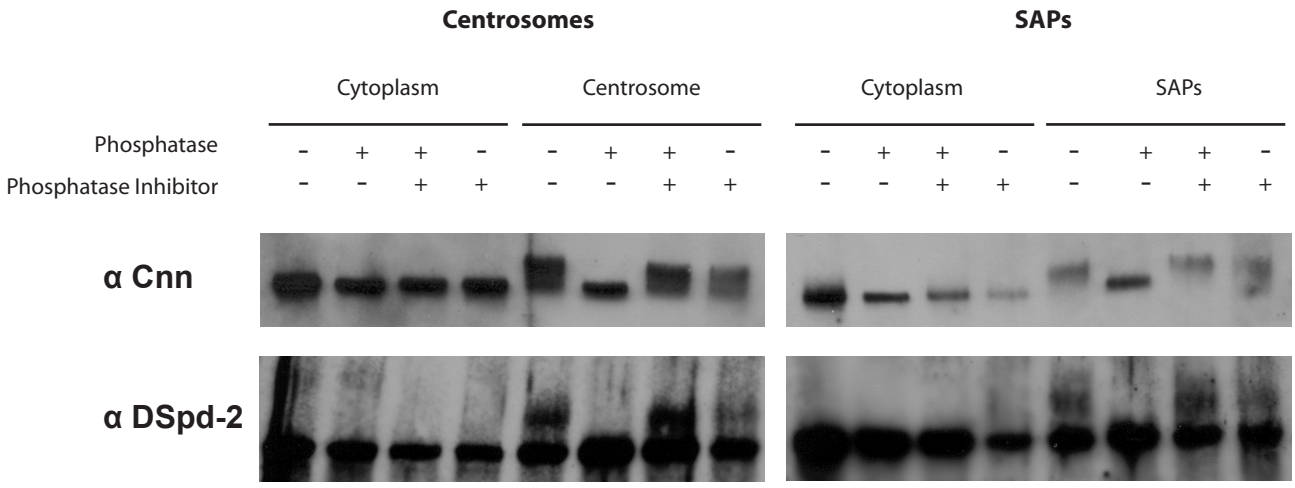


Figure 3.4 PCM proteins are phosphorylated at centrosomes and SAPs
Western blots show the behaviour of Cnn or DSpd-2 that were purified from either the cytoplasmic or centrosome/SAP fractions and subjected to phosphatase—(+/-) phosphatase inhibitors-treatment (as indicated above each blot). The mobility shift observed for both proteins in the centrosome/SAP fractions is due to phosphorylation, as the higher MW bands return to their lower forms upon phosphatase treatment, and this changes is abolished by phosphatase inhibitors.

way. DSpd-2 was present as a single band in the cytoplasmic fractions and was partially shifted to a higher band in both the centrosome and SAP fractions (Fig.3.3B).

In order to confirm that the shifts observed in the SAPs were due to phosphorylation, as had been observed at the centrosome, I exposed the SAP fraction to a phosphatase treatment (in collaboration with Alan Wainman). As shown in Fig.3.4, when exposed to phosphatases the higher band of Cnn at the SAPs shifts back to the lower band observed at the cytoplasm. This effect is specific to phosphorylation, as treatment with phosphatase inhibitor restores Cnn to its higher form. This mirrors the behaviour of Cnn at the centrosome when exposed to the same treatment. A similar behaviour is observed for DSpd-2 (Fig.3.4).

I conclude that the fractions of Cnn and DSpd-2 that are recruited to the SAPs are being post-translationally modified in a similar way to the fractions of these proteins that are recruited to centrosomes.

3.2.3 PCM proteins have the same dynamics in SAPs as in centrosomes

My initial characterisation of the SAPs showed that they organise a PCM containing all tested PCM components (section 3.2.1), and that the PCM components Cnn and DSpd-2 are post-translationally modified at SAPs in a

similar manner to their modification at centrosomes (section 3.2.2). These experiments, however, do not address whether the PCM of SAPs exhibits a similar dynamic behaviour to the PCM of centrosomes. In particular, there were two aspects of PCM dynamics in embryos that I was interested in investigating. The first was PCM flaring. Flaring is the detachment of PCM particles (that do not contain centrioles) from the edges of the PCM. This process is particularly active during telophase and interphase, and is dependent on MTs (Megraw et al., 2002). Both Cnn (Megraw et al., 2002) and DTACC (Lee et al., 2001) have been shown to flare, and many flares contain both Cnn and DTACC (Megraw et al., 2002). As SAPs contain Cnn and organise MT asters, I asked whether Cnn flares at the SAPs. Interestingly, in 30% of the eggs examined, the detachment of Cnn flares from the SAPs could be observed (Fig.3.5). This low percentage may reflect a difference between eggs and embryos. Nevertheless this observation suggested that the PCM of SAPs is to some extent dynamic.

I next wanted to examine the dynamics of PCM proteins in the SAPs in more detail. Recent work in the lab used FRAP to analyse the dynamics of PCM proteins and showed that the behaviour of Cnn was unusual. In Conduit et al*.,* 2010, GFP-tagged versions of several PCM proteins were bleached and their fluorescence recovery over time was analysed. Specifically, the percentage of fluorescence recovery in the central versus more peripheral pixels of the PCM was compared. For most PCM components (DTACC, Msps, γ-tubulin, DGrip75 and EB1) fluorescence recovery took place at a similar rate in both these areas of the PCM.

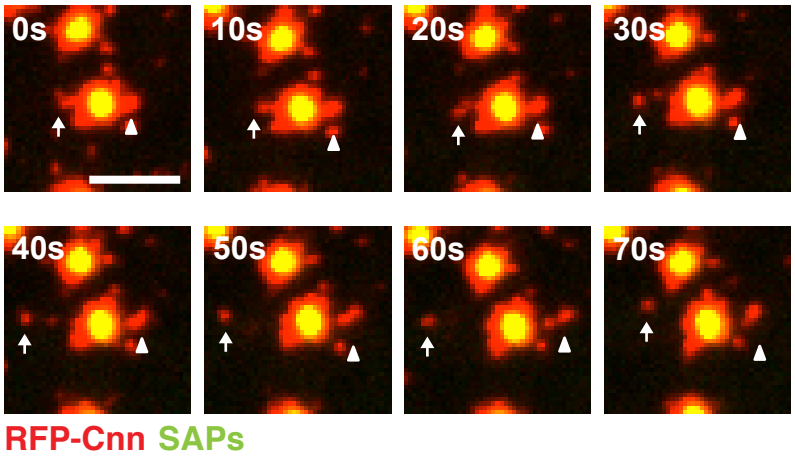


Figure 3.5 Cnn 'flaring' can be observed in SAPs
Live imaging of SAPs in eggs expressing GFP-DSas-6 and GFP-Ana2 (green) and RFP-Cnn (red). Time in seconds is shown in the corner of each picture. Arrow and arrow-heads mark the position of 2 different flares over time. Note how Cnn 'flares'. Scale bar=5μm.

Interestingly, however, Cnn recovers its fluorescence faster at the centre of the PCM (generally corresponding to the area where the centriole is localised) and slower at the edges. It was proposed that Cnn may be incorporated into the PCM close to the centriole, and then move slowly outwards, away from the centrioles.

I wanted to test whether Cnn in SAPs might also show this unusual behaviour. As a control I first looked at the recovery pattern of RFP-DTACC following bleaching. As was observed in centrosomes, RFP-DTACC appears to recover at an equal rate throughout the PCM (Fig.3.6A). RFP-Cnn, however, does indeed seem to recover its fluorescence in the central regions around the SAP faster than the peripheral regions (Fig.3.6B), suggesting a similar dynamic behaviour of this protein in both centrosomes and SAPs.

To quantify this behaviour I employed a new method recently developed in the lab (Paul Conduit, personal communication). By drawing a line of defined length through the centre of the PCM it is possible to generate a line plot of fluorescence intensity across the PCM (see Chapter 2, section 2.5.5). Such line plots can be generated for all time points of the FRAP experiment, both before and after bleaching. By normalising and overlaying the line plots obtained at different time points it is then possible to compare the shape of the FRAP recovery curves over time. Using this method it was possible to see that at the centrosome Cnn recovers its fluorescence initially in a curve that is narrower than its pre-bleach curve. The recovery curve then moves

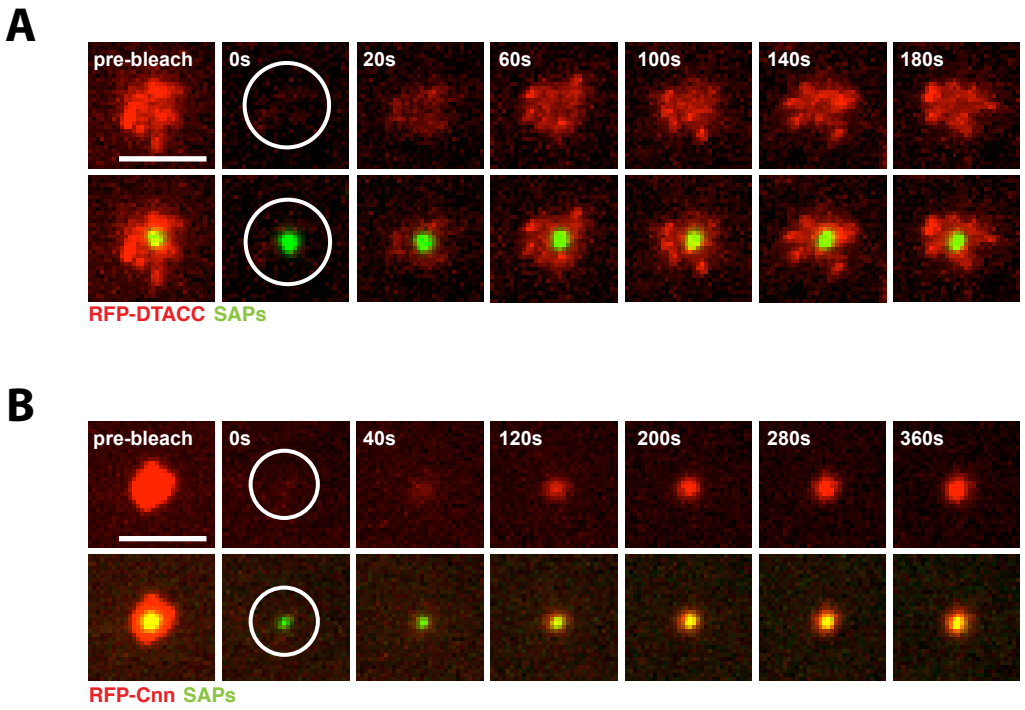


Figure 3.6 FRAP analysis of PCM proteins in SAPs
Images show the dynamic behaviour of (**A**) RFP-DTACC (red) or (**B**) RFP-Cnn (red) at SAPs (green) formed in eggs expressing GFP-DSas-6 and GFP-Ana2 before (pre-bleach) and after (time in seconds) bleaching. Note how RFP-DTACC recovers its fluorescence throughout the PCM, while RFP-Cnn recovers its fluorescence in an area much smaller than its initial, pre-bleach, distribution. Circle represents area bleached at t=0s. Scale bars=5μm.

outwards over time, eventually matching the pre-bleach curve. This supports the earlier hypothesis that Cnn is being incorporated in the centrosome by binding close to the centriole, and then spreads outwards. On the other hand, the recovery curve of other PCM components such as Aurora-A, γ-tubulin and DTACC always matches their pre-bleach curve in shape. This suggests that, unlike Cnn, these proteins bind to sites distributed throughout the PCM.

I applied this quantification method to the FRAP data obtained from SAPs. I first analysed the dynamic behaviour of RFP-DTACC (Fig.3.7). As was the case in centrosomes, the recovery curve of RFP-DTACC always matched the shape of its pre-bleach curve, suggesting that fluorescence recovery of this molecule happens equally throughout the PCM. In contrast, and as is the case in centrosome, Cnn recovers its fluorescence at the SAPs in a narrower curve than its pre-bleach distribution (Fig.3.8). However, Cnn fluorescence recovery in the SAPs is much slower than in centrosomes. In centrosomes, Cnn recovers to its pre-bleach values within 3.5 min, while in the time scale of this experiment (over 6 min) Cnn at SAPs only recovers 20% of its pre-bleach values. Probably due to this slow recovery rate, the recovery curve of Cnn at the SAPs does not move outwards significantly during the course of this experiment. However, it is important to note that at 20% of recovery, Cnn at the centrosome also has not yet moved outwards significantly (Paul Conduit, personal communication). This presumably reflects the fact that Cnn incorporation and subsequent movement

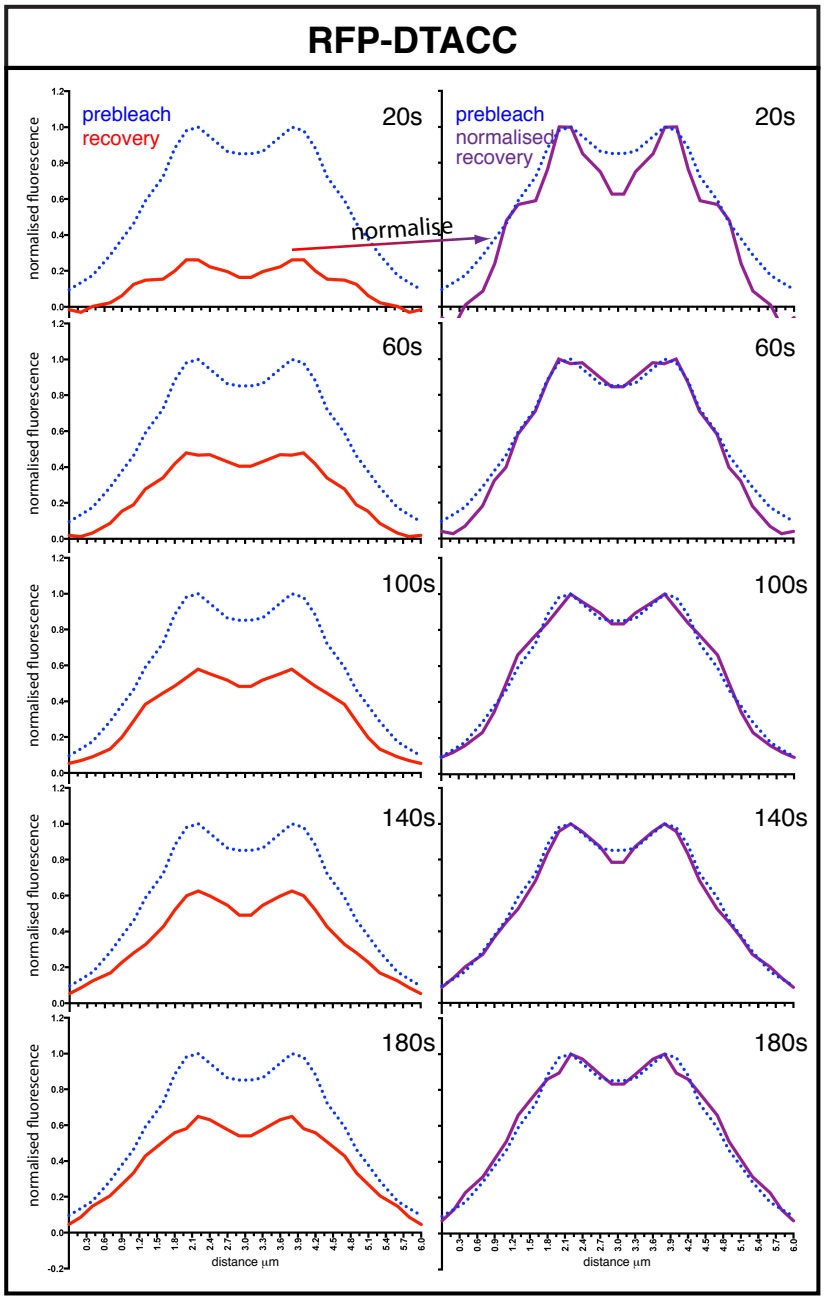


Figure 3.7 Quantitative analysis of RFP-DTACC FRAP behaviour in SAPs
Graphs show the average fluorescence intensity along lines drawn through the centre of several SAPs (n=20) at selected time-points after photobleaching of RFP-DTACC. Graphs on the left display pre-bleached profiles (dotted blue lines) and “raw” recovery profiles (red lines). Graphs on the right display recovery profiles (purple lines) that have been normalized so that their peak intensity is equal to the peak intensity of the pre-bleached profile (dotted blue line). Note how the normalised RFP-DTACC recovery curve closely matches the shape of the pre-bleach curve at all timepoints.

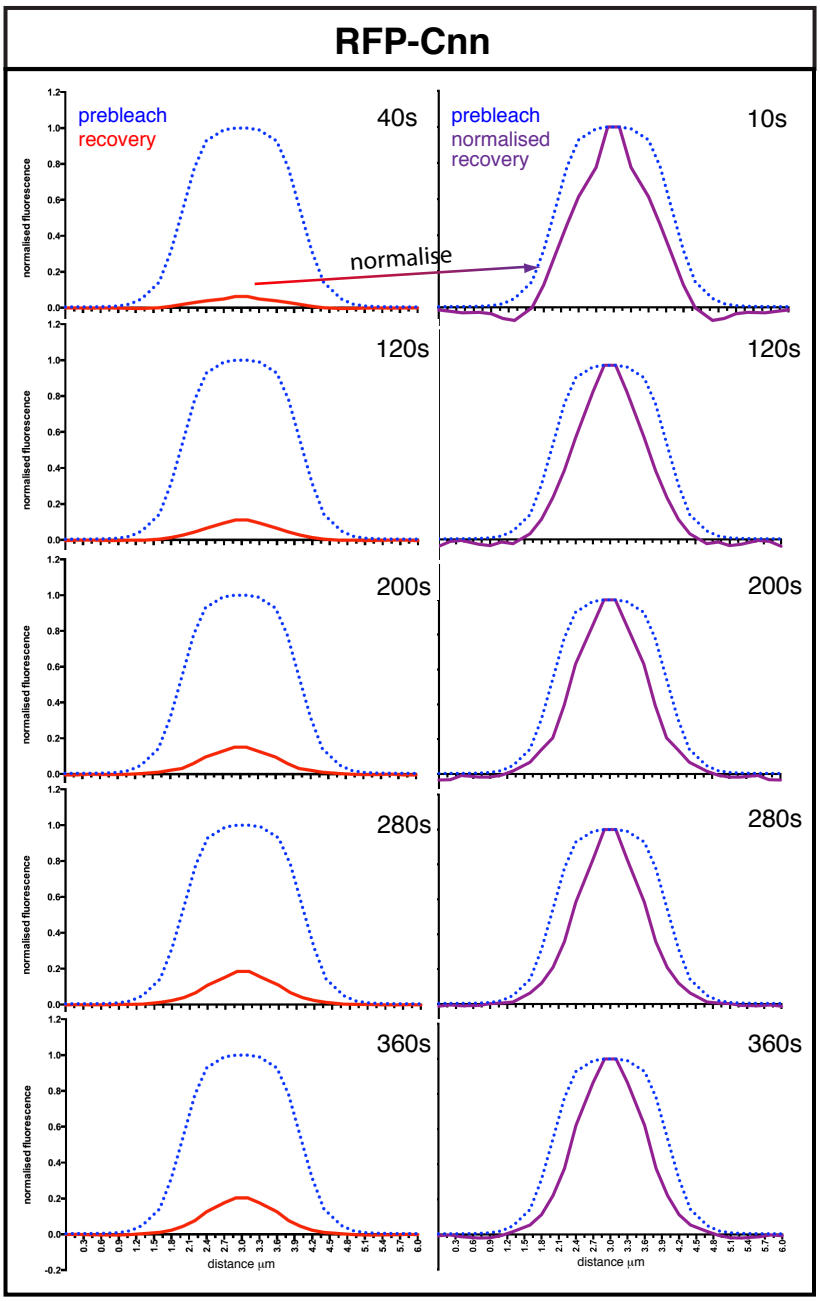


Figure 3.8 Quantitative analysis of RFP-Cnn FRAP behaviour in SAPs
Graphs show the average fluorescence intensity along lines drawn through the centre of several SAPs (n=16) at selected time-points after photobleaching of RFP-Cnn. Graphs on the left display pre-bleached profiles (dotted blue lines) and “raw” recovery profiles (red lines). Graphs on the right display recovery profiles (purple lines) that have been normalized so that their peak intensity is equal to the peak intensity of the pre-bleached profile (dotted blue line). Note how the normalised RFP-Cnn recovery curve remains narrower than its pre-bleach curve at all time points.

throughout the PCM is much slower in unfertilised eggs than in developing embryos. Nevertheless, these observations indicate that the PCM assembled around SAPs exhibits a similar dynamic behaviour to the PCM assembled around the centrioles. PCM components like D-TACC appear to have binding sites distributed throughout the PCM in centrosomes and SAPs, while Cnn is only incorporated into the PCM at binding sites that are distributed close to the centriole or SAP surface.

3.2.4 SAPs cluster in the surface of eggs over time

Previously published work has shown that most neuroblasts that contain too many centrosomes due to overexpression of SAK still divide via a bipolar spindle (Basto et al., 2008). This is possible due to the clustering of supernumerary centrosomes in two poles. Similar clustering behaviour has also been observed in cancers characterised by excessive numbers of centrosomes (Godinho et al., 2009). A recent RNAi screen of the *Drosophila* genome identified several different mechanisms by which this clustering may occur, including MT motor proteins (Kwon et al., 2008).

Previous work (Stevens et al., 2010a) and my own (Fig.3.2) has shown that SAPs can organise MT asters. As these microtubules asters presumably have such motor proteins, I wondered whether SAPs might cluster over time. This hypothesis was also motivated by my observation that *de novo*

centrosomes formed by the overexpression of SAK in unfertilised eggs appear to cluster over time (Fig.3.9A).

Staining of fixed eggs showed that there were both eggs with unclustered SAPs and eggs with clustered SAPs (Fig.3.9B). Live imaging showed that unclustered SAPs tend to move to the surface of the egg over time, forming clusters similar to those observed by *de novo* formed centrosomes (Fig.3.9C). I therefore conclude that, like centrosomes, SAPs are able to cluster over time.

3.2.5 When injected into developing embryos, SAPs can respond to cell cycle cues and form spindle poles

My experiments so far examined SAP behaviour in unfertilised eggs. However, unfertilised eggs are not an ideal model, as they are arrested in development. I wondered how SAPs would behave dynamically in the context of a developing embryo, and in particular whether they would be able to respond to cell cycle cues in a similar manner to centrosomes.

In the early stages of *Drosophila* development the embryo is a syncytium where nuclei divide in near synchrony (Foe and Alberts, 1983). In these early stages, the cell cycle consists exclusively of successive rounds of S and M phase, with no intervening G1 and G2. In this context, the centrosome duplication cycle is slightly different from that described in

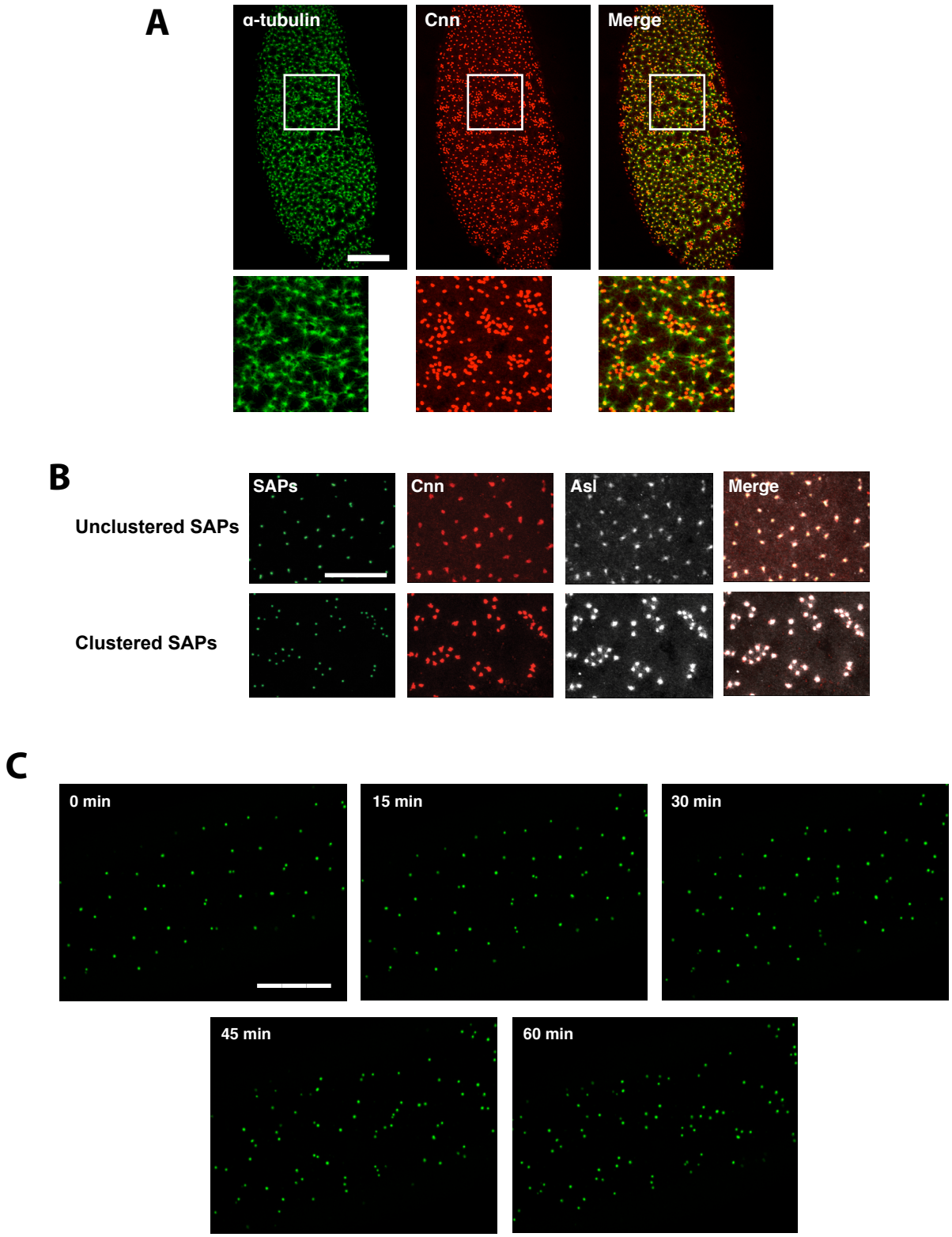


Figure 3.9 SAPs cluster over time.
(A) Images show *de novo* centrosomes formed by overexpression of SAK in unfertilised eggs stained with antibodies for α-tubulin and Cnn. Note how the *de novo* centrosomes in this egg are clustered at the surface. Scale bar = 50μm. **(B)** SAPs in unfertilised eggs stained with antibodies for Cnn and Asl. Note how some eggs contain SAPs that are unclustered, while other eggs shows clustered SAPs similar to the clustered centrosomes in A. Scale bar=20μm **(C)** Live imaging of SAPs at the surface of egs. Time in minutes is shown in the corner of each panel. Note how over time SAPs move to the surface of the egg and cluster. Representative images from 1 egg, 9 eggs imaged in total. Scale bar=30μm

Chapter 1, and centrioles disengage and start to duplicate immediately after mitosis is finished. As the cell cycles are quick and dozens of centrosomes can be seen undergoing the cell cycle in synchrony, the *Drosophila* early embryo is a good system in which to assess SAP behaviour in the context of the cell cycle.

It is not possible to examine the behaviour of SAPs in embryos expressing GFP-DSas-6 and GFP-Ana2 because these embryos arrest in development very quickly. Therefore, in order to examine the behaviour of SAPs in the context of the embryonic cell cycle, I adapted a technique routinely used in the lab to inject antibodies in developing embryos (see Chapter 2). Briefly, I used a glass needle to pierce an unfertilised egg containing SAPs (donor egg) and then extracted the SAPs into the needle. I then injected these SAPs into a developing embryo (acceptor embryo) expressing the MT binding protein Jupiter (Karpova et al., 2006) tagged to mCherry. This allowed me to follow the cell cycle and assess MT dynamics. Injected SAPs are visible in the acceptor embryo as they are formed by GFP-DSas6 and GFP-Ana2. As a control I also performed this experiment with centrioles extracted from developing embryos expressing GFP-DSas-6 to label the centrosome (done in collaboration with Zsofia Novak). Importantly, GFP-DSas-6 does not appear to exchange at centrosomes or at SAPs, so these structures remain labelled with GFP-DSas-6 throughout the time-course of my experiments.

The injected SAPs are able to nucleate MT asters (arrow head, Fig.3.10A). MT asters organised by centrosomes are dynamic structures that change in density during the cell cycle (Kuriyama and Borisy, 1981). As can be seen in Fig.3.10A, the behaviour of the MTs organised by the SAP (arrowhead) mimics that of the MTs organised by the endogenous centrosomes (arrows): the MT asters are of medium size during interphase, are at their shortest during mitosis and then dramatically increase in size during anaphase/telophase. Control injected centrosomes showed a similar MT aster behaviour to injected SAPs (Fig.3.10B). SAPs therefore nucleate MT asters dynamically and in synchrony with the endogenous centrosomes.

In addition, when in close proximity to a spindle, SAPs are able to interact with endogenous centrosomes, leading to the formation of multipolar spindles (Fig.3.11A). Although the early *Drosophila* embryo is a syncytium, each spindle is contained within its own compartment that is bordered by an actin network (Karr and Alberts, 1986). Interestingly, injected SAPs are also able to organise such compartments, as is obvious when embryos injected with SAPs are imaged close to the edges of the embryo (Fig.3.11B).

Very interestingly, I often observed the injected SAPs separating into 2 individual SAP structures, reminiscent of centriole duplication. SAP separation was observed in 80% of the movies made, but was mostly restricted to larger SAPs. Remarkably, this sometimes occurred in synchrony with the separation of the endogenous centrosomes at the end of

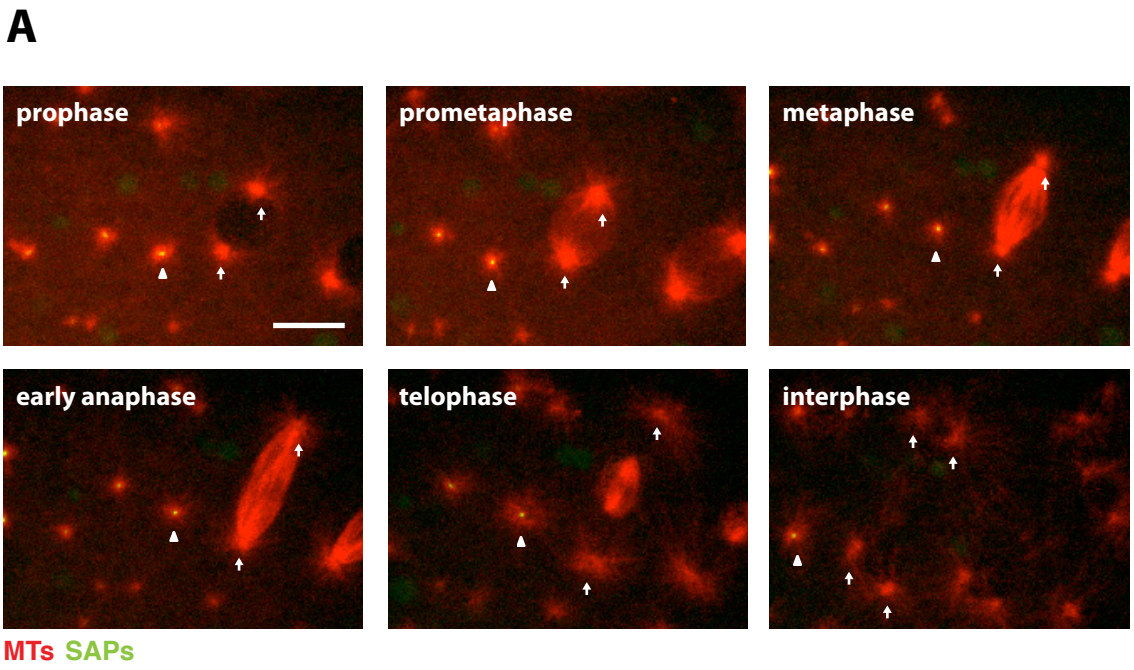


Figure 3.10 MT asters organised by SAPs injected into developing embryos mimic those organised by endogenous centrosomes and by exogenously injected centrosomes
SAPs (visible by GFP fluorescence, green) were removed from unfertilised eggs and injected into developing embryos that expressed Jupiter-mCherry (red) to allow MT visualization. **(A)** Injected SAPs (arrow-head) organise MT asters that change their dynamics in synchrony with the endogenous centrosomes (arrows). Note how centrosomal and injected SAP asters expand and contract synchronously during mitosis. Scale bar=10μm **(B)** Injected centrosomes (labelled with GFP-DSas-6, green) and injected SAPs (labelled with both GFP-DSas-6 and Ana2-GFP, green) both organise MT asters (red) that cycle in synchrony with the endogenous centrosomes (not shown here): MTs are short in prophase, but dramatically elongate in late anaphase/telophase. Scale bar=5μm.

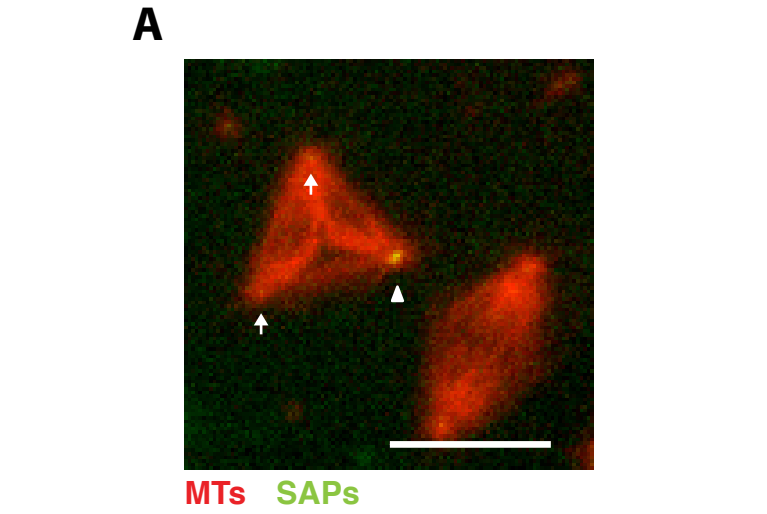


Figure 3.11 SAPs injected in developing embryos can form spindles and organise their own cytoplasmic compartments

SAPs (visible by GFP fluorescence, green) were injected into developing embryos expressing Jupiter-mCherry (red) to allow MT visualization. (**A**) Injected SAP (arrow-head) interacting with 2 endogenous centrosomes (arrows) to form a tripolar spindle. (**B**) Imaging at the edge of the embryo shows that SAPs organise individual 'compartments' similar to those organised by centrosomes. Scale bars=10μm. Note how a very small amount of GFP-DSas-6 can be detected on the endogenous centrosomes. This is because some cytoplasmic GFP-DSas-6 is transferred into the recipient embryo when SAPs are injected, and this becomes incorporated into the endogenous centrosomes close to the site of injection.

mitosis (Fig.3.12A), although occasionally it was a little delayed (Fig.3.12B). The significance of this observation is, however, unclear. As SAPs do not have the characteristic structural organisation of centrioles, it is unlikely that they are undergoing canonical duplication.

In conclusion, when SAPs are injected into developing embryos they display many features of centrosomes, organising dynamic asters that cycle in synchrony with the endogenous centrosomal asters and can organise actin domains, and interacting with the endogenous chromosomes to form multipolar spindles.

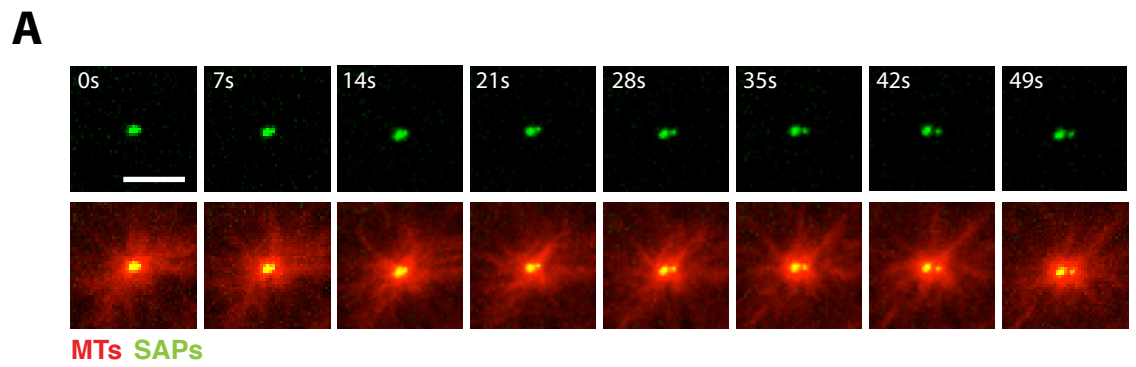


Figure 3.12 SAPs injected into developing embryos can occasionally separate
SAPs (visible by GFP fluorescence, green) were injected into developing embryos expressing Jupiter-mCherry (red) to allow MT visualization. **(A,B)** in the two examples shown here the single SAP eventually formed two SAPs, and this usually occurred at the same time (A) or shortly after (B) the endogenous centrosomes (not shown here) separated from one another at the end of mitosis. Scale bar=5μm.

3.3 Discussion

In this chapter I used a variety of methods to probe the similarities and differences between SAPs and centrosomes. As I will describe in Chapters 4 and 5, I have used SAPs to analyse in detail the pathway of centrosome assembly. The conclusions from those experiments rely on the validity of using SAPs as a model for centrosomes, making this initial characterisation work very important.

Encouragingly, SAPs appear to be very similar to centrosomes. I show that SAPs have a similar composition to centrosomes, and that the PCM proteins Cnn and DSpd-2 are post-translationally modified in the SAPs in a similar way to that seen in centrosomes. In addition I show that SAPs are dynamic structures and that two PCM components, Cnn and DTACC, exhibit similar dynamics to that seen in centrosomes, as assessed by FRAP: DTACC is incorporated throughout the PCM, while Cnn is only incorporated at the SAP surface. Perhaps most importantly, I show that SAPs behave in a very similar manner to centrosomes when injected into cycling embryos: the MT asters nucleated by SAPs respond to cell cycle cues in synchrony with the endogenous centrosomes; the SAPs can compete with the endogenous centrosomes for attachment to the endogenous chromosomes, leading to the formation of multipolar spindles; SAPs can organise their own actin-dependent domains around themselves. The similarity of SAPs to centrosomes probably explains why embryos expressing Ubq-GFP-DSas-6 and Ubq-GFP-Ana2 arrest very early in development: the rapid formation of

so many centrosome-like structures in the cytoplasm is presumably incompatible with normal development.

SAPs do, however, differ from centrosomes, for example with regards to the extent of PCM flaring. While flaring is a common behaviour of the PCM organised by centrioles, flaring in SAPs was only observed in 30% of the eggs expressing GFP-DSas6 and GFP-Ana2. However, flaring at centrosomes is cell cycle dependent, most likely due to the changes in MT dynamics in different stages of the cell cycle. It is therefore likely that SAPs are not seen flaring more often because they have been examined in unfertilised eggs, which are arrested in development.

Another difference observed in PCM dynamics regards the organisation of Cnn at the SAPs. Like centrosomes, I showed that Cnn in SAPs recovers its fluorescence after bleaching with a distribution that is narrower than its pre-bleach distribution. However, and unlike centrosomes, during the time course of the experiment I did not observe Cnn fluorescence moving outwards. Again, it is important to note that the FRAP analysis at the centrosome was performed in a developing embryo, while my FRAP analysis was performed in an unfertilised egg that was not developing. Cnn dynamics do vary during the cell cycle (Conduit et al., 2010), so it is possible that they may simply be much slower in unfertilised eggs. This hypothesis could be tested in the future by assessing the FRAP dynamics of Cnn organised by *de novo* centrosomes generated in unfertilised eggs by the overexpression of Sak.

Surprisingly, when injected into developing embryos, SAPs can sometimes be seen to separate into 2 SAPs. This often occurred at the end of mitosis, when the endogenous mother and daughter centrioles that duplicated in the previous cycle separate. This poses the interesting question of whether SAPs might be able to duplicate. The fact that this separation often occurs in synchrony with centriole disengagement suggests that this might be the case. However, SAPs in eggs do not to have the typical well-organised structure of a centriole. Canonical, template-driven duplication is therefore unlikely. A possible explanation is that SAPs might simply break apart in synchrony with centrosome separation as a consequence of the highly dynamic behaviour of the MTs during this period in the cell cycle. It will be very interesting to determine whether new DSas-6 and Ana2 are incorporated into one, or both, of the SAP structures as they separate. This type of analysis could be performed, for example, by injecting SAPs into developing embryos expressing RFP-DSas-6 or RFP-Ana2. While I suspect that this process of SAP “duplication” is unlikely to be of physiological relevance, it is certainly intriguing and worthy of further analysis.

In summary, the experiments presented in this Chapter strongly suggest that SAPs are very similar to centrosomes in both composition and behaviour. I am therefore confident that SAPs can be used as a useful model system to analyse the pathway of centrosome assembly.

Chapter 4: Using SAPs to elucidate the pathway of PCM recruitment

4.1 Summary

Previous work has identified many of the major players in the assembly of the mitotic PCM. In an RNAi screen in fly S2 cells (Dobbelaere et al., 2008), 11 proteins were identified as being involved in PCM assembly, while an additional 9 proteins were identified as candidates in both centriole duplication and PCM assembly. Recent studies in the Raff laboratory have strongly suggested that three proteins, Asl, DSpd-2 and Cnn play a particularly important role in initiating the mitotic assembly of the PCM (Conduit et al., manuscript under review). Embryos lacking Cnn have centrosomes and develop up to cellularisation and an important role for Cnn in the assembly of embryonic centrosomes has already been established (Megraw et al., 1999; Vaizel-Ohayon and Schejter, 1999; Lucas and Raff, 2007).

The role of Asl and DSpd-2 in the embryonic centrosome is, however, less clear. Embryos laid by *dspd-2* mutant females arrest very early in embryonic development, prior to pronuclear fusion. Pronuclear fusion is initiated when the paternal centriole on the fertilising sperm starts to recruit PCM and nucleate MTs, forming the first centrosome in the embryo. The MTs

organised by this first centrosome are required to move the paternal and maternal pronuclei together for fusion. *dspd-2* mutants fail at this stage of embryonic development. Mutants of *asl* are lethal due to centriole duplication defects. However, weaker hypomorphs of *asl* can still undergo centriole duplication, showing however defects in PCM recruitment. I will refer to these mutants as *asl* PCM recruitment mutants. Though *asl* PCM recruitment mutants are not lethal, the embryos that are laid by these flies also fail in pronuclear fusion due to PCM recruitment defects. The specific role of DSpd-2 and Asl in PCM recruitment in the embryo is therefore still unclear.

In this chapter I analyse the pathway of PCM recruitment in the embryo using SAPs. I first confirm that SAPs are good models for analysis of the pathway of PCM recruitment by showing that SAPs lacking Cnn show the same composition as centrosomes lacking Cnn. I then analyse the composition of SAPs in the absence of DSpd-2, and in a PCM recruitment mutant of *asl*. I also compare the composition of SAPs in these two mutant backgrounds with the composition of *de novo* formed centrosomes in eggs in the same mutant backgrounds. My data suggests that Asl is the most upstream of these three players in the recruitment pathway. I also show that both DSpd-2 and Cnn can independently recruit PCM components, but that DSpd-2 is the most important of the two in the embryo, as DSpd-2 is also involved in recruiting Cnn. My data allow me to propose a pathway for the recruitment of the PCM in centrosomes in embryos.

4.2 Results

4.2.1 SAPs in Cnn mutants show the same composition as centrosomes lacking Cnn

In the previously published RNAi screen for centrosomal components, Cnn was one of the highest scorers for candidates involved in PCM recruitment. The role of Cnn at centrosomes has been analysed in neuroblasts, suggesting an important role in PCM recruitment (Megraw et al., 2001). The role of Cnn in PCM recruitment in embryonic centrosomes has also been previously analysed. Embryos lacking Cnn can partially develop, undergoing several cell cycles, and arresting eventually prior to cellularisation. Centrosomes lacking Cnn in embryos have been shown to fail to recruit several PCM components, or to be able to recruit them only at lower levels (Megraw et al., 1999; Vaizel-Ohayon and Schejter, 1999; Lucas and Raff, 2007). Cnn is therefore thought to be an important player in PCM recruitment in the embryo.

In Chapter 3 I showed that SAPs are very similar to centrosomes in composition and dynamics. To test whether SAPs can potentially be used to analyse the role of different PCM proteins in PCM assembly I decided to analyse the composition of SAPs in the absence of Cnn to determine whether the composition of centrosomes and SAPs lacking Cnn was similar.

I imaged SAPs formed in either WT or *cnn*^{hk21}/*cnn*^{f04547} eggs expressing GFP-DSas6 and GFP-Ana2 and quantified the recruitment of several centrosomal proteins (Fig.4.1A,B). Both mutant alleles have been shown not to produce any protein (Megraw et al, 1999; Lucas and Raff, 2007) In *cnn*^{hk21}/*cnn*^{f04547} SAPs, the levels of centriolar component Asl were shown to be increased when compared with WT SAPs (p=0.0039, n=30), while DSas-4 levels were unchanged (p=0.6057, n=28). This is similar to the composition of Cnn mutant centrosomes in embryos, where an increase in the levels of Asl has been previously observed (Paul Conduit, personal communication). The levels of PCM proteins in *cnn* SAPs, however, were reduced. The levels of DSpd-2 were reduced to ~80% of WT (p=0.0136; n=30 eggs) while the levels of γ-tubulin were reduced to ~20% of WT (p<0.0001; n=30 eggs). This is consistent with previous data available for centrosomes lacking Cnn in the embryo, where both DSpd-2 (Paul Conduit, personal communication) and γ-tubulin (Megraw et al., 1999; Vaizel-Ohayon and Schejter, 1999) are present at reduced levels. As PCM components are still present at lower levels, *cnn*^{hk21}/*cnn*^{f04547} SAPs still organise MT asters, albeit of smaller size than WT SAPs.

In conclusion, in the absence of Cnn, SAPs recruit normal, or even slightly increased, levels of centriolar components, but reduced levels of PCM components. This is consistent with the composition of centrosomes formed in *cnn* mutant embryos. I am therefore confident that the analysis of SAP composition in different centrosome mutants can be used to draw conclusions on the pathway of centrosome assembly.

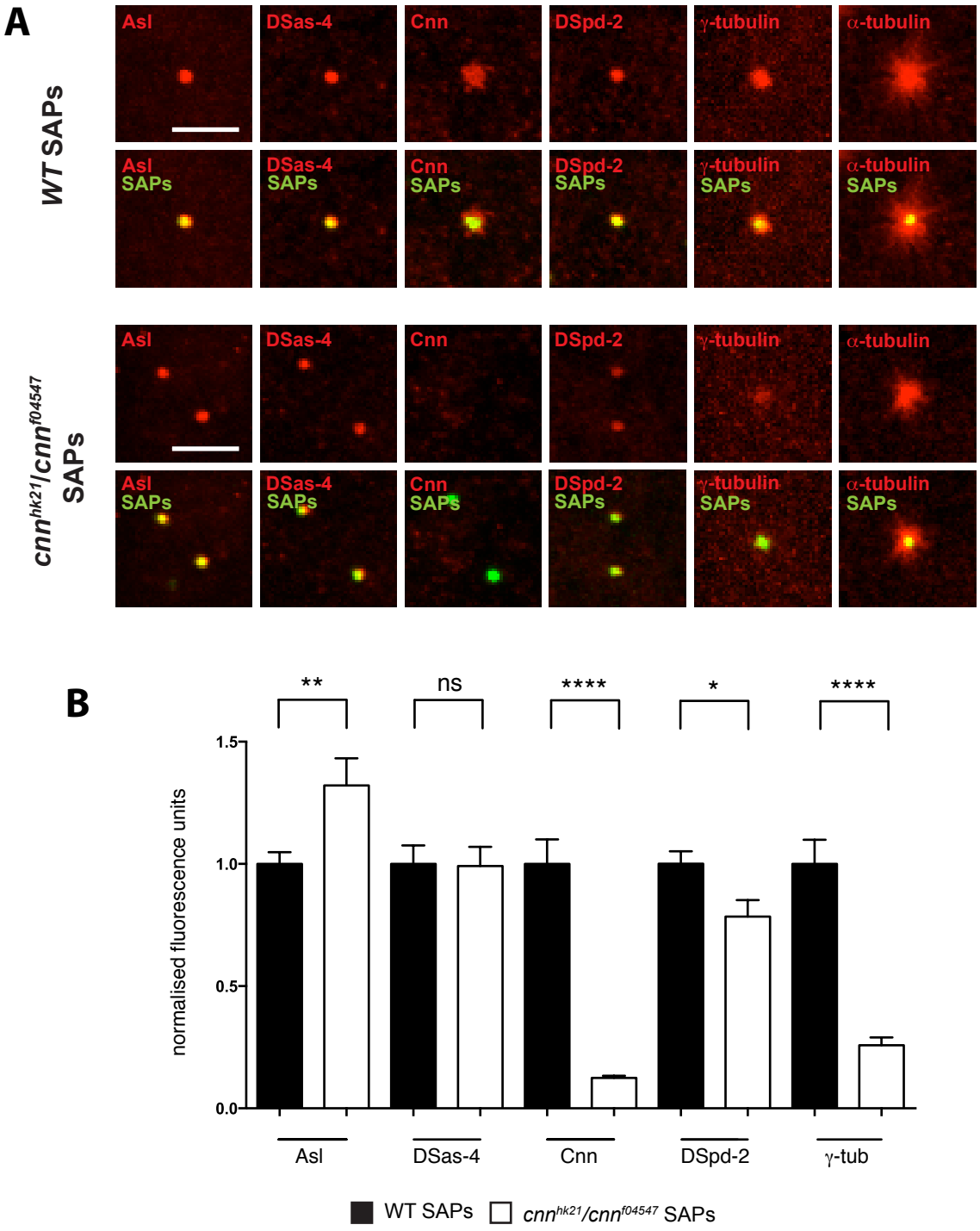


Figure 4.1 SAP composition in the absence of Cnn

An analysis of SAP composition in eggs expressing Ubq-GFP-DSas-6 and Ubq-GFP-Ana2 in either a WT or a *cnn*^{hk21}/*cnn*^{f04547} background. **(A)** Representative images of WT SAPs and *cnn*^{hk21}/*cnn*^{f04547} SAPs stained with antibodies for different centrosomal proteins, as indicated. Scale bar=5μm. **(B)** Graph shows the quantification of protein levels in WT (black) and *cnn*^{hk21}/*cnn*^{f04547} (white) SAPs. Note how *cnn*^{hk21}/*cnn*^{f04547} SAPs have normal or increased levels of centriolar components (Asl and DSas-4), but reduced levels of PCM components (DSpd-2 and γ-tubulin). Bars represent the average SAP fluorescence of at least 27 eggs. Error bars in this and all subsequent graphs show the standard error of the mean (SEM) unless otherwise indicated. Statistical significance in this and all subsequent experiments was calculated by a two tailed Student's t-test or the corresponding non-parametric equivalent, as appropriate, unless otherwise indicated. Levels of significance are indicated above each each comparison (p value: *<0.05; **<0.01; ***<0.001; ****<0.0001).

4.2.2 *de novo* centrosomes form in the absence of DSpd-2, but recruit very little PCM

DSpd-2 mutants in *Drosophila* have been described, and shown not to produce any protein (Giansanti et al., 2008) and DSpd-2 has been implicated in PCM recruitment in neuroblasts and spermatocytes (Dix and Raff, 2007; Giansanti et al., 2008). It is unclear, however, if a similar function is performed in embryos. After fertilisation, paternal centrioles fail to recruit PCM in *dspd-2* embryos, suggesting a PCM recruitment function. However, the inability of the paternal centriole to recruit PCM leads to a failure in pronuclear fusion (Dix and Raff, 2007). As a consequence, it has not been possible to analyse the composition of centrosomes formed in *dspd-2* embryos.

Centriole formation is normally templated by a pre-existing mother centriole (see Chapter 1). As *Drosophila* oocytes do not have centrioles, all centrioles in the embryo are derived from the basal body in the sperm, via successive rounds of duplication. However, Rodrigues-Martins et al*.,* 2007b, showed that centriole biogenesis is possible in the absence of a template centriole. By overexpressing the kinase Sak in the unfertilised egg, the authors showed that *de novo* centriole formation can take place. These *de novo* formed centrioles showed the same structure (shown by EM) and composition (shown by DSas-4 and DSas-6 stainning) as canonical centrioles. In addition, they could organise a functional PCM, as shown by the presence of γ-tubulin, D-PLP and the organisation of MT asters. These

structures were therefore thought to be *de novo* formed centrosomes. For the remainder of this thesis I will therefore refer to centrosomes formed in unfertilised eggs by the overexpression of Sak as *de novo* centrosomes.

DSpd-2 in *Drosophila* does not appear to be required for centriole duplication (Dix and Raff, 2007). I therefore hypothesised that *de novo* centrosomes might be able to form in the absence of DSpd-2. I overexpressed Sak in unfertilised eggs in both WT and *dspd-2*$^{z3\text{-}5711}$*/dspd-2*^{df} mutant backgrounds. As hypothesized, *de novo* centrosomes could indeed form in the absence of DSpd-2 (Fig.4.2A). In the absence of DSpd-2, *de novo* centrosomes showed very low levels (almost undetectable) of the PCM components Cnn ($p<0.0001$; $n>19$ eggs) and γ-tubulin ($p<0.0001$; $n=21$ eggs), and could organise only very small MT asters (Fig.4.2A and B). This is consistent with reports in other tissues that DSpd-2 is involved in PCM recruitment. The centriolar components Asl and DSas-4 were recruited to *de novo* centrosomes formed in the absence of DSpd-2. Unexpectedly, however, their levels were dramatically increased ($p<0.0001$ $n= 21$ for both).

4.2.3 SAPs in a *dspd-2* mutant background recruit little PCM but, unlike *de novo* centrosomes, have reduced levels of Asl

I was interested in knowing if SAPs, which also form *de novo* in unfertilised eggs, had a similar composition to *de novo* centrosomes in the absence of DSpd-2. I therefore imaged SAPs formed in *dspd-2*$^{z3\text{-}5711}$*/dspd-2*^{df} eggs

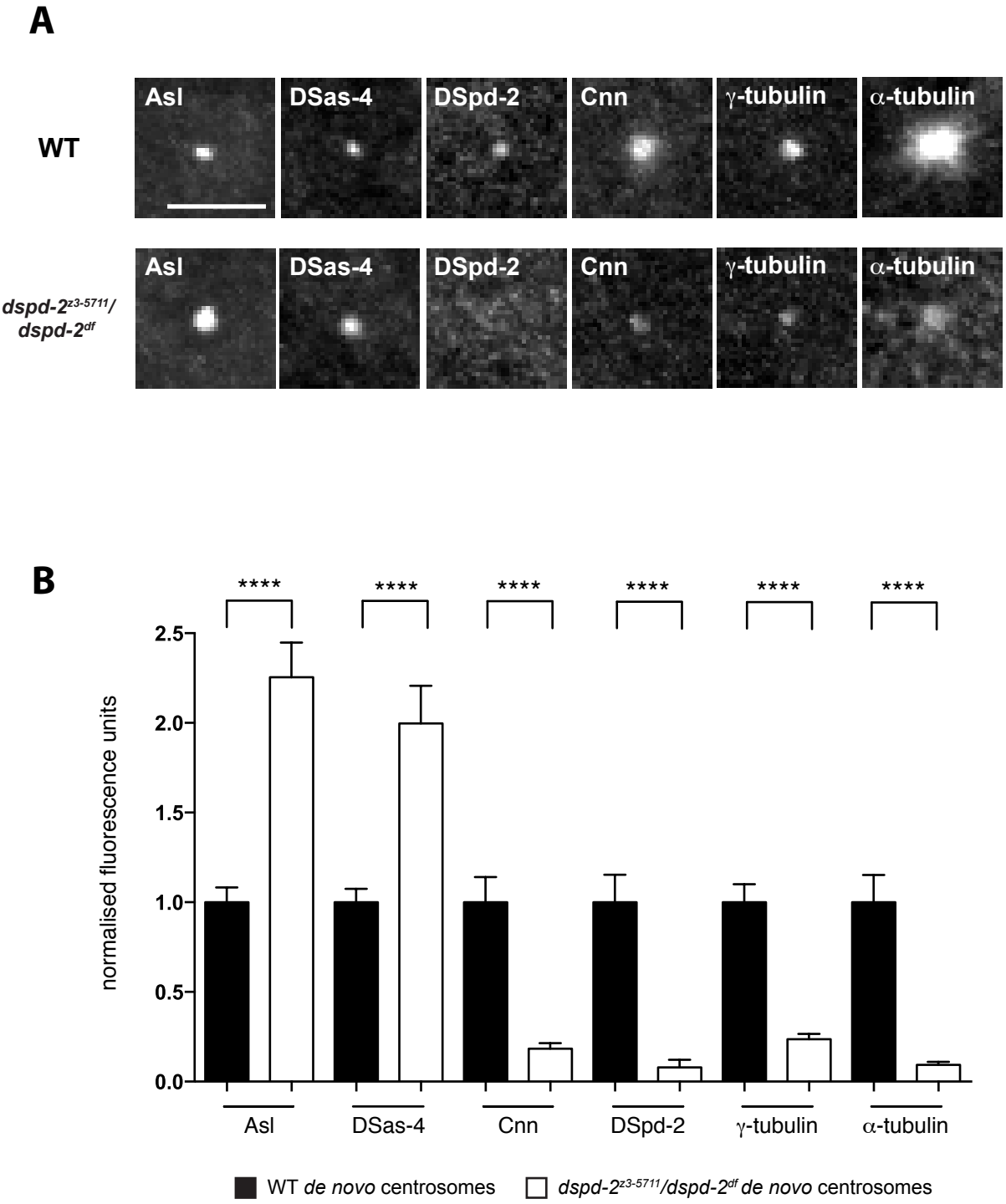


Figure 4.2 Centrosomes can form *de novo* in the absence of DSpd-2
An analysis of the composition of centrosomes formed *de novo* by the overexpression of Sak, in WT and *dspd-2$^{z3-5711}$/dspd-2^{df}* unfertilised eggs. **(A)** Representative images of *de novo* formed centrosomes in WT (top) and *dspd-2$^{z3-5711}$/dspd-2^{df}* (bottom) unfertilised eggs stained with antibodies for different centrosomal proteins, as indicated. Scale bar=5μm. **(B)** Graph showing the quantification of different protein levels in WT (black) and *dspd-2$^{z3-5711}$/dspd-2^{df}* (white) *de novo* formed centrosomes. Note how *dspd-2$^{z3-5711}$/dspd-2^{df} de novo* centrosomes have increased levels of Asl and DSas-4, but reduced levels of all tested PCM components. Bars represent the average centrosome fluorescence of at least 19 eggs.

expressing GFP-DSas6 and GFP-Ana2. As a control I also imaged SAPs in WT eggs expressing GFP-Sas6 and GFP-Ana2.

SAPs without DSpd-2 showed very low (almost undetectable) levels of the PCM components Cnn ($p<0.0001$, n=25) and γ-tubulin ($p<0.0001$, n=32), when compared with WT SAPs (Fig.4.3A and B). In fact, this reduction in PCM recruitment seemed to be even more dramatic than in *de novo* centrosomes, as MT aster formation was only very rarely observed (1/35 eggs).

Interesting differences were observed when comparing SAPs and *de novo* centrosomes formed in the absence of DSpd-2 with regards to centriolar proteins. While DSas-4 was increased in *dspd-2*$^{z3\text{-}5711}$*/dspd-2*^{df} *de novo* centrosomes (when compared with WT *de novo* centrosomes), DSas-4 levels were slightly lower in *dspd-2*$^{z3\text{-}5711}$*/dspd-2*^{df} SAPs, although this difference was just below the level of statistical significance ($p=0.0624$; n=25). In addition, while Asl levels were greatly increased in *dspd-2*$^{z3\text{-}5711}$*/dspd-2*^{df} de novo centrosomes (when compared with its respective WT control), the levels of Asl were reduced in *dspd-2*$^{z3\text{-}5711}$*/dspd-2*^{df} SAPs ($p< 0.0001$; n=25).

During the course of this analysis I noticed that even in eggs laid by WT females expressing GFP-DSas-6 and GFP-Ana2, some of the eggs contained SAPs that were not stained with antibodies against centrosomal components and some eggs contained a mixture of SAPs, where some

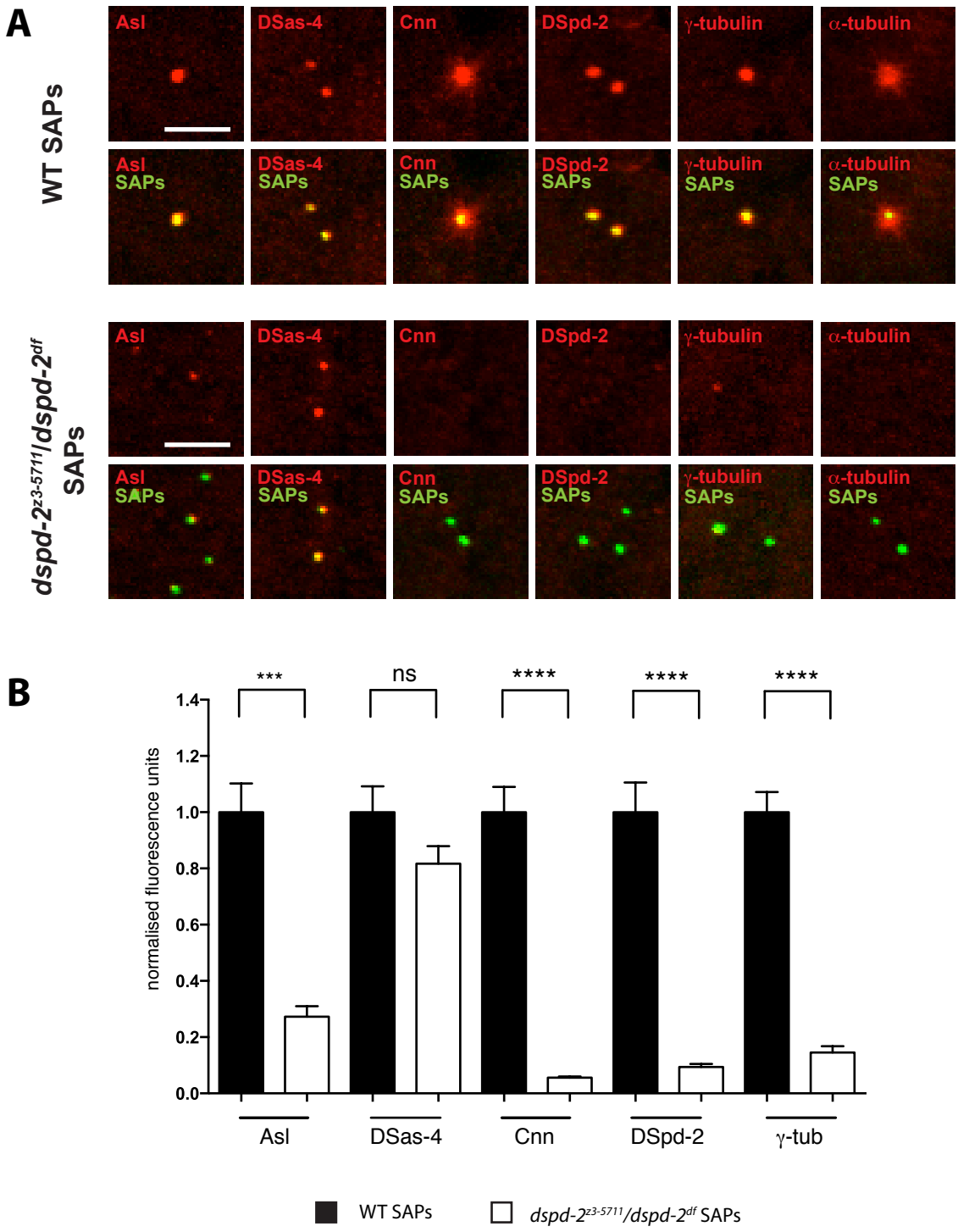


Figure 4.3 SAP composition in the absence of DSpd-2
An analysis of SAP composition in eggs expressing Ubq-GFP-DSas-6 and Ubq-GFP-Ana2 in either a WT or a *dspd-2*$^{z3\text{-}5711}$*/dspd-2*^{df} background. **(A)** Representative images of WT SAPs and *dspd-2*$^{z3\text{-}5711}$*/dspd-2*^{df} SAPs stained with antibodies for different centrosomal proteins, as indicated. Scale bar=5μm. **(B)** Graph showing the quantification of different protein levels in WT (black) and *dspd-2*$^{z3\text{-}5711}$*/dspd-2*^{df} (white) SAPs. Note how *dspd-2*$^{z3\text{-}5711}$*/dspd-2*^{df} SAPs have normal levels of DSas-4, but reduced levels of Asl. They also show very reduced levels of all tested PCM components, and do not seem to organise MT asters. Bars represent the average SAP fluorescence of at least 25 eggs.

SAPs were stained with antibodies against centrosomal components and others were not (Fig. 4.4A, black bars). This may reflect problems with antibody penetrance or a natural variation in the ability of SAPs to recruit centrosomal proteins. I found that the proportion of *dspd-2*$^{z3\text{-}5711}$*/dspd-2*^{df} eggs in which no SAPs stained for Asl, or in which some of the SAPs did not stain for Asl was higher than in WT (Fig.4.4A, white bars). Interestingly, however, even when only eggs in which all SAPs were seen to stain for Asl were included in the analysis, the levels of Asl in *dspd-2*$^{z3\text{-}5711}$*/dspd-2*^{df} SAPs were still lower than in WT SAPs (Fig.4.4B). I am therefore confident that the levels of Asl are reduced in SAPs that lack DSpd-2.

4.2.4 *de novo* centrosomes cannot form in *asl*^{1} eggs

Asl was identified in an RNAi screen for centrosomal proteins as an important player in both centriole duplication and PCM recruitment (Dobbelaere et al., 2008). Early studies have identified *asl*^{1} as a hypomorphic allele of Asl (Bonaccorsi et al., 1998)- Asl protein is produced (Blachon et al., 2008), but a nonsense mutation means that the C-terminal 140 amino acids are missing (Jennifer Richens, personal communication). In the presence of only this truncated version of Asl, centrosomes in spermatocytes show strong defects in PCM recruitment (Bonaccorsi et al., 1998; Giansanti et al., 2001; Varmark et al., 2007; Blachon et al., 2008). In stronger hypomorphic alleles (such as *asl*^{mecD}) the PCM recruitment defect

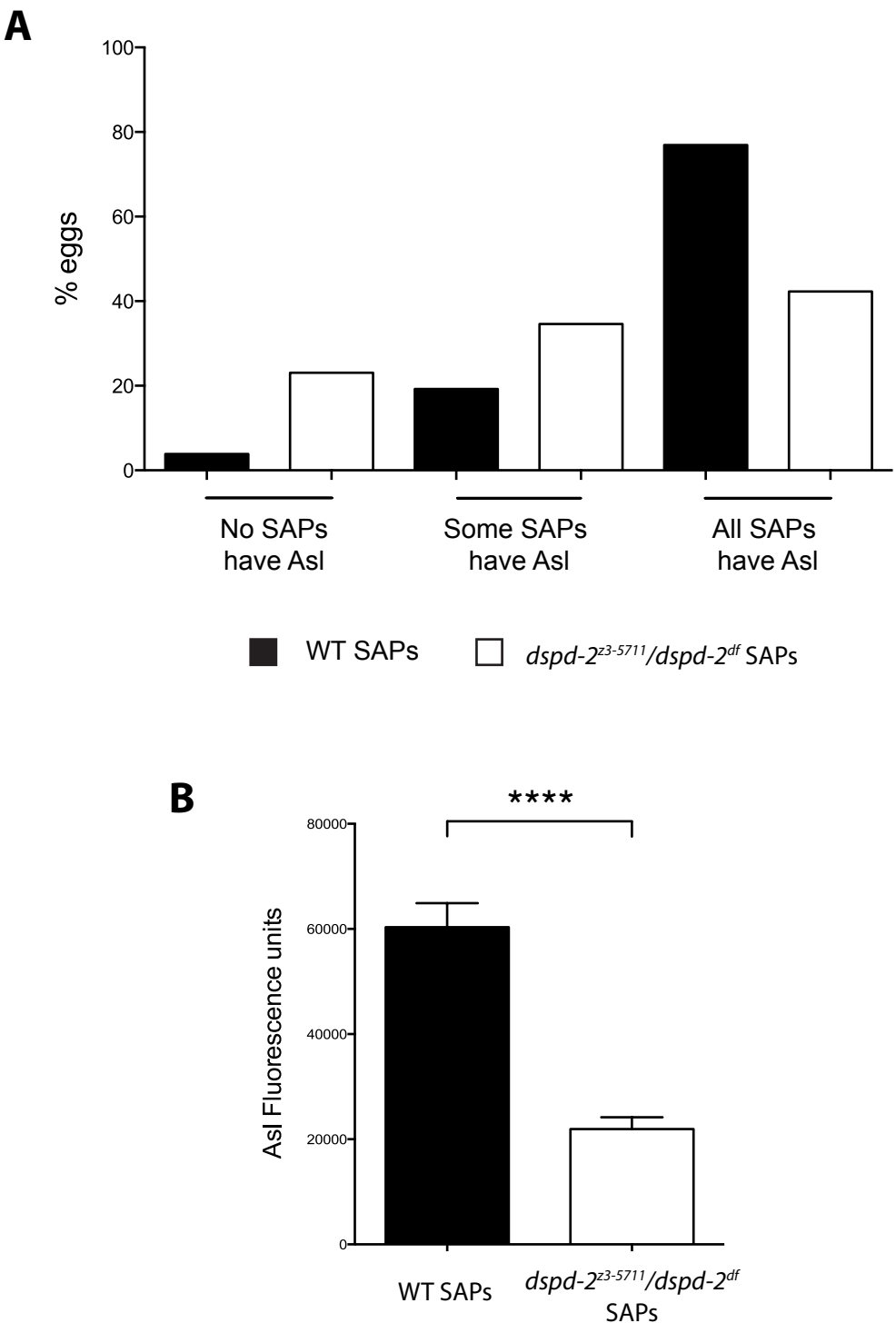


Figure 4.4 Detailed analysis of Asl behaviour in *dspd-2$^{z3-5711}$/dspd-2^{df}* SAPs
An analysis of Asl levels in SAPs in either a WT (black) or a *dspd-2$^{z3-5711}$/dspd-2^{df}* (white) background **(A)** Graph shows the proportion of eggs where none of the SAPs contain Asl, some of the SAPs contain Asl or where all of the SAPs contain Asl, as indicated. Note how a higher proportion of *dspd-2$^{z3-5711}$/dspd-2^{df}* eggs contain all or some SAPs that lack Asl. Also note that all the SAPs in all the eggs I included in this quantification were positive for DSas-4 staining. **(B)** Asl fluorescence comparison in SAPs from WT (n=19, black) and *dspd-2$^{z3-5711}$/dspd-2^{df}* (n=11, white) eggs expressing GFP-DSas-6 and GFP-Ana2, but including only the eggs in which all SAPs showed Asl stainning. Note how even in this case the levels of Asl are lower in *dspd-2$^{z3-5711}$/dspd-2^{df}* SAPs than in WT SAPs.

is not apparent as centriole duplication also fails, so no centrosomes are present (Blachon et al., 2008). The composition of centrosomes in *asl*^{1} embryos has not, however, been analysed probably because, as is the case with *dspd-2* mutants, *asl*^{1} embryos fail in pronuclear fusion (Varmark et al., 2007). As *asl*^{1} spermatocytes have morphologically normal centrioles (Bonaccorsi et al., 1998), and *asl*^{1} flies are not uncoordinated, I hypothesised that, as is the case in *dspd-2* mutant eggs, *de novo* centrosomes might be able to form in *asl*^{1} eggs.

I overexpressed Sak in unfertilised eggs in both WT and *asl*^{1}/*asl*^{df} mutant backgrounds. To allow the identification of *de novo* centrosomes I also expressed GFP-Ana2 as a marker of centrosomes. Unexpectedly, however, no *de novo* centrosomes could be detected in *asl*^{1}/*asl*^{df} eggs (Fig.4.5, n=40 eggs). It should be noted, however, that this experiment has only been performed once, so this result needs to be confirmed.

4.2.5 SAPs in an *asl*^{1} background do not recruit any PCM

As Sak appeared unable to drive the formation of *de novo* centrosomes in *asl*^{1}/*asl*^{df} eggs I was interested in knowing if SAPs might be able to form in these eggs. I therefore imaged SAPs formed in *asl*^{1}/*asl*^{df} eggs expressing GFP-DSas6 and GFP-Ana2. As a control I also imaged SAPs in eggs expressing only GFP-Sas6 and GFP-Ana2.

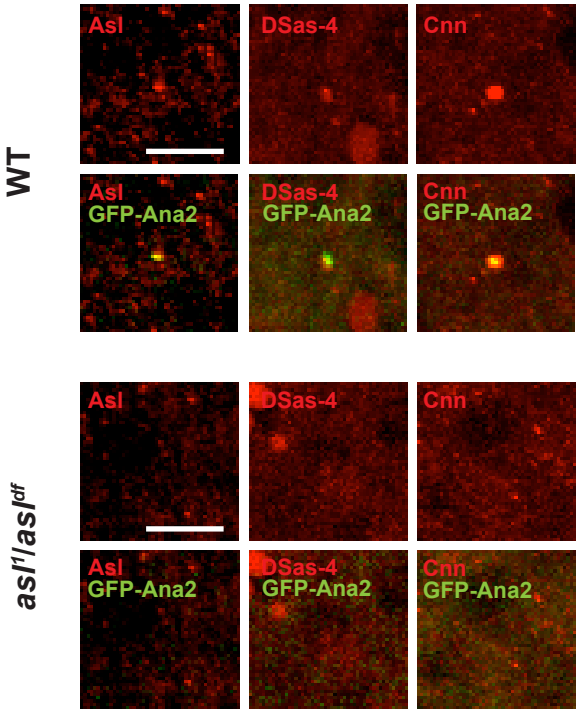


Figure 4.5 *de novo* centrosomes cannot form in an *asl*^{1}/*asl*^{df} background
Representative images of centrosomes formed *de novo* by the overexpression of Sak, in WT (top) and *asl*^{1}/*asl*^{df} (bottom) unfertilised eggs. n>33 eggs. Scale bar=5μm. Note how no *de novo* centrosomes formed in *asl*^{1}/*asl*^{df} eggs.

Unlike *de novo* centrosomes, SAPs were able to form in asl^{1}/asl^{df} eggs. SAPs in asl^{1}/asl^{df} eggs showed very low (virtually undetectable) levels of the PCM components Cnn ($p<0.0001$, $n>14$) and DSpd-2 ($p<0.0001$, $n>14$), when compared with WT SAPs (Fig.4.6A and B). DSas-4 levels, however, were unchanged ($p=0.3163$, $n>14$). This data suggests that Asl is very important in the recruitment of the PCM components Cnn and DSpd-2 in the *Drosophila* embryo. Moreover, it provides direct evidence that Asl is not required to recruit DSas-4 to SAPs and so, by inference, to centrosomes. This result is potentially of great significance as it challenges the previously held view that Asl/Cep152 acts as a scaffold for DSas-4/CPAP assembly (Cizmecioglu et al., 2010; Dzhindzhev et al., 2010).

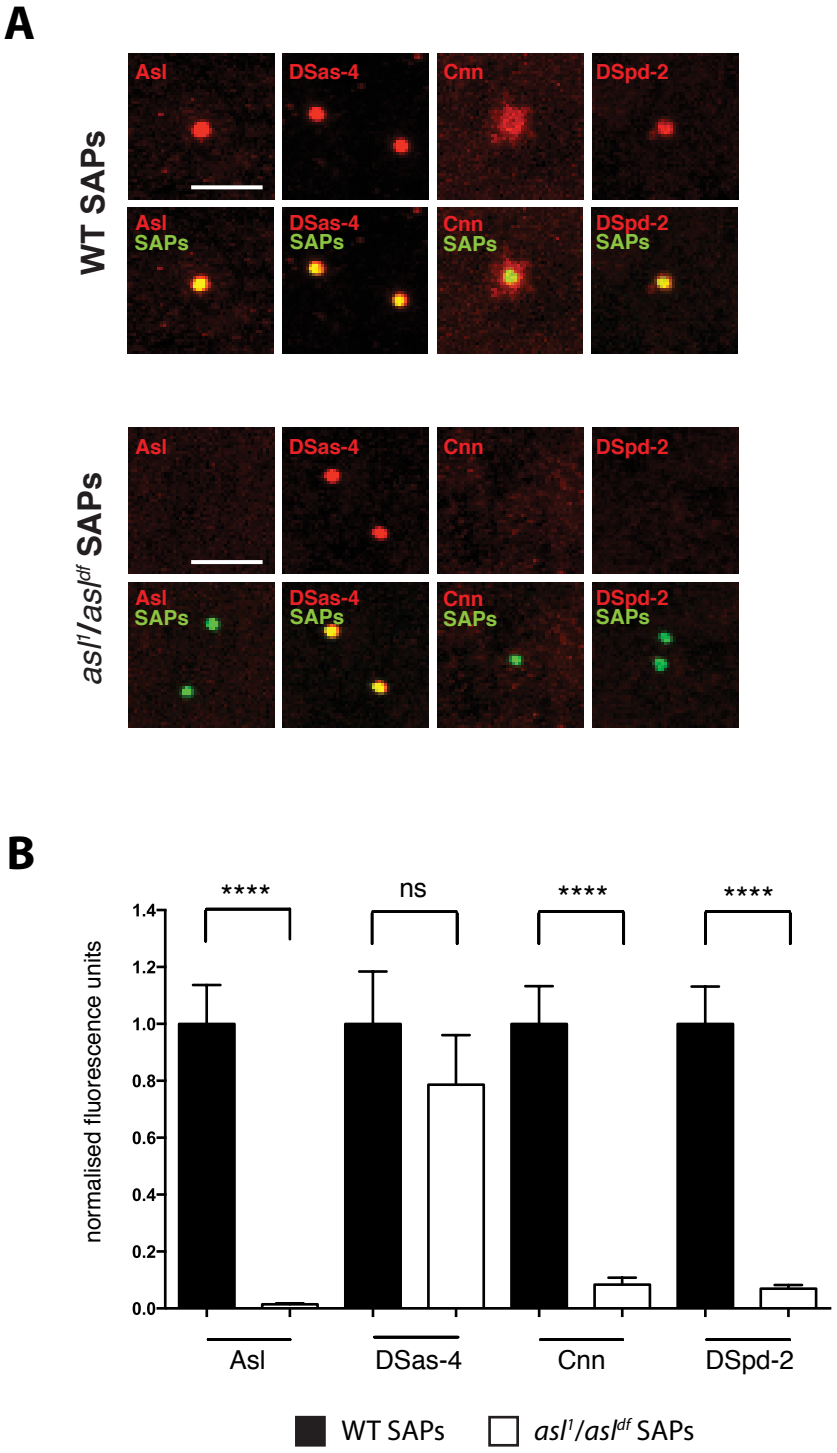


Figure 4.6 SAP composition in *asl*^{1} eggs
Analysis of SAP composition in eggs expressing Ubq-GFP-DSas-6 and Ubq-GFP-Ana2 in either a WT or a *asl*^{1}/*asl*^{df} background. **(A)** Representative images of WT SAPs and *asl*^{1}/*asl*^{df} SAPs stained with antibodies for different centrosomal proteins, as indicated. Scale bar = 5μm. **(B)** Graph shows the quantification of different protein levels in WT (black) and *asl*^{1}/*asl*^{df} (white) SAPs. Note how *asl*^{1}/*asl*^{df} SAPs have normal levels of DSas-4, but very low levels of the PCM components Cnn and DSpd-2. Bars represent the average SAP fluorescence of at least 14 eggs.

4.3 Discussion

Several proteins have been implicated in PCM recruitment in *Drosophila* (see Chapter 1 for detailed description). Of these, three players are thought to be the most important in recruiting the PCM in the *Drosophila* embryo: the PCM components Cnn and Dspd-2 and the centriolar component Asl.

The composition of *cnn* mutant centrosomes in the embryo has been analysed in detail (see below). However, centrosome composition in DSpd-2 mutants and PCM recruitment mutants of Asl have not been characterised in the embryo. This is mainly due to the fact that these mutants fail in pronuclear fusion, arresting very early in embryonic development. To overcome these limitations I examined the composition of SAPs in different PCM mutant backgrounds. SAPs are a good model for the analysis of the pathway of PCM recruitment in embryos because SAPs can form in unfertilised eggs, independently of pronuclear fusion. For comparison purposes I also analysed the composition of *de novo* formed centrosomes in the same PCM mutants. In this section I discuss my results starting with the most inward localised of the proteins analysed (Asl) and then successively following the pathway of PCM recruitment to the most outward protein analysed (Cnn).

4.3.1 Asl is recruited downstream of DSas-4

Asl is a protein that has been implicated both in PCM recruitment and centriole duplication (Bonaccorsi et al., 1998; Giansanti et al., 2001; Varmark et al., 2007; Blachon et al., 2008; Dobbelaere et al., 2008; Dzhindzhev et al., 2010). In this chapter I was interested exclusively in the role of Asl in PCM recruitment, and as such I focused my analysis on the *asl*^{1} mutant allele. *asl*^{1} was initially identified in a screen for mutants affecting male sterility (Bonaccorsi et al., 1998): Asl protein is still produced, but the C-terminal 140 aa are missing (Varmark et al., 2007; Jennifer Richens, personal communication). *asl*^{1} mutants are not uncoordinated, and centriole duplication is not affected (Bonaccorsi et al., 1998; Varmark et al., 2007). However, PCM recruitment defects have been reported in *asl*^{1} spermatocytes where there is a dramatic reduction in the centrosomal levels of Cnn, γ-tubulin and MTs (Bonaccorsi et al., 1998; Varmark et al., 2007). Surprisingly, *asl*^{1} mutants do not appear to have a PCM recruitment phenotype in brain cells (Blachon et al., 2008). I was therefore very interested in determining the *asl*^{1} PCM phenotype in embryos.

As *asl*^{1} mutants fail in pronuclear fusion (Varmark et al., 2007), I analysed the composition of SAPs and *de novo* formed centrosomes in *asl*^{1} mutant unfertilised eggs. Unexpectedly, no *de novo* centrosome formation was observed in *asl*^{1} mutant eggs expressing Sak. This result suggests that, unlike in other tissues, the *asl*^{1} mutant might lead to problems in centriole

formation in the embryo. However, it should be noted that this experiment was only performed once, and hence needs to be repeated.

While *de novo* centrosome formation did not occur in an asl^{1}/asl^{df} background, SAP formation was unaffected. Analysis of SAP composition showed that in these conditions no DSpd-2 or Cnn were recruited, while DSas-4 recruitment was normal. This suggests that, as in spermatocytes, Asl has an important role in PCM recruitment in embryos. These data allow me to position Asl in a putative pathway of PCM recruitment in embryos. Asl must be downstream of the centriolar component DSas-4 (which is recruited to SAPs normally in the absence of Asl), but upstream of PCM components such as DSpd-2 and Cnn (Fig.4.7). This is consistent with previous observations that Asl is localised in the interface between the centriole and the PCM, as shown by immuno-EM (Varmark et al., 2007; Blachon et al., 2008), and more recently by 3D-SIM (Fu and Glover, 2012; Mennella et al., 2012). These results are, however, in disagreement with recent work claiming a direct involvement of DSas-4 in PCM recruitment (Gopalakrishnan et al., 2012). My data suggest that the role of DSas-4 in PCM recruitment is most likely indirect, via its role in recruiting Asl.

As the Asl protein produced from the asl^{1} allele is lacking the C-terminal domain of the protein, we can conclude that this region must be involved in recruiting downstream PCM proteins in spermatocytes and embryos. This is consistent with Y2H data suggesting that only the C-terminal region of Asl interacts with DSpd-2 (Jennifer Richens, personal communication). It is also

interesting to note that in *asl*^{1} eggs no Asl could be detected at SAPs, even though I used an antibody raised against the N-terminal region of the protein. This suggests that, without its C-terminal region, Asl cannot be recruited to SAPs/centrioles, perhaps because this region may be required to interact with DSas-4 (Dzhindzhev et al., 2010). This is unlike the case in brains, where Asl is detectable in *asl*^{1} centrosomes. The different localisation of the Asl^{1} protein, as well as the observation that PCM recruitment is only mildly impaired in *asl*^{1} neuroblasts (Blachon et al., 2008) highlights some potentially interesting tissue specific differences between PCM recruitment in brains and spermatocytes/embryos.

4.3.2 DSpd-2 is recruited downstream of Asl

DSpd-2 was another candidate PCM recruitment factor highlighted in an RNAi screen in S2 cells (Dobbelaere et al., 2008). The extent to which DSpd-2 is involved in PCM recruitment, however, also seems to be tissue dependent. In spermatocyte centrosomes, very reduced Cnn localisation is observed in the absence of DSpd-2, and MT nucleation is severely impaired (Giansanti et al., 2008). In larval brains, however, the absence of DSpd-2 seems to have a milder effect on PCM recruitment. In *dspd-2* neuroblasts, centrosomes can still recruit Cnn, γ-tubulin, Grip91, D-TACC and Dgp71WD, albeit at reduced levels (Dix and Raff, 2007; Giansanti et al., 2008; Paul Conduit, personal communication). As *dspd-2* mutants fail in pronuclear fusion, it had not previously been possible to analyse the requirement for

DSpd-2 in PCM recruitment in the embryo. However, analysis of the dynamic behaviour of DSpd-2 in the embryo suggests that it may be an important player. When FRAP experiments were performed in a variety of PCM recruitment candidates, DSpd-2 was the only component besides Cnn that showed a characteristic in-to-out dynamic recovery behaviour (Paul Conduit, personal communication). This suggests that DSpd-2 and Cnn might both be involved in setting the structure of the PCM.

I addressed this question by analysing the composition of SAPs and *de novo* formed centrosomes in *dspd-2* unfertilised eggs. In both cases a very strong reduction in the levels of Cnn and γ-tubulin was observed, suggesting that DSpd-2 is a very important PCM recruitment factor in the embryo. In addition, this observation provided strong genetic support for the idea (proposed on the basis of antibody injection experiments) that DSpd-2 is involved in the recruitment of Cnn to centrosomes (Conduit et al., 2010). This interaction may be direct as DSpd-2 can interact with Cnn in Y2H experiments (Jennifer Richens, personal communication). Second, when GFP-Cnn is bleached in embryos, its initial recovery profile matches the distribution of DSpd-2, before it eventually moves outwards (Paul Conduit, personal communication). This behaviour suggests that DSpd-2 might provide an important initial binding site for Cnn.

In the absence of DSpd-2, centriolar components such as Asl and DSas-4 are still present in both SAPs and *de novo* centrosomes. This places DSpd-2 downstream of these proteins in my putative pathway of PCM recruitment

in the embryo. Two observations support the hypothesis that Asl and DSpd-2 might interact directly. First, Y2H data shows that DSpd-2 can interact with the C-terminal region of Asl. This is consistent with my observation that no recruitment of DSpd-2 is observed in *asl*^{1} mutant SAPs or *de novo* centrosomes. Second, when GFP-DSpd-2 is bleached in embryos, its initial recovery profile matches the distribution of Asl, before eventually moving outwards (Paul Conduit, personal communication). This behaviour suggests that Asl might provide the initial binding site for DSpd-2.

While my data in Chapter 3 strongly suggested that SAPs are an excellent system for studying PCM recruitment, my analysis of *dspd-2* mutants has highlighted a striking difference between SAPs and *de novo* formed centrosomes. In the absence of DSpd-2, the levels of centriolar DSas-4 and Asl both increase in *de novo* formed centrioles (Fig.4.2), but remain largely unchanged or decrease, respectively, in SAPs (Fig.4.3). The reason for this discrepancy is unclear, but there are several plausible explanations. My favoured hypothesis is that the centriolar fractions of DSas-4 and DSpd-2 both compete for limited binding sites at the centriole (DSas-6 or Ana2 would be excellent candidates for such binding sites, and DSas-4 is known to bind to Ana2- Tang et al*.,* 2011). In the absence of DSpd-2, more DSas-4 is recruited to centrioles and this can then recruit more Asl. In SAPs, the binding sites for DSas-4 and DSpd-2 are not limiting (as the SAPs are largely composed of extended tubules of DSas-6 and Ana2). Thus, in the absence of DSpd-2 there is no extra recruitment of DSas-4 and so no extra recruitment of Asl.

This model does not explain, however, why Asl levels decrease in SAPs that lack DSpd-2. One possible explanation comes from the observation that there are two fractions of Asl at the centrosome: a fraction that does not exchange (and that seems to be recruited by DSas-4) and a fraction that does exchange and that seems to be recruited by the non-exchange fraction of Asl (Zsofi Novak, unpublished data). There is some evidence that the exchange fraction of Asl is dependent on DSpd-2 and Cnn for its proper maintenance at centrosomes. Thus, a lack of DSpd-2 and Cnn in the SAPs may destabilise the exchange fraction of Asl, and this loss of Asl is not compensated for by extra recruitment of the non-exchange fraction of Asl. In centrioles, the destabilisation of the exchange-fraction of Asl in the absence of DSpd-2 is compensated for by the extra recruitment of the non-exchange fraction of Asl. Clearly more work is required to understand these complex behaviours. Nevertheless, I believe these explanations are plausible and that the differences between SAPs and *de novo* centrioles that I identify here do not negate the use of SAPs as a model system for studying PCM assembly.

4.3.3 Cnn is required for the maintenance, but not recruitment, of DSpd-2

Cnn has long been known to be an important player in PCM recruitment. Cnn's role in PCM recruitment was initially studied in the embryo, where *cnn* mutants were observed to have defective mitotic spindles, eventually

leading to a failure in embryo cellularisation. Cnn was shown to be important for the proper recruitment of PCM components like γ-tubulin, CP190, CP60 (Megraw et al., 1999; Vaizel-Ohayon and Schejter, 1999), and to a lesser extent Aurora A, D-TACC, Dgp71WD (Lucas and Raff, 2007) and DSpd-2 (Paul Conduit, personal communication). SAPs in *cnn* mutant eggs showed results consistent with this: reduced levels of γ-tubulin were observed, and MT asters could form, but were smaller than those formed by WT SAPs. As was the case in centrosomes, SAPs lacking Cnn still contained DSpd-2. This is consistent with DSpd-2 being localised upstream of Cnn in the pathway of PCM recruitment in the embryo, (discussed above), and with the observation that in *cnn* centrosomes the DSpd-2 rate of incorporation is the same as in WT centrosomes (Paul Conduit, personal communication). However, the levels of DSpd-2 were slightly reduced in *cnn* centrosomes and in SAPs. This suggests a role for Cnn in maintaining DSpd-2 at the centrosome.

The reduction in the levels of γ-tubulin and MT nucleation ability in the absence of Cnn might be simply a consequence of this small reduction in the levels of DSpd-2. However, this result most likely reflects the ability of Cnn to recruit PCM components directly. Cnn has been shown to have a particular region in its N-terminal half, Centrosomin motif 1 (CM1), which is known to be required for the recruitment of PCM components such as γ-tubulin and D-TACC (Zhang and Megraw, 2007). Data from brains also suggests that Cnn has the ability to recruit PCM components independently of DSpd-2. In the absence of DSpd-2 or Cnn individually, several PCM

components are still recruited to significant levels to centrosomes. Only in double mutant brains is PCM recruitment completely abolished to interphase levels (Paul Conduit personal communication). However, as DSpd-2 is involved in both recruiting Cnn and other PCM components in the embryo, DSpd-2 is probably a more dominant PCM recruitment factor in the embryo than Cnn.

4.3.4 The pathway of PCM recruitment in the embryo

Figure 4.7 summarises the pathway of PCM recruitment in *Drosophila* embryos based on the results presented in this chapter. In this pathway, Asl is downstream of centriolar components such as DSas-4. Asl is the most upstream component in the recruitment of the PCM, and is involved in recruiting DSpd-2 into the PCM. In the absence of Cnn, DSpd-2 can still be recruited, and hence we can safely place Cnn downstream of DSpd-2. However, Cnn recruitment feedbacks on DSpd-2 recruitment, probably because it is required to properly maintain DSpd-2 in the PCM. Both DSpd-2 and Cnn are most likely involved in the recruitment of PCM components and subsequent MT nucleation.

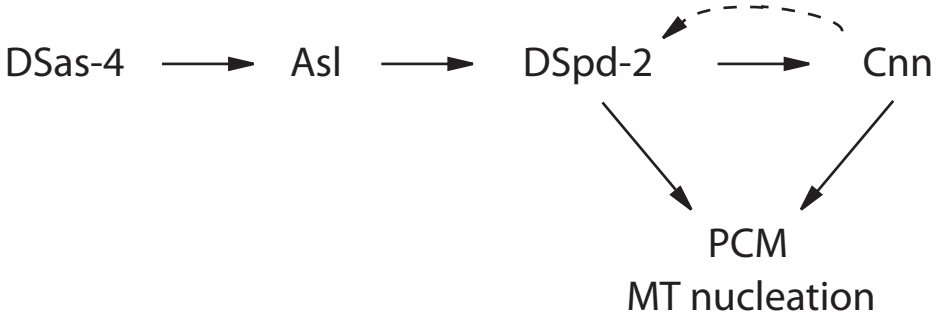


Figure 4.7 Model of the pathway of PCM recruitment in *Drosophila* embryos.

Chapter 5: Using SAPs to elucidate the pathway of centriole assembly

5.1 Summary

In the previous chapter I used SAPs and *de novo* centrosomes to analyse the pathway of PCM assembly in embryos. I had to use these models instead of template-formed centrosomes due to the fact that many of the important mutants I wanted to analyse failed in pronuclear fusion. Those experiments did not address, however, how the centriole assembles. Five proteins have been identified as being essential for centriole duplication in *Drosophila*: Ana2, Asl, DSas-4, DSas-6 and Sak (Bettencourt-Dias et al., 2005; Basto et al., 2006; Peel et al., 2007; Rodrigues-Martins et al., 2007b; Basto et al., 2008; Blachon et al., 2008; Dobbelaere et al., 2008; Dzhindzhev et al., 2010; Stevens et al., 2010a). Although some published work provides clues as to the position of these proteins in the pathway of centriole assembly—most notably in worms (Delattre et al., 2006; Pelletier et al., 2006)- a detailed genetic analysis in most systems is lacking. This is largely because mutations in the genes encoding these proteins block centriole assembly and so mutant cells have no centrioles that can be analysed.

SAPs in eggs do not have fully formed centrioles at their core, as shown by EM (Fig.3.1). It is not clear how their formation is nucleated, but is likely that DSas-6 and Ana2 form a platform on which other components can then bind, forming structures that are ultimately very similar to centrosomes in composition and behaviour. As this structure is not as well organised as a centriole, I reasoned that it might be able to form even in the absence of components that are essential for the assembly of *bona fide* centrioles. If this is the case, then SAPs could be an invaluable tool with which to study the pathway of centriole assembly.

In this chapter, I analyse the pathway of centriole formation using SAPs. I first develop a methodology that allows me to analyse the composition of SAPs in the absence of essential centriolar proteins. I also show that it is very hard to analyse *de novo* formed centrosomes in the absence of essential centriolar components using this methodology. I then apply this method to mutants of *asl*, *dsas-4* and *sak*. Based on these results I propose a pathway for centriole formation in the *Drosophila* embryo.

5.2 Results

5.2.1 Developing an *in vitro* activation protocol to analyse SAP composition in the absence of essential centriole components

Mutant flies for important players in centriole formation are morphologically normal and are able to eclose from pupae (Basto et al., 2006; Bettencourt-Dias and Glover, 2007; Peel et al., 2007). However, they lack functional cilia. They are therefore uncoordinated and usually die shortly after eclosion as they cannot feed nor move in a coordinated fashion and quickly become stuck in the food. By keeping these flies in dry conditions and manually feeding them, it is possible to keep them alive for several days, although they are unable to mate or to lay eggs. This therefore presents a major technical barrier to analysing the composition of SAPs in centriolar mutants: SAPs have so far only been analysed in laid eggs, and these mutants, even if kept alive and fed by hand, cannot lay eggs.

The transition from a mature stage 14 oocyte to an unfertilised egg occurs upon egg activation. Activation is the process by which the oocyte is primed for embryonic development, and involves a variety of morphological and biochemical changes (described in Chapter 6). In *Drosophila,* activation occurs independently of fertilisation, and stage 14 oocytes are activated simply by the physical laying of the oocyte. The mechanical stimulation of egg laying allows calcium entrance, which triggers a variety of signalling

pathways leading to egg activation (Horner and Wolfner, 2008b). While this is the normal process of oocyte activation, recent work has shown that the transition from oocyte to egg can be mimicked, albeit less efficiently, by incubation of stage 14 oocytes in a calcium-containing activation buffer (Page and Orr-Weaver, 1997).

Oogenesis is unaffected in *dsas-4* mutant flies (Stevens et al., 2007) and I found that this was also the case in all the other centriole-duplication mutants (data not shown). However, SAPs in oocytes do not seem to behave as centrosomes, as they are not able to recruit PCM components or nucleate MTs (see Chapter 6). I wondered therefore whether analysis of SAPs in centriolar mutant eggs might be possible by the *in vitro* activation of mutant stage 14 oocytes. I made activation buffer as described in Horner and Wolfner, 2008a. To test activation efficiency I incubated the "activated" oocytes in bleach. This is an easy and accurate measure of activation efficiency because activation leads to the cross-linking of the vitelline membrane, making laid eggs (and successfully activated oocytes) resistant to bleach; non-activated oocytes are quickly destroyed (LeMosy and Hashimoto, 2000; Heifetz et al., 2001). In my hands no WT stage 14 oocytes survived in bleach, while typically ~98% of laid eggs and ~74% of activated oocytes survived bleach treatment (Fig.5.1A). This is very similar to the ~70% survival rate for activated oocytes described by Horner and Wolfner. Hereafter, I refer to these activated oocytes as activated eggs, to distinguish them from laid eggs.

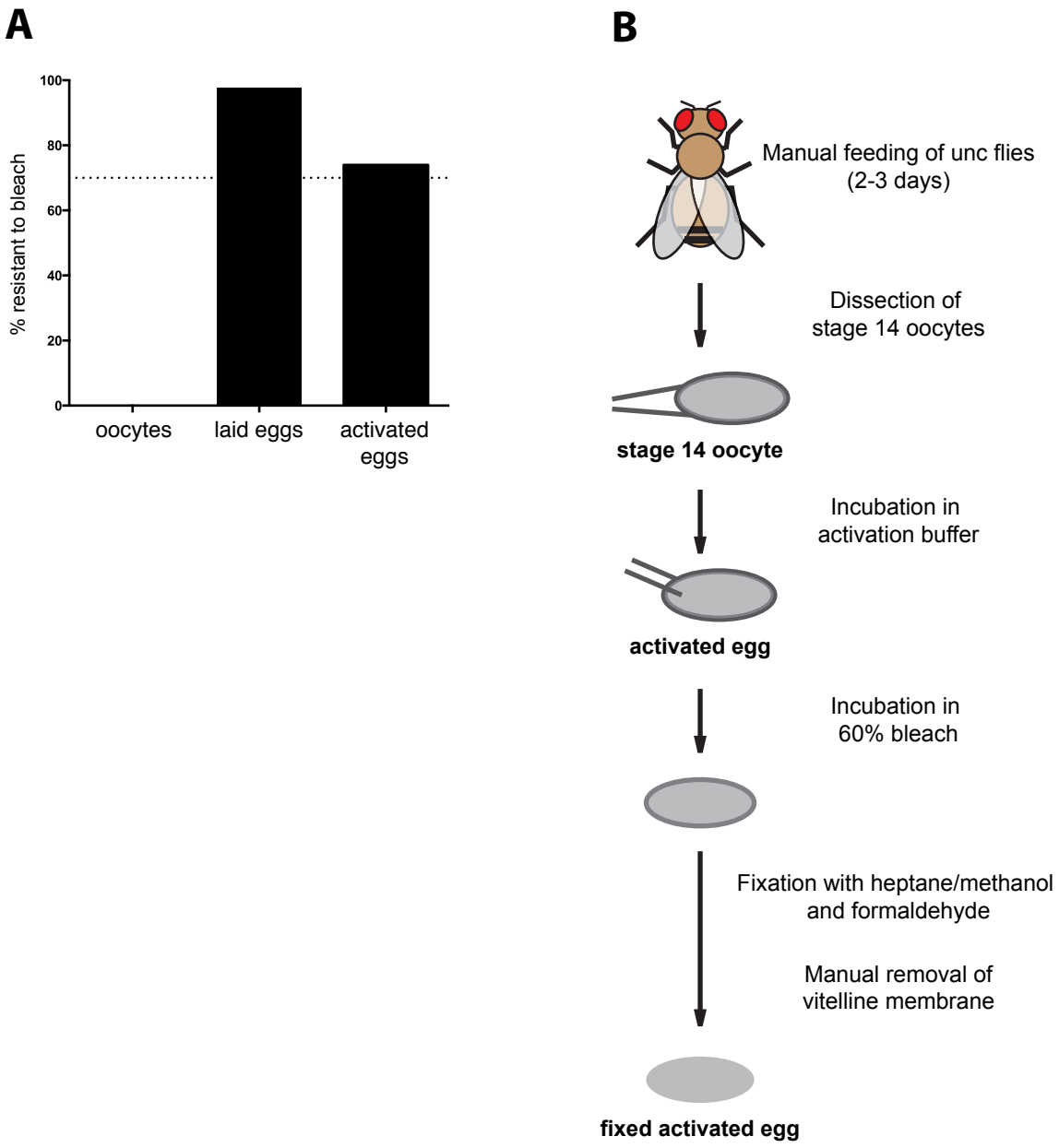


Figure 5.1 Developing an *in vitro* activation protocol for SAP analysis
(A) Percentage of eggs/oocytes remaining after incubation in 60% bleach. Note how no oocytes (0%, n=23) can survive in bleach, while the vast majority of laid eggs are resistant to bleach (98%, n=43). The majority of activated eggs are resistant to bleach (74%, n=68) which is close to the previously reported efficiency (dotted line). **(B)** Protocol developed for the *in vitro* activation and fixation of stage 14 oocytes from uncoordinated flies.

To test whether these activated eggs were able to form SAPs that behaved like centrosomes I activated *in vitro* stage 14 oocytes that expressed GFP-DSas-6 and GFP-Ana2, as well as the MT-binding protein Jupiter-mCherry. Approximately 95% (18/19) of these activated eggs had SAPs that could organise MT asters, confirming that SAPs can successfully form in eggs activated *in vitro*. As ~74% of stage 14 oocytes incubated in activation buffer for 20 min are successfully activated, and ~94% of these show functional SAPs, I conclude that ~70% of stage 14 oocytes activated *in vitro* can form functional SAPs.

Having shown proof-of-principle that functional SAPs can be formed in eggs activated *in vitro,* I developed a protocol that would allow me to analyse the pathway of centriole formation in a reliable manner. A schematic of this protocol is shown in Fig.5.1B. Briefly, pupae of the desired genotype are collected and placed in petri dishes, so that uncoordinated flies do not get stuck in their food upon eclosion. These flies are then manually fed for 2-3 days (see Chapter 2). This allows some oocytes to mature to the last stage of oogenesis (stage 14). Stage 14 oocytes are then dissected in activation buffer, and incubated in this buffer for 1 hour (with activation buffer changed every 5 min). As activation efficiency was shown to increase with incubation time (Horner and Wolfner, 2008a), I chose this long incubation period to maximise activation efficiency. I then placed activated eggs in 60% bleach. This performs two functions: first, it destroys any eggs that have not been completely activated; second, it removes the chorion, allowing the activated

eggs to be analysed by microscopy (or to be fixed prior to staining and further analysis).

Ultimately, I wanted to stain SAPs in different centriolar mutants with antibodies for different centriolar and centrosomal proteins. I therefore experimented with several methods of fixation. I found that incubation in a heptane/methanol interphase followed by incubation in 4% formaldehyde to be most successful. The heptane/methanol fixation allowed MTs to be preserved, while the post-fixation formaldehyde incubation was required to harden the activated eggs, allowing the subsequent manual removal of the vitelline membrane. This protocol is described in detail in Chapter 2.

Having successfully developed this protocol, I was interested in knowing whether SAPs formed in activated eggs were similar to SAPs formed in laid eggs. Having used SAPs in laid eggs for analysis of the pathway of PCM recruitment, I therefore compared the PCM composition of SAPs formed in activated eggs and SAPs formed in laid eggs. For this analysis I obtained fixed activated eggs as described in Fig.5.1B. For comparison purposes I obtained laid eggs and exposed them to same bleach incubation and fixation protocol. I then stained both types of eggs with antibodies for Cnn and DSpd-2 (Fig.5.2A and B). I found that the levels of Cnn and DSpd-2 were not significantly different between SAPs in laid eggs and SAPs in activated eggs ($p=0.3068$ for Cnn, Mann-Whitney test, $p=0.7576$ for DSpd-2, $n>12$, t-test). Thus, SAPs in activated eggs appear to be very similar to

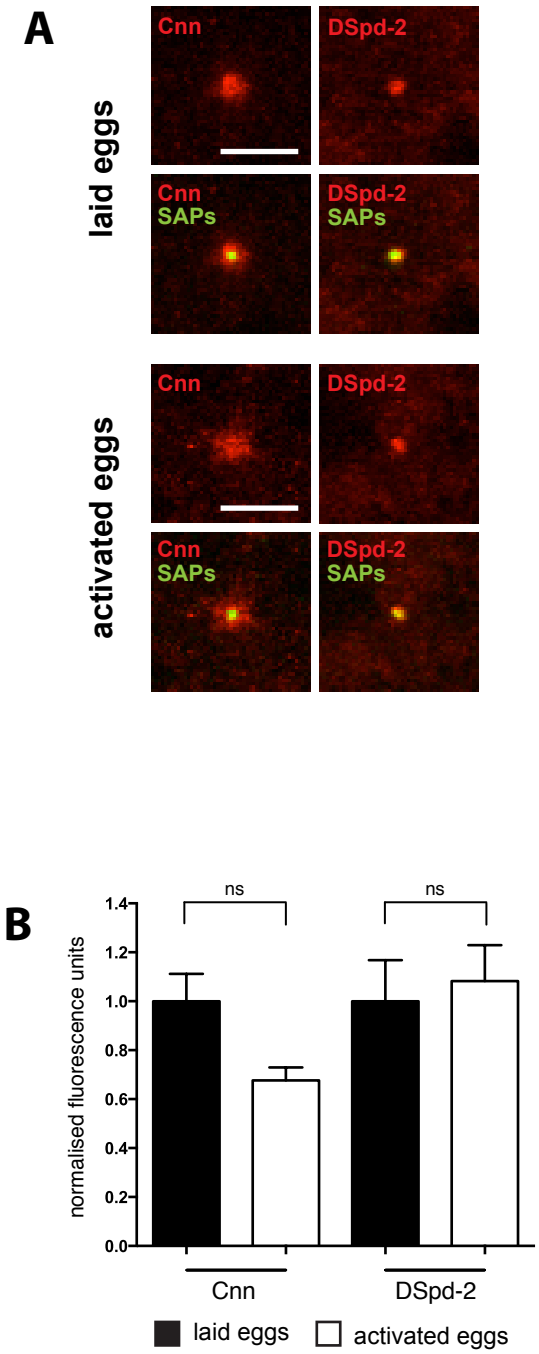


Figure 5.2 SAPs formed in activated eggs are similar to those formed in laid eggs
(A) Representative images of SAPs formed in laid eggs and in activated eggs, stained with antibodies for the proteins indicated. **(B)** Graph shows the quantification of PCM protein levels in SAPs formed in laid (black) and activated (white) eggs. There is no significant difference between the two groups. Scale bar=5μm.

those formed in laid eggs, and so can be used to study the pathway of centriole formation.

5.2.2 The *in vitro* activation protocol cannot be applied to analyse *de novo* centrosome formation in the absence of essential centriole components

In Chapter 4 I used centrosomes formed *de novo* by the overexpression of UAS-Sak in unfertilised eggs to analyse the pathway of PCM recruitment. As such I was able to compare the composition of SAPs and of *de novo* formed centrosomes in different PCM recruitment mutants. I therefore addressed the feasibility of forming *de novo* centrosomes in activated eggs. I first analysed whether *de novo* formed centrosomes formed in laid eggs with the same efficiency as previously reported (Rodrigues-Martins et al., 2007b). I made timed collections of laid eggs expressing UAS-Sak under the V32a promoter, and quantified the percentage of eggs that showed no, a few or many *de novo* centrosomes (Fig.5.3A). The results obtained resembled those published (Rodrigues-Martins et al., 2007b), with the vast majority of laid eggs only showing many *de novo* formed centrosomes after 3-4 hour collections.

I then examined how well *de novo* centrosomes formed in activated eggs. As shown in Fig.5.3A, and as previously reported (Rodrigues-Martins et al., 2007b), the number of *de novo* centrosomes increases over time in laid

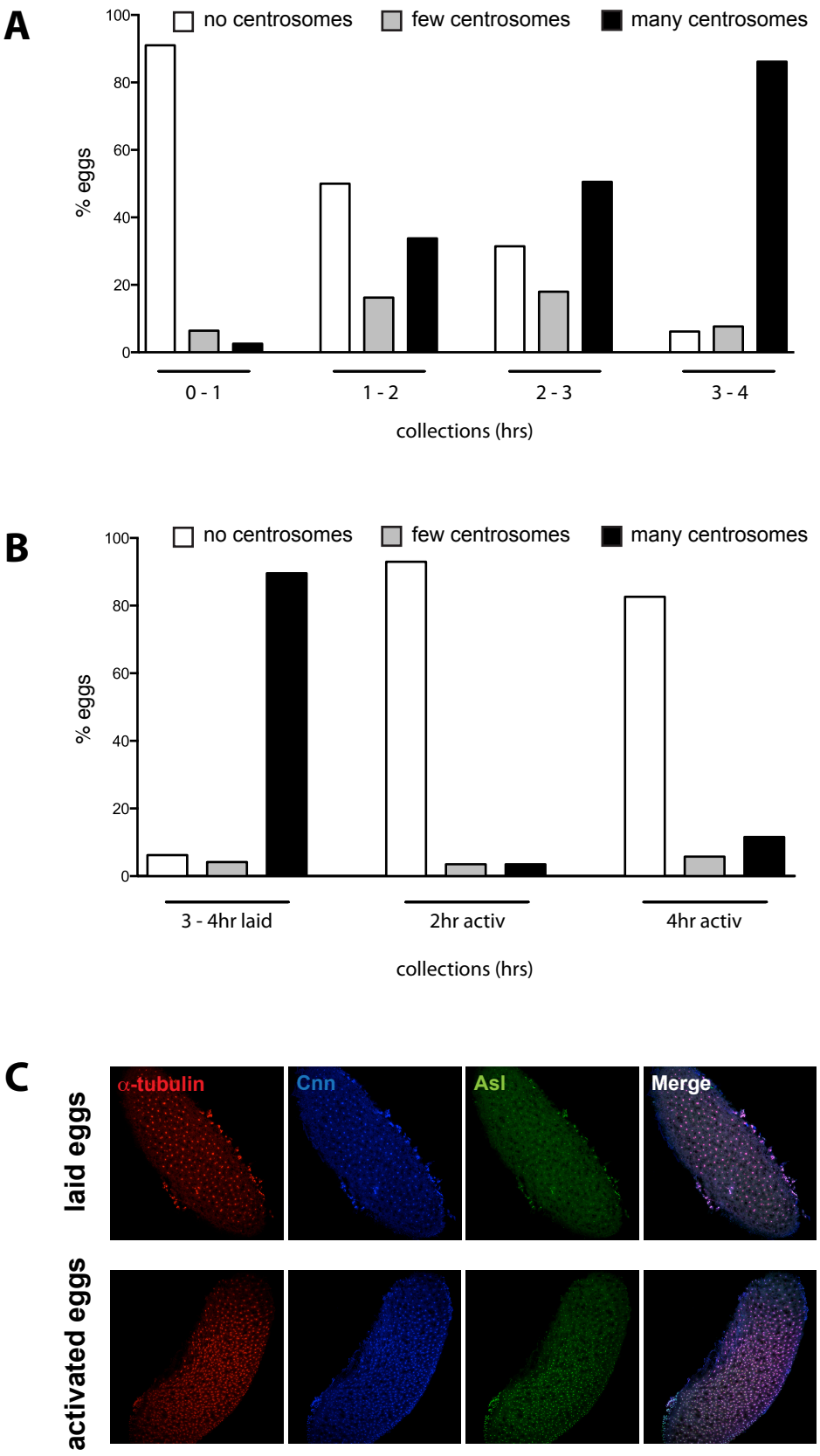


Figure 5.3 *de novo* centrosome formation in *in vitro* activated eggs
(A and B) Graphs show the percentage of eggs containing no (white), few (grey) and many (black) *de novo* centrosomes formed by the overexpression of Sak, collected for the time periods indicated (n>48 eggs for each category), from (A) laid and (B) laid or activated eggs as indicated. **(C)** Examples of laid (top) and activated (bottom) eggs overexpressing Sak stained with antibodies for the proteins indicated. Scale bar=5μm.

eggs. In fact, in 0-1 hour collections the number of eggs with *de novo* centrosomes is very low (9%). This led to the suggestion that a small number of centrosomes form *de novo* in eggs, and these *de novo* centrosomes then duplicate canonically, eventually filling most of the egg (Rodrigues-Martins et al*.,* 2007b). This is in contrast with SAPs, where the majority of laid eggs form SAPs after only a few minutes (e.g. 0-30min collection shows nearly 90% of eggs with SAPs, see Chapter 6). Anticipating this slower kinetics, I incubated stage 14 oocytes expressing UAS-Sak for 2 or 4 hours in activation buffer (with buffer changes every 15 minutes), as opposed to the 1 hour incubation performed with SAPs. Otherwise, the protocol of bleaching and fixation was the same as used with SAPs. I then stained the eggs for different centrosomal components (Fig.5.3C). After a 2 hour incubation in activation buffer, only ~8% of the eggs showed *de novo* centrosomes (Fig.5.3B). Incubation for 4 hours showed only a mild improvement, with ~18% of the activated eggs showing *de novo* centrosomes. As ~74% of oocytes are successfully activated, we can conclude that ~13% of stage 14 oocytes processed *in vitro* can form *de novo* centrosomes.

As I started to use my activation protocol to analyse SAP composition in centriole assembly mutants (see below) I realised that to complete the composition analysis of one mutant, at least two slides of activated eggs stained with different antibodies are required. To account for variability and allow statistical comparison between mutant and wild type activated eggs, each slide must contain between 40 to 50 activated eggs (meaning 60 to 70

stage 14 oocytes must be dissected from uncoordinated females). On average, 0.8 oocytes of the appropriate stage can be obtained from each uncoordinated female that I fed manually (my unpublished data). Thus, to complete a statistically significant analysis of one centriolar mutant, ~170 uncoordinated females must be manually fed for several days. In practice, this procedure meant that it took me around 2 months to generate enough material to analyse SAP formation in any single centriole duplication mutant. This time scale applies to SAPs, where ~70% of stage 14 oocytes activated *in vitro* can be used. I realised that to generate enough material to analyse *de novo* centriole formation in any one of these mutants (where only ~13% of stage 14 oocytes activated *in vitro* can form *de novo* centrosomes) would take over 5 months. I concluded, therefore, that the analysis of *de novo* centriole formation using this *in vitro* activation protocol was not feasible in the time scale of my thesis.

In conclusion, I developed a protocol that allows the analysis of SAPs in the absence of essential centriolar proteins by the *in vitro* activation of stage 14 oocytes. This protocol is inefficient, and so cannot be used to analyse the pathway of *de novo* centriole formation. I can therefore use only SAPs to analyse the pathway of centriole formation with this assay.

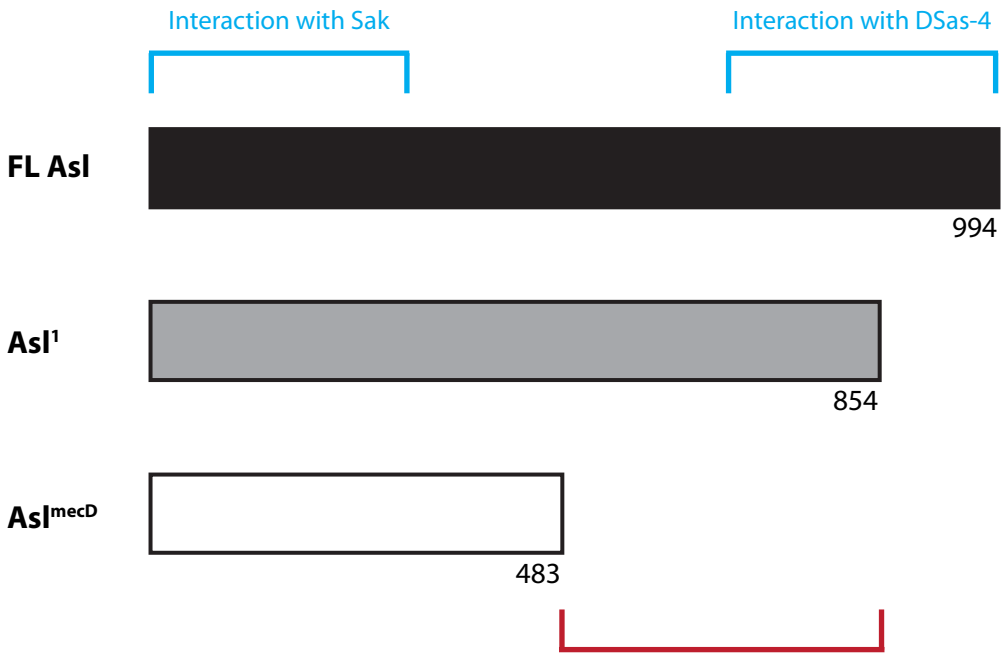


Figure 5.4 A schematic representation of different Asl proteins
A schematic illustration of the different Asl proteins produced from different *asl* alleles analysed in this thesis. The number of amino acids in each protein is shown underneath. Red box indicates the region of Asl that is potentially involved in inhibiting the alternative pathway of PCM recruitment. Regions in blue correspond to the regions of Asl that are throught to interact with Sak and DSas-4, as indicated. FL- full length. Adapted from Dzhindzhev et al., 2010.

5.2.3 *asl*^{mecD} mutant SAPs can still occasionally recruit Cnn

In Chapter 4 I analysed the composition of SAPs (and *de novo* centrosomes) in an *asl*^{1} background. As mentioned previously, the *asl*^{1} allele is a hypomorph, as Asl protein is still produced though lacking part of its C-terminus (Fig.5.4, Blachon et al., 2008). *asl*^{1} flies are not uncoordinated and still have centrioles, and therefore *asl*^{1}, at least to some extent, allows the analysis of Asl's role in PCM recruitment separately from its role in centriole duplication. However, stronger alleles of *asl* have been identified, which do interfere with centriole duplication. The strongest published allele is thought to be *asl*^{mecD}, identified in a screen for mechanosensory mutants (Blachon et al., 2008). This allele has a nonsense mutation in position 1718, and produces a very small (but detectable) amount of Asl that lacks essentially the C-terminal half of the protein (Blachon et al., 2008, Fig.5.4). Centriole duplication fails in these flies, and as a consequence they lack centrosomes and functional cilia.

The exact position of Asl in the pathway of centriole formation in the *Drosophila* embryo has not yet been precisely determined and remains a controversial point (section 1.2.1, Chapter 1). This is due to two factors. First, because as *asl*^{mecD} flies lack cilia they are uncoordinated and cannot lay eggs. Second, as Asl is an important centriole duplication factor, even if fertilisation and egg laying was possible, it is unlikely that the paternal centriole would be able to duplicate in the absence of Asl.

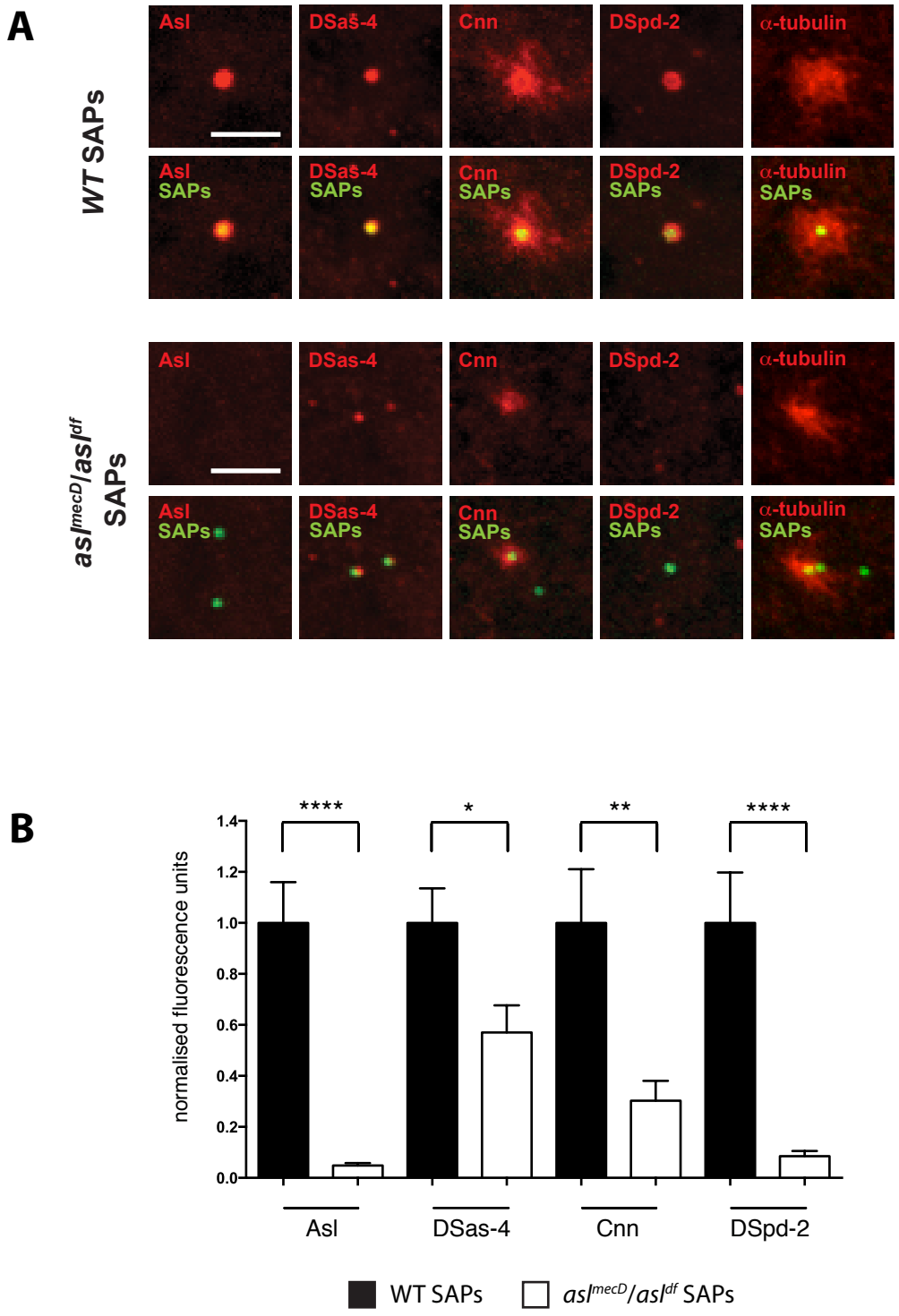


Figure 5.5 SAP composition in *asl*^{mecD} activated eggs

Analysis of SAP composition in *in vitro* activated eggs expressing Ubq-GFP-DSas-6 and Ubq-GFP-Ana2 in either a WT or a *asl*^{mecD}/*asl*^{df} background. **(A)** Representative images of WT SAPs and *asl*^{mecD}/*asl*^{df} SAPs stained with antibodies for different centrosomal proteins, as indicated. Scale bar=5μm. **(B)** Graph shows the quantification of different protein levels in WT (black) and *asl*^{mecD}/*asl*^{df} (white) SAPs. Note how *asl*^{mecD}/*asl*^{df} SAPs have reduced levels of DSas-4 and Cnn, and very low levels of DSpd-2. Bars represent the average SAP fluorescence of at least 26 eggs.

As SAPs do not have centrioles at their core, I asked whether SAPs might be able to form in an *asl*^{mecD} background. I therefore expressed GFP-DSas-6 and GFP-Ana2 in *asl*^{mecD}/*asl*^{df} activated eggs. For comparison purposes, I also looked at SAPs formed in a WT background. Excitingly, and unlike centrosomes, SAPs could still form in an *asl*^{mecD} background confirming my hypothesis that SAPs can be used as models to study the pathway of centriole formation (Fig.5.5A). *asl*^{mecD} SAPs still had DSas-4, albeit at lower levels ($p=0.0151$, $n>26$ eggs). DSpd-2 levels, however, were very strongly reduced, almost down to undetectable levels ($p<0.0001$, $n>26$). This is similar to the result obtained in an *asl*^{1} background, where DSas-4 could still be detected at SAPs, whereas DSpd-2 was not detected (section 4.2.5, Chapter 4).

SAPs formed in an *asl*^{1} background were also characterised by the absence of any Cnn recruitment. Surprisingly, a more complex scenario was observed when considering Cnn recruitment in *asl*^{mecD} SAPs. When simply considering the average SAP fluorescence per egg (Fig.5.5B), a strong reduction in Cnn levels was observed ($p=0.0054$, $n>26$). However, and as shown in Fig.5.5A, careful examination revealed that not all SAPs had low levels of Cnn, but rather that some *asl*^{mecD} SAPs showed Cnn recruitment, while others did not. I decided to examine whether a mix of SAPs recruiting and not recruiting Cnn could be observed in all *asl*^{mecD} activated eggs expressing GFP-DSas-6 and GFP-Ana2. As shown in Figure 5.6A, the majority of *asl*^{mecD} eggs (60%) had no SAPs that recruited Cnn. Approximately 40% of *asl*^{mecD} eggs had some SAPs that could and some

SAPs that could not recruit Cnn. No *asl*^{mecD} eggs were found in which all the SAPs could recruit Cnn. It seems therefore that Cnn recruitment in *asl*^{mecD} SAPs only occurs occasionally. I also noticed that in *asl*^{mecD} eggs, the SAPs that can recruit Cnn also are the ones that can nucleate MT asters (Fig.5.5A), suggesting that when Cnn recruitment occurs, it does so at functionally significant levels. In addition, I noticed that those SAPs that can recruit Cnn and nucleate MTs tend to localise to the surface of the eggs (Fig.5.6B). This suggests that, as hypothesised, the movement to the surface and subsequent clustering of SAPs (Chapter 3) most likely relies on the ability of SAPs to nucleate MTs.

38% of WT eggs also showed SAPs that did not recruit any Cnn (Fig.5.6A). This is not totally unexpected. In order not to bias my analysis, I imaged all eggs that showed GFP signal, without considering the signal in other channels. As a consequence, these 38% most likely correspond to eggs that could survive the bleach test but were not successfully activated. A proportion of these eggs may also correspond to aggregates of only GFP-DSAs-6 or only GFP-Ana2, as they have also been observed in laid eggs.

My analysis of the pathway of PCM recruitment (Chapter 4) suggested that DSpd-2 is the main recruiter of Cnn to the SAPs (and hence centrosomes) in the embryo. I therefore wondered whether some DSpd-2 was still present in the *asl*^{mecD} SAPs that organised Cnn. My analysis of average (per egg) DSpd-2 levels in *asl*^{mecD} SAPs suggested that DSpd-2 levels are very low (Fig.5.5B)- indeed, the reduction in levels when compared with WT SAPs

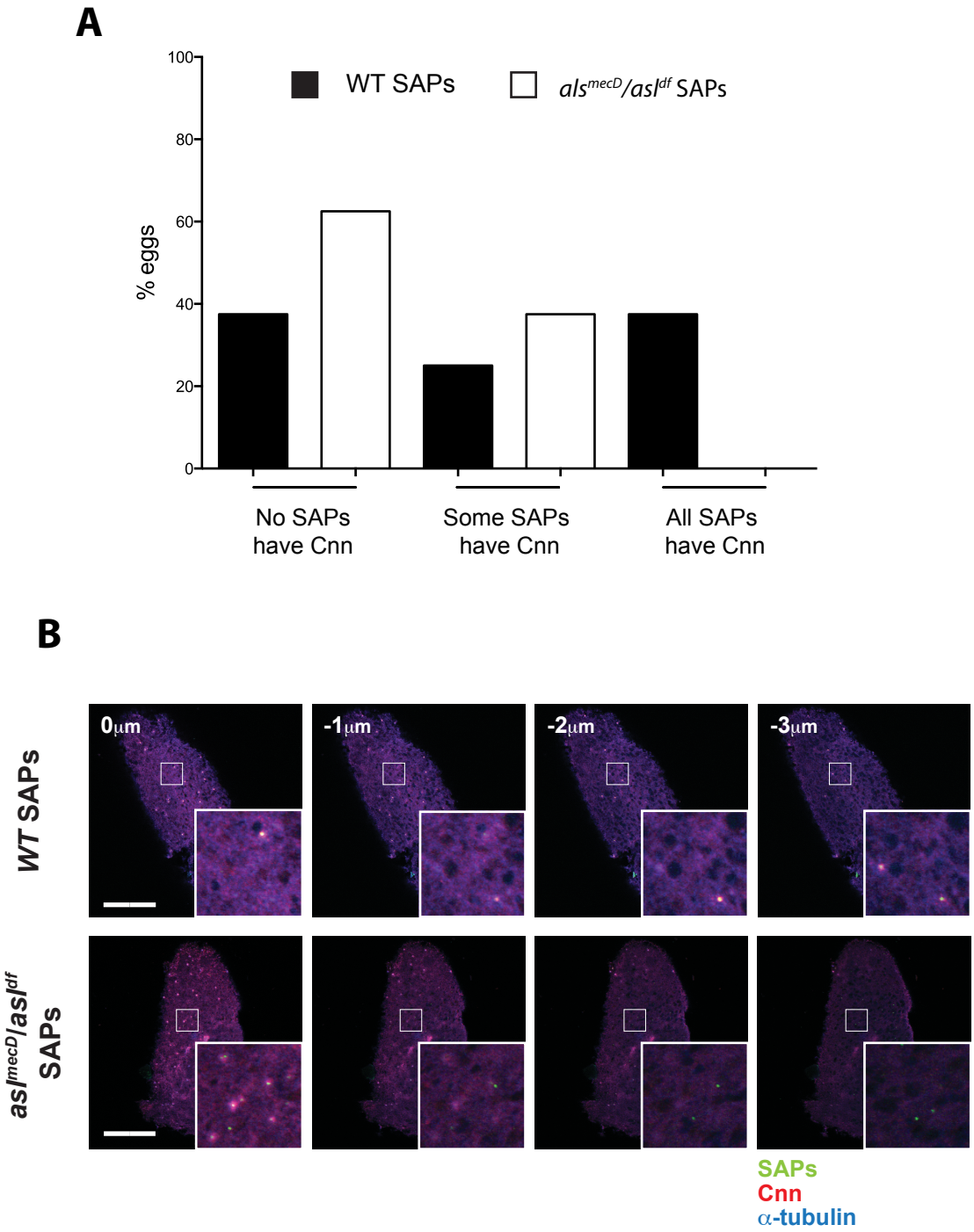


Figure 5.6 Proportions and distribution of *asl*^{mecD} SAPs that recruit Cnn.
(A) Graph shows the proportion of *in vitro* activated eggs expressing GFP-DSas-6 and GFP-Ana2 in a WT (black) or *asl*^{mecD}/*asl*^{df} background (white) with no SAPs stainning for Cnn, some SAPs stainning for Cnn and all SAPs staining for Cnn, as indicated (n>26). Note how 40% of *asl*^{mecD}/*asl*^{df} eggs had some SAPs recruiting Cnn, while none had all SAPs recruiting Cnn. **(B)** Distribution of SAPs in eggs expressing GFP-DSas-6 and GFP-Ana2 in a WT (top) or *asl*^{mecD}/*asl*^{df} (bottom) background, stained for the proteins indicated. Note how the SAPs that recruit Cnn in *asl*^{mecD}/*asl*^{df} eggs tend to localise at the surface of the eggs. Depth in the egg relative to the first figure of the panel is shown in the top left corner. A magnified view of the regions within small boxes of each figure are shown in the bottom right corner. Scale bar=100μm.

was very similar to that observed for Asl itself. However, on close examination it was sometimes possible to observe extremely low levels of DSpd-2 at the *asl*^{mecD} SAPs that could still recruit Cnn (Fig.5.7A). Perhaps more convincingly, I found that DSpd-2 levels correlate significantly with those of Cnn in WT SAPs ($r=0.7614$, $p<0.0001$, Fig.5.7B), and this was also the case in *asl*^{mecD} SAPs, although, perhaps unsurprisingly when the signal is so low, the correlation was not as strong ($r=0.4621$, $p<0.0001$, Fig.5.7B).

In conclusion, my data suggests that SAPs can form in *asl*^{mecD} eggs, but they usually have severely impaired PCM recruitment. However, they can still occasionally recruit Cnn, and this recruitment might be due to the presence of DSpd-2 at very low levels. This result is particularly surprising in view of my earlier observation that both DSpd-2 and Cnn recruitment are essentially abolished in the eggs laid by females homozygous for the "weaker" *asl*^{1} allele.

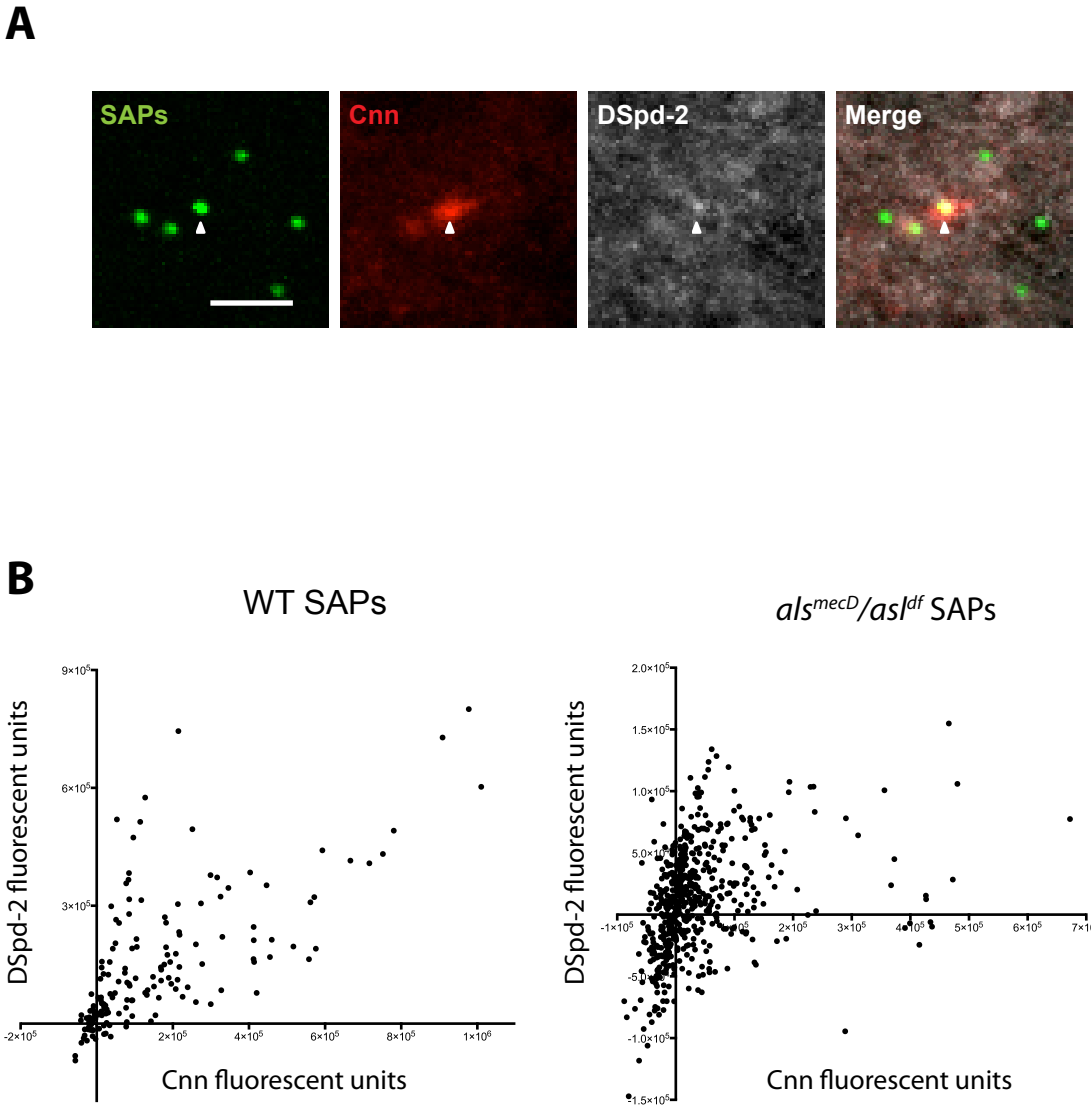


Figure 5.7 *asl*^{mecD} SAPs that recruit PCM may still have some DSpd-2
(A) Panels show *asl*^{mecD}/*asl*^{df} SAPs stained for the antibodies indicated. Note how a very small amount of DSpd-2 can be observed in the SAP recruiting most Cnn. Scale bar=5μm. **(B)** Correlation analysis of DSpd-2 and Cnn levels in SAPs in eggs expressing GFP-DSas-6 and GFP-Ana2 in a WT (left, r=0.7614, p<0.0001, n=161 SAPs) and *asl*^{mecD}/*asl*^{df} (right, r=0.4621, p<0.0001, n=564 SAPs) background.

5.2.4 SAPs formed in a new *asl* allele background show the same results as in a *asl*^{mecD} background

In an *asl*^{1} background, SAPs cannot recruit Cnn or DSpd-2. In *asl*^{mecD} background, however, Cnn recruitment can still occasionally occur. This result was unexpected because *asl*^{1} is seen as a weaker hypomorph, while *asl*^{mecD} gives a strong centriole and cilia phenotype, with uncoordinated flies and lacking centrioles (Blachon et al., 2008). However, although the Asl protein produced from the *asl*^{mecD} allele is truncated and unstable, low levels of the N-terminal fragment of Asl can still be detected by western blot (Blachon et al., 2008). I therefore wondered if the partial Cnn recruitment observed in *asl*^{mecD} SAPs might be due to the fact that the truncated Asl being produced was still able to localise to the SAPs and induce the recruitment of DSpd-2 and/or Cnn. During the course of my PhD, a new *asl* allele, *asl*^{B46}, was generated in the lab (Zsofia Novak and Alan Wainman, personal communication). This allele was generated by the imprecise excision of a P-element located 0.35Kb upstream of *asl*, which removed the first half of the coding region of the gene, and part of its 5' UTR. Unlike in *asl*^{mecD}*,* no Asl protein can be detected by western blot by either N- or C-terminal antibodies in *asl*^{B46} samples, and no centrioles can be observed in immunofluorescence studies, suggesting that this was an even stronger, and possibly genetically null, allele (Zsofia Novak, personal communication). I therefore asked whether the composition of SAPs in *asl*^{B46} activated eggs was the same as in *asl*^{mecD} activated eggs.

I expressed GFP-DSas-6 and GFP-Ana2 in *asl*^{B46}/*asl*^{df} activated eggs. For comparison purposes, I also looked at SAPs formed in a WT background. Unfortunately, I found that I could not identify which *asl*^{B46}/*asl*^{df} flies expressed both GFP-DSas-6 and GFP-Ana2 based on their wing phenotype, most likely because some unknown secondary genetic interaction was suppressing the curly wing phenotype in the appropriate mutant flies (see Fig.5.8 for more detailed description). However, as both *asl*^{1} and *asl*^{mecD} SAPs were able to recruit DSas-4 (see Fig.4.6 and Fig.5.5), I realised I could use DSas-4 co-localisation with the GFP signal as a marker to identify real SAPs (as I found DSas-4 was also still localised to SAPs in *asl*^{B46}/*asl*^{df} activated eggs). Therefore, for all experiments described here using *asl*^{B46}/*asl*^{df} activated eggs, a SAP was identified as such by both its GFP and DSas-4 signal. It is important to note that this is a different strategy to the one used for all other SAP experiments in this thesis where, to avoid bias, all eggs showing GFP signal were imaged and quantified.

As shown in Fig.5.9 A and B, as was the case in *asl*^{mecD} SAPs, *asl*^{B46} SAPs also show recruitment of DSas-4, albeit at reduced levels ($p<0.0001$, n=30). *asl*^{B46} SAPs also showed a strong average reduction in the levels of Cnn recruitment ($p<0.0001$, n=30), although, as was the case in *asl*^{mecD} SAPs, there was a mixture of SAPs with and SAPs without Cnn signal (Fig.5.6A). A similar analysis of the proportion of *asl*^{B46}/*asl*^{df} activated eggs containing all, none or some SAPs recruiting Cnn showed that 53% of the *asl*^{B46}/*asl*^{df} activated eggs have only SAPs that cannot recruit Cnn, while 43% have a mixture of SAPs that can and SAPs that cannot recruit Cnn (Figure 5.10A).

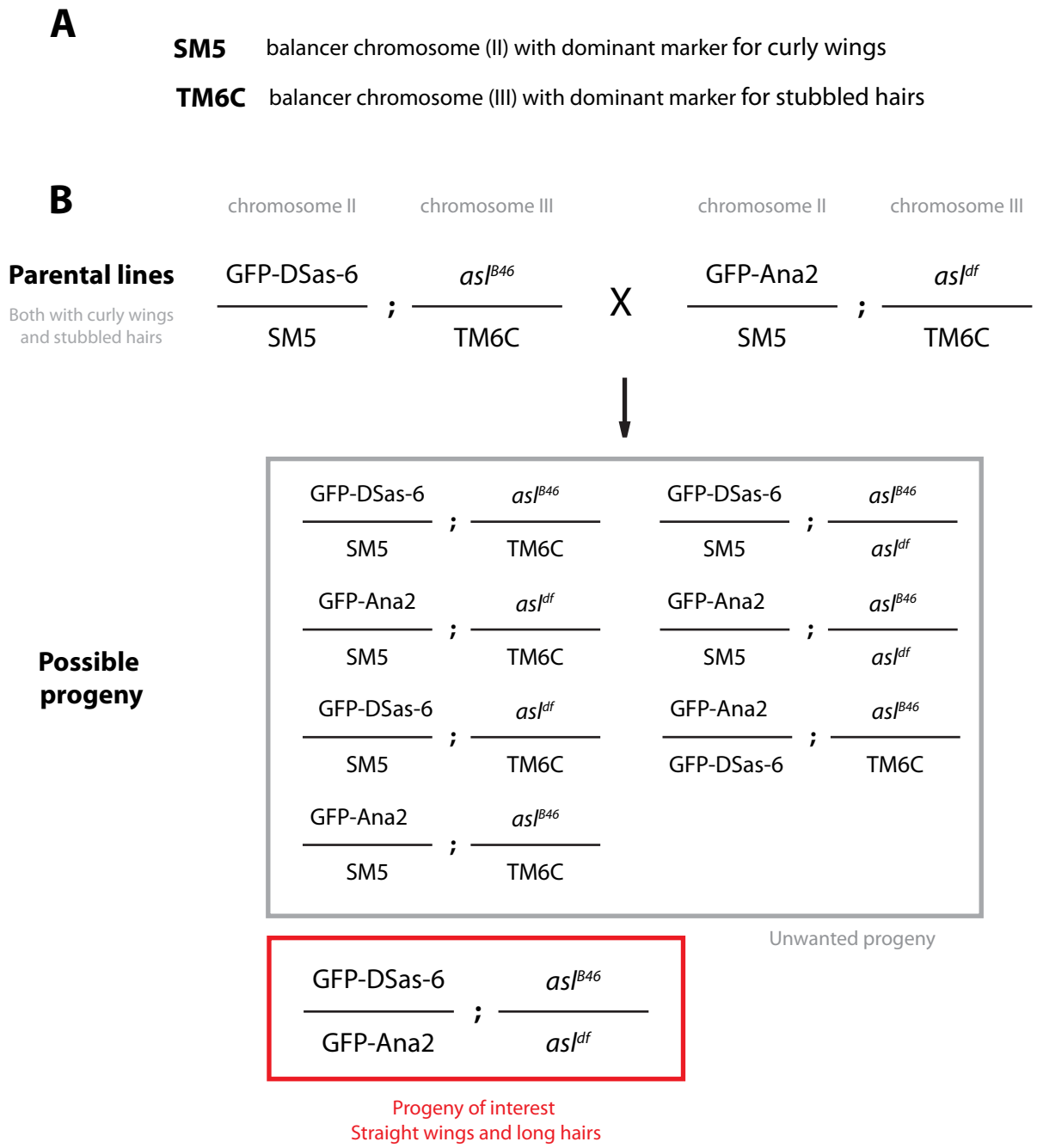


Figure 5.8 A schematic illustration of the genetic crossing scheme used to generate SAPs in a *asl*^{B46}/*asl*^{df} background

Transgenes and mutations of interest can be traced in a cross by the presence or absence of a marker on the chromosome of interest. **(A)** The 'balancer' chromosome for chromosome II is SM5, which carries a dominant marker that makes the wings of the fly curly. The 'balancer' chromosome for chromosome III is TM6C, and carries a dominant marker that makes the hairs of the fly stubbled. **(B)** The two parental lines of interest have both these 'balancer' marker chromosomes, so the flies from both lines have curly wings and stubbled hairs. When these two lines are crossed, it is possible to identify the progeny of interest, i.e. those containg both GFP-DSas-6 and GFP-Ana2 in an *asl*^{B46}/*asl*^{df} mutant background (red box), because these flies will not contain any balancer chromosomes, and hence will have straight wings and normal hairs. Due to a secondary genetic interaction (of unknown nature), however, I found it was not possible to assess the wing morphology clearly in the progeny of this cross, and as such it was not possible to distinguish the progeny of interest from some of the other possible progeny. As described in the text, this has important implications for how the experiment performed with these flies is assessed.

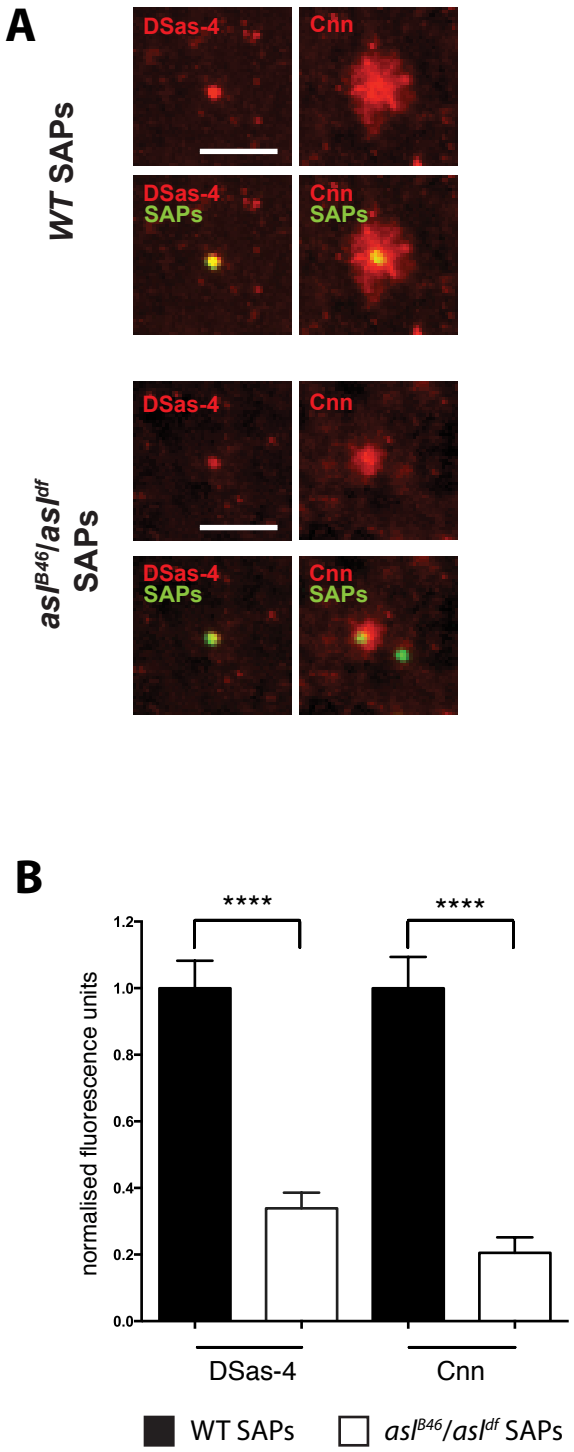


Figure 5.9 SAP composition in *asl*^{B46} activated eggs
An analysis of SAP composition in *in vitro* activated eggs expressing Ubq-GFP-DSas-6 and Ubq-GFP-Ana2 in either a WT or a *asl*^{B46}/*asl*^{df} background. **(A)** Representative images of WT SAPs and *asl*^{B46}/*asl*^{df} SAPs stained with antibodies for different centrosomal proteins, as indicated. Scale bar=5μm. **(B)** Graph shows the quantification of different protein levels in WT (black) and *asl*^{B46}/*asl*^{df} (white) SAPs. Note how *asl*^{B46}/*asl*^{df} SAPs have reduced levels of both DSas-4 and Cnn. Bars represent the average SAP fluorescence of 30 eggs. Note that all the SAPs analysed in this experiment were positive for DSas-4.

Note that when only SAPs that also recruit DSas-4 are considered in this analysis, no WT eggs are seen to have SAPs that cannot recruit Cnn. This confirms that, as hypothesized, the proportion of WT eggs in Fig.5.6A in which all SAPs did not recruit Cnn probably corresponded to eggs that had not completely activated and so did not contain real SAPs, or corresponded to aggregates of only GFP-DSas-6 or GFP-Ana2.

The observation that SAPs in an *asl*^{B46}/*asl*^{df} background can still occasionally recruit Cnn is similar to that observed in a *asl*^{mecD}/*asl*^{df} background. I decided therefore to examine this observation in more detail by asking whether the *asl*^{B46}/*asl*^{df} SAPs that can recruit Cnn do so at the same levels as WT SAPs. I therefore compared the Cnn fluorescence levels in only those *asl*^{B46}/*asl*^{df} SAPs that had a detectable level of Cnn recruitment and compared them with levels of Cnn in WT SAPs (Fig.5.10B). The results of this analysis show that *asl*^{B46}/*asl*^{df} SAPs that recruit Cnn do so that at lower levels of Cnn than WT SAPs ($p<0.0001$, $n>101$ SAPs).

I then asked whether DSpd-2 staining could be observed in *asl*^{B46} SAPs. As was the case with *asl*^{mecD} SAPs, DSpd-2 staining was mostly absent, although sometimes some weak staining could be observed. The levels of DSpd-2 staining also seemed to be proportional to the level of Cnn recruited (Fig.5.10C).

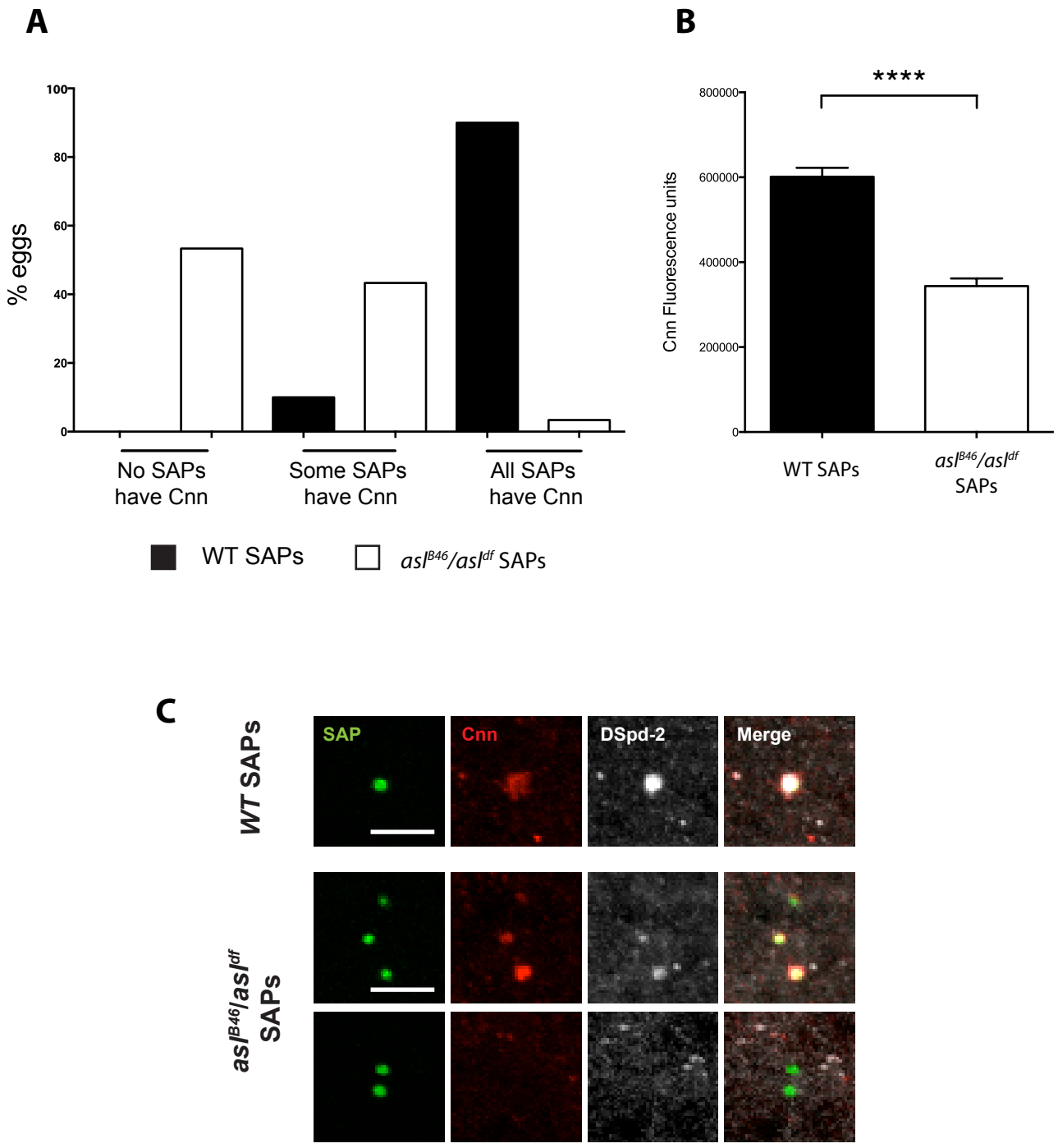


Figure 5.10 *asl*^{B46} SAPs that recruit PCM may still have DSpd-2
(A) Graph show the proportion of *in vitro* activated eggs expressing GFP-DSas-6 and GFP-Ana2 in a WT (black) or *asl*^{B46}/*asl*^{df} (white) background with no SAPs staining for Cnn, some SAPs staining for Cnn and all SAPs staining for Cnn, as indicated (n=30). Note how 43% of *asl*^{B46}/*asl*^{df} eggs have some SAPs recruiting Cnn. **(B)** A comparison of Cnn fluorescence levels in WT (n=301, black) and *asl*^{B46}/*asl*^{df} (n=101, white) SAPs that had at least some detectable Cnn staining. Note that unlike other graphs in this thesis, the average fluorescence of individual SAPs, rather than the average SAP fluorescence per egg, is shown here. Note how the levels of Cnn in *asl*^{B46}/*asl*^{df} are lower than in WT SAPs. **(C)** Panels show WT and *asl*^{B46}/*asl*^{df} SAPs stained for the antibodies indicated. Note how DSpd-2 staining seems to be proportional to the level of Cnn recruited. Scale bar=5μm. Note that all the SAPs analysed in this experiment were positive for DSas-4.

In conclusion, analysis of SAPs in two mutants of *asl* with centriolar defect phenotypes shows very similar results. In both the case of asl^{mecD} SAPs and of asl^{B46} SAPs, a reduction in the ability to recruit Cnn was observed, with most SAPs lacking Asl being unable to recruit Cnn. Interestingly, around 40% of eggs had some SAPs that were still able to recruit Cnn at robust levels (see section 5.3 for discussion). DSpd-2 recruitment seems to be almost absent in SAPs lacking Asl. However, extremely low levels of DSpd-2 might be responsible for the occasional recruitment of Cnn by *asl* SAPs.

5.2.5 DSas-4 is required for efficient SAP formation

DSas-4 is an essential centriolar protein. Mutants for *dsas-4* have been shown to lack centrioles, both by immunostaining and by EM (Basto et al., 2006). As is the case with centriolar mutants of *asl*, *dsas-4* mutant flies are uncoordinated and cannot lay eggs. I was curious therefore to investigate whether SAPs can form in the absence of DSas-4.

I expressed GFP-DSas-6 and GFP-Ana2 in $dsas\text{-}4^{S2214}$ activated eggs. This mutant allele is known not to produce any detectable protein (Basto et al., 2004). For comparison purposes, I also looked at SAPs formed in a WT background. Interestingly, I noticed that the vast majority (80%) of eggs expressing GFP-DSas-6 and GFP-Ana2 in a $dsas\text{-}4^{S2214}$ background did not contain any SAPs (Fig.5.11A), as compared with WT eggs, where only 17% of the eggs did not contain SAPs. The remaining 20% of $dsas\text{-}4^{S2214}$ eggs

had SAPs which did not stain for any centrosomal components (Fig.5.11B and Fig.5.12A and B). Interestingly, this is very similar to the percentage of eggs in WT background that contained SAPs that also did not stain for any centrosomal components (Fig.5.11A and B). A similar small percentage of non-active SAPs (i.e. not recruiting PCM components or organising MTs) in the WT background was also described in section 5.2.3. The similar proportion of non-active SAPs in the *dsas-4*^{S2214} background and WT suggests that the small percentage of structures observed in the *dsas-4*^{S2214} background are unlikely to be genuine SAPs, and are most likely aggregates of only GFP-Ana2 (and Ana2) or GFP-DSas-6 (and DSas-6). In fact, the occasional observation of GFP aggregates forming upon GFP-DSas-6 overexpression that are not MTOCs (among other MTOCs) has been previously reported (Peel et al., 2007; Rodrigues-Martins et al., 2007a). Staining these GFP aggregates for antibodies for Ana2 and DSas-6 would be a possible way of confirming this hypothesis. However, and even if these are genuine SAPs, their low formation frequency still suggests that DSas-4 is very important for the efficient formation of SAPs.

I can therefore conclude that in the absence of DSas-4 SAPs cannot form, or can only do so at very low efficiency, and cannot stain for any centrosomal components.

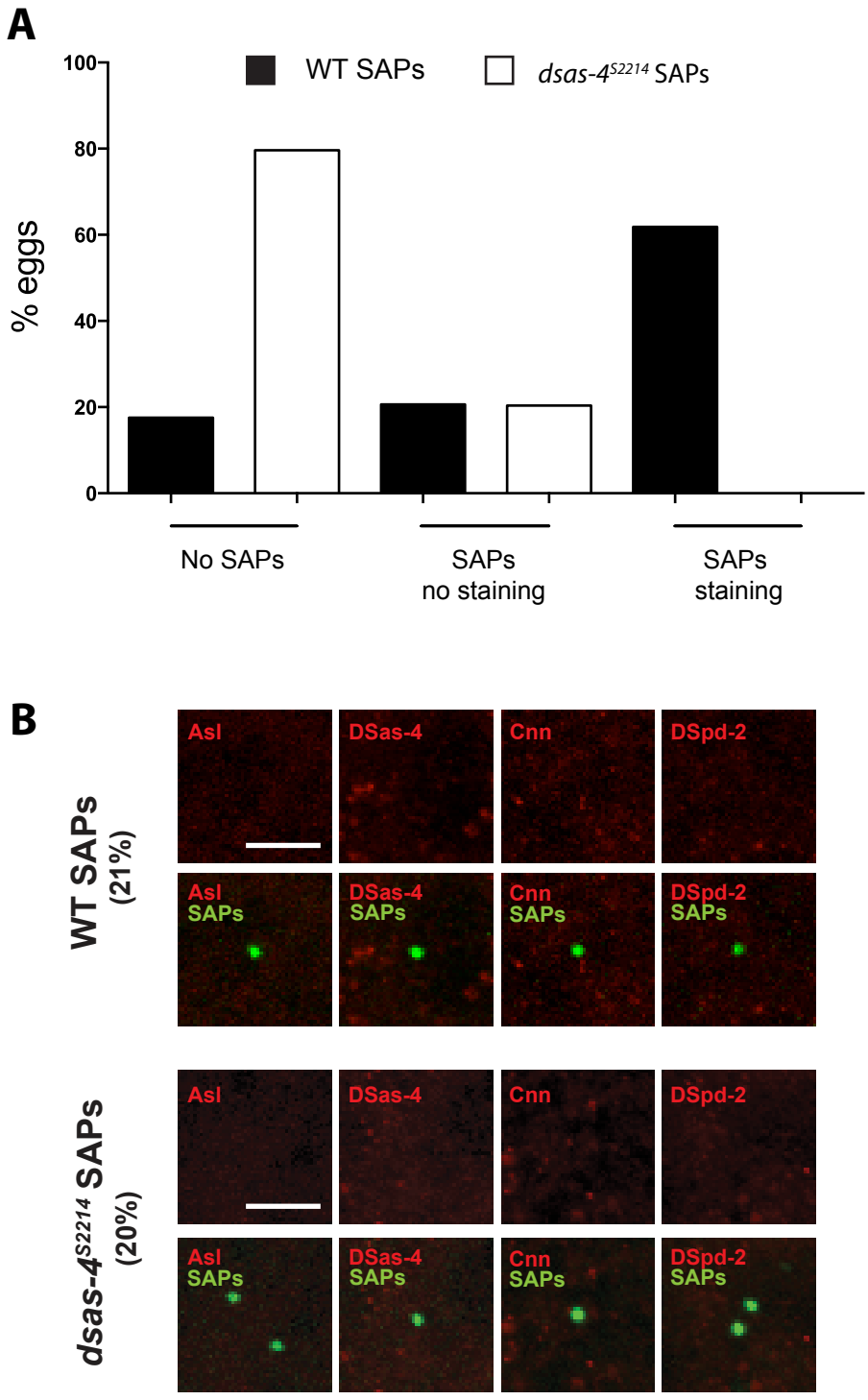


Figure 5.11 Analysis of *dsas-4*^{S2214} activated egg proportions
(A) Graphs show the proportion of eggs expressing GFP-DSas-6 and GFP-Ana2 in a WT (black) or *dsas-4*^{S2214} background (white) with no SAPs, SAPs showing no staining and SAPs showing some staining for centrosomal components (n>97 eggs). Note how the proportion of eggs containing SAPs that do not stain for centrosomal components is the same for WT and for *dsas-4*^{S2214} eggs. **(B)** Images of WT SAPs that do not behave like centrosomes and *dsas-4*^{S2214} SAPs stained with antibodies for different centrosomal proteins, as indicated. Scale bar=5μm.

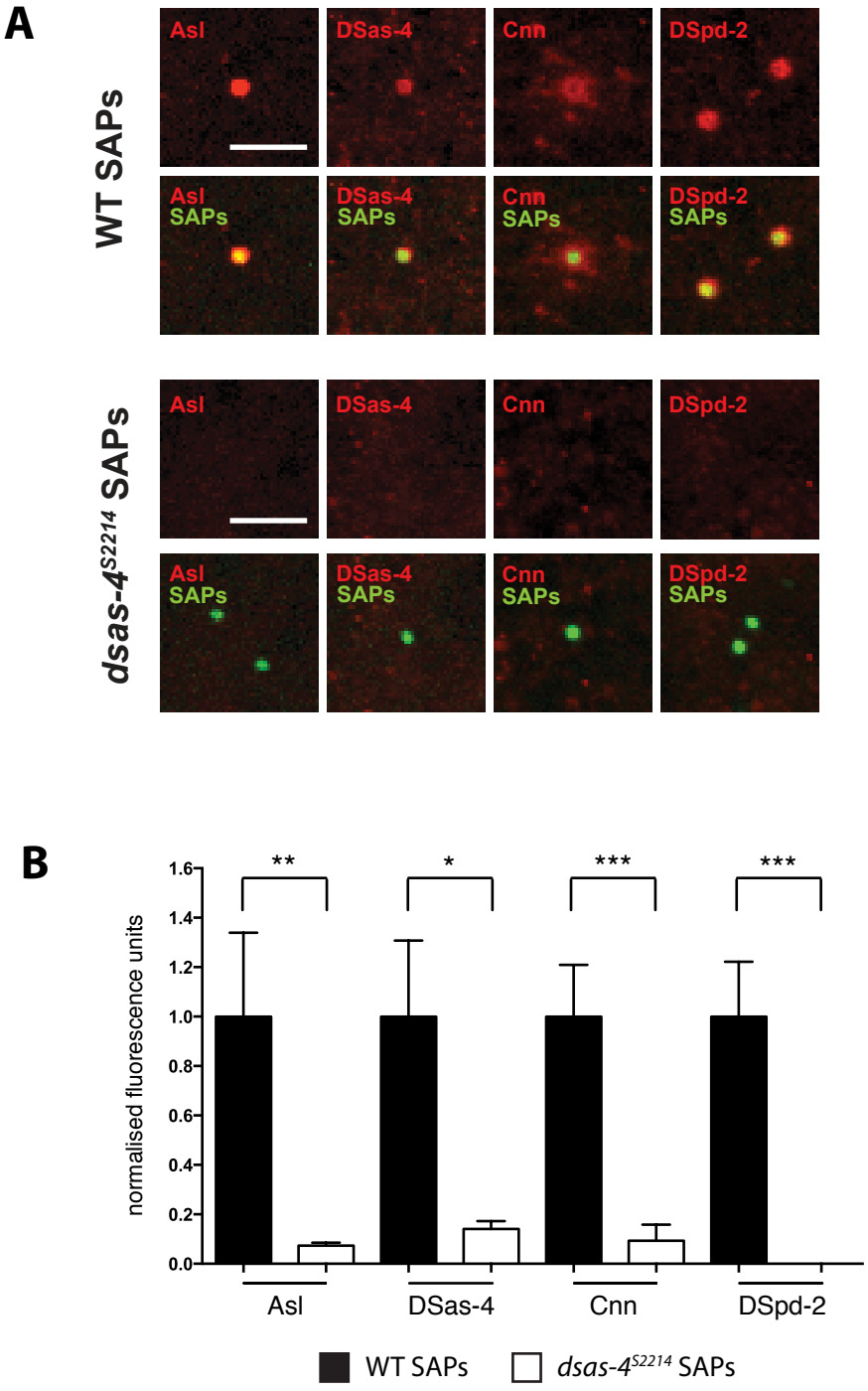


Figure 5.12 SAP composition in *dsas-4*^{S2214} activated eggs
Analysis of SAP composition in *in vitro* activated eggs expressing Ubq-GFP-DSas-6 and Ubq-GFP-Ana2 in either a WT or a *dsas-4*^{S2214} background. **(A)** Representative images of WT SAPs and *dsas-4*^{S2214} SAPs stained with antibodies for different centrosomal proteins, as indicated. Scale bar=5μm. **(B)** Graph shows the quantification of different protein levels in WT (black) and *dsas-4*^{S2214} (white) SAPs. Note how *dsas-4*^{S2214} SAPs have very reduced levels of all tested centrosomal components. Bars represent the average SAP fluorescence of at least 7 eggs.

5.2.6 Sak is not required to form SAPs

The kinase Sak is an important centriole duplication factor. It was shown to be important for centriole duplication in human cells and in flies (Bettencourt-Dias et al., 2005; Habedanck et al., 2005; Dobbelaere et al., 2008) and when overexpressed in unfertilised eggs it can generate *de novo* centrioles (Rodrigues-Martins et al., 2007b). Crucially, the homologue of SAK in *C. elegans,* Zyg-1, is thought to be a very upstream protein in the pathway of centrosome formation (Pelletier et al., 2006).

The only published *Drosophila* mutant allele of *sak* is a weak hypomorph caused by a P-element insertion into the coding region of the gene (Bettencourt-Dias et al., 2005). Mutant flies are viable and fertile, which was an unexpected result, as mutants for other important centriole duplication proteins are uncoordinated (Basto et al., 2006; Peel et al., 2007; Rodrigues-Martins et al., 2007a; Blachon et al., 2008). However, during the course of my PhD a *sak* null allele was generated by members of the lab (Janina Baumbach and Alan Wainman, personal communication). The sak^{Aa74} allele was generated by excision of a P-element upstream of *sak*, which resulted in the deletion of the promoter region and one third of the coding region (including the entire kinase domain). Flies homozygous for this allele are uncoordinated and lack centrioles in larval brains. Both phenotypes are fully rescued by expression of Sak-GFP (Janina Baumbach and Alan Wainman, personal communication).

I was interested in knowing whether SAP formation in the absence of Sak is possible. I therefore expressed GFP-DSas-6 and GFP-Ana2 in sak^{Aa74}/sak^{df} activated eggs. For comparison purposes, I also looked at SAPs formed in a WT background. This experiment was done in collaboration with Alan Wainman and Janina Baumbach. Surprisingly, I found that SAPs behave like WT SAPs (i.e. as centrosomes) in the absence of Sak (Fig.5.13). *sak* SAPs showed similar levels of Asl ($p= 0.3006$, $n>11$ eggs) and DSas-4 ($p= 0.3763$, $n>11$ eggs) as WT SAPs. In addition, *sak* SAPs were able to nucleate MT asters of similar properties to those nucleated by WT SAPs ($p=0.5958$, $n>8$ eggs). I can therefore conclude that SAP composition and MT nucleating ability does not require Sak.

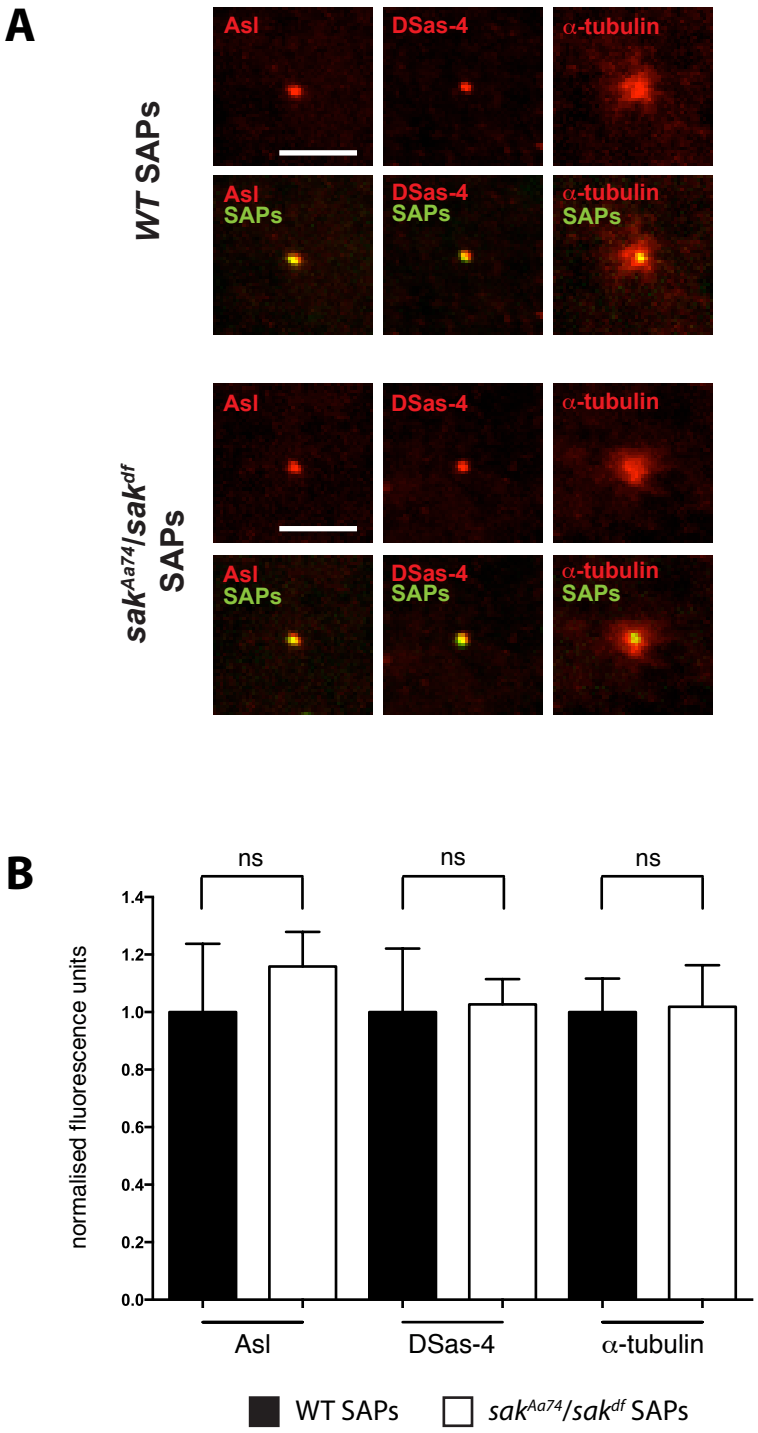


Figure 5.13 SAP composition in *sak^{Aa74}* activated eggs
Analysis of SAP composition in *in vitro* activated eggs expressing Ubq-GFP-DSas-6 and Ubq-GFP-Ana2 in either a WT or a *sak^{Aa74}/sak^{df}* background. **(A)** Representative images of WT SAPs and *sak^{Aa74}/sak^{df}* SAPs stained with antibodies for different centrosomal proteins, as indicated. Scale bar=5μm. **(B)** Graph shows the quantification of different protein levels in WT (black) and *sak^{Aa74}*/sak^{df} (white) SAPs. Note how *sak^{Aa74}/sak^{df}* SAPs have the same protein composition and levels as WT SAPs. y axis shows the normalised fluorescent values (except for α-tubulin levels, in which bars represent α-tubulin/GFP normalised fluorescent values). Bars represent the average SAP fluorescence of at least 8 eggs.

5.3 Discussion

Five proteins have been identified in *Drosophila* as being essential for centriole duplication: Ana2, Asl, DSas-4, DSas-6 and Sak. These proteins have been identified as important centriolar players because they fullfill the following two criteria: a) mutants for these proteins have no centrioles b) overexpression of these proteins in unfertilised eggs leads to the formation of MTOCs which, in the case of Asl and Sak, have *de novo* formed centrioles at their core (Basto et al., 2006; Peel et al., 2007; Rodrigues-Martins et al., 2007a; Rodrigues-Martins et al., 2007b; Blachon et al., 2008; Dzhindzhev et al., 2010; Stevens et al., 2010a).

In this chapter I have examined the pathway of centriole assembly by analysing the composition of SAPs in the absence of Asl, DSas-4 and Sak. As flies lacking these proteins are uncoordinated but undergo normal oogenesis, I used an *in vitro* activation protocol to convert stage 14 oocytes into eggs that are able to form functional SAPs. Unlike my analysis of the pathway of PCM recruitment (Chapter 4), I did not also perform this analysis with *de novo* formed centrosomes. There are two reasons for this. First, as described in section 5.2.2, the kinetics and efficiency of *de novo* centrosome formation in activated eggs made this experiment too time consuming to be completed within the time scale of my thesis. Second, as *de novo* centrosomes contain fully formed centrioles in their core, it seemed unlikely that they would be able to form in the absence of these essential centriole assembly proteins.

5.3.1 SAP composition in two different *asl* mutants

I first analysed the position of Asl in the pathway of centriole assembly by assessing the composition of SAPs in two different mutants: *asl*^{mecD}, in which a short, unstable N-terminal fragment of the protein is produced; and *asl*^{B46}, a new allele recently generated in the lab that appears to be at least close to a genetic null. SAP composition in these mutant backgrounds was very similar, suggesting that the short protein produced by the *asl*^{mecD} is largely not functional. Excitingly, I found that SAPs can form in the absence of Asl. This has confirmed the potential utility of using SAPs to analyse the pathway of centriole assembly, as centrioles cannot form in the absence of Asl (Blachon et al., 2008). SAPs therefore provide a unique system in which the role of different important centriole players can be dissected.

SAPs formed in *asl* mutants contained DSas-4, albeit at reduced levels when compared with WT SAPs. This is similar to the result obtained when analysing the composition of SAPs in an *asl*^{1} background. The results from these three mutants of *asl* suggest, therefore, that DSas-4 is upstream of Asl in the pathway of centriole assembly. In a recent paper, Dzhindzhev and colleagues showed by co-IP and Mass Spectrometry techniques that DSas-4 is able to interact with Asl. Specifically they were able to map residues 648 to 994, in the C-terminus of Asl as the interacting region with residues 101 to 650 in the N-terminus of DSas-4 (Fig.5.4). They also showed that overexpression of a form of Asl lacking part of its N-terminus in the unfertilised egg was able to generate MTOCs that also contained DSas-4.

Antibody injection experiments looking at Asl recruitment upon injection of antibodies against DSas-4, as well as work on the dynamic behaviour of these two proteins, also support this (Zofia Novak, personal communication). I can therefore conclude that DSas-4 is the recruiter of Asl to the centriole. The reduction in the levels of DSas-4 in the absence of Asl suggests a role for Asl in stabilising DSas-4. Interestingly, data in human cells suggests that the recruitment might be reversed in that system, i.e. the homologue of Asl, Cep152 might be the recruiter of CPAP (the homologue of DSas-4) to the centriole in human cells (Cizmecioglu et al., 2010; Dzhindzhev et al., 2010).

In the previous chapter I showed that SAPs formed in an asl^{1} background cannot recruit Cnn and DSpd-2, therefore establishing Asl's central role in PCM recruitment. In the absence of Asl (asl^{mecD} and asl^{b46} analysis) the majority of SAPs were also not able to recruit PCM components. Surprisingly, however, occasionally the recruitment of Cnn was still observed, albeit at reduced levels when compared with WT SAPs. This recruitment seemed to be sufficient to initiate the formation of a functional PCM, as smaller MT asters were nucleated by these SAPs. A similar observation has been previously reported. Blachon and colleagues took advantage of the perdurance of some maternally contributed centrioles in *asl* mutant spermatocytes to examine the PCM composition of these centrioles in the absence of Asl. While in an asl^{1} background they observed a strong reduction in PCM recruitment by these centrioles, in meiotic spermatocytes the recruitment of PCM was not impaired (and was in fact

increased) in an *asl*^{mecD} background. This effect was specific to meiotic cells though, as in *asl*^{mecD} mature spermatocytes an impairment in PCM recruitment is now observed (albeit not completely). It seems therefore that at least in embryos (my work reported here) and in meiotic spermatocytes (Blachon et al., 2008) PCM recruitment is possible, to a limited extent, in the absence of Asl (but not in the presence of the truncated version of Asl present in the *asl*^{1} allele – see below).

My analysis of the pathway of PCM recruitment (Chapter 4) led me to conclude that Asl is most likely involved in recruiting DSpd-2, while DSpd-2 is most likely the recruiter of Cnn. An important question, therefore, is whether Cnn is being recruited to SAPs lacking Asl because some DSpd-2 can still be recruited there. My analysis was not conclusive on this point, although it suggested that some DSpd-2 could be recruited to some SAPs in the absence of Asl. To confirm this, an analysis of SAP composition in an *asl, dspd-2* double mutant background is required. If in this context no Cnn recruitment is observed, we can conclude that some DSpd-2 can still be recruited to centrioles in the absence of Asl, and that it likely recruits Cnn. If some Cnn is still recruited in the double mutant combination, then there must be an alternative pathway of Cnn recruitment that does not rely on either protein. In the first scenario an unknown protein must recruit DSpd-2 to centrioles in the absence of Asl; in the second scenario, an unknown protein must recruit Cnn to centrioles in the absence of Asl and DSpd-2. In either case, my results demonstrate that the real pathway of PCM assembly is not as simple as Asl recruits DSpd-2, which in turn recruits Cnn. One way

to address either question would by making use of the antibody injection techniques developed by the lab. It would be interesting to inject antibodies for potential recruiters in *asl* null eggs that have SAPs that can recruit Cnn, and observe whether the recruitment of Cnn is any way impaired or interfered with.

It is very interesting that in an asl^{1} background no PCM recruitment takes place, while in an asl^{mecD} and asl^{B46} allele background PCM recruitment can occasionally take place. As shown in Fig.5.4 the proteins produced in the asl^{1} and asl^{mecD} genetic backgrounds differ by 371 amino acids. Perhaps this specific region can in some way inhibit the alternative PCM recruitment pathway that operates in the stronger asl alleles. The mechanism or target of this potential inhibition is not known, but are interesting points for future analysis. Another interesting point is whether this inhibition is taking place at the centriole or in the cytoplasm. As previously mentioned, Asl^{1} was not detected at SAPs, suggesting that it cannot be recruited to the centriole. This might suggest that inhibition takes place in the cytoplasm. It is also worth considering that Asl^{mecD} is thought to be relatively unstable, explaining why the phenotype observed in asl^{medD} (truncation) and asl^{B46} (very likely a null) is very similar.

5.3.2 DSas-4 is required for the interaction between DSas-6 and Ana2

While DSas-4 fulfils the 2 criteria of an important centriole duplication player, its role in centriole formation appears to be less central than the other core duplication proteins. While the overexpression (UAS promoter) of GFP-DSas-6 and GFP-Sak is able to generate *de novo* MTOCs at very high efficiency in eggs (>90% of laid eggs contain MTOCs), overexpression of GFP-DSas-4 was less efficient (~58% of laid eggs contain MTOCs) (Peel et al., 2007). In addition, while the overexepression of Asl and Sak can generate *de novo* centrosomes containing fully formed centrioles, overexpression of GFP-DSas-4 generates MTOCs that do not have centrioles (Rodrigues-Martins et al., 2007b; Dzhindzhev et al., 2010). Most importantly, unlike other important centriole duplication players, the overexpression of DSas-4 is not able to induce centriole overduplication (Peel et al., 2007).

I found that SAPs were not able to form, or formed at very low efficiency, in the absence of DSas-4. Previous work has shown that DSas-6 and Ana2 are able to interact, and that this interaction is functionally relevant. Indeed, while SAPs can form when GFP-DSas-6 and GFP-Ana2 are expressed in eggs, such efficient MTOC formation was not observed with any other combination of centriole duplication proteins (Stevens et al., 2010a). In addition, GFP-DSas-6 and GFP-Ana2 expression in testis generates highly ordered tubules that resemble the central cartwheel (Stevens et al., 2010b). DSas-6 and Ana2 interact directly in Y2H experiments and can be co-

immunoprecipitated with one another in fly and human cell extracts (Stevens et al., 2010a; Tang et al., 2011). My analysis of SAPs in activated eggs strongly suggests that DSas-4 is required for these proteins to interact in a functionally meaningful way. This conclusion is also supported by the analysis of SAPs in testis: the highly ordered tubules observed in a WT background are much harder to discern in the absence of DSas-4 (Helio Roque, personal communication).

A role for DSas-4 in promoting the interaction between DSas-6 and Ana2 is supported by recent data showing that DSas-4 is able to interact with Ana2 (Tang et al., 2011). The crystal structure of this interaction interface in flies and zebrafish has recently been solved, and a mutational analysis has shown that the interaction is essential for centriole duplication in flies (Cottee et al., 2013 *in press*). An interaction between DSas-6 and DSas-4 has not been reported, but in human cells STIL (homologue of Ana2), CPAP (homologue of DSas-4) and hSas-6 (homologue of DSas-6) are able to immunoprecipitate as a complex (Tang et al., 2011). It is therefore very likely that DSas-4 plays a role in promoting or stabilising the interaction between DSas-6 and Ana2, although it will be interesting to explore in more detail the structural role of DSas-4 in this process in the future.

5.3.3 Sak is not required for SAP formation

Sak is thought to be one of the most important centriole duplication proteins. Sak mutant flies lack centrioles, centrosomes and cilia (Janina Baumbach and Alan Wainman, personal communication), and the overexpression of Sak/Plk4 can drive centriole overduplication (Peel et al., 2007; Rodrigues-Martins et al., 2007b; Basto et al., 2008) and *de novo* centriole formation in unfertilised eggs (Rodrigues-Martins et al., 2007b). In addition, the functional homologue of Sak in *C. elegans,* Zyg-1, is thought to be one of the most upstream components in the pathway of centriole assembly in this organism (discussed in Chapter 7). Although Sak is thought to play an important role in initiating centriole duplication, it is unclear whether this role is restricted to the initiation process, or whether it has additional roles in guiding later stages of centriole, or perhaps PCM, assembly. Until now, this question has been impossible to address as the lack of Sak/Plk4 leads to a lack of centrioles and centrosomes.

I found that SAPs were able to recruit PCM and form functional MTOCs at nearly normal levels in the absence of Sak. This result is perhaps surprising, and has important implications for the role of Sak in centriole assembly. Clearly, at least some (and possibly all) centriolar and PCM proteins can be recruited to the SAPs to form a functional MTOC in the absence of Sak. There are two interpretations for this result. The first interpretation is that Sak's role in centriole duplication is to provide a nucleation point for the formation of the new centriole. This explains why Sak is the first component

to be recruited to the place in which the procentriole is formed, as recently shown by structured illumination for the vertebrate homologue of Sak, Plk4 (Sonnen et al., 2012). It also explains why *de novo* centriole formation is possible in unfertilised eggs upon Sak overexpression: the increase in local concentration may be enough to sporadically form nucleation points where the process of centriole assembly can be initiated. In eggs expressing GFP-DSas-6 and GFP-Ana2, the DSas-6/Ana2 (and potentially DSas-4) association may form a platform to which downstream components can be recruited, therefore making Sak obsolete. Alternatively, Sak role might be very specific to the building of a centriole with all its intricacy and complexity. For example, Sak might be involved in phosphorylating a centriolar component that is structurally essential for the formation of a normal centriole, but whose phosphorylation is not necessary when an unstructured platform is being preformed due to the high levels of DSas-6 and Ana2.

My results also suggest that the phosphorylation of a substrate by Sak is not necessary to recruit any downstream components, as a functional SAP can form without Sak. Future work on the role of Sak, and in particular its phosphorylation target, will hopefully be able to elucidate this mystery. As discussed in Chapter 1 (section 1.2.1), the target of Sak is currently unknown, and recent work in *C. elegans* has shown that SAS-6 is not its target but that the kinase activity of Zyg-1 (the worm homologue of Sak) is required for SAS-6 localisation. In light of my results shown in this chapter, it is likely that a target of Sak might be Ana2 or DSas-4.

5.3.4 The relationship between Asl and Sak

One of the most surprising results from these experiments was the mild effects on SAP formation observed in the absence of Asl and Sak. In the absence of both Asl and Sak, SAPs can form as normal and they contain centriolar components that organise a PCM (although this PCM recruitment ability is limited in *asl* mutant SAPs due to Asl's separate role in PCM recruitment). Thus, centriolar and PCM components can recruit each other and come together to form a functional structure in the absence of Sak and Asl. As discussed above, Sak most likely has a role that is very specific to defining the place and initiating the formation of the procentriole. As SAPs are unstructured and are formed on a pre-existing platform of DSas-6 and Ana2, they do not require Sak.

Asl is also an essential centriole duplication component and a recent paper by Dzhindzhev and colleagues suggested that Asl is a vital scaffold for centriole duplication, partially due to its role in recruiting Sak to the mother centriole. But is Asl essential for centriole duplication only because it is the upstream recruiter of Sak, or does it play a more active role? The similarity in phenotypes in SAP formation in the absence of Asl and Sak suggests that indeed Asl's role in centriole duplication is limited to the upstream recruitment of Sak, at least as far as the recruitment of downstream centriole components is concerned. In addition, Asl is localised to the full extent of the mother centriole wall, while Sak is only recruited to a very specific region where the procentriole will be formed, suggesting that Asl

alone is probably not even sufficient to recruit Sak to the mother centriole in its normal restricted localisation pattern. It cannot be excluded, however, that Asl might still play an additional essential role in the formation of the centriole proper in addition to recruiting Sak. One way to address this question would be by overexpressing Sak in *Drosophila* eggs that lack Asl. A similar experiment has previously been attempted, but it was performed in cell culture and using RNAi, rather than full mutants, and the results are therefore not conclusive (Dzhindzhev et al., 2010). If Asl is only necessary to increase Sak local concentration in the mother centriole wall, then the artificial increase in Sak concentration generated by overexpression should be able to induce the formation of *de novo* fully structured centrioles. These would be able to recruit downstream centriole components, but would not be able to recruit a PCM, due to Asl's additional role in PCM recruitment. If, however, Asl has an additional essential role in the formation of the centriole, increasing the local concentration of Sak in the absence of Asl will not be able to generate *de novo* centrioles. Such experiments would be able to clarify the role of Asl in centriole duplication and its relationship with Sak.

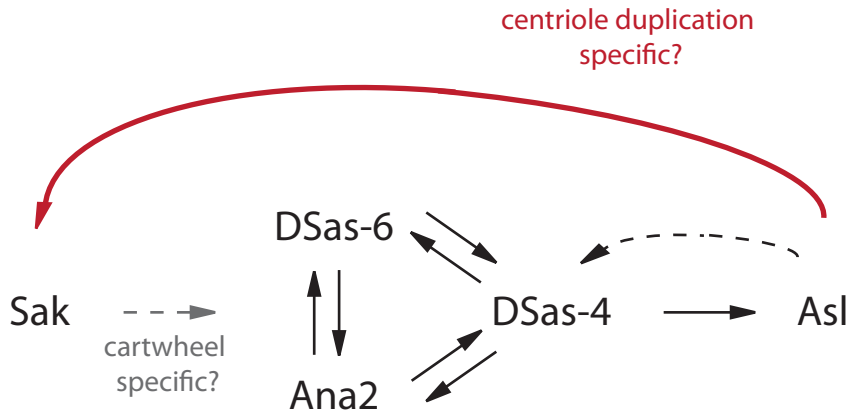


Figure 5.14 Model of the pathway of centriole assembly in *Drosophila* embryos.

5.3.5 The pathway of centriole formation in the embryo

In conclusion, in this chapter I have analysed the formation and composition of SAPs in three different centriolar mutants. My data allows me to propose the following pathway (Fig.5.14): Sak is most likely the most upstream component in the pathway of centriole assembly, but its role might be specific to the assembly of a cartwheel. Downstream of Sak, a very integral interaction must occur between DSas-6, Ana2 and DSas-4, although the exact nature of this interaction is unknown. DSas-4 is then able to recruit Asl, and Asl is most likely involved in the maintenance of DSas-4 at the centriole. In centriole duplication Asl is also involved in recruiting Sak to the mother centriole to initiate procentriole formation (red arrow).

Chapter 6: Examining centrosome formation during activation

6.1 Summary

Activation is the process by which an oocyte is primed for embryonic development. It includes a variety of processes, from modification of the outer layers of the oocyte to translation of proteins. While in marine invertebrates and vertebrates fertilisation triggers these events, in insects such as *Drosophila* activation and fertilisation are separate, independent events (Doane, 1960). In *Drosophila*, activation takes place during ovulation, as the mature stage 14 oocyte travels through the female reproductive tract to the uterus (Heifetz et al., 2001). Activation is induced by both osmotic stress and by the pressure applied to the oocyte as it travels through the oviduct (Horner and Wolfner, 2008a); these mechanisms allow the entrance of external calcium to the oocyte, even if no sperm fertilises the egg during its passage through the oviduct (Horner and Wolfner, 2008a). If fertilisation does not take place, the activated oocyte, or egg, is laid by the female but it does not develop.

The entrance of calcium in the oocyte during activation is thought to induce signalling pathways that trigger several events. The most obvious change is the swelling of the oocyte. This is accompanied by the cross-linking of

structural components of the vitelline membrane, which makes the egg impermeable to small molecules (LeMosy and Hashimoto, 2000). During *Drosophila* oogenesis, the oocyte enters early prophase I in region 2A of the germarium and arrests in diplotene in stage 5. Meiotic maturation takes place in stage 13, and by stage 14 oocytes are arrested in metaphase I (Von Stetina and Orr-Weaver, 2011). Meiosis is only resumed again upon activation (Doane, 1960). Also during activation some maternal mRNAs start to be translated, while others are repressed or are selectively degraded (Tadros and Lipshitz, 2005). In addition, several maternally deposited proteins are phosphorylated upon activation (Krauchunas et al., 2012).

As is the case in many species, centrosomes and centrioles are somehow inactivated during oogenesis in *Drosophila*. As a consequence, stage 14 oocytes do not contain centrosomes or centrioles. The mechanism by which centrosomes are cleared during oogenesis is currently unknown. It is apparent, however, that this mechanism is incredibly powerful. As mentioned in previous chapters, the overexpression of any of the 5 main centriole duplication players is able to induce centriole overduplication and *de novo* centriole and centriole-like structure formation in many tissues, including in oocytes (Peel et al., 2007; Rodrigues-Martins et al., 2007b; Dzhindzhev et al., 2010). All these supernumerary centrioles and centriole-like structures are also cleared by stage 14 of oogenesis (Peel et al., 2007; Rodrigues-Martins et al., 2007b). However, this clearance mechanism seems to be turned off upon activation: unfertilised, activated eggs overexpressing these same 5 components are now able to generate *de*

novo centrosome and centrosome-like structures (Rodrigues-Martins et al., 2007b).

In this chapter I show that “immature” SAPs (iSAPs) are present in oocytes, but they do not recruit any centrosomal proteins and organise no MTs. Upon oocyte activation, the iSAPs rapidly “mature” to form mSAPs, acquiring the ability to recruit downstream centrosomal components and organise MTs. I hypothesise that SAP maturation might be triggered by a rapid post-translational modification of one of the three core components of the SAPs (DSas-6, Ana2 and DSas-4) during oocyte activation. This putative modification could be the trigger that not only allows SAPs to “mature” upon activation, but that also normally allows a centrosome to form around the fertilising sperm centriole- so over-riding the mechanism that normally inactivates centrioles and centrosomes during oogenesis.

In this chapter I explore two alternative methodologies to identify these potential modifications. As SAPs are able to form in stage 14 oocytes, but are unable to recruit centrosomal proteins, I first examine the possibility of isolating iSAPs from oocytes and comparing post-translational modifications in its centrosomal proteins with those in mSAPs in activated eggs by Mass Spectrometry. I show that this strategy is not immediately feasible because iSAPs are too light to be successfully separated from the cytoplasm using our standard mSAP/centrosome purification protocols. I then explore the possibility of identifying post-translational modifications that may occur on the cytoplasmic fractions of specific centrosomal proteins upon egg

activation. In particular, I develop a methodology that allows me to assess whether specific changes to cytoplasmic DSas-4, Ana2 and DSas-6 occur upon activation. Excitingly, I identify a partial shift in the gel mobility of the protein DSas-4 that occurs specifically upon egg activation. Future work will be required to assess whether this shift is of relevance to the process of centrosome formation post-activation, or indeed to the mechanism of centriole/centrosome inactivation in oocytes. All the work performed in this chapter was done in collaboration with Alan Wainman, who performed the fly crosses.

6.2 Results

6.2.1 SAPs are able to form in oocytes, but can only recruit other centrosomal proteins upon activation

The overexpression of UAS-Sak was shown to promote *de novo* centriole (and centrosome) formation in laid eggs (Rodrigues-Martins et al., 2007b). That same overexpression does not, however, generate *de novo* centrosome formation in late stage oocytes. This observation suggests that the mechanism of centrosome clearance is powerful enough to over-ride even the centriole-forming effect of extra Sak. I was interested in knowing whether, as is the case in *de novo* centrosomes, SAPs are also not able to form in stage 14 oocytes. I therefore imaged live stage 14 oocytes that expressed GFP-DSas-6 and GFP-Ana2 in addition to different centrosomal components tagged with red markers (Fig.6.1). Interestingly I found that SAPs could be observed in stage 14 oocytes. However, they were not able to recruit any RFP-Cnn or nucleate MTs, and they seem to contain only very low levels of RFP-DSas-4 when compared with SAPs formed in laid eggs. From here onwards I will therefore refer to SAPs formed in oocytes as immature SAPs (iSAPs) and SAPs formed in laid eggs as mature SAPs (mSAPs).

It seems that these iSAPs can “mature” very quickly after egg activation into mSAPs that organise PCM proteins and MTs- after a 30 minute collection of

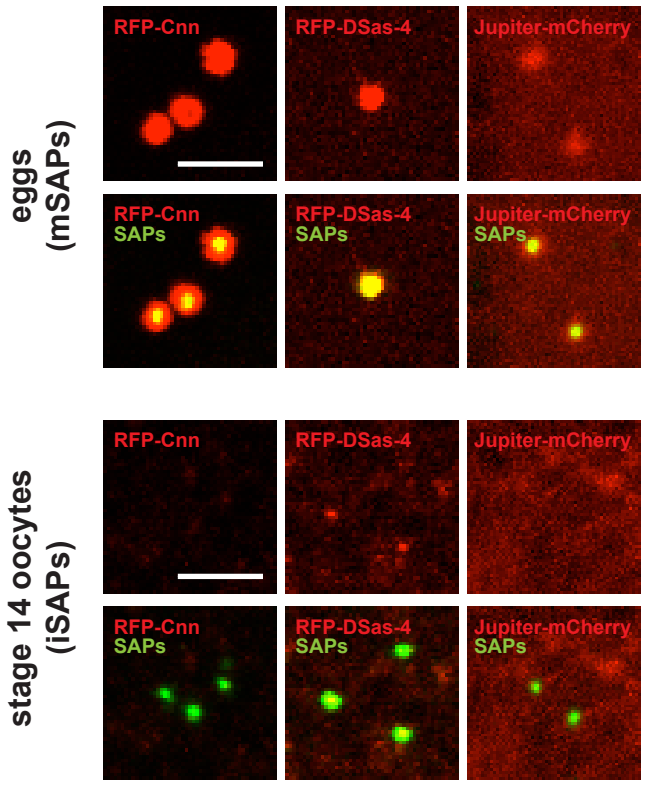


Figure 6.1 SAPs resemble centrosomes in eggs but not in stage 14 oocytes
Panels show still images taken from movies of SAPs (green) in living eggs and stage 14 oocytes expressing different centrosomal proteins tagged with fluorescent proteins (red), as indicated. Note how in eggs SAPs recruit Cnn and DSas-4 and can nucleate MTs, while in stage 14 oocytes none of these components are recruited robustly, although there is some weak recruitment of RFP-DSas-4. Scale bar=5μm.

eggs expressing GFP-DSas-6 and GFP-Ana2, nearly 90% of eggs already have mSAPs that can organise MT asters (Fig.6.2). I wondered whether this process is so quick because mSAPs do not form *de novo* upon activation, but rather mature directly from the iSAPs present in stage 14 oocytes. To examine this process I therefore imaged stage 14 oocytes expressing GFP-DSas-6 and GFP-Ana2, as well as RFP-Cnn, that were incubated in activation buffer. As shown in Fig.6.3, the iSAPs do not disassemble upon activation, but rather start recruiting RFP-Cnn as the oocyte transitions to an activated egg. I conclude that iSAPs directly mature into mSAPs during activation, acquiring the ability to recruit other centrosomal components as they do so.

6.2.2 Immature SAPs cannot be feasibly isolated from stage 14 oocytes

The rapid conversion of iSAPs to mSAPs upon egg activation raised the possibility that one or more of the components essential for centriole/centrosome assembly might be rapidly modified upon activation, most likely via a post-translational modification, allowing mSAP assembly. In particular, as SAPs in oocytes appear to be formed only of DSas-6, Ana2 and DSas-4, these three proteins are excellent candidates for this modified component(s). One way to identify these modifications would be by isolating iSAPs and mSAPs, and comparing the migration behaviour of their DSas-6, Ana2 and DSas-4 on an SDS gel. Any shifts observed could indicate an important activation post-translational modification, as modifications such as

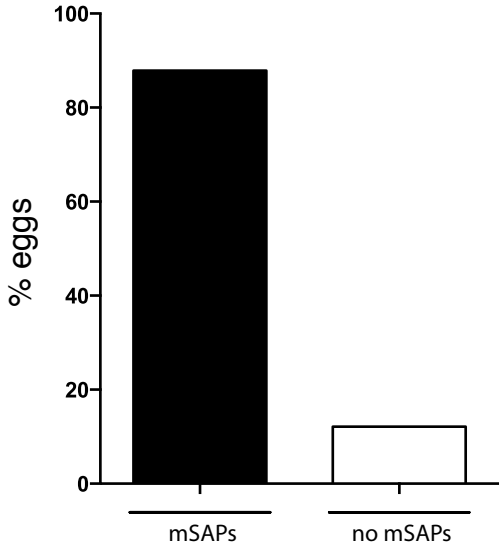


Figure 6.2 mSAPs form very quickly upon egg activation
Graph shows the percentage of eggs containing mSAPs (black) or no mSAPs (white) after a 30 minute collection of eggs expressing GFP-DSas-6, GFP-Ana2 and Jupiter-mCherry. Presence of mSAPs was scored based on SAPs' ability to nucleate MT asters. n=33 eggs.

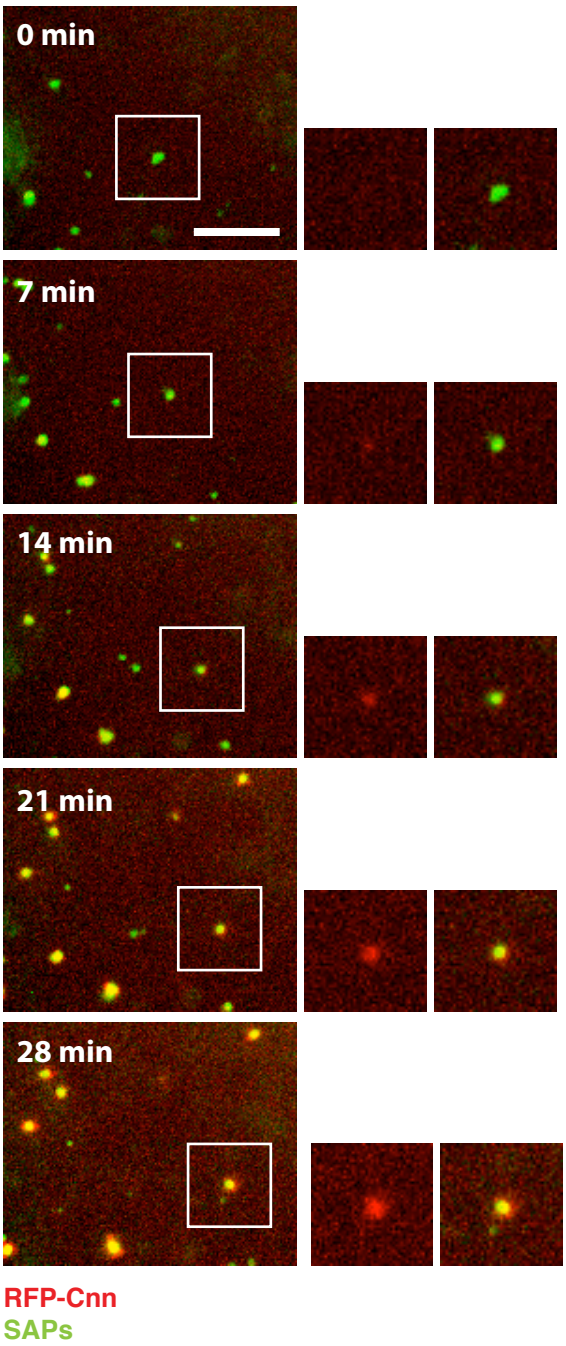


Figure 6.3 SAPs start recruiting Cnn at, or soon after, egg activation
Panels show still images taken from movies of stage 14 oocytes expressing Ubq-GFP-DSas-6 and Ubq-GFP-Ana2 (green) and RFP-Cnn (red), during incubation in activation buffer. Note that SAPs do not appear to assemble *de novo* on activation, but rather the immature SAPs (iSAPs) present in oocytes seem to gradually acquire the ability to recruit RFP-Cnn. Smaller images on the right of the larger panels correspond to magnified views of the areas within the white boxes. Time in minutes is indicated in the main panels. Scale bar=10μm.

phosphorylation and ubiquitination are known to affect protein migration rates in these gels. The proteins could then be isolated and the modification potentially identified by Mass Spectrometry.

As described in Chapter 3, SAPs (mSAPs) can be easily separated from the cytoplasm using sucrose gradients as a fractionation method. I therefore assessed the feasibility of isolating iSAPs from stage 14 oocytes using a similar method. Stage 14 oocytes can be isolated by manual dissection from ovaries. However this process is very time-consuming, and therefore not feasible to obtain the large quantities of material required for Mass Spectrometry analysis. The alternative method described in the literature involves the homogenisation of large numbers of females, and successive filtration of the resulting material with meshworks of different sizes (Page and Orr-Weaver, 1997). I attempted this method several times, and although the final sample was enriched for stage 14 oocytes, it also included a significant amount of additional tissue, including activated eggs. As a consequence, this final sample was significantly contaminated with centrosomes, making this protocol inadequate to isolate iSAPs from stage 14 oocytes for subsequent analysis.

6.2.3 *sarah* mutant females lay eggs that fail to activate properly and iSAPs are not converted to mSAPs

The process of activation involves a variety of signalling pathways. Different mutants affecting the activation process have been identified. These mutant females usually lay eggs that can partially activate as, for example, they can swell and harden their vitelline membrane, but they do not fully activate and in other aspects they are very similar to stage 14 oocytes. These partially activated eggs can therefore be collected and processed in a similar way to WT eggs, but biochemically retain some of the properties of stage 14 oocytes. I therefore explored the possibility of using activation mutant eggs instead of stage 14 oocytes for biochemical work.

I first examined *cortex* mutants. Cortex is a cdc20/cdh1-related protein (Swan and Schupbach, 2007). It is thought to form a complex with the APC complex and to be involved in meiosis progression (Chu et al., 2001; Pesin and Orr-Weaver, 2007). *cortex* mutant females lay eggs that are morphologically very similar to WT eggs. However, several aspects of activation are disrupted in the absence of Cortex. *cortex* eggs arrest in meiosis II and the MTs within *cortex* eggs resemble those of stage 14 oocytes (Lieberfarb et al., 1996; Page and Orr-Weaver, 1996). Normal activation-dependent RNA processing is also disrupted (Lieberfarb et al., 1996; Bashirullah et al., 1999).

In order to assess whether *cortex* mutant eggs are similar to stage 14 oocytes for the purpose of my analysis, I examined whether SAPs behave like centrosomes in the absence of cortex. I expressed GFP-DSas-6 and GFP-Ana2 in $cor^{[qw55]}/cor^{df}$ laid eggs. For comparison purposes, I also looked at SAPs formed in a WT background. As *cortex* mutant eggs arrest in meiosis II, I first analysed SAP composition in fertilised eggs (Fig.6.4A). Interestingly, I found that SAPs formed in the absence of cortex are similar to mSAPs, as they are still able to recruit PCM components, such as Cnn, and can organise robust MT asters.

I wondered whether the reason why SAPs behaved like WT in the *cortex* mutant might be due to the fact that I had analysed their composition in fertilised eggs. The *cortex* mutant allele used is only a hypomorph, with low levels of cortex mRNA still present, and a low percentage of eggs has been reported to complete meiosis (Chu et al., 2001). I therefore examined the composition of SAPs in a *cortex* mutant background in unfertilised eggs. In this context *cortex* mutant SAPs also recruited centriolar and centrosomal components, i.e. resembled mSAPs (Fig.6.4B). I conclude that SAPs behave like WT in *cortex* laid eggs, and as such *cortex* mutant eggs are not good surrogates of stage 14 oocytes for my iSAP analysis.

I next examined *sarah* mutants. Sarah is a calcipressin protein, an inhibitor of the calcium-dependent phosphatase calcineurin (Takeo et al., 2006; Takeo et al., 2010; Takeo et al., 2012). *sarah* mutant eggs fail in certain aspects of activation. The eggs remain arrested in meiosis I, show elevated

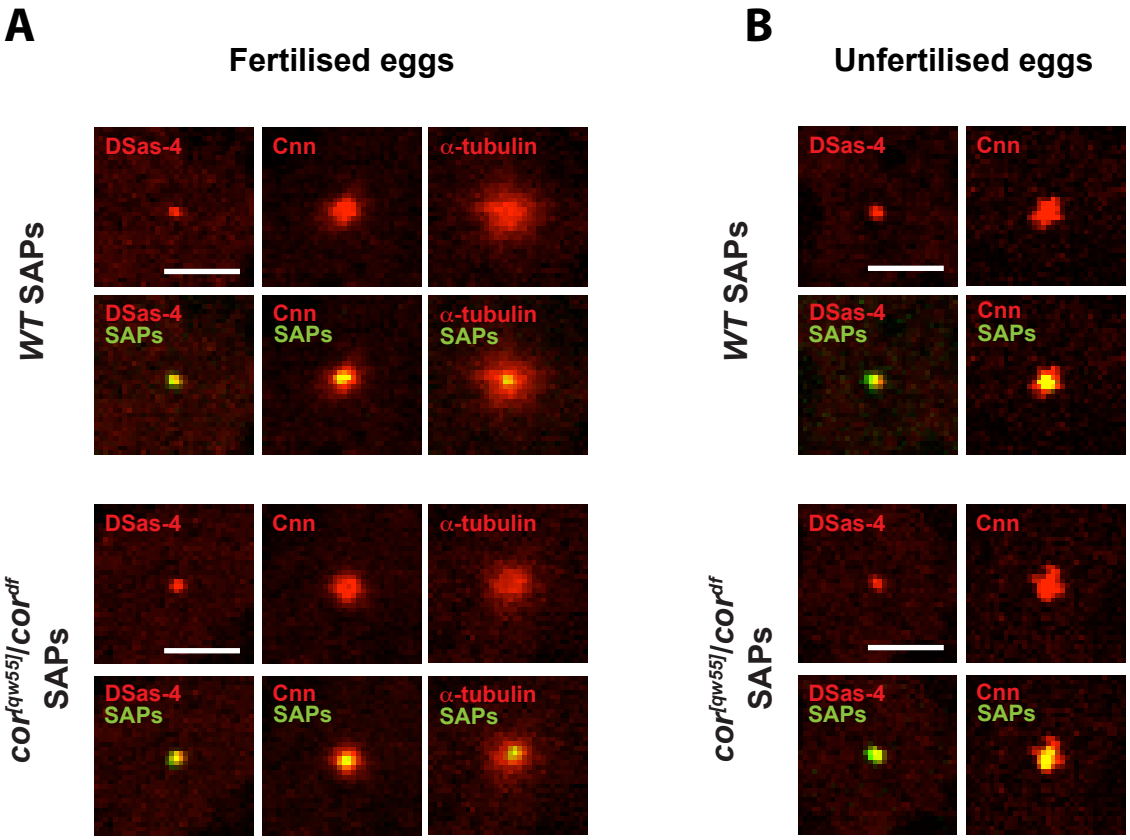


Figure 6.4 SAP composition in *cor*$^{[qw55]}$/*cor*^{df} mutant eggs
An analysis of SAP composition in **(A)** fertilised and **(B)** unfertilised eggs expressing Ubq-GFP-DSas-6 and Ubq-GFP-Ana2 in either a WT or a *cor*$^{[qw55]}$/*cor*^{df} background, stained with antibodies for different centrosomal proteins, as indicated (n>15). Note how *cor*$^{[qw55]}$/*cor*^{df} SAPs behave as WT (mSAPs). In these figures the WT SAPs were obtained from unfertilised eggs. Scale bar=5μm.

levels of cyclin B and show several defects in RNA processing (Horner et al., 2006; Takeo et al., 2006). However, and like *cortex* mutant eggs, *sarah* mutant flies are able to complete certain aspects of activation, as laid eggs also swell and show hardening of the outer layers (Horner et al., 2006; Takeo et al., 2006).

I examined whether the SAPs formed in eggs laid by *sarah* mutant females resembled those formed in stage 14 oocytes. I expressed GFP-DSas-6 and GFP-Ana2 in sra^{KO}/sra^{A426} laid eggs. Both these mutant alleles have been shown not to produce any protein (Takeo et al., 2006; Horner et al., 2006). For comparison purposes, I also looked at SAPs formed in a WT background. Excitingly, SAPs were present in *sarah* mutant laid eggs, but they did not recruit detectable levels of centriolar components such as Asl and DSas-4, nor PCM components such as Cnn and DSpd-2 (Fig.6.5A). This is similar to what I observed in stage 14 oocytes, although in WT stage 14 oocytes very low levels of DSas-4 were detectable in the iSAPs. It is important to note, however, that in the iSAP analysis shown in Fig.6.1A, I assessed the localisation of RFP-DSas-4 in live samples, while the SAP analysis in the *sarah* mutant background shown in Fig.6.5A was performed in fixed samples using antibody staining. It is possible that the difference observed is due to a weaker signal of the anti-DSas-4 antibody in the fixed samples, when compared to the signal strength of RFP-DSas-4 in live samples. It is therefore vey likely that SAPs in *sarah* mutant, as is the case in iSAPs, contain very low levels (undetectable in fixed samples) of DSas-4.

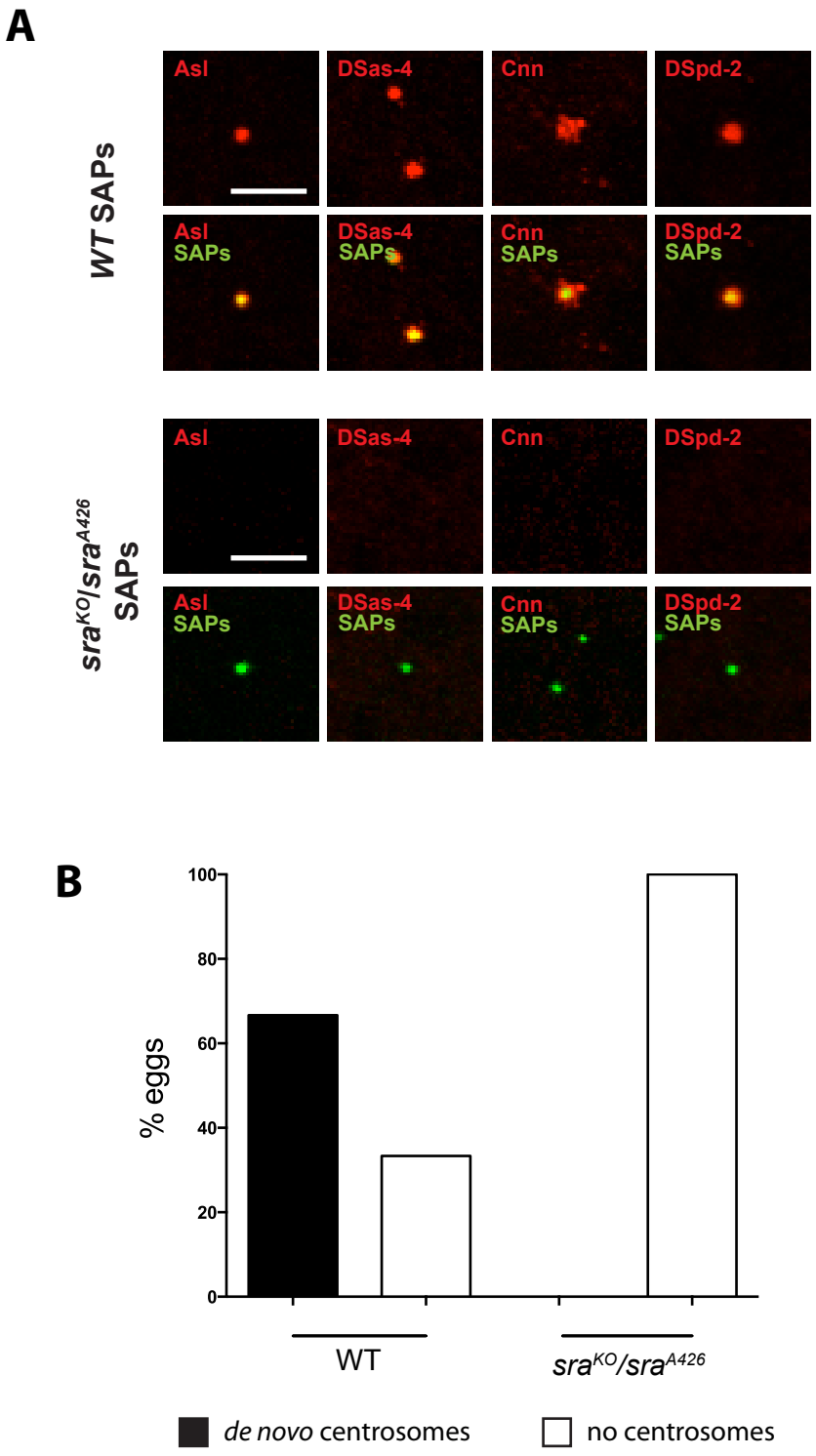


Figure 6.5 Analysis of SAPs and *de novo* formed centrosomes in sra^{KO}/sra^{A426} eggs
(A) An analysis of SAP composition in eggs expressing Ubq-GFP-DSas-6 and Ubq-GFP-Ana2 in either a WT or a sra^{KO}/sra^{A426} background. Representative images of WT SAPs (mSAPs) and sra^{KO}/sra^{A426} SAPs (n=20 eggs). SAPs stained with antibodies for different centrosomal proteins, as indicated. Note how SAPs in a *sarah* mutant background seem to have not matured and resemble iSAPs. Scale bar=5μm. **(B)** Graphs showing the percentage of eggs that contained *de novo* formed centrosomes (black) and that did not contain *de novo* centrosomes (white) in eggs expressing UAS-Sak in a WT (n=75 eggs) and in a sra^{KO}/sra^{A426} (n=50 eggs) background.

Future analysis of RFP-DSas-4 in *sarah* mutant SAPs should be able to confirm this conclusion.

To further confirm that *sarah* eggs are indeed similar to stage 14 oocytes, I also assessed the ability of *de novo* centrosomes to form in the absence of *sarah.* I therefore expressed UAS-Sak in sra^{KO}/sra^{A426} eggs. In a 0-3 hour collection, 60% WT eggs contained *de novo* centrosomes. In eggs lacking *sarah,* however, no *de novo* centrosomes were detected (Fig.6.5B). This is identical to the case in stage 14 oocytes, where *de novo* centrosomes could not be detected upon Sak overexpression (Rodrigues-Martins et al., 2007b). I conclude that, as far as centrosome and SAP formation is concerned, *sarah* mutant eggs are good models for stage 14 oocytes. *sarah* mutant eggs can therefore be used instead of stage 14 oocytes for biochemical analysis of iSAPs.

6.2.4 Immature SAPs cannot be isolated using a sucrose gradient

I showed in the previous section that *sarah* mutant eggs resemble stage 14 oocytes as far as their ability to form centrosomes and SAPs is concerned. Unlike stage 14 oocytes, however, *sarah* mutant eggs are laid and undergo certain activation processes, such as outer layer hardening. As such *sarah* mutant eggs can undergo the same biochemistry protocols as WT laid eggs.

I tested whether it would be possible to isolate iSAPs from *sarah* mutant eggs using the sucrose gradient protocol described in Chapter 3. Briefly, an egg extract is loaded on top of a 55% and 70% sucrose cushion. Ultracentrifugation then forces the heavy SAP particles to move towards the bottom of the tube, moving through the 55% cushion and eventually getting "trapped" in the 70% sucrose cushion. The lighter cytoplasmic components remain in the top layer of the tube as they are not heavy enough to be forced through the 55% sucrose cushion. I performed this protocol in eggs expressing GFP-DSas-6 and GFP-Ana2 in a WT or in a sra^{KO}/sra^{A426} background. I then obtained fractions along the sucrose gradient, as shown in the diagram in Figure 6.6A. These fractions were then run in a gel for western blotting, and the membrane was blotted for a variety of proteins (Fig.6.6B).

I first confirmed that the sucrose gradient had successfully separated the cytoplasm from heavier components of the eggs by blotting the membrane for actin and for α-tubulin, which, under the conditions in the extract, would have been in their "light" monomeric and dimeric (with β-tubulin) forms, respectively. As expected, actin and α-tubulin (two bottom panels of Fig.6.6B) are only detected in the input (first lane) and cytoplasmic fractions (last lane) in both WT and sra^{KO}/sra^{A426} eggs (Fig.6.6B). I then blotted the membranes with antibodies against DSas-6 and Ana2, in order to identify which fractions contained SAPs (top two panels of Fig.6.6B). As expected, WT SAPs containing DSas-6 and Ana2 were present in the heaviest fractions. Unexpectedly, however, DSas-6 and Ana2 were not detected in

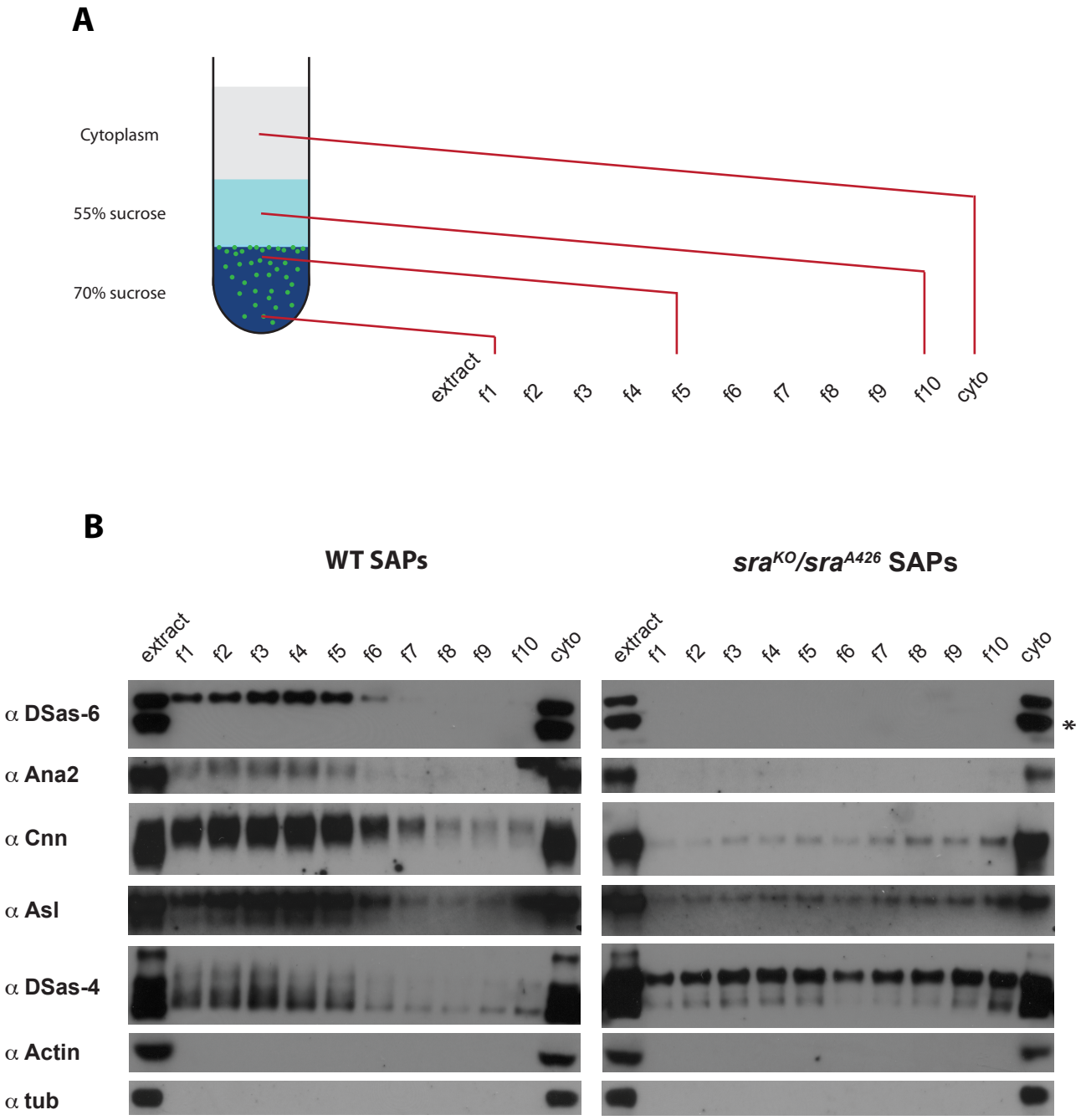


Figure 6.6 SAPs formed in *sra^{ko}/sra^{A426}* eggs are too light to be separated from the cytoplasmic fraction

(A) Schematic representation of a sucrose step-gradient (as described in Fig.3.3A) indicating the regions corresponding to the different fractions shown in B. **(B)** Western blot of fractions obtained from a sucrose step-gradient fractionation of extracts from eggs expressing GFP-DSas-6 and GFP-Ana2 in a WT background (left) or in a *sra^{ko}/sra^{A426}* background (right). Antibodies used for blotting are indicated on the left of the panels. Note how no SAPs can be identified in the heavy fractions of the *sra^{ko}/sra^{A426}* eggs, indicating that the SAPs in these eggs are too light to be separated from the cytoplasmic fractions using this fractionation method. Also note how DSas-4 in a *sarah* mutant background migrates slower than DSas-4 present in mSAPs in a WT background. Asterisk indicates lower weight background band detectable upon blotting with anti-DSas-6 antibodies.

any of the heavy *sra*^{KO}/*sra*^{A426} fractions loaded. This absence of signal is not due to the absence of DSas-6 and Ana2 in the mutant extracts, as DSas-6 and Ana2 can be detected in both the extract and cytoplasmic fraction lanes. I also blotted the membranes for other centrosomal components: Cnn, Asl and DSas-4 (Fig.6.6B). As expected, a stronger signal for these 3 proteins could be detected in those fractions shown to contain SAPs in the WT eggs. In *sra*^{KO}/*sra*^{A426} eggs, however, no specific enrichment of these proteins could be detected in the heavy fractions. DSas-4, however, exhibited an unusual behaviour in the mutant extracts as it was present in all of the fractions. Moreover, I noticed that the major form of DSas-4 in these fractions also seemed to run more slowly on the SDS gels than the major form of DSas-4 found in the WT SAPs (Fig.6.6B).

In conclusion, the SAPs formed in *sra*^{KO}/*sra*^{A426} eggs (that do not seem to mature upon egg activation) cannot be isolated using our standard sucrose fractionation protocol, because these SAPs appear to be too light to sediment into the higher concentration sucrose layers. This is most likely because SAPs in *sarah* mutants have not matured, and so have not recruited the centriole and centrosomal components that presumably make the mSAPs heavy.

6.2.5 Dsas-4 is the only centrosomal protein studied that changes its gel mobility in the cytoplasm upon activation

As *sarah* mutant SAPs cannot be isolated from the cytoplasm using a sucrose gradient, it is not possible to isolate iSAPs, and hence compare the post-translational modifications in DSas-6, Ana-2 and DSas-4 in iSAPs and mSAPs. However, I thought it likely that the post-translational modifications that allow centrosome formation upon egg activation also modify the proteins that are present in the cytoplasm (as SAPs are not normally present in eggs that are being activated). I therefore decided to compare the behaviour of centrosome proteins in the cytoplasm before and after activation.

First I wanted to determine whether stage 14 oocytes contain all the centrosomal proteins that are present in eggs and in embryos. I therefore made extracts from stage 14 oocytes, activated eggs and fertilised embryos from WT females and probed these by western blotting with antibodies raised against several centrosomal proteins. As shown in Fig.6.7A, all the centrosomal proteins tested were present in stage 14 oocytes, in eggs and in embryos. Interestingly, a slight increase in protein levels seems to be seen for most centrosomal proteins upon activation (in relation to the actin loading control), although the extent of this increase will have to be precisely determined in the future using quantitative western blotting. This increase in levels suggests that new translation of these proteins, for example from mRNA deposited in the oocytes, may be triggered by activation, as is the

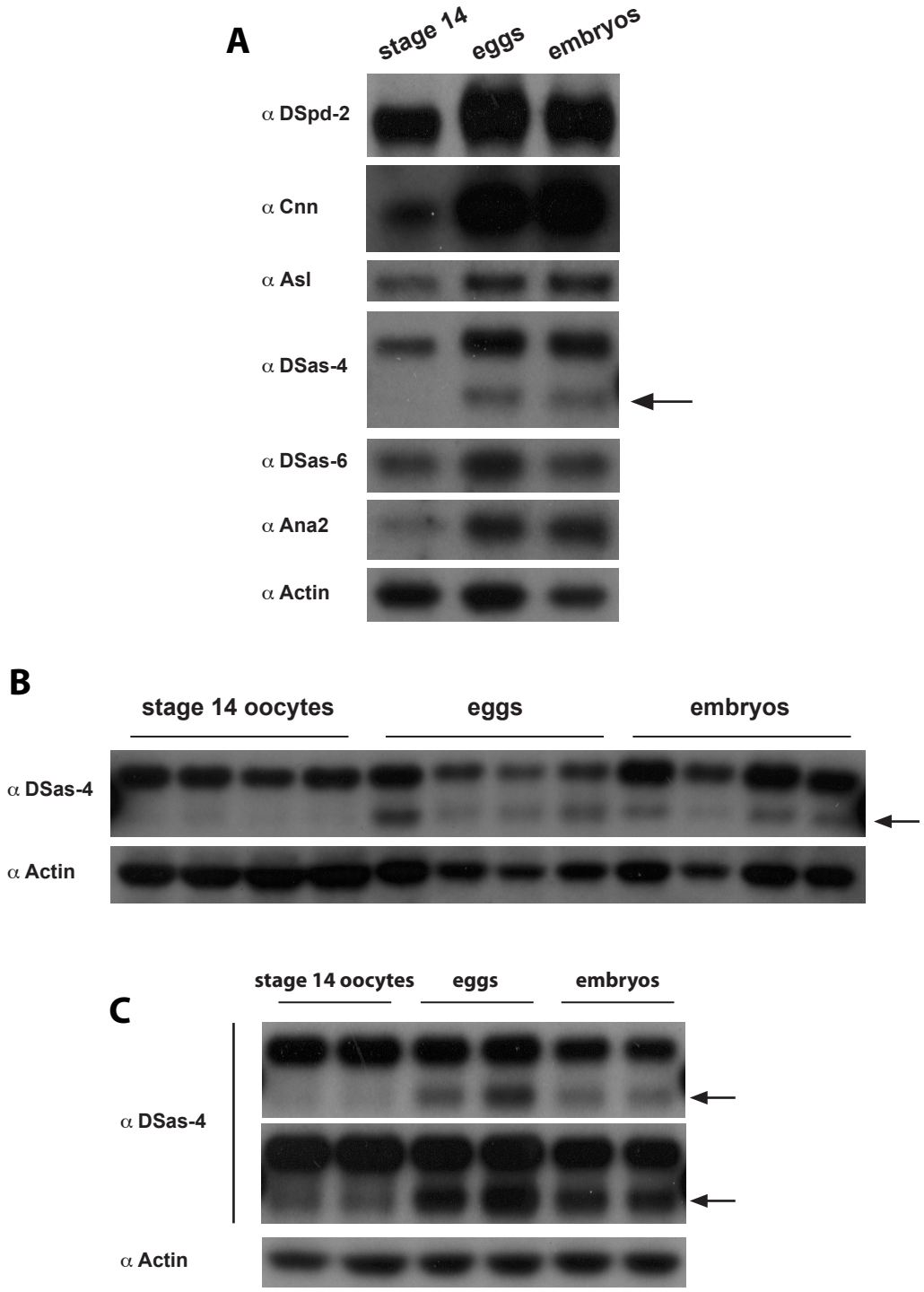


Figure 6.7 A comparison of centrosomal protein behaviour in stage 14 oocytes, eggs and embryos

(A) Western blot of WT stage 14 oocytes, eggs and embryos. Antibodies used for blotting are indicated on the left of the panels. Note how all the centrosomal proteins tested are present in all 3 samples, with an increase in protein levels after activation apparent in some cases. Also notice that of all proteins tested, only DSas-4 obviously changes its behaviour upon activation, with a fraction of the protein shifting to a lower MW band upon activation (arrow). **(B)** Western blot of several different preparations of WT stage 14 oocytes, eggs and embryos blotted for the proteins indicated. Note that the eggs and embryos shown here were fixed in methanol/heptane before processing for western blotting. **(C)** Western blot of several individual preparations of WT stage 14 oocytes, eggs and embryos blotted for DSas-4 and actin (2 different exposures are shown for DSas-4). Note that the eggs and embryos shown here were not fixed before processing for western blotting. Note how in all tested cases shown in B and C the partial shift of DSas-4 upon activation is observed.

case with other proteins (Krauchunas and Wolfner, 2013). It should be noted that the egg and embryo samples used corresponded to 0-3 hour collections. As zygotic transcription in *Drosophila* initiates ~2 hours after embryo development starts (McKnight and Miller, 1976), the increase I observe may instead correspond to this later zygotic expression. Whatever the mechanism, however, all the key centriole and centrosome proteins that I have examined here are clearly present in stage 14 oocytes. Though these data cannot exclude the possibility that it is an increase in protein translation that allows centrosome formation, an increase in levels is unlikely to be fast enough to explain how centrosomal formation occurs so quickly upon egg activation.

Stage 14 oocyte and egg samples contain no centrosomes. As such, comparison of protein behaviour at these two stages can provide a clue to their behaviour upon activation in the cytoplasm. Only one of the proteins blotted for in this gel showed an unusual behaviour upon activation: DSas-4 (Fig.6.7A). While in stage 14 oocytes DSas-4 is present as a single band, upon activation a fraction of the protein shifts to a lower weight band (arrow). Although this is a subtle difference, I thought it potentially interesting as my previous analysis of DSas-4 behaviour in WT and *sarah* mutants had revealed that a faster migrating form of DSas-4 seemed to be specifically enriched in WT SAPs, and this form was greatly reduced in the *sarah* mutants (Fig.6.6B)

I wanted to determine whether this partial shift in DSas-4 mobility upon egg activation was reproducible. I therefore collected 4 samples of stage 14 oocytes, eggs and embryos in different occasions and examined them by western blotting. As shown in Fig.6.7B, in all the samples examined the behaviour of DSas-4 was the same as described before: in stage 14 oocytes it is present as a single band, that partially shifts into a lower weight band upon activation, and remains as a double band even after fertilisation. All these comparisons were made using eggs and embryos that had been dechorionated and fixed in methanol/heptane before being processed for western blotting. The stage 14 oocyte samples, however, had not been dechorionated nor fixed. I wondered whether this double band was an artefact of fixation. I therefore prepared new egg and embryo samples that were processed for western blotting in a similar way to stage 14 oocytes, without any fixation. Even when no fixation takes place, DSas-4 is still present as a double band in eggs and embryos (Fig.6.7C). I conclude that DSas-4 partially, but reproducibly, shifts to a higher gel mobility form upon activation.

6.2.6 Centrosomal proteins in the cytoplasm of *sarah* mutant eggs behave like proteins in the cytoplasm of stage 14 oocytes

In *sarah* mutant eggs iSAPs fail to mature into mSAPs (Fig.6.5), so I wondered whether the faster migrating form of DSas-4 might also fail to be generated in these mutant eggs. I therefore compared the behaviour of

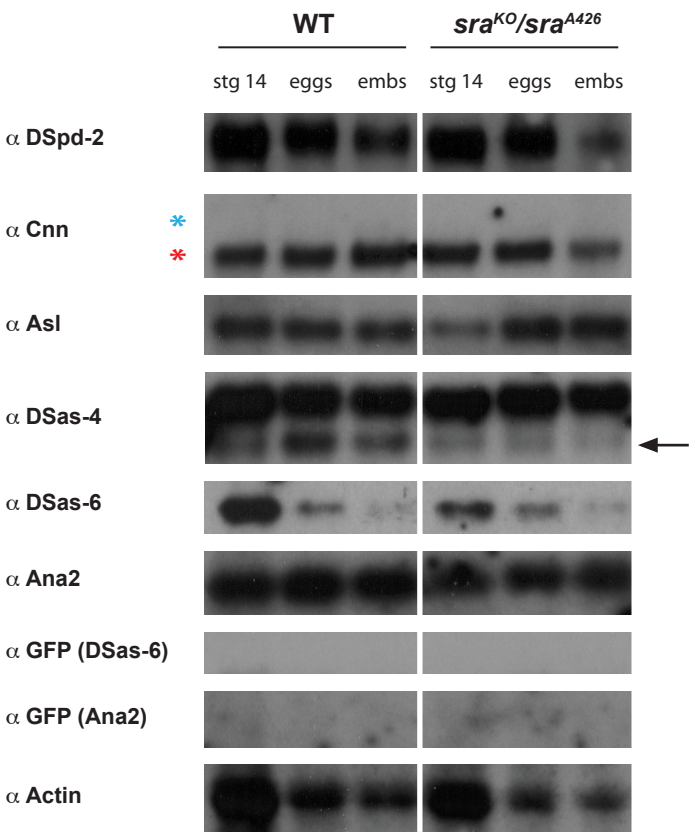


Figure 6.8 Centrosomal protein composition in stage 14 oocytes, eggs and embryos without SAPs

Western blot of stage 14 oocytes, eggs and embryos in a WT (left panel) and *sra*^{KO}/*sra*^{A426} (right panel) background. Antibodies used for blotting are indicated on the left of the panels. Note how protein behaviour in *sra*^{KO}/*sra*^{A426} stage 14 oocytes, eggs and embryos mirrors that of WT stage 14 oocytes. Red asterisk indicates migration position of the cytoplasmic Cnn form, while blue asterisk indicates migration position of the centrosome-specific Cnn form. Arrow indicates the position of the activation-specific DSas-4 migrating band.

several centriole and centrosome proteins in stage 14 oocytes, activated eggs and fertilised embryos laid by WT and *sarah* mutant females by western blotting (Fig.6.8). Most of these proteins behaved very similarly to WT in the *sarah* mutant samples, but I noticed that the faster migrating form of DSas-4 was much less pronounced in eggs and embryos in the *sarah* mutants. This suggested that this form of DSas-4 was not being formed properly in these mutant eggs that fail to activate properly (arrow, Fig.6.8).

In these blots, I noticed that I could not detect the shift in mobility of Cnn or DSpd-2 that occurs exclusively in centrosomes or SAPs (shown in Fig.3.3). This is not surprising in oocytes or eggs (where no centrosomes are formed), but I thought that it might have been possible to detect this shift in fertilised embryos, where lots of centrosomes are being formed. I realised that I might be able to detect this shift if I drove SAP formation in eggs or embryos—by driving the formation of many hundreds of SAPs I would drive PCM formation and so a greater proportion of the Cnn and DSpd-2 should be modified (and this shift would not occur in oocytes, as they do not form mSAPs). I reasoned that if the shift in DSas-4 mobility was in some way related to the ability of DSas-4 to participate in centrosome or SAP maturation, then driving SAP formation might also drive the formation of the modified form of DSas-4 that normally occurs upon egg activation.

Excitingly, I found that this was indeed the case (Fig.6.9). In the WT situation, driving the formation of SAPs drove the formation of the modified form of Cnn in eggs and embryos (but not oocytes, as expected), and also

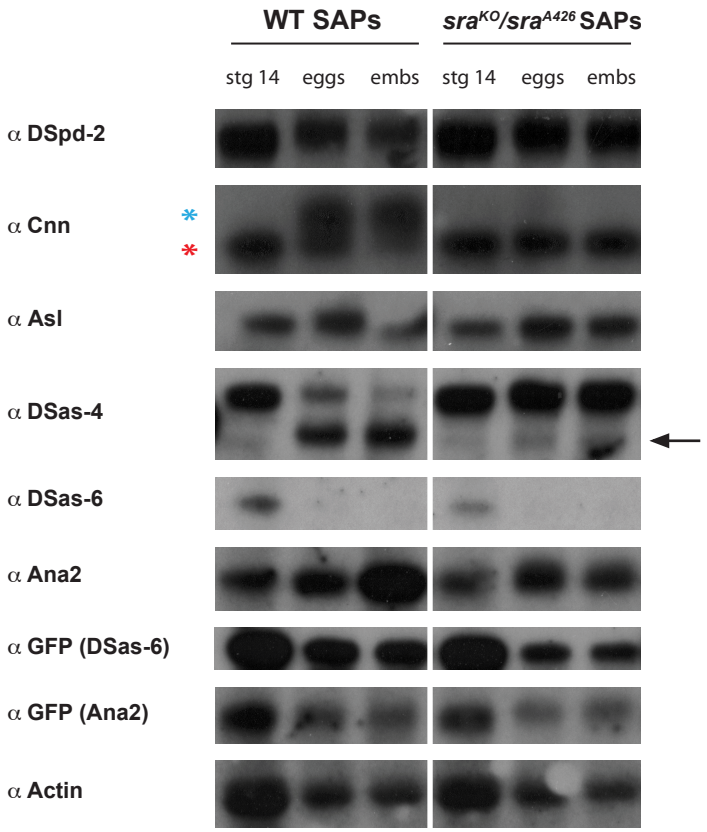


Figure 6.9 Centrosomal protein composition in stage 14 oocytes, eggs and embryos containing SAPs

Western blot of stage 14 oocytes, eggs and embryos expressing GFP-DSas-6 and GFP-Ana2 in a WT (left panel) and *sra*^{KO}/*sra*^{A426} (right panel) background. Antibodies used for blotting are indicated on the left of the panels. Note how protein behaviour in *sra*^{KO}/*sra*^{A426} stage 14 oocytes, eggs and embryos is very similar to those in WT stage 14 oocytes. Also note that in the presence of SAPs, the majority of Cnn migrates in its centrosome-specific form, while no such change in migration is observed for DSpd-2. Red asterisk indicates migration position of the cytoplasmic Cnn form, while blue asterisk indicates migration position of centrosome-specific Cnn form. Arrow indicates the position of the activation-specific DSas-4 migrating band.

drove the formation of the modified form of DSas-4, which now became the dominant form of the protein in the extract. Even more excitingly, I could not detect the formation of the modified form of either Cnn or DSas-4 in the *sarah* mutant eggs or embryos, thus confirming that the production of both forms was dependent on proper egg activation and the proper maturation of iSAPs to mSAPs. I could detect no difference in the migration of any of the other proteins (including DSpd-2, presumably because only a small fraction of this protein is converted to the slower migrating form in the PCM, whereas essentially all the Cnn is converted to the slower migrating form in the PCM- see Fig.6.9).

These observations provide a tantalising hint that the modification of DSas-4 that occurs upon egg activation may indeed be linked to the ability of DSas-4 to participate in the maturation of iSAPs to mSAPs (and so, by analogy, to the process that normally allows centrosomes to form in activated eggs but not stage 14 oocytes). Moreover, my observation that the maturation of iSAPs to mSAPs does not occur in *sarah* mutants means that I now potentially have a way to biochemically analyse the nature of this modification (and to potentially look for the modification of other key proteins). In theory, any modification that is required for iSAP maturation to mSAPs will occur in WT eggs, but not in *sarah* mutant eggs.

6.2.7 Developing a methodology to identify the post-translational modifications that may occur to cytoplasmic centrosome proteins during activation

In the previous sections I showed that all tested centrosome proteins are present in stage 14 oocytes and in eggs, and that it is therefore very likely that upon activation one (or several) of these proteins is modified in such a way that centrosome formation becomes possible. My analysis of the pathway of centriole assembly suggests that iSAPs containing DSas-6, Ana2 and some DSas-4 can form in stage 14 oocytes, but these cannot recruit any PCM components or organise any MTs until the eggs have been activated. These three proteins are all therefore excellent candidates for proteins that might be modified in some way to allow mSAPs (and so, by analogy, centrosomes) to form upon egg activation. As described above, DSas-4 seems a particularly attractive candidate as a fraction of this protein is modified upon egg activation, and I have some evidence that this modification may be linked to the formation of mature SAPs (see Chapter 5). I therefore decided to concentrate my efforts in DSas-4, DSas-6 and Ana2.

I reasoned that if I could obtain enough DSas-4, Ana2 and DSas-6 from WT and *sarah* mutant egg extracts I should be able to compare these proteins by Mass Spectrometry, and so potentially identify any modifications that occur in WT (where the extracts are competent for mSAP/centrosome assembly) but not in *sarah* mutants (where the extracts are not competent for mSAP/centrosome assembly). From previous experience in the lab (Paul

Conduit, Janina Baumbach, personal communication) I knew that most centriole duplication proteins are expressed at very low levels in embryos, making this sort of analysis very challenging. I reasoned, however, that if I overexpressed each of these three proteins individually at high levels, and then concentrated them by co-immunoprecipitation, I might be able to obtain enough material to perform this analysis.

I decided to overexpress these proteins using a UAS promoter driven by the female germline driver V32 as this gives very high levels of expression in the oocyte and egg/embryo (Peel et al., 2007; Stevens et al., 2010a). In order to standardise the procedures, and to facilitate subsequent co-immunoprecipitation, I expressed GFP-tagged versions of these proteins. As an essential first step, I confirmed that the GFP tagged versions of DSas-4, Ana2 and DSas-6 behaved upon activation in a similar way to their untagged counterparts. I expressed UAS-DSas-4-GFP, UAS-Ana2-GFP and UAS-GFP-DSas-6 in a WT background and collected stage 14 oocytes, eggs and embryo samples for western blot analysis. It is important to note that the overexpression of these proteins does not generate centrosome or MTOC formation in stage 14 oocytes, suggesting that the “inactivation” mechanism is very efficient even in the presence of excess DSas-4, Ana2 or DSas-6. As shown in Fig.6.10A, even when highly overexpressed, DSas-4-GFP partially shifts to a faster migrating form upon egg activation. As was the case for endogenous Ana2 and DSas-6, no shift was observed upon activation for the highly overexpressed Ana2-GFP or GFP-DSas-6

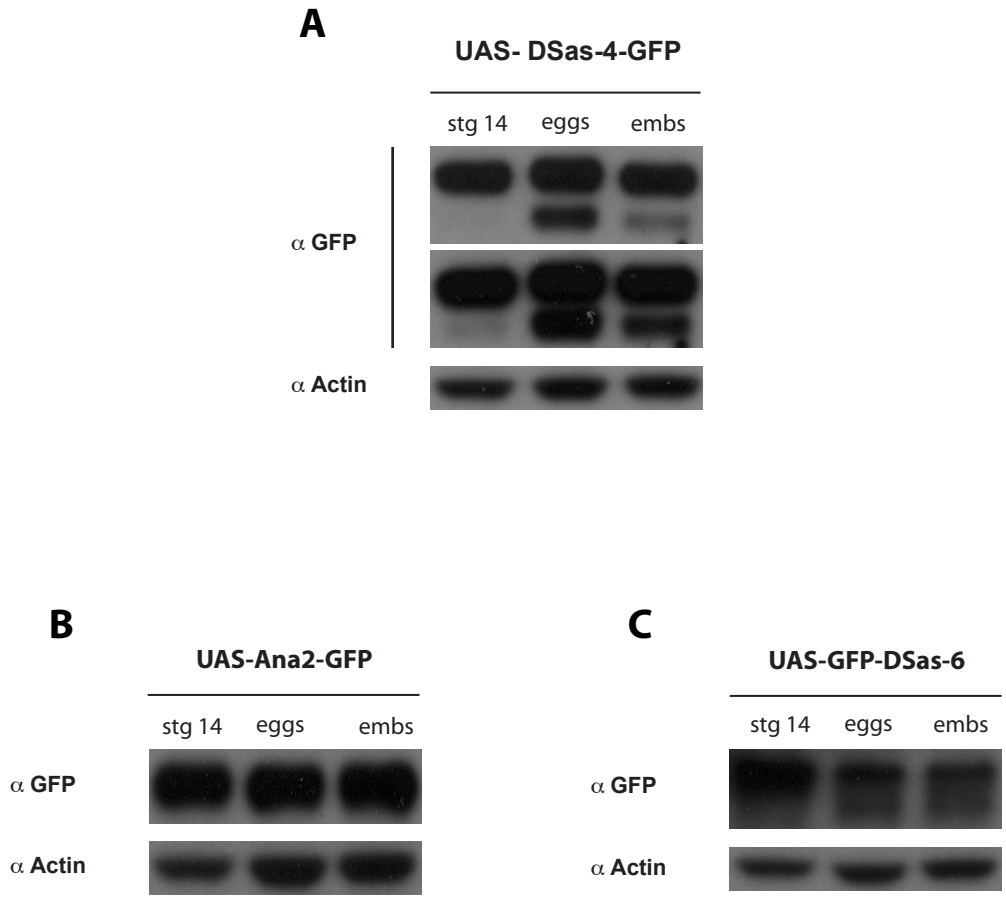


Figure 6.10 UAS DSas-4-GFP, Ana2-GFP and DSas-6-GFP expression
Western blot of stage 14 oocytes, eggs and embryos expressing **(A)** UAS-DSas-4-GFP **(B)** UAS-Ana2-GFP and **(C)** UAS-DSas-6-GFP, blotted with antibodies against GFP and actin (two exposures for DSas-4 blot). Note how DSas-4-GFP is the only protein showing a clear shift in behaviour upon activation, as previously shown.

(Fig.6.10B and C). I am therefore confident that I can use these overexpressed tagged proteins in my analysis.

I have now collected WT and *sra*^{KO}/*sra*^{A426} eggs overexpressing DSas-4-GFP, Ana2-GFP and GFP-DSas-6. In the future it will be interesting to co-immunoprecipitate these proteins from both extracts and to use Mass Spectrometry to try to compare the extent of any potential modifications that might occur upon egg activation.

6.3 Discussion

In this chapter I was interested in examining how egg activation is linked to centrosome formation. During oogenesis in flies, centrosomes are normally inactivated, and no centrosomes are detectable by stage 14 (Fig.6.11A and B). However, several observations suggest that after egg activation something changes in the cytoplasm to allow subsequent centrosome formation. This normally occurs around the centriole brought into the egg with the fertilising sperm (Fig.6.11A), but can also occur around the *de novo* centrioles generated by the overexpression of Sak (Fig.6.11C), or around the SAPs generated by the over-expression of GFP-DSas-6 and GFP-Ana2 (Fig.6.11D). These observations suggest that, if a suitable nucleation platform is present, centrosome formation will occur once the egg has been activated.

The change that allows centrosome formation upon egg activation is likely to occur quickly. While *de novo* centrosome formation upon activation is a slow process, mSAP maturation after activation is very fast: in collections of as little as 30 minutes the vast majority (nearly 90%) of eggs already have numerous SAPs behaving like centrosomes. The slow kinetics of *de novo* centrosome formation are unlikely to reflect a slow activation process, but rather the fact that the structurally complex centrioles simply take time to assemble from cytoplasmic components *de novo*. As SAPs are already preformed in oocytes, they can presumably start recruiting downstream components immediately after activation.

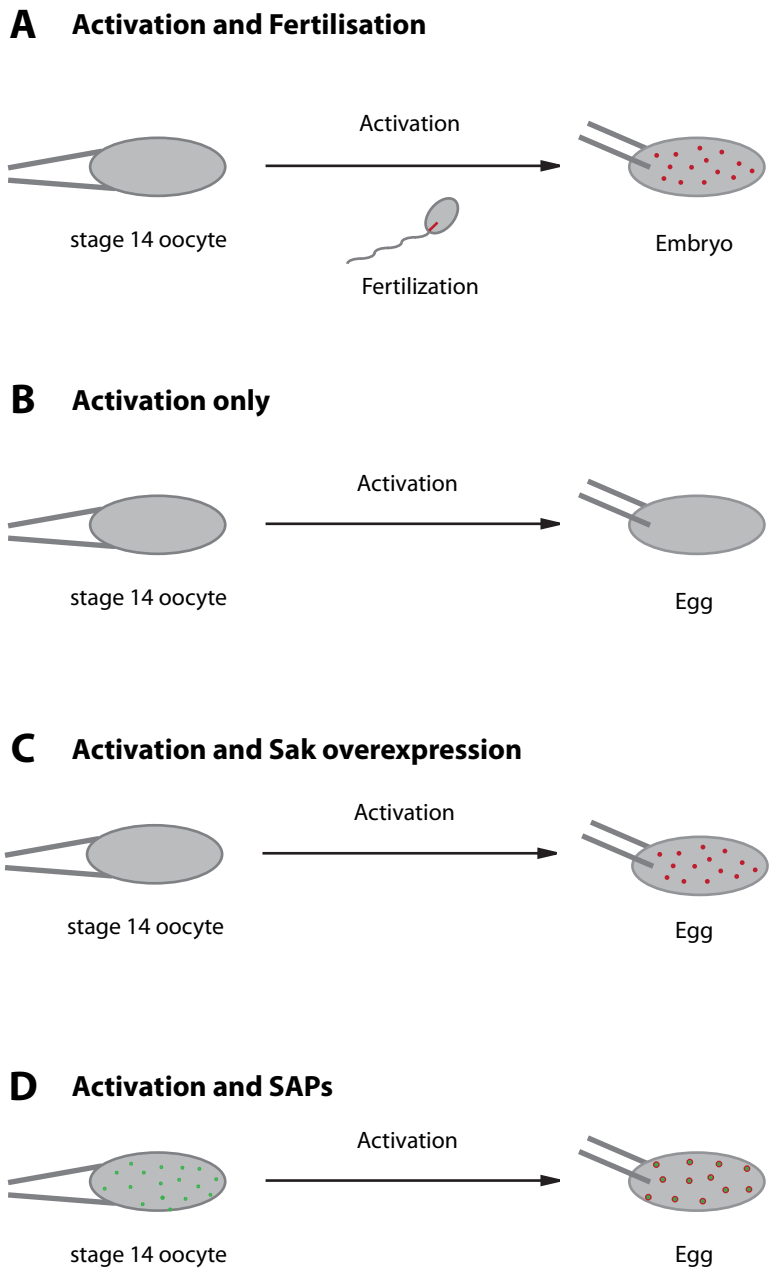


Figure 6.11 Activation and centrosome formation
Schematic representation of the events taking place following egg activation **(A)** When activation takes place concurrently with fertilisation, the centriole brought in by the sperm undergoes duplication and embryonic development can take place. **(B)** In the absence of fertilisation, activation takes place but the laid egg does not develop as no centrosomes are present. **(C)** If Sak is overexpressed, no centrioles are formed in stage 14 oocytes. However, after activation, *de novo* centrosome formation takes place (but is a slow process). **(D)** When GFP-DSas-6 and GFP-Ana2 are overexpressed, stage 14 oocytes have immature SAP (iSAPs), but these cannot recruit centrosomal components. Upon activation these SAPs quickly mature into mature SAPs (mSAPs) and start recruiting centrosomal components. Red indicates centrosomal components, green dots represent SAPs.

My data suggests that the trigger that allows centrosome assembly upon activation is not a sudden burst of translation of a key centrosomal protein, as all the known key centriolar and PCM components are already present in both stage 14 oocytes and eggs (although a small increase in protein translation upon activation was observed). Thus, it seems most likely that one of the key regulators of centriole and centrosome formation is in some way post-translationally modified upon activation.

Post-translational modification of proteins has been suggested as a fast and efficient method to activate/inactivate proteins during the process of activation (Krauchunas et al., 2012). Phosphorylation, in particular, has been studied in some detail. Three important proteins in the activation process, Giant Nuclei, Young Arrest and Spindly are dephosphorylated during activation (Yu et al., 1999; Renault et al., 2003; Krauchunas et al., 2013). In addition, a recent paper used a variety of proteomic methods to identify 311 proteins whose phosphorylation state changes upon activation in *Drosophila* (Krauchunas et al., 2012). Therefore the post-translational modification of a key centrosome protein, for example by phosphorylation, is a plausible mechanism by which centrosomal formation potential could be regulated during activation.

Five proteins have been so far identified in *Drosophila* as being essential for centriole formation: Asl, Ana2, DSas-4, DSas-6 and Sak. It is feasible that a modification that affects the function of one or several of these proteins would be capable of potentiating or not the formation of centrosomes.

However, we can exclude Sak from the list of potential targets of activation. I have shown that Sak is not required for the formation of SAPs that can behave like centrosomes, and therefore is an unlikely candidate (Chapter 5). Asl could in theory be the target protein of activation, as it is the first centrosomal component to be loaded to the SAPs upon activation. However, as my data suggest that SAP formation requires the integral interaction between DSas-4, DSas-6 and Ana2, these three proteins are the most likely potential targets for modification during activation. The post-translational modification that may take place in any or all of these three proteins is unlikely to be necessary for their structural association, as SAPs containing all three components can form in oocytes (iSAPs). These immature SAPs are just unable to recruit downstream components. It should be noted, however, that the methodology developed here can be easily applied to investigate potential activation-dependent modifications in any protein of interest. Hence future experiments could use this methodology to examine activation-dependent modifications in Asl, but also in Cnn and DSpd-2, as part of their essential role in establishing the structure of the PCM.

My initial strategy to try to identify potential modifications in DSas-4, DSas-6 and Ana2 proteins was to isolate iSAPs from oocytes and mSAPs from eggs and compare DSas-4, Ana2 and DSas-6 in both by Mass Spectrometry. Due to technical difficulties, namely the inability to biochemically isolate iSAPs, I was not able to pursue this strategy. I therefore changed my strategy to try to compare the modifications that occur in these three proteins in the cytoplasm of oocytes and laid (activated) eggs. However, I found it very

difficult to obtain sufficient amounts of sufficiently pure stage 14 oocytes to realistically attempt this experiment. I therefore explored the possibility of using mutant females to lay eggs that failed in activation. Such “activation” mutants lay eggs that are morphologically similar to activated eggs, but fail to complete some aspects of activation. As the eggs are morphologically normal, they can be collected and processed in a similar manner to WT eggs, therefore bypassing the difficulties in obtaining enough pure stage 14 oocyte material. As different aspects of activation are disrupted in different activation mutants, I examined whether the pathway of SAP/centrosome maturation is disrupted in two different activation mutants, *cortex* and *sarah*.

I identified the *sarah* mutant as being suitable for my analysis, as these mutants laid eggs, but the SAPs formed could not recruit any additional centrosomal components, as is the case with iSAPs. Interestingly SAPs could still recruit centrosomal components in eggs laid by *cortex* mutant females. It is interesting to compare the difference in SAP behaviour in these two mutants, as they share similarities in their activation phenotype. In both cases certain aspects of activation take place, e.g. eggs can be laid normally and vitelline membrane cross-linking takes place as usual; while others do not, such as completion of meiosis (Lieberfarb et al., 1996; Page and Orr-Weaver, 1996; Horner et al., 2006; Takeo et al., 2006). However, a more detailed analysis of the phenotypes displayed by these mutants shows that it is not unexpected that these two activation mutants have a different phenotype with respect to centrosome potentiation. While meiosis is not completed in both these mutants, it does progress to different stages: while

a *cortex* mutant egg will resume meiosis until metaphase or anaphase II (Lieberfarb et al., 1996; Page and Orr-Weaver, 1996), *sarah* mutant eggs appear to arrest earlier, and only progress as far as anaphase I (Horner et al., 2006; Takeo et al., 2006). In addition, it has been recently described how *bicoid* mRNA undergoes normal redistribution upon egg laying in *cortex* eggs, while in *sarah* mutant eggs it remains organised in small foci, similar to its behaviour in stage 14 oocytes (Weil et al., 2008). These observations, suggest that Sarah is probably a more upstream component of the activation signalling pathway, and a recent paper assessing the effects of different activation mutants on the phosphorylation status of maternal proteins is consistent with the positioning of Sarah upstream of Cortex (Krauchunas et al., 2013). Sarah is known to be a calcipressin, involved in the activation of calcineurin, a calcium-dependent phosphatase (Takeo et al., 2006; Takeo et al., 2010; Takeo et al., 2012). Sarah is presumably normally activated by the calcium that enters the oocyte upon activation and then triggers downstream events that are essential for subsequent SAP/centrosome maturation. I therefore used *sarah* mutant eggs as biochemically tractable models of stage 14 oocytes for my analysis.

As I decided to focus my analysis on the 3 structural components of the SAPs (DSas-4, DSas-6 and Ana2), I generated flies that overexpress UAS-DSas-4-GFP, UAS-Ana2-GFP and UAS-DSas-6-GFP in both WT and *sarah* mutant eggs. The ultimate objective would be to isolate the three proteins in enough quantities from both backgrounds to perform Mass Spectrometry analysis, and hence identify potential activation-dependent post-translational

modifications. Although I was not able to definitively determine whether any or all of the these three candidates are modified upon activation during the time-scale of my PhD, my preliminary experiments identify DSas-4 as an attractive candidate. Specifically, of all centrosomal proteins tested, DSas-4 was the only one where a significant fraction of the protein was observed to alter its behaviour (seen as a shift in mobility in SDS gels) upon egg activation. The fact that only a proportion of the protein shifts in behaviour does not necessarily exclude the possibility that it might be the target of activation, as it is possible that only a certain threshold of protein needs to be altered in order to allow centrosome formation. Moreover, by driving the formation of SAPs in WT activated eggs I was able to increase the proportion of DSas-4 converted to this faster migrating form, and this did not occur in *sarah* mutants that cannot form mSAPs, providing some evidence that this modification may indeed be linked to SAP/centrosome maturation.

The reason for the DSas-4 mobility shift is yet to be determined. An exciting possibility is that it is due to a post-translational modification such as phosphorylation or de-phosphorylation, as such modifications are well known to often modify the mobility of proteins in SDS gels. However, it is also possible that upon activation DSas-4 is simply more susceptible to cleavage, e.g. if certain protease activities are turned on upon activation. As western blotting experiments with eggs expressing GFP-DSas-4 with an antibody for GFP also showed this double band it would suggest that if such cleavage is taking place, it is most likely cleaving part of the protein's C-terminus. *A priori*, it perhaps seems unlikely that cleaving DSas-4 would be

required to “activate” the protein for SAP/centrosome maturation: the role of SAS-4 proteins in centriole and centrosome formation has been studied extensively in several systems, and functionally relevant cleavage of this proteins has not been reported. Thus, if it is the case that a fraction of DSas-4 is simply being cleaved upon egg activation, it seems unlikely that this modification would be linked to SAP/centrosome maturation. Future Mass Spectrometry analysis will hopefully resolve this important issue.

In conclusion, in this chapter I have described how I have attempted to identify the post-translational modifications that are likely to be involved in allowing centrosome formation upon egg activation in *Drosophila*. I hypothesise that these modifications are likely to be happening to either one, or all, of the following centriolar proteins: DSas-4, Ana2 and DSas-6. In particular, I developed a strategy involving the overexpressing of these three proteins in *sarah* mutant eggs and WT eggs which should allow me to obtain enough cytoplasmic DSas-4, Ana2 and DSas-6 for Mass Spectrometry analysis. Future work will hopefully complete this analysis and identify any post-translational modifications in these proteins that take place during activation. Future work could also examine whether the reversal of these modifications might be involved in the process of centrosome inactivation that normally occurs during oogenesis.

Chapter 7: Discussion

7.1 Summary

The centrosome has been the subject of extensive molecular analyses for several decades. Hundreds of proteins have now been identified as potential components of the centrosome (Andersen et al., 2003; Bettencourt-Dias and Glover, 2007; Muller et al., 2010; Jakobsen et al., 2011; Jakobsen et al., 2013) and recent studies have identified a relatively small "core" of proteins that seem to be essential for centriole duplication and PCM recruitment. In *C. elegans* an assembly pathway for the main players involved in centriole and centrosome assembly has already been proposed (Fig.7.1A), but such a detailed analysis has been lacking in other systems. In this thesis I have examined this pathway in *Drosophila* using SAPs as proxy models of centrosomes, allowing me to propose a pathway for centriole and centrosome assembly in *Drosophila* (Fig.7.1B).

In this chapter, I discuss my work in a wider context. I first assess the usefulness of SAPs in eggs as a proxy model for centrosomes, and examine some of the advantages and disadvantages of this system. I then consider the pathway of centrosome assembly in *Drosophila* that has emerged from my studies and compare it with the available data in *C. elegans* and in

humans. Finally I consider some of the interesting points emerging from my work and how these might lead on to interesting studies in the future.

7.2 SAPs as models to study the pathway of centrosome assembly

In this thesis I used SAPs formed in eggs as proxy models for centrosomes in order to assess the pathway of centrosome formation. As shown in Chapter 3, SAPs closely resemble centrosomes, as assessed by a variety of criteria. However, SAPs are not centrosomes. The most striking difference between a SAP and a centrosome is that a SAP is, by its nature, relatively unstructured: unlike a centrosome, it does not contain at its core a highly ordered centriole. The absence of a centriole in SAPs constitutes both the main advantage and the main disadvantage of this system.

Many studies of the pathway of centrosome assembly have been performed in cell culture, particularly those analysing this pathway in human and other vertebrate cells. In these cells, different centrosomal proteins are often depleted using RNAi, and subsequently the composition of centrosomes is analysed. There are several drawbacks in using this approach to study the importance of different proteins at the centrosome (in addition to the potential problems of incomplete depletion and off-target effects inherent to this type of analysis). When centriole duplication factors are depleted, for example, procentriole formation is largely prevented. As such, the only centrosomal recruitment that is being assessed in this context is the recruitment of proteins to pre-formed centrosomes. As these structures are already fully formed, they will at least initially contain all necessary centrosomal components, and the RNAi applied will only have an effect if

the target protein normally turns over at the centrosome. The true effect on downstream recruitment would only be achieved if the protein of interest exchanges completely with the cytoplasmic pool. For proteins whose depletion does not prevent procentriole formation, the use of super-resolution microscopy techniques would be required to examine which proteins can be recruited to the procentriole in the absence of the target protein while avoiding misinterpretation of the recruitment to the already pre-existing centriole. Such studies are only now starting to be applied to the centrosome (Fu and Glover, 2012; Lawo et al., 2012; Mennella et al., 2012; Sonnen et al., 2012).

The alternative to using RNAi approaches is to assess centrosome behaviour in genetically tractable cell lines (such as chicken DT40 cells) or model organisms (such as worms and flies). Such approaches can have important advantages. In *Drosophila* eggs laid by homozygous mutant mothers, for example, there are no concerns regarding maternally contributed protein and RNA, and, depending on the nature of the mutation, the protein of interest may be said to be completely absent. The main problem with this experimental set up, however, is that in the absence of any of the main five core centriole duplication factors, centrioles cannot form making it impossible to analyse the pathway of centriole, or subsequent centrosome, assembly.

Analysing the pathway of centrosome assembly using SAPs potentially bypasses this problem. Because SAPs are not centrioles (and lack a

defined structure), I found that they can form in eggs lacking most centriolar proteins (see Chapter 5). In addition, as SAP formation occurs *de novo*, there are no concerns regarding the presence of structures that were preformed in the presence of the depleted protein. Finally, as SAPs are formed in unfertilised eggs that are not developing, they provide an environment for centrosome assembly that is independent of additional complicating variables such as cell cycle stage.

The absence of centrioles, however, means that there is a limit to the information that can be obtained from studying the pathway of centrosome formation in SAPs. The centriole and the centrosome are complex structures formed of many proteins. SAP formation is initiated by the interaction of excess DSas-6 and Ana2 (with DSas-4), which seems to form an unstructured platform to which all downstream components can be recruited in a similar manner to that observed in centrosomes. The formation of centrioles, however, is initiated by components upstream of DSas-6 and Ana2. In addition, centrioles have a very intricate structure. This means that SAPs can be used to assess the order in which different centriolar proteins are recruited downstream of DSas-6 and Ana2, and the order in which the PCM components are recruited to form a functional dynamic structure. It cannot, however, assess the stepwise process necessary to form the intricate ultrastructure of the centriole, because there is not one. As such, SAPs can most usefully be used to identify the sequential pathway of recruitment of the various centriole and centrosome

components, and not how these interactions, or any additional interactions, are required to form a proper structure.

A good example of both the advantages and disadvantages of the SAP system is the result obtained with SAPs formed in the absence of Sak. Sak/Plk4 is generally considered to be one of the most crucial centriole duplication proteins. However, I found that SAPs formed in the absence of Sak behave like WT (Chapter 5). This demonstrates that Sak is not required for DSas-6 and Ana2 to interact in a manner that allows them to initiate the recruitment of other centriole and centrosome proteins—a potentially very important result. Nevertheless, Sak clearly must be required for a specific step in the process of forming the precise ultrastructure of centrioles, as centrioles cannot duplicate without Sak (Bettencourt-Dias et al., 2005; Janina Baumbach and Alan Wainman, personal communication). I suspect that Sak may normally be required to increase the efficiency of the interaction between DSas-6 and Ana2, and the concentration of Sak to a specific region of the mother centriole (Sonnen et al., 2012) ensures that this is normally the only site where daughter centrioles assemble. By overexpressing both DSas-6 and Ana2 in eggs, the requirement for Sak is bypassed, and SAPs rapidly assemble throughout the egg cytoplasm. Therefore the analysis of SAPs in a Sak mutant background is able to both assess some aspects of Sak function that are not possible to examine in centrosomes in the same background, but the fact that they do not have centrioles means that other aspects of its function are not being assessed.

SAPs may, however, eventually prove to be useful in testing the role of the different centriole proteins in the formation of the centriole structure. Unlike SAPs in eggs, SAPs in testis have a much more well defined ultrastructure and are formed from elongated tubules that resemble the centriole inner cartwheel, although these SAPs do not recruit any PCM components and organise no MTs (Stevens et al., 2010b). Like SAPs in eggs, SAPs in testis are able to assemble in the absence of essential centriolar components and of centrioles (Helio Roque, personal communication). A postdoctoral fellow in the lab (Helio Roque) has started to assess the ultrastructure of testis SAPs in the absence of different centriolar components, and this is shedding some light on the role of the different centriolar components in the formation of the cartwheel structure. These data complement my pathway analysis in eggs and overcomes some of the shortcomings of using SAPs in eggs as a model.

One additional shortcoming from using SAPs in eggs as an experimental model in the context of essential centriolar protein mutants concerns the nature of these mutations. Mutant flies lacking any of the 5 essential centriolar components are uncoordinated (Basto et al., 2006; Peel et al., 2007; Rodrigues-Martins et al., 2007a; Blachon et al., 2008), and hence are not able to lay eggs and die by getting stuck in the food. The inability of these mutant flies to lay eggs was overcome in this project by the *in vitro* activation of stage 14 oocytes dissected from these flies. However, and as described in Chapter 5, this a very time-consuming process, and several months were necessary to analyse the composition of SAPs in each of the

centriolar mutants shown in this thesis. As a consequence, this protocol is not very high throughput and the number of eggs and proteins examined is limited. However, a new strategy is currently being developed in the lab that may be able to overcome these difficulties. By overexpressing the essential centriolar protein that is absent in the mutant fly exclusively in the mechanosensory neurons (containing the cilia that are necessary for coordination), it may be possible to rescue the uncoordinated phenotype. Flies would therefore be coordinated, and hence able to lay eggs, while the eggs laid (and all other tissues in these flies) would be in a mutant background. Promising preliminary data shows that it might be possible to implement this strategy in the future.

7.3 The pathway of centrosome assembly across evolution

In this project I have analysed the composition of SAPs in mutant eggs for different centrosomal proteins. Based on these results I propose a pathway of centrosome assembly in *Drosophila* eggs (Fig.7.1B). The pathway of centrosome assembly in *C. elegans* has been studied in detail, and is shown in Fig.7.1A. A detailed analysis of the assembly pathway of the human homologues of these seven centrosomal proteins is still largely lacking, but a preliminary pathway can be drawn, as shown in Fig.7.1C. For ease of comparison, functional homologues from each species are shown in the same colour. The data leading to the drawing of the pathway of

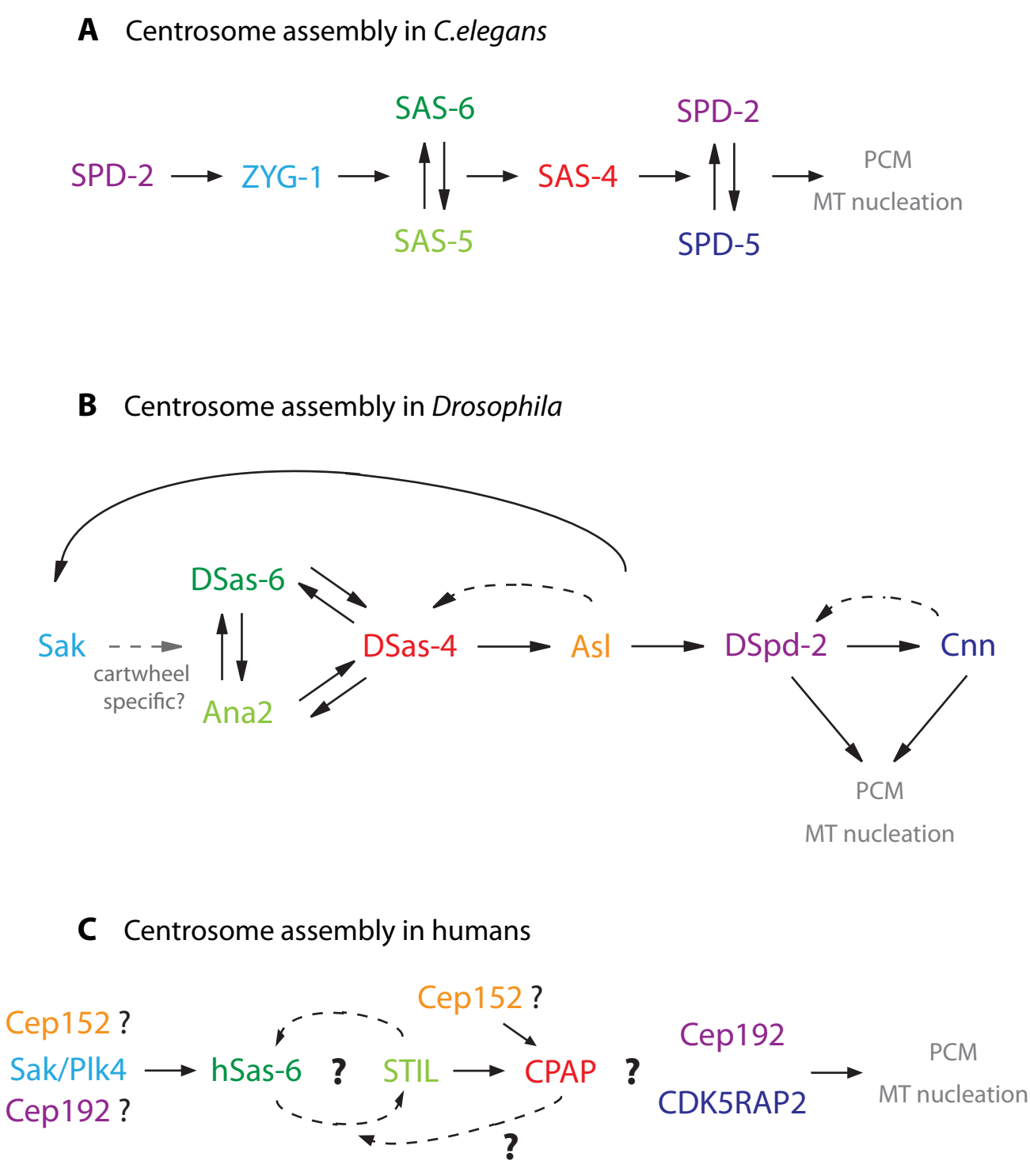


Figure 7.1 The pathway of centrosome assembly in *C. elegans, Drosophila* and humans
A schematic illustration of the likely pathways of centrosome assembly in *C. elegans* **(A)**, *Drosophila* **(B)** and humans **(C)**. A and C are based on previously published data, B is based on published data and the work in this thesis. Homologues and functional orthologues are color-coded in the same way.

centrosome assembly in *C. elegans* and humans is discussed in detail in Chapter 1.

Comparison of this pathway across evolution highlights the importance of a core of 4 proteins in the pathway of centriole assembly in all species: Zyg-1/Sak/Plk4, SAS-6/DSas-6/hSas-6, SAS-5/Ana2/STIL and SAS-4/DSas-4/CPAP. In all organisms Zyg-1/Sak/Plk4 is consistently placed in one of the most upstream positions in the pathway of centriole formation (Bettencourt-Dias et al., 2005; Habedanck et al., 2005; Delattre et al., 2006; Pelletier et al., 2006; Kleylein-Sohn et al., 2007; Rodrigues-Martins et al., 2007b; Basto et al., 2008; Dzhindzhev et al., 2010). This is closely followed by the contribution of SAS-6/DSas-6/hSas-6, not surprising considering its conserved structural role in the formation of the centriole cartwheel (Kitagawa et al., 2011c; van Breugel et al., 2011).

In all three organisms the interplay between SAS-6/DSas-6/hSas-6, SAS-5/Ana2/STIL and SAS-4/DSas-4/CPAP is important in centriole formation downstream of Zyg-1/Sak/Plk4. However, the exact nature of this relationship seems to have changed across evolution. In *C. elegans* the co-dependence between SAS-6 and SAS-5 is well established (Delattre et al., 2006; Pelletier et al., 2006). In *Drosophila* DSas-6 and Ana2 have been shown to physically interact (Stevens et al., 2010a; Tang et al., 2011), and evidence of the biological relevance of this interaction has been demonstrated by the ability of these proteins to form SAPs in eggs and testis at relatively moderate expression levels (Stevens et al., 2010a;

Stevens et al., 2010b). This has lead to the suggestion that Ana2 might be involved in playing an important role in stabilising the cartwheel structure formed by DSas-6 (Cottee et al., 2011). However, my work in SAPs in eggs has shown that in *Drosophila* this interaction is probably dependent on DSas-4. This is in contrast with its *C. elegans* homologue, SAS-4, which seems to be recruited downstream of SAS-5 and SAS-6 (Delattre et al., 2006; Pelletier et al., 2006).

In human cells, hSAS-6 and STIL (the human homologue of SAS-5/Ana2) are only partially required for each other's localisation, and there is no evidence so far for a direct interaction between these proteins (Tang et al., 2011; Arquint et al., 2012). However, the human homologue of DSas-4, CPAP, is known to directly interact with STIL (Tang et al., 2011) and recent work from my lab shows that the interaction between Ana2 and DSas-4, and between STIL and CPAP is structurally conserved (Cottee et al*.,* 2013 *in press*). It is very likely that, as is the case in *Drosophila*, the human homologue of DSas-4, CPAP, is the protein that establishes the connection between hSAS-6 and STIL. Future work is necessary to address how similar the interaction between these three proteins is conserved between *Drosophila* and humans.

Another interesting similarity observed across evolution is the existence of a centrosomal player that is both required for centriole duplication and for PCM recruitment. In *C. elegans* this role seems to be performed by SPD-2 (Kemp et al., 2004; Pelletier et al., 2004; Delattre et al., 2006; Pelletier et al.,

2006). My work in the *Drosophila* pathway has confirmed previous data suggesting that the homologue of SPD-2, DSpd-2, is involved exclusively in PCM recruitment. My most persuasive result supporting this conclusion is the observation that centrioles seem to be able to form *de novo* in the absence of DSpd-2 (Chapter 4). Therefore, instead of DSpd-2, the dual role in centriole duplication and in PCM recruitment is performed by a protein not present in worms: Asl. Human cells possess both a homologue of SPD-2/DSPd-2 (Cep192) and of Asl (Cep152). Both these proteins have also been implicated in PCM recruitment (Gomez-Ferreria et al., 2007; Zhu et al., 2008; Dzhindzhev et al., 2010). In addition, work published very recently shows that both these proteins are also involved in centriole duplication in humans (Sonnen et al., 2013). It seems therefore that a dual role in centriole duplication and PCM recruitment has changed across evolution: this role is performed in *C. elegans* by SPD-2, in *Drosophila* by Asl, and in humans in cooperation by the homologues of these two proteins: Cep192 and Cep152, respectively.

The conserved requirement for a protein that is able to perform both functions is most likely a consequence of the fact that both centriole duplication and PCM assembly are initiated from the exterior wall of the centriole. A component localised to the external wall of the centriole, such as SPD-2 or Asl, would be ideally place to guarantee that both processes are seeded from the same external wall. The specificity of one or the other process will, of course, be dependent on process-specific modifications and interactions with different proteins. In the case of centriole duplication, the

interaction partner is most likely Zyg-1/Sak/Plk4. In *C. elegans* Zyg-1 is dependent on SPD-2 for its localisation in the centriole, while the inverse is not observed (Delattre et al., 2006; Pelletier et al., 2006). In *Drosophila*, recent work has shown a similar dependence for Asl and Sak, and the direct interaction regions for these two proteins have been mapped (Dzhindzhev et al., 2010). In human cells the interaction between Cep152 and Plk4 (Cizmecioglu et al., 2010; Dzhindzhev et al., 2010; Hatch et al., 2010), and Cep192 and Plk4 have also been demonstrated (Sonnen et al., 2013). In PCM assembly, the interaction partner of this dual-role protein is likely to be another essential PCM recruitment factor. Work in *C. elegans* suggests an interaction with SPD-5 (Kemp et al., 2004; Pelletier et al., 2004; Srayko et al., 2005; O'Toole et al., 2012), while recent work in *Drosophila* has shown that DSpd-2 is able to interact with Cnn, the potential fly homologue of SPD-5, and indeed PCM recruitment in *Drosophila* is completely abolished in the absence of these two proteins (Paul Conduit, personal communication).

7.4 Future work

SAPs are very close models of centrosomes. The lack of centrioles at their core has made it possible to assess the pathway of centrosome recruitment in several essential centrosomal mutants, allowing me to propose an improved description of this pathway. However, SAPs are only proxy models of centrosomes. They have allowed me to identify novel features of the pathway of centrosome assembly, but these will have to be confirmed and further studied using real centrosomes.

One of the most interesting points arising from my work is the observation that Sak is not required for the recruitment and function of any downstream components. As discussed previously, this observation does not exclude a very important role in establishing the formation of the cartwheel, or another centriole structure-specific role. It does highlight, however, that many of the subsequent steps can happen in its absence, and that it most likely performs a very specific role. Future work is necessary to clarify the specific role of Sak in centriole duplication in light of my results.

Another interesting point is the requirement for DSas-4 in the interaction between DSas-6 and Ana2. The interaction between DSas-4 and Ana2 has been described in molecular detail (Cottee et al*.,* 2013 *in press*). My data predicts a possible interaction between DSas-6 and DSas-4, and it would be interesting to explore this possibility in more detail in the future. Structural studies will play an important part in this investigation, but SAPs are a

potentially very useful assay in which to assess some of these interactions. As SAP formation requires the successful interaction between DSas-6, DSas-4 and Ana2, assessing SAP formation in different point mutants of these three proteins would be able to identify and/or confirm regions of interactions between them. In addition, this set-up could also be used to identify regions of interaction with downstream components.

I have already explored in some detail another interesting hypothesis arising from the core interaction between DSas-6, Ana2 and DSas-4. In Chapter 6 I have described how different observations suggest that the mechanism by which centrosome formation is potentiated upon egg activation is likely to be a quick post-translational modification. The observation that SAPs mature upon activation, has led me to propose that this modification could take place in one of these three core proteins: DSas-6, Ana2 and DSas-4. DSas-4 is a particularly exciting candidate, as I have shown that its gel mobility is modified upon activation, and was able to place this modification in the context of the pathway of activation (Fig.7.2). I have developed a methodology to try to identify this and other modifications, and future work will be able to explore this further.

With regards to the assembly of the PCM (Chapter 4), the most interesting observation in my data is the existence of an alternative pathway of PCM recruitment that bypasses Asl (and potentially DSpd-2). It would be interesting to determine the alternative centriolar component that is involved in this recruitment, and proteins such as DSas-4 and DPLP are potential

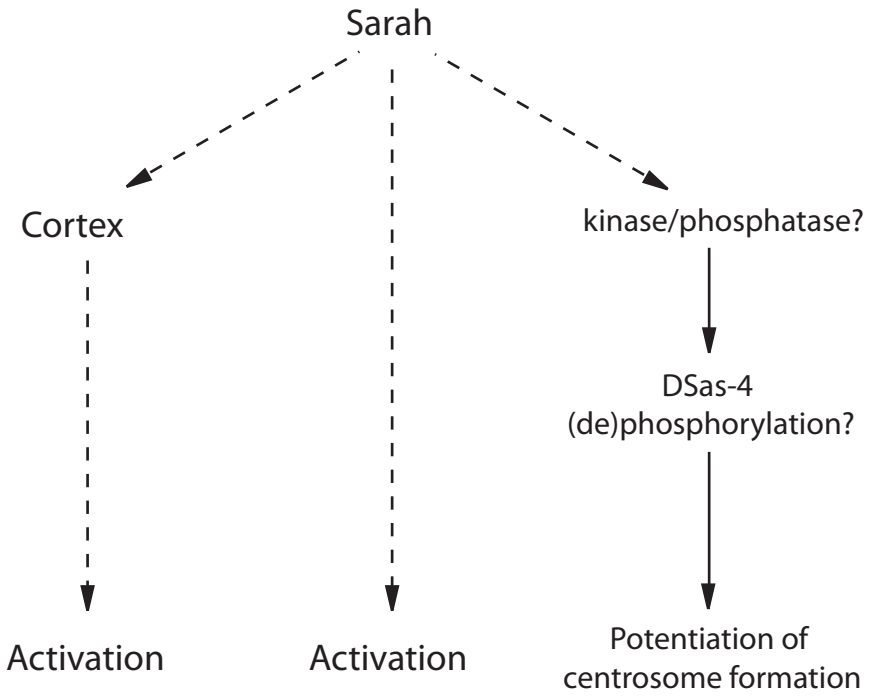


Figure 7.2 The position of a potential DSas-4 post-translational modification in the activation pathway
Dashed interactions are likely to be indirect.

candidates (Martinez-Campos et al., 2004; Gopalakrishnan et al., 2012). My data also suggest that the 483-854 aa region of Asl might be involved in preventing the activity of this additional pathway. Further work is required to clarify if this is the case in centrosomes as well, and the mechanism of this inhibition. If this result is confirmed it would provide an explanation for some of the contradictory results observed with different *asl* mutant alleles.

7.5 Conclusion

In this project I used SAPs as proxy models of centrosomes and analysed their composition in the absence of different essential centrosomal components. This work has allowed me to propose a pathway of centrosome assembly in *Drosophila*. I have described several novel relationships between some of the most important players in the centrosome assembly pathway, and future work will test whether these interactions occur in the same way in real centrosomes, and their importance to the assembly process. I note, however, that this was not an extensive analysis. The centrosome is composed of hundreds of proteins, and only seven of these were analysed in detail in this project. SAPs offer an attractive system in which the position of additional proteins in the pathway can be determined, and I hope that my work will lay the foundation for future studies that may one day lead to a complete understanding of how centrosomes are assembled.

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