

# **Centriole biogenesis in early murine development**

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I, Katie Anwen Howe confirm that the work presented in this thesis is my own. Where information has been derived from other sources it has been indicated in the thesis.

## **ABSTRACT**

Centrosomes are the major microtubule organising centre of most cells. A centrosome consists of a pair of perpendicular centrioles surrounded by a cloud of pericentriolar material. Centrioles must be duplicated once per cell cycle to ensure each daughter cell inherits the correct number of centrioles. In somatic cells this process is controlled by centriole replication proteins including SAS-6 and Plk4. Centrioles have an unusual life-cycle in early mouse development. Both the sperm and the egg lack centrioles. Therefore the first few divisions in mouse embryos take place without centrioles. New centrioles are then formed ‘*de novo*’ in early developing embryos. In this thesis the molecular basis for this unusual scenario is examined. Firstly, a transgenic mouse model was used to confirm centrioles are formed *de novo* at the mouse blastocyst stage. This model was then used to examine the influence of centriole emergence on microtubule organisation. Secondly, overexpression of the centriole replication protein Plk4 was found to drive precocious formation of centriole-like structures in all stages of oocyte and embryo examined, which cause abnormal spindles. However, this does not appear to affect embryo development or chromosome segregation. Finally, overexpression of the downstream centriole component SAS-6 was also found to drive the formation of foci in embryos but strikingly, SAS-6 fails to arrange into centriole-like foci in immature or mature unfertilised oocytes. These experiments document the presence of an inducible *de novo* centriole formation pathway in mammalian oocytes and embryos, and show that the pathway is more resistant to activation prior to fertilisation.

## **LIST OF ABBREVIATIONS**

ACA	anti-centromere antigen
APC	Anaphase promoting complex
Asl	Asterless
ATP	Adenosine Trisphosphate
BSA	Bovine serum albumin
<i>C. elegans</i>	<i>Caenorhabditis elegans</i>
<i>C. reinhardtii</i>	<i>Chlamydomonas reinhardtii</i>
CCD	Charge-coupled device
Cdk	cyclin-dependent kinase
cki	cyclin-dependent kinase inhibitor
CaMKII	calmodulin dependent protein kinase II
CETN-2	Centrin-2
CPAP	centrosomal P4.1-associated protein
CSF	Cytostatic factor
dpc	days post coitum
DNA	Deoxyribonucleic acid
<i>Drosophila</i>	<i>Drosophila melanogaster</i>
EB1	end binding protein 1
eGFP	enhanced Green Fluorescent Protein
EM	Electron microscopy
Emi	Early mitotic inhibitor
EtOH	Ethanol
FISH	Fluorescence <i>in-situ</i> hybridisation
FSH	Follicle-stimulating hormone
Fucci	Fluorescent ubiquitin-based cell cycle indicator
GAP	GTPase activating protein
GEF	GTP exchange factor
GFP	Green Fluorescent Protein
GV	Germinal vesicle

GVBD	Germinal vesicle breakdown
$\gamma$ -TuRC	$\gamma$ -tubulin ring complex
hCG	human Chorionic Gonadotrophin
H-KSOM	Hepes-buffered-potassium simplex optimized media
HURP	Hepatoma upregulated protein
IBMX	3-isobutyl-1-methylxanthine
ICM	Inner cell mass
IP3	inositol 1,4,5-trisphosphate
IP3R	inositol 1,4,5-trisphosphate receptor
IU	International units
IVF	<i>In vitro</i> fertilisation
KSOM	Potassium simplex optimized media
LH	Luteinizing hormone
MI	Metaphase I
MII	Metaphase II
MAP7	Microtubule-associated protein 7
MAP kinase	Mitogen-activated protein kinase
MCAK	Mitotic centromere-associated kinesin
MO	Morpholino
MPF	Maturation promoting factor
MT	Microtubule
MTOC	Microtubule organising centre
NEB	New England BioLabs
NEBD	Nuclear envelope breakdown
PACT	pericentrin-AKAP450 centrosomal targeting
PAF	Paraformaldehyde
Pb	Polar body
PBS	Phosphate buffered saline
PCM	Pericentriolar material
PCNA	Proliferating cell nuclear antigen

PLC	Phospholipase C
Plk4	Polo-like kinase 4
PMSG	Pregnant mares' serum gonadotrophin
RFP	Red Fluorescent Protein
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
SAC	Spindle Assembly Checkpoint
Sak	Snk akin kinase
SAKOE	Sak-overexpressing ( <i>Drosophila</i> cell line)
SAS-4/5/6	Spindle assembly abnormal 4/5/6
SCF	Skp1-cullin1-F box protein ubiquitin ligase
SCNT	Somatic cell nuclear transfer
siRNA	small interfering ribonucleic acid
TE	Trophoectoderm
TPX2	Targeting Protein for the <i>Xenopus</i> kinesin xklp2
TACC3	Transforming Acidic Coiled Coil protein 3
UTR	Untranslated region
<i>Xenopus</i>	<i>Xenopus Laevis</i>

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# **1. INTRODUCTION**

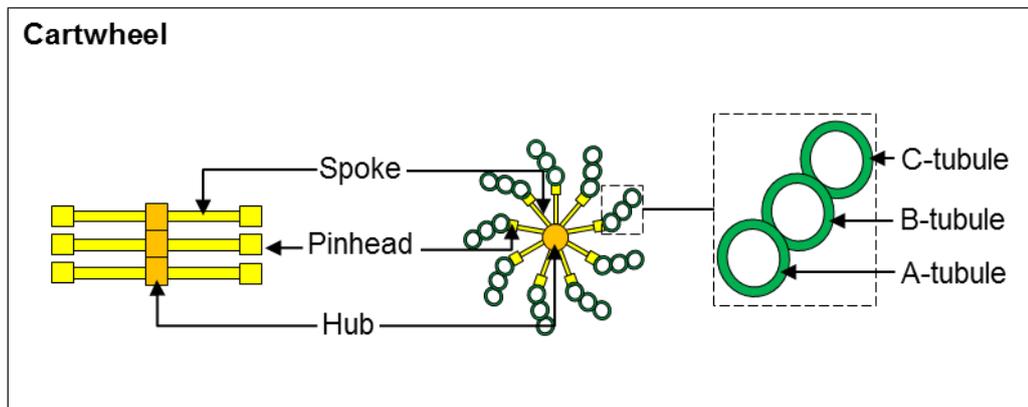
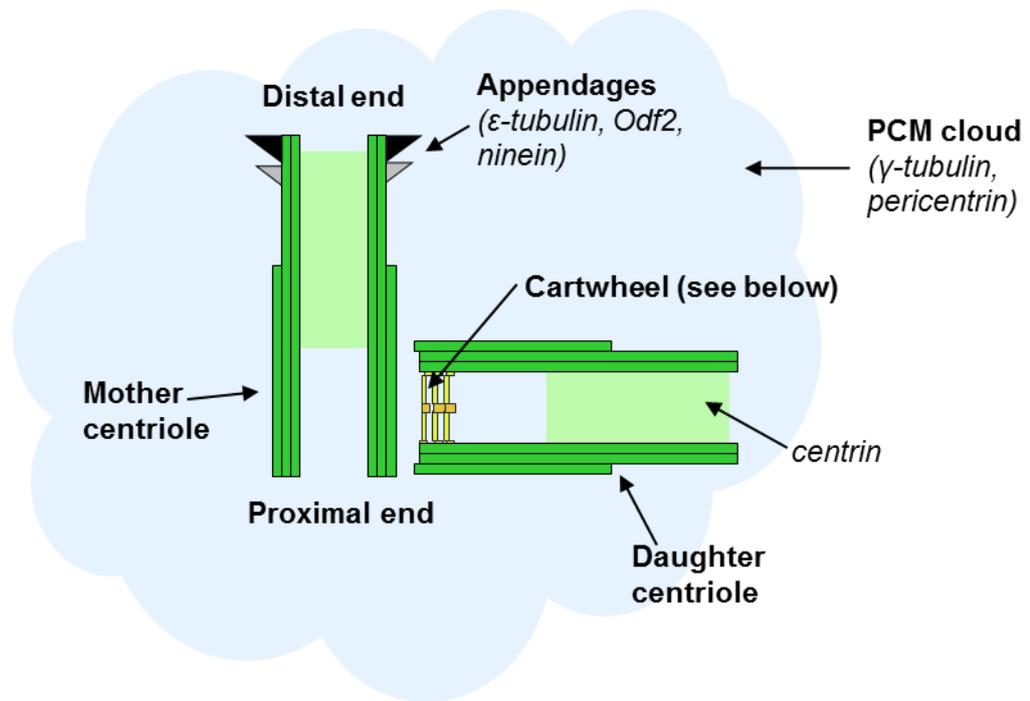
Centrosomes are the main microtubule organisers of most animal cells. They consist of a pair of perpendicular centrioles and a surrounding cloud of pericentriolar material (PCM). Centrioles normally replicate once per cell cycle, in order that each daughter cell receives one pair. Centrioles can also form ‘*de novo*’ in a select number of cellular settings including in early mouse embryos.

The experiments presented in this thesis have been designed to investigate the mechanism of *de novo* centriole biogenesis in early murine development. This introduction will therefore introduce topics relevant to the experiments presented herein. Firstly, the structure and function of centrosomes will be discussed. Next, the canonical as well as the *de novo* mode of centriole replication will be covered. Following a review of the mechanisms employed to regulate centrosome number, the implications of any numerical deviations will be addressed. The subsequent section will address the different pathways of centriole reduction during gametogenesis. This will be followed by a review of spindle assembly pathways in the presence and absence of centrioles. Finally, the unique physiology and cell biology of mouse oocytes and early embryos will be addressed.

## **1.1. Structure and function of the centrosome**

### **1.1.1. Structure of the centrosome**

The centrosome consists of a pair of centrioles surrounded by a cloud of electron-dense pericentriolar material (PCM). These centrioles are highly stable, barrel-shaped organelles arranged in an orthogonal configuration. The outer wall of the barrel is composed of a nine-fold symmetric array of microtubule (MT) triplets referred to as A-, B- and C-tubules. In higher organisms, these MT blades are arranged around a cartwheel structure at the proximal end of the centriole. The cartwheel is made up of a central hub from which project nine radial spokes. These spokes are terminated by pinhead structures which bind to the A-tubule thereby connecting the MT blades to the central cartwheel ((Azimzadeh and Marshall, 2010; Brito et al., 2012); see Fig 1.1 for a diagram of the structural features of the centrosome). The C-tubule does not span the entire length of the centriole barrel. Thus, the distal end of the centriole is composed of a MT doublet (see Fig 1.1; (Azimzadeh and Marshall, 2010; Bettencourt-Dias et al., 2011; Brito et al., 2012)). In lower organisms, the MT blades are composed of singlet (e.g. *C. elegans*) or doublet MTs (e.g. *Drosophila*) suggesting that the original evolutionary centriole precursor contained a



**Figure 1.1. Structural features of the centrosome.** A centrosome consists of two cylindrical centrioles surrounded by a cloud of pericentriolar material (PCM). Each pair of centrioles is composed of a mature mother centriole (which is characterised by its distal and sub-distal appendages) and an immature daughter centriole. Centrioles are composed of nine microtubule (MT) blades assembled around a symmetric central cartwheel structure. In human cells, the cartwheel structure is only present at the proximal end of the daughter centrioles. Each MT blade consists of three MTs bound to the cartwheel pinhead via the A-tubule. Adapted from Azinzadeh and Marshall 2010 and Brito et al., 2012.

single triplet blade (Marshall, 2009). In addition, the size of the centriole varies from ~100 x 150 nm in *C. elegans* to ~200 x 500 nm in mammals. Despite these variations, the nine-fold symmetry and cylindrical shape are highly conserved.

The PCM which surrounds the centriole pair has been visualised as a fibrous lattice (DICTENBERG et al., 1998) and has been found to harbour numerous proteins. These include components for MT nucleation such as pericentrin and  $\gamma$ -tubulin (DICTENBERG et al., 1998), as well as predicted coiled-coil domain proteins (ANDERSEN et al., 2003; DOXSEY et al., 2005; JACKMAN et al., 2003). Recent work has shown that the size of the PCM correlates with its MT nucleating ability which increases during centrosome maturation (CONDUIT et al., 2010; CONDUIT and RAFF, 2010; KIRKHAM et al., 2003). Centrioles have been shown to regulate the incorporation of the key protein centrosomin into the PCM, which in turn defines centrosome size and MT nucleation capacity (CONDUIT et al., 2010; CONDUIT and RAFF, 2010; GOMEZ-FERRERIA and PELLETIER, 2010). The centriole pair is crucial for the structure and function of the centrosome as a whole, since disassembly of the centriole causes dispersion of the PCM (ABAL et al., 2005; BOBINNEC et al., 1998a). Therefore, the number of centrioles determines the number of centrosomes. Conversely, the PCM cloud controls the formation and stabilization of daughter centrioles (DAMMERMANN et al., 2004; LONCAREK et al., 2008), illustrating the close relationship which exists between the PCM and the centriole pair.

Each centriole pair comprises a mature (mother) and immature (daughter) centriole (PIEL et al., 2000), the daughter centriole being about 80% of the length of the mother centriole (CHRETIEN et al., 1997). The mother centriole can also be identified by its distal and sub-distal appendages (PAINTRAND et al., 1992). Several appendage proteins have now been characterized including  $\epsilon$ -tubulin (CHANG et al., 2003), Odf2 (ISHIKAWA et al., 2005) and ninein (MOGENSEN et al., 2000). Appendages play important roles in anchoring centrioles at the plasma membrane when they act as basal bodies (distal) as well as in controlling MT nucleation (subdistal).

### **1.1.2. Function of centrosomes**

Centrosomes fulfill many key cellular functions. They are responsible for organising MTs throughout the cell cycle; they form the basis for the formation of cilia and flagella as well as being important for cell

cycle progression. In addition, they help propagate the assembly of new centrioles (see section 1.2 for a detailed discussion of the mechanisms of centriole assembly).

### *Centrosomes as organisers of MTs*

Centrosomes are the major site of MT nucleation and anchoring in most cells. This nucleation function is mediated by the PCM via  $\gamma$ -tubulin containing ring complexes ( $\gamma$ TuRCs). These are ring-like multiprotein complexes which are required for the assembly of MTs onto the outer wall of the centriole cartwheel (Guichard et al., 2010). In addition, several proteins associated with the mother centriole's sub-distal appendages such as ninein (Mogensen et al., 2000) act to anchor the minus ends of MTs at the centrosomes. Therefore, although MTs are nucleated via the PCM associated with both centrioles, only the mother centriole is able to anchor MTs.

In interphase, the centrosome organises MT asters which control cell shape, cell motility and cell polarity (Bornens, 2002; Doxsey, 2001). During mitosis, centrosomes must organise a bipolar spindle to ensure chromosomes are segregated accurately between the daughter cells. They are also important in determining the position of the cleavage plane during cytokinesis, thereby ensuring asymmetric cell divisions and morphogenesis (Basto et al., 2008; Bornens, 2012; Conduit et al., 2010; Conduit and Raff, 2010; Januschke et al., 2011; Rebollo et al., 2007; Rusan and Peifer, 2007). However, some plant cells lack centrosomes but can still organise normal bipolar spindles and undergo accurate cell division (Karsenti and Vernos, 2001). Furthermore, experiments have shown that centrosomes are not essential for spindle assembly in various cell types (Heald et al., 1997; Hinchcliffe et al., 2001; Khodjakov et al., 2000), suggesting that many cells can also operate a non-centrosomal pathway of spindle assembly. (The mechanisms contributing to centrosomal and acentrosomal spindle assembly will be discussed further in section 1.6).

Centrosomes also nucleate astral MTs which play a crucial role in cleavage furrow formation. Positioning of the furrow depends on dynein-mediated interaction of the cell cortex with astral MTs (O'Connell and Wang, 2000). In yeast, astral MTs also influence spindle positioning. A checkpoint prohibits completion of cytokinesis until spindles are correctly positioned in the neck bud (Shaw et al., 1997). Thus, a key role of the centrosome is to enable proper cytokinesis through the function of astral MTs.

### *Centrosomes in ciliogenesis*

Centrosomes can also form a scaffold for basal bodies, the structures that initiate the formation of cilia and flagella. Cilia form in non-dividing cells when the centrioles migrate to the plasma membrane and the mother centriole forms a basal body. The distal end of the mature centriole is then encapsulated by a Golgi-derived vesicle and pre-existing MTs elongate into a single ciliary axoneme (Satir and Christensen, 2008). The appendages of the mature centriole may also play a role in anchoring the cilium at the plasma membrane but their exact function is unknown. The assembly of the cilium depends on the trafficking of membrane vesicles to the cell periphery which requires the small GTPase Rab8 (Yoshimura et al., 2007). Basal bodies therefore act as recruitment centres for molecules involved in ciliogenesis (Deane et al., 2001).

Cilia can appear either as immotile primary cilia or as motile cilia and flagella (Dawe et al., 2007). Motile cilia usually contain nine MT doublets which surround a central pair of singlet MTs (9+2). In contrast, immotile cilia lack the central MT singlets (9+0; (Satir and Christensen, 2008)). These distinct structures mean that cilia can fulfill a range of cellular functions. Motile cilia enable the movement of whole organisms, as well as providing a beating function to clear debris in epithelial tissues such as the trachea (Afzelius, 1976). Non-motile primary cilia have now been found to be involved in several signal transduction pathways including *sonic hedgehog* (Goetz and Anderson, 2010) and Wnt signalling (Wallingford and Mitchell, 2011). Both types of cilia may also function as sensory receptors. Dysfunction of cilia can lead to a broad spectrum of diseases termed ciliopathies (Bettencourt-Dias et al., 2011; Nigg and Raff, 2009). These include Bardet-Biedl (Ansley et al., 2003), Meckel-Gruber (Frank et al., 2008) and Kartagener's syndrome (Afzelius, 1976).

Importantly, the presence of centrioles is strictly correlated with the presence of cilia in all eukaryotes. All species that have centrioles always possess cilia and vice versa. For example, some lower plants such as mosses and ferns lack both centrioles and cilia in most cells. However, both are manufactured during spermatogenesis (Carvalho-Santos et al., 2011; Marshall, 2009). Therefore, centrioles are universally required for ciliogenesis (Pedersen and Rosenbaum, 2008).

### *Centrosomes as cell cycle regulators*

Accumulating evidence now suggests that centrosomes are important in ensuring the fidelity of cell cycle progression. Many cell cycle regulatory proteins have been found to be localized to the centrosome suggesting that the centrosome may act as a platform from which to control cell cycle events (Basto and Pines, 2007). For example, centrosomes have recently been found to play a role in promoting nuclear envelope breakdown at prophase. The role of the centrosome in this process is independent of its role as a MT organiser and instead centrosomes promote mitotic entry as the site of active Aurora-A kinase (Hachet et al., 2007; Portier et al., 2007). Although some cells can undergo cell division in the absence of centrosomes (Basto et al., 2006; Hinchcliffe et al., 2001; Khodjakov et al., 2000; Khodjakov and Rieder, 2001), centrosomes appear to facilitate the timely entry of cells into mitosis.

Furthermore, depletion or inhibition of a range of centrosome components led to cell cycle arrest in G1 (Mikule et al., 2007). This G1 arrest phenotype was found to depend on p38 kinase, the tumour suppressor p53 and p21. Moreover, depletion of several PCM proteins in human cells induced p53-dependent cell cycle exit (Srsen et al., 2006). These findings suggest a strong link between defective centrosomes and cell cycle arrest. However, another study reported that human cells can progress through G1 after microsurgical removal of existing centrosomes unless they are subjected to additional stresses such as exposure to blue light (Uetake et al., 2007). Thus, it is not the absence of centrosomes which triggers the G1 arrest but in fact a p38-kinase-p53-dependent stress response which is triggered in cells with abnormal centrosomes. Thus, the status of the centrosome can influence cell cycle progression although a fully functional centrosome is not required for G1 arrest.

Several reports now indicate that DNA repair proteins are concentrated at the centrosomes (Doxsey et al., 2005; Shimada and Komatsu, 2009). For example, ATM/ATR kinases and BRCA1 which regulate the homologous recombination repair pathway, are both enriched at centrosomes and knockdown of either protein can cause centrosome overduplication (Ko et al., 2006; Starita et al., 2004). Moreover, the centriole replication protein Plk4 has been found to phosphorylate Cdc25C, a key protein involved in mitotic entry as well as Chk2 which is a key transducer in the DNA damage response pathway (Bonni et al., 2008; Petrinac et al., 2009).

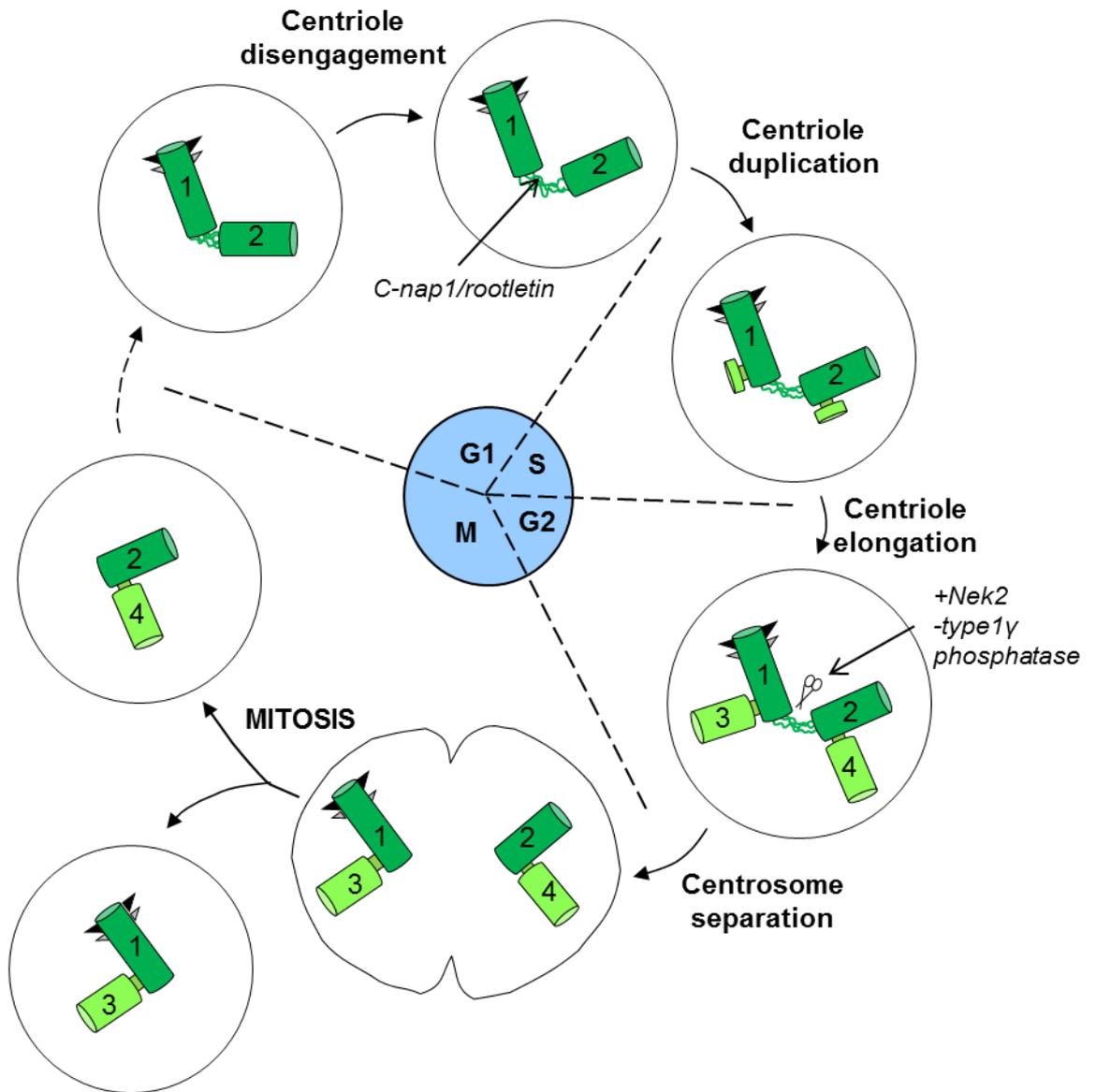
In summary, the above data suggest that as well as its traditional functions as a MT organiser or initiator of ciliogenesis, the centrosome also plays a crucial role in regulating the cell cycle.

## **1.2. The centriole replication pathway**

### **1.2.1 Structural features of the centriole replication pathway**

A single centrosome is present in interphase where it is responsible for nucleation of MTs. However, in mitosis, two centrosomes are required to organise the mitotic spindle poles and ensure accurate segregation of chromosomes. Therefore, the centrosome must be replicated during S-phase in order to allow one complete centrosome (a pair of centrioles and surrounding PCM) to organise each spindle pole in mitosis. Centrosomes are then segregated equally to the daughter cells to re-establish the original copy number (as illustrated in Fig 1.2). In most proliferating cells, the assembly of new centrioles occurs via a so-called canonical pathway in which newly-formed daughter centrioles (procentrioles) assemble in close association with existing mother centrioles within the PCM cloud.

The structural features of centriole duplication were first revealed by electron microscopy (EM) work in the 1980s (Chretien et al., 1997; Guichard et al., 2010; Kuriyama and Borisy, 1981; Vorobjev and Chentsov, 1982). Firstly, in early G1 phase, the mother and daughter centriole lose their orthogonal arrangement and separate from each other slightly. This disengagement of mother and daughter centrioles allows the centrioles to become permissive for duplication (Freed et al., 1999; Piel et al., 2000). Upon entry to S-phase, a procentriole begins to form at the proximal end of each mother centriole (Chretien et al., 1997; Kuriyama and Borisy, 1981). This initial formation of procentrioles in *C. elegans* is characterised by the emergence of a symmetric central tube structure which is thought to be functionally equivalent to the cartwheel in higher organisms (Pelletier et al., 2006). It is now apparent that the central tube/cartwheel both appear to play an important role in determining the number and position of the centriolar MTs (Gopalakrishnan et al., 2010; Hiraki et al., 2007; Kitagawa et al., 2011; Nakazawa et al., 2007; van et al., 2011). In some species, the cartwheel structure is not detected in mature centrioles (Alvey, 1986), suggesting that the central tube/cartwheel is a key structure in specifying the nine-fold symmetry of a forming centriole. However, how the cartwheel structure is destroyed in mature centrioles is unknown.



**Figure 1.2. The canonical centriole replication pathway.** During G1 phase, the two centrioles within the pair are connected by a flexible tether. At the G1-S transition, the centrioles become disengaged, licensing replication which occurs in S-phase with new centrioles (3 and 4) forming perpendicular to the mother centrioles (1 and 2). At G2 phase the two newly-formed pairs separate from each other in order to form the two poles of the mitotic spindle. One centriole pair is then separated into each of the two daughter cells. Appendages are then acquired on the mother centriole in the following G2 phase. Adapted from Delattre and Gonczy 2004 and Nigg and Stearns 2011.

In the subsequent G2 phase, newly-formed procentrioles elongate until they reach approximately the same length as their mothers (Chretien et al., 1997). The two new centriole pairs remain tethered at the proximal end of the mother centrioles by a flexible linker which includes the proteins C-nap1 (Fry et al., 1998) and rootletin (Bahe et al., 2005). This structure containing two pairs of centrioles is often referred to as the 'diplosome'. Early in mitosis, the two centrosomes must then be separated by the severing of the proteinaceous link between the two mothers. This occurs once Nek2 kinase activity exceeds that of the antagonistic type 1 $\gamma$  phosphatase and results in the phosphorylation of the linker proteins, rootletin and C-nap1 (Bahe et al., 2005; Fry et al., 1998; Mayor et al., 2000). The newly-separated centriole pairs, each surrounded by its own cloud of PCM, can then form opposite poles of the mitotic spindle. As a result, one functional centrosome is inherited into each daughter cell and the correct centrosome number is maintained (see Fig 1.2).

In the G2 phase of the following cell cycle, the centriole formed in the previous cell cycle becomes fully mature through acquisition of its distal and sub-distal appendages (Paintrand et al., 1992). A dividing cell therefore possesses three generations of centrioles, as illustrated in Fig 1.2. A grandmother centriole (1), a mother centriole (2) and two daughter centrioles (3 and 4), which have varying abilities to nucleate MTs and form cilia (Nigg and Stearns, 2011). This asymmetry in centriole age has been proposed to be important in asymmetric cell divisions in germline stem cells and in *Drosophila* neuroblasts (Conduit et al., 2010; Conduit and Raff, 2010; Januschke et al., 2011; Piel et al., 2000; Rebollo et al., 2007; Wang et al., 2009; Yamashita et al., 2007). Interestingly, in *Drosophila* most mother centrioles lack appendages (Callaini and Riparbelli, 1990). However, centrioles can still behave asymmetrically suggesting that mother and daughter centrioles can still be distinguished in the absence of appendages.

### **1.2.2. Key molecules in the centriole replication pathway**

The nematode *C. elegans* has played a pivotal role in understanding centrosome biology. Its centrioles are small and structurally more simple than higher organisms being composed of a central tube surrounded by MT singlets (Pelletier et al., 2006). In fact, the mature *C. elegans* centriole has been proposed to be structurally analogous to immature procentrioles in higher organisms (Dammermann et al., 2004). Nematodes therefore provide an excellent system in which to examine the centriole assembly pathway. Although the structural morphogenesis of centriole replication was first described in the 1980s (Chretien

et al., 1997; Guichard et al., 2010; Kuriyama and Borisy, 1981; Vorobjev and Chentsov, 1982), it was another 20 years before extensive genetic and RNAi-based screens carried out in *C. elegans* identified a small number of proteins necessary for centriole replication: the kinase ZYG-1 (O'Connell et al., 2001), as well as the coiled-coil proteins SPD-2 (Kemp et al., 2004; Pelletier et al., 2004), SAS-4 (Kirkham et al., 2003), SAS-5 (Delattre et al., 2004) and SAS-6 (Dammermann et al., 2004; Leidel et al., 2005). Homologues of these *C. elegans* centriole replication proteins have now been identified in several other eukaryotes. SPD-2, SAS-4 and SAS-6 all have homologues in human cells termed Cep192 (Pelletier et al., 2004), CPAP/CENPJ (Hung et al., 2000) and hsSAS-6 (Leidel et al., 2005) respectively. These proteins feature heavily in this thesis therefore see Figure 1.3 for a table of the key centriole replication proteins and their functional homologues which has been compiled for reference. In addition, see Appendix 1 for a more detailed table describing the effect of overexpression or disruption of each of these proteins.

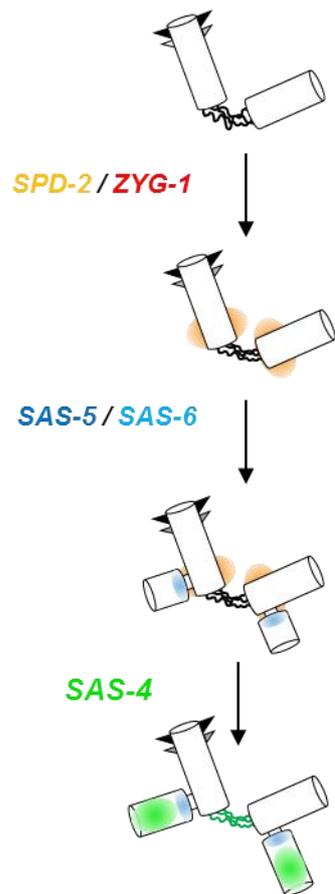
Although more than 100 proteins have been found to be enriched at the centrosomes (Keller et al., 2005), it is unclear how many of these proteins are absolutely required for centrosome function. A recent genome-wide RNAi screen in *Drosophila* has now indicated that just nine proteins are required for centriole duplication in flies (Dobbelaere et al., 2008). These include functional homologues of the five proteins found to be necessary for centriole formation in *C. elegans* as well as three proteins which have previously been implicated in centriole duplication on the basis of the anastral spindle phenotype observed when depleted from *Drosophila* somatic cells (Ana1-3 (Goshima et al., 2007)). Therefore, it is clear that of the numerous proteins localised to the centrosome, only a small subset of these are required for centriole replication and these appear to be conserved between species. As well as the role of the conserved centriole replication proteins in the assembly of new centrioles, proteins which localise to the PCM have also been found to be important in controlling the formation of new centrioles. Indeed, knockdown of the PCM proteins  $\gamma$ -tubulin and SPD-5 causes centriole assembly defects in *C. elegans* (Dammermann et al., 2004).

Electron tomography-based experiments in *C. elegans* embryos have now helped delineate the sequence in which these five proteins are recruited to the centrioles to initiate replication (Delattre et al., 2006; Pelletier et al., 2006). Firstly, the coiled-coil protein SPD-2 is recruited to each mother centriole

A)

<i>C.elegans</i>	<i>Drosophila</i>	Human	<i>Xenopus</i>
SPD-2	D-SPD-2	Cep192	
ZYG-1	SAK	Plk4	Plx4
SAS-6	D-SAS-6	hsSAS-6	
SAS-5	Ana2	STIL	
SAS-4	D-SAS-4	CPAP/CENPJ	

B)



**Figure 1.3.** (A) Nomenclature of key centriole replication proteins and their functional homologues. (B) Schematic diagram showing the involvement of the key *C. elegans* proteins in centriole replication.

(Kemp et al., 2004; Pelletier et al., 2004). Interestingly, SPD-2 is vital in *C. elegans* centriole biogenesis (Kemp et al., 2004; Pelletier et al., 2004) but not in *Drosophila* (Dix and Raff, 2007; Giansanti et al., 2008). In human cells, controversy still remains as to whether the SPD-2 homologue, Cep192 is required for centriole replication ((Gomez-Ferreria et al., 2007; Zhu et al., 2008); see Appendix 1). As well as playing a role in the assembly of new centrioles, SPD-2 has also been shown to be required for PCM recruitment (Kemp et al., 2004; Pelletier et al., 2004).

SPD-2 is required for the centrosomal recruitment of the kinase ZYG-1 (O'Connell et al., 2001). Although ZYG-1 does not have a structural homologue in humans, Plk4/SAK has been found to fulfill a functionally analogous role in human cells (Habedanck et al., 2005) as well as *Drosophila* (Bettencourt-Dias et al., 2005). In all species examined, ZYG-1/SAK/Plk4 was found to be necessary for centriole replication (Bettencourt-Dias et al., 2005; Habedanck et al., 2005; Kuriyama et al., 2009; O'Connell et al., 2001; Rodrigues-Martins et al., 2008). Thus, Plk4 is considered the master regulator of centriole replication and is thought to fulfill a regulatory rather than structural role in the assembly of new centrioles. The role of Plk4 in centriole biogenesis in early murine development is addressed in Chapter 4. A more detailed description of Plk4 and its mechanism of action is therefore provided in section 4.1.

In *C. elegans*, activation of ZYG-1 at the centrosome is necessary for the recruitment of the structural proteins SAS-5 and SAS-6 which form part of the central tube. It has now been proposed that ZYG-1-mediated phosphorylation of SAS-6 at Ser123 is essential for formation of the central tube structure and the maintenance of SAS-6 at this central tube (Kitagawa et al., 2009). Similarly, in human cells, hsSAS-6 is recruited but not maintained at centrioles in Plk4-depleted cells (Strnad et al., 2007). Thus, ZYG-1 is required for the recruitment of SAS-6 to forming centrioles. Based on the localization to the proximal end of the centriole and the phenotypes observed following its disruption (see Appendix 1), SAS-6 (and its homologues) were proposed to participate in the establishment of the nine-fold radial symmetry of the cartwheel and in imparting this symmetry to the growing centriole (Gopalakrishnan et al., 2010; Hiraki et al., 2007; Leidel et al., 2005; Nakazawa et al., 2007; Rodrigues-Martins et al., 2007a; Strnad et al., 2007; van et al., 2011). Recent structural data has now confirmed that SAS-6 oligomerisation is the key to the establishment of this symmetric structure (Gopalakrishnan et al., 2010; Kitagawa et al., 2011; van et al., 2011). Structural homologues of SAS-5 have remained elusive, however recently, the human STIL

protein and *Drosophila* Ana2 have been found to interact with SAS-6 and have therefore been suggested to be functionally equivalent to the *C. elegans* SAS-5 (Arquint et al., 2012; Pelletier et al., 2006; Stevens et al., 2010a; Stevens et al., 2010b; Tang et al., 2011; Vulprecht et al., 2012). Indeed, structures similar to the central cartwheel were assembled in *Drosophila* spermatocytes upon co-expression of SAS-6 and Ana2 (Stevens et al., 2010a). In Chapter 5 of this thesis, the role of SAS-6 in centriole biogenesis in mouse oocytes and embryos is addressed and therefore SAS-6 is covered in more detail in section 5.1.

Finally, SAS-4 is recruited by SAS-6-SAS-5 and is required for the assembly of MT blades onto the outer wall of the centriole cylinder via the A-tubule (Guichard et al., 2010). Initially, SAS-4 associates loosely with the nascent procentriole but upon  $\gamma$ -tubulin-mediated addition of MTs it becomes stably incorporated (Dammermann et al., 2004). MT attachment in other species depends upon the action of SAS-4 homologues CPAP and D-SAS-4, in humans (Kohlmaier et al., 2009; Schmidt et al., 2009; Tang et al., 2009) and *Drosophila* respectively (Basto et al., 2006; Kohlmaier et al., 2009; Schmidt et al., 2009; Stevens et al., 2007; Tang et al., 2009). In line with this, knockdown of these proteins impairs centriole replication (Basto et al., 2006; Kirkham et al., 2003; Kohlmaier et al., 2009; Leidel and Gonczy, 2003; Stevens et al., 2007) as well as Plk4-induced centriole amplification (Kleylein-Sohn et al., 2007; Tang et al., 2009). Both SAS-4 and CPAP have found to interact with tubulin and have been proposed to sequester tubulin dimers which promotes MT incorporation at the distal ends of centriolar MTs in human cells (Cormier et al., 2009; Kohlmaier et al., 2009; Schmidt et al., 2009; Tang et al., 2009). Intriguingly, only procentrioles (and not mature mother centrioles) can elongate suggesting a centriole-intrinsic length limiting method. Several recent studies now illustrate that the final length of the centriole is determined by the antagonistic actions of CP110 and CPAP. Overexpression of CPAP/SAS-4 was found to induce extra long centrioles, whereas CP110 appears to limit MT extension by serving as a protective cap (Chen et al., 2002; Kohlmaier et al., 2009; Schmidt et al., 2009; Tang et al., 2009). Consistent with this CP110 (and its binding partners Cep290 and Cep76) can also suppress ciliogenesis (Spektor et al., 2007).

In addition to these components, some additional proteins have now been implicated in centriole replication in humans and *Drosophila* including Cep135/Bld10 (Dobbelaere et al., 2008; Kim et al., 2008; Kleylein-Sohn et al., 2007; Mottier-Pavie and Megraw, 2009; Ohta et al., 2002) and Cep152/Asl (Blachon et al., 2008; Dzhindzhev et al., 2010; Hatch et al., 2010; Stevens et al., 2010a; Stevens et al., 2010b) and

disruption of these proteins led to errors in centriole assembly. In *Drosophila* and humans, Asl/Cep152 is necessary for Plk4 recruitment to the centriole and forms a scaffold between Plk4 and D-SAS-4/CPAP which is required for centriole assembly (Cizmecioglu et al., 2010; Dzhindzhev et al., 2010). All of these proteins and their role in centriole replication in various species are summarized in the table shown in Appendix 1.

### **1.3. *De novo* centriole formation**

Replication of pre-existing centrioles usually occurs via the canonical pathway (Fig 1.2). However, procentrioles can also assemble via an alternative *de novo* pathway, in which centrioles appear in the absence of a pre-existing mother centriole.

#### **1.3.1. Artificial activation of the *de novo* centriole formation pathway**

In the early 2000s, a series of elegant experiments by Khodjakov and co-workers showed that the *de novo* pathway could be activated in cycling or S-phase-arrested somatic cells if resident centrioles were removed by laser ablation or microsurgery (Khodjakov et al., 2002; La et al., 2005; Uetake et al., 2007). Thus, pre-existing centrioles are not strictly required for centriole assembly and animal cells have the capacity to generate centrioles '*de novo*'. However, a variable number of procentrioles are produced on activation of *de novo* centriole assembly in somatic cells and some of these procentrioles exhibit structural aberrations (Khodjakov et al., 2002). Therefore, centrioles, when present, restrict the number of daughter centrioles formed in each cell cycle. If cells which have formed multiple centrioles *de novo* are then allowed to enter mitosis this leads to aberrant cell divisions (Khodjakov et al., 2002). Evidently this experimentally-induced *de novo* centriole formation pathway is not as rigorously controlled as the canonical centriole replication pathway. Therefore, it follows that *de novo* centriole formation is normally suppressed by the presence of a single centriole in the cytoplasm (La et al., 2005; Marshall et al., 2001; Uetake et al., 2007). Interestingly, the phenomenon of *de novo* centriole formation upon laser ablation was first thought to be unique to cancer cells. However, Uetake et al., (2007) demonstrated that untransformed cells could also generate centrioles *de novo* when existing centrioles were laser ablated (Uetake et al., 2007). The number of extra centrioles which can form *de novo* in untransformed cells is less than in cancer cells suggesting that cancer cells lack an additional control mechanism which restricts the number of centrioles (Uetake et al., 2007). *De novo*-formed centrioles may also represent a possible

origin of the extra centrioles commonly observed in cancer cells. However, this hypothesis remains to be tested.

*De novo* formation of functional centrioles can also be triggered by overexpression of centriole replication factors in acentriolar *Drosophila* eggs (Dzhinzhev et al., 2010; Peel et al., 2007; Rodrigues-Martins et al., 2007a; Rodrigues-Martins et al., 2007b; Stevens et al., 2010a) or mature *Xenopus* eggs/egg extracts (Eckerdt et al., 2011). However, it is worth noting that high level overexpression of SAK or SAS-6 cannot activate the *de novo* pathway in centriole-containing *Drosophila* somatic cells even though subunit pools exist to manufacture multiple centrioles (Peel et al., 2007; Rodrigues-Martins et al., 2007b). Again, the presence of an endogenous centriole blocks the *de novo* centriole assembly pathway, presumably to prevent the deleterious effects of supernumerary centrioles. On the other hand, physical removal of the centriole induces *de novo* centriole assembly in the presence of endogenous levels of centriole replication proteins. The finding that *de novo* centriole formation does not depend on supplementation of a centriole replication protein, implies that inhibition of this pathway is due to an alternative regulatory mechanism rather than the shortage of a particular centriole replication protein (Khodjakov et al., 2002; La et al., 2005; Uetake et al., 2007). Furthermore, *de novo* centriole formation is prevented if entry to S-phase is blocked (La et al., 2005; Uetake et al., 2007). Collectively, these results imply that both *de novo* and canonical centriole replication are dependent on the same molecular requirements and it has been suggested that rather than providing a template for centriole assembly, the mother centriole acts as a platform for the recruitment and assembly of centriole replication proteins (Rodrigues-Martins et al., 2007b). Importantly, although centrioles can form *de novo* upon overexpression of centriole replication factors or following laser ablation, the rate of assembly is significantly slower than if an existing centriole is present (La et al., 2005; Marshall et al., 2001; Peel et al., 2007; Rodrigues-Martins et al., 2007b). This slower rate of construction could be deleterious in dividing cells as *de novo* centriole assembly is unlikely to keep up with the rate of cell division, providing further reasons why *de novo* centriole assembly is only permitted if canonical centriole replication is not possible (Pelletier, 2007).

As well as experimentally-induced *de novo* centriole assembly (Khodjakov et al., 2002; La et al., 2005; Uetake et al., 2007), some biological systems can sometimes assemble centrioles *de novo* despite the fact

that the canonical replication pathway is the dominant one. Examples include *Drosophila*, sea urchin or rabbit embryos in which parthenogenesis is artificially activated (Kallenbach, 1983; Riparbelli and Callaini, 2003; Szollosi and Ozil, 1991).

### **1.3.2. The role of the PCM in *de novo* centriole formation**

The PCM has been proposed to play an important role in preventing *de novo* centriole formation in centriole-containing cells (Loncarek and Khodjakov, 2009; Song et al., 2008). Since daughter centrioles normally form within the PCM cloud as opposed to in direct contact with the mother centriole itself, the principal role of the mother centriole may be in concentrating centriole assembly factors within the PCM which in turn promotes daughter centriole formation (Dammermann et al., 2004; Loncarek et al., 2008). Therefore, in canonical centriole duplication the PCM may serve to focus centriole assembly proteins allowing duplication to proceed in the vicinity of the mother centriole. Centriole assembly in proximity to a pre-existing centriole may be kinetically preferred over *de novo* assembly in the cytoplasm and therefore the lower fidelity *de novo* formation pathway would only be activated in the absence of intact centrioles. In *de novo* centriole formation, the random aggregation of PCM may allow centriole formation when a critical mass is reached. However, this hypothesis remains to be tested (Loncarek and Khodjakov, 2009; Song et al., 2008). Nevertheless, this theory also explains why an absence of the PCM proteins SPD-5 and  $\gamma$ -tubulin in *C. elegans* embryos leads to centriole assembly defects (Dammermann et al., 2004). Moreover, *de novo* centriole formation in somatic cells in which endogenous centrioles have been artificially removed is preceded by the appearance of  $\gamma$ -tubulin foci (Khodjakov et al., 2002), adding further weight to the notion that the PCM plays a crucial role in dictating the assembly of new centrioles via the canonical or *de novo* pathway.

### **1.3.3. Examples of physiological *de novo* centriole formation**

There are also a small number of physiological examples of *de novo* centriole formation. For example the formation of multiple basal bodies for ciliogenesis in multiciliated cells (Beisson and Wright, 2003; Vladar and Stearns, 2007), in the production of flagellated sperm in plants (Renzaglia and Maden, 2000), in early embryos of parthenogenetic species (Callaini et al., 1999; Riparbelli et al., 1998) and interestingly, in the early mouse embryo (Abumuslimov et al., 1994; Gueth-Hallonet et al., 1993; Szollosi et al., 1972). Strikingly, when *de novo* centriole formation occurs in a natural setting the process is always

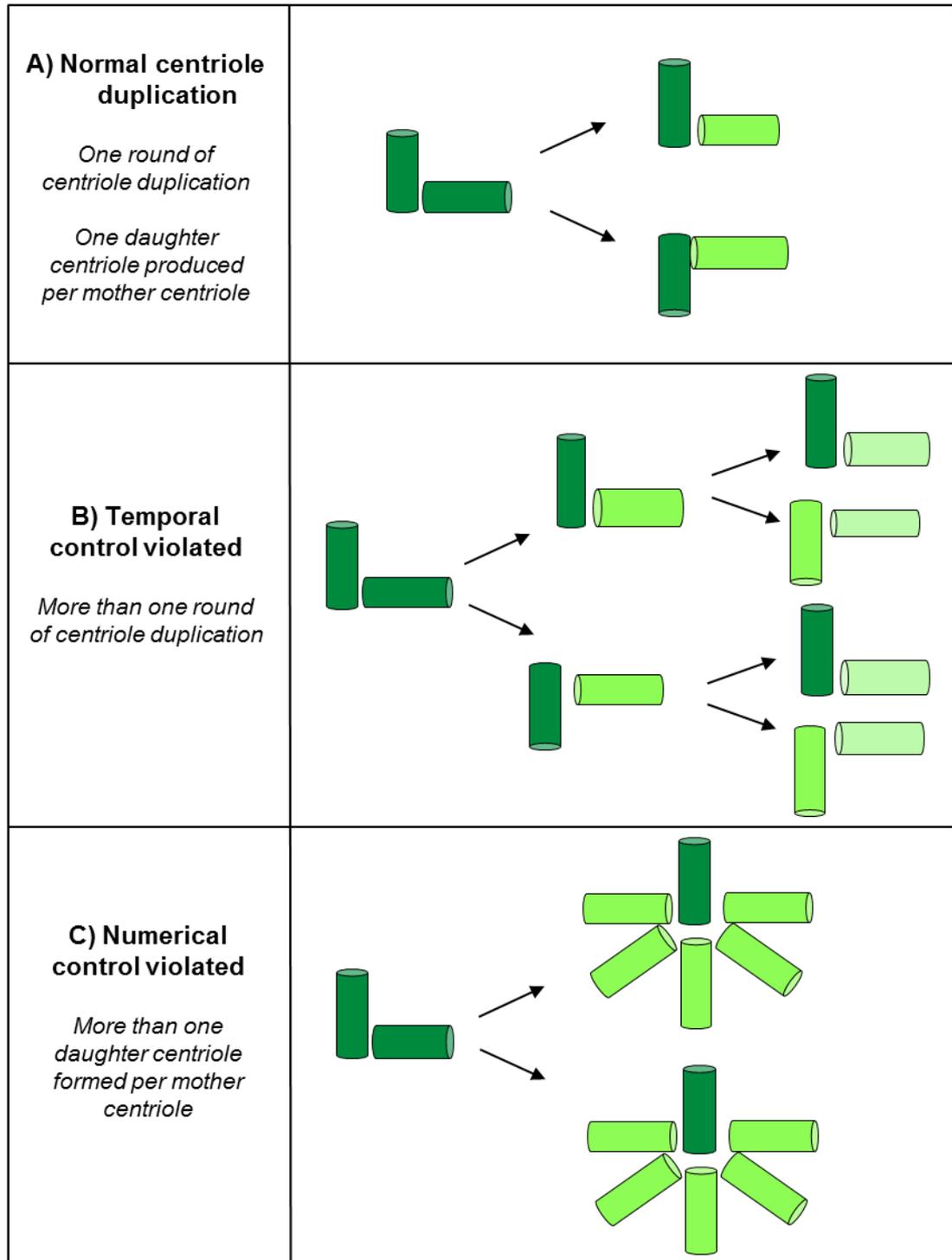
tightly regulated and results in the correct number of centrioles being produced. Although, artificially-induced *de novo* centriole formation has previously been studied (Dzhinzhev et al., 2010; Khodjakov et al., 2002; La et al., 2005; Peel et al., 2007; Rodrigues-Martins et al., 2007a; Rodrigues-Martins et al., 2007b; Stevens et al., 2010a; Uetake et al., 2007), the molecular requirements for physiological *de novo* centriole assembly remain poorly understood.

## **1.4. Controlling centriole number**

In order to maintain fidelity of chromosome segregation and to avoid causing genomic instability, it is imperative that the correct number of centrosomes is maintained throughout the cell cycle. An increase in centrosome number is well known to promote genetic instability (Bettencourt-Dias et al., 2011; Nigg, 2002; Nigg, 2006). The mechanisms underlying this process will be discussed in more detail in section 1.4.3. Since the number of centrioles determines the number of centrosomes (Bobinnec et al., 1998a), it is vital that centriole replication is precisely controlled. Deviations in the number of centrioles can result from a failure of cytokinesis or cell-cell fusion (which would generate a tetraploid cell with four centrosomes (Holland and Cleveland, 2009)). In addition, numerical centrosome aberrations could arise through deregulation of the centrosome cycle either as a result of assembly of multiple procentrioles around a single mother or more than one replication event per cell cycle (Nigg, 2007; Song et al., 2008). Therefore, several different levels of control operate to limit the number of centrioles, which will be discussed in the following sections (see Fig 1.4 for a diagram illustrating these two rules controlling centriole number).

### **1.4.1. Temporal control of centriole replication**

Temporal control ensures that only one centriole duplication event occurs in each S-phase (see Fig 1.4B). In cancer cells or cells that lack the tumour suppressor p53, centrosomes are able to over-replicate during a prolonged S-phase arrest (Balczon et al., 1995; Bennett et al., 2004; Tarapore and Fukasawa, 2002). However, in normal cells, centrosomes cannot re-duplicate if they are arrested for long periods of time in S-phase (Wong and Stearns, 2003). Thus, untransformed cells possess a mechanism for inhibiting re-duplication of centrioles. Centrosomes and chromosomes must both be replicated in a semi-conservative fashion, once and only once per cell cycle. Therefore, these two pathways are tightly coordinated. Indeed, defective synchronisation between the two cycles is a familiar feature of many cancer cells in which



**Figure 1.4. Two rules controlling centriole number.** (A) In a normal cell division, each of the two resident centrioles replicates once and produces one daughter centriole. Thus, the cell contains four centrioles (two centrosomes) in total at mitosis. (B) The first rule ensures that centriole replication must happen only once per cell cycle after passage through M-phase. If this temporal control is violated centrioles re-duplicate in S or G2 phase resulting in extra centrioles. (C) The second rule ensures only one procentriole is formed in each replication event. If this numerical control is violated multiple procentrioles are formed in association with a mother centriole. Adapted from Nigg, 2007.

centriole numbers as well as chromosome numbers are frequently deregulated (Kramer et al., 2004; Nigg, 2002). In addition, centriole replication and DNA replication can be uncoupled in various scenarios. For example, repeated duplications of the same mother centriole can occur upon expression of SAK or SAS-6 in human or *Drosophila* cells as well as in human tumour cells following expression of the viral E7 oncogene (Duensing et al., 2007; Duensing and Munger, 2003; Kleylein-Sohn et al., 2007; Strnad et al., 2007).

Similar to chromosome replication, assembly of the daughter centriole is also under the control of the cell cycle proteins Cdk2/cyclin E or Cdk2/cyclin A which confines both of these replication events to S-phase (Hinchcliffe et al., 2001; Hinchcliffe and Sluder, 2001). Centrosomal accumulation of three Cdk2 substrates (nucleophosmin, CP110 and Mps1) has been found to be crucial for centriole replication (Tsou and Stearns, 2006a). Thus, Cdk2-mediated phosphorylation of specific substrates at S-phase onset coincides with centriole replication (Lacey et al., 1999; Matsumoto et al., 1999; Meraldi et al., 1999). However, cyclin E1/E2 has been found to be dispensable for centriole duplication in mice and humans (Geng et al., 2003). Moreover, cell fusion studies revealed that duplicated centrioles (G2) returned to S-phase (where Cdk2/cyclin E/A activity is high) could not re-duplicate. In contrast, unduplicated G1 centrioles were able to duplicate in an S-phase cytoplasm (Wong and Stearns, 2003). These results suggested that Cdk2/cyclin E/A activity alone is not sufficient to authorize centriole re-duplication and instead points to a centrosome-intrinsic block to the re-duplication of centrioles within a single cell cycle (Wong and Stearns, 2003). Combined with ultrastructural data, these studies led to the proposal of a licensing model in which the tight orthogonal association of newly-replicated centrioles blocks further duplication until disengagement at the end of mitosis licenses the two centrioles for a further round of duplication (Tsou and Stearns, 2006a). Thus, newly-duplicated centrosomes must pass through mitosis before they are able to duplicate again. Further support for this model was obtained from laser ablation experiments in HeLa cells arrested in S-phase, which normally do not re-duplicate their centrioles. However, upon ablation of an engaged daughter centriole, the mother centriole initiates re-duplication, illustrating that engagement of the mother centriole determines its ability to replicate (Loncarek et al., 2008).

Centriole disengagement can be blocked by non-degradable forms of either the anaphase inhibitor securin or Cdk1/cyclin B1 (Tsou and Stearns, 2006a; Tsou and Stearns, 2006b). Since both of these proteins block the proteolytic activity of separase, this protease has been implicated in centriole disengagement. Indeed, separase in cooperation with the polo-like kinase 1 (Plk1) has been found to regulate the centriole licensing event in human cells (Tsou et al., 2009). Separase is also the well known regulator of sister chromatid separation in mitosis and meiosis. Sister chromatids are paired by the tripartite cohesin complex (Scc1-Smc1-Smc3). In mitosis, separase cleaves the Scc1 subunit of the cohesin complex and sister chromatids are separated (Hauf et al., 2001; Tomonaga et al., 2000; Uhlmann et al., 1999). Prior to anaphase this cohesion is protected by shugoshin 1 and protein phosphatase 2A (Kitajima et al., 2004; Kitajima et al., 2006; Kudo et al., 2006; McGuinness et al., 2005; Riedel et al., 2006). In meiosis I, Rec8 – the meiotic counterpart of Scc1 – is cleaved at chromosome arms (but maintained at centromeres) which induces the separation of homologous chromosomes (Kitajima et al., 2004; Kitajima et al., 2006; Kudo et al., 2006; Kudo et al., 2009; Riedel et al., 2006). In meiosis II, centromeric Rec8 cohesion is cleaved by separase and sister chromatids are separated (Kitajima et al., 2003).

Cohesin has now also been identified as forming part of the link between mother and daughter centrioles (Schockel et al., 2011). Indeed, disengagement was triggered when human separase was added to human centrioles in *Xenopus* extracts (Schockel et al., 2011). Furthermore, depletion of a splice variant of shugoshin (sSgo1) triggers aberrant centriole separation (Wang et al., 2008), suggesting that sSgo1 protects the cohesive factor linking mother and daughter centrioles preventing untimely disengagement. The fact that the same proteins are involved in both sister chromatid separation and centriole disengagement ensures that the processes of DNA replication and centriole duplication are tightly synchronised in the cell cycle and ensures that a daughter cell will receive one full set of chromosomes and one full centrosome (Schockel et al., 2011).

Recently, a novel centrosomal protein Cep76 was identified which specifically prevents centrioles from undergoing multiple rounds of duplication within a single cell cycle (Tsang et al., 2009). Cep76 overexpression was found to suppress centriole amplification generated through multiple rounds of duplication in S-phase-arrested cancer cells. Interestingly, overexpression is not able to suppress centriole amplification mediated by Plk4 overexpression (in which parental centrioles give rise to multiple

daughters (Tsang et al., 2009)). In addition, Cep76 does not affect normal centriole duplication. Since this phenotype is specific to cancer cells, it is likely that non-transformed cells possess additional mechanisms for suppressing centrosome amplification and a role for Cep76 is only revealed if another (as yet unidentified) regulatory protein is lost or mutated in tumourigenesis (Tsang et al., 2009).

#### **1.4.2. Numerical control of centriole replication**

Cells also possess mechanisms to limit the number of daughter centrioles formed to one per duplication event (Fig 1.4C). In ciliogenesis, multiple daughter centrioles can form simultaneously in tracheal cells (Dawe et al., 2007; Dirksen, 1991). Furthermore, physical removal of a daughter centriole in S-phase-arrested cells causes the mother centriole to form multiple daughters. If these daughter centrioles are then ablated, multiple daughters are formed again although this number does not always match the number that were ablated (Loncarek et al., 2008), indicating that daughter centrioles do not appear to require one specific site near the wall of the mother for their assembly.

Plk4 is now known to be a key regulator of centriole replication in humans (Habedanck et al., 2005) and *Drosophila* (Bettencourt-Dias et al., 2005). Overexpression of Plk4 in human cells results in the formation of multiple daughter centrioles around a single mother in a characteristic “rosette-like” arrangement (Habedanck et al., 2005; Kleylein-Sohn et al., 2007) and on the other hand a lack of Plk4 causes a progressive loss of centrioles through impaired duplication (Bettencourt-Dias et al., 2005; Habedanck et al., 2005). Therefore, Plk4 levels are closely correlated with centriole number. Furthermore, Plk4-mediated centriole over-replication is now used as a model for the loss of numerical control phenotype (Ganem et al., 2009) and a system in which to test the effect of protein depletion on centriole replication (Dzhindzhev et al., 2010; Kleylein-Sohn et al., 2007; Tsang et al., 2009).

In order to prevent centriole over-replication, cells possess mechanisms to limit the availability of centriole replication proteins. The first evidence for proteasomal degradation as a mechanism for controlling Plk4 levels came from studies in U2OS cells where inhibition of the proteasome led to Plk4-dependent centriole over-replication in a ‘rosette’ arrangement (Duensing et al., 2007), reminiscent of that observed upon Plk4 overexpression (Habedanck et al., 2005; Kleylein-Sohn et al., 2007). Studies have now shown that centriole amplification is prevented by degradation of Plk4 mediated by the SCF (SKP1-

CUL1-E-box protein) ubiquitin ligase (Cunha-Ferreira et al., 2009b; Rogers et al., 2009). Accumulating evidence from several groups now points towards autophosphorylation events as a key determinant of Plk4 kinase stability (Guderian et al., 2010; Holland et al., 2010; Sillibourne et al., 2010). Additionally, PP2A phosphatase has been shown to counteract Plk4's stabilizing autophosphorylation activity (Brownlee et al., 2011). These data illustrate the importance of proteasomal degradation pathways in controlling the number of daughter centrioles formed. It has now been proposed that Plk4 overexpression marks additional assembly sites on mother centrioles and provides the 'seed' which initiates the rapid growth of single procentrioles (Loncarek et al., 2007; Rodrigues-Martins et al., 2007b).

Formation of centriolar rosettes also occurs following overexpression of SAS-6 (Leidel et al., 2005; Strnad et al., 2007). In line with this, human SAS-6 levels are regulated by proteasomal degradation directed by the APC<sup>Cdh1</sup> and this control is vital for limiting SAS-6 levels and preventing the formation of supernumerary daughter centrioles (Strnad et al., 2007). Since overexpression of either Plk4 or SAS-6 can cause cells to lose their numerical control over centriole duplication, the number of procentrioles formed in each S-phase may be dictated by limiting amounts of active Plk4 that recruits limiting amounts of SAS-6 to the mother centriole (Nigg and Raff, 2009; Rodrigues-Martins et al., 2007b). Moreover, SAS-6 has been shown to be regulated by the upstream Plk4 in humans and *C. elegans* (Kitagawa et al., 2009; Puklowski et al., 2011), therefore strengthening the idea that these two key proteins act in concert to regulate procentriole formation.

Although two conceptually distinct rules for controlling centriole number have been described in sections 1.4.1 and 1.4.2 above, it is likely that these two means of control are integrated at a molecular level as adherence to both rules is vital to limit the number of centrosomes and therefore maintain genomic stability (Nigg, 2007; Song et al., 2008). In the next section, the consequences of any violations of these important rules will be addressed.

### **1.4.3. The impact of deviations in centrosome number**

Theodor Boveri was the first to postulate a link between numerical centrosomal aberrations and disease in the early 20<sup>th</sup> century (Boveri, 2008). He proposed that deviations in centrosome number could lead to chromosome segregation errors. Indeed, most cancer cells (as well as many pre-malignant lesions) that

have been examined have been found to possess extra centrosomes, in addition to deviations in chromosome number (Lingle et al., 2005; Lingle and Salisbury, 1999; Nigg, 2002; Pihan et al., 2003; Salisbury et al., 2004). Furthermore, a variety of diseases (including cancer) have now been linked to mutations or an absence of specific centrosomal proteins (Bettencourt-Dias et al., 2011; Nigg and Raff, 2009) and similarly, the dysfunction of several oncogenes and tumour suppressors is known to alter centrosome numbers (Fukasawa, 2007; Nigg, 2002; Nigg, 2006). Despite these striking correlations, it remains unclear whether supernumerary centrosomes contribute to carcinogenesis or are simply a consequence of cellular transformation (Nigg, 2002; Nigg, 2006). The following section reviews the state of knowledge with respect to the impact of numerical centriole aberrations and the link between these aberrations and cancer progression.

#### ***Centrosome amplification can initiate tumourigenesis***

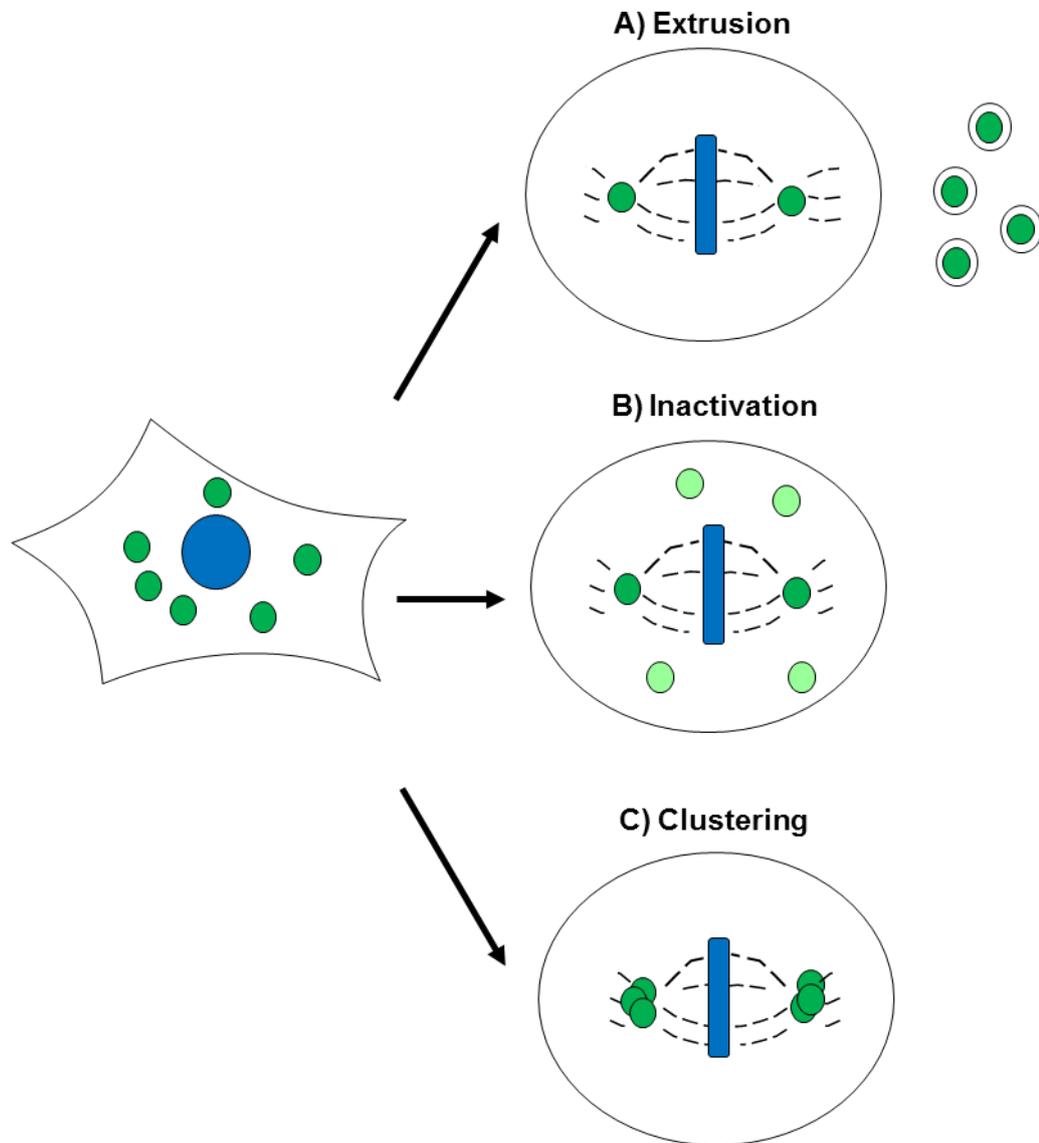
Recent work has now uncovered a causal link between extra centrosomes and tumourigenesis in flies (Basto et al., 2008; Castellanos et al., 2008). In the first of these landmark studies, the centriole replication protein SAK was overexpressed in *Drosophila* cell lines (*SAKOE*) and was found to drive centriole over-replication in ~60% of somatic cells (Basto et al., 2008). This transgenic line was then used to investigate the long-term effects for an organism with too many centrosomes. When larval brain cells harbouring extra centrioles were transplanted into normal hosts, this induced the formation of metastatic tumours which rapidly killed host flies (Basto et al., 2008). Similarly, another study using the same transplantation assay, showed that mutations causing centrosome defects including a complete absence of centrosomes can also cause tumours in wild type host flies (Castellanos et al., 2008). Importantly, the major consequence of extra centrosomes in flies appears to be disruption of asymmetric cell divisions which increases the proportion of proliferating progenitor cells, rather than chromosome mis-segregation (Basto et al., 2008). Nevertheless, these studies illustrate that extra centrosomes are sufficient to initiate tumour formation in flies and provides an important advance in our understanding of the relationship between extra centrosomes and cancer.

#### ***Cells with supernumerary centrosomes can undergo bipolar mitoses***

According to Boveri's early model, centrosome aberrations were predicted to promote tumour formation by causing multipolar spindles which would in turn lead to chromosomal instability upon cell division

(Boveri, 2008; Nigg, 2002). However, although extra centrosomes can initiate tumourigenesis in flies, the resulting tumors only display a small increase in aneuploidy and low rates of multipolar mitoses (Basto et al., 2008). In addition, the frequencies of multipolar divisions in cancer cells are not high enough to explain the observed rates of chromosome segregation errors (Ganem et al., 2009; Silkworth et al., 2009). Furthermore, the progeny of the few multipolar divisions which do occur are inviable which is not expected to favour the clonal expansion of a tumour. These findings are also in line with previous studies in which chromosome mis-segregation reduces cellular viability (Kops et al., 2004; Thompson and Compton, 2008; Williams et al., 2008). Thus, extra centrosomes do not cause aneuploidy through multipolar cell divisions as resulting daughter cells would not be viable.

In order to avoid multipolar mitoses, several mechanisms exist to enable cells with supernumerary centrosomes to undergo bipolar divisions. These include centrosome elimination, centrosome inactivation or centrosome clustering ((Godinho et al., 2009); see Fig 1.5 for a diagrammatic summary of mechanisms employed to evade multipolar mitoses). Centrosome elimination regularly occurs in gametogenesis ((Manandhar et al., 2005; Schatten, 1994); see section 1.5). However, the mechanism by which this is achieved remains poorly understood. Alternatively, centrosomes may be ejected from cells as in *Dictyostelium* cells, which remove extra centrosomes by cytoplasm formation (Graf et al., 2003). Inactivation of supernumerary centrosomes represents a further mechanism to ensure that supernumerary centrosomes are not able to form multiple spindle poles (Fig 1.5B). In this process only two centrosomes are able to function as MTOCs. This is observed in *Drosophila* cells with too many centrosomes, where some centrosomes gradually organise less  $\gamma$ -tubulin and MTs as mitosis progresses (Basto et al., 2008). However, how this inactivation is mediated at a molecular level, and how the centrosomes to be inactivated are distinguished from those which will form the spindle poles, remains unclear (Godinho et al., 2009). An alternative strategy for avoiding multipolar mitoses involves the coalescence of extra centrosomes into two dominant spindle poles (see Fig 1.5C). This clustering process has been observed in several cancer (and non-cancer) cells with extra centrosomes (Ganem et al., 2009; Kwon et al., 2008; Quintyne et al., 2005; Silkworth et al., 2009; Yang et al., 2008) and enables cells to undergo bipolar divisions. This mechanism gives cells with extra centrosomes a selective advantage over cells with intolerably high rates of aneuploidy caused by multipolar mitoses (Basto et al., 2008; Godinho et al., 2009).



**Figure 1.5. Mechanisms to avoid multipolar cell divisions in cells containing supernumerary centrosomes.** (A) Centrosome extrusion: Centrosomes (green discs) can be extruded by the formation of centrosome-containing cytoplasts. (B) Centrosome inactivation: The MTOC activity of additional centrosomes is silenced. (C) Centrosome clustering: Extra centrosomes coalesce into two groups to form a bipolar spindle. DNA is shown in blue. Adapted from Godinho et al., 2009.

Centrosome clustering can also occur in *Drosophila* cells in which extra centrosomes have been artificially induced. Here, multipolar spindles are initially formed, but ultimately resolve into bipolar spindles before cell division (Basto et al., 2008) by clustering of supernumerary centrosomes. This explains why adult flies with extra centrosomes are viable and also why only a small increase in chromosomal instability was observed in these flies with extra centrosomes (Basto et al., 2008). The data above suggest that centrosome clustering is not a phenomenon unique to cancer cells but can also occur in normal cells in response to centrosome amplification and therefore represents a general means which cells employ to avoid the deleterious effects of multipolar cell divisions.

### ***Mechanisms facilitating centrosome clustering***

Recent studies have uncovered multiple mechanisms required for the efficient clustering of extra centrosomes. These include the activity of the spindle assembly checkpoint (SAC), various molecular motors and also the actin cytoskeleton.

Many cancer cells display a high mitotic index which may be explained by an extended metaphase (Lambert, 1913). Moreover, cells with extra centrosomes are often delayed in mitosis. This delay has now been found to be maintained by the SAC (Basto et al., 2008; Kwon et al., 2008; Yang et al., 2008). The SAC monitors whether kinetochores are attached to MTs before allowing cells to undergo anaphase (Musacchio and Salmon, 2007). Thus, unconnected kinetochores activate the SAC which negatively regulates the ability of Cdc20 to activate APC-mediated polyubiquitination, and subsequent degradation, of two substrates (securin and cyclin B1) required for anaphase entry. Cyclin B1 degradation inactivates Cdk1 which promotes mitotic exit. Likewise, securin is an inhibitor of the cysteine protease separase which is required to cleave the cohesin complex which holds sister chromatids together. An active SAC therefore prevents entry to anaphase and allows time for all kinetochores to become attached to MTs. When all kinetochores are attached, the SAC is satisfied and anaphase ensues (Musacchio and Salmon, 2007). Interestingly, if the SAC component Mad2 is mutated in *Drosophila*, flies are still viable and fertile. Thus, the SAC is not strictly required for normal cell divisions in flies (Buffin et al., 2007). However, Mad2 mutant flies which also have extra centrosomes (Mad2, *SAKOE*) are embryonically lethal and die as pupae with high levels of spindle multipolarity and chromosomal instability (Basto et al., 2008). Furthermore, inhibition of the SAC component Mad2 accelerated anaphase onset in cells with

extra centrosomes and prevented centrosome coalescence and spindle bipolarisation (Basto et al., 2008; Kwon et al., 2008; Yang et al., 2008). Therefore, the SAC is vital for cells with extra centrosomes, presumably because it allows extra time for supernumerary centrosomes to cluster into two dominant poles (Basto et al., 2008). It has now been shown that a metaphase delay (not necessarily SAC-mediated) suppresses multipolar divisions as a transient treatment with a proteasome inhibitor can also prevent multipolar divisions (Kwon et al., 2008).

Basto et al., (2008) further show that the minus-end-directed kinesin 14 motor protein Ncd is required for efficient centrosome clustering (Basto et al., 2008). Ncd is needed for focusing of acentrosomal female meiotic spindle poles in flies (Goshima et al., 2005) but, as for *Drosophila* Mad2 mutants, Ncd mutant flies are also viable so Ncd is not essential for normal mitoses (Endow and Komma, 1998; Skold et al., 2005). However, Ncd is indispensable for efficient centrosome clustering in *Drosophila* cells with extra centrosomes (Basto et al., 2008). The minus end-directed motor dynein has also been shown to play an important role in the clustering of supernumerary centrosomes (Quintyne et al., 2005). Thus, minus-end-directed motor proteins appear to be necessary for organising a bipolar spindle in cells with too many centrosomes but are dispensable for normal divisions. Finally, the actin-based machinery which drives centrosome separation and spindle positioning in normal cells has been proposed to facilitate the organisation of supernumerary centrosomes (Kwon et al., 2008; Quintyne et al., 2005).

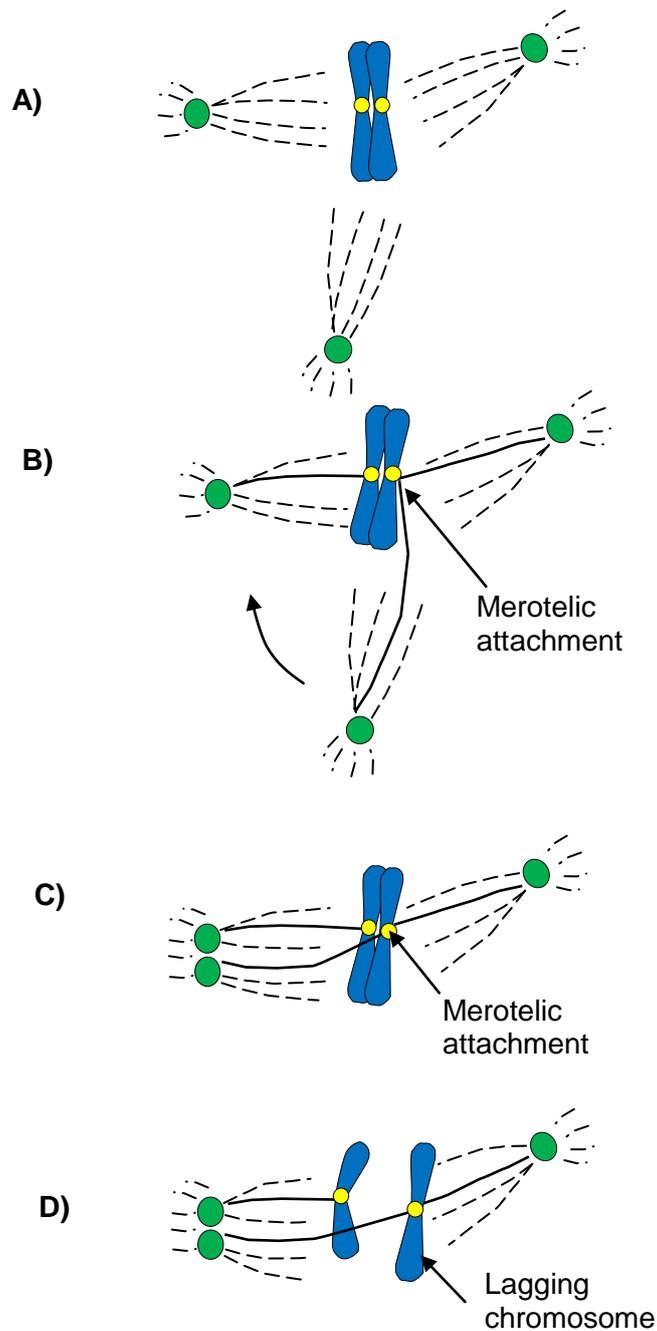
The clustering of supernumerary centrosomes into bipolar spindles is advantageous to tumour cells as it prevents lethality caused by multipolar divisions. Therefore, inhibition of the proteins necessary for centrosome clustering might provide an effective strategy for the selective elimination of cancer cells containing extra centrosomes by induction of an inviable multipolar mitosis (Fielding et al., 2011; Ganem et al., 2009; Kwon et al., 2008; Nigg, 2002). Possible targets include motor proteins such as Ncd (HSET in vertebrates). Ncd is vital for the ability of *Drosophila* cells with supernumerary centrosomes to form bipolar spindles (Basto et al., 2008). But crucially, it is dispensable for normal cell divisions (Buffin et al., 2007; Endow and Komma, 1998; Skold et al., 2005). Accordingly, inhibition of this protein could target malignant cells whilst leaving normal cells unaffected. In support of this, perturbation of HSET function in human tumour cell lines successfully eliminated cells with high levels of centrosome amplification but not those with the normal centrosome complement (Kwon et al., 2008). Since centrosomal abnormalities

usually increase as tumours develop, this suggests that more aggressive cancers may be more susceptible to this chemotherapeutic strategy.

Collectively, these data demonstrate that the presence of extra centrosomes does not necessarily lead to multipolar mitoses. In fact, cells with extra centrosomes appear to be relatively well tolerated in *Drosophila* due to the cooperation of several pathways which allow multiple centrosomes to resolve into bipolar spindles.

### ***A mechanism connecting supernumerary centrosomes and chromosomal instability in cancer cells***

Despite the finding that cells with extra centrosomes can undergo bipolar mitoses (Basto et al., 2008; Kwon et al., 2008; Quintyne et al., 2005; Yang et al., 2008), cancer cells with extra centrosomes still display high rates of chromosome mis-segregation, even when centrosomes cluster into two poles (Ganem et al., 2009; Silkworth et al., 2009). Recent work using time-lapse microscopy has now revealed that centrosome amplification in cancer cells leads to chromosome mis-segregation errors, not through the formation of multipolar spindles, but due to increased rates of ‘merotely’, where one sister kinetochore is simultaneously attached to two (or more) spindle poles (Ganem et al., 2009; Silkworth et al., 2009). These merotelic attachments arise through the formation of transient multipolar spindle intermediates which form as extra centrosomes are coalescing into two dominant poles. Merotelic attachments are not detected by the SAC as all kinetochores are connected to a spindle pole and under tension (Cimini et al., 2001; Musacchio and Salmon, 2007). Unresolved merotely gives rise to lagging chromosomes at anaphase (i.e. chromosomes which travel slowly towards the spindle pole and lag behind their counterparts). This raises the possibility of chromosome segregation errors and the incidence of chromosomal instability ((Ganem et al., 2009; Silkworth et al., 2009); see Fig 1.6 for a schematic illustration of the mechanism leading to chromosomal instability in cells with extra centrosomes). Lagging chromosomes have previously been found at high levels in many cancer cell types and have been implicated as the major cause of chromosomal instability in cancer (Bakhoum et al., 2009; Reing et al., 2004; Saunders et al., 2000; Thompson and Compton, 2008).



**Figure 1.6. Schematic illustration of the mechanism by which extra centrioles can lead to chromosomal instability (proposed in Silkworth et al., 2009; Ganem et al., 2009).** (A) In cells with extra centrosomes (green discs), transient multipolar spindles assemble before centrosome clustering occurs. (B) In this configuration single kinetochores (yellow) are more likely to become attached to two spindle poles generating a merotelic attachment. (C) Multipolar spindles then bipolarize via centrosome coalescence before the onset of anaphase. (D) Merotelic attachments are not detected by the SAC so anaphase can commence with mis-attached kinetochores. More multipolar spindle intermediates leads to more merotelic attachments which give rise to lagging chromosomes in anaphase and hence chromosome segregation errors and aneuploidy. Chromosomes are shown in blue. Adapted from Silkworth et al., 2009.

Ganem et al., (2009) further showed that the artificial induction of extra centrioles in normal cells (by overexpression of Plk4 or induction of tetraploidisation by inhibiting cytokinesis) also led to an increased incidence of lagging chromosomes and chromosomal instability (Ganem et al., 2009). Importantly however, the frequency of chromosomal instability in diploid cells engineered to have extra centrosomes did not reach the levels seen in cancer cells, suggesting that cancer cells with chromosomal instability may have additional defects in the machinery which detects and repairs kinetochore-MT misattachments. In agreement with this, many cancer cells with the regular two centrosomes also exhibit increased rates of lagging chromosomes (Ganem et al., 2009). Together these data indicate that extra centrosomes induce chromosomal instability by generating transient multipolar spindles which increases the incidence of kinetochore-MT misattachments. Therefore, although centrosome coalescence prevents multipolar mitoses which would lead to inviable progeny, it represents a major mechanism contributing to chromosomal instability in cancer (Ganem et al., 2009; Silkworth et al., 2009). In addition, it is highly likely that other mechanisms may also contribute to chromosome instability in cancer cells such as a decreased efficiency of correction mechanisms for kinetochore-MT misattachments (Bakhoum et al., 2009). Nevertheless, the mechanism described above can explain a large incidence of chromosomal instability errors occurring in cancer cells.

Although chromosomal instability may appear to be unfavourable for cell viability and tumor growth, it has been suggested that cancer cells may be inherently more tolerant to low levels of aneuploidy whilst gross chromosomal instability is detrimental (Thompson and Compton, 2008; Weaver et al., 2007; Weaver and Cleveland, 2008). However, the means by which this is achieved is unclear. Indeed, it has been shown that chromosomally-unstable cells can promote cancer progression in mouse models (Schvartzman et al., 2010). Moreover, induction of chromosomal instability independent of centrosome amplification did not lead to tumourigenesis in flies (Castellanos et al., 2008). Hence, it is probable that extra centrosome-mediated chromosomal instability contributes to tumourigenesis.

In summary, cells with extra centrosomes employ many mechanisms to avoid the deleterious effects of a multipolar cell division. However, cells with extra centrosomes can still lead to chromosomal instability through the formation of multipolar spindle transients which increase the likelihood of lagging chromosomes and subsequent chromosome mis-segregation.

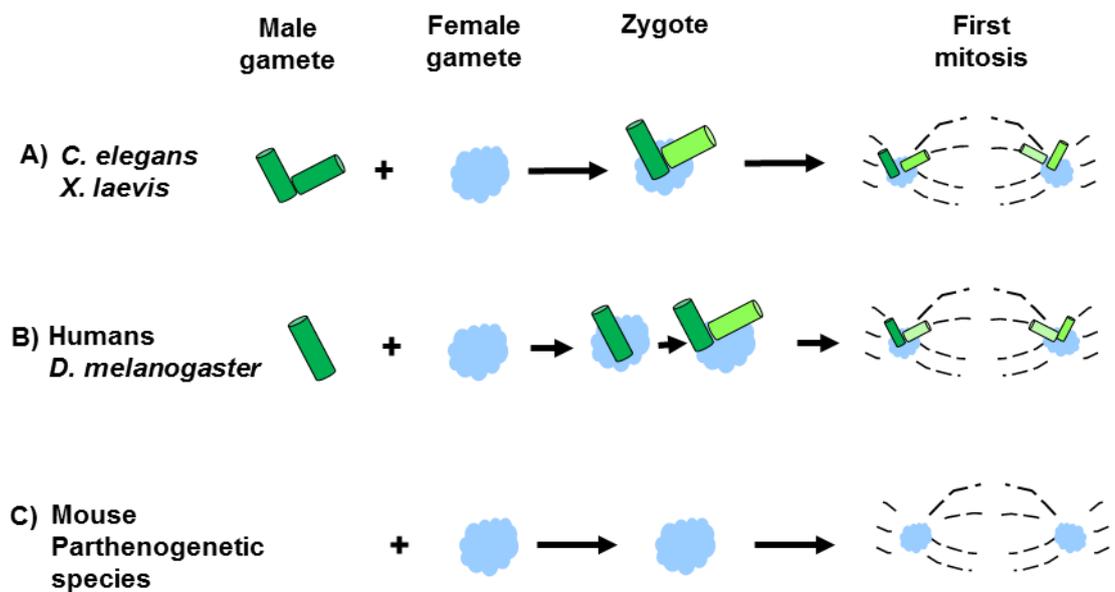
## 1.5. Centrosome reduction during gametogenesis

If each gamete were to contribute one full centrosome at fertilisation, there would be two centrosomes in the newly-formed zygote. In the first mitotic S-phase, each centrosome would then be replicated, leading to four centrosomes at the first mitosis. Given the detrimental effects of extra centrosomes (described above in section 1.4.3 and reviewed in (Bettencourt-Dias et al., 2011; Nigg, 2002; Nigg, 2006; Nigg and Raff, 2009)), a complementary centrosomal contribution from each gamete is required to ensure that the correct number of centrosomes is maintained following fertilisation (reviewed in (Delattre and Gonczy, 2004; Manandhar et al., 2005; Schatten, 1994)).

### 1.5.1. The diversity of differential centrosome contributions at fertilisation

In the clam *S. solidissima* or the brown algae *F. distichus*, both the egg and the sperm contain centrioles (Nagasato et al., 1999; Wu and Palazzo, 1999). However, the maternal centriole pair is silenced in the zygote and is not able to replicate or nucleate MTs (Wu and Palazzo, 1999). In most other species, centrosomes are degenerated in a reciprocal manner between oocytes and sperm enabling a functional centrosome to be generated following fertilization (see Fig 1.7 for a diagrammatic summary of the different parental centrosomal contributions in different species). In *C. elegans* and *Xenopus*, centrioles disappear completely in oogenesis, leaving PCM components in the oocyte cytoplasm (Albertson and Thomson, 1993). Conversely, the centrosome is reduced to a single pair of centrioles during spermatogenesis (Wolf et al., 1978). Thus, at fertilisation the centriole pair is contributed by the sperm which organises the PCM present in the ooplasm, thereby reconstituting one full centrosome (Karsenti et al., 1984b; Wolff et al., 1992). This newly-created centrosome then continues to replicate normally in the following S-phase.

In *Drosophila* and humans, centrioles are eliminated in oocytes and the sperm introduces a single paternal centriole at fertilisation (reviewed in (Callaini et al., 1999; Manandhar et al., 2000)). In flies, this single centriole is the result of a lack of centrosome duplication in meiosis II (Gonzalez et al., 1998). In primates, the mother centriole forms the basal body of the sperm flagellum but is eventually destroyed leaving a single daughter centriole (Manandhar et al., 2000; Sathananthan et al., 1996). The sperm centriole then assembles a MT aster in the zygote and the female pronucleus is able to migrate towards



**Figure 1.7. Differential parental centrosomal contributions at fertilisation.** (A) In *C. elegans* and *X. laevis* the sperm contributes a pair of centrioles (green tubes) which then recruit PCM components (blue cloud) from the oocytes in the zygote. (B) In *Drosophila* and humans, the oocyte lacks centrioles and the sperm contributes a single centriole at fertilisation. The single centriole then duplicates twice so that four centrioles are present at the first mitosis. (C) In mouse and parthenogenetic species, no centrioles are present at fertilisation and spindles are assembled in the absence of centrioles in early embryos. Adapted from Delattre and Gonczy 2004.

the male pronucleus along these astral MTs (Sathananthan et al., 1991; Schatten, 1982; Schatten and Schatten, 1986). The single paternally-contributed centriole must then duplicate twice to give rise to four centrioles (i.e. two centrosomes) prior to the first mitotic division. Hence, sperm centrioles are the predecessor for all centrioles in the offspring. Most interestingly, in mouse, both the unfertilised egg and the fertilising sperm lack centrioles. In maturation of mouse sperm,  $\gamma$ -tubulin, centrin and finally centrioles completely disappear (Manandhar et al., 1998; Manandhar et al., 1999). The lack of centrioles in mouse sperm raises the question of how sperm motility is controlled. However, centrioles are lost in mature epididymal sperm so the flagellated tail has already been assembled (Manandhar et al., 1998; Manandhar et al., 1999). Likewise, mouse oocytes contain centrioles until the pachytene stage of meiosis I (which occurs during fetal development (Pepling, 2006)), but lack them in subsequent stages (Szollosi et al., 1972). As two acentriolar gametes fuse, the first few cell divisions take place in the absence of centrioles before centrioles are generated *de novo* at the blastocyst stage (Abumuslimov et al., 1994; Gueth-Hallonet et al., 1993). The unusual inheritance of centrioles in mouse early embryos is a major focus of this thesis and is discussed further in section 1.8.

### **1.5.2. Evolutionary basis for differential centrosome contributions**

Most mammals, including humans, operate a paternal mode of centrosome inheritance as described above in section 1.5.1 (Manandhar et al., 1998; Manandhar et al., 1999; Manandhar et al., 2005; Schatten, 1994). The basis for the retention of (at least one) centriole(s) in the sperm of most species may be explained by the fact that sperm cells require centrioles to organise the axoneme of the tail (Manandhar et al., 2005; Schatten, 1994). However, some species which lack flagellated sperm (e.g. nematodes) still contain centrioles but these do not act as basal bodies (Ward et al., 1981; Wolf et al., 1978) and conversely, mouse sperm lack centrioles but are still capable of organising motile flagella. On the other hand, oocyte centrosome reduction may have evolved as a mechanism to suppress parthenogenetic development. Schatten (1994) proposes that the introduction of the sperm centriole represents a mechanism to ensure biparental fertilisation (Schatten, 1994). Thus, mechanisms are likely to exist within the oocyte to prevent spontaneous assembly of centrosomes and thereby ensure genetic variability by blocking parthenogenesis. In line with this, microinjection of exogenous centrosomes into *Xenopus* oocytes can trigger successful parthenogenesis (Maller et al., 1976a; Tournier et al., 1989; Tournier et al., 1991). Furthermore, the sperm of some species of stick insects do not contain centrioles and the oocyte

provides the embryonic centrosome (Marescalchi et al., 2002). This has resulted in a high incidence of parthenogenesis in this species.

In mouse, centrioles are eliminated in spermiogenesis (Manandhar et al., 1998) and the oocyte contributes PCM components at fertilisation. Therefore, mouse embryonic development is unique in that it begins in the absence of centrioles. However, biparental reproduction still appears to be the preferred pathway of activation in this mammal. A possible explanation for this unusual scenario was proposed by (Schatten, 1994). Schatten suggested that with the evolution of genomic imprinting (in which maternal and paternal DNA are differentially methylated), the centrosome's contribution to avoiding parthenogenesis has become less important, meaning that, in some mammals such as mice, a paternal pattern of centrosome inheritance is no longer strictly necessary (Schatten, 1994). In line with this hypothesis, parthenogenetic development of mice is possible until the time in development when imprinted genes become activated (Kono, 2009).

### **1.5.3. Mechanism of centrosome elimination**

Centriole MTs are highly stable structures (Bobinnec et al., 1998b; Khodjakov et al., 2000; Piperno and Fuller, 1985) being resistant to cold and MT depolymerisers, meaning that centriole elimination in gametes is unlikely to be the result of a global MT-destabilizing environment (Manandhar et al., 2005). The finding that in species in which a single centriole is retained following spermiogenesis this tends to be the daughter centriole suggests that older centrioles are somehow more susceptible to degradation (Callaini et al., 1999; Manandhar et al., 2000). Despite this observation, the molecular basis for centriole elimination has remained poorly studied.

Manandhar et al., (2005) suggest that centriole elimination could be related to a deficiency of a key protein required for centriole assembly (Manandhar et al., 2005). However, when centriole replication factors such as SAS-6 and Plk4 are depleted in various cell types, this leads to a progressive reduction in the number of centrioles as new centrioles are not assembled in the following S-phases (Bettencourt-Dias et al., 2005; Habedanck et al., 2005; Leidel et al., 2005; Peel et al., 2007; Rodrigues-Martins et al., 2007a). In contrast, centriole elimination in gametes is a rapid process in which existing centrioles are degraded, suggesting that absence of a key centriole manufacture protein is not sufficient to explain the

loss of centrioles in gametes. As far as I am aware the only molecular clue as to how centriole elimination is mediated comes from studies of cell cycle regulators in nematodes (Kim and Roy, 2006). In *C. elegans*, knockdown of the cdk inhibitor cki-2 caused the maternal centrosome to persist. Interestingly, reduction of Cyclin E or cdk2 levels ameliorates centriole elimination suggesting regulation of Cyclin E-cdk2 is important for appropriate centriole reduction in oocytes (Kim and Roy, 2006). However, ultrastructural evidence would be needed to confirm centriole endurance in oocytes from cki-2-depleted worms. Moreover, it remains possible that the persistent centrosome-like foci observed in cki-2-depleted embryos were in fact duplicated centrosomes of paternal origin.

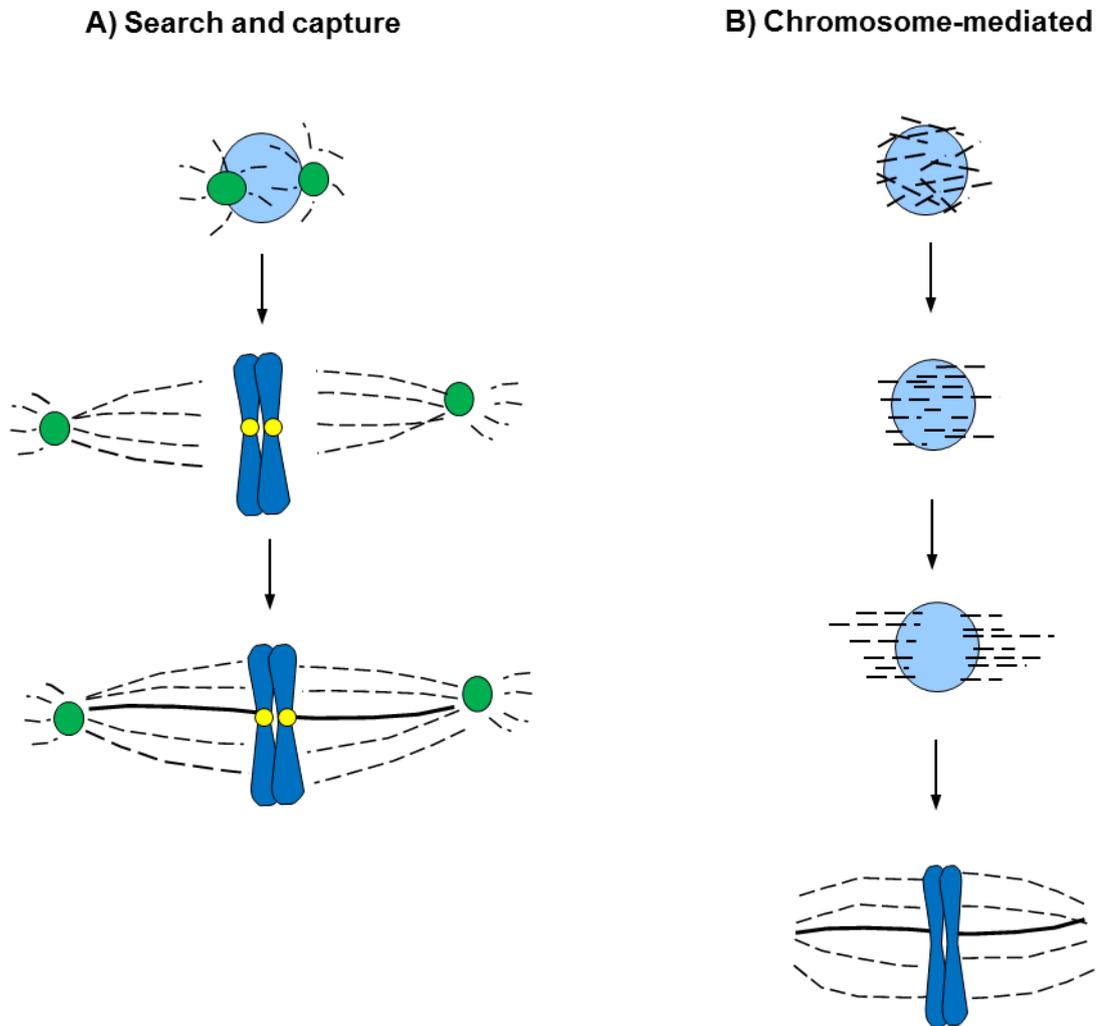
## **1.6. Pathways of spindle assembly**

Since some cells lack centrosomes (such as oocytes - see section 1.5 above), and are capable of undergoing successful cell divisions, it is clear that centrosomes are not absolutely essential for spindle assembly. The following section will therefore review the mechanisms of spindle formation in both centrosomal and acentrosomal cells.

### **1.6.1. The centrosomal spindle assembly pathway**

Centrosomes are indispensable for mitosis in many systems. This is illustrated by several studies in early *Xenopus* embryos (Klotz et al., 1990). In *Xenopus*, centrioles are normally eliminated during oogenesis and the sperm contributes a centriole pair to the zygote (Albertson and Thomson, 1993). If *Xenopus* oocytes are artificially activated by pricking with a needle, this triggers several rounds of DNA replication but the embryo fails to cleave between cycles. However, if exogenous centrosomes are introduced at the time of activation the egg can now divide correctly. In fact some of these parthenogenetically-activated embryos can develop into morphologically normal tadpoles. Thus, in *Xenopus*, the centrosome is required for the embryo to divide (Klotz et al., 1990; Maller et al., 1976b). Similarly, in *Drosophila* and *C. elegans*, centrosomes are absolutely essential for early embryonic development and spermatogenesis (Bettencourt-Dias et al., 2005; Kirkham et al., 2003; Leidel and Gonczy, 2003; Rodrigues-Martins et al., 2008; Stevens et al., 2007).

In cells with centrosomes, the spindle is thought to be assembled via a ‘search-and-capture’ mechanism first put forward by Kirschner and Mitchison ((Kirschner and Mitchison, 1986); see Fig 1.8A for a



**Figure 1.8. Pathways of spindle assembly.** Adapted from Karsenti and Vernos 2001 and Varmark, 2004. (A) The search and capture model: dynamic MTs are nucleated by a pair of centrosomes (green discs). MTs are progressively captured and stabilized by kinetochores (yellow) and a bipolar spindle forms. (B) The chromosome-mediated model: MTs are initially nucleated in close proximity to the chromatin. Molecular motors then enable MTs to congress around the chromatin before the MT array is focussed at the spindle poles resulting in a bipolar spindle. DNA is shown in blue.

cartoon diagram of the classical search-and-capture mode of spindle assembly). In this pathway, a radial array of MTs emanates from each of the two centrosomes in the cell. The plus ends of these MTs switch between polymerisation and depolymerisation states resulting in the growth, shrinkage and regrowth of MTs (a property termed dynamic instability; (Mitchison and Kirschner, 1984). In doing so, MTs may make contact with a kinetochore which results in 'capture' of the associated chromosome. The chromosome in question then becomes stably attached to the spindle pole. Over time the proportion of kinetochore-MTs increases whilst the proportion of astral-MTs decreases, resulting in the typical fusiform spindle structure with chromosomes aligned on the metaphase plate (Kirschner and Mitchison, 1986). In this model the chromosomes are passive and await capture by MTs. Direct demonstration of kinetochore capture by MT plus ends in newt lung cells added further support to this model (Rieder and Alexander, 1990). It is now established that various molecular motor proteins are also required for spindle morphogenesis in centrosome-containing cells. Plus end-directed kinesin-like motors are required for the separation of the spindle poles (Blangy et al., 1995; Heck et al., 1993; Sawin et al., 1992; Wilson et al., 1997). This separation activity is balanced by minus end-directed kinesin-like motors (e.g. Ncd) which pull the asters together (Nedelec, 2002; Sharp et al., 2000). In addition, the structural protein NuMA maintains focussed spindle poles (Merdes et al., 2000).

### **1.6.2. The acentrosomal spindle assembly pathway**

Despite the finding that centrosomes are necessary for some cell divisions, it is evident that centrosomes are not strictly required for cell divisions in many cell types. Indeed, centrosomes are naturally absent in some higher plant cells (Carvalho-Santos et al., 2011; Debec et al., 2010) as well as in female germ cells of many species (reviewed in (Delattre and Gonczy, 2004; Manandhar et al., 2005; Schatten, 1994); Fig 1.7)). Furthermore, in the early 2000s, a series of experiments demonstrated that vertebrate cells were still capable of bipolar mitoses even after centrioles were artificially removed. Thus, centrosome-independent spindle formation pathways exist even in cells which normally contain this organelle (Hinchcliffe et al., 2001; Khodjakov et al., 2000; Khodjakov and Rieder, 2001). The identification of several acentrosomal systems which are capable of successfully organising a spindle pointed towards an alternative mode of spindle assembly. In these acentrosomal cells, the chromosomes appear to initiate assembly of the bipolar spindle, thereby compensating for the lack of centrosomes (see Fig 1.8B for a model of chromosome-mediated spindle assembly). This was first appreciated after artificial chromosomes were added to

*Xenopus* egg extracts and this resulted in the nucleation of MTs near the DNA (Heald et al., 1996). Studies in insects have also shown that meiotic chromosomes in spermatocytes are also able to stabilize MTs (Fuge, 1999). In addition, kinetochores were found to be dispensable in this process (Karsenti et al., 1984a). Thus, in acentrosomal cells the formation of spindle MTs is initiated at the chromosomes and extends outwards in an 'inside-out' mechanism. This is in stark contrast to centrosomal cells, in which the 'search and capture' model states that the spindle is formed from the poles inwards ('outside-in'; (Varmark, 2004; Wadsworth and Khodjakov, 2004)). A similar 'inside out' mechanism of acentrosomal spindle morphogenesis is also observed in meiosis I of mouse, *C. elegans* and *Drosophila* oocytes (Albertson and Thomson, 1993; Brunet et al., 1998; Brunet et al., 1999; Theurkauf and Hawley, 1992). Recent advances in understanding the mechanism of spindle assembly in mammalian oocytes will be discussed further in section 1.6.4.

Further experiments in *Xenopus* egg extracts have revealed a key role for the small GTPase Ran in acentrosomal spindle assembly. RanGTP was found to be present in a concentration gradient around the chromosomes (Kalab et al., 2002) as its GTP exchange factor (GEF) RCC1 localises to chromatin and GTPase activating protein (GAP) localises to the cytoplasm (Hetzer et al., 2000). This gradient is thought to establish a local concentration of MT stabilizing factors which promote capture of MTs (Bastiaens et al., 2006) and stimulate MT nucleation in the vicinity of the chromatin (Karsenti and Vernos, 2001). The crucial nature of the Ran gradient in *Xenopus* spindle assembly was highlighted in experiments in which the ratio of key regulators was perturbed. This altered the size of the gradient and resulted in the loss of spindle asymmetry and fewer chromosome-MT attachments (Caudron et al., 2005).

Work in *Xenopus* and *Drosophila* eggs has now revealed that several molecular motor proteins are also involved in spindle self-organisation in the absence of centrosomes (Scholey et al., 2003; Walczak et al., 1998). MTs which are randomly nucleated around the chromosomes are aligned by the action of the plus end-directed kinesin Eg5 which creates antiparallel MT arrays (Sawin et al., 1992; Sharp et al., 1999). This process also requires the activity of Ran-GTP (Wilde et al., 2001). In addition, Xklp1 which is a chromatin-associated plus end motor protein moves MT minus ends away from the chromatin (Vernos et al., 1995; Vernos and Karsenti, 1995). Finally, dynein acts to focus the MT minus ends into poles (Heald et al., 1997).

Another feature of acentrosomal spindles is that they lack astral MTs. Astral MTs are important in allowing the spindle to respond to changes in cell shape. When endogenous centrioles were ablated in vertebrate cells, astral MTs rapidly disappeared. However, bipolar spindles could still form (Khodjakov and Rieder, 2001). In control cells, the spindle is able to respond to shape changes and reposition the spindle axis (Khodjakov and Rieder, 2001; O'Connell and Wang, 2000). Conversely, in ablated cells, cytokinesis often fails as the spindle is unable to reposition itself if the cell changes shape and the cell divides with the axis of the spindle positioned perpendicular to the long axis of the cell. Therefore, although centrosomes are not necessary for chromosome segregation they play an important role in ensuring accurate cytokinesis. In addition, when the centrosome is artificially removed from cultured cells in S or G2 phase, the acentrosomal cells can complete mitosis but their resultant daughter cells arrest in G1 (Hinchcliffe et al., 2001; Khodjakov and Rieder, 2001). Thus, it appears that the centrosome is necessary for cell cycle progression. However the precise components which trigger this arrest remain to be elucidated. Interestingly, experimentally-induced acentrosomal cell divisions appear to more error prone than physiological ones (Khodjakov et al., 2000; Khodjakov and Rieder, 2001), suggesting that cells which naturally lack centrioles can be able to respond to cortical cues even in the absence of astral MTs. However, how this is achieved is unknown.

The first indication that animal development could proceed without functional centrosomes came from the centrosomin mutant in *Drosophila*. Centrosomin mutant flies develop normally to adulthood without any structural aberrations, although they are sterile (Megraw et al., 2001). Although, centrosomin mutant centrosomes have a reduced ability to nucleate MTs, there were some doubts about the non-functional status of centrosomin-depleted centrosomes. More recently Basto et al (2006) studied *Drosophila* mutants lacking the key centriole reproduction factor D-SAS-4. Strikingly, D-SAS-4 mutant flies could develop to adulthood with a complete lack of centrioles. However, the adult flies were devoid of cilia and therefore died early as the lack of mechanosensory organs caused them to be severely uncoordinated (Basto et al., 2006). Thus, centrioles are essential for fly survival, not because they are required for mitotic divisions, but because they form the basis for cilia. Therefore, centrioles *per se* are dispensable for most stages of *Drosophila* development. Importantly, D-SAS-4 mutant flies are produced from heterozygous mothers (as homozygous flies die early). Therefore, early embryos contain centrioles until maternally supplied D-SAS-4 protein is exhausted. Thus, centrioles may be necessary for early *Drosophila* embryogenesis.

Indeed, several groups demonstrated that centrioles are required for the asymmetric divisions in *Drosophila* syncytial embryos (Rodrigues-Martins et al., 2008; Stevens et al., 2007; Varmark et al., 2007) and *Drosophila* sperm (Bettencourt-Dias et al., 2005; Dix and Raff, 2007; Rodrigues-Martins et al., 2008). Moreover, although flies can develop normally in the absence of D-SAS-4, asymmetric cell division is perturbed in ~30% of larval neuroblasts (Basto et al., 2006). This is thought to be due to the lack of astral MTs in acentrosomal cells, which normally would contact the cell cortex and ensure the mitotic spindle (and thus the cleavage furrow) is positioned correctly within the cell (Betschinger and Knoblich, 2004; Cowan and Hyman, 2004; Gonczy, 2002). Thus, centrosomes are important for enabling efficient asymmetric cell divisions (Basto et al., 2006; Debec et al., 2010; Rodrigues-Martins et al., 2008; Yamashita et al., 2007). This is in line with previous findings that artificially-induced acentrosomal vertebrate cells fail to respond to changes in cell shape and thus often undergo failed cytokinesis (Khodjakov and Rieder, 2001). In *Drosophila* neuroblasts which lack centrioles, ~70% of cells undergo successful asymmetric cell division (Basto et al., 2006). In these cells, acentrosomal spindles extended across the cell such that the spindle poles were closely associated with the cell cortex (Basto et al., 2006). This likely enables acentrosomal spindles to interact with cortical cues even in the absence of astral MTs.

Although flies can develop in the absence of centrioles, a lack of centrioles may have a more devastating role in vertebrate cells where primary cilium are present and a larger number of chromosomes must be segregated (Basto et al., 2006). It also remains unclear whether *Drosophila* development does not depend on centrioles at all or alternatively, that development normally depends on centrioles but the lack of centrioles can be compensated for (Basto et al., 2006). Together these data illustrate that centrosomes are not absolutely required for organisation of a functional spindle. However, they can fulfill other important roles in the cell cycle.

### **1.6.3. Cooperative mechanisms of spindle assembly in centrosome-containing cells**

The two modes of spindle MT nucleation have now been found to be influenced by many common factors. Analyses of chromosome-directed spindle assembly in *Xenopus* extracts have suggested that MT organisation in the absence of centrioles relies on several of the motor proteins which are also involved in centrosomal spindle assembly (reviewed in (Varmark, 2004)). In addition, the Ran-GTPase which was previously shown to be important in establishing a gradient for MT stabilisation around chromosomes in

acentrosomal cells has now been found to be important in centrosome-containing cells as well (Odde, 2005; Wollman et al., 2005). Computer-based modelling revealed that the random probing of the cytoplasm by MTs in the “search and capture” mechanism would take an average of ~ 2 hours to capture all 92 kinetochores in human cells (Wollman et al., 2005). However, in reality HeLa cells can undergo mitosis in only 30 minutes. Thus, the Ran gradient is important in centrosomal cells as it provides an area of MT stabilisation in the vicinity of the chromosomes such that centrosome-nucleated MTs are guided to kinetochores. However, in HeLa cells once the bipolar spindle is established the Ran gradient appears to be largely dispensable for the maintenance of spindle integrity (Wollman et al., 2005). In contrast, in *C. elegans* embryos where Ran-GTP was disrupted a bipolar spindle failed to form (Askjaer et al., 2002; Bamba et al., 2002). Thus, the relative contribution of Ran-regulated pathways in spindle function differs between the acentrosomal and centrosomal pathways of spindle assembly.

Although the two modes of spindle assembly described in sections 1.6.1 and 1.6.2 above were initially believed to be mutually exclusive, evidence now points to a cooperative mode of spindle assembly where centrosome-nucleated and non-centrosome-nucleated MTs act in concert to assemble the spindle in cells which contain centrosomes (O'Connell and Khodjakov, 2007; Varmark, 2004). The finding that both pathways are dependent on the same regulatory molecules described above, coupled with the finding that bipolar spindles can still assemble following artificial removal of resident centrosomes in vertebrate cells (Hinchcliffe et al., 2001; Khodjakov et al., 2000; Khodjakov and Rieder, 2001), might suggest that centrosome-containing cells possess an acentrosomal mechanism for spindle assembly and this is only revealed when existing centrosomes are removed. Alternatively, these data could suggest that bipolar spindles in centrosome-containing cells might contain both centrosome-nucleated MTs as well as a population of MTs nucleated at the chromosomes. The two populations of MTs could then cooperate to form a bipolar spindle structure (Gruss et al., 2002). Variations in the relative contributions of the two pathways (centrosome-nucleated MTs and chromosome-nucleated MTs) proposed in the latter hypothesis, could explain the differential requirements for centrosomes in different cell types and organisms.

#### **1.6.4. Recent advances in understanding spindle formation in mammalian oocytes and early embryos**

Unlike in mitosis where spindle assembly is largely directed by the centrosomes, in female mammalian meiosis I, spindles are assembled without centrosomes (see Fig 1.8B for a diagrammatic summary of spindle assembly in the absence of centrosomes). However, in mouse both the sperm and egg lack centrioles ((Manandhar et al., 2005; Schatten, 1994); see section 1.5). Therefore, murine early embryos must also divide in the absence of centrioles. In this section, the current state of knowledge regarding spindle assembly and function in mouse early development will be discussed.

In acentriolar mouse oocytes, chromosomes and MTs direct spindle assembly. In studies of mouse oocyte fragments lacking chromosomes (termed cytoplasts), MTs can assemble into stable bipolar MI spindles due to the activities of motor proteins and MAPs (MT-associated proteins; (Brunet et al., 1998)). However, in cytoplasts several bipolar spindles may form suggesting that chromosomes are necessary to limit MT organisation to their vicinity. This is important for the formation of a spindle that is very small compared to the volume of the oocyte. Furthermore, the size of the spindle can vary between cytoplasts whereas spindles from untreated oocytes are all a similar size (Brunet et al., 1998). Therefore, chromosomes are necessary to control spindle size. These data therefore reveal a crucial role for chromosome-directed spindle assembly in mouse oocytes.

Recently, live fluorescence microscopy of mouse oocytes revealed that the bipolar spindle is derived from over 80 cytoplasmic MTOCs (Schuh and Ellenberg, 2007). These MTOCs arise *de novo* from a cytoplasmic MT network and initially surround a MT ball shortly after germinal vesicle breakdown (GVBD). In the first few hours following GVBD the small GTPase Ran was found to concentrate around the chromosomes in its GTP bound form (Dumont et al., 2007). This promotes a massive increase in MT nucleation in the region of the chromosomes at this stage (Schuh and Ellenberg, 2007). Ran-GTP gradients also exist in many other acentrosomal as well as centrosomal systems. Ran-GTP is usually concentrated at the chromosomes where it releases spindle assembly factors such as TPX2 (Targeting Protein for the Xenopus kinesin xklp2) from sequestration by importins (Gruss et al., 2001). The Ran-GTP effector TPX2 has now been shown to regulate acentrosomal spindle pole integrity in mouse oocytes

via TACC3 (Transforming Acidic Coiled Coil protein) phosphorylation at MTOCs (Brunet et al., 2008). Indeed, TPX2 depletion in oocytes leads to a marked reduction in MT density and aberrant spindles (Brunet et al., 2008). Interestingly, perturbation of this Ran-GTP gradient did not prevent the formation of a functional spindle in female meiosis I of mouse or *Xenopus* (Dumont et al., 2007; Schuh and Ellenberg, 2007). Thus, formation of the female meiotic spindle in mouse depends on the regulation of Ran target TPX2, rather than regulation of the Ran-GTP gradient itself. It is possible that Ran-GTP is not required for the protracted first meiotic division as there is sufficient time for alternative pathways to complete spindle assembly (Dumont et al., 2007). In agreement with this theory, Ran-GTP is crucial for the shorter second meiotic division in vertebrate eggs (Dumont et al., 2007).

As meiosis I progresses, the multiple MTOCs progressively cluster into two poles under the control of the plus end-directed MT motor kinesin 5 (Kif11 – the mouse orthologue of *Xenopus* Eg5; (Schuh and Ellenberg, 2007)). The establishment and maintenance of meiotic spindle bipolarity has now been shown to be impaired in HURP (hepatoma upregulated protein) knockout mice (Breuer et al., 2010). However, interestingly, HURP is dispensable for mitosis (Koffa et al., 2006; Sillje et al., 2006; Tsai et al., 2008). HURP is a MT-associated protein and Ran GTPase effector and was shown to accumulate on interpolar MTs in close proximity to the chromosomes in meiosis I. Kinesin 5 was also found to be required to recruit HURP to central domain of the meiotic spindle (Breuer et al., 2010). Interestingly, kinesin 5 is required for the movement of MTs towards the spindle poles (termed poleward flux), in metaphase spindles of mouse oocytes and early embryos. However, spindles become resistant to kinesin-5 inhibition in morulae and blastocysts (Fitzharris, 2009), suggesting a gradual transition to kinesin-5 independence in pre-implantation development. These data illustrate that in mouse oocytes, multiple MTOCs functionally replace centrosomes and that Ran pathways and HURP activity are important for spindle bipolarization in meiosis.

Once a bipolar meiosis I spindle axis is established, chromosomes attain stable attachments to the spindle poles by the anchoring of MTs to kinetochores. In contrast to mitotic cells, kinetochore-MTs in mouse oocytes are only stabilized following a long prometaphase which leads to chromosome congression at the metaphase plate (Brunet et al., 1999). Recently, kinetochore tracking experiments in oocytes revealed that ~90% of all homologous chromosomes in meiosis I undergo several rounds of incorrect kinetochore-MT

attachments and subsequent Aurora-A-dependent error correction before proper biorientation is established (Kitajima et al., 2011). Therefore, most kinetochore-MT attachments are incorrect at prometaphase (Kitajima et al., 2011). The high levels of erroneous kinetochore-MT attachments may help to explain the high levels of aneuploidies resulting from female meiosis I especially in older oocytes (Hassold and Hunt, 2001; Kitajima et al., 2011). A series of recent papers also pointed to the role of weakened centromeric cohesion in age-related aneuploidy in oocytes (Chiang et al., 2010; Lister et al., 2010; Revenkova et al., 2010). In order to avoid segregation errors in meiosis I and II, cohesion at centromeres must remain intact until sister chromatids are separated in anaphase II. However, the centromere-associated cohesin Rec8 and shugoshin2 (which protects centromeric cohesion in meiosis I; (Lee et al., 2008)) were found to be depleted in older mice causing an increase in the inter-kinetochore distance at meiosis I and II (Chiang et al., 2010; Lister et al., 2010). In addition, Revenkova et al., (2010) show that SMC1 $\beta$  cohesin laid down in the dictyate stages of oocyte development is sufficient for sister chromatid cohesion in adult mice (Revenkova et al., 2010). Therefore, the finding that centromeric cohesion is reduced in older mice (Hodges et al., 2005; Revenkova et al., 2004) is likely to be a result of a gradual degradation of SMC1 $\beta$ -mediated cohesion, rather than a failure to continually express and reload SMC1 $\beta$  (Revenkova et al., 2010).

Recent work investigated the mechanism of chromosome segregation in anaphase in mouse MII eggs. Anaphase was found to be initially driven by spindle elongation (anaphase B) followed by the movement of chromosomes towards the spindle poles (anaphase A; (Fitzharris, 2012)). Importantly, anaphase was found to be kinetochore-led unlike in *C. elegans* oocytes (Dumont et al., 2010), suggesting that oocytes are prone to the same types of chromosome segregation errors as somatic cells.

Another mechanism which was considered as a possible contributing factor to female age-related aneuploidy was the dysfunction of the spindle assembly checkpoint (SAC; see section 1.4.3 and (Musacchio and Salmon, 2007) for a review of SAC function). It was suggested that mammalian oocytes may lack this important mechanism in meiosis I which would render the oocyte vulnerable to chromosome segregation errors (reviewed in (Homer, 2011)). However, depletion of the SAC components Mad2, Bub1 and Bub3 caused an increase in chromosome mis-segregation in MII eggs due to premature initiation of anaphase (Homer et al., 2005; Li et al., 2009; McGuinness et al., 2009),

showing that the SAC is critical for accurate chromosome disjunction in female meiosis I in mouse. Furthermore, a recent study has now revealed that oocytes from old mice arrest in meiosis I when the SAC is perturbed by the addition of MT poisons and in addition, oocytes which exhibit high levels of aneuploidy did not show an accelerated meiosis I (Duncan et al., 2009). These data indicate that dysfunction of the SAC is not likely to be a major cause of maternal age-related aneuploidy. The SAC protein BubR1 has now been shown to be important for prophase I arrest and prometaphase progression via stabilisation of the APC cofactor Cdh1 (Homer 2009). Depletion of BubR1 causes an increase in securin levels due to reduced APC<sup>Cdh1</sup> activity and arrest at meiosis I (likely due to the sequestration of separase (the protease responsible for sister chromatid separation) by excess securin). These results therefore uncover a role for SAC proteins in sustaining meiotic progression as well as in preventing cell cycle progression until a bipolar spindle has been accurately assembled.

Although much work has been carried out to establish mechanisms of spindle assembly in mouse oocytes, spindle formation and function in mouse embryos has been less well studied. However, since early mouse embryos are acentriolar (by virtue of centriole degradation in mouse spermiogenesis and oogenesis), one might speculate that the mechanisms driving spindle assembly in mouse embryos share some common features with those in oocytes as both are assembled in the absence of centrioles. In line with this, spindle bipolarity becomes independent of kinesin-5 activity in pre-implantation development (Fitzharris, 2009). This switch occurs at the morula stage so it is unlikely that the molecular basis of this transition depends on the appearance of centrioles (which are first detected at the blastocyst stage; (Abumuslimov et al., 1994; Gueth-Hallonet et al., 1993)). However, interphase PCM foci begin to appear at the morula stage (Calarco-Gillam et al., 1983; Gueth-Hallonet et al., 1993; Palacios et al., 1993) and therefore might be important in modifying spindle behaviour at this stage.

## **1.7. The physiology and cell biology of early murine development**

### **1.7.1. Oogenesis**

In mouse fetal development, female primordial germ cells migrate to the genital ridge and then proliferate by mitosis as they colonize the ovary (Monk and McLaren, 1981). The germ cells, now termed oogonia, divide by mitosis until approximately 13.5 days post coitum (dpc). Incomplete cytokinesis means that daughter cells remain attached and develop in clusters called cysts (reviewed in (de et al., 1997)). Oogonia

then begin to enter meiosis and become oocytes. Most oocytes enter meiosis during fetal development between 13.5 and 15.5dpc. However, some may not commence meiosis until after birth (Bristol-Gould et al., 2006). Oocytes then progress through prophase I before becoming arrested in the diplotene stage which occurs between 17.5 dpc and post natal day 5 (BORUM, 1961; McLaren, 2000). Entry to meiosis is accompanied by the breakdown of germ cell cysts into individual oocytes which then become enclosed by a single layer of pre-granulosa cells to form a primordial follicle. During meiotic arrest, oocytes grow in size eventually reaching approximately 80  $\mu\text{m}$  in diameter ( $\sim 270$  pL in volume). In addition, the nucleus also expands resulting in a large nucleus termed the germinal vesicle (GV) in fully grown oocytes (Chouinard, 1975). During this growth phase, the oocyte synthesizes the cortical granules and a glycoprotein coat termed the zona pellucida, both of which later regulate sperm penetration at fertilisation (Bleil and Wassarman, 1980a; Bleil and Wassarman, 1980b; Green, 1997). Oocyte growth depends on robust communication between oocytes and granulosa cells. Indeed, the number of granulosa cells contacting the oocyte directly affects the oocyte's growth rate (Brower and Schultz, 1982). In response to follicle stimulating hormone (FSH) or at sexual maturity, granulosa cells secrete fluid within the follicle to form an antral, or Graafian follicle, which migrates to the ovarian cortex.

### **1.7.2. Oocyte maturation**

Oocytes remain arrested in the diplotene stage until meiotic resumption and ovulation is triggered by a surge in levels of luteinising hormone (LH). This causes the rupture of several follicles and the release of occupant eggs into the oviduct. Each egg is surrounded by several layers of granulosa cells (termed cumulus cells). This LH surge triggers resumption of meiosis and the final stage of oogenesis, termed oocyte maturation. This is first characterised by the breakdown of the germinal vesicle (germinal vesicle breakdown; GVBD) within 2-3 hours, condensation of chromosomes and MT association with the chromosomes (Combelles and Albertini, 2001). After  $\sim 6$  hours, a metaphase I (MI) spindle is evident and by 9-10 hours the spindle has migrated to the cortex (Verlhac et al., 2000a). Homologous chromosomes are then segregated in anaphase and moved towards opposing spindle poles. Upon cytokinesis a highly asymmetric cell division takes place resulting in the formation of a small polar body (which degenerates shortly after formation) and a much larger cell (the oocyte). The oocyte then forms a second meiotic spindle at the oocyte cortex and becomes arrested at metaphase II (MII) until fertilisation. The MII-arrested oocyte is the only stage of oogenesis which should be referred to as a mature egg (see Fig 1.9 for

images of mouse oocytes and pre-implantation embryos throughout development; reviewed in (Brunet and Maro, 2005)).

*In vitro* meiotic resumption can also be triggered by mechanical removal of the cumulus cells that surround the immature oocyte (see Chapter 2). A normal mouse ovulation results in the release of 8-12 eggs. However, superovulation can be achieved following administration of gonadotrophins (see Chapter 2).

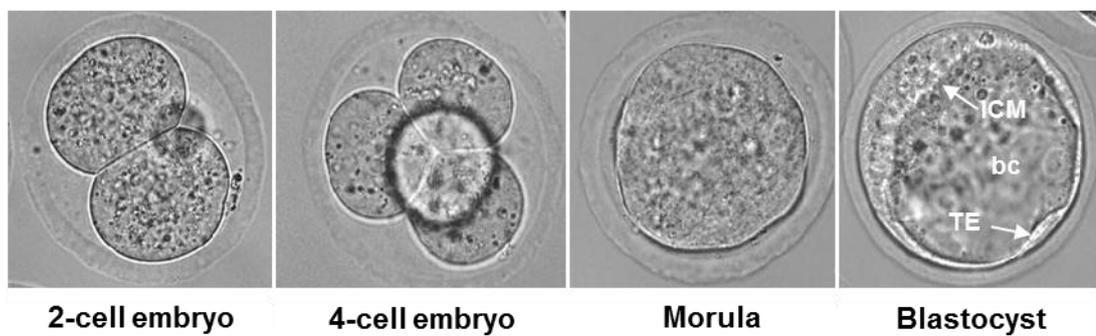
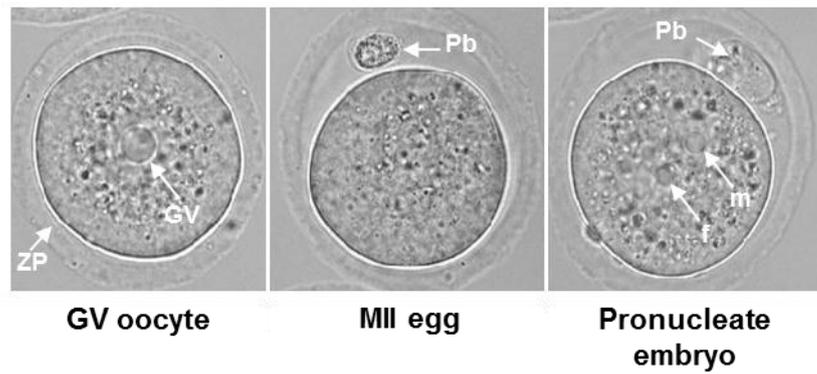
### **1.7.3. Fertilisation**

Multiple sperm migrate through the layer of cumulus cells before binding to the zona pellucida proteins ZP2 and ZP3. It was initially believed that sperm binding to ZP3 triggers an influx of  $\text{Ca}^{2+}$  into the sperm cytosol which in turn initiates the acrosome reaction, in which hydrolytic enzymes such as hyaluronidase are released which is required for zona penetration (Wassarman, 2002). However, recent work reveals that in mouse most fertilising sperm begin the acrosome reaction prior to contact with the zona (Jin et al., 2011). Having passed through the zona, the single successful sperm binds to and fuses with the egg plasma membrane.

### **1.7.4. Early pre-implantation development**

Within a few minutes of sperm-egg fusion, exocytosis of cortical granules commences. This results in the release of hydrolytic enzymes into the perivitelline space, which target the zona pellucida proteins ZP2 and ZP3. This forms a block to polyspermy and prevents any further penetration of the zona (reviewed in (Liu, 2011)).

Following sperm-egg fusion the oocyte becomes activated to re-enter meiosis. Newly-fertilised zygotes exit MII arrest and sister chromatids are separated in anaphase II. This occurs about 90 minutes after activation and a second asymmetric division results in the formation of the second polar body. Around 4 hours after activation, the maternal and paternal chromosomes become surrounded by two individual nuclear membranes, or pronuclei. The pronuclei then migrate towards the centre of the cell pending the first mitotic division at ~17-20 hours post activation (Howlett and Bolton, 1985).



**Figure 1.9. Bright field images illustrating the morphology of early murine development.** Key features are indicated with arrows. Abbreviations used: ZP: zona pellucida; GV: Germinal vesicle; Pb: polar body; ICM: inner cell mass; TE: trophoectoderm; bc: blastocoele cavity. m and f denote the male and female pronucleus respectively. See text for more details.

The second embryonic division occurs 46-54 hours after fertilisation and the embryo typically reaches the 8-cell stage by around 60 hours. Several cleavage divisions then occur producing a morula stage embryo of around 32 cells. The increase in cellular adhesion which occurs at this stage is known as compaction. Three to four days after fertilisation the embryo differentiates into an outer layer called the trophoectoderm (TE) and an accumulation of cells at one pole known as the inner cell mass (ICM). This is called a blastocyst. The ICM contributes cells which will eventually become the embryo proper whilst the TE forms extra-embryonic tissues such as the placenta. The TE surrounds the ICM and a fluid-filled blastocoele cavity. During the formation of this cavity, water enters embryos via an osmotic gradient which is the result of an accumulation of  $\text{Na}^+$  on the basolateral side of the TE, induced by  $\text{Na}^+/\text{K}^+$  ATPases (reviewed in (Cockburn and Rossant, 2010; Watson et al., 2004)). Four to five days after fertilisation the blastocyst stage embryo (Fig 1.9) hatches from the zona pellucida and will implant into the wall of the uterus.

#### **1.7.5. Cell cycle control in early murine development**

The events of meiotic maturation and early embryonic development described above are dependent on the interplay between cell cycle machinery and the cytoskeleton. The following section will therefore review the current understanding of mechanisms that control early murine development.

##### ***Maturation promoting factor (MPF) activity during early murine development***

Landmark studies in frog oocytes illustrated that transfer of cytoplasm from progesterone-treated maturing frog oocytes caused recipient immature GV stage oocytes to undergo maturation (Masui and Markert, 1971; Smith and Ecker, 1971). Thus, M-phase entry requires a cytosolic activity termed maturation promoting factor or MPF (reviewed in (Masui, 2001)). MPF activity was later observed in amphibian embryos suggesting that MPF may play a role in both meiotic and mitotic cell cycle progression (Wasserman and Smith, 1978). MPF activity is now attributed to the activity of the kinase Cdk1 and its regulatory subunit cyclin B1 (Doree and Hunt, 2002; Gautier et al., 1990; Labbe et al., 1989; Lohka et al., 1988). MPF activation depends on modulation of cyclin B1 levels and dephosphorylation of Cdk1. Cdk1 is maintained in an inactive state by phosphorylation by the kinases Wee1 and Myt1 (Mueller et al., 1995a; Mueller et al., 1995b). Inactivation of Wee1 and Myt1 and dephosphorylation of cdk1 by the phosphatase Cdc25 therefore activates MPF and M-phase ensues (Ohi and Gould, 1999).

As well as its role in promoting M-phase entry in mitotic cells, MPF has also been shown to fulfill a similar role in meiosis. At the GV stage in mouse, oocytes are arrested in prophase I of meiosis with low MPF activity. Inactive 'pre-MPF' is located at cytoplasmic MTOCs surrounding the GV (Marangos and Carroll, 2004b). In mouse, synthesis of new cyclin B1 is not necessary for resumption of meiosis since protein synthesis inhibition fails to block GVBD (Clarke and Masui, 1983; Hashimoto and Kishimoto, 1988; Ledan et al., 2001). Instead, dephosphorylation of its cofactor cdk1 triggers activation of MPF at GVBD. Immediately prior to GVBD, cyclin B1 is translocated to the nucleus (Marangos and Carroll, 2004b), which is thought to be important for the rapid activation of MPF and is consistent with the role of cdk1-cyclin B1 in disassembly of the nuclear envelope (Peter et al., 1990). After GVBD, cyclin B1 synthesis is upregulated which stimulates entry into M-phase of meiosis I. Synthesis of cyclin B1 then reaches a plateau at the end of the first meiotic metaphase (Ledan et al., 2001; Verlhac et al., 1994). This surge in MPF activity is required to organise a bipolar spindle structure at MI and to allow capture of kinetochores and stabilisation of kinetochore-MTs (Brunet et al., 1999; Polanski et al., 1998). Once MPF activity has reached this threshold level at metaphase I, the anaphase promoting complex (APC) is activated (Tsurumi et al., 2004). The APC is an E3 ubiquitin ligase which is required for proteasome-mediated degradation of securin and cyclin B1 at the onset of anaphase (Taylor et al., 2004). The APC is not activated until all chromosomes are bioriented on the spindle, thus ensuring that cyclin B1 is degraded in a timely manner (Brunet et al., 2003; Homer et al., 2005; Li and Murray, 1991; Tsurumi et al., 2004; Wassmann et al., 2003). Accordingly, APC is subject to several regulatory mechanisms. APC activity requires one of two cofactors - Cdc20 or Cdh1 (Chang et al., 2004; Kramer et al., 1998; Lorca et al., 1998; Raff et al., 2002). It is also positively regulated by MPF-dependent phosphorylation, thereby providing a feedback loop that ensures the rapid onset of anaphase and polar body extrusion (Felix et al., 1990; Golan et al., 2002). Finally, the spindle assembly checkpoint proteins Mad2, Bub1 and BubR1 complex with Cdc20 to ensure the APC remains inactive until all chromosomes are stably attached to kinetochores (Fang et al., 1998; Fang, 2002; Homer et al., 2005).

A transient decrease in MPF activity mediated by degradation of cyclin B1 accompanies anaphase I and extrusion of the first polar body. MPF then increases again and drives the oocyte into metaphase II where it remains arrested until fertilisation. At fertilisation, sperm-induced  $\text{Ca}^{2+}$  transients (see section below for more details) trigger cyclin B1 destruction and the loss of MPF activity which continues until the

formation of pronuclei (Marangos and Carroll, 2004a; Nixon et al., 2002). MPF activity then rises again during the first mitotic division (Carroll, 2001). A diagram depicting changes in MPF levels during early mouse development is shown in Fig 1.10.

### ***Maintenance of MII arrest: cytostatic factor***

The MII egg remains arrested with chromosomes aligned at the metaphase plate for several hours before fertilisation. This metaphase arrest is mediated via the activity of cytostatic factor (CSF) which prevents premature egg activation. The presence of CSF activity was first demonstrated in vertebrate oocytes when transfer of cytoplasm from a MII-arrested oocyte to a cleaving frog or mouse embryo (Kubiak et al., 1993; Masui and Markert, 1971) caused cell cycle arrest in mitosis (reviewed in (Masui, 2001)). CSF activity in MII eggs is responsible for the stabilisation of the cdk1/cyclinB1 complex (i.e. MPF) and maintenance of MII arrest.

Until the late 1980s the molecular identity of CSF remained unknown. However, Sagata and co-workers found striking similarities in behaviour between CSF and the proto-oncogene Mos. Mos was detected at high levels in MII eggs. However, upon fertilisation expression is reduced such that Mos is undetectable in pronucleate stage embryos (Paules et al., 1989; Wu et al., 1997). In addition, supplementation of Mos mRNA causes metaphase arrest in cleaving *Xenopus* embryos and conversely, immunodepletion of Mos from *Xenopus* egg extracts prevents the arrest phenotype when the *Xenopus* extract was injected into recipient cells (Sagata et al., 1989). Furthermore, mouse oocytes in which Mos had been depleted fail to arrest in MII (Colledge et al., 1994; Hashimoto et al., 1994; O'Keefe et al., 1989). Thus, Mos contributes to CSF activity in mouse oocytes.

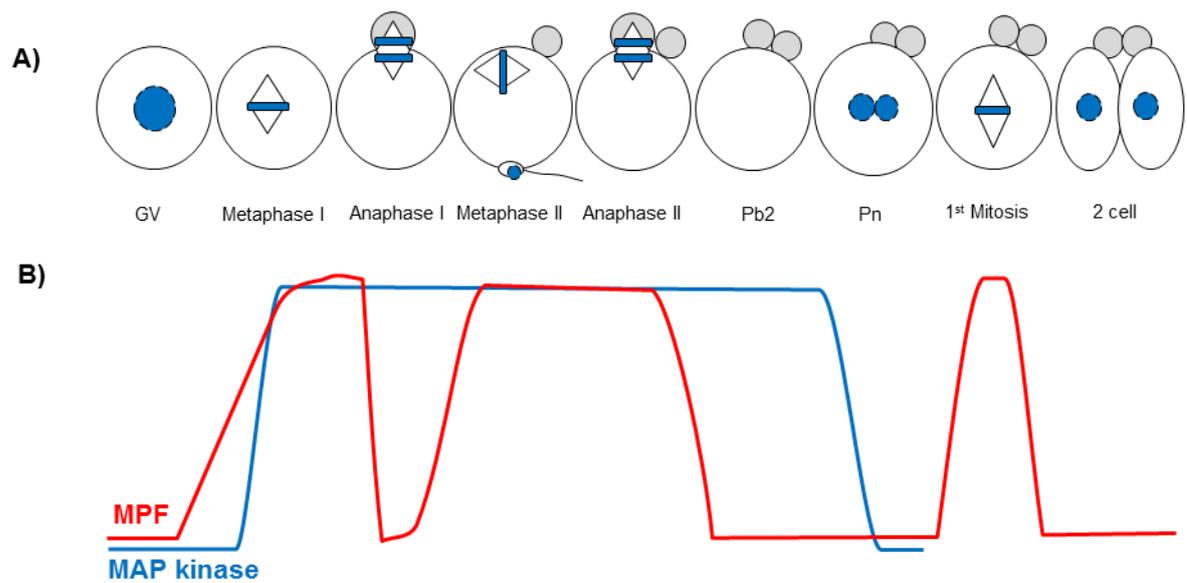
More recently the mitogen activated protein (MAP) kinase pathway was found to be necessary for the activity of Mos in both *Xenopus* and mouse eggs (Haccard et al., 1993; Kosako et al., 1994; Verlhac et al., 2000b). MAP kinase is activated at GVBD and remains active until MII (Verlhac et al., 1993) - a similar activation profile as for Mos. In addition, in Mos<sup>-/-</sup> oocytes, MAP kinase fails to activate (Verlhac et al., 1996), providing more direct evidence for the involvement of the Mos-MAP kinase pathway in maintaining MII arrest. *In vitro* studies have also revealed that Mos can phosphorylate and activate an upstream regulator of MAP kinase (a MAP kinase kinase; (Posada et al., 1993). Thus, Mos acts as a MAP

kinase kinase kinase. More recently, MAP kinase itself has been shown to possess CSF activity, since constitutively-active MAP kinase is able to induce a metaphase arrest when injected into *Xenopus* embryos (Haccard et al., 1993). Moreover, the MAP kinase inhibitor UO126 can activate mouse eggs and produce parthenogenetically-activated embryos with a phenotype similar to *Mos*<sup>-/-</sup> embryos (Phillips et al., 2002). Fig 1.10 shows the timecourse of MAP kinase activity in mouse early development.

The early mitotic inhibitor 2 (Emi2) has now been suggested to be a component of CSF in mouse oocytes. Depletion of endogenous Emi2 elicited precocious meiotic exit in MII eggs (Paronetto et al., 2004; Shoji et al., 2006) and conversely, recombinant Emi2 reduced the propensity of MII eggs to enter anaphase in response to activating stimuli (Madgwick et al., 2006; Shoji et al., 2006). Furthermore, Emi2 is phosphorylated upstream of the Cdc20 binding region thus stabilising their interaction (Paronetto et al., 2004). Therefore, CSF activity is established through stabilisation of the APC-inhibitory complex Emi2-Cdc20. Furthermore, Emi1-dependent regulation of the APC<sup>Cdh1</sup> is important for the arrest of the oocyte at prophase I and progression through meiosis I (Marangos et al., 2007). Collectively, these data illustrate that several players contribute to CSF activity and act to maintain high cdk1-cyclin B1 activity at MII to prevent premature egg activation.

### ***Release of Ca<sup>2+</sup> at fertilisation***

Fertilisation elicits a series of Ca<sup>2+</sup> oscillations which are key for the resumption of meiosis. The first evidence that repetitive Ca<sup>2+</sup> transients at fertilisation were necessary for the meiotic resumption came from studies in which Ca<sup>2+</sup> was able to trigger egg activation (Fulton and Whittingham, 1978; Kline and Kline, 1992; Steinhardt et al., 1974; Swann, 1994). Conversely, the Ca<sup>2+</sup> chelator BAPTA was able to block activation in mouse eggs (Kline and Kline, 1992; Xu et al., 1996). Later, the oscillation frequency of the Ca<sup>2+</sup> transients was found to affect cell cycle progression (Bos-Mikich et al., 1997; Ducibella et al., 2002; Ozil and Huneau, 2001). Therefore, Ca<sup>2+</sup> oscillations are a prerequisite for egg activation. The Ca<sup>2+</sup> oscillations at fertilisation appear to be mediated by the IP3 (inositol trisphosphate) receptor since oscillations were abrogated when the IP3 receptor was inhibited or downregulated (Xu et al., 2003) and furthermore, microinjection of IP3 stimulates Ca<sup>2+</sup> release in mammalian oocytes (Miyazaki, 1993). In addition, fertilisation leads to down-regulation of IP3 receptors (Brind et al., 2000; Jellerette et al., 2000) pointing to sperm-induced IP3 production in mammalian eggs.



**Figure 1.10. MPF and MAP kinase levels in early development.** Adapted from Masui, 2001, Carroll, 2001. MPF and MAP kinase activity are initially low in GV oocytes. Following GVBD, the activities of both factors increase. MPF activity then briefly declines in the transition from metaphase I to metaphase II and decreases again around the time of polar body extrusion (Pb2). MPF is then reactivated during the first mitotic division. MAP kinase activity remains high until pronucleus formation (Pn). See text for further details.

The means by which sperm trigger  $\text{Ca}^{2+}$  release remained a source of debate for many years (Runft et al., 2002; Swann et al., 2006; Swann and Yu, 2008; Whitaker and Smith, 2008). Most recently, a novel sperm-specific phospholipase C isoform termed PLC zeta has been identified and proposed as a soluble sperm-borne factor which elicits  $\text{Ca}^{2+}$  oscillations (Cox et al., 2002; Saunders et al., 2002). Exogenous PLC zeta triggers  $\text{Ca}^{2+}$  transients similar to those seen at fertilisation and at levels approximately equivalent to a single sperm and conversely, PLC zeta immunodepletion eliminates  $\text{Ca}^{2+}$  releasing ability from sperm homogenates (Saunders et al., 2002). Moreover, release of PLC zeta from sperm occurs within the same time frame over which physiological  $\text{Ca}^{2+}$  oscillations occur (Larman et al., 2004; Yoon and Fissore, 2007). There is now evidence to suggest that PLC zeta is required for normal male fertility in mice (Knott et al., 2005).

$\text{Ca}^{2+}$  release at fertilisation has now been shown to trigger destruction of cyclin B1, which is crucial for anaphase entry (Marangos and Carroll, 2004b). Cyclin B is destroyed at the onset of  $\text{Ca}^{2+}$  oscillations (Marangos and Carroll, 2004b; Nixon et al., 2002) and furthermore, the proteasome, which is responsible for cyclin proteolysis, has been demonstrated to be sensitive to  $[\text{Ca}^{2+}]$  (Aizawa et al., 1996; Kawahara and Yokosawa, 1994). In addition, calmodulin-dependent protein kinase II (CaMKII) may also mediate cyclin destruction following fertilisation. CaMKII activity is upregulated at fertilisation (Markoulaki et al., 2004) and this can indirectly activate the APC (Jones, 2007). Studies in *Xenopus* and mouse demonstrate that  $[\text{Ca}^{2+}]$  stimulates cyclin destruction at fertilisation through activation of CaMKII (Lorca et al., 1993; Madgwick et al., 2005). Moreover, constitutively-active CaMKII can also inactivate cdk1-cyclin B and cause parthenogenetic activation whilst inactivation of CaMKII with an inhibitory peptide prevents  $\text{Ca}^{2+}$ -activated cdk1-cyclinB inactivation (Lorca et al., 1993). In mouse, egg activation can be prevented by a calmodulin inhibitor (Xu et al., 1996), suggesting a key role for CaMKI in  $\text{Ca}^{2+}$  release-mediated cyclin B destruction at fertilisation.

*In vitro* parthenogenetic activation can also be induced by treatments which increase the intracellular  $\text{Ca}^{2+}$  concentration. For example, ethanol treatment induces a single large influx of  $\text{Ca}^{2+}$  which lasts 5-10 minutes (Colonna et al., 1989; Ozil and Swann, 1995; Steinhardt et al., 1974). This monotonic  $\text{Ca}^{2+}$  increase is often seen in frog or sea urchin eggs but is not very efficient at activating mammalian eggs. This is because, despite cyclin B degradation and polar body emission after the single  $\text{Ca}^{2+}$  transient, the

continued synthesis of cyclin B can lead to re-establishment of meiotic arrest (Ducibella et al., 2002). In contrast, eggs activated by repetitive  $\text{Ca}^{2+}$  oscillations (which can be induced by treatment with  $\text{Sr}^{2+}$ ) develop better after implantation than those activated by a single transient (Rogers et al., 2006). Furthermore, the pattern of oscillations influences the size and morphology of embryos (Ozil et al., 2006; Ozil and Huneau, 2001).

## 1.8. Synopsis

As both mouse sperm and oocytes lack centrioles, the mouse zygote does not initially contain centrioles and the first few embryonic divisions are acentrosomal. Well-defined interphase foci of the PCM component  $\gamma$ -tubulin start to appear in compacting morulae (~32 cell stage; (Calarco-Gillam et al., 1983; Gueth-Hallonet et al., 1993; Palacios et al., 1993) but centrioles themselves are first detected by EM in blastocysts (~64 cell stage; (Abumuslimov et al., 1994; Gueth-Hallonet et al., 1993). Thus, the appearance of centrioles is preceded by the appearance of acentriolar centrosomes by approximately one cell cycle. Thereafter, centrioles continue to duplicate via the canonical pathway. In contrast to laser-ablated cells or following overexpression of centriole replication proteins, where an unpredictable number of centrioles are generated (Dzhindzhev et al., 2010; Khodjakov et al., 2002; La et al., 2005; Peel et al., 2007; Rodrigues-Martins et al., 2007b; Stevens et al., 2010a; Uetake et al., 2007), the emergence of centrosomes and centrioles *de novo* in the mouse embryo occurs in a tightly coordinated manner.

The *de novo* centriole assembly is much less well documented than the canonical centriole duplication pathway. However, the *de novo* pathway provides an excellent model system in which centriole biogenesis can be studied, without being obscured by centriole duplication (Loncarek and Khodjakov, 2009). Investigating the mechanism of *de novo* centriole formation and importantly, how this pathway is suppressed will increase understanding of the control of centriole number and the mechanism and coordination of centriole and centrosome biogenesis. As the only known example of *de novo* centriole formation in a non-ciliated mammalian cell, early murine development represents an excellent system in which to study centriole biogenesis.

The aim of this thesis is to investigate the mechanism of centriole biogenesis in early murine development. Experiments presented in Chapter 3 examine the time course of centriole formation and MT

behaviour in pre-implantation mouse embryos. The aim of Chapter 4 is to investigate the specific role of the initiator protein of centriole replication, Plk4, in mouse oocytes and early embryos. Continuing in this theme, in Chapter 5 the role of the structural centriole replication protein SAS-6 in centriole biogenesis in mouse oocytes and early embryos is addressed. Finally, Chapter 6 summarises the data presented herein and highlights some of the remaining questions in the field.

## **2. MATERIALS AND METHODS**

### **2.1. Mice**

Oocytes, eggs and embryos were recovered from 4-6 week old female MF1 mice (Charles River, UK). GFP-CETN2 transgenic mice were used where stated in order to label centrioles in embryos (obtained from Jackson laboratory, Bar Harbour, ME). These mice ubiquitously express a GFP-tagged copy of the centriolar protein centrin-2 in cells of every organ and the phenotype of the mice is indistinguishable from wild type (Higginbotham et al., 2004). To obtain GFP-CETN-2-expressing embryos, wild type female MF1 mice were mated with CB6 males carrying the transgene. All mice were kept in a light/dark cycle with free access to food and water. All animal procedures were carried out under license from the UK Home Office.

### **2.2. Oocyte and embryo collection**

Germinal vesicle (GV) stage oocytes were obtained from female MF1 mice administered 48 hours previously with 7 IU (international units) of pregnant mares' serum gonadotrophin (PMSG; Intervet) by intraperitoneal injection. Mice were culled by cervical dislocation and ovaries collected into warm M2 medium (Sigma, UK) supplemented with 200  $\mu$ M 3-isobutyl-1-methylxanthine (IBMX; Sigma) to prevent germinal vesicle breakdown. Oocytes were released by puncturing the ovary with a 27-gauge needle and placed in drops of media under oil to prevent evaporation. Only oocytes with an intact layer of cumulus cells were selected and cumulus cells were subsequently removed by repeated pipetting up and down through a narrow pipette.

Metaphase II (MII) eggs were obtained from mice administered with PMSG and 7.5 IU human Chorionic Gonadotrophin (hCG, Intervet) at a 48 hour interval. 12-14 hours after hCG administration the oviducts were collected in M2 medium and cumulus masses released by tearing the oviduct using forceps. Hylaronidase (300  $\mu$ g/ml, Sigma) was used to remove cumulus cells and eggs were subsequently washed through 3 drops of hylaronidase-free media.

To recover pronucleate stage embryos, female MF1 mice were administered with PMSG and hCG at a 48 hour interval and mated with male MF1 mice of at the time of hCG administration. 1-cell stage embryos

were then collected from the oviduct 27-28 hours after hCG and mating. Embryos were collected in hepes-buffered KSOM medium (Lawitts and Biggers, 1993) supplemented with 1 mg/ml BSA (Sigma, UK). 2-cell stage embryos were recovered 48 hours after hCG and mating. 4-8-cell embryos, morulae, early blastocysts and late blastocysts were obtained by culturing in KSOM medium (Millipore (Erbach et al., 1994)) at 37 °C in 5% CO<sub>2</sub> in air for 24, 48, 72 or 96 hours respectively.

### **2.3. Parthenogenetic activation**

In order to produce parthenogenetically-activated embryos, cumulus-free MII eggs (19 hours post hCG) were exposed to 7% solution of ethanol in M2 media for 7 minutes at 25 °C. Cells were then thoroughly washed in ethanol-free media. Activated eggs were subsequently identified by second polar body extrusion and formation of a single pronucleus. Embryos were then cultured in KSOM media to the appropriate stage.

### **2.4. Use of fluorescent proteins**

GFP-AKAP450 (gift from Sean Munro, Cambridge, UK) was received in a COS cell vector containing a CMV promoter followed by a myc epitope tag and the insertion site. The construct was excised by PCR using the following primers which added a 5' *Xho*I site and a 3' *Eco*RI site:

*Forward:* 5'CTCGAGATGAGTAAAGGAGAACTTTTCACT3'

*Reverse:* 5'GAATTCTTATCTTCTCATGCCAGCATG3'

The PCR product was then subcloned into pcDNA3.1/*myc*-His(-) (Invitrogen) using *Xho*I and *Eco*RI restriction enzymes (New England BioLabs; NEB).

EB1-GFP (gift from Lynn Cassimeris, Lehigh, PA; (Piehl and Cassimeris, 2003) was received in pEGFP-N1 vector (Clontech) and subcloned into pcDNA 3.1/*myc*-His(-) using the restriction enzymes *Nhe*I and *Not*I (NEB).

eGFP-Plk4 and eGFP-(D154A)Plk4 (gifts from Erich Nigg, Basel, Germany; (Habedanck et al., 2005)) were received in pEGFP-C2 vector (Clontech) and subcloned into pcDNA3.1/*myc*-His(-) using the restriction enzymes *Nhe*I and *Bam*HI (NEB). Subcloning of Plk4 and EB1 was carried out by Jenny Bormann. eGFP-hsSAS-6 (gift from Pierre Gönczy, Lausanne, Switzerland; (Leidel et al., 2005)) was

received in pEGFP-C1 vector (Clontech) and subcloned into pcDNA3.1/*myc*-His(-) (Invitrogen) using *Nhe*I and *Xba*I restriction enzymes (NEB).

MAP7 fragment-RFP (gift from Alex McDougall, Villefranche, France; mouse MAP7 N-terminal fragment-BC052637; (Prodon et al., 2010)) was received in Gateway DEST vector pRN3 (pSPE3). eGFP only (gift from Thomas Mayer, Konstanz, Germany) was received in pCS2 vector.

Plasmids were amplified using a miniprep kit (Qiagen, UK) and linearised using an appropriate restriction enzyme which cuts at the 3' end of the insert. Polyadenylated RNA was subsequently produced by *in vitro* transcription using the mMESSAGE mMACHINE kit (T3, T7 or SP6; Ambion) following manufacturer's instructions. Polyadenylation of the RNA transcript was then carried out using the Poly(A) tailing kit (Ambion). A summary of RNA manufactured in this thesis is shown in Table 2.1.

## 2.5. Use of morpholino antisense oligonucleotides

In order to specifically deplete SAS-6, a morpholino antisense oligonucleotide was used. Morpholinos are designed to bind to a complementary sequence in the selected mRNA and block translation. Morpholino antisense oligonucleotides were designed against the 5' untranslated region (UTR) of the mouse SAS-6 mRNA by GeneTools (Philomath, OR). A control morpholino with no target and no significant biological activity was also used in this study. Morpholinos were resuspended in water to a concentration of 1.5 mM and microinjected to a final concentration of 75-150  $\mu$ M. The morpholino sequences were as follows (underlined bases correspond to the initiation codon on mouse SAS-6 mRNA):

*SAS-6 morpholino*: 5' GCTGCTGGAACAGAACTTGACTCAT 3'

*Control morpholino*: 5'CCTCTTACCTCATTACAATTTATA 3'

## 2.6. Microinjection

Fine microinjection pipettes were produced by pulling borosilicate glass capillary tubing in a micropipette puller (LIST medical; Model L/M-3P-A). Polyadenylated RNA (see section 2.4) and morpholino oligonucleotides (see section 2.5) were microinjected using Narishige micromanipulators mounted on a Leica DMI4000 inverted microscope. Oocytes/embryos were placed in a drop of media covered in mineral oil to prevent evaporation. Cells were immobilised with a holding pipette custom-made using a

microforge (Narishige). A short pulse of negative capacitance allowed the injection pipette to pierce the plasma membrane. A controlled injection volume was delivered through injection pipettes using a picopump (World Precision Instruments) and estimated at 5-10% of the total cell volume by cytoplasmic displacement.

## **2.7. Chemicals**

Chemicals used and their concentrations are shown in Table 2.2. DMSO was used as a vehicle control where necessary. Drugs were reconstituted in DMSO (Sigma, UK) to produce 1000x stock solutions and stored at -20 °C until required. Resuspended UO126 is stable for no more than a week so was used immediately.

## **2.8. Immunofluorescence**

Several fixation methods were used in order to optimise staining for each antibody used. Oocytes, eggs and embryos were briefly permeabilised with 0.25% Triton X-100 in PHEM solution (10 mM EGTA, 2 mM MgCl<sub>2</sub>, 60 mM PIPES, 25 mM HEPES, pH 6.9) for 5 seconds, prior to fixing using 3.7% paraformaldehyde (PFA) in PHEM for 40 minutes. Alternatively, oocytes/embryos were fixed directly in 3.7% PFA in phosphate buffered saline (PBS). Oocytes/embryos were subsequently permeabilised for 10 minutes in 0.25% Triton X-100. In the final method employed, oocytes were fixed and permeabilised in PHEM buffer containing 3.7% PFA and 0.5% Triton X-100. Cells in all fixation groups were then blocked overnight at 4 °C in 3% bovine serum albumin (BSA). Primary antibodies used are shown in Table 2.3.

Following three washes in PBS with 1% BSA, cells were labelled with appropriate Alexa Fluor-labelled secondary antibodies (1:1000 in 1% BSA; Invitrogen). Fixed chromatin was labelled by a 5-minute exposure to 5 µg/ml Hoechst 33342 (Invitrogen).

## **2.9. Epifluorescence imaging**

Epifluorescence microscopy was used for analysis of relative fluorescence levels throughout development. Epifluorescence microscopy was carried out on a Leica DMI4000 epifluorescence

microscope fitted with a charge-coupled device (CCD) camera and controlled using LAS AF software. GFP was imaged using a 510 nm dichroic mirror. GFP was excited using a 480/50 nm bandpass filter, and emitted light monitored with a 520/35 nm bandpass filter (Fig 2.1A). Relative GFP fluorescence following microinjection of eGFP-hsSAS-6 or eGFP alone was measured using LAS AF lite software.

## **2.10. Confocal microscopy**

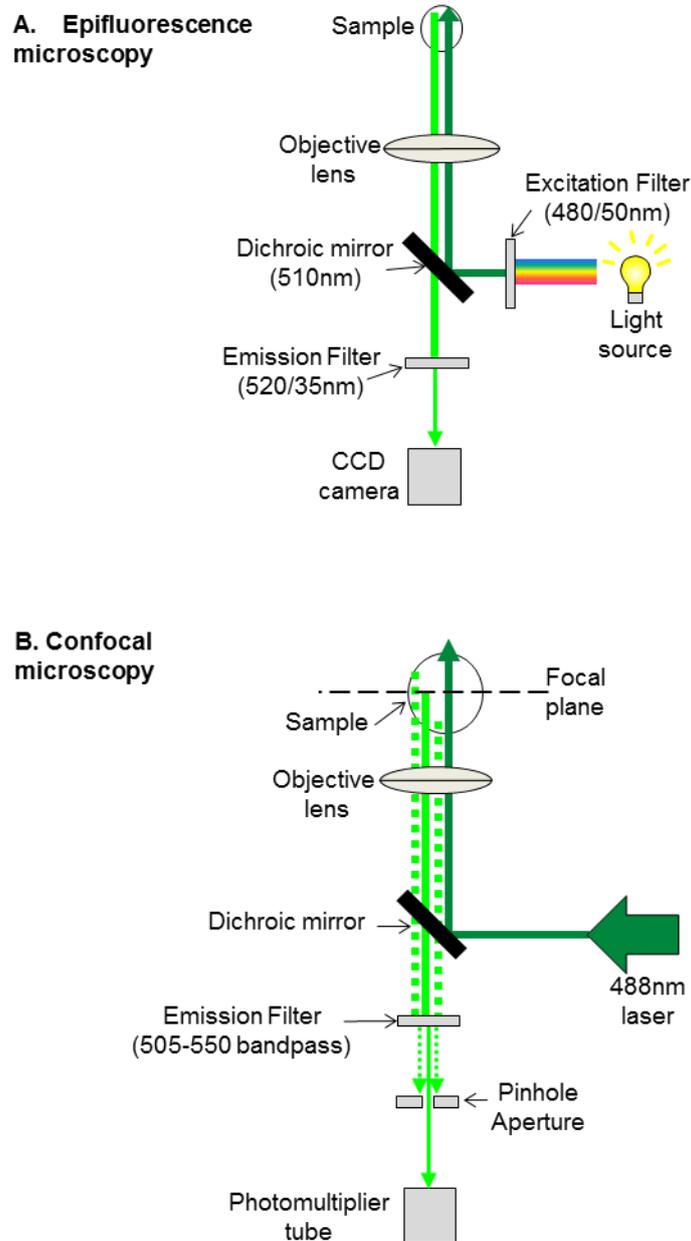
The advantage of confocal microscopy is that it can collect light from a single plane of a specimen whilst rejecting out of focus blur by use of a pinhole conjugated to the focal plane. The pinhole therefore obstructs light from objects outside the plane and only light from in-focus planes can reach the detector (Fig 2.1B). Confocal imaging was therefore used in preference to epifluorescence microscopy for detection of sub-cellular structures.

Confocal imaging was performed on a Zeiss LSM 700 confocal microscope using a 63x oil immersion lens with a numerical aperture of 1.40. For imaging of fixed cells, oocytes/embryos were imaged in a small drop of 1% BSA in a glass-bottomed plastic culture dish. For imaging of live cells, oocytes/embryos were imaged in a chamber with a glass coverslip base and maintained at 37 °C throughout imaging by use of a heated stage.

eGFP was imaged using a 488-nm laser and a 505- to 550-nm bandpass emission filter. RFP was imaged using a 555-nm laser and a 560- to 615-nm bandpass emission filter. Far red fluorophores were excited using a 639-nm laser and emitted light collected using a 650-nm longpass filter. Hoechst was excited using a 405-nm laser, and emitted light collected with a 420- to 480-nm bandpass filter. A MBS (main beam splitter) dichroic 405/488/555/639 was used to image fluorophores. For all confocal imaging experiments a pinhole of 3.03 airy units was used, giving a calculated optical slice of 2 µm.

## **2.11. Embryo karyotyping**

Initially, several methods were employed in order to optimise the method used for the counting of chromosomes. Late 4-cell stage embryos were treated with MG132 (25 µM) for 4 hours in order to arrest individual cells in metaphase. Spindles were then collapsed using the drugs nocodazole (10 µM; 10 mins



**Figure 2.1. Schematic illustrating epifluorescence and confocal microscope set up.** (A) Epifluorescence microscope: Excitation light is provided by a mercury lamp which passes through an excitation filter to obtain the correct wavelength. Light shorter than 510nm is reflected by a dichroic mirror and onto the specimen by the objective lens. Emitted light (longer than 510nm) can the pass through the dichroic mirror and is then filtered and collected by a CCD camera. (B) Confocal microscope: A laser is used to provide high intensity excitation light which is reflected by the dichroic mirror and onto the sample. Emitted light then passes through the dichroic and is focussed onto the pinhole. The light that passes through the pinhole represents the in focus plane (solid green line) and is detected by the photomultiplier tube. Light emitted from out of focus planes is rejected (dotted green lines). Note that the only the green channel is shown for clarity.

or 30 mins) or monastrol (100  $\mu\text{M}$ ; 15 mins, 30 mins, 45 mins or 60 mins). A 30 minute pulse of monastrol (100  $\mu\text{M}$ ) was found to produce the most effective chromosome dispersal, allowing the resolution of all 80 kinetochores and was therefore used in subsequent experiments involving Plk4 overexpression. Embryos were fixed and permeabilised in PHEM buffer containing 3.7% PFA and 0.5% Triton X-100. Embryos were labelled with CREST (anti-ACA) antibodies (gift from William Earnshaw, Edinburgh, UK) and Hoechst before being mounted on glass slides. Serial Z-sections were acquired using a 1.1  $\mu\text{m}$  optical slice and 0.5  $\mu\text{m}$  intervals. To obtain a chromosome count for each cell, serial sections were analyzed to determine the total number of kinetochores.

In conventional chromosome spread analysis, only hyperploid cells are counted. The reason for this is that individual chromosomes may have been lost in preparation of the spread. Therefore, hypoploid cells are normally dismissed as an artefact of the chromosome spreading technique. In the method of embryo karyotyping described here, intact embryos are spread on slides thus minimising the risk of chromosome loss. However, since 80 kinetochores must be resolved, it remained possible that close apposition of CREST signals could cause some kinetochores to be ‘missed’ in the counting process. Thus, as for traditional chromosome spread analysis, only hyperploid cells were counted as aneuploid in the method used here.

## **2.12. Image analysis**

For experiments to detect centriole/centrosome emergence the number of cells with spots was quantified by careful examination of serial Z-sections to detect GFP-CETN2/ $\gamma$ -tubulin foci. Following overexpression of eGFP-hsSAS-6 or eGFP-Plk4 in oocytes/embryos, Z-stack projections were created using LSM Image Browser and the number of cytoplasmic accumulations produced was quantified using the ‘*analyse particles*’ function in ImageJ software. The average + three standard deviations of the image fluorescence was used to set the lower threshold. Ultrastructural studies have revealed that centrioles are 100-150 nm in diameter and 100-400 nm in length (Chretien et al., 1997; Kuriyama and Borisy, 1981). Therefore only foci of more than 0.1  $\mu\text{m}^2$  and with a circularity of 0.4-1.00 were counted. For experiments to determine the number of MTOCs in MII eggs, serial Z-sections were carefully examined to detect  $\beta$ -tubulin-labelled MTOCs. Images were scored blind.

### **2.13. EB1 microtubule growth velocity analysis**

Confocal images of live EB1-expressing embryos were obtained at 1.936 second intervals. Images were then converted to maximum intensity projections and an ROI used to select comets of interest. Only comets which were visible for at least three consecutive frames were analyzed. The ROI was restored onto the original Z-series and the '*multiple kymograph*' plug-in for ImageJ software then used to produce a kymograph of the EB1 comet in question. The comet was visible as a distinct 'edge' in the kymograph which was then selected and the '*read velocities from tsp*' macro used to calculate the average speed. This value was then converted to  $\mu\text{m}/\text{min}$  using Microsoft Excel.

### **2.14. $\gamma$ -tubulin nucleation analysis**

Confocal Z-stack images of morulae and early blastocyst stage embryos were obtained as previously. The '*plot profile*' function in ImageJ was then used to select foci of interest. Values were then imported into Microsoft Excel where the peak height and width was calculated. The area under the curve was calculated using the trapezoid rule. The baseline was set at the average + one standard deviation of the fluorescence of the corresponding slice.

### **2.15. Statistical analysis**

Experiments were performed on a minimum of two experimental days. Error bars shown on graphs represent the standard error of the mean (sem). T-tests are two-tailed and are based upon two samples (equal variance). T-tests were used to compare significance between two groups and ANOVA followed by Tukey-Kramers post-hoc test used to compare significance between three or more groups. Chi squared tests were used to compare significance between two groups of binary values. A p-value of  $<0.05$  was considered statistically significant. Different letters where shown on graphs indicate statistically significant differences at  $p < 0.05$ .

**Table 2.1. RNA manufactured in this study**

<b>Protein</b>	<b>Vector</b>	<b>RNA promoter used</b>
GFP-AKAP450	pcDNA3.1/ <i>myc</i> -His(-), Invitrogen	T7
EB1-GFP	pcDNA3.1/ <i>myc</i> -His(-), Invitrogen	T7
eGFP-Plk4	pcDNA3.1/ <i>myc</i> -His(-), Invitrogen	T7
eGFP-Plk4 (D154A)	pcDNA3.1/ <i>myc</i> -His(-), Invitrogen	T7
eGFP-hsSAS-6	pcDNA3.1/ <i>myc</i> -His(-), Invitrogen	T7
eGFP only	pCS2	SP6
MAP7-RFP	Gateway DEST vector pRN3 (pSPE3)	T3

**Table 2.2. Drugs used in this study**

<b>Drug</b>	<b>Source</b>	<b>Final Concentration</b>
IBMX	Sigma, UK	200 $\mu$ M
MG132	Merck Biosciences, Germany	25 $\mu$ M
Monastrol	Merck Biosciences, Germany	100 $\mu$ M
Nocodazole	Merck Biosciences, Germany	10 $\mu$ M
UO126	Promega, UK	50 $\mu$ M

**Table 2.3. Primary Antibodies used in this study**

<b>Antibody</b>	<b>Concentration</b>	<b>Source</b>
mouse anti- $\gamma$ tubulin	1:1000	T5326, Sigma
mouse anti- $\beta$ -tubulin	1:1000	T4026, Sigma
mouse anti-C-nap1	1:500	BD biosciences
rabbit anti-hsSAS-6	1:200	gift from Pierre Gönczy, Lausanne, Switzerland
rabbit anti-Centrin-2	1:200	N17; Santa Cruz
rabbit anti-Centrin-1/2	1:200	H40; Santa Cruz
mouse anti-Oct 4	1:300	C10; Santa Cruz
mouse anti-GT335	1:1000	gift from Carsten Janke, Paris, France (Wolff et al., 1992)
rabbit anti-Kif2A	1:1000	gift from Duane Compton, Dartmouth, NH (Ganem and Compton, 2004)
rabbit anti-Plk4	1:200	gift from Alex Dammermann, Vienna, Austria (unpublished)
rabbit anti-cleaved caspase 3	1:500	#9661, Cell Signalling Technology
human anti-ACA (CREST)	1:300	gift from William Earnshaw, Edinburgh, UK (Earnshaw and Rothfield, 1985)

### **3. CENTROSOME AND CENTRIOLE EMERGENCE IN MOUSE PRE-IMPLANTATION EMBRYOS AND THE EFFECT ON MICROTUBULE BEHAVIOUR AND ORGANISATION**

#### **3.1. Introduction**

Centrioles are usually duplicated once per cell cycle via the canonical duplication pathway (Delattre et al., 2006; Pelletier et al., 2006; Strnad and Gonczy, 2008). However, fertilisation presents a particular problem in terms of controlling centriole number. If each gamete contributed one pair of centrioles there would be a surplus of MTOCs at the first mitosis. It is well established that supernumerary centrioles can lead to chromosome segregation errors and have been associated with cancer (Ganem et al., 2009; Nigg, 2006; Silkworth et al., 2009). Therefore, in order to ensure the correct centriolar complement is maintained following fertilisation most species require a differential contribution from each gamete (reviewed by (Schatten, 1994)). In *C. elegans* the sperm contributes the centriole pair (Wolf et al., 1978) while the oocyte retains only the PCM (Albertson and Thomson, 1993). Thus, one functional MTOC is reconstituted from the two gametes. In humans and flies, sperm cells retain a single centriole which helps organise the basal body of the flagellum (Callaini et al., 1999; Manandhar et al., 2000; Schatten, 1994). Following fertilisation, the single paternally-contributed centriole must replicate twice prior to the first mitosis in order to produce the two centriole pairs necessary for cell division. Interestingly, in mouse, centrioles are eliminated in spermiogenesis (Manandhar et al., 1998; Manandhar et al., 1999) as well as oogenesis (Szollosi et al., 1972) and as such two acentriolar gametes combine at fertilisation. EM analysis first suggested that the first few embryonic divisions in mice occur in the absence of centrioles before centrioles become detectable at the blastocyst stage (Abumuslimov et al., 1994; Gueth-Hallonet et al., 1993; Szollosi et al., 1972). This suggests that centrioles are generated *de novo* late in pre-implantation development. Therefore, mouse embryos represent one of the few examples of *de novo* centriole formation in a physiological setting.

Early EM work showed that centrioles are first detectable at the blastocyst (~64 cell) stage in mouse embryos (Abumuslimov et al., 1994; Gueth-Hallonet et al., 1993; Szollosi et al., 1972). However, more recently, changes in centrosomal appearance have been observed by immunofluorescence at the morula

stage (~32 cell) when the PCM component  $\gamma$ -tubulin first appears (Calarco-Gillam et al., 1983; Gueth-Hallonet et al., 1993; Palacios et al., 1993). These data suggest that the appearance of centrioles is preceded by the appearance of acentriolar centrosomes by approximately one cell cycle. Similarly, after centriole removal in somatic cells, the appearance of a PCM focus precedes the emergence of centrioles (Khodjakov et al., 2002). Furthermore, overexpression of the PCM component pericentrin can induce the formation of extra daughter centrioles (Loncarek et al., 2008). These studies imply a key link between the PCM cloud and centriole formation. The presence of acentriolar centrosomes is unusual since the PCM is usually defined by its association with the centrioles. Indeed, upon centriole removal the PCM is gradually dispersed (Bobinnec et al., 1998a).

Although EM has previously been used to monitor centriole emergence in mouse embryos (Abumuslimov et al., 1994; Gueth-Hallonet et al., 1993; Szollosi et al., 1972), EM analysis is very time-consuming, making it impractical to assess the presence or absence of centrioles in each individual cell of blastocysts. To readdress this question and to provide a tool for examining the effect of interventions such as protein overexpression and depletion, a method of labelling centrioles fluorescently was required. Various markers of centrioles have been used in other cellular settings, including antibodies for polyglutamylated tubulin (Bobinnec et al., 1998b), C-nap1 (Kohlmaier et al., 2009; Leidel et al., 2005) and centrin (Habedanck et al., 2005; Loncarek et al., 2008; Strnad et al., 2007) as well as fluorescently-tagged PACT domain proteins (Basto et al., 2006; Basto et al., 2008; Peel et al., 2007; Rodrigues-Martins et al., 2007a). In this chapter, a variety of approaches for the labelling of centrioles in mouse embryos are tested as potential markers of centrioles in embryos. Having established a robust method for detecting centrioles, the question of when precisely centrioles appear in mouse embryos is revisited. Given that early mouse embryos are acentriolar and mouse embryos appear at the blastocyst stage (Abumuslimov et al., 1994; Gueth-Hallonet et al., 1993; Szollosi et al., 1972), this model also provides the opportunity to examine the impact of centriole emergence on MT function in a physiological setting.

## **AIMS**

The aims of the experiments presented in this chapter were threefold. Firstly, it was important to establish reliable methods for detecting centrioles and centrosomes in both live and fixed mouse embryos. This would then allow investigation of the effects of interventions, such as protein overexpression, on centriole

and centrosome emergence in the following chapters. Secondly, these labelling methods were used to produce a time course of centrosome and centriole emergence in mouse early embryos. Finally, having established the stage at which centrioles and centrosomes appear, any differences in MT organisation and behaviour in mouse embryos were investigated and related to the presence/absence of centrioles in specific stages.

## **3.2. Results**

### **3.2.1. $\gamma$ -tubulin foci first emerge in morula stage embryos**

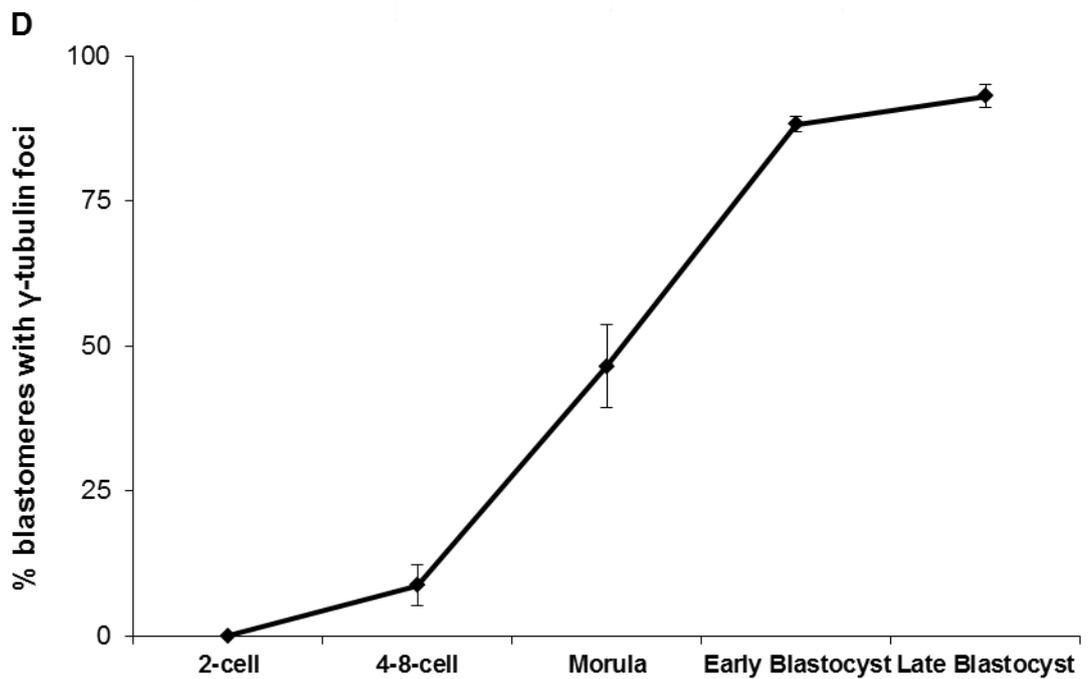
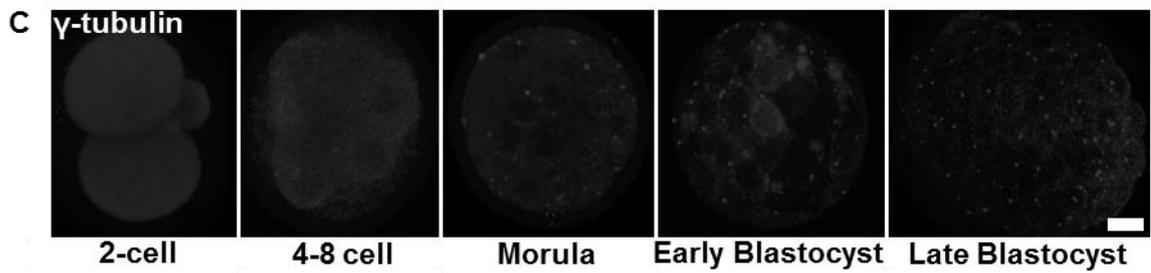
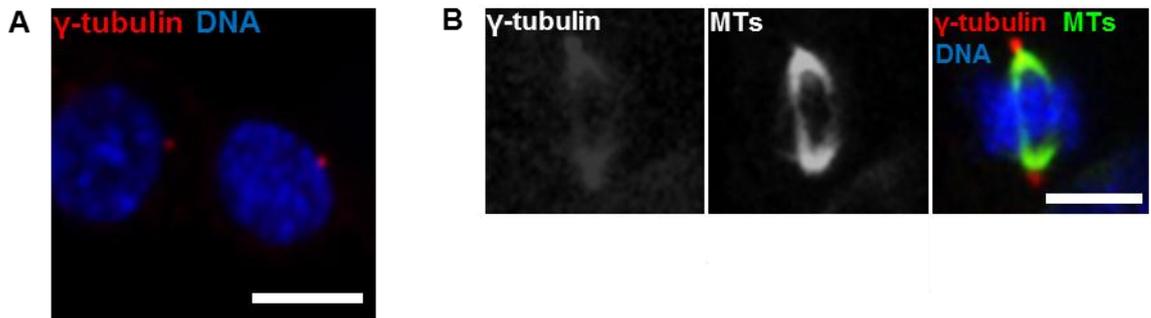
To establish a method for the labelling of the PCM, an antibody raised against the known PCM marker  $\gamma$ -tubulin was tested. Early studies employed a human auto-immune serum from a scleroderma patient termed #5051 (Calarco-Gillam et al., 1983; Houliston et al., 1987) to label the PCM. However, more recently,  $\gamma$ -tubulin antibodies have been shown to effectively label interphase centrosomes/MTOCs in mouse embryos/oocytes (Gueth-Hallonet et al., 1993; Palacios et al., 1993; Schuh and Ellenberg, 2007).

The antibody was initially tested in late blastocysts as previous work has shown that centrosomes will be present by this stage (Gueth-Hallonet et al., 1993). Blastocysts were obtained by culturing 2-cell embryos for four days. Embryos were subsequently fixed and labelled with a  $\gamma$ -tubulin antibody. The antibody was found to effectively label interphase foci in late blastocysts (Fig 3.1A) and, as expected, was also capable of labelling the spindle pole (and to a lesser extent the spindle MTs) in mitosis (Fig 3.1B). Consequently, this antibody represents a reliable method for labelling the PCM in mouse embryos.

It was next important to determine when  $\gamma$ -tubulin foci first emerge in mouse early development. Embryos at various different developmental stages were obtained as described in the materials and methods section, fixed at different stages and stained with a  $\gamma$ -tubulin antibody. Z-stack images were acquired on a confocal microscope and the number of cells with foci per embryo quantified (Fig 3.1C-D). Clear interphase  $\gamma$ -tubulin foci were frequently observed in morulae and blastocysts, but not in 2- or 4-8-cell stage embryos. Thus, early mouse embryos lack interphase centrosomes for the first ~4-5 cell divisions.

### **3.2.2. A polyglutamylated tubulin antibody fails to specifically label centrioles in mouse blastocysts**

It was next important to establish a suitable method for labelling centrioles in mouse embryos. Centriole MTs are highly stable structures due to the large number of tubulin modifications such as acetylation (Piperno and Fuller, 1985), detyrosination (Gundersen and Bulinski, 1986) and glutamylation (Edde et al., 1990). Thus, one of these modifications may represent a potential marker of centrioles. A monoclonal



**Figure 3.1.  $\gamma$ -tubulin foci first appear at the morula stage of mouse embryo development.** (A) Representative confocal Z-section showing  $\gamma$ -tubulin-labelled foci in mouse blastocysts. (B) Confocal Z-projection illustrating  $\gamma$ -tubulin labelling in a blastomere in mitosis. Note that  $\gamma$ -tubulin labels the spindle poles as well as the spindle. (C) Confocal Z-projections of  $\gamma$ -tubulin-labelled early embryos. (D) Quantification of  $\gamma$ -tubulin foci in mouse embryos (6-12/stage examined). Quantification was performed by examining serial Z-sections to establish the proportion of nuclei which had an associated  $\gamma$ -tubulin focus. Scale bar 10  $\mu$ m.

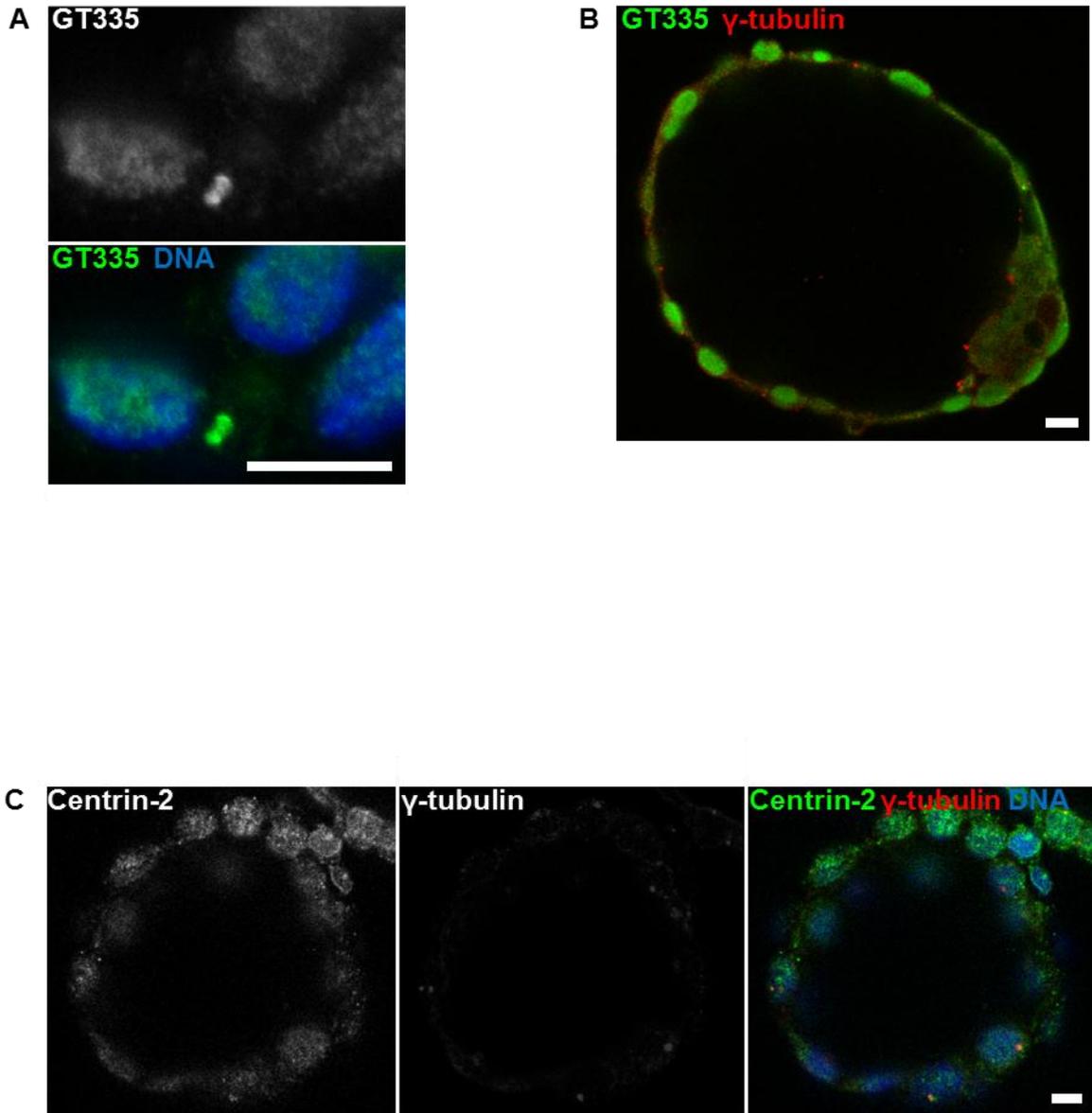
antibody raised against polyglutamylated tubulin (GT335; (Wolff et al., 1992)) has been used effectively as a centriole marker in somatic cells (Bobinnec et al., 1998b) and therefore may represent a possible method for labelling centrioles in mouse embryos. Blastocyst stage embryos were fixed and stained with a GT335 antibody (gift from Carsten Janke, Paris, France). In a small number of blastomeres staining was visible in a characteristic pair suggesting labelling of the centrioles (Fig 3.2A). However, the GT335 antibody also appears to stain the DNA very strongly (Fig 3.2A,B). This unexpected nuclear staining combined with inconsistent labelling of centriole pair means that it would be difficult to use GT335 to robustly monitor centriole appearance.

### **3.2.3. Centrin antibodies fail to specifically label centrioles in mouse blastocysts**

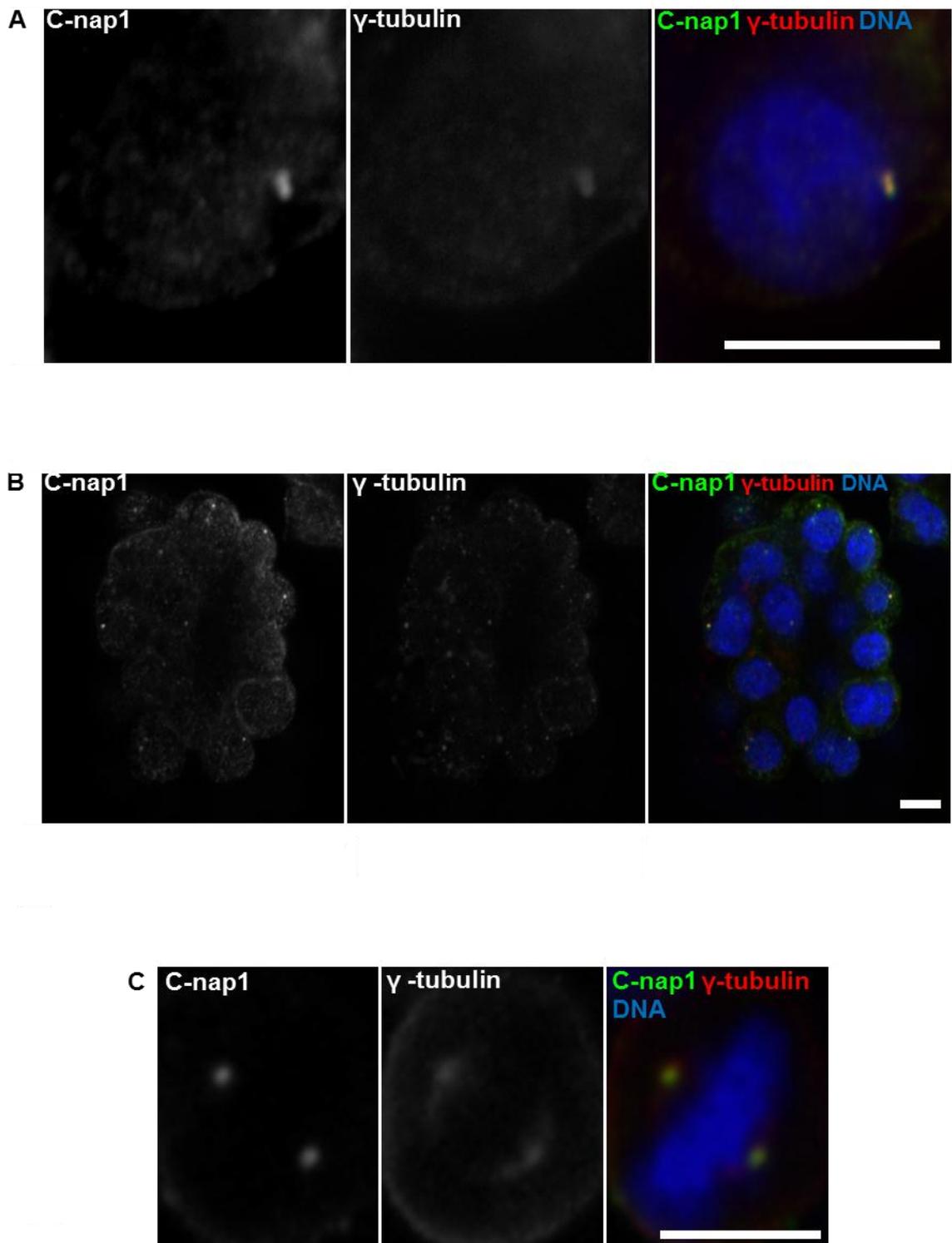
Centrin is specifically located in the distal lumen of the centriole (Paoletti et al., 1996; Piel et al., 2000) and is a widely used marker of centrioles in somatic cells with a discrete centrin focus identifying a single centriole (Habedanck et al., 2005; Kleylein-Sohn et al., 2007; Loncarek et al., 2008; Strnad et al., 2007). Two different centrin antibodies were therefore used to attempt to label centrioles in late blastocysts. Since disassembly of the centriole causes disappearance of the PCM (Bobinnec et al., 1998a), I expect that centrioles will always be found in association with the PCM in blastocysts. Therefore,  $\gamma$ -tubulin (a component of the PCM, which is known to effectively label centrosomes in mouse embryos) was also stained. Both centrin antibodies stained sites distinct from those sites stained by  $\gamma$ -tubulin and also produced a large amount of background staining (Fig 3.2C). These antibodies will therefore not provide an adequate method for observing centriole emergence in mouse embryos. Despite this finding, anti-centrin antibodies have previously been used in mouse embryos in some contexts (Zhong et al., 2005). This will be discussed further in section 3.3.1.

### **3.2.4. A C-nap1 antibody unexpectedly binds to the spindle poles during mitosis**

Another marker which has previously been used successfully to label centrioles is C-nap1 (Habedanck et al., 2005; Leidel et al., 2005; Strnad et al., 2007). C-nap1 is a centriole-specific protein which forms part of the structural link between parental centrioles. It is phosphorylated in G2 which triggers its degradation and allows the subsequent splitting of newly-formed centriole pairs or “diplosomes” (Fry et al., 1998; Mayor et al., 2000). Blastocysts stained with an anti-C-nap1 antibody show discrete spots in the majority of cells (Fig 3.3B). To confirm these spots represent centriolar staining, embryos were co-labelled with C-



**Figure 3.2.** *GT335 and centrin antibodies fail to robustly label centrioles in mouse blastocysts. Single confocal Z-section of (A) a small area of a blastocyst showing GT335 apparently labelling a centriole pair and (B) a whole blastocyst showing GT335 and  $\gamma$ -tubulin labelling discrete sites. Note that GT335 also labels the DNA. (C) Representative confocal Z-section of a whole blastocyst showing centrin (N17) antibody staining. Note that centrin does not colocalise with  $\gamma$ -tubulin and produces a high-level of non-specific staining. Similar results were obtained with two different centrin antibodies. Scale bar 10  $\mu$ m.*



**Figure 3.3.** *C-nap1* is not reliable marker of centrioles in mouse blastocysts. Typical confocal Z-sections showing colocalisation of  $\gamma$ -tubulin and *C-nap1* in (A) a single blastomere and (B) a whole blastocyst. (C) Confocal Z-section of a mitotic blastomere labelled with *C-nap1* and  $\gamma$ -tubulin. Note the unexpected localisation of *C-nap1* at the spindle poles. Scale bar 10  $\mu$ m.

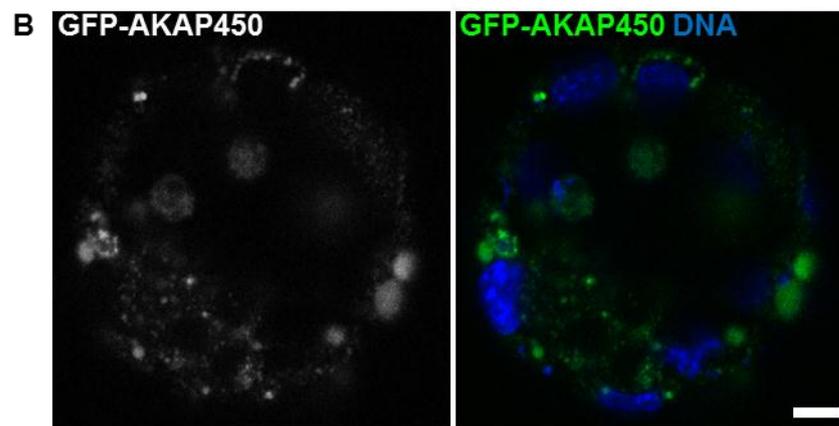
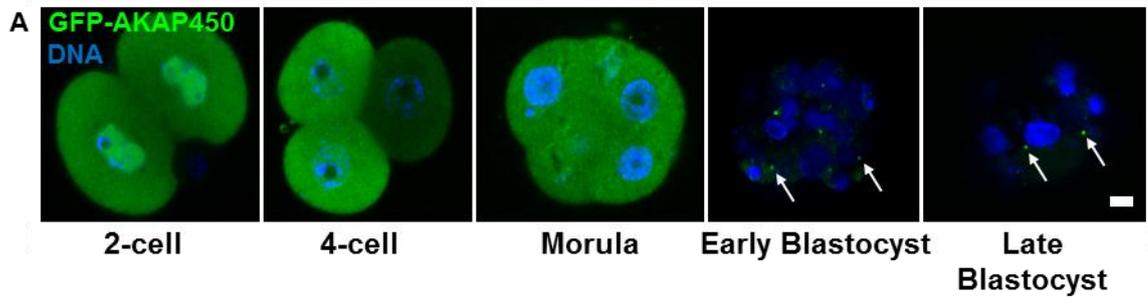
nap1 and  $\gamma$ -tubulin which has already been established to be a reliable marker of the PCM. C-nap1 and  $\gamma$ -tubulin spots colocalise in most cases (Fig 3.3A,B).

C-nap1 forms part of a cohesive structure between centrosomes which is disassembled to allow parental centrioles to separate and form spindle poles (Fry et al., 1998; Mayor et al., 2000). Consistent with this model, C-nap1 staining is diminished upon formation of mitotic spindle poles in human cells (Mayor et al., 2000). Interestingly however, C-nap1 was found to strongly label the spindle poles in mitotic mouse embryo blastomeres (Fig 3.3C). Although the reason for this was unclear and was not addressed further here, this raised doubts regarding the specificity of the antibody. Thus, a more reliable method for the labelling of centrioles was sought.

### **3.2.5. GFP-AKAP450 fails to specifically label centrioles in mouse embryos**

AKAP450 and pericentrin are large coiled-coil domain proteins which contain conserved regions near their C-termini. This conserved region has been named the pericentrin-AKAP450 centrosomal targeting (PACT) domain as fusion to a transporter protein confers a centrosomal localisation (Gillingham and Munro, 2000). However, the only PACT domain protein in *Drosophila* (D-PLP) has been found to be most strongly associated with the centrioles (Martinez-Campos et al., 2004) and has been used extensively to label centrioles in *Drosophila* embryos (Basto et al., 2006; Basto et al., 2008; Peel et al., 2007; Rodrigues-Martins et al., 2007a). Furthermore, differential PACT fluorescence can distinguish between mother and daughter centrioles in *Drosophila* embryos and neuroblasts (Conduit et al., 2010; Conduit and Raff, 2010). The PACT domain therefore represented a promising method as live embryos could be examined for the presence of centrioles without the need for manipulation.

A GFP-tagged version of the C-terminal PACT domain of AKAP450 (GFP-AKAP450) was obtained from Sean Munro (Cambridge, UK) and RNA corresponding to the protein microinjected into each blastomere of 2-cell embryos. The embryos were subsequently cultured to blastocyst stage and confocal imaging of live embryos carried out at set time points throughout development. A pair of GFP-AKAP450 cytoplasmic foci was, in some cases, found to first appear at the early blastocyst stage (Fig 3.4A). This corresponds to previous EM studies (Gueth-Hallonet et al., 1993) and suggests that GFP-AKAP450 has potential as a centriole-labelling tool. However, although discrete foci were observed in some embryos,



**Figure 3.4. GFP-AKAP450 is not a consistent and robust marker of centrioles in mouse blastocysts.** (A) Single confocal slices of live embryos at different stages of development after injection with GFP-AKAP450 at the 2-cell stage. Note that in some embryos cytoplasmic foci first appear at the early blastocyst stage (arrows). (B) Single confocal slice of a live blastocyst after injection of GFP-AKAP450. Note the non-specific staining. Scale bar 10  $\mu$ m.

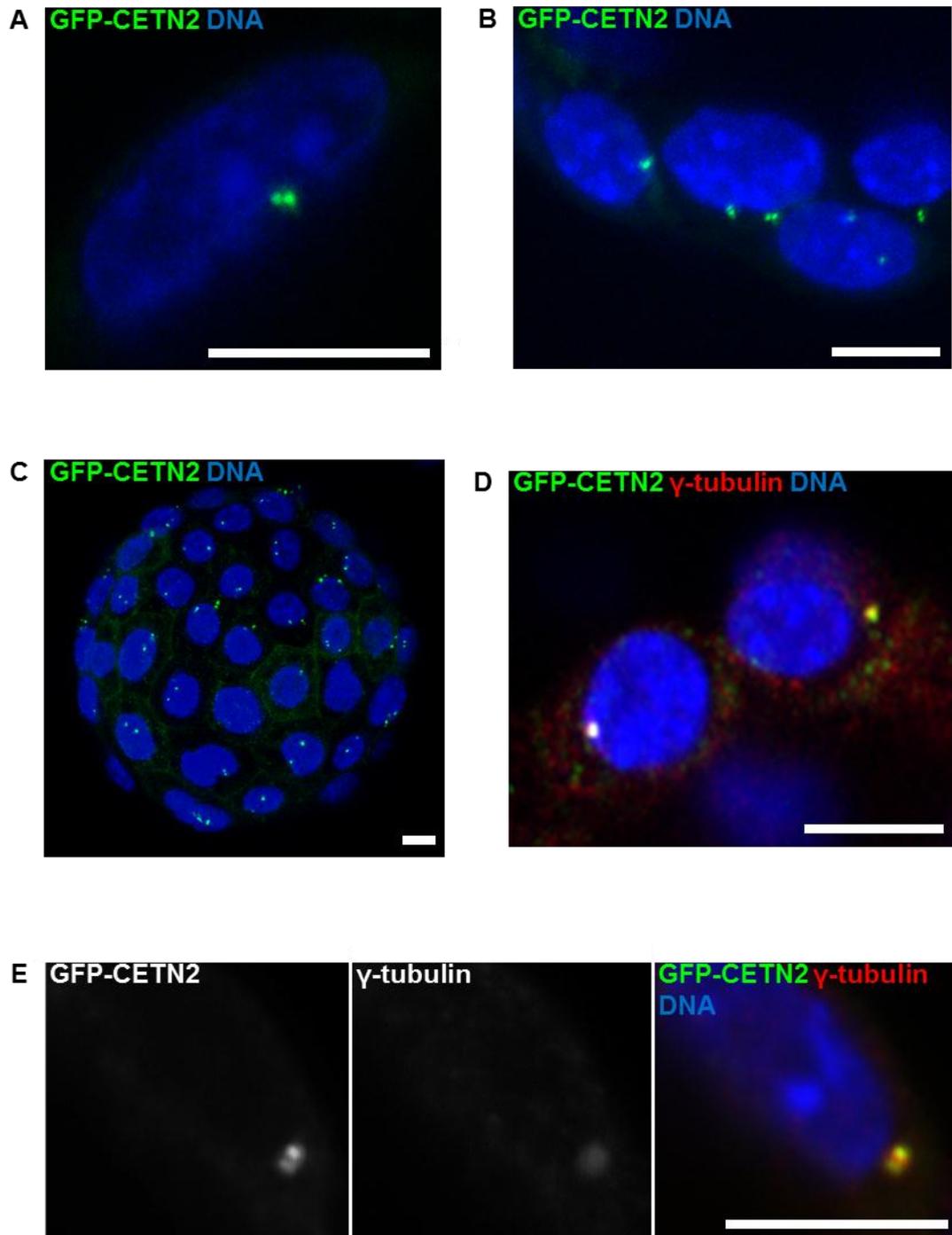
the majority showed non-specific localisation (Fig 3.4B). Although there have been many examples of PACT domain proteins being used as centriole markers in *Drosophila* cells (Basto et al., 2006; Bettencourt-Dias et al., 2005; Conduit et al., 2010; Conduit and Raff, 2010; Peel et al., 2007; Rodrigues-Martins et al., 2007a), only a few studies have reported the use of a PACT protein to label centrioles in mammalian cells (Gillingham and Munro, 2000), raising questions concerning suitability for use in mouse embryos. In addition, it has been reported that overexpression of the PACT domain from AKAP450 in HeLa cells can displace endogenous AKAP450 (Gillingham and Munro, 2000; Keryer et al., 2003) and impair centriole duplication and cell cycle progression (Keryer et al., 2003). Therefore, the use of GFP-AKAP450 will not provide a suitable method for monitoring the emergence of centrioles in large numbers of mouse embryos.

### **3.2.6. A GFP-CETN2 transgenic mouse model provides a robust method for centriole labelling in mouse embryos**

GFP-tagged centrin has been widely used to label centrioles in various somatic cell lines (Habedanck et al., 2005; Loncarek et al., 2008; Strnad et al., 2007). Recently, a transgenic mouse model which constitutively expresses a GFP-tagged copy of the centriolar protein centrin-2 was engineered (Higginbotham et al., 2004). Cultured cell lines stably expressing GFP-CETN2 had previously been created, indicating that expression of GFP-CETN2 has no deleterious effect on cell cycle progression or centriole function (White et al., 2000). In agreement with this, GFP-CETN2 mice have no observable phenotype and possess a single pair of GFP-labelled centrioles in every cell. Furthermore, the presence of the transgene does not affect the number of centrosomes per cell (Higginbotham et al., 2004).

To investigate whether this system was suitable for the labelling of centrioles in mouse blastocysts, which are expected to contain centrioles (Abumuslimov et al., 1994; Gueth-Hallonet et al., 1993; Szollosi et al., 1972), CB6 male mice possessing the transgene were mated with wild type MF1 females (see Materials and Methods). 2-cell embryos were then collected and cultured to the blastocyst stage before confocal imaging of live embryos was performed.

As in somatic cells (Higginbotham et al., 2004), GFP-CETN2 was arranged into pairs of tightly-focussed spots associated with each nucleus (Fig 3.5A-C). To determine whether these pairs of GFP-CETN2-



**Figure 3.5. GFP-CETN2 labels distinct foci in late blastocysts which colocalise with  $\gamma$ -tubulin.** (A) Confocal slice of a single blastomere showing a pair of centrin spots. (B) Zoomed image of a small area of a blastocyst showing a pair of centrin spots associated with each blastomere. (C) Projection of a whole blastocyst showing centrin spots in most cells. Confocal slice of a (D) small area of a blastocyst and (E) a single blastomere showing a pair of centrin spots surrounded by a  $\gamma$ -tubulin 'cloud'. Scale bar 10  $\mu$ m.

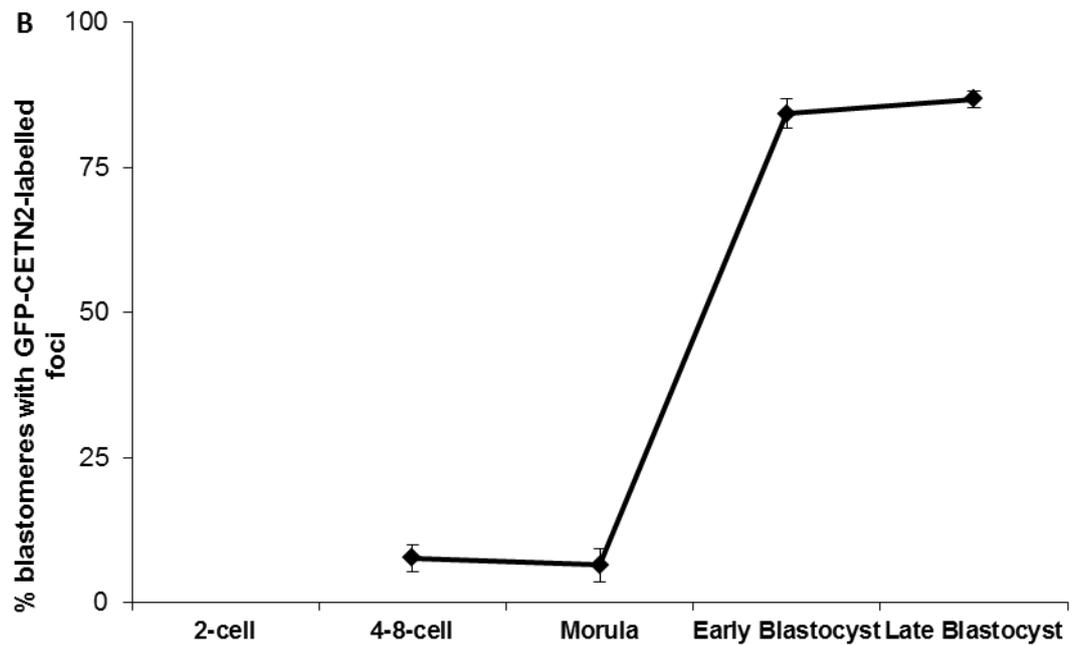
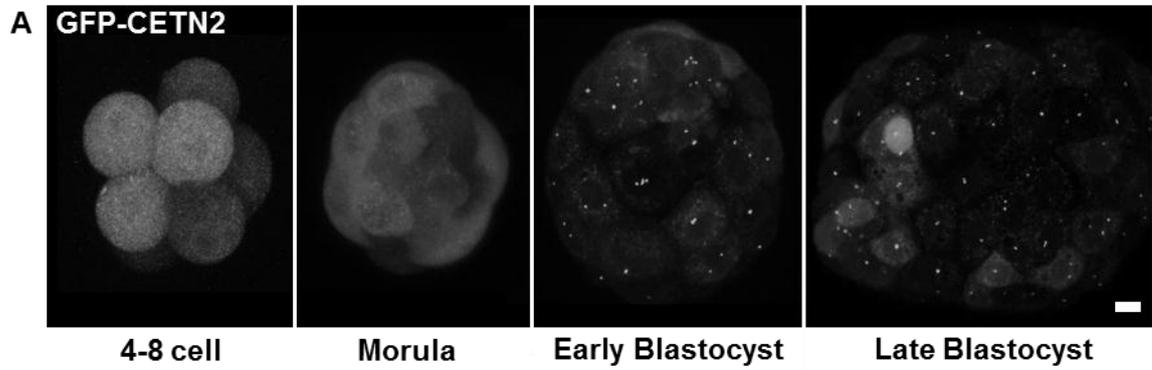
labelled foci represent part of the centrosome, GFP-CETN2-expressing blastocysts were fixed and stained with a  $\gamma$ -tubulin antibody. As expected, GFP-CETN2-labelled centrioles colocalised with the PCM component  $\gamma$ -tubulin, confirming centrosomal localisation (Fig 3.5D,E).

### **3.2.7. Centrioles first appear at the blastocyst stage in mouse embryos**

Next, confocal imaging of live embryos was used to detect centriole emergence throughout the development of mouse embryos possessing the GFP-CETN2 transgene. Z-stack images were obtained and quantification of spots carried out as for  $\gamma$ -tubulin (Fig 3.1). GFP-CETN2 fluorescence was homogeneous in almost all blastomeres of 4-8 cell stage embryos and morulae (~16-32 cell stage), whereas prominent GFP-CETN2-labelled centrioles accompanied 86.71 $\pm$ 1.36% of nuclei in expanded blastocysts (Fig 3.6). Notably however, GFP-CETN2-labelled centrioles were absent in morulae and 4-8 cell embryos, suggesting that the first few mitotic divisions in mouse embryos are acentriolar (Fig 3.6). The finding that centrioles first emerge in blastocysts concurs with previous EM data (Abumuslimov et al., 1994; Gueth-Hallonet et al., 1993; Szollosi et al., 1972) and suggests that the GFP-CETN2 mouse model will provide an excellent tool for the study of centriole biogenesis in mouse embryos.

### **3.2.8. GFP-CETN2-labelled centrioles first emerge preferentially in the TE lineage of mouse blastocysts**

The above data reveals that centrioles (as labelled by GFP-CETN2) first appear at the blastocyst stage (Fig 3.6). The morula to blastocyst transition involves the establishment of two distinct cell lineages - the inner cell mass (ICM) which develops into the embryo proper and the trophoectoderm (TE) which gives rise to the trophoblast. Previous studies have used EM analysis to suggest that centrioles emerge preferentially in the basal part of the TE in mouse blastocysts (Abumuslimov et al., 1994; Gueth-Hallonet et al., 1993). Further studies make use of the human auto-immune antibody #5051 (Calarco-Gillam et al., 1983) and  $\gamma$ -tubulin antibodies (Palacios et al., 1993) to monitor centrosome emergence in mouse embryos. Similar to centriole emergence, both studies report that centrosome-positive foci were first evident in interphase cells of the TE (Calarco-Gillam et al., 1983; Palacios et al., 1993; Palazzo et al., 1992). Two of these previous studies were carried out using EM (Abumuslimov et al., 1994; Gueth-Hallonet et al., 1993). Due to its time-consuming nature, EM analysis allows only a small number of cells to be examined and is therefore not suitable for experiments designed to establish the absence or presence



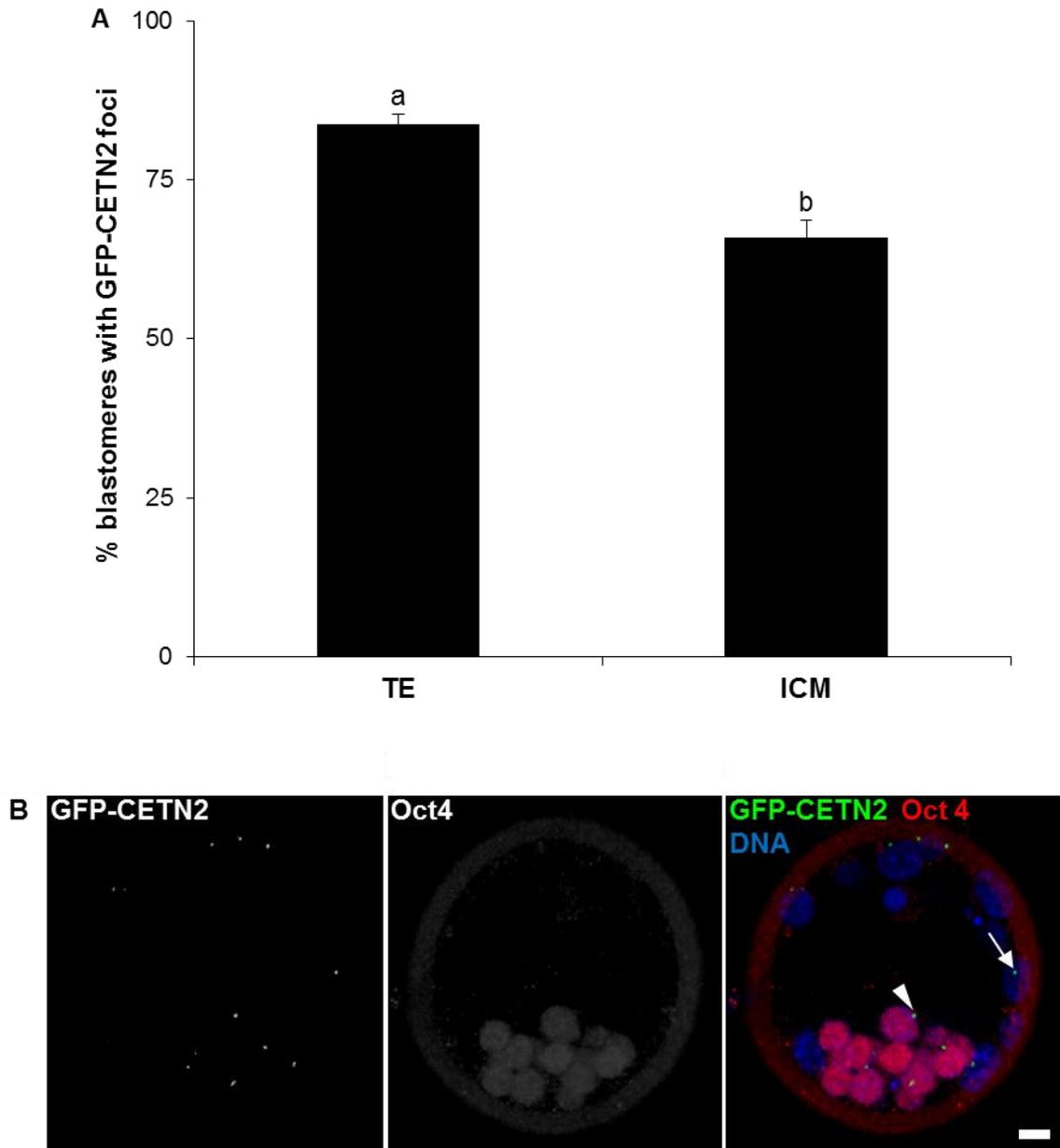
**Figure 3.6. Mouse embryos are acentriolar until the blastocyst stage.** (A) Representative confocal Z-projections of GFP-CETN2-labelled early embryos. (B) Quantification of GFP-CETN2-labelled foci in embryos (10-23 embryos/stage examined). Quantification was performed by examining serial Z-sections to determine the % of nuclei with an associated GFP-CETN2-labelled centriole pair. Note that centrioles are first evident in blastocysts. Scale bar 10  $\mu$ m.

of a particular cellular structure. Furthermore, all the studies described above rely on cell position rather than a specific cell lineage marker to distinguish between the two cell lines. Using the GFP-CETN2 mouse model and a reliable ICM marker Oct4, the question of whether centrioles appear *de novo* in an asynchronous manner within mouse blastocysts was revisited. Oct4 is an ICM-specific transcription factor and this antibody has previously been used in our lab to robustly distinguish between ICM and TE cells (Fitzharris, 2009). In order to determine in which lineage centrioles first emerge, early blastocyst stage embryos expressing GFP-CETN2 were fixed and stained with an antibody against Oct4. In early blastocyst stage embryos, 83.74 $\pm$ 1.67% of TE cells have GFP-CETN2-labelled foci, whereas only 65.98 $\pm$ 2.69% of ICM cells possess centrioles ( $p=00010$ ; Fig 3.7). Therefore, centrioles appear asynchronously in the two cell lineages in mouse embryos with TE cells preferentially displaying GFP-CETN2-labelled centrioles in early blastocysts.

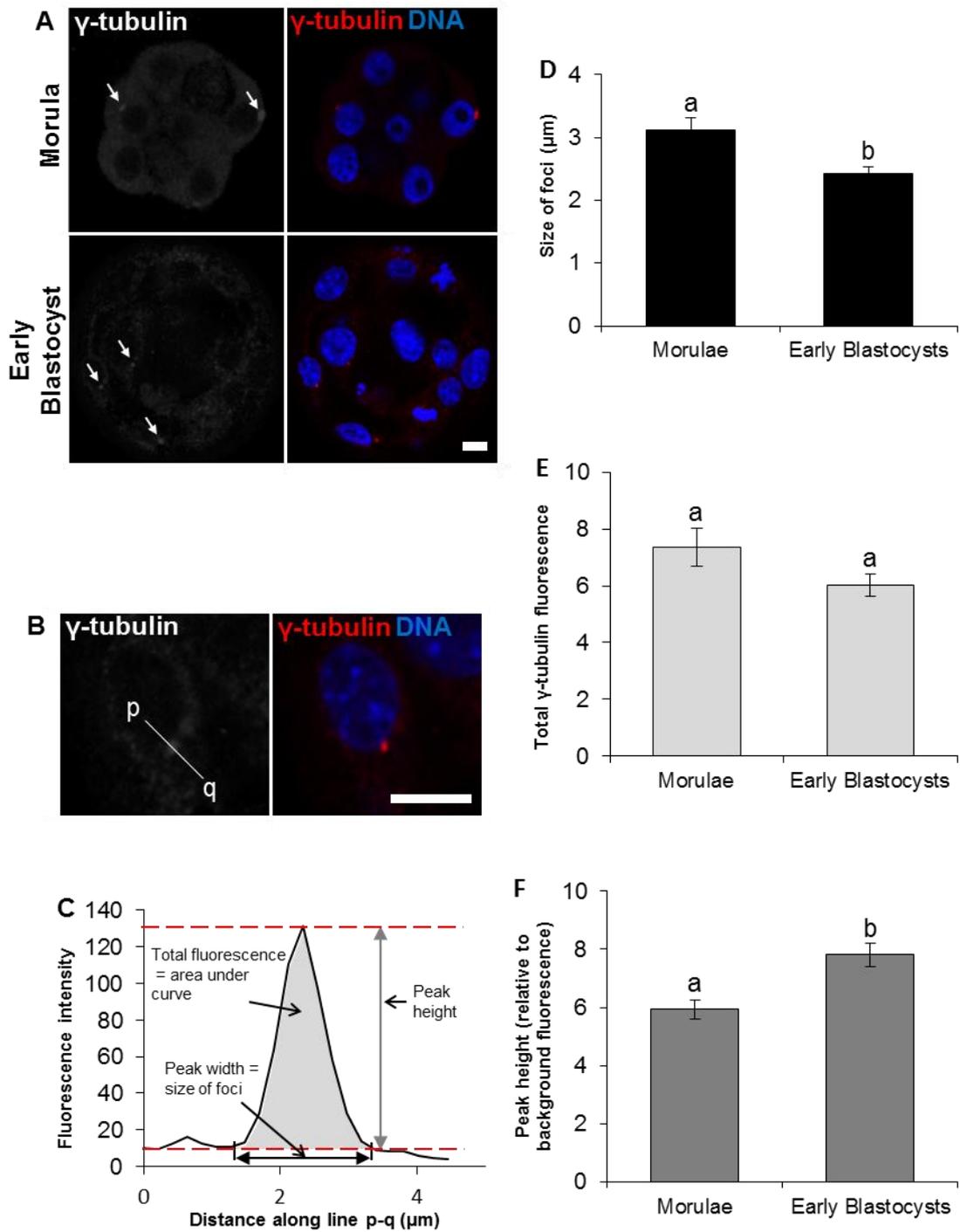
### **3.2.9. $\gamma$ -tubulin is more focussed in the presence of centrioles**

Having established that centrioles emerge at the blastocyst stage, I next set out to determine if centriole emergence impacts the centrosome. Therefore, the organisation of  $\gamma$ -tubulin was examined in the absence (morulae) and presence (early blastocysts) of centrioles. Morulae and early blastocyst stage embryos were fixed and stained with antibodies against  $\gamma$ -tubulin. The width of the  $\gamma$ -tubulin foci and the intensity of the foci (peak height) and total  $\gamma$ -tubulin fluorescence (area under the curve) were then compared between the two stages (Fig 3.8C; see also Materials and Methods for  $\gamma$ -tubulin foci analysis methods).

$\gamma$ -tubulin foci were found to be significantly more focussed in early blastocysts (peak width: 2.43 $\pm$ 0.11  $\mu$ m) compared to in morulae (3.12 $\pm$ 0.19  $\mu$ m;  $p<0.001$ ; Fig 3.8A,D). The average amount of  $\gamma$ -tubulin at each focus was similar in morulae (7.37 $\pm$ 0.66 relative to background fluorescence) and blastocysts (6.02 $\pm$ 0.38 relative to background fluorescence;  $p>0.05$ ; Fig 3.8A,E). However, the relative  $\gamma$ -tubulin peak height was significantly greater in blastocysts (7.81 $\pm$ 0.40x relative to background) than morulae (5.94 $\pm$ 0.32x relative to background;  $p<0.01$ ; Fig 3.8A,F). These data reveal that there was no significant difference in the total amount of  $\gamma$ -tubulin at foci between morulae and blastocysts. However,  $\gamma$ -tubulin foci become more focussed at the blastocyst stage.



**Figure 3.7. Centrioles emerge preferentially in the TE lineage of early mouse blastocysts.** (A) Quantification of centrioles in the two cell lineages (18 early blastocyst stage embryos examined). Quantification was performed by examining serial Z-sections to establish the proportion of cells in each cell lineage with an associated GFP-CETN2-labelled centriole pair. (B) Representative confocal Z-section of a early mouse blastocyst showing centrioles (as labelled by GFP-CETN2) in the TE (arrow) and the ICM (arrowhead). Note the robust Oct4 labelling of the ICM. Scale bar 10  $\mu$ m.

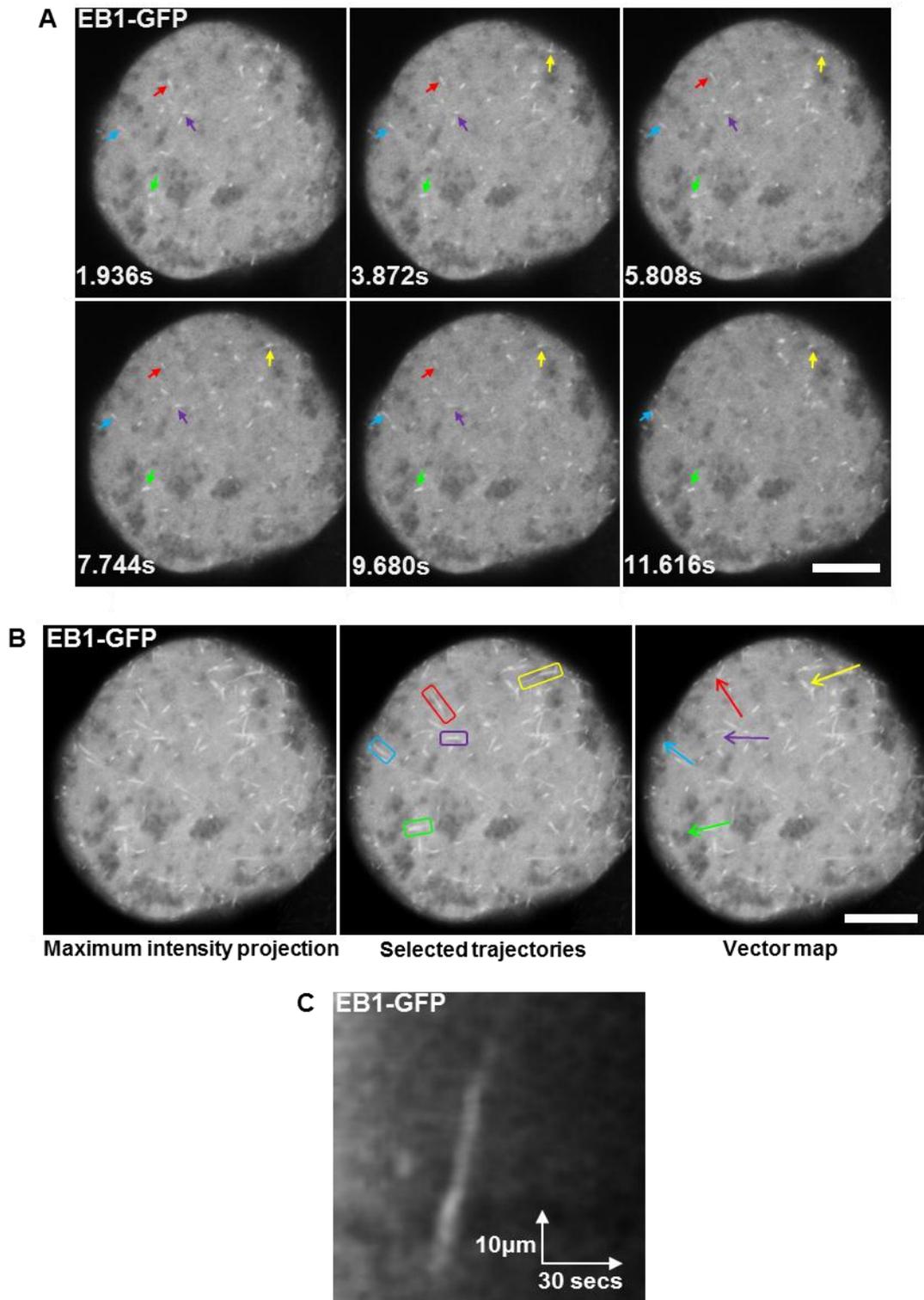


**Figure 3.8.**  $\gamma$ -tubulin foci become more focussed at the blastocyst stage. (A) Typical confocal slices showing  $\gamma$ -tubulin foci in morulae and early blastocysts. Arrows indicate  $\gamma$ -tubulin foci. (B) Example of plot profile function in Image J. (C) Illustration of analysis methods for profile shown in B. (D) Graph showing size of  $\gamma$ -tubulin foci in morulae and blastocysts. (E) Graph showing average total  $\gamma$ -tubulin fluorescence at foci. (F) Graph showing intensity of  $\gamma$ -tubulin foci in morulae and blastocysts. 27-48 foci from 5-8 embryos examined per stage. Scale bar 10  $\mu$ m.

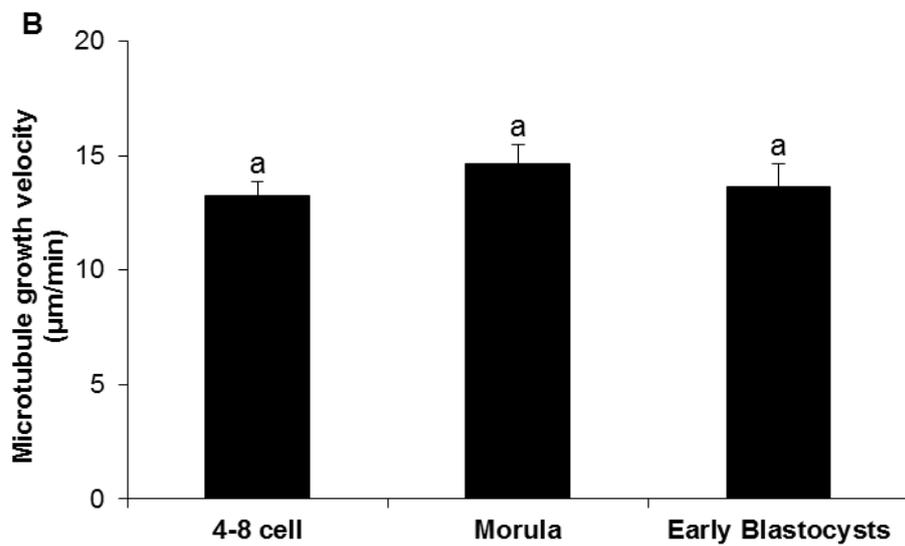
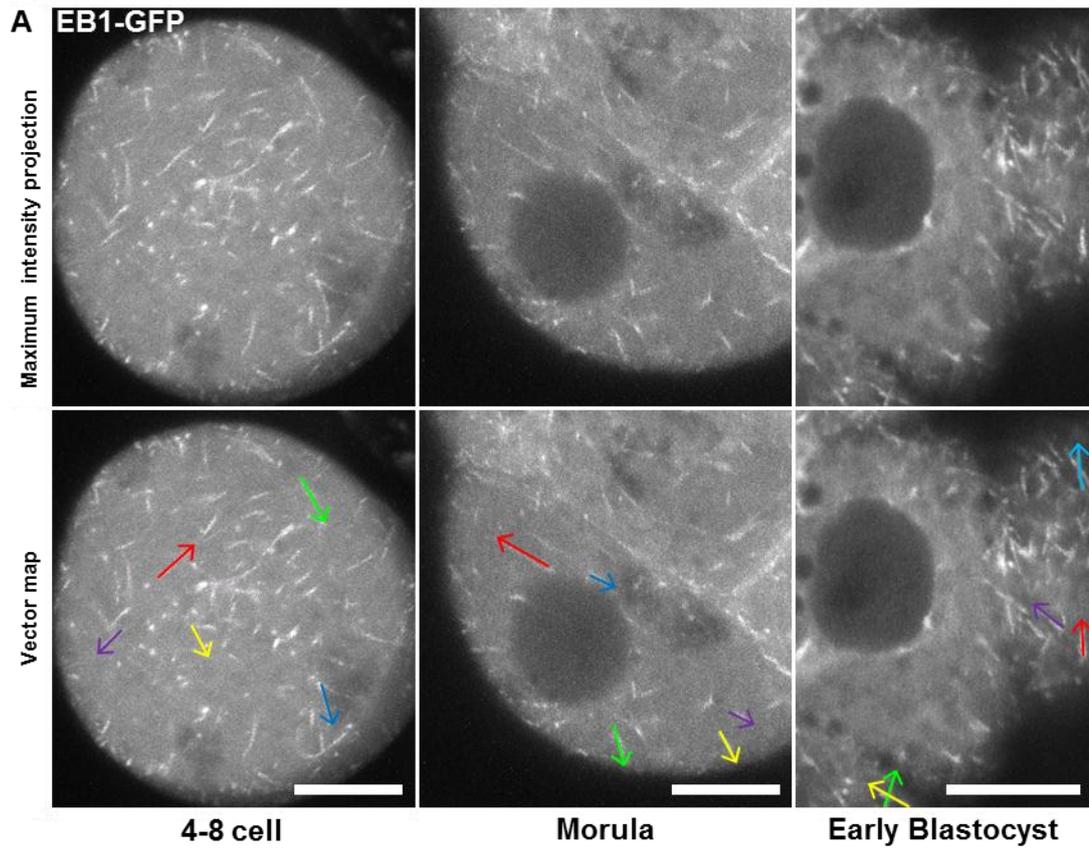
### **3.2.10. There is no difference in MT growth velocity in different stage mouse pre-implantation embryos**

I next wondered whether the emergence of centrioles at the blastocyst stage and the emergence of centrosomes at the morula stage, was accompanied by a major change in the ability of centrosomes to nucleate MTs. To do this a GFP-tagged EB1 probe was used (gift from Lynn Cassimeris, Lehigh, PA). Direct observation of MT nucleation in living cells using MT markers has previously proven difficult due to the high density of cytoplasmic MTs in close proximity to MTOCs. EB1 is a MT plus tip-binding protein which allows visualisation of growing MT plus ends without affecting cellular function (Piehl and Cassimeris, 2003). The movement of EB1-GFP comets have been used to detect sites of MT nucleation and to measure rates of MT nucleation and growth in various cell types (Piehl et al., 2004; Piehl and Cassimeris, 2003; Tirnauer et al., 1999). Therefore, this provided a tool for comparing MT nucleation in morulae and blastocysts.

To ascertain whether the emergence of centrioles in mouse blastocysts affects MT dynamics, MT growth velocities were measured by analysing the apparent movement of EB1-GFP comets. It has previously been established that in somatic cells MT growth velocities can increase from ~1.1 - 2 fold in prophase cells versus interphase cells (Belmont et al., 1990; Hayden et al., 1990; Rusan et al., 2001). Therefore, only interphase cells were compared in this analysis. EB1-GFP-expressing embryos were obtained by microinjection with EB1-GFP RNA at the 2-cell stage and subsequent culturing to the appropriate stage. Live embryos expressing EB1 were then transferred to a confocal microscope and a series of images obtained at 1.936 second intervals. Maximum intensity projections were then produced and kymograph analysis of individual EB1-GFP trajectories performed (see Materials and Methods and Fig 3.9). Kymograph analysis revealed that there was no significant difference in the MT growth velocity between 4-8-cell, morulae and early blastocysts ( $p > 0.05$ , ANOVA). The growth velocity ranged from  $13.27 \pm 0.59$   $\mu\text{m}/\text{min}$  (4-8-cell) to  $14.66 \pm 0.82$   $\mu\text{m}/\text{min}$  (morulae; Fig 3.10). Thus, despite the emergence of  $\gamma$ -tubulin foci at the morula stage (Fig 3.1) and centrioles at the blastocyst stage (Fig 3.6), MT growth velocities are similar in 4-8-cell stage embryos through to early blastocysts. Further observations relating to the organisation of MTs in mouse embryos are discussed in section 3.3.3.



**Figure 3.9. Examples of EB1 analysis methods.** (A) Representative confocal time series of EB1-GFP comets in a 4-8 cell stage embryo. Each coloured arrow follows a single EB1-GFP comet. (B) Maximum intensity projections of the entire time series shown in A. Coloured arrows follow the trajectory of the comets shown in A. (C) Kymograph analysis of an example comet. The comet is visible as a distinct edge in the kymograph. The gradient of this line was then calculated using the 'read velocities from tsp' macro which provides the microtubule growth velocity.



**Figure 3.10.** There is no difference in microtubule growth velocity in 4-8 cells, morulae and early blastocysts. (A) Representative confocal Z-projection of EB1-GFP comet trajectories in embryos of different stages. In the lower panel each coloured arrow shows the trajectory of a single EB1-GFP comet. (B) Graph showing microtubule growth velocities in 4-8 cells, morulae and blastocysts. 25-33 comets examined from 5-9 embryos per stage. Scale bar 10 µm.

### 3.3. Discussion

This chapter describes the development of methods to monitor the appearance of centrioles and centrosomes in mouse embryos. Several methods, which had previously been used to label centrioles/centrosomes effectively in somatic cells, were tested. However, differences in the efficacy of various antibodies in mouse embryos meant some of these methods were not suitable for use in this system. The GFP-CETN2 transgenic mouse was found to be the most robust method for the labelling of centrioles and using this model centrioles were found to appear at blastocyst stage in agreement with previous work (Abumuslimov et al., 1994; Gueth-Hallonet et al., 1993; Szollosi et al., 1972). Furthermore, centrioles were found to be generated asynchronously in the two cell lineages. Having established these methods, the emergence of centrioles/centrosomes and the influence on MT behaviour and organisation was investigated. The following discussion will therefore focus on three main topics. Firstly, the challenges associated with the visualisation of centrioles in mouse embryos will be considered. Secondly, the role of the PCM as a possible centriole precursor in embryos will be addressed. Finally, the impacts of centriole emergence will be discussed.

#### 3.3.1 Visualisation of centrioles in mouse embryos

##### *Challenges of antibody-based detection of centrioles*

A number of antibodies were trialled as possible centriole markers in embryos, each of which has been successfully used in somatic cells, namely centrin, C-nap1 and GT335 (Bobinnec et al., 1998b; Habedanck et al., 2005; Kleylein-Sohn et al., 2007; Leidel et al., 2005; Loncarek et al., 2008; Paoletti et al., 1996; Piel et al., 2000; Strnad et al., 2007). However, antibody markers which are successful in somatic cells may not necessarily prove effective for the labelling of the same structure in mouse oocytes/embryos. The large size of a mouse oocyte/early embryo (~100  $\mu\text{m}$ ) means that antibodies may have more difficulty in penetrating the centre of the embryos compared to cells grown in culture. Moreover, non-specific binding of antibodies to the zona pellucida can sometimes occur. Therefore, despite having been used as a marker of centrioles in somatic cells, the same methods may not be effective in mouse embryos. In this study, various methods were tested before a suitable method for the labelling of centrioles in mouse embryos was established. Nonetheless, this gave rise to some interesting observations which are discussed further below.

In particular, the failure of two different centrin antibodies, each trialled several times with three different fixation protocols (see Materials and Methods) provides an interesting observation. A recent study in mouse has suggested that centrioles from somatic cells are degraded immediately after transfer into an enucleated egg by somatic cell nuclear transfer (SCNT; (Zhong et al., 2005)). This result was based on the absence of detectable centrin labelling in SCNT-reconstructed eggs. However, a positive control showing that the antibodies were able to detect centrioles in oocytes or embryos of any stage (i.e. blastocysts) was not presented (Zhong et al., 2005). Therefore, it is not clear whether the investigators would have been able to detect centrioles had they been present. Consequently, the fate of exogenously-introduced centrioles in mouse oocytes and embryos remains to be investigated. This important question is discussed further in Chapter 6.

#### *Advantages and disadvantages of electron microscopy for addressing de novo centriole biogenesis in mouse embryos*

Several previous studies have investigated the presence/absence of centrioles in mouse embryos using EM methods (Abumuslimov et al., 1994; Gueth-Hallonet et al., 1993). EM is a valuable technique as it allows the ultrastructure of cellular organelles to be examined. Centrioles have a very distinctive molecular architecture being made up of nine triplet MTs arranged around a central hub structure (Chretien et al., 1997; Kuriyama and Borisy, 1981). In addition, centrioles are found in an orthogonal mother-daughter pair throughout the cell cycle. These unique features mean that centrioles can be identified relatively easily by the experienced manipulator. However, EM analysis requires ultra-thin sections of ~100-200 nm to allow electrons to penetrate the sample (Marco et al., 2004). Since a mouse blastocyst is typically more than 100  $\mu\text{m}$  in diameter, it is infeasible to prepare and image the 500-1000 serial sections which would be required to exhaustively section one embryo. Moreover, biological samples need chemical fixation, followed by dehydration and embedding in a resin (Marco et al., 2004) to enable these ultra-thin slices to be cut, further increasing the time required to process samples.

Ultrastructural analysis of centriole-like foci induced following overexpression of centriole replication factors, reveals that some of these foci represent structurally abnormal centrioles (Peel et al., 2007; Rodrigues-Martins et al., 2007a; Rodrigues-Martins et al., 2007b). These findings demonstrate that EM remains a useful technique to confirm the ultrastructural identity of centriole-like foci even if they are

capable of organising centrosome components. However, many of these studies were carried out in cells grown in culture where it is not necessary to analyse every individual cell by EM. In order to detect the first appearance of centrioles in mouse embryos, it is imperative that all cells are thoroughly examined for the presence of centrioles at each stage of development, which is not feasible by EM. One study reports that centrioles are first evident by EM in TE cells in mouse embryos (Gueth-Hallonet et al., 1993). However, since serial sections representing entire embryos were not obtained, it remains possible that some centrioles were in fact present in ICM cells at this stage. In summary, whilst EM remains invaluable for examining the ultrastructure of centrioles, the GFP-CETN2 transgenic mouse model is a vastly superior tool for examining the timing of centriole emergence in the entire blastocyst.

### **3.3.2. Is the PCM a precursor for centriole biogenesis in mouse embryos?**

In most cells the PCM is defined by its association with the centriole since disassembly of the centriole causes disappearance of the PCM (Bobinnec et al., 1998a). However, in early mouse embryos, distinct  $\gamma$ -tubulin foci are apparent at the morula stage (this work: Fig 3.1; (Gueth-Hallonet et al., 1993; Palacios et al., 1993)), approximately one cell cycle before the appearance of centrioles (this work: Fig 3.6; (Abumuslimov et al., 1994; Gueth-Hallonet et al., 1993)). The finding that  $\gamma$ -tubulin foci appear one cell cycle before centrioles, suggests that these PCM accumulations may be acting as centriole precursors. Indeed, the *de novo* formation of centrioles in somatic cells is also preceded by the appearance of a PCM focus (Khodjakov et al., 2002). Furthermore, knockdown of the PCM protein  $\gamma$ -tubulin in *C. elegans* or *Drosophila* caused a defect in the centriolar MTs (O'Toole et al., 2012; Raynaud-Messina et al., 2004) and in addition, recruitment of the  $\gamma$ TuRC ( $\gamma$ -tubulin ring complex) to the centrosome is necessary for centriole duplication in cultured cells (Haren et al., 2006). These findings suggest that  $\gamma$ -tubulin may play a part in centriole formation and could explain why the appearance of centrioles in mouse blastocysts is preceded by the appearance of  $\gamma$ -tubulin foci.

Recent work has also shown that amplification of the PCM by overexpression of the PCM component pericentrin induced the formation of numerous daughter centrioles (Loncarek et al., 2008). This suggests that the size of the PCM cloud restricts the size of the environment in which new centrioles can form by concentrating replication proteins (Loncarek et al., 2008). In view of this finding it would be interesting to test whether overexpression of a PCM component, such as  $\gamma$ -tubulin, in acentriolar early mouse embryos

could circumvent the tight controls which ensure the appropriate number of centrioles are generated at the blastocyst stage and drive precocious *de novo* centriole biogenesis in early embryos.

### **3.3.3. The impact of centriole emergence**

#### ***Impact of centriole emergence upon lineage specification***

The GFP-CETN2 transgenic mouse model reveals that centrioles emerge preferentially in the TE lineage of mouse early blastocysts. This is in agreement with previous studies which state that centrioles are first evident in polar trophoblasts (Abumuslimov et al., 1994; Calarco-Gillam et al., 1983; Gueth-Hallonet et al., 1993). TE cells form extra-embryonic tissues such as the placenta whereas the ICM gives rise to the embryo proper. Blastomeres isolated from the ICM are pluripotent and various transcription factors including Oct4 maintain their undifferentiated state. Since ICM cells are less differentiated than TE cells at the blastocyst stage, it is possible that an interphase MT array organised by a centrosome is not necessary (Calarco-Gillam et al., 1983). Calarco-Gillam et al., (1983) suggest that the centriole is the dominant organiser of the centrosome and is important in controlling the axis of division which becomes more important in more fully differentiated cells such as those in the TE layer.

#### ***Impact of centriole emergence upon MT organisation - centrosome focussing***

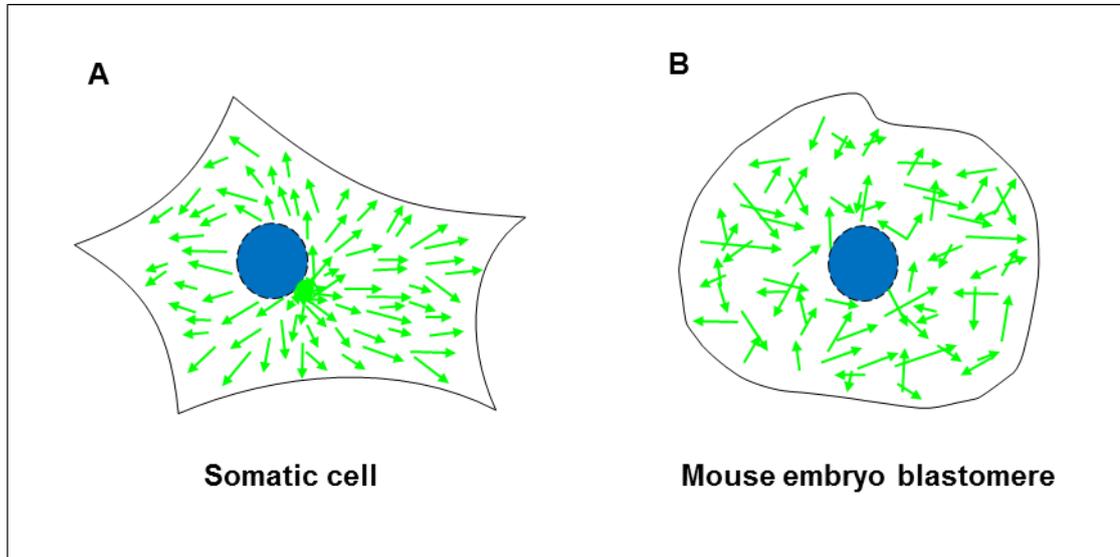
The GFP-CETN2 mouse model allowed investigation the impact of centriole emergence upon centrosome focusing. The total amount of  $\gamma$ -tubulin at foci was found to be similar in morulae and blastocysts. However, the  $\gamma$ -tubulin peak height was found to be greater in blastocyst stage embryos (Fig 3.8). Combined with the finding that the  $\gamma$ -tubulin peak width is reduced in blastocysts, these results suggest that additional  $\gamma$ -tubulin is not manufactured at the blastocyst stage but existing  $\gamma$ -tubulin becomes more focussed around the emerging centriole. This is in agreement with Bobinnec et al., (1998a) who show that in the absence of centrioles the PCM cloud becomes unstable and gradually disperses (Bobinnec et al., 1998a). Other studies have also suggested a key link between the centriole and the regulation of centrosome size. Modification of the size of the centriole in *C. elegans* embryos by depletion of core centriole proteins, leads to corresponding changes in the size of the PCM (Delattre et al., 2006; Kirkham et al., 2003). Furthermore, modulation of the levels of the PCM protein centrosomin (Lucas and Raff, 2007; Megraw et al., 1999) in *Drosophila* embryos was found to affect the size of the centrosome which in turn defines its MT nucleation capacity (Conduit et al., 2010; Conduit and Raff, 2010). Since centrosomin homologues exist in many species, this may represent a conserved mechanism for the

maintenance of PCM size (Conduit et al., 2010). The work described here shows that in mouse embryos an important relationship also exists between the centriole and PCM size (Fig 3.8). Therefore, it would be interesting to test the requirement for centrosomin in the regulation of centrosome size in *de novo* centriole emergence in mouse embryos.

It is crucial that centrosome size and PCM incorporation are tightly controlled so that cell division can occur accurately (Conduit et al., 2010; Conduit and Raff, 2010). Abnormally large centrosomes have been linked to genomic instability and defects in human centrosomin homologues can lead to neurological defects and microencephaly (Barrera et al., 2010; Buchman et al., 2010). Therefore, upon centriole emergence in mouse blastocysts (Fig 3.6),  $\gamma$ -tubulin foci become more focussed (Fig 3.8), perhaps to avoid any deleterious effects of a large centrosome on cell division.

#### ***Impact of centriole emergence upon MT organisation - MT architecture***

In order to investigate the effect of PCM/centriole emergence in mouse embryos on MT nucleation, an EB1 probe was used to track growing MT plus ends. No difference was found in growth velocity between developmental stages (Fig 3.10). However, analysis of these EB1 movies provides interesting preliminary information about the role of centrosomes in determining MT layout in early development. In interphase somatic cells, the centrosome is the dominant MTOC. In maximum intensity projections of somatic cells expressing EB1-GFP, a distinct focus of EB1 nucleation (usually in the vicinity of the nucleus) is obvious (Piehl et al., 2004). This focus represents the centrosome, from which new MTs are nucleated (see Fig 3.11 for a cartoon representation of MT nucleation strategies in somatic cells and mouse embryos). Therefore, it was expected that in blastocysts, MTs would primarily be nucleated from the newly-generated centriole. However, observation of EB1-GFP comets suggested that there is no dominant MTOC in interphase mouse embryos of any stage (Fig 3.9;3.10). Instead, in mouse embryos, EB1 comets seem to be randomly oriented in the cytoplasm and do not appear to be nucleated from a single site (see Fig 3.11). This implies that the centrosome is not acting as a dominant organiser of MTs in mouse morulae/blastocysts. Further investigation of this would require a control somatic cell for comparison of the randomness of EB1 comet trajectories. Nonetheless, it appears that there is no dominant MTOC in mouse embryos of any stage despite the appearance of  $\gamma$ -tubulin at the morula stage and centrioles at the



**Figure 3.11. Cartoon illustrating microtubule organisation strategies in somatic cells and mouse embryo blastomeres.** (A) Somatic cell: Note that EB1 comets (represented by green arrows) are nucleated by a centrosome near the interphase nucleus (blue circle). Based on examples of EB1-GFP expressing LLCPK cells (Piehl et al., 2003; Piehl and Cassimerris 2004). (B) Mouse embryo blastomere: EB1 comets do not appear to be organised by a dominant MTOC in interphase and instead are randomly distributed and oriented throughout the cytoplasm. Based on the results presented in figure 3.9 and 3.10.

blastocyst stage, suggesting that newly-formed centrosomes in mouse embryos do not yet exhibit the same behavior as canonically-generated centrosomes.

It would also be interesting to use the EB1 probe to generate MT vector diagrams within spindles, to compare M-phase MT organisation in the presence and absence of centrioles. These vector diagrams show the direction of MT growth - away or towards the chromosomes. It is well established that cells without centrosomes operate a chromosome-mediated pathway for spindle formation (Karsenti and Vernos, 2001; Schuh and Ellenberg, 2007) and this is characterized by a decrease in the number of astral MTs which grow away from the centrosome and towards the cell periphery (Mahoney et al., 2006). It will therefore be interesting to investigate whether mouse embryos switch to the canonical centrosome-mediated mode of spindle assembly (and manufacture more astral MTs) at the morula stage when  $\gamma$ -tubulin foci are first evident or at the blastocyst stage when centrioles appear. Since astral MTs are understood to be important for responding to cortical signals and positioning the mitotic spindle (and thus the cleavage furrow), particularly during asymmetric cell divisions (Basto et al., 2006; Betschinger and Knoblich, 2004; Cowan and Hyman, 2004; Gonczy, 2002), this may help explain why acentrosomal cells such as oocytes and embryos often mis-segregate their chromosomes (Hassold and Hunt, 2001).

#### ***Impact of centriole emergence on MT organisation - MT growth velocity***

Given that  $\gamma$ -tubulin is the main source of MT nucleation activity from the centrosome (Wiese and Zheng, 2006), it was expected that MT growth rates may be correlated with the total amount of  $\gamma$ -tubulin at foci. In agreement with this prediction, there was no significant difference in the total amount of  $\gamma$ -tubulin observed at interphase foci in morula and blastocysts (Fig 3.8) and likewise, rates of MT growth were similar in both stages (Fig 3.10). However, 4-8 cell stage embryos, which lack interphase  $\gamma$ -tubulin foci (Fig 3.1), display a MT growth velocity similar to morulae and blastocysts (Fig 3.10). This suggests that interphase MT nucleation in early mouse embryos involves  $\gamma$ -tubulin-independent mechanisms.

Interestingly, RNAi-mediated knockdown of the *C. elegans*  $\gamma$ -tubulin gene *tbg-1*, resulted in a significant reduction but, notably, not a total loss of centrosomal MTs (Bobinnec et al., 2000; Hannak et al., 2002; Srayko et al., 2005; Strome et al., 2001). Similarly, after  $\gamma$ -tubulin disruption in developing *Drosophila* ovaries, cells are still competent to nucleate MTs (Tavosanis and Gonzalez, 2003). Finally, studies using

EB1-GFP have shown that although *tbg-1*-depleted *C. elegans* embryos have only ~60% of the wild type centrosomal MT levels, the existing MTs display normal growth rates and polarity (Srayko et al., 2005). These results suggest that  $\gamma$ -tubulin-independent methods for organisation of MTs may also function even if the dominant centrosomal MT organisation pathway depends on the presence of  $\gamma$ -tubulin (Hannak et al., 2002). Therefore,  $\gamma$ -tubulin-independent methods of MT organisation may also be operating in mouse embryos and this may explain why MT growth rates are similar from 4-8 cells through to blastocysts in mouse embryos despite significant differences in  $\gamma$ -tubulin organisation over this time course (Fig 3.8;3.10). In interphase acentrosomal *Drosophila* cells, MT re-growth following MT depolymerisation required several components including the plus-end tip proteins CLIP-190 and EB1, the MT-associated protein (MAP) mini-spindles as well as the cytoplasmic motor protein dynein and  $\gamma$ -tubulin (Rogers et al., 2008). This suggests that all of these proteins cooperate to assemble an interphase MT array and explains why depletion of  $\gamma$ -tubulin had little effect on MT nucleation in these cells. (Rogers et al., 2008). A similar mode of interphase MT nucleation involving some of the factors required for MT regrowth described above may also be operating in mouse embryos which could explain why MT elongation velocities are not correlated with the total amount of  $\gamma$ -tubulin present at MTOCs.

In many *Drosophila* cell types,  $\gamma$ -tubulin-containing centrosomes can only nucleate MTs in mitosis. Whereas in interphase, cells lack a  $\gamma$ -tubulin-containing MTOC and although MTs are still organised, they do not emanate from a single distinct MTOC (Rogers et al., 2008). In line with this, interphase MT levels as well as MT growth and nucleation rates were unaffected by  $\gamma$ -tubulin depletion (Rogers et al., 2008). Therefore, although there was no difference in the interphase MT growth velocity in different developmental stages, it is still possible that mitotic mouse embryo blastomeres may display different rates of MT growth or MT nucleation in the presence/absence of centrioles.

### **3.4. Summary**

In Chapter 3, the time course of centriole and centrosome emergence in mouse pre-implantation development was examined and the effect of these processes on MT behaviour investigated. This chapter first described the development of a unique model system with which to examine the mechanism of centriole biogenesis in mouse embryos - namely the GFP-CETN2 transgenic mouse. The GFP-CETN2 mouse was used to establish that centrioles are first manufactured at the early blastocyst stage in

agreement with previous data. In addition, this model was critical in allowing unambiguous determination of the TE as the lineage in which centrioles preferentially appear at the blastocyst stage. Moreover, interphase  $\gamma$ -tubulin foci first appear in morula stage embryos. These foci become more focussed at the blastocyst stage coincident with the appearance of centrioles. Thus, these PCM accumulations are more tightly organized in the presence of centrioles and likely act as precursors for the formation of centrioles in the following cell cycle. Finally, the use of a MT plus-end tracker, EB1, revealed that no difference in MT elongation velocity was observed between 4-8 cells, morulae or blastocysts suggesting that MTs can also be effectively organised in the absence of centrosomes and/or centrioles. Having established methods and confirmed the time course of the centriole emergence programme, the experiments in Chapter 4 and 5 will next use molecular approaches to attempt to manipulate the programme.

## **4. ROLE OF THE CENTRIOLE REPLICATION PROTEIN PLK4 IN CENTRIOLE BIOGENESIS IN MOUSE OOCYTES AND EMBRYOS**

### **4.1. Introduction**

Plk4 is a member of the polo-like kinase family of proteins. Four polo-like kinase family members - Plk1, Plk2 (Snk), Plk3 (Fnk) and Plk4 (Sak) - are expressed in mammalian cells. Plk1 is the best characterised of these polo-like kinases and is known to play a vital role in the segregation of chromosomes and cell division (Barr et al., 2004; Glover, 2005).

The four polo family members share common features including an N-terminal catalytic domain that contains a distinctive ATP binding site G-X-G-X-F-A (Donohue et al., 1995; Simmons et al., 1992; Yamashita et al., 2001). Another feature common to the polo-like kinases is a conserved 64 amino acid 'polo-box motif' at their C-termini which dictates the substrate specificity of the kinase (Elia et al., 2003a; Elia et al., 2003b). Plks 1-3 harbour two polo-box motifs which are able to form intramolecular heterodimers and recognize a phosphorylated serine-threonine binding domain (Elia et al., 2003a). This dimerisation is thought to induce a conformational change which allows the N-terminal catalytic domain to access its substrates (Elia et al., 2003a; Elia et al., 2003b).

Plk4 is the most divergent of the polo-like kinases (Johnson et al., 2007). It possesses only a single polo-box motif at its C-terminus, in addition to a larger 'crypto' polo-box which has little homology to the polo-box domain (Leung et al., 2002; Lowery et al., 2004). Plk4 is therefore unable to form intramolecular dimers as with other family members and may therefore bind to substrates in a different manner to that described for Plks 1-3 (Leung et al., 2002; Swallow et al., 2005). Nevertheless, the Plk4 polo-box is able to form intermolecular homodimers and this process is important for the regulation of Plk4 kinase activity (Leung et al., 2002). Another difference between Plk4 and other family members is the large (500 amino acid) linker between the kinase domain and the single polo-box. However, the function of this domain remains to be elucidated (Lowery et al., 2004). Finally, Plk4 also possesses three

PEST sequences (domains rich in proline, aspartate, glutamate, serine and threonine) which are important for its stability (Fode et al., 1994).

Plk4 was first identified in mice as a kinase with homology to the *Drosophila* polo-like kinase and murine Snk (and was therefore named Sak (Snk akin kinase); (Fode et al., 1994)). Knockout mouse studies show that Sak <sup>-/-</sup> embryos arrest after gastrulation with an increased number of apoptotic and late mitotic cells (Hudson et al., 2001) revealing a crucial role for Plk4 in development and mitotic progression. Furthermore, adult Sak <sup>+/-</sup> heterozygous mice frequently develop liver and lung tumours (Ko et al., 2005). Inspection of dividing hepatocytes from these mice revealed an increase in tri- or tetra-polar spindles. Similarly, embryonic fibroblasts from Sak <sup>+/-</sup> mice have supernumerary centrosomes and have a higher level of aneuploidy than their wild type counterparts (Hudson et al., 2001). Ultrastructural studies have now revealed that Plk4 plays a key role in an early step of procentriole formation (Bettencourt-Dias et al., 2005; Habedanck et al., 2005; Kleylein-Sohn et al., 2007). Furthermore, depletion of individual proteins implicated in centriole biogenesis including SAS-6, CPAP and  $\gamma$ -tubulin did not abrogate Plk4 accumulation at the centriole (Kleylein-Sohn et al., 2007), suggesting that Plk4 is involved high up in a regulatory hierarchy. Together, these data point to a vital role for Plk4 in controlling centriole replication.

Experiments in cultured cells reveal that Plk4 overexpression results in the formation of multiple procentrioles around each daughter centriole in a characteristic ‘rosette’ arrangement (Habedanck et al., 2005; Kleylein-Sohn et al., 2007). This ‘rosette-like’ phenotype is the result of the overriding of the centriole licensing mechanism which normally limits the number of daughter centrioles formed in each round of replication to one (Tsou and Stearns, 2006a; Tsou and Stearns, 2006b). Plk4 overexpression has since been used as a model for this centriole amplification phenotype (Ganem et al., 2009) and a system in which to test the effect of protein depletion on centriole replication (Dzhindzhev et al., 2010; Kleylein-Sohn et al., 2007; Tang et al., 2009; Tsang et al., 2009). By contrast, RNAi depletion of Plk4 prevents centriole duplication ((Bettencourt-Dias et al., 2005; Habedanck et al., 2005); see Appendix 1).

Aberrant centriole replication can lead to abnormal spindles and mitotic defects (Brito et al., 2012; Nigg, 2002; Nigg, 2006). It is therefore crucially important that Plk4 levels are tightly controlled so that centriole duplication can proceed normally. Plk4 overexpression has also been found to induce the *de*

*de novo* formation of centrioles in *Drosophila* oocytes (Peel et al., 2007; Rodrigues-Martins et al., 2007b) and more recently, the *Xenopus* Plk4 homologue, Plx4, has been found to drive *de novo* centriole biogenesis in activated eggs or egg extracts (Eckerdt et al., 2011). Therefore, Plk4 overexpression can drive both *de novo* centriole formation and centriole over-replication suggesting that the same proteins are involved in both pathways.

In order to avoid centriole amplification Plk4 levels must be tightly regulated (Habedanck et al., 2005; Kleylein-Sohn et al., 2007). Plk4 levels are now understood to be controlled by the SKP1-CUL1-F-box protein (SCF) ubiquitin ligase which targets Plk4 for proteasomal degradation (Cunha-Ferreira et al., 2009b; Rogers et al., 2009). In addition, accumulating evidence from several groups now points towards autophosphorylation events as a key determinant of Plk4 kinase stability (Guderian et al., 2010; Holland et al., 2010; Sillibourne et al., 2010).

Since its initial identification in the mouse, homologues of Plk4 have been identified in many organisms including human (Habedanck et al., 2005), *Drosophila* (Bettencourt-Dias et al., 2005) and *Xenopus* (Eckerdt et al., 2011). Interestingly, nematode worms have no direct Plk4 homologue. However, the kinase ZYG-1 is an upstream regulator of centriole duplication in *C. elegans* (Delattre et al., 2006; O'Connell et al., 2001; Pelletier et al., 2006) and has therefore been suggested to be the functional equivalent of Plk4 in worms (O'Connell et al., 2001). All Plk4 homologues which have so far been identified have been found to play a crucial role in the control of centriole number ((Bettencourt-Dias et al., 2005; Eckerdt et al., 2011; Habedanck et al., 2005; Kleylein-Sohn et al., 2007; O'Connell et al., 2001); see Appendix 1) indicating conserved function.

## **AIMS**

Plk4 and its homologues can drive both canonical and *de novo* centriole replication in many cell types (Bettencourt-Dias et al., 2005; Eckerdt et al., 2011; Habedanck et al., 2005; Kleylein-Sohn et al., 2007; O'Connell et al., 2001; Peel et al., 2007; Rodrigues-Martins et al., 2007b). However, the mechanism of Plk4-induced *de novo* centriole formation in mammalian cells has not yet been studied. Since both murine gametes lack centrioles (Manandhar et al., 1998; Szollosi et al., 1972), mouse oocytes and early embryos

lack centrioles (Abumuslimov et al., 1994; Gueth-Hallonet et al., 1993) and therefore represent an excellent model system for addressing this question.

In this chapter the role of Plk4 in mouse oocytes and embryos was investigated using overexpression approaches. The long term effects of Plk4 overexpression in embryos were then examined. This chapter finds evidence for a Plk4-inducible pathway for *de novo* centriole formation in all stages examined and reveals that Plk4 overexpression can lead to abnormal spindle structures. Furthermore, it appears that despite these aberrant spindles, embryos can develop normally.

## **4.2. Results**

### **4.2.1. Exogenous Plk4 self-organises into numerous cytoplasmic foci in 2-cell embryos**

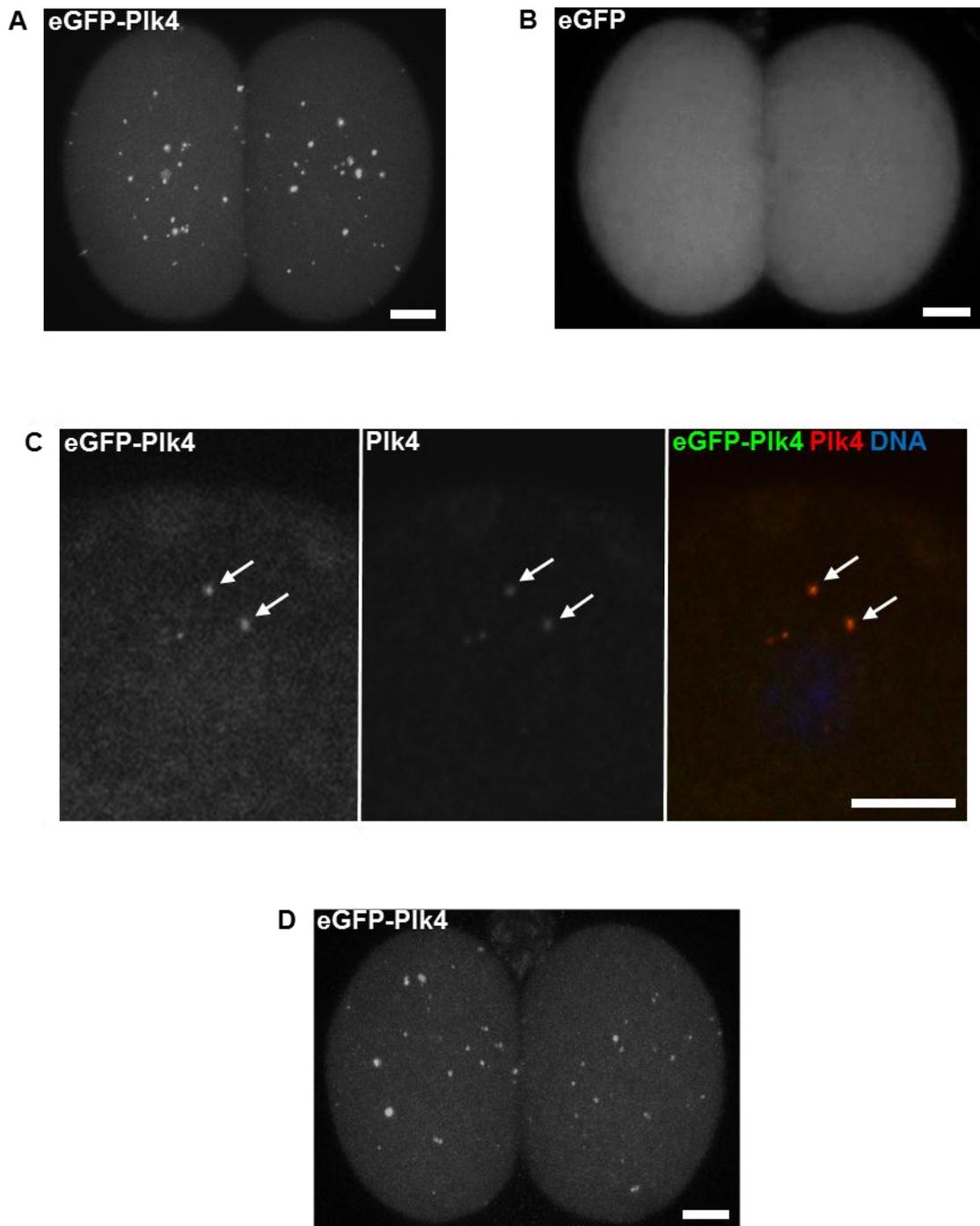
Firstly, the effect of Plk4 overexpression was investigated in 2-cell embryos which are normally acentriolar. eGFP-Plk4 was overexpressed by microinjection of the corresponding mRNA into each blastomere of 2-cell mouse embryos. After 2-3 hours the eGFP-Plk4-overexpressing embryos were transferred to a confocal microscope for examination. Numerous punctuate accumulations were observed throughout the cytoplasm (Fig 4.1A). Importantly, overexpression of eGFP alone did not cause such foci suggesting that accumulations were attributable to exogenous Plk4 (Fig 4.1B). eGFP-Plk4-overexpressing embryos were fixed and stained with an antibody against human Plk4 (gift from Alex Dammermann, Vienna, Austria, unpublished). eGFP-Plk4 foci were also found to be labelled by the Plk4 antibody indicating that these are in fact accumulations of exogenous Plk4. (Fig 4.1C).

### **4.2.2. Cytoplasmic foci can still form in the presence of the MT depolymeriser nocodazole**

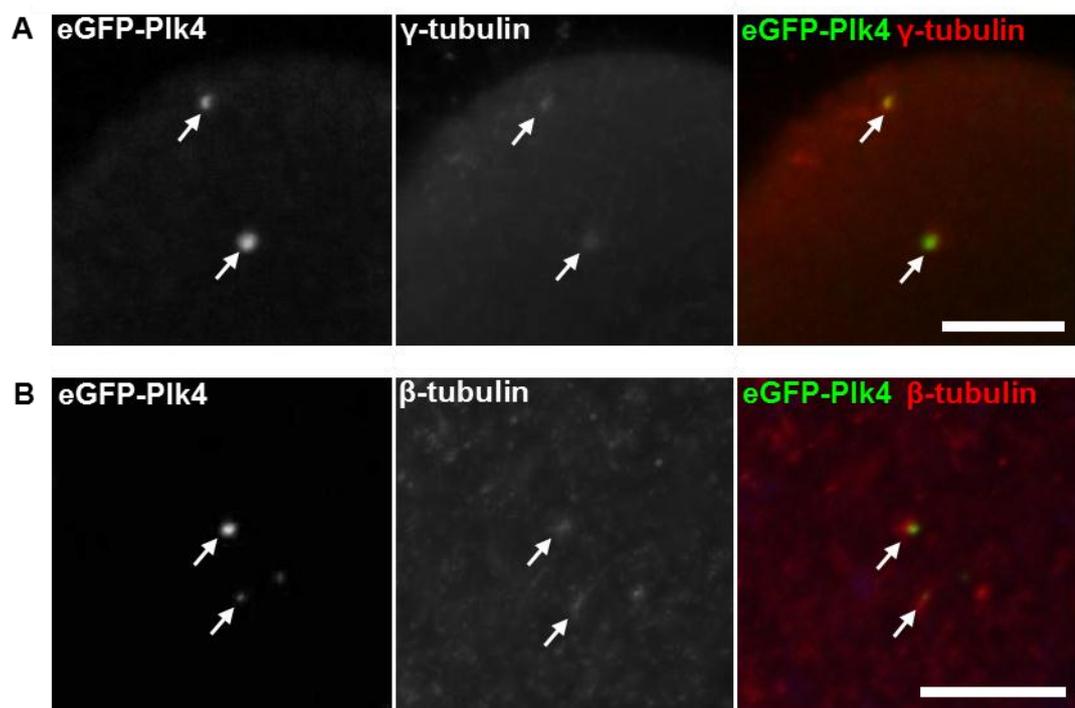
To investigate whether the formation of these foci depended on the presence of an intact network of MTs, 2-cell embryos were treated with the MT depolymeriser nocodazole ((Jordan et al., 1992); 10  $\mu$ M) to disrupt the MT network at the time of eGFP-Plk4 overexpression. Discrete cytoplasmic foci were still observed in nocodazole-treated embryos (Fig 4.1D). Nocodazole-induced spindle collapse in MII eggs from the same experimental day provided a positive control. Therefore, formation of these foci does not depend on the presence of a cellular MT network.

### **4.2.3. Exogenous Plk4 accumulations can organise $\gamma$ -tubulin and MTs**

In order to examine whether eGFP-Plk4-induced foci had any centriole-like function, 2-cell embryos were fixed after 3 hours of eGFP-Plk4 expression and stained with antibodies against  $\gamma$ -tubulin and  $\beta$ -tubulin. The exogenous Plk4 foci were colocalised with  $\gamma$ -tubulin in most cases (80.43% colocalisation) and in some cases were colocalised with MTs (41.67% colocalisation; Fig 4.2). Thus, exogenous Plk4 foci are capable of organising both  $\gamma$ -tubulin and MTs suggesting that eGFP-Plk4 foci have MTOC properties.



**Figure 4.1. Overexpression of eGFP-Plk4 drives the formation of cytoplasmic foci.** (A) Typical confocal Z-projection of a whole 2-cell embryo showing numerous cytoplasmic accumulations of exogenous *Plk4*. (B) Typical confocal Z-projection showing that no foci are observed in 2-cell embryos injected with eGFP only. (C) Representative confocal Z-section showing colocalisation of exogenous *Plk4* (green) with *Plk4* antibody (red). DNA is shown in blue. Arrows indicate colocalisation. (D) Typical confocal Z-projection of a 2-cell embryo treated with nocodazole at the time of *Plk4* overexpression. Note that characteristic cytoplasmic accumulations are still observed. Scale 10  $\mu$ m.



**Figure 4.2. eGFP-Plk4 foci can function as MTOCs.** Single confocal slices showing exogenous eGFP-Plk4 (green) colocalising with (A)  $\gamma$ -tubulin and (B) microtubules (red) after 3 hours of expression in 2-cell embryos. Arrows indicate colocalisation. Scale bar 10  $\mu$ m.

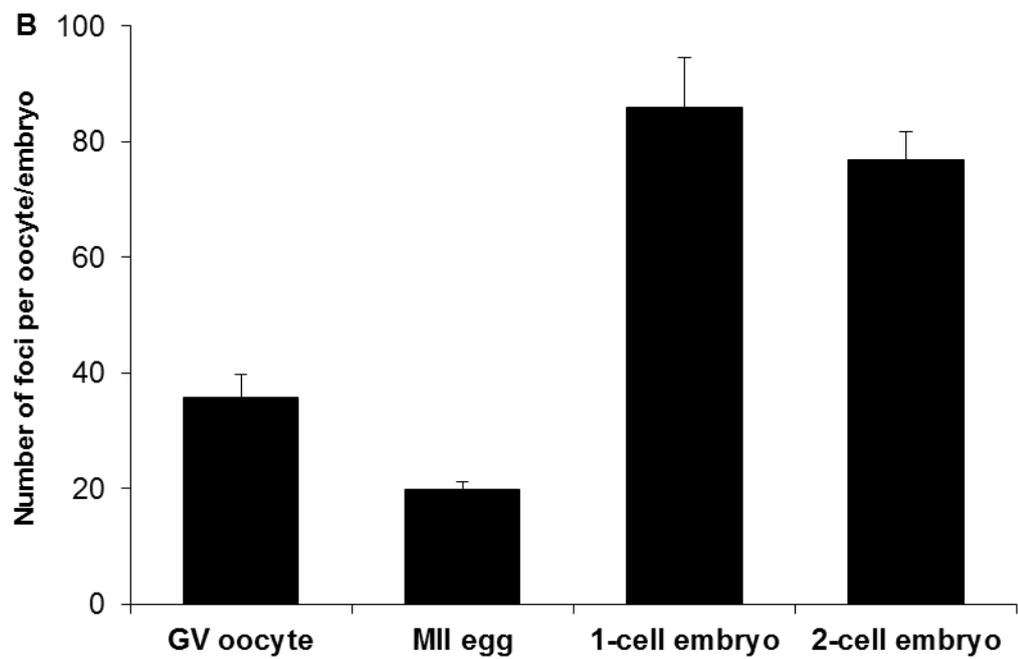
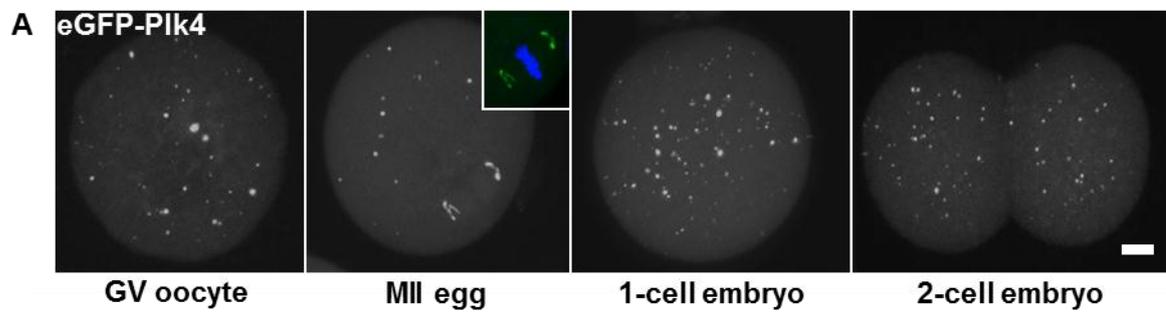
#### **4.2.4. Acentriolar oocytes and embryos of different developmental stages are able to support Plk4-induced foci formation**

Since Plk4 overexpression is able to induce the formation of centriole-like structures in 2-cell embryos, it was important to establish whether other stages of development could also support the formation of these accumulations. Thus, eGFP-Plk4 was overexpressed in immature GV oocytes, mature MII eggs and also in fertilised 1- and 2-cell embryos. Overexpression of Plk4 caused numerous cytoplasmic foci in all stages examined ( $19.92 \pm 1.33$  to  $85.9 \pm 8.53$  foci per oocyte/embryo; Fig 4.3). Foci were found throughout the cytoplasm in all stages. In addition, accumulations were located adjacent to the nuclear membrane in interphase embryos and GV oocytes and also decorated spindle poles in MII eggs (Fig 4.3A, inset). Therefore, eGFP-Plk4 overexpression induced the formation of foci in all stages of oocyte and embryo examined.

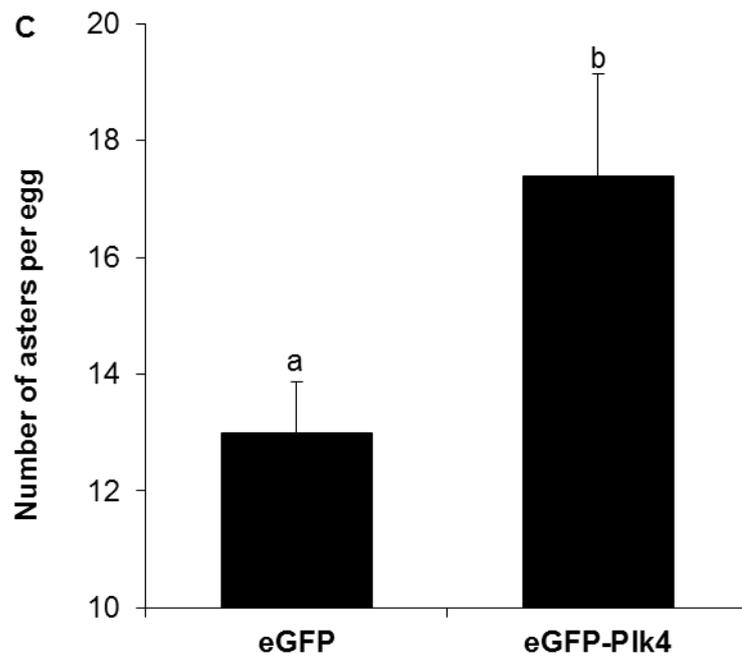
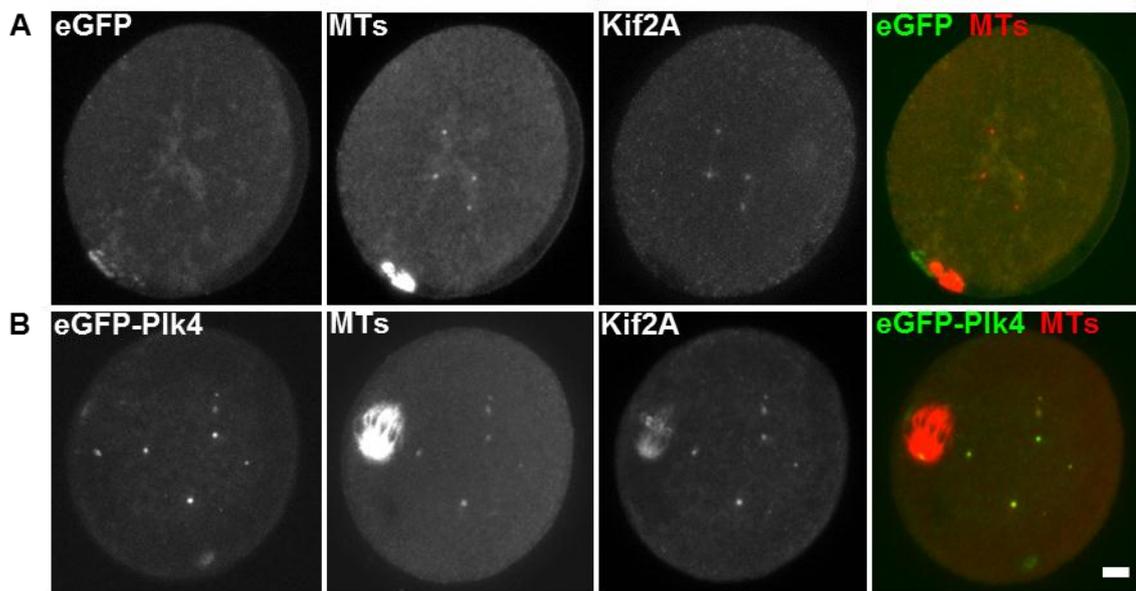
#### **4.2.5. Plk4 overexpression can generate new MTOCs in MII eggs as well as binding to pre-existing MTOCs**

Given that overexpression of Plk4 was found to drive the formation of multiple cytoplasmic accumulations as well as decorating the poles of the MII spindle (Fig 4.3A), it was important to distinguish whether eGFP-Plk4-overexpression induces the formation of new MTOCs *de novo* or simply associates with existing MT structures. To that end eGFP-Plk4 or eGFP-alone was overexpressed in unfertilised MII eggs which usually possess several discrete MTOCs (Combelles and Albertini, 2001), and the number of  $\beta$ -tubulin-labelled MT asters counted. Kif2A, a MT-depolymerising motor protein which binds MT minus ends, has previously been used in our lab to label MTOCs in oocytes (Pirmadjid and FitzHarris, unpublished data; (Illingworth et al., 2010)). Antibodies against Kif2A also colocalise with  $\beta$ -tubulin asters in MII eggs confirming its ability to label MTOCs in eggs (Fig 4.4). Therefore, eggs expressing eGFP-alone or eGFP-Plk4 were stained with antibodies against Kif2A as well as  $\beta$ -tubulin to label MTOCs and the number of MTOCs per egg then counted in a blinded manner.

In eGFP-Plk4-expressing eggs, all  $\beta$ -tubulin-labelled MTOCs possessed an eGFP-Plk4 focus (Fig 4.4A), suggesting that Plk4 associates with pre-existing MTOCs in unfertilised eggs. However, eggs expressing eGFP-Plk4 possessed significantly more MTOCs per cell than control (eGFP-expressing) eggs (eGFP-Plk4:  $17.39 \pm 1.74$  asters/egg; eGFP:  $13.00 \pm 0.87$  asters/egg;  $p < 0.05$ ; Fig 4.4B,C), revealing that eGFP-



**Figure 4.3. Exogenous Plk4 can organise foci in oocytes and embryos.** (A) Typical confocal Z-projections of oocytes and embryos after 3 hours expression of eGFP-Plk4. (B) Graph showing average number of spots per oocyte/embryo after overexpression of eGFP-Plk4. 10-13 oocytes/embryos examined per stage. Scale bar 10  $\mu$ m.



**Figure 4.4. *Plk4* overexpression increases the number of MTOCs in MII eggs.** Typical examples of (A) a control (eGFP alone) and (B) an eGFP-*Plk4*-expressing egg with  $\beta$ -tubulin-labelled MTOCs. Antibodies against the kinesin 13 Kif2A were used to further confirm MTOC identity. (B) Blinded quantification of the number of asters in eGFP-*Plk4*-expressing eggs ( $n=18$ ) compared to control eggs ( $n=21$ ). The data presented in this figure and figure 5.6 were conducted contemporaneously. Therefore, statistical analysis was performed using ANOVA and Tukey-Kramers post-hoc test. Scale bar 10  $\mu$ m.

Plk4 overexpression increases the number of MTOCs. Thus, eGFP-Plk4 binds to pre-existing MTOCs, but also assembles into new foci which function as additional MTOCs.

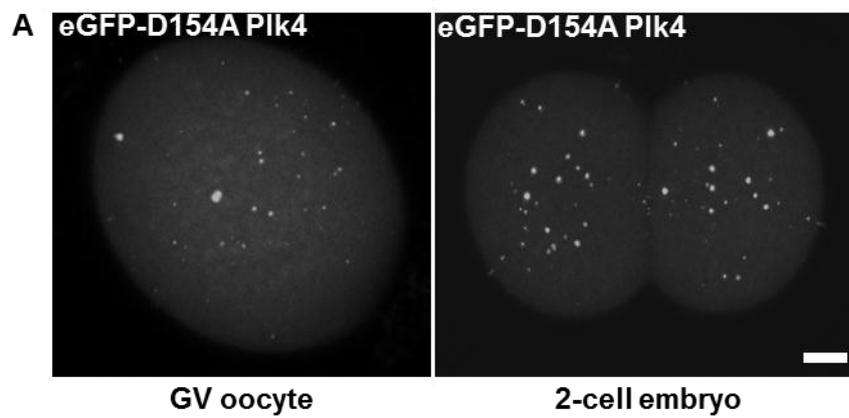
#### **4.2.6. Kinase-dead Plk4 can also drive the formation of cytoplasmic foci in oocytes and embryos**

Perhaps unexpectedly, recent data show that overexpressed kinase-dead Plk4 can induce centriole overduplication in somatic cells, similar to overexpression of wild type Plk4 (Guderian et al., 2010; Habedanck et al., 2005; Holland et al., 2010). Having found that wild type Plk4 is able to drive the *de novo* formation of centriole-like foci in mouse oocytes/embryos (Fig 4.3;4.4), just as wild type Plk4 can drive centriole overduplication in somatic cells, I wondered whether kinase-dead Plk4 might be sufficient for *de novo* formation of centrioles in mouse oocytes/embryos. The kinase-dead form of Plk4 (D154A;(Habedanck et al., 2005)) was therefore overexpressed in GV stage mouse oocytes and 2-cell embryos. Overexpression of eGFP-Plk4-D154A in oocytes or embryos was able to drive the formation of multiple cytoplasmic foci in both cases (Fig 4.5). Therefore, Plk4's kinase activity is not necessary for Plk4-induced *de novo* formation of centrioles in mouse early development.

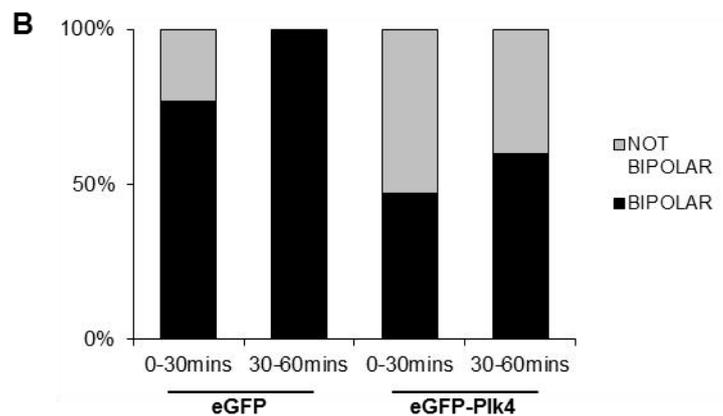
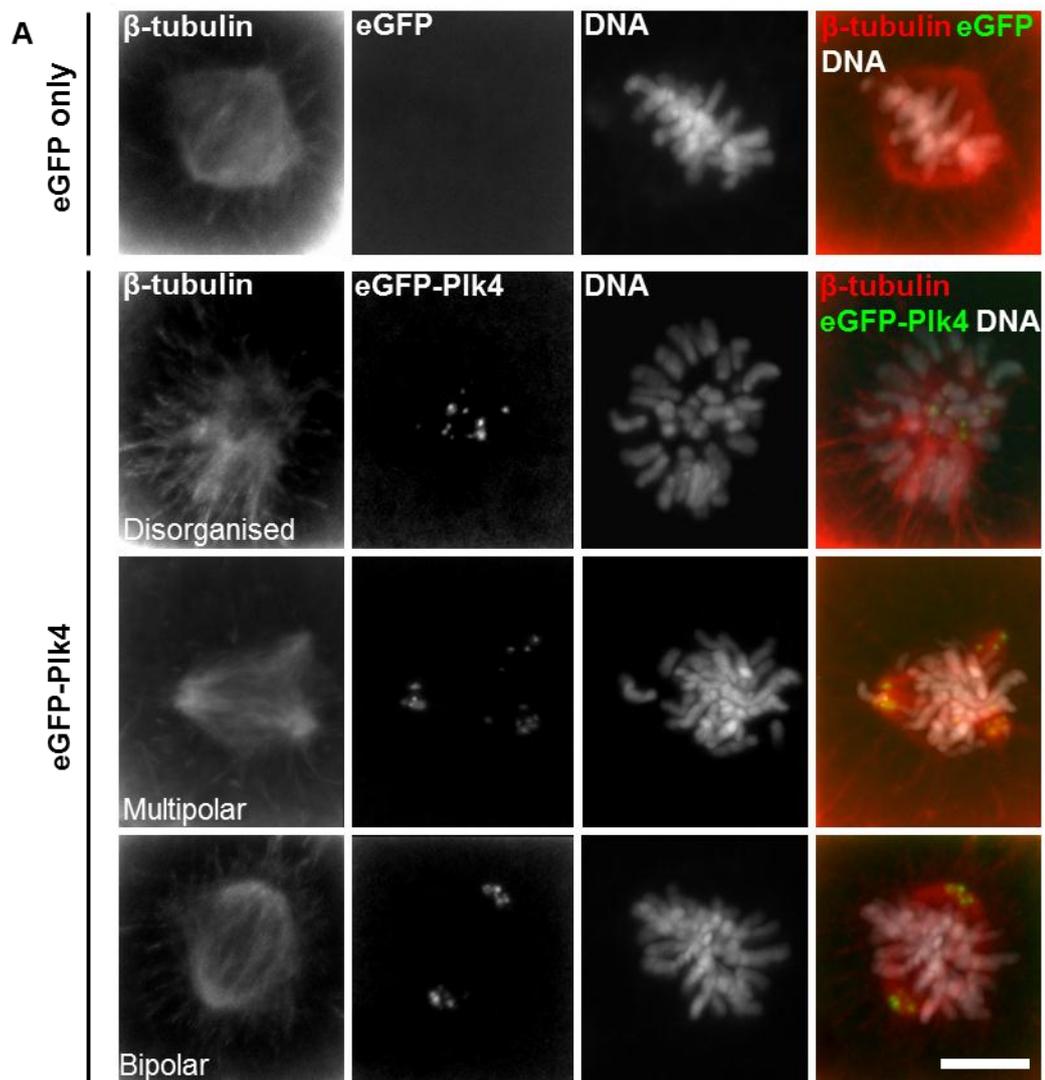
The physiological explanation for this result is discussed further in sections 4.3.2 and 5.3.3. Nonetheless, this experiment shows that the ability of overexpressed Plk4 to drive *de novo* centriole biogenesis is independent of the kinase domain, similar to the ability of Plk4 to drive over-replication in somatic cells.

#### **4.2.7. eGFP-Plk4 overexpression causes aberrant spindle formation in 2-cell embryos**

The impact of the Plk4-induced MTOCs upon mitotic spindle function was next investigated. eGFP-Plk4 was therefore overexpressed in 2-cell embryos that were then allowed to progress into M-phase. Embryos were monitored for nuclear envelope breakdown (NEBD) every 30 minutes and fixed either immediately upon observation of NEBD (to observe early mitosis) or 30 minutes after observation of NEBD (to observe late mitosis). Spindle structure was then examined on a confocal microscope following staining with a  $\beta$ -tubulin antibody and Hoechst.



**Figure 4.5. Overexpression of the kinase-dead *Plk4-D154A* can induce centriole-like foci in GV oocytes and 2-cell embryos.** (A) Typical confocal Z-projections of a GV oocyte and a 2-cell embryo expressing eGFP-*Plk4-D154A*. Note that cytoplasmic accumulations are evident in both stages. Scale bar 10  $\mu$ m.



**Figure 4.6. Plk4 overexpression decreases the proportion of bipolar spindles.** (A) Z-projections of 2-cell embryos fixed and immunolabelled 0-30 or 30-60 mins after M-phase entry, including examples of multipolar or disorganised spindles characteristic of eGFP-Plk4-expressing embryos. (B) Blinded quantification of the impact of eGFP-Plk4 upon spindle morphology. 27 eGFP-Plk4 and 19 eGFP-expressing embryos examined. Scale bar 10  $\mu$ m.

Bipolar spindles were observed in almost all eGFP-expressing (control) embryos (84.21%) in both early and late mitosis. However, interestingly, about half of eGFP-Plk4-expressing embryos possessed disorganised MT-arrays or multipolar spindles in early or late mitosis (48.15%,  $\text{CHI}^2$   $p=0.023$ ; Fig 4.6B). eGFP-Plk4 foci were found at the spindle poles or at the focal point of MT arrays (Fig 4.6A). The proportion of eGFP-Plk4 embryos possessing multipolar or disorganised spindles was decreased in late mitosis (early mitosis: 52.94%; late mitosis: 40%; Fig 4.6B) suggesting that multipolar spindle intermediates resolve into bipolar spindles to complete mitosis, similar to in somatic cells (Ganem et al., 2009; Silkworth et al., 2009).

#### **4.2.8. eGFP-Plk4-overexpressing embryos develop to the blastocyst stage without obvious defects**

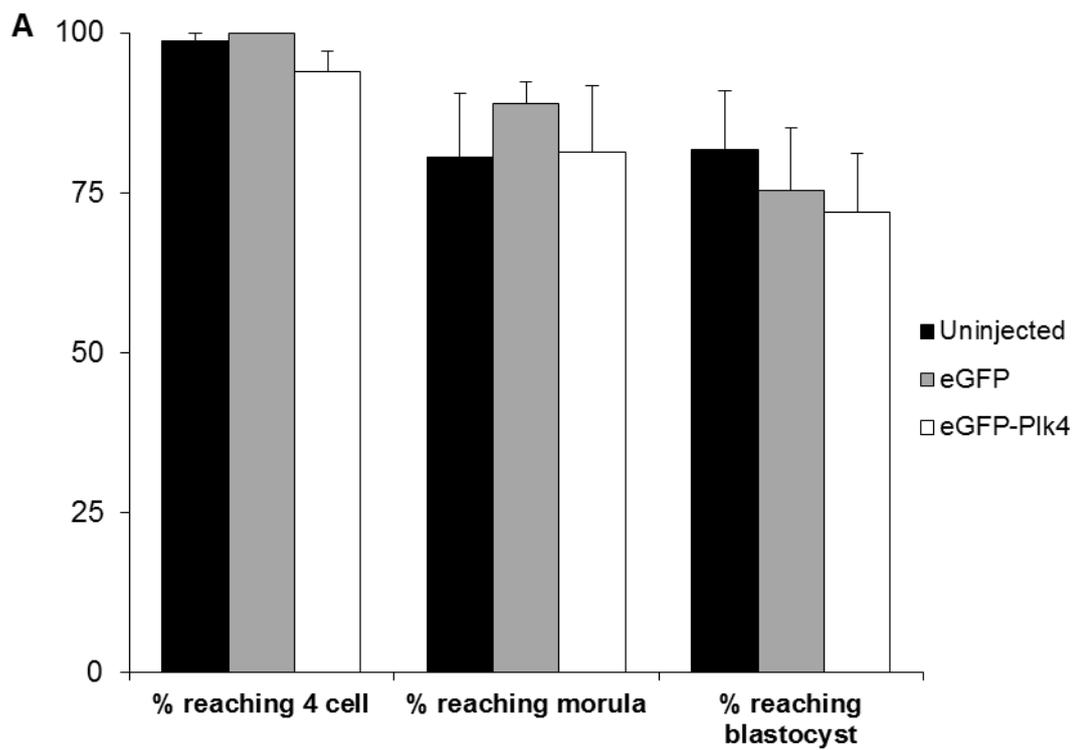
Recent work has shown that extra centrosome-mediated chromosomal instability is not a result of multipolar divisions, but in fact results from an increased chance of merotelic attachments and consequent lagging chromosomes in bipolar cell divisions (Ganem et al., 2009; Silkworth et al., 2009). Since an increased proportion of eGFP-Plk4-overexpressing embryos exhibit abnormal spindles (Fig 4.6), it was possible that these could be leading to chromosome segregation errors which may have adverse effects on embryo development. Therefore, the long term effects of Plk4 overexpression were investigated.

##### ***eGFP-Plk4 overexpression does not affect development to blastocyst***

Embryos were injected with eGFP-Plk4 or eGFP-alone at the 2-cell stage and developmental progression examined. eGFP-Plk4-injected embryos developed normally to the blastocyst stage, similar to their control counterparts (ANOVA,  $p>0.05$ ; Fig 4.7). Plk4-overexpressing embryos therefore displayed normal morphology with compaction and cavitation occurring at the expected times.

##### ***eGFP-Plk4 overexpression does not affect cell number at blastocyst***

Since, compaction and cavitation are not dependent on a minimum cell number and instead take place a defined time after fertilisation (Samake and Smith, 1996), it was possible that Plk4-overexpressing embryos could contain a reduced number of cells despite undergoing compaction and cavitation at the expected times (Cockburn and Rossant, 2010). Therefore, the number of cells in Plk4-overexpressing embryos was investigated. Embryos were fixed 5 days post hCG and the number of Hoechst-labelled



**Figure 4.7. Long term *Plk4* overexpression does not affect developmental progression.** (A) Graph showing normal developmental progression of *Plk4*-overexpressing embryos. (ANOVA,  $p > 0.05$ ). Repeated on 6 experimental days.

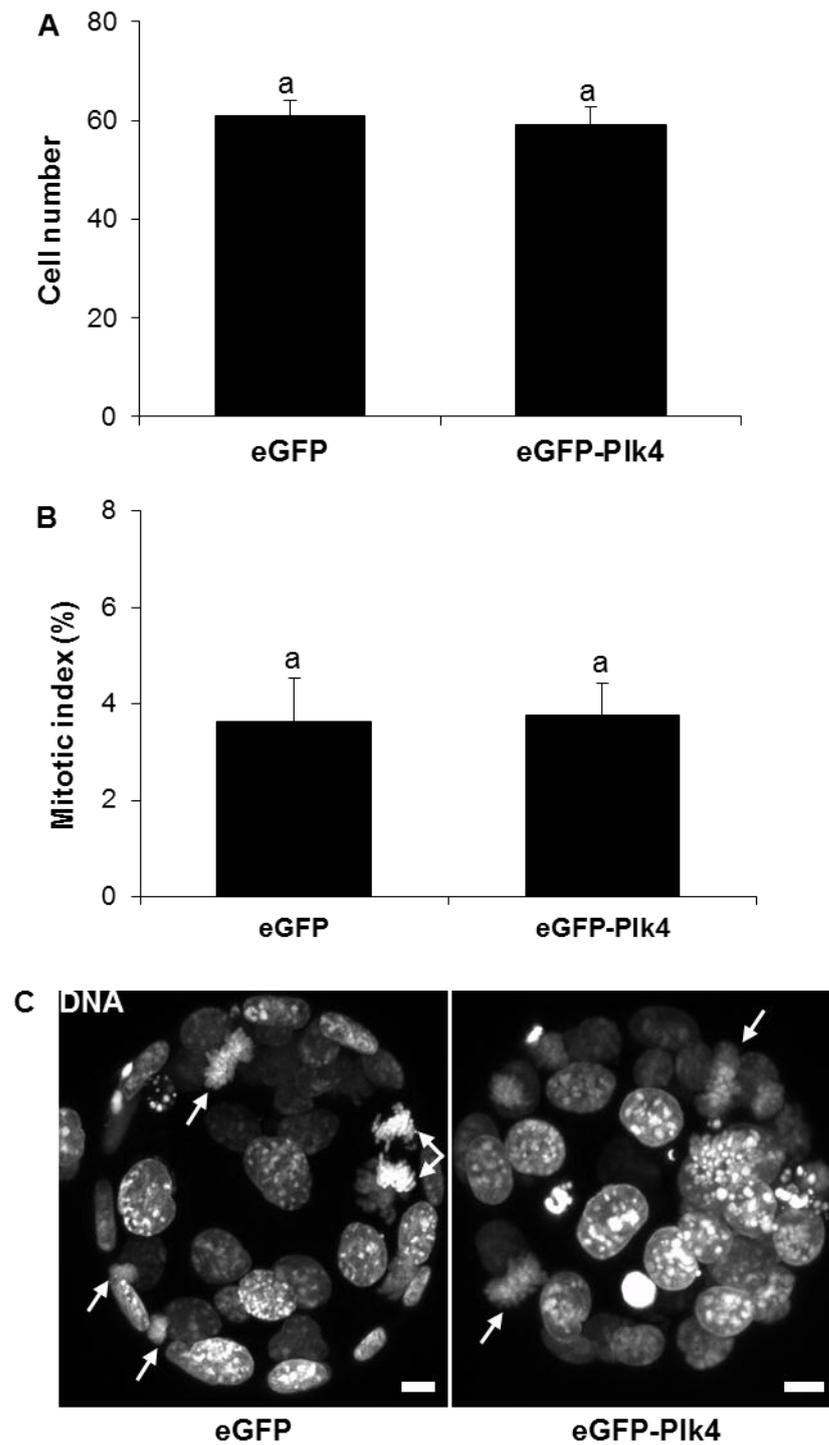
nuclei counted. No significant difference was observed between the total number of cells in eGFP-Plk4-overexpressing (59.05 $\pm$ 3.66) and control embryos (60.85 $\pm$ 3.26;  $p=0.71$ ; Fig 4.8A,C), revealing that the rate of cell division was unaffected by Plk4 overexpression.

#### ***eGFP-Plk4 overexpression does not affect mitotic index***

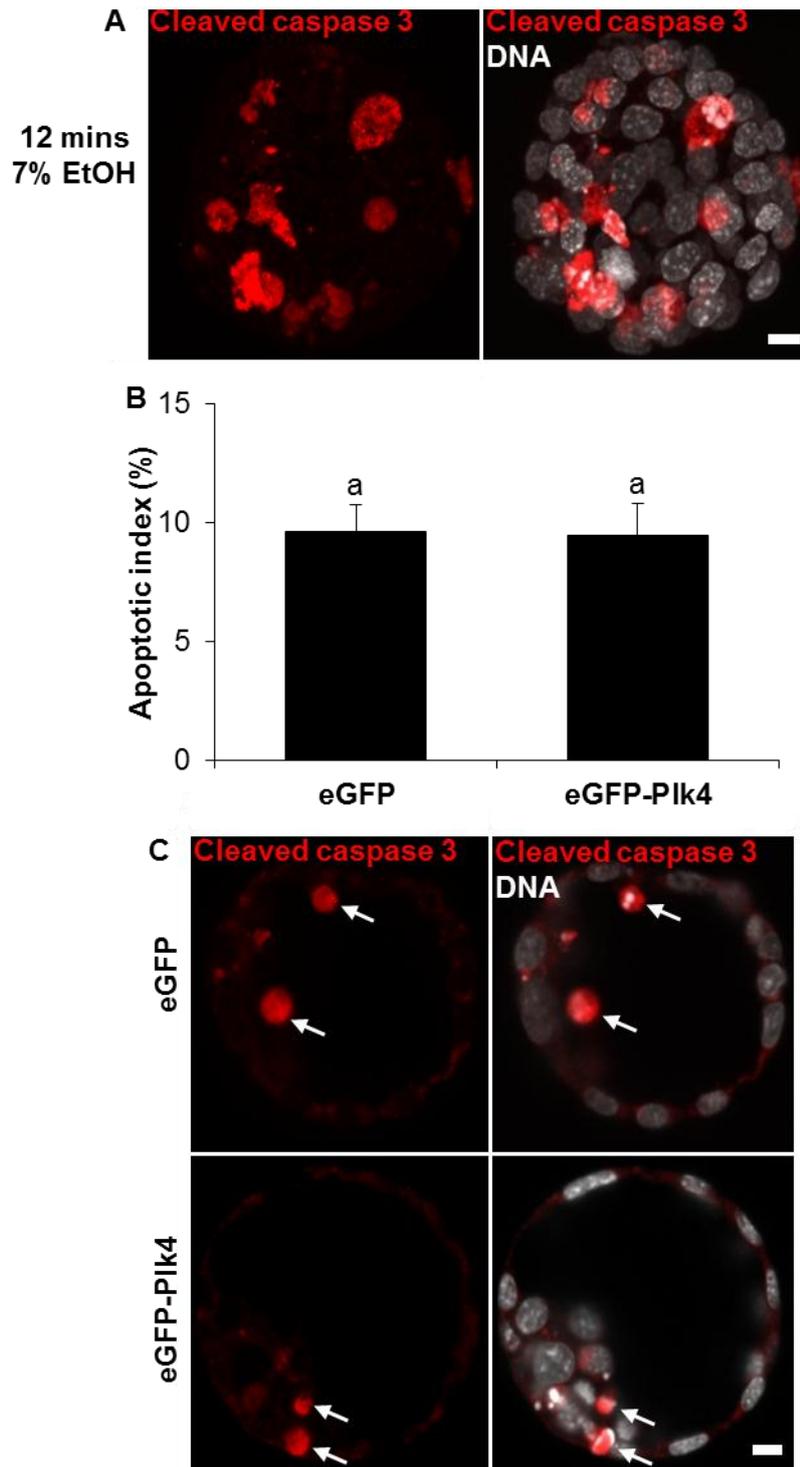
Cells with extra centrosomes are found to have a high mitotic index due to activation of the spindle assembly checkpoint (SAC; (Basto et al., 2008; Kwon et al., 2008; Yang et al., 2008)). This allows supernumerary centrosomes sufficient time to cluster around spindle poles before the onset of anaphase. Multiple cytoplasmic foci (Fig 4.1;4.3) and abnormal spindles (Fig 4.6) were observed upon overexpression of Plk4 in mouse embryos so it was possible that activation of the SAC in Plk4-overexpressing embryos could be causing an increased mitotic index. Therefore, the proportion of mitotic cells in Plk4-overexpressing blastocysts was examined. 5 days post hCG, embryos expressing eGFP-Plk4 or eGFP alone were fixed, stained with Hoechst and the mitotic index then calculated for both groups. However, no significant difference in the proportion of mitotic cells was observed between eGFP-Plk4 (3.63% $\pm$ 0.91) and eGFP-expressing embryos (3.78 $\pm$ 0.67,  $p=0.90$ ; Fig 4.8B,C), suggesting that Plk4-overexpressing blastocysts do not appear to spend longer in mitosis than their control counterparts.

#### ***eGFP-Plk4 overexpression does not affect apoptotic index***

Mouse blastocysts developed in vivo typically have an apoptotic index of 1-10% (Brison, 2000). This process of programmed cell death is thought to eliminate abnormal or defective cells from the pre-implantation embryo so a viable fetus can form (Brison, 2000). In somatic cells, apoptosis can occur in response to chromosomal abnormalities which arise due to errors in mitosis. Since it has previously been established that Plk4-overexpression in mouse embryos leads to the formation of abnormal spindles (Fig 4.6), the effect of Plk4 overexpression on the level of apoptosis was investigated. The apoptotic index was therefore compared in eGFP-Plk4-expressing embryos and eGFP-expressing controls. Positive control embryos (12 mins exposure to 7% EtOH at 25 $^{\circ}$ C which induces a monotonic rise in intracellular Ca<sup>2+</sup> concentration (Steinhardt et al., 1974) and causes apoptosis by triggering aberrant gene expression and accelerated development (Rout et al., 1997; Stachecki et al., 1994)) were first used to confirm that the cleaved caspase 3 antibody could recognize cells undergoing programmed cell death (Fig 4.9A). Having validated the technique, the percentage of apoptotic cells per embryo was examined 5 days post hCG. The



**Figure 4.8. Long term *Plk4* overexpression does not affect cell number at blastocyst or mitotic index.** (A) Graph showing no significant difference between the number of cells in blastocysts (5 days post hCG) injected with either eGFP or eGFP-*Plk4*. (B) Graph showing similar apoptotic index in blastocysts injected with either eGFP-alone (control; n=21) or eGFP-*Plk4* (n=19) at the 2-cell stage. (C) Typical confocal Z-projections showing blastocysts injected with eGFP or eGFP-*Plk4* at the 2-cell stage. Arrows show cells in mitosis. Double arrow denotes a cell in anaphase. 19 eGFP-*Plk4*-expressing and 21 control embryos examined. Scale bar 10  $\mu$ m.



**Figure 4.9. Long term *Plk4* overexpression does not affect apoptotic index.** (A) Typical Z-projection of a whole blastocyst treated with 7% EtOH for 12 mins before fixation (positive control). (B) Graph showing no significant difference in apoptotic index between eGFP-*Plk4*-expressing ( $n=19$ ) and eGFP-expressing ( $n=21$ ) embryos. Repeated on 3 experimental days. (C) Typical Z-projections of *Plk4*-overexpressing and control embryos labelled with Hoechst and cleaved caspase 3. Arrows indicate apoptotic cells. Scale bar  $10\mu\text{m}$ .

apoptotic index did not differ significantly between control (9.63% $\pm$ 1.13) and Plk4-overexpressing embryos (9.44% $\pm$ 1.37;  $p=0.92$ ; Fig 4.9B,C). Thus, despite inducing multipolar spindles, long-term overexpression of Plk4 in embryos does not appear to affect embryo development, mitotic index or apoptosis levels as determined by blastocyst development rates, cell number counts and apoptotic index.

#### **4.2.9. Development of a method for karyotyping of 4-cell embryos**

Approximately half of eGFP-Plk4-overexpressing 2-cell embryos possess disorganised MT-arrays or multipolar spindles (48.15%; Fig 4.6). However, the proportion of eGFP-Plk4 embryos possessing these non-bipolar spindles was decreased later in mitosis (early mitosis: 52.94%; late mitosis: 40%; Fig 4.6), suggesting that multipolar spindles are resolved before the onset of anaphase. The reorganisation of multipolar spindle intermediates into bipolar spindles also occurs in somatic cells (Ganem et al., 2009; Silkworth et al., 2009) and can lead to an increased incidence of lagging chromosomes and a consequent increase in the rate of aneuploidy in daughter cells.

Embryos are understood to be unusually tolerant to aneuploidy (Lightfoot et al., 2006; van Echten-Arends et al., 2011; Vanneste et al., 2009). Thus, despite the finding that Plk4-overexpressing embryos appear to develop normally to the blastocyst stage (Fig 4.7;4.8;4.9), it was possible that Plk4-overexpression could have adverse effects on chromosome segregation and lead to aneuploidy. To investigate this hypothesis, a method for counting the number of chromosomes in embryos was required.

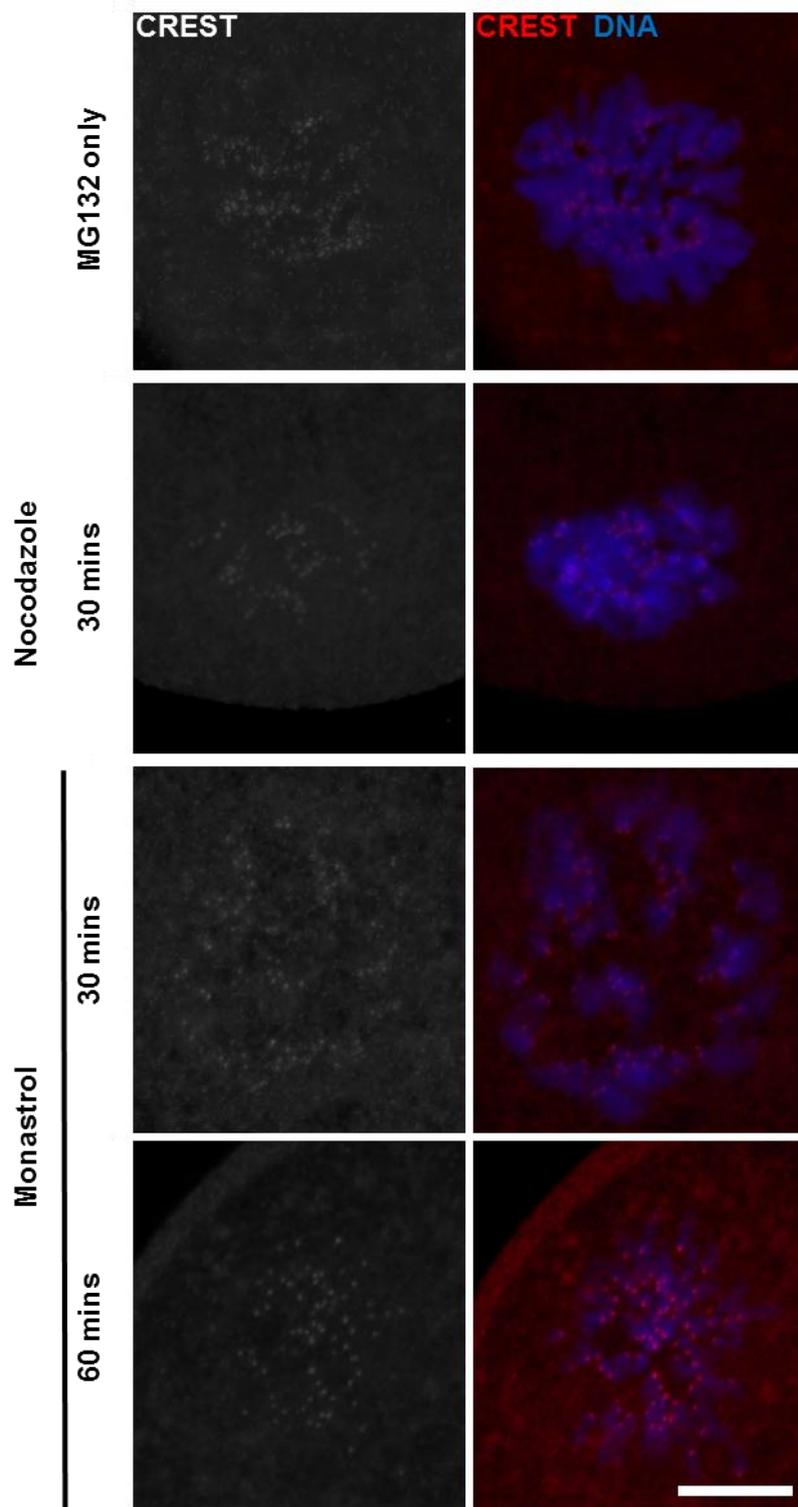
Fluorescence *in-situ* hybridisation (FISH) has previously been used to determine the rate of aneuploidy in mouse embryos (Sabhnani et al., 2011). In this method two fluorescent probes which are targeted to specific chromosomes are used. A euploid cell will display two fluorescent foci denoting two copies of that specific chromosome (one maternal and one paternal). Reprobing can increase the total number of chromosomes analysed to four but the signal tends to be weaker which can make interpretation difficult (Sabhnani et al., 2011). Since in mouse a maximum of four chromosomes can be analysed, this technique may not provide an accurate representation of the overall aneuploidy rate.

Recently, a novel method to enable the counting of chromosomes *in-situ* in mouse oocytes has been developed (Duncan et al., 2009). In this method the spindle is collapsed in MII eggs using monastrol to

allow the dispersal of chromosomes. The number of CREST-labelled kinetochore pairs can then be easily counted with a euploid mouse oocyte containing 20 pairs of kinetochores. This approach has now been used successfully in several labs (Chiang et al., 2010; Duncan et al., 2009; Lane et al., 2010; Merriman et al., 2012), including ours (Illingworth et al., 2010). However, this method of karyotyping has not previously been attempted in mitotic mouse embryos. As such it was necessary to adapt this technique for the counting of 40 sister chromatid pairs (80 kinetochores) in mouse embryos with a view to employing this method to test the effect of Plk4 overexpression on aneuploidy rates in embryos.

In order to arrest individual blastomeres in mitosis, uninjected late 4-cell stage embryos were first treated with MG132 (25  $\mu$ M) for 4 hours. This step is unnecessary in MII eggs which are already arrested at metaphase. Prolonged MG132 treatment had a deleterious effect on spindle structure causing chromosomes to aggregate in clumps. However, following a 4 hour arrest period, approximately 1/3 of blastomeres were arrested in metaphase with bipolar spindles. After embryos had been arrested in metaphase, the spindle was collapsed to enable visualisation of kinetochores. Embryos were then treated for different lengths of time with either the kinesin-5 inhibitor monastrol (which causes the mitotic spindle to collapse into a monoaster (Mayer et al., 1999)) or nocodazole (which depolymerises MTs causing spindle collapse (Jordan et al., 1992)). Embryos were then stained using Hoechst and CREST antibodies to label the DNA and kinetochores respectively, before compression between a coverslip and glass slide to further aid the visualisation of individual kinetochores.

MG132 treatment alone was not an effective method for the counting of chromosomes due to small separation between the individual kinetochores within a pair at the metaphase plate. This was observed even when embryos were not compressed on slides and instead visualised in a glass-bottomed dish (Fig 4.10). Treatment with the MT depolymeriser nocodazole (10  $\mu$ M; 30 minutes) caused spindle disruption but the tight focussing of chromosomes meant that the total number of kinetochores could not be accurately counted (Fig 4.10). 60 minutes of treatment with the monastrol (100  $\mu$ M) was previously used to enable the counting of the 40 CREST-labelled kinetochores present in a mouse oocyte (Chiang et al., 2010; Duncan et al., 2009; Illingworth et al., 2010; Lane et al., 2010). However, the close apposition of the chromosomes in a monopolar spindle after 60 minutes of monastrol treatment in embryos made it difficult to resolve 80 individual kinetochores of a mouse embryo unambiguously (Fig 4.10). A shorter 30



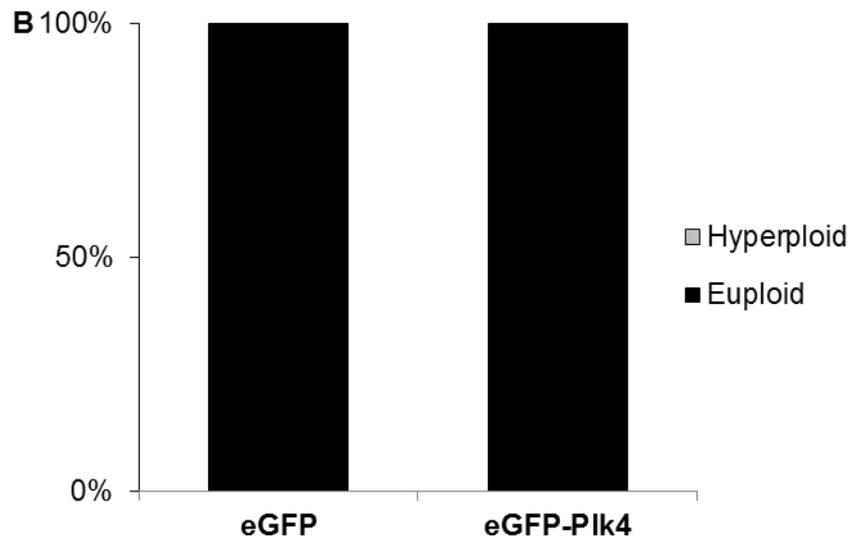
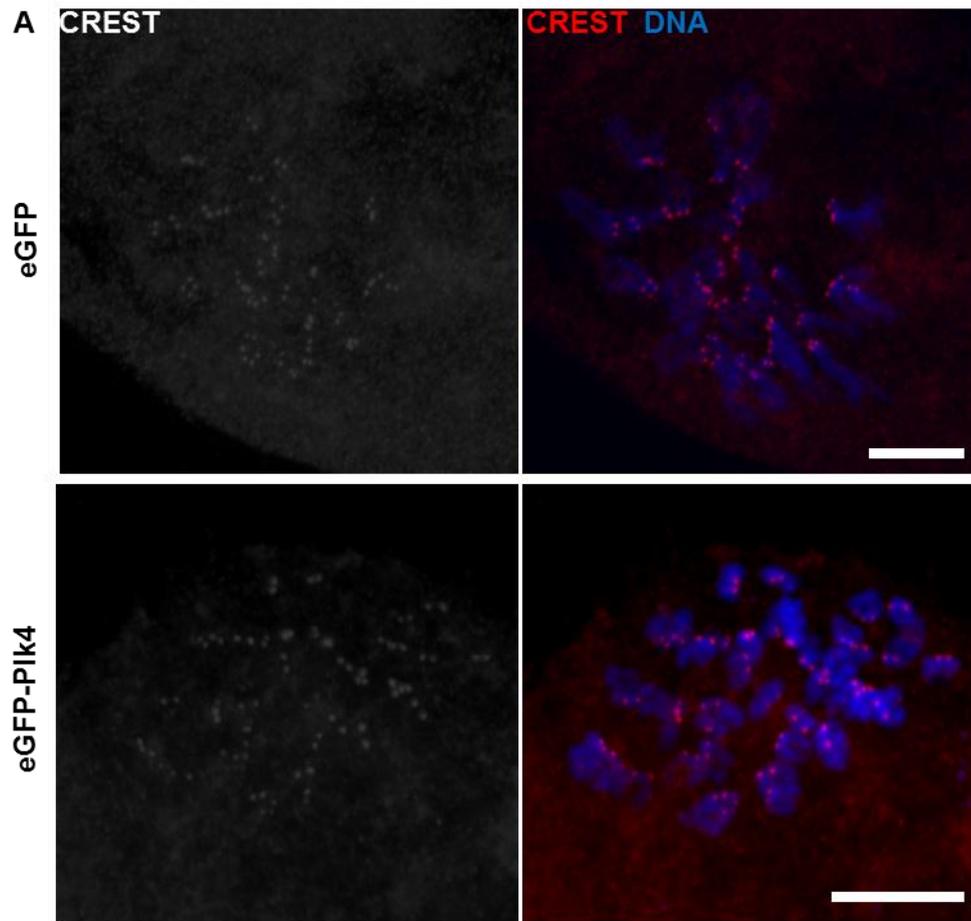
**Figure 4.10. Development of a method to enable karyotyping of 4-cell embryos.** (A) Typical confocal Z-projections of chromosome dispersal induced after various drug treatments. Note the close apposition of CREST-labelled kinetochore pairs in embryos treated with MG132 only, 60 minutes monastrol or nocodazole. A 30 minute pulse of monastrol allows the 80 individual kinetochores to be resolved accurately. See text for more details. Scale bar 10  $\mu\text{m}$ .

minute pulse of monastrol treatment (100  $\mu$ M) produced a collapsing spindle in which individual kinetochores could be more easily resolved (Fig 4.10). In summary, a short (30 minute) pulse of monastrol following MG132 treatment represents the most effective method to enable the counting of 80 kinetochores in mouse embryos.

#### **4.2.10. eGFP-Plk4 overexpression does not cause aneuploidy in embryos**

Having optimised the method for the karyotyping of chromosomes in embryos, eGFP-Plk4 or eGFP (control) was expressed in 2-cell embryos by microinjection. Embryos were cultured to the 4-cell stage before the addition of MG132 (25  $\mu$ M) for 4 hours to arrest the cell in the third mitotic division. Embryos were then subjected to a short 30 minute pulse of monastrol (100  $\mu$ M) to disrupt the spindle. Following staining with CREST auto-immune serum and Hoechst, tight confocal Z-sections were obtained and the kinetochore pairs counted. eGFP-Plk4-overexpressing embryos were found to possess the expected 80 kinetochores similar to control (eGFP-expressing) embryos (Fig 4.11). Thus, long term overexpression of eGFP-Plk4 in mouse embryos does not result in aneuploidy.

In summary, Plk4 overexpression causes aberrant spindle formation. However these resolve into bipolar spindles, which avoids chromosome mis-segregation and means that Plk4 overexpression has no detectable lasting impact on development.



**Figure 4.11. *Plk4* overexpression does not cause aneuploidy in embryos.** (A) Typical confocal Z-projections showing chromosome spreads obtained following a 30 minute pulse of monastrol. Note that 80 individual kinetochores can be resolved in both cases. (B) Graph showing chromosomal status of embryos overexpressing eGFP-*Plk4* or eGFP-alone. 16 eGFP- and 23 eGFP-*Plk4*-expressing embryos examined. Scale bar 10  $\mu$ m.

### 4.3. Discussion

In this chapter the role of the centriole replication protein Plk4 in the regulation of centriole appearance in mouse oocytes and embryos was investigated. Overexpression of Plk4 was found to trigger the *de novo* formation of centriole-like foci in all stages of oocytes and embryos examined (Fig 4.3). Consistent with the finding that Plk4-induced foci have MTOC properties (Fig 4.2), an increased proportion of Plk4-overexpressing embryos exhibited multipolar spindle intermediates or disorganised multipolar MT arrays (Fig 4.6). Despite possessing aberrant spindles, Plk4-overexpressing embryos exhibited the normal euploid number of chromosomes (Fig 4.11), revealing that the abnormal spindle phenotype did not lead to aneuploidy. Moreover, embryo development and rates of apoptosis were apparently normal in embryos overexpressing Plk4 (Fig 4.7;4.8;4.9). The discussion below will focus on three main topics. Firstly, the ability of Plk4 to form centriole-like foci in mouse oocytes and embryos will be discussed. Next, the potential role of Plk4 in *de novo* centriole formation at the blastocyst stage is addressed. Finally, the possible mechanisms that enable mouse embryos to tolerate highly abnormal spindles will be considered.

#### 4.3.1. Plk4 overexpression causes the formation of centriole-like foci

Plk4 is considered to be a crucial controller of centriole number and overexpression can lead to the formation of extra daughter centrioles in a ‘rosette’ arrangement (Habedanck et al., 2005; Kleylein-Sohn et al., 2007). Furthermore, overexpression of Plk4 in *Drosophila* and *Xenopus* oocytes can lead to the *de novo* formation of centriole-like structures (Eckerdt et al., 2011; Peel et al., 2007; Rodrigues-Martins et al., 2007b). Here, I found that Plk4 overexpression in mouse oocytes and embryos can also lead to the formation of centriole-like structures (Fig 4.1;4.2;4.3) and these foci represent new *de novo*-formed MTOCs as illustrated by Fig 4.4. Interestingly overexpression of the Plk4 homologue (Plx4) in *Xenopus* induced the *de novo* formation of centriole-like structures in activated eggs, but not in immature/mature oocytes (Eckerdt et al., 2011). Eckerdt and colleagues (2011) suggest that this change in the ability of Plk4 to induce centriole-like foci is mediated by the Mos-MAPK pathway which blocks Plx4-dependent *de novo* centriole formation in the unfertilised egg, thereby preventing the maternal gamete from contributing centrioles at fertilisation. In the experiments presented herein, overexpression of Plk4 could drive the formation of new centrioles in all mouse oocytes and embryos examined, irrespective of developmental stage (Fig 4.3). Therefore, Plk4 is not only a vital regulator of the canonical centriole replication pathway (Habedanck et al., 2005; Kleylein-Sohn et al., 2007) and the *de novo* centriole

formation pathway in *Drosophila* (Peel et al., 2007; Rodrigues-Martins et al., 2007b) and *Xenopus* (Eckerdt et al., 2011), but can also drive the *de novo* formation of centrioles in mammalian cells (this work). However, it appears that a regulatory mechanism that suppresses the Plk4-dependent *de novo* centriole formation pathway in oocytes is not conserved between mouse and *Xenopus*.

The *de novo*-formed structures observed upon overexpression of Plk4 are able to organise  $\gamma$ -tubulin and MTs (Fig 4.2), therefore exhibiting centriole-like behaviour. In somatic cells relieved of their resident centrioles, new centrioles are able to form *de novo* albeit with numerous structural defects (Khodjakov et al., 2002). Overexpression of various centriole replication proteins in *Drosophila* oocytes also drives the *de novo* formation of MTOCs (Dzhinzhev et al., 2010; Peel et al., 2007; Rodrigues-Martins et al., 2007a; Rodrigues-Martins et al., 2007b). EM analysis of these structures reveals that the *de novo*-formed MTOCs observed upon overexpression of Plk4 and Asl contain structurally normal centrioles (Dzhinzhev et al., 2010; Rodrigues-Martins et al., 2007b). In contrast, SAS-6-induced, *de novo*-formed MTOCs do not contain centrioles but instead possess a tube-like intermediate (Rodrigues-Martins et al., 2007b). It would therefore be interesting to examine the ultrastructure of these Plk4-induced centriole-like structures in mouse embryos to discover if they exhibit the regular nine-fold symmetry of normal centrioles.

Plk4 (and several other centriole replication proteins) contain coiled-coil domains (Dammermann et al., 2004; Delattre et al., 2004; Habedanck et al., 2005; Kemp et al., 2004; Leidel et al., 2005; Leidel and Gonczy, 2003; Leung et al., 2002; O'Connell et al., 2001). These regions are important in oligomerisation due to their specific interaction. Co-immunoprecipitation experiments reveal that Plk4 dimerizes via its C-terminal coiled coil region (Guderian et al., 2010; Habedanck et al., 2005; Leung et al., 2002) and Plk4 can also interact with other centriole replication proteins including Asterless (Asl) via coiled-coil domains (Stevens et al., 2010b). Therefore, coiled-coil domains may be instrumental in the oligomerisation of exogenous Plk4, thereby causing cytoplasmic accumulations.

Plk4-induced centrosome-like accumulations were also observed if 2-cell embryos were treated with the MT inhibitor nocodazole (Fig 4.1D). Therefore, an intact MT network is not necessary for the formation of MTOC-like structures in mouse embryos. If the cytoplasmic MT network is disassembled in somatic cells freed of their centrioles, well-defined clouds of PCM can reform when the cells are arrested in S-

phase. However, these PCM foci do not contain centrioles (Khodjakov et al., 2002). In contrast, in the presence of a MT network,  $\gamma$ -tubulin foci form rapidly and after 24 hours these foci are found to contain *de novo*-formed centrioles (Khodjakov et al., 2002). This suggests that the early stages of centrosome assembly can take place in the absence of MTs but MTs are required to complete the formation of a new centriole. Similarly, if the MT network is disrupted in CHO cells arrested in S-phase, centriole overduplication is prevented but  $\gamma$ -tubulin foci can still form (Collins et al., 2010). Interestingly, if MT inhibitors are washed out of S-phase-arrested CHO cells, functional centrioles can then form within these  $\gamma$ -tubulin-containing accumulations (Collins et al., 2010) supporting the idea that centrosome precursors can form in the absence of MTs, but MTs are necessary for the formation of a new centriole. In line with this, Plk4-induced foci were observed in nocodazole-treated 2-cell embryos (Fig 4.1D). It will therefore be interesting to examine the difference in ultrastructure and MT organising properties between Plk4-induced foci formed in the presence and absence of MTs in mouse embryos. By analogy with other systems (Collins et al., 2010; Khodjakov et al., 2002), I speculate that the cytoplasmic foci induced upon overexpression of Plk4 in the absence of an intact MT network will not represent bonafide centrioles with characteristic nine-fold symmetry.

Upon siRNA depletion of the structural centriole proteins hsSAS-6 or CP110, the percentage of cells showing multiple centrioles upon Plk4 overexpression in human cells is reduced (Habedanck et al., 2005). Similarly, knockdown of the downstream centriole replication factors SAS-6 or SAS-4 has been found to prevent SAK-mediated centriole biogenesis in *Drosophila* eggs and embryos (Rodrigues-Martins et al., 2007b). Thus, SAS-6, SAS-4 and CP110 are required for Plk4-induced centriole amplification. Raff and colleagues (Peel et al., 2007) suggest that the reason that individual overexpression of several centriole replication proteins can drive the formation of new centrioles in *Drosophila* eggs is because endogenous centriole components in unfertilised eggs tend to assemble into centriole precursors and following overexpression, these structures are stabilized and centrioles are manufactured. Therefore, depletion of one of these endogenous proteins means that these new centrioles cannot form (Peel et al., 2007). In light of this theory and since Plk4 is involved at an early step in centriole formation it would be interesting to consider if knockdown of other centriole replication proteins (such as SAS-6) affects the ability of Plk4 to induce centriole-like structures in mouse oocytes and embryos.

#### **4.3.2. Is Plk4 the rate-limiting step in centriole emergence in blastocysts?**

Plk4 depletion in human cells causes a progressive reduction in centriole numbers (Habedanck et al., 2005), in addition to suppressing centrosome amplification in aphidicolin-treated cells (Habedanck et al., 2005), thus indicating that Plk4 is necessary for centriole reproduction. Therefore, it is possible that an absence of Plk4 could prevent centrioles appearing until the blastocyst stage of mouse development. Indeed, overexpression of Plk4 alone is able to drive the formation of new centriole-like structures in all stages of oocyte and embryo examined (Fig 4.3), supporting this hypothesis. It would therefore be valuable to examine endogenous levels of Plk4 throughout mouse development to elucidate if an absence of Plk4 has a role in preventing precocious centriole appearance in early mouse embryos.

#### ***Western blots may prove infeasible for examination of Plk4 levels in mouse embryos***

Western blot analysis of centriolar proteins in *Drosophila* have previously been found to be unsuccessful (Peel et al., 2007). In these experiments 40 *Drosophila* embryos were used (which would be expected to have a total cytoplasmic volume of approximately 360nL, based on the average volume of *Drosophila melanogaster* eggs being approximately  $9.02 \times 10^{-3} \text{ mm}^3$  (Markow et al., 2009)), and still no signal was obtained. Since the volume of a mouse oocyte is approximately 270 pL, more than one thousand mouse oocytes would be required to obtain an equivalent cytoplasmic volume. Consequently, Western blot analysis of Plk4 in mouse oocytes and embryos is not feasible and were therefore not attempted. However, RT-PCR (reverse transcriptase PCR) may enable us to at least examine endogenous levels of Plk4 RNA throughout early mouse development.

#### ***Overexpression of kinase-dead Plk4 suggests that Plk4 activity is not the rate limiting step***

Measuring Plk4 kinase activity levels in oocytes and embryos may provide an alternative method to help elucidate any role for Plk4 in preventing the untimely appearance of centrioles in mouse early development. However, the absence of a specific substrate for Plk4 means that a direct approach for measuring kinase activity is not possible. Nevertheless, the finding that overexpression of the kinase-dead form of Plk4 (D154A) can drive the *de novo* formation of centrosome-like structures in oocytes and embryos (Fig 4.5), provides indirect evidence for the presence of endogenous Plk4 at these stages. In somatic cells, kinase-dead Plk4 is able to drive centriole over-replication but can only do so in the presence of endogenous Plk4 (Guderian et al., 2010). Indeed, concomitant expression of the kinase-dead

Plk4 (D154A) and knockdown of endogenous Plk4 significantly reduced the centriole over-replication phenotype (Guderian et al., 2010). The explanation for this kinase-dead Plk4-induced centriole overduplication is not yet fully understood but likely results from the disruption of Plk4 *trans*-autophosphorylation by kinase-dead Plk4 (Guderian et al., 2010; Sillibourne et al., 2010). This is thought to protect endogenous Plk4 from proteasomal degradation mediated by the F-box protein  $\beta$ -TrCP (Guderian et al., 2010). Since eGFP-Plk4-D154A overexpression can drive the *de novo* formation of centrosome-like foci in oocytes and embryos, this suggests that at least some endogenous Plk4 protein must be present at these stages. Although indirect, these findings tend to oppose the idea that a complete absence of Plk4 is preventing the untimely formation of centrioles until the blastocyst stage. However, it remains possible that a more subtle increase in the amount of endogenous Plk4 or Plk4 activity could permit formation the *de novo* of centrioles in blastocysts.

#### **4.3.3. How do Plk4-induced multipolar spindles in mouse embryos manage to segregate chromosomes correctly?**

A major finding of this chapter is that Plk4 overexpression produced excess centriole-like structures, which promoted multipolar spindles (Fig 4.6). Such multipolar spindles are now well known to be a risk factor for chromosome mis-segregation and aneuploidy (Ganem et al., 2009; Silkworth et al., 2009). Strikingly however, the novel karyotyping method developed here revealed that these multipolar spindles did not cause aneuploidy (Fig 4.11), nor were there any lasting effects upon development or overall embryo health (Fig 4.7;4.8;4.9). Thus, importantly, these experiments show that the early embryo can withstand relatively major spindle insults whilst preventing chromosome mis-segregation. It is well known that mammalian embryos frequently mis-segregate chromosomes (Lightfoot et al., 2006; van Echten-Arends et al., 2011; Vanneste et al., 2009). Therefore, it may not have been predicted that the mouse embryo would cope with such spindle aberrations and remain euploid. The discussion which follows will address the possible mechanisms mouse embryos may employ to deal with these insults.

#### ***The clustering of supernumerary centrosomes***

Several groups have now shown that supernumerary centrosomes can coalesce into two dominant poles, allowing cells with extra centrosomes to undergo bipolar divisions and avoid the detrimental effects of multipolar mitoses (Basto et al., 2008; Kwon et al., 2008; Quintyne et al., 2005; Yang et al., 2008).

Following overexpression of Plk4 in mouse embryos, an increased proportion of abnormal spindles were observed (Fig 4.6). However, the proportion of non-bipolar spindles was found to have decreased later in mitosis (Fig 4.6B). Closer examination of these bipolar spindles reveals that multiple Plk4-foci cluster around the two poles (Fig 4.6A, bottom panel), reminiscent of the phenotype observed in cancer cells or flies with extra centrosomes (Basto et al., 2008; Kwon et al., 2008; Quintyne et al., 2005; Yang et al., 2008). This data implies that the clustering of supernumerary centrioles into two dominant poles is a mechanism to prevent multipolar mitoses employed by *de novo*-formed centrioles as well as those produced due to de-regulation of the canonical duplication pathway.

### ***Mechanisms facilitating centrosome clustering***

Extra centrosomes have been shown to prolong mitosis which allows cells time to cluster centrosomes before the onset of anaphase (Basto et al., 2008; Kwon et al., 2008; Yang et al., 2008). Indeed, an intact SAC is crucial for the viability of flies with supernumerary centrosomes (Basto et al., 2008). Although, analysis of cells in Plk4-overexpressing blastocysts did not reveal any increase in mitotic index (Fig 4.8), it would be interesting to examine the time course of this rearrangement of supernumerary centrosomes using live fluorescence microscopy to detect any difference in the length of mitosis between Plk4-overexpressing and control embryos. By analogy with other cell types it is likely that Plk4-overexpressing embryos will take longer to complete mitosis allowing time for this centrosome clustering to occur. Furthermore, depletion of Mad2 would identify the role of the SAC in Plk4-overexpressing embryos.

Centrosome coalescence has also been found to rely upon motor proteins which organise the spindle poles such as the kinesin 14 family member Ncd/HSET and the NuMA/dynein complex (Basto et al., 2008; Kwon et al., 2008; Quintyne et al., 2005). It would therefore be of value to test the requirement of these motor proteins for centrosome clustering following Plk4-induced centriole amplification in mouse embryos.

### ***Correction of kinetochore-MT misattachments***

Two recent landmark papers demonstrate that cancer cells with extra centrosomes pass through a multipolar spindle intermediate which gives rise to an increased proportion of merotelic attachments and increases the likelihood of lagging chromosomes, which are a major risk factor for aneuploidy (see Fig

1.7; (Ganem et al., 2009; Silkworth et al., 2009)). The absence of aneuploidy in 4-cell embryos following Plk4 overexpression (Fig 4.11), despite the observation of highly abnormal spindles (Fig 4.6), raises the possibility that an error correction mechanism(s) may be operating in order to avert chromosomal instability in Plk4-overexpressing embryos. In somatic cells, MT motor proteins of the kinesin-13 family play a critical role in correcting kinetochore-MT misattachments by depolymerising MTs at their plus ends, thereby promoting re-attachment and increasing the chance of achieving a correct attachment (Moore and Wordeman, 2004). This raises the possibility that these motor proteins may also be playing a role in error-correction in mouse embryos allowing them to tolerate highly abnormal spindles.

MCAK (Kif2C) is the most well characterised member of the kinesin 13 family of MT depolymerisers. Overexpression of MCAK suppresses the incidence of lagging chromosomes in chromosomally unstable cells (Bakhom et al., 2009) and conversely, depletion or inhibition of MCAK (or its *Xenopus* homologue) causes abnormal spindles and defects such as chromosome misalignment, lagging chromosomes and chromosome mis-segregation (Kline-Smith et al., 2004; Maney et al., 1998; Walczak et al., 1996). Recent data from our lab has shown that although MCAK is indispensable for regulating chromosome alignment in mouse oocyte meiosis, it is not required for preventing aneuploidy in this setting (Illingworth et al., 2010). Illingworth et al., (2010) propose that the late establishment of kinetochore-MT attachments in meiosis (Brunet et al., 1999), might reduce the need for MCAK-dependent correction of kinetochore-MT misattachments in mouse oocytes (Illingworth et al., 2010). However, it is possible that in mouse embryos MCAK may play an important role in promoting the correction of merotelic attachments. Indeed, MCAK depletion in 2-cell stage mouse embryos leads to subsequent poor embryo development and increased levels of apoptosis (Illingworth and FitzHarris, unpublished data). However, MCAK-depleted embryos display a mitotic index similar to controls indicating that the poor development is not likely to be due to activation of the SAC (Illingworth and FitzHarris, unpublished data). Work is ongoing to establish whether MCAK-depleted embryos are chromosomally abnormal. However, this preliminary data supports the notion that MCAK-dependent error correction may be important in embryos and would also explain why Plk4 overexpression fails to cause aneuploidy in embryos. To investigate this hypothesis, Plk4 could be overexpressed in MCAK-depleted embryos. One would predict that an absence of MCAK-dependent error correction will disrupt the ability of Plk4-induced foci to rearrange into bipolar spindles which could lead to defects in

chromosome segregation and a resultant increase in aneuploidy levels. In addition, overexpression of another of the kinesin 13 family of error-correcting kinesins, Kif2b, has been shown to reduce the incidence of chromosome segregation errors in cancer cells by increasing MT turnover (Bakhom et al., 2009). Thus, it will also be interesting to examine the effect of Kif2b knockdown on the ability of Plk4-overexpressing embryos to deal with aberrant spindles. In summary, the mechanisms of error correction which embryos employ to avoid chromosome mis-segregation remain unclear.

### ***Error correction methods in human embryos***

In most systems, cells with severe levels of chromosome segregation errors undergo programmed cell death by apoptosis. However, embryos are thought to be surprisingly tolerant to aneuploid cells. In fact, only 9% of human pre-implantation embryos have been found to have a normal karyotype in all blastomeres (Vanneste et al., 2009). Importantly, IVF success rates at the fertility centre from which the embryos were obtained are more than 20% per embryo transferred (Harper et al., 2008). This implies that mosaic embryos can result in chromosomally-normal fetuses suggesting that aneuploid cells may be eliminated after implantation (Sandalinas et al., 2001) or survive as part of the placenta with indirect effects on embryonic development (Ledbetter, 2009). The very high levels of aneuploidy observed in human embryos (van Echten-Arends et al., 2011; Vanneste et al., 2009) suggest that error correction methods may be absent or repressed in human pre-implantation embryos. This is in stark contrast to mouse embryos, in which embryos are euploid despite major spindle aberrations following overexpression of Plk4, suggesting that error correction methods are likely to be active.

The embryos studied in Vanneste et al., (2009) were from healthy young couples undergoing IVF (*in vitro* fertilisation) for genetic risks rather than infertility (Vanneste et al., 2009). Although there was no evidence for aberrant culture conditions in the clinic from which the embryos used in the Vanneste study were obtained (Harper et al., 2008), it remains possible that this highly abnormal phenotype is a consequence of *in vitro* culture and ovarian hyperstimulation as these have previously been found to affect aneuploidy frequency in embryos (Munne et al., 1997; Weghofer et al., 2008). In order to investigate this possibility it would be necessary to compare *in vivo*-conceived pre-implantation embryos for chromosomal abnormalities. However, these are not available for research.

Since an increase in female meiosis I aneuploidy rate is strongly correlated to advancing maternal age (Hassold and Hunt, 2001), it is interesting to speculate that mitotic aneuploidy rates may also be increased in pre-implantation embryos from older mothers, possibly due to a decreased ability to detect or remove cells with chromosome aberrations at this stage. However, it is not possible to compare naturally conceived embryos from young and old human mothers.

#### **4.4. Summary**

In this chapter, overexpression of Plk4 was found to drive the *de novo* formation of centrosome-like structures in all stages of oocyte and embryo examined. This data indicates for the first time that Plk4 can drive *de novo* formation of centrosome-like structures in mammalian cells. In addition, Plk4 overexpression in embryos can induce the formation of abnormal spindles. However, these appear to be resolved into bipolar spindles before the onset of anaphase. This provides evidence that *de novo*-formed extra centrioles, as well as those formed by centriole over-replication, are subject to similar controls to limit the deleterious effects of supernumerary centrosomes. Interestingly, although abnormal spindles were formed following Plk4 overexpression, there were no adverse effects on development or chromosome segregation, illustrating that mouse embryos can withstand major spindle aberrations. The mechanisms underpinning this phenotype remain uninvestigated, though one might speculate that in the presence of extra *de novo*-formed centrosomes, several extra levels of control and error-correction methods are required to ensure chromosome segregation can still proceed faithfully.

# **5. ROLE OF THE STRUCTURAL CENTRIOLE REPLICATION PROTEIN SAS-6 IN CENTRIOLE BIOGENESIS IN MOUSE OOCYTES AND EMBRYOS**

## **5.1. Introduction**

SAS-6 is a structural centriole replication protein which is required for the establishment of the characteristic nine-fold radial symmetry of forming centrioles (Nakazawa et al., 2007), and for controlling the number of procentrioles formed in each S-phase (Peel et al., 2007; Rodrigues-Martins et al., 2007b; Strnad et al., 2007). SAS-6 was first identified and characterised in *C. elegans* (Dammermann et al., 2004; Leidel et al., 2005) where it was found to be necessary for centriole replication. SAS-6 homologues have since been found to be essential for procentriole formation in several species including *Drosophila* (Peel et al., 2007; Rodrigues-Martins et al., 2007a), *C. reinhardtii* (Nakazawa et al., 2007), zebrafish (Yabe et al., 2007) and human cells (Strnad et al., 2007). SAS-6 is involved downstream of Plk4 in the centriole replication pathway (Delattre et al., 2006; Pelletier et al., 2006). In human cells and *C. elegans*, SAS-6 is found to be recruited but not maintained at centrioles in Plk4-depleted cells (Kitagawa et al., 2009; Strnad et al., 2007). Thus, Plk4 is the master regulator of centriole replication and SAS-6 is involved at a later stage of the pathway.

Electron tomograph reconstructions of procentriole assembly in *C. elegans* embryos have revealed that the first step in the manufacture of a new centriole is the formation of a central tube or ‘cartwheel’ structure at the proximal end of each mother centriole (Delattre et al., 2006). SAS-6 has been found to be recruited at the onset of cartwheel formation indicating a structural role for SAS-6 in the centriole replication pathway (Pelletier et al., 2006). The cartwheel consists of a central hub from which nine radial spokes protrude (see Fig 1.1). Following assembly of the cartwheel, MTs are then assembled at the tips of these spokes, which allows the daughter centriole to elongate until it reaches a size similar to that of the pre-existing mother centriole (Pelletier et al., 2006). Accumulating evidence suggests that SAS-6 plays a key role in determining the symmetrical structure of the forming centriole by organising the central cartwheel structure (Hiraki et al., 2007; Nakazawa et al., 2007; Rodrigues-Martins et al., 2007a). In *C. reinhardtii*, basal bodies in null mutants of the SAS-6 homologue bld12, have several ultrastructural

defects including an abnormal number of triplet MTs, as well as a lack of the central hub structure (Nakazawa et al., 2007). Furthermore, open centrioles with disorganized or missing triplet MTs are observed in D-SAS-6 mutant flies (Rodrigues-Martins et al., 2007a). Conversely, tubules reminiscent of the cartwheel hub could be assembled in *Drosophila* spermatocytes by co-expression of D-SAS-6 and its binding partner Ana2 (Stevens et al., 2010a; Stevens et al., 2010b). Thus, SAS-6 stabilizes the nine-fold symmetry of the new centriole by determining the radial structure of the cartwheel in several different species.

Rodrigues Martin et al., (Rodrigues-Martins et al., 2008) proposed that SAS-6 brings together nine pre-centriolar units called ‘enatosomes’ which form a tube-like centriole precursor thereby imparting nine-fold radial symmetry to the nascent procentriole. Recently, it has been shown that recombinant and native SAS-6 can self-oligomerise into stable structures which form the key building blocks of the central tubule in *Drosophila* centrioles, supportive of a direct role for SAS-6 in dictating the nine-fold symmetry of the new centriole (Gopalakrishnan et al., 2010). Structural evidence has now provided further clues as to how SAS-6 determines this symmetry (Kitagawa et al., 2011; van et al., 2011). Characterisation of SAS-6 from *C. elegans*, *C. reinhardtii* (Kitagawa et al., 2011) and zebrafish (van et al., 2011), all reveal N-terminal head group- as well as coiled-coil domain-interactions, which were found to be necessary for SAS-6 to arrange itself into circular oligomers. Despite the fact that the primary structure is highly divergent between the three species, the three structures exhibit a high degree of structural conservation. These studies therefore confirm that the ability of SAS-6 to self-oligomerise is crucial for the assembly of a symmetric cartwheel structure and the subsequent formation of a symmetric daughter centriole.

In addition to its role in determining the symmetry of the emerging daughter centriole, SAS-6 is also an important factor in controlling the number of new centrioles formed in each cell cycle. Upon siRNA inactivation of SAS-6 in human cells a decreased number of centrioles were observed leading to a higher incidence of monopolar spindles and failed cytokineses (Leidel et al., 2005; Strnad et al., 2007). Conversely, overexpression of SAS-6 can induce centriole over-replication or *de novo* centriole formation in *Drosophila* eggs ((Peel et al., 2007; Rodrigues-Martins et al., 2007b; Strnad et al., 2007); see Appendix 1). This in turn can lead to a dangerous centriole excess and cause chromosome segregation errors (Ganem et al., 2009; Nigg, 2002; Silkworth et al., 2009). It is therefore crucial that SAS-6 levels are

tightly regulated throughout the cell cycle. Accordingly, the human SAS-6 homologue (hsSAS-6) possesses a KEN box thereby targeting it for degradation by the APC<sup>Cdh1</sup> (Strnad et al., 2007) and ensuring only one procentriole forms alongside each mother centriole. Indeed, overexpression of a non-destructible form of SAS-6 leads to centriole overduplication in human cells highlighting the importance of regulating the amount of centriole proteins in the cell (Strnad et al., 2007).

## **AIMS**

Since it has previously been established that SAS-6 is important for canonical centriole replication (Leidel et al., 2005; Peel et al., 2007; Rodrigues-Martins et al., 2007a; Rodrigues-Martins et al., 2007b; Strnad et al., 2007) as well as *de novo* centriole formation in *Drosophila* oocytes (Peel et al., 2007; Rodrigues-Martins et al., 2007b), it was important to establish whether SAS-6 may also be involved in *de novo* centriole formation in mammalian cells. Mouse embryos and oocytes represent an ideal model for this since both murine gametes lack centrioles (Manandhar et al., 1998; Szollosi et al., 1972) and consequently early mouse embryos are acentriolar.

In this chapter, the role of SAS-6 in mouse oocytes and embryos was addressed using three approaches: Firstly, the endogenous localisation of SAS-6 was determined using SAS-6 antibodies. Secondly, the effect of SAS-6 overexpression in various developmental stages was examined. An important and interesting feature of the data presented in this chapter was that SAS-6 overexpression was not equally potent in all stages of development. Finally, depletion of SAS-6 using a morpholino oligonucleotide attempts to uncover any requirement for SAS-6 in mouse embryos.

## 5.2 Results

### 5.2.1. SAS-6 is recruited to $\gamma$ -tubulin hotspots at the time of centriole formation

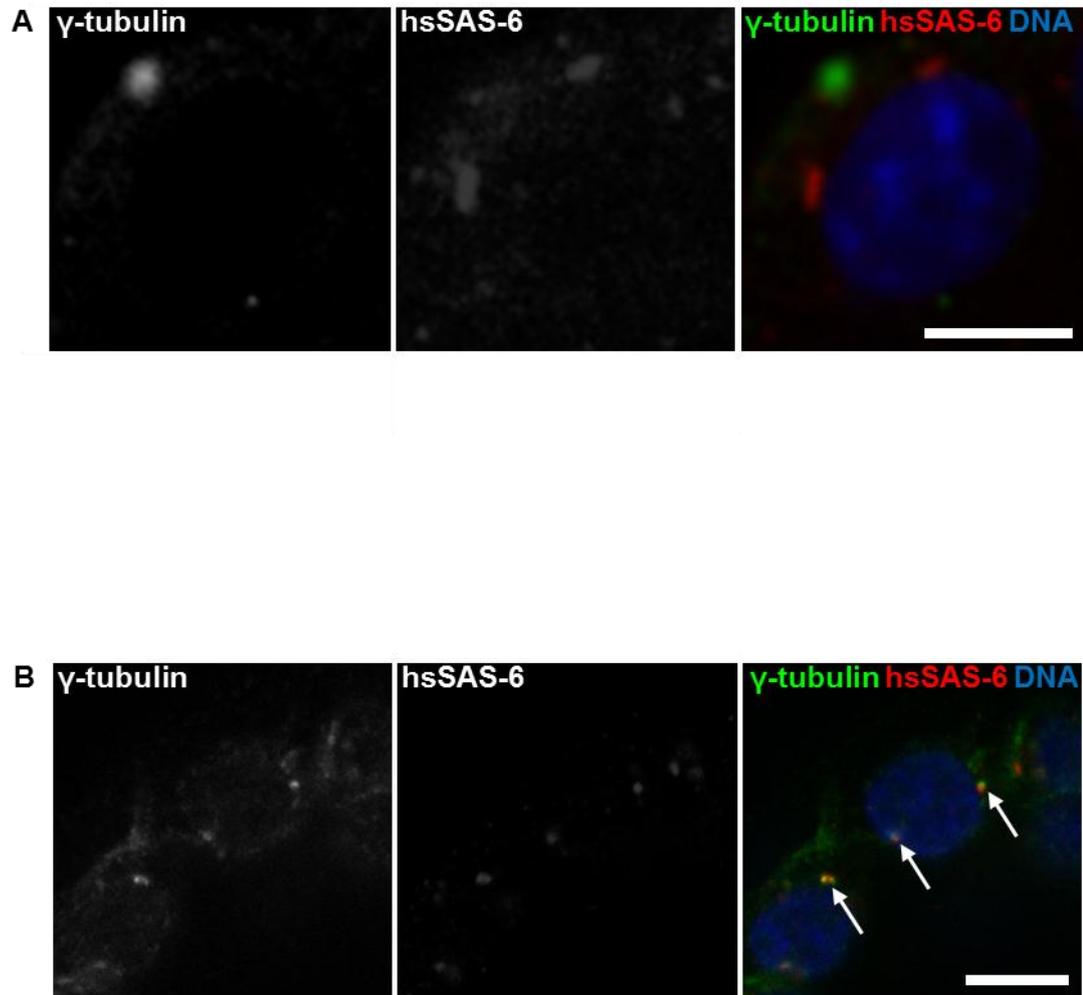
Firstly, the presence and location of endogenous SAS-6 protein was investigated in morulae and blastocysts. Morula and blastocyst stage embryos were fixed and stained with antibodies raised against hsSAS-6 (gift from Pierre Gönczy, Lausanne, Switzerland) and  $\gamma$ -tubulin. At the morula stage SAS-6 was evident as extra-centrosomal accumulations (Fig 5.1A). However, by the blastocyst stage SAS-6 was localised to the  $\gamma$ -tubulin-labelled centrosomes (Fig 5.1B). The identity of these extra-centrosomal structures to which SAS-6 localises in morula was not investigated further here but this data suggests that SAS-6 is indeed present in mouse embryos and relocates to the centrosome by the blastocyst stage. The re-localisation of SAS-6 to the centrosome at the time of blastocyst formation (when centrioles are manufactured; (Abumuslimov et al., 1994; Gueth-Hallonet et al., 1993)), suggests that SAS-6 antibodies associate with the centrioles, rather than non-specifically with the centrosomal material.

### 5.2.2. Exogenous SAS-6 organises into numerous cytoplasmic foci

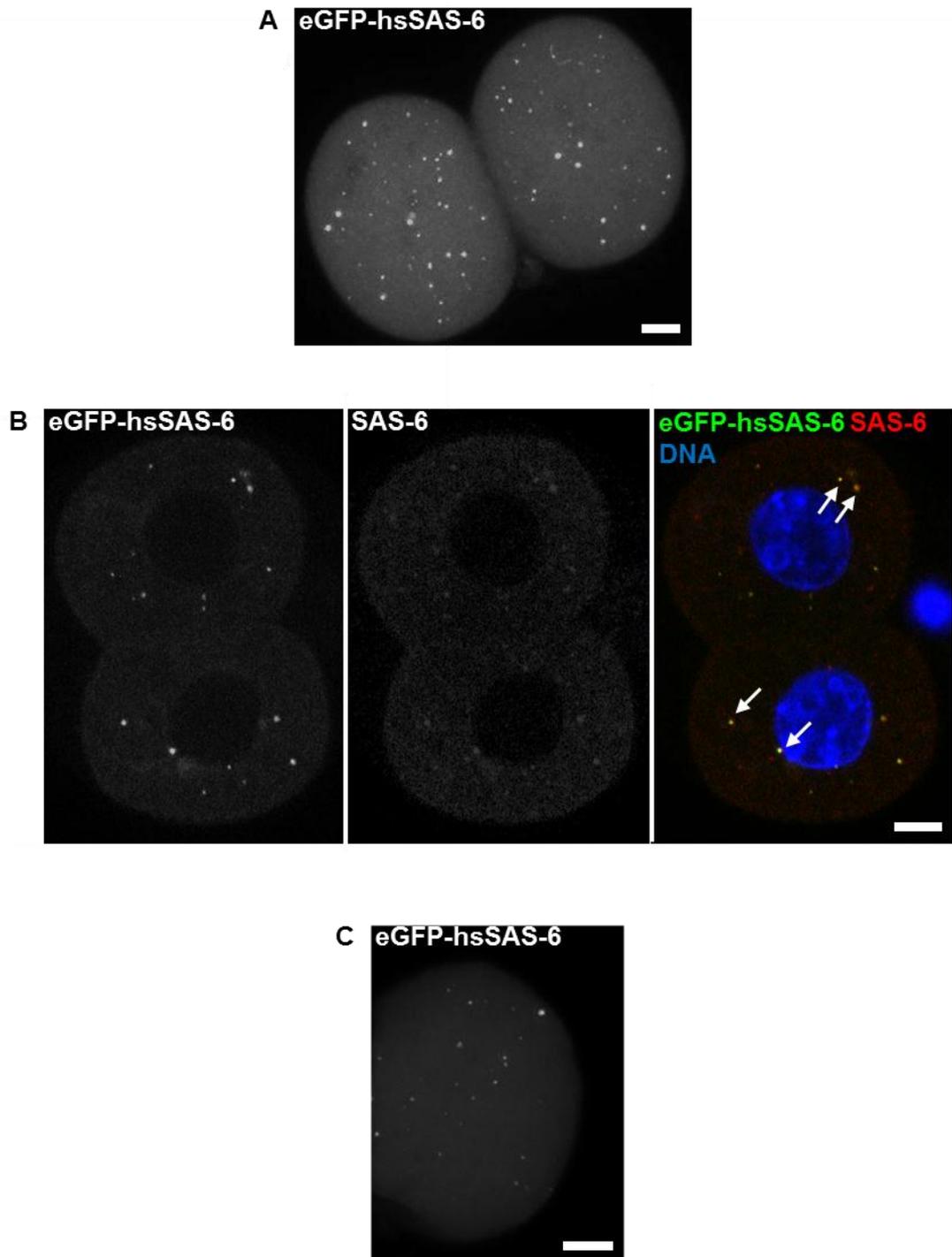
To further examine the role of SAS-6 in *de novo* centriole formation, confocal microscopy was used to examine 2-cell embryos overexpressing eGFP-hsSAS-6. As for previous experiments, (see Chapter 3 & 4), SAS-6 was overexpressed in 2-cell embryos by microinjection of mRNA into each blastomere. 2-3 hours after microinjection the exogenous eGFP-hsSAS-6 was seen to have assembled into numerous discrete foci throughout the cytoplasm (Fig 5.2A). As previously (Fig 4.1B), overexpression of eGFP alone did not cause such accumulations. The eGFP-hsSAS-6 foci co-label with hsSAS-6 antibodies confirming that these spots are indeed accumulations of SAS-6 (Fig 5.2B). These experiments reveal that exogenous SAS-6 organises into cytoplasmic foci in acentriolar 2-cell embryos.

### 5.2.3. Cytoplasmic foci can still form in the absence of an intact MT network

To determine whether the arrangement of exogenous eGFP-hsSAS-6 into foci was dependent upon MTs, nocodazole was used to disrupt the MT network (Jordan et al., 1992). Characteristic foci were still observed in 2-cell embryos, which were treated with the MT depolymeriser nocodazole (10  $\mu$ M) at the time of SAS-6 overexpression (Fig 5.2C). As a positive control, MII eggs were also treated with nocodazole on the same day. Spindle collapse confirmed that nocodazole was effective. These data show that the formation of these SAS-6-induced foci is not dependent on the presence of an intact MT network.



**Figure 5.1.** *SAS-6* relocates to the centrosomes at the blastocyst stage. (A) Confocal slice of a interphase blastomere from a morula. Note that  $\gamma$ -tubulin and *SAS-6* are found at distinct sites. (B) Confocal slice of small area of blastocyst showing colocalisation of  $\gamma$ -tubulin and *SAS-6*. Arrows indicate colocalisation. Scale bar 10  $\mu$ m.



**Figure 5.2. Exogenous eGFP-hsSAS-6 arranges into cytoplasmic foci.** (A) Typical confocal Z-projection of a whole 2-cell embryo showing numerous cytoplasmic foci of exogenous SAS-6. (B) Single confocal slice showing colocalisation of exogenous SAS-6 (green) with hsSAS-6 antibody (red). DNA is shown in blue. (C) Z projection of a 2-cell embryo treated with nocodazole at the time of SAS-6 overexpression. Note that the characteristic cytoplasmic foci are still observed. Scale bar 10  $\mu$ m.

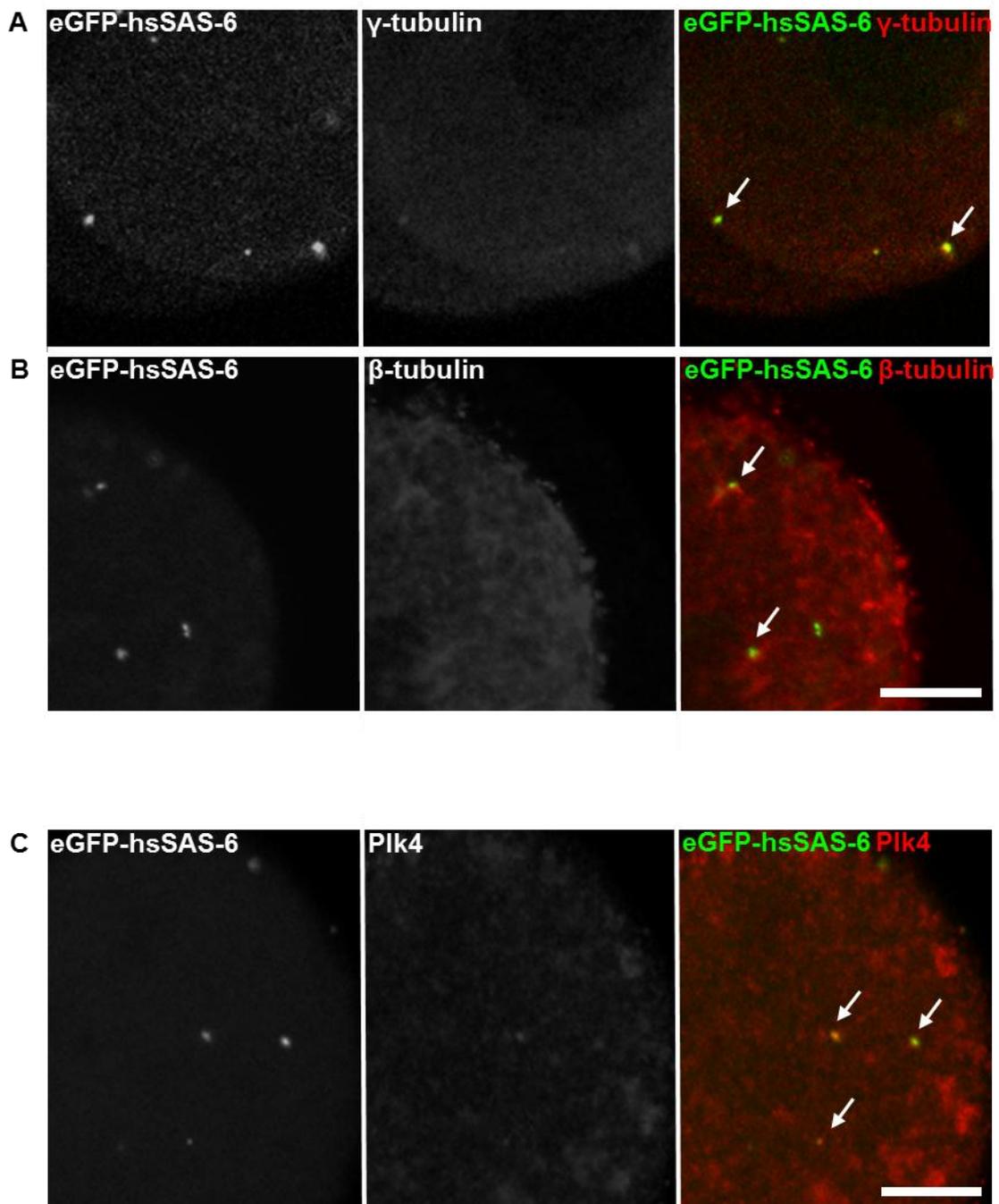
#### **5.2.4. Exogenous SAS-6-induced 'spots' can organise $\gamma$ -tubulin, MTs and Plk4**

These SAS-6 accumulations were next examined for any centrosome-like function. After three hours of expression, eGFP-hsSAS-6-injected 2-cell embryos were fixed and stained with antibodies against  $\gamma$ - and  $\beta$ -tubulin. Exogenous SAS-6 accumulations were colocalised with  $\gamma$ -tubulin in most cases (Fig 5.3A; 83.5% of spots colocalised with  $\gamma$ -tubulin) and in some cases were able to organise MTs (Fig 5.3B; 28.3% colocalisation). Therefore, exogenous SAS-6 causes the formation of foci with at least some centrosome-like function. I also wondered whether exogenous SAS-6 foci could organise other centriole replication proteins. Therefore, eGFP-hsSAS-6-expressing embryos were fixed after three hours of expression and stained with antibodies against Plk4 (gift from Alex Dammermann, Vienna, Austria, unpublished). Plk4 is a regulatory centriole replication protein involved upstream of SAS-6, at the initial stages of centriole replication (Habedanck et al., 2005; Kleylein-Sohn et al., 2007; Sillibourne and Bornens, 2010). Some of the eGFP-hsSAS-6 foci were found to be colocalised with Plk4 (Fig 5.3C). Thus, exogenous SAS-6 foci are capable of recruiting other centriole replication proteins as well as centrosome components.

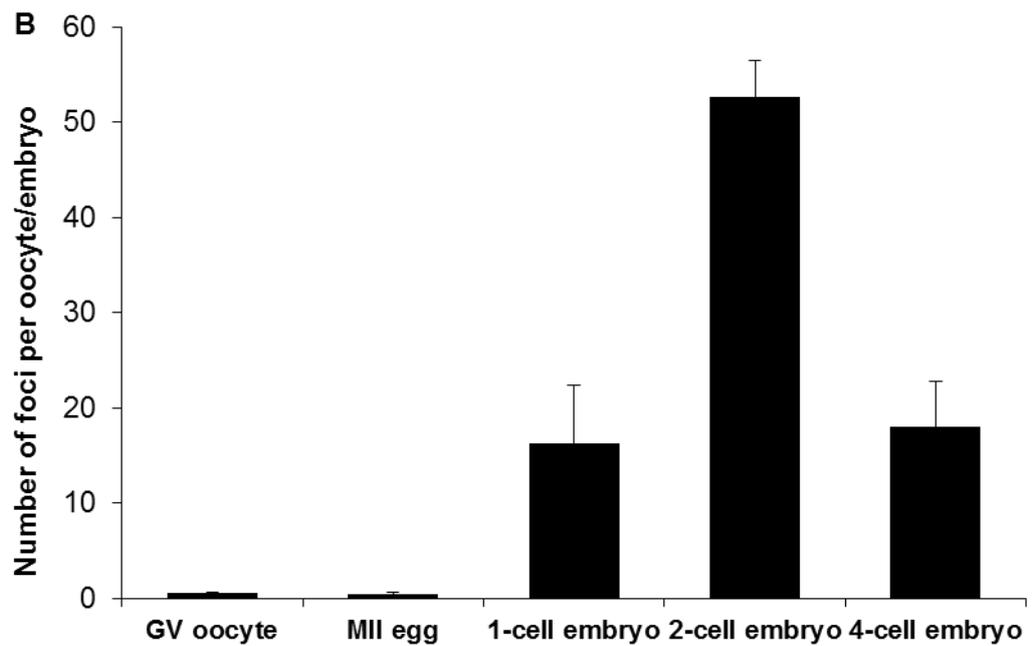
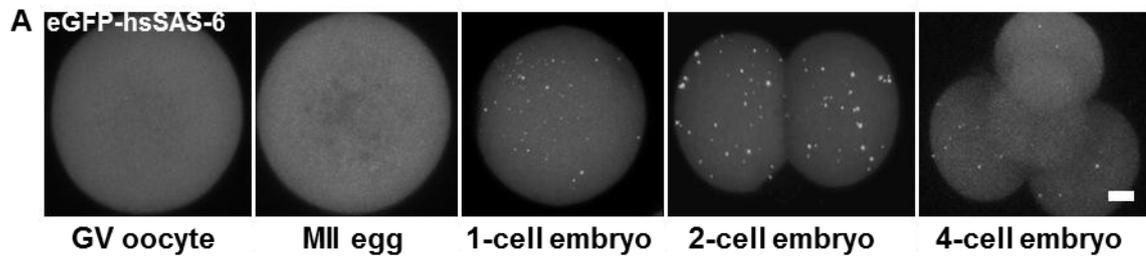
#### **5.2.5. Different developmental stages have different propensities for SAS-6-induced foci formation**

The finding that overexpression of eGFP-hsSAS-6 can induce the formation of centriole-like structures in 2-cell embryos provoked the question of whether such accumulations could also be observed following overexpression of eGFP-hsSAS-6 in other acentriolar stages of development. After overexpression of eGFP-hsSAS-6 in fertilised embryos of different developmental stages, numerous centriole-like foci were observed in 1-, 2- and 4-cell embryos (Fig 5.4; 16.23 $\pm$ 6.14 to 52.60 $\pm$ 3.88 foci per embryo). Next, the effect of SAS-6 overexpression in oocytes was investigated. Interestingly, foci were not observed upon overexpression of eGFP-hsSAS-6 in GV oocytes or MII eggs (Fig 5.4; 0.39 $\pm$ 0.27 to 0.53 $\pm$ 0.16 foci per oocyte).

To exclude the possibility that the inability of SAS-6 to induce foci in oocytes was due to inadequate expression, eGFP-hsSAS-6 was overexpressed in oocytes and embryos on the same day for comparison side-by-side. Characteristic foci were not formed in oocytes expressing far greater levels of eGFP-hsSAS-6 than eGFP-hsSAS-6-expressing 2-cell embryos which always formed foci (data not shown). Similarly,



**Figure 5.3. eGFP-hsSAS-6 foci can organise  $\gamma$ -tubulin and microtubules.** Single confocal slices showing exogenous eGFP-hsSAS-6 colocalising with (A)  $\gamma$ -tubulin and (B) microtubules after 3 hours of expression. (C) In addition, some eGFP-hsSAS-6 foci are able to organise the centriole replication factor Plk4. Arrows indicate colocalisation. Scale bar 10  $\mu$ m.



**Figure 5.4. eGFP-hsSAS-6 overexpression is able to induce foci in embryos but not in immature or mature oocytes.** (A) Confocal Z-projections of oocytes and embryos after 3 hours expression of eGFP-hsSAS-6. 9-19 oocytes/embryos examined per stage. (B) Graph showing average number of spots per oocyte/embryo following overexpression of eGFP-hsSAS-6. Scale bar 10  $\mu$ m.

eGFP-Plk4-expressing oocytes were able to assemble numerous MTOCs whereas eGFP-hsSAS-6-expressing oocytes were unable to do so despite higher levels of protein expression (data not shown). These experiments show that eGFP-hsSAS-6 overexpression fails to induce foci in oocytes and confirm that the differing ability of embryos and oocytes to permit the formation of SAS-6 foci was not a concentration-dependent effect.

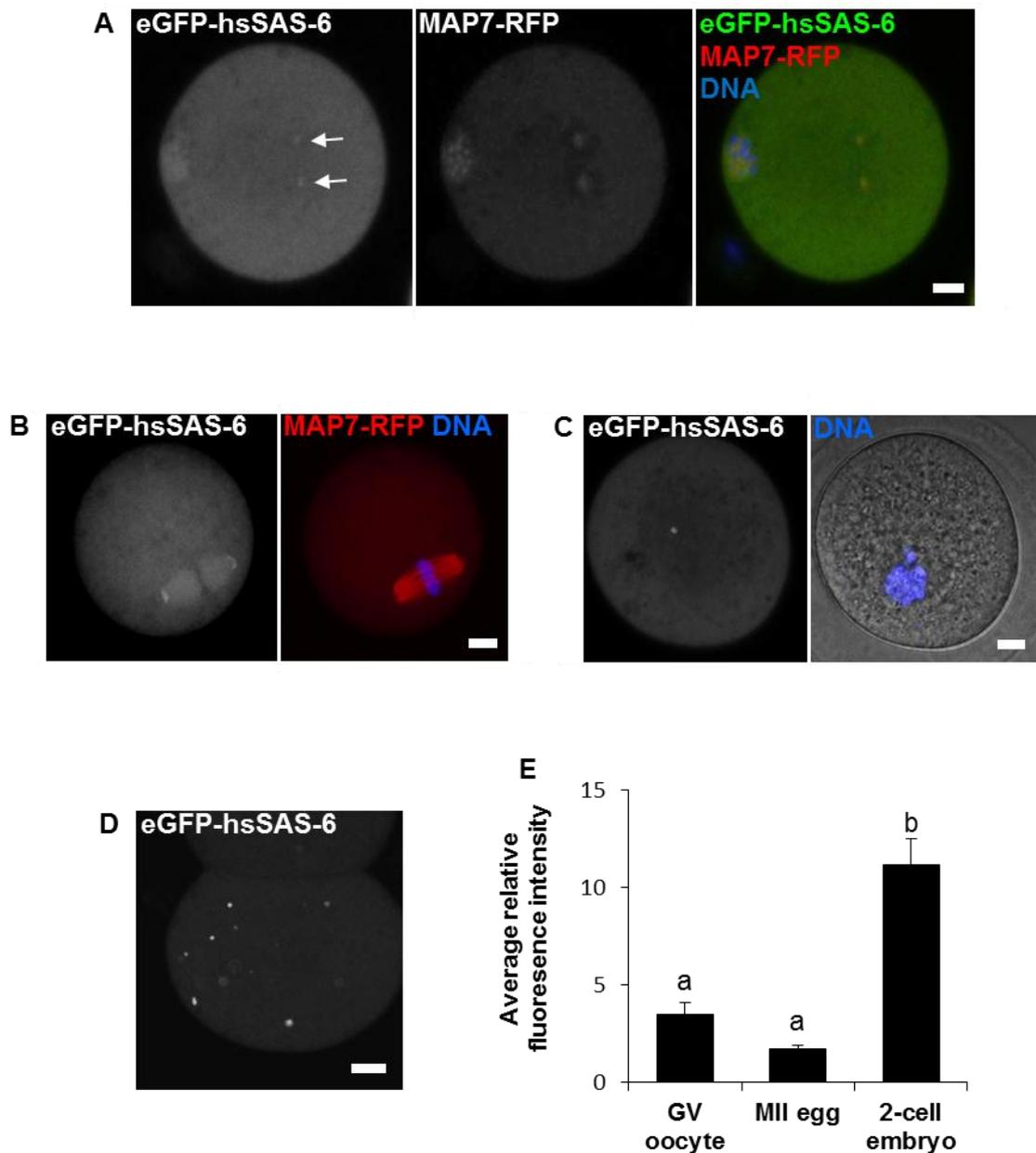
Following the prolonged expression of exogenous SAS-6 in MII eggs, faint staining was sometimes observed at the spindle poles (Fig 5.5B) as well as occasional diffuse patches around existing cytoplasmic MTOCs (Fig 5.5A). In addition, in ~50% of immature oocytes examined, a single accumulation of eGFP-hsSAS-6 was observed in close proximity to the GV (Fig 5.5C). However, discrete foci such as those seen in eGFP-hsSAS-6-overexpressing embryos (average relative fluorescence intensity of foci: 11.12 $\pm$ 1.37; Fig 5.5D,E) were not observed in GV oocytes or MII eggs (average relative fluorescence intensity of foci: 3.47 $\pm$ 0.62 and 1.73 $\pm$ 0.20 respectively,  $p < 0.01$  ANOVA + Tukey-Kramers; Fig 5.5E). Therefore, SAS-6 overexpression is able to drive the formation of discrete foci in embryos, but not oocytes.

Together, these experiments reveal that different developmental stages exhibit different propensities for SAS-6-induced foci formation, suggesting a shift in the responsiveness of the cytoplasm to the formation of new centrioles at fertilisation. Furthermore, since overexpression of eGFP-hsSAS-6 in GV oocytes or MII eggs did not produce centrosome-like foci, SAS-6 alone is not sufficient to drive formation of centrioles in this setting.

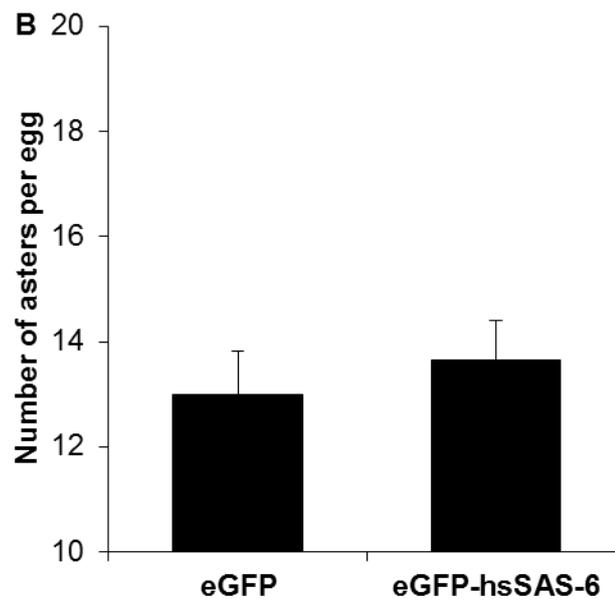
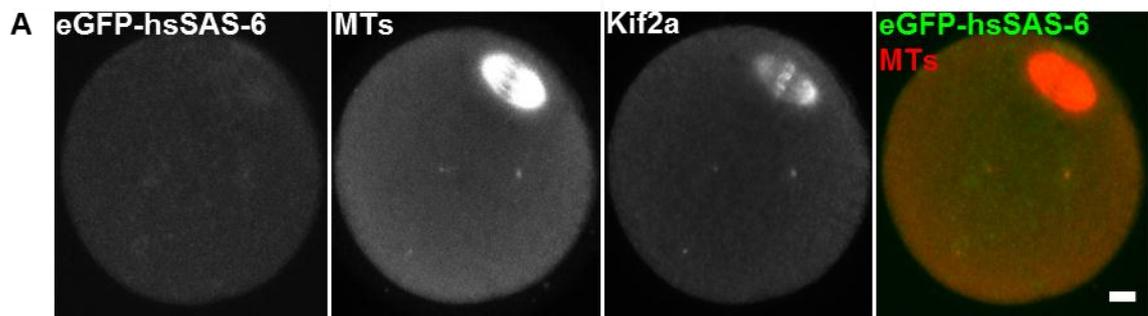
#### **5.2.6. SAS-6 overexpression is unable to drive the formation of new MTOCs in MII eggs.**

Since exogenous SAS-6 localised to the poles of the MII spindle and it is well established that MII eggs possess numerous discrete MTOCs (Combelles and Albertini, 2001), it was possible that these faint and diffuse cytoplasmic patches of eGFP-hsSAS-6 were localising to pre-existing MTOCs and as such were not influencing MTOC number. To investigate this hypothesis the number of  $\beta$ -tubulin-labelled MT asters was counted in fixed MII eggs expressing either eGFP-alone or eGFP-hsSAS-6.

Antibodies against the MT motor protein Kif2A have previously been shown to label cytoplasmic MTOCs in oocytes (Pirmadjid and FitzHarris, unpublished data; Illingworth et al., 2010) and colocalise



**Figure 5.5. Exogenous SAS-6 occasionally formed diffuse patches around cytoplasmic MTOCs in MII eggs.** (A) Single confocal slice of a MII egg expressing eGFP-hsSAS-6 and MAP7-RFP exhibiting eGFP-hsSAS-6 patches around pre-existing MTOCs. Such accumulations were observed in approximately half (7/15) of eggs examined. (B) Example of accumulation of eGFP-hsSAS-6 at spindle poles in MII eggs. (C) Confocal slice of an eGFP-hsSAS-6-expressing GV oocyte illustrating the single accumulation of eGFP-hsSAS6 seen directly adjacent to the nucleus in ~50% of cases. Numerous cytoplasmic foci such as are caused by eGFP-Plk4 were never observed in eGFP-hsSAS-6-expressing GV oocytes. (D) A 2-cell-stage embryo expressing similar levels of eGFP-hsSAS-6 is shown for comparison. (E) Graph showing average relative fluorescence intensity of foci in GV oocytes, MII eggs and 2-cell embryos. Scale bar 10 $\mu$ m.



**Figure 5.6. SAS-6 overexpression does not affect the number of MTOCs in MII eggs.** (A) An eGFP-hsSAS-6-expressing egg with  $\beta$ -tubulin-labelled MTOCs. Antibodies against the kinesin 13 Kif2a were used to confirm MTOC identity. (B) Blinded quantification of the number of asters in eGFP-hsSAS-6 eggs ( $n=17$ ) compared to control eggs ( $n=21$ ). The data presented in this figure and figure 4.4 were conducted contemporaneously. Therefore statistical analysis was performed using ANOVA and Tukey-Kramers post-hoc test. Scale bar  $10\mu\text{m}$ .

with  $\beta$ -tubulin asters in MII eggs (Fig 5.6; Fig 4.4) confirming the ability of Kif2A to label MTOCs. Therefore, eggs expressing eGFP-alone or eGFP-hsSAS-6 were stained with antibodies against Kif2A as well as  $\beta$ -tubulin. The number of asters per egg was then scored in a blinded manner. No significant difference was observed between the number of MTOCs observed in eGFP- (13.00 $\pm$ 0.87 asters/egg) and eGFP-hsSAS-6-expressing eggs (13.65 $\pm$ 1.01 asters/egg;  $p > 0.05$ ; Fig 5.6). This result was also verified by counting the number of MAP7-RFP-labelled MTOCs in live eggs expressing eGFP-hsSAS-6 compared to control (eGFP-expressing) eggs. Similarly, no significant difference in the number of MAP7-labelled asters was observed between control and eGFP-hsSAS-6-expressing eggs (data not shown;  $p = 0.27$ ). Hence, in sharp contrast to Plk4, eGFP-hsSAS-6 fails to arrange into foci and is unable to drive the formation of new MTOCs in MII eggs.

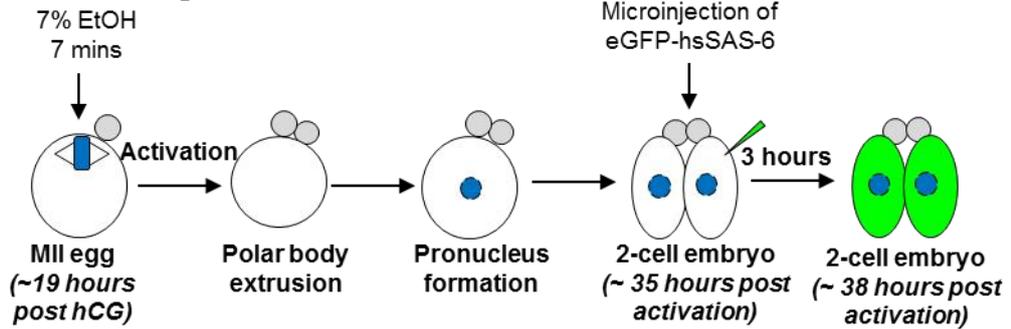
### **5.2.7. Activation of the SAS-6-dependent centriole formation pathway is not dependent on fertilisation**

In view of the finding that SAS-6 triggers new MTOC formation in embryos but not in oocytes, it was possible that the change in sensitivity of the *de novo* centriole formation pathway at the egg-embryo transition was dependent on fertilisation. To address this possibility, the ability of SAS-6 to form foci was investigated in parthenogenetically-activated 2-cell embryos, created by briefly exposing MII eggs to 7% ethanol (see Fig 5.7A for experimental procedure). Most SAS-6-overexpressing parthenogenetically-activated 2-cell embryos (~38 hours post activation) displayed multiple foci (Fig 5.7B; 19 out of 28 parthenogenetically-activated embryos examined had 29.78 $\pm$ 4.10 foci per embryo) similar to *in vivo*-fertilised embryos of the equivalent developmental stage (~52 hours post hCG (assumes fertilisation takes place ~14 hours after hCG administration); 37.00 $\pm$ 3.32;  $p = 0.19$ ). This data indicates that loss of suppression of the SAS-6-dependent pathway is not dependent on the fertilising sperm and is instead controlled by a maternal developmental programme.

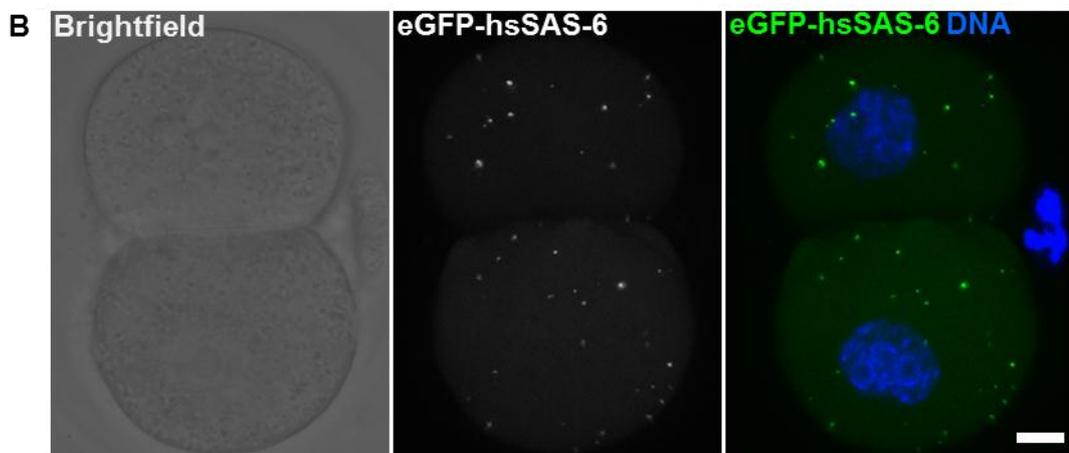
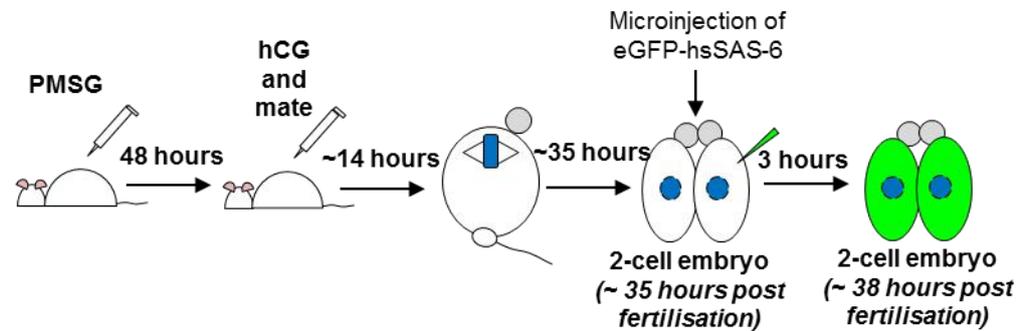
### **5.2.8. Activation of the SAS-6-dependent centriole formation pathway is not attributable to MAPK activity levels**

Recently it was found that overexpression of the Plk4 homologue (Plx4) in *Xenopus* leads to the *de novo* formation of MT asters in activated eggs but not in oocytes (Eckerdt et al., 2011). The authors proposed that high levels of MAP kinase activity in eggs (Eckerdt et al., 2011; Tunquist and Maller, 2003) are

### A.i Parthenogenetic activation



### A.ii Fertilisation



**Figure 5.7. Activation of the SAS-6-dependent centriole formation pathway is not dependent on fertilisation.** (A) Cartoon illustrating the experimental protocol for obtaining parthenogenetically-activated and fertilised 2-cell embryos expressing eGFP-hsSAS-6. (B) Typical confocal Z-projection of a 2-cell-stage parthenogenetically-activated embryo expressing eGFP-hsSAS-6. Numerous cytoplasmic eGFP-hsSAS-6 foci were observed in 19 of 28 parthenogenetic embryos examined. Scale bar 10  $\mu$ m.

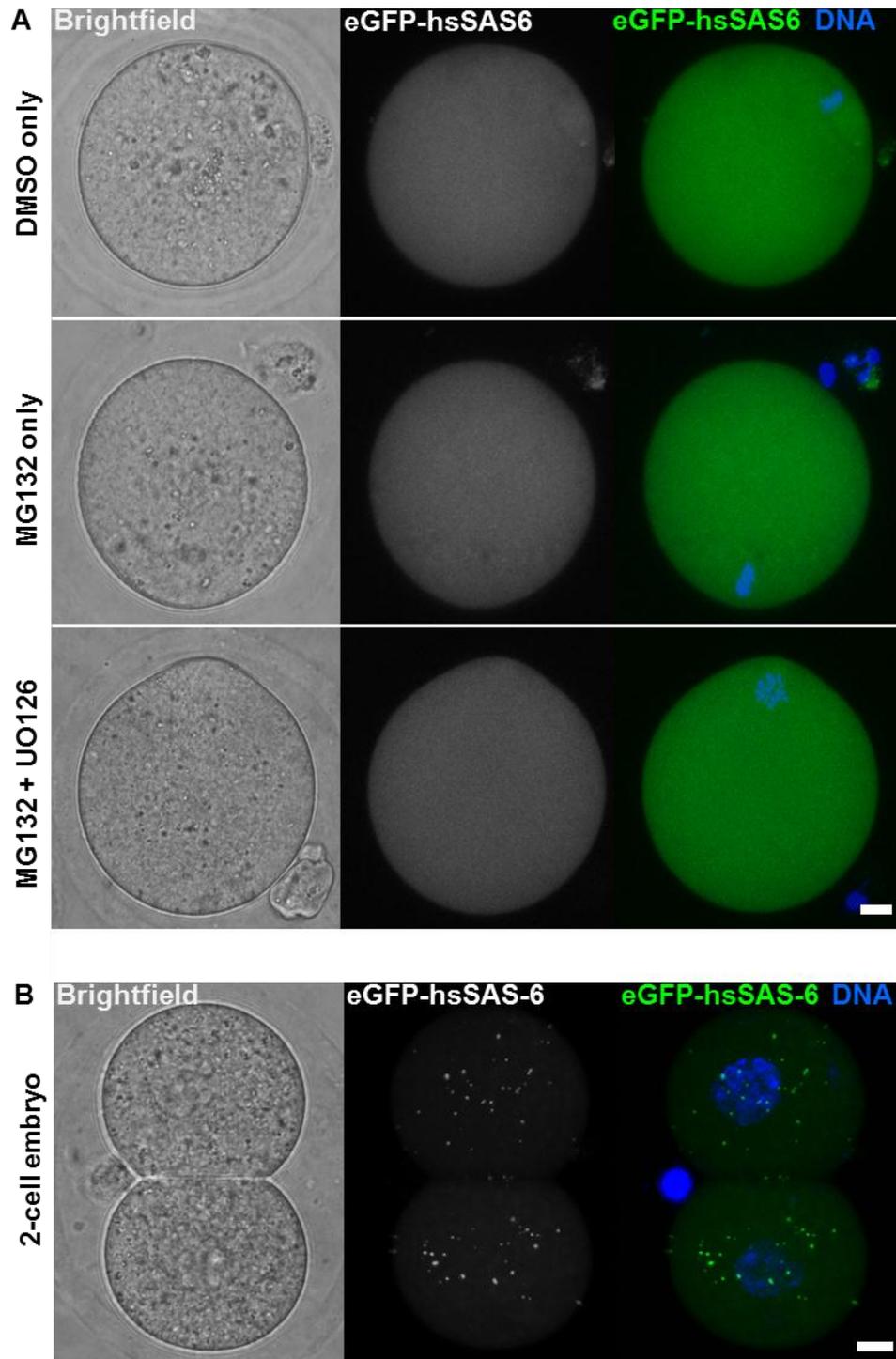
responsible for the inability of Plk4 to form MTOCs in oocyte cytoplasm (Eckerdt et al., 2011). Eckerdt and colleagues (2011) therefore reported a cytoplasmic shift in the ability of the centriole-replication protein Plk4 to trigger the formation of MTOCs in *Xenopus*. The same shift is not observed in mouse embryos, since overexpression of Plk4 was found to drive the formation of foci in all developmental stages examined (Fig 4.3). However, the shift in *Xenopus* is somewhat reminiscent of the shift in the ability of SAS-6 to induce centriole-like structures which was observed in mouse oocytes and embryos (Fig 5.4). Therefore, the role of MAP kinase in the shift in the ability of SAS-6 to induce foci between oocytes and embryos was investigated.

In order to address whether differences in MAP kinase activity were responsible for the inability of SAS-6 to induce foci in mouse eggs, UO126 was used to inhibit MAP kinase in eGFP-hsSAS-6-expressing eggs. UO126 has been previously used by our lab and others to rapidly deplete MAP kinase activity to ~30% of original levels within 3 hours (Marangos et al., 2003; Phillips et al., 2002). Since UO126 is known to trigger meiotic resumption of MII eggs (Phillips et al., 2002), the ability of UO126 to activate eggs was confirmed as a positive control on each experimental day. Three hours of UO126 treatment (50  $\mu$ M) did not cause eGFP-hsSAS-6 to form MTOCs (Fig 5.8), thus revealing that the inability of SAS-6 to induce foci in mouse eggs cannot be attributed to MAP kinase activity.

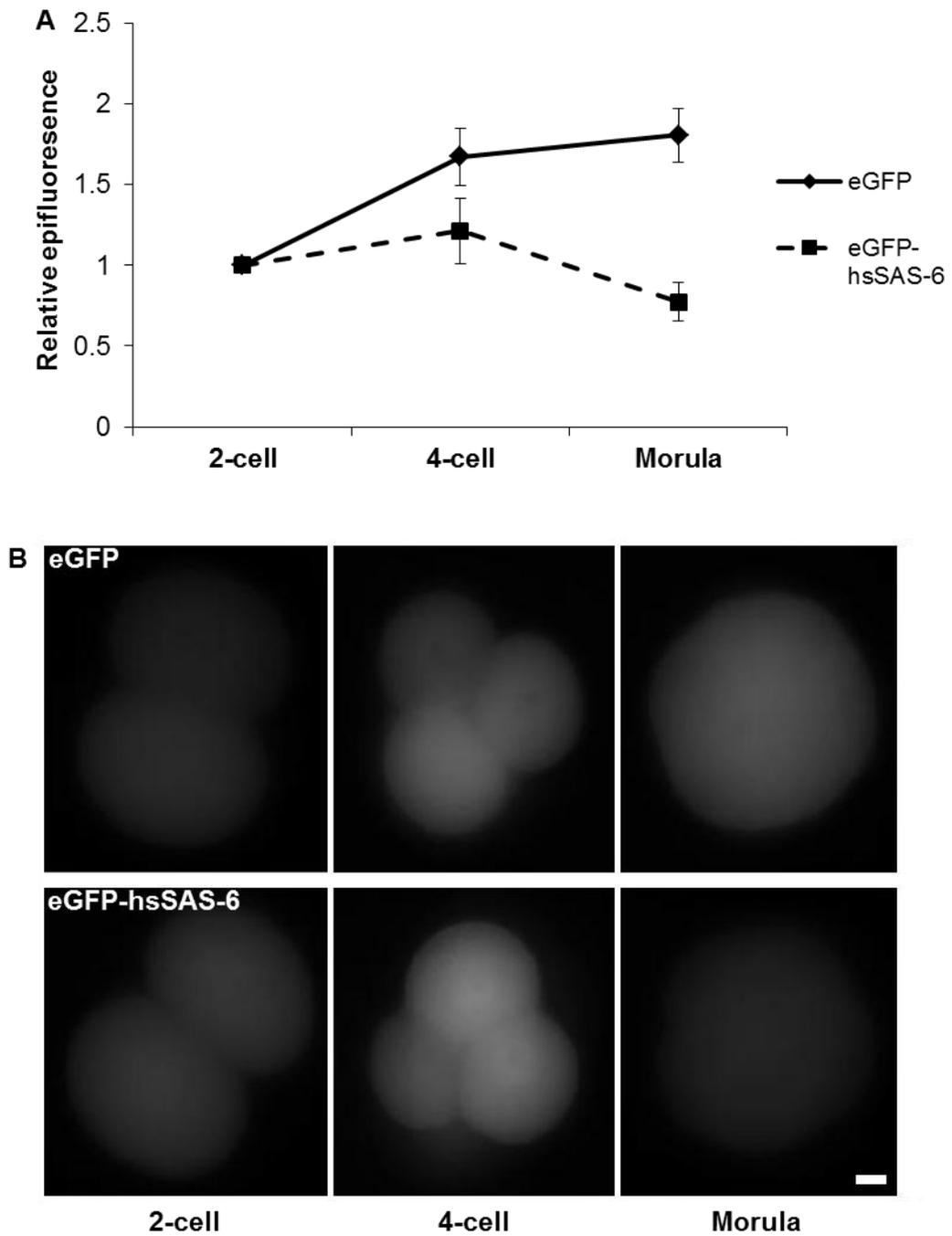
### **5.2.9. SAS-6 protein is degraded**

Having established that overexpression of exogenous SAS-6 can cause numerous cytoplasmic foci, it was next important to examine the effect of SAS-6 depletion in mouse embryos. Before attempting to knockdown SAS-6, the behaviour of SAS-6 protein in embryos was first examined.

SAS-6 has been shown to be targeted for degradation by the APC cofactor Cdh1 in human cells (Strnad et al., 2007). Therefore, it was possible that this degradation pathway may also play a role in SAS-6 destruction in mouse embryos. To investigate this possibility, relative eGFP-hsSAS-6 fluorescence levels were quantified over several cell cycles. After 2-3 hours of SAS-6 expression in 2-cell embryos, eGFP fluorescence was evident in the cytoplasm. eGFP fluorescence was quantified and the relative epifluorescence level then examined during each subsequent cell cycle. Having expressed to a maximum level by the 4-cell stage (Fig 5.9; eGFP-hsSAS-6: 1.21 $\pm$ 0.20 relative to 2-cell; eGFP: 1.67 $\pm$ 0.18



**Figure 5.8. eGFP-hsSAS-6 does not form MTOCs in eggs in the presence of U0126.** (A) Eggs were transferred to media containing DMSO, MG132 (50 $\mu$ M), or MG132 and U0126 (50 $\mu$ M) shortly after eGFP-hsSAS-6 mRNA injection, and imaged 3-5 hours later. MG132 was used to prevent U0126 from activating eggs. Characteristic centriole-like foci were not observed. Repeated on four experimental days. (B) Same-day eGFP-hsSAS-6-expressing 2-cell embryo shown for comparison. Scale bar 10  $\mu$ m.



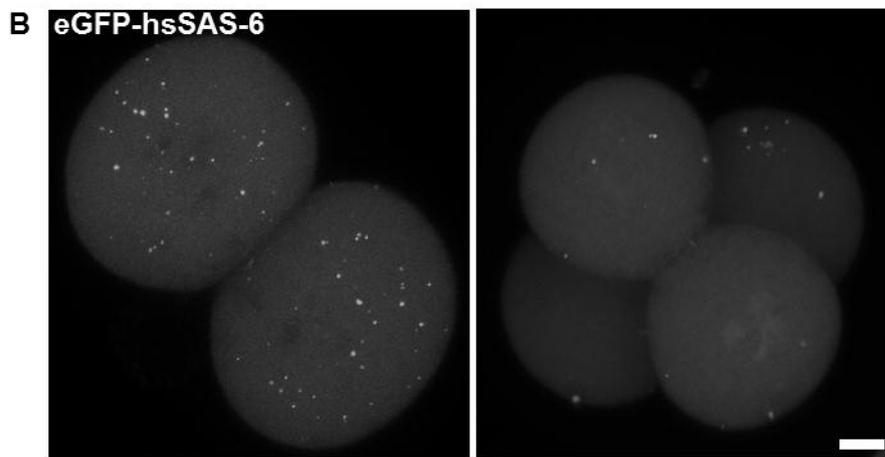
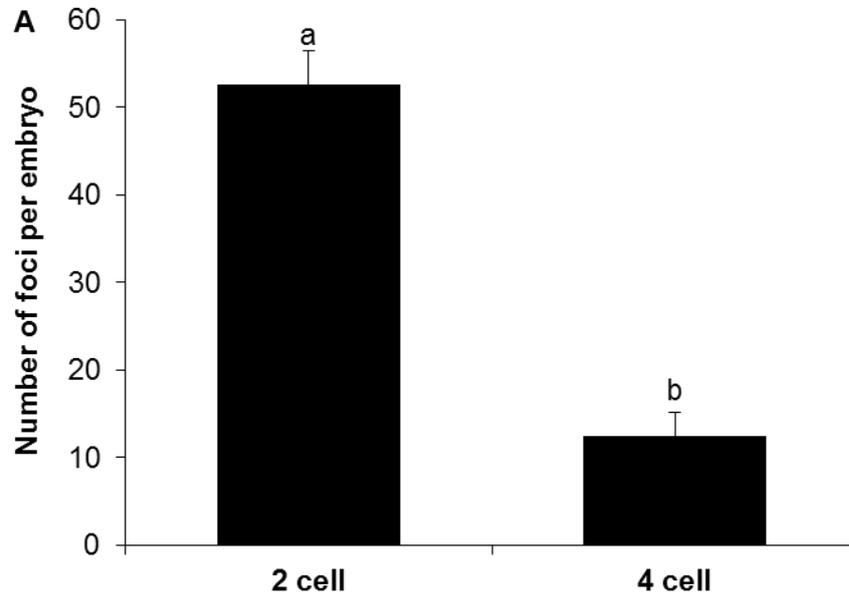
**Figure 5.9. SAS-6 protein is degraded.** (A) Relative fluorescence decreases from 4-cell to morula in eGFP-hsSAS-6 injected embryos compared to an eGFP controls. (B) Representative examples of epifluorescence in eGFP-hsSAS-6 injected embryos and eGFP-injected controls at the 2-cell, 4-cell and morula stage. 8-18 embryos per developmental stage examined. Scale bar 10  $\mu$ m.

relative to 2-cell,  $p=0.16$ ), the eGFP-hsSAS-6 fluorescence levels in morulae were found to have decreased dramatically compared to an eGFP-injected control (Fig 5.9; eGFP-hsSAS-6:  $0.77\pm 0.12$  relative to 2-cell; eGFP:  $1.80\pm 0.16$  relative to 2-cell,  $p=0.037$ ). This suggests that SAS-6 protein is destroyed over time. Transient overexpression of SAS-6 has been shown to induce multiple cytoplasmic foci in 2-cell embryos (Fig 5.2). Therefore, the fate of these spots after long term overexpression was next explored. Interestingly, if 2-cell embryos in which SAS-6 had been overexpressed were cultured for 24 hours to the 4-cell stage, the number of cytoplasmic accumulations was significantly reduced (Fig 5.10;  $12.47\pm 2.72$  foci per 4-cell embryo compared to  $52.6\pm 3.88$  per 2-cell embryo,  $p<0.0001$ ). Together these data suggest that as exogenous eGFP-hsSAS-6 is depleted, SAS-6 foci are also disassembled by exchange with the cytoplasmic pool.

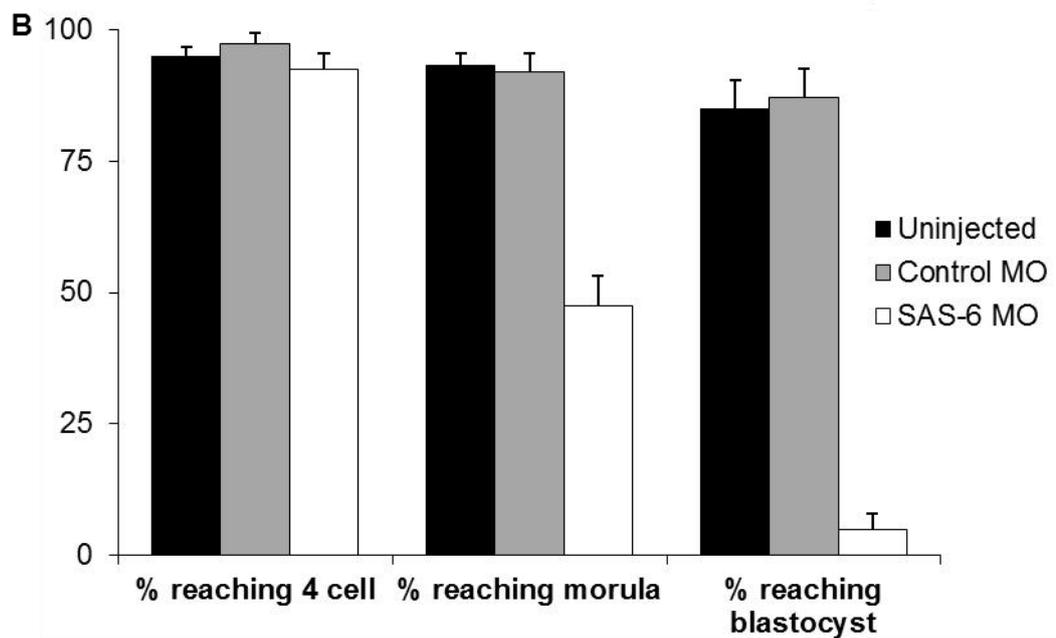
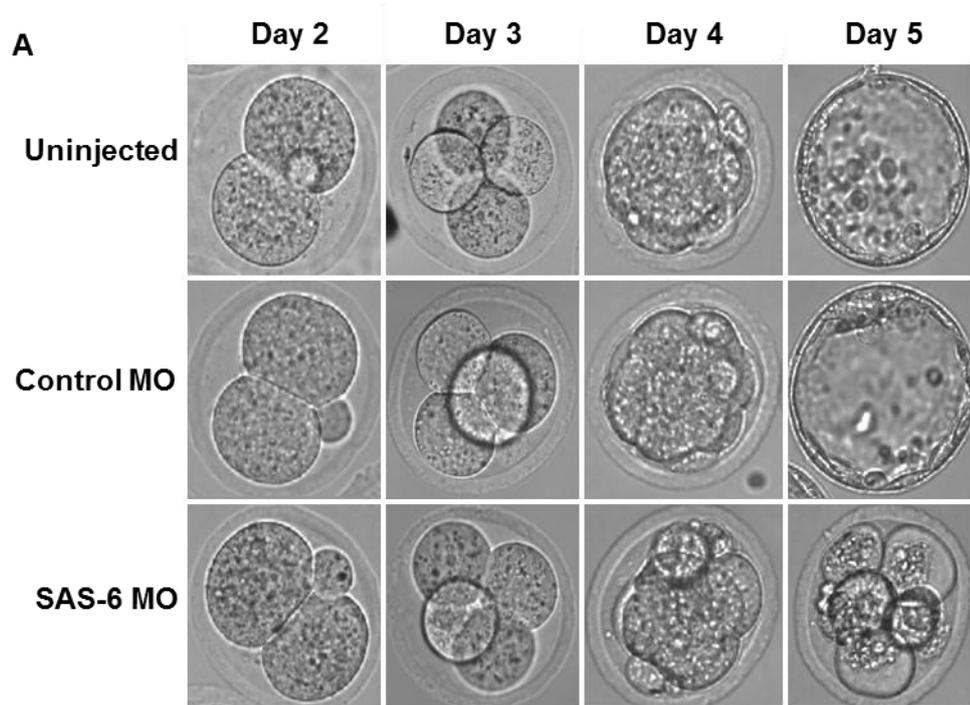
#### **5.2.10. Knockdown of SAS-6 induces prevents development to morula and blastocyst**

This rapid turnover of SAS-6 suggests that SAS-6 represents a suitable target for depletion using a morpholino (MO) oligonucleotide. Therefore, a MO oligonucleotide directed against mouse SAS-6 was microinjected into 2-cell embryos and developmental progression examined. In uninjected and control MO-injected groups, embryos develop normally to the blastocyst stage (uninjected:  $84.90\pm 5.53\%$  and control MO:  $87.05\pm 5.46\%$  reaching blastocyst). However, SAS-6 MO-injected embryos began to show abnormal development 4 days post hCG when only  $47.47\pm 5.70\%$  of embryos compacted (compared to  $91.88\pm 3.64\%$  of control MO-injected embryos;  $p<0.01$ ; ANOVA + Tukey-Kramers; Fig 5.11). Furthermore, SAS-6 MO-injected embryos consistently failed to cavitate and form blastocysts (SAS-6 MO:  $4.90\pm 3.09\%$  reaching blastocyst,  $p<0.01$ ; ANOVA + Tukey-Kramers; Fig 5.11).

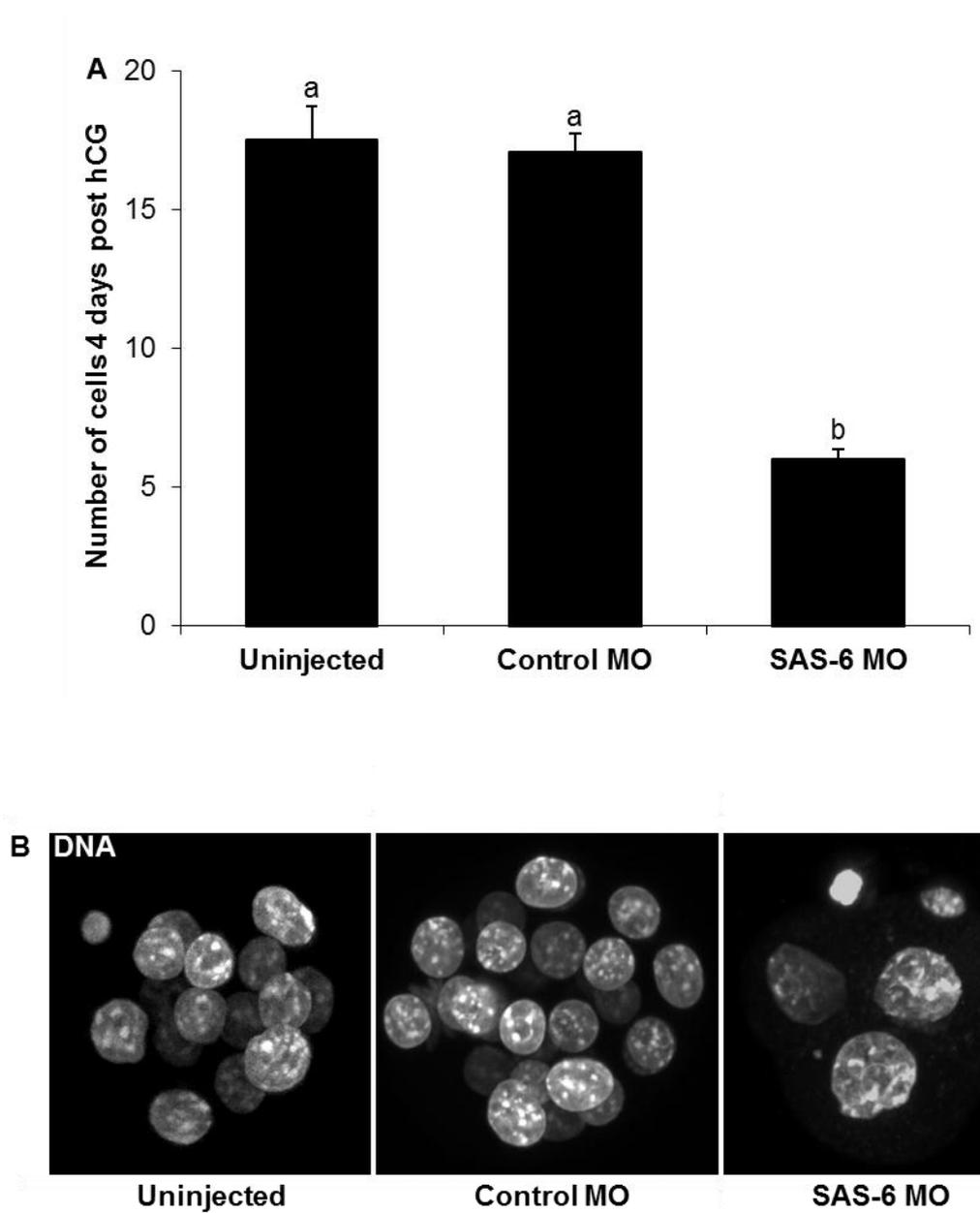
Since the embryonic failure phenotype occurs at the morula/blastocyst transition, I hypothesized that SAS-6 may be critical for the establishment and/or maintenance of polarity. However, it is well established that embryo compaction is a timed process which can occur regardless of the number of cells (Samake and Smith, 1996), therefore it was possible that those which had compacted did so with a reduced cell number. To distinguish between these possibilities, the number of labelled nuclei were counted in SAS-6 MO-injected embryos 4 days post hCG (i.e. normally morula stage). SAS-6 knockdown embryos were found to have significantly fewer cells (control MO:  $17.07\pm 0.66$  cells, SAS-6 MO:  $6.04\pm 0.36$  cells;  $p<0.01$ , ANOVA + Tukey-Kramers; Fig 5.12). Embryos depleted of SAS-6



**Figure 5.10. SAS-6 foci are not immortal.** Graph (A) and representative confocal Z-projections (B) showing number of foci induced after overexpression of eGFP-hsSAS-6 in 2-cell embryos (~3 hours expression) and ~24 hours of expression (4-cell). 15 embryos examined per stage. Scale bar 10  $\mu$ m.



**Figure 5.11. SAS-6 knockdown prevents development to the morula and blastocyst stage.** (A) Representative examples of embryo morphology at different stages of development (days post hCG) in uninjected, control MO- injected and SAS-6 MO-injected embryos. (B) Uninjected embryos and those injected with a control MO develop normally to blastocyst stage. However, 47.47 $\pm$ 5.70% of SAS-6 MO-injected embryos fail to compact and only 4.90 $\pm$ 3.09% form blastocysts. N=6 experiments.



**Figure 5.12.** *SAS-6 MO-injected compacted ‘morula’ have significantly fewer cells than controls. (A) Graph showing number of cells in control and SAS-6 MO-injected embryos 4 days post hCG. (B) Typical confocal Z-projections showing Hoechst-labelled nuclei used to count the number of cells in control and SAS-6 MO-injected embryos 4 days post hCG. Repeated on 6 experimental days. Scale bar 10µm.*

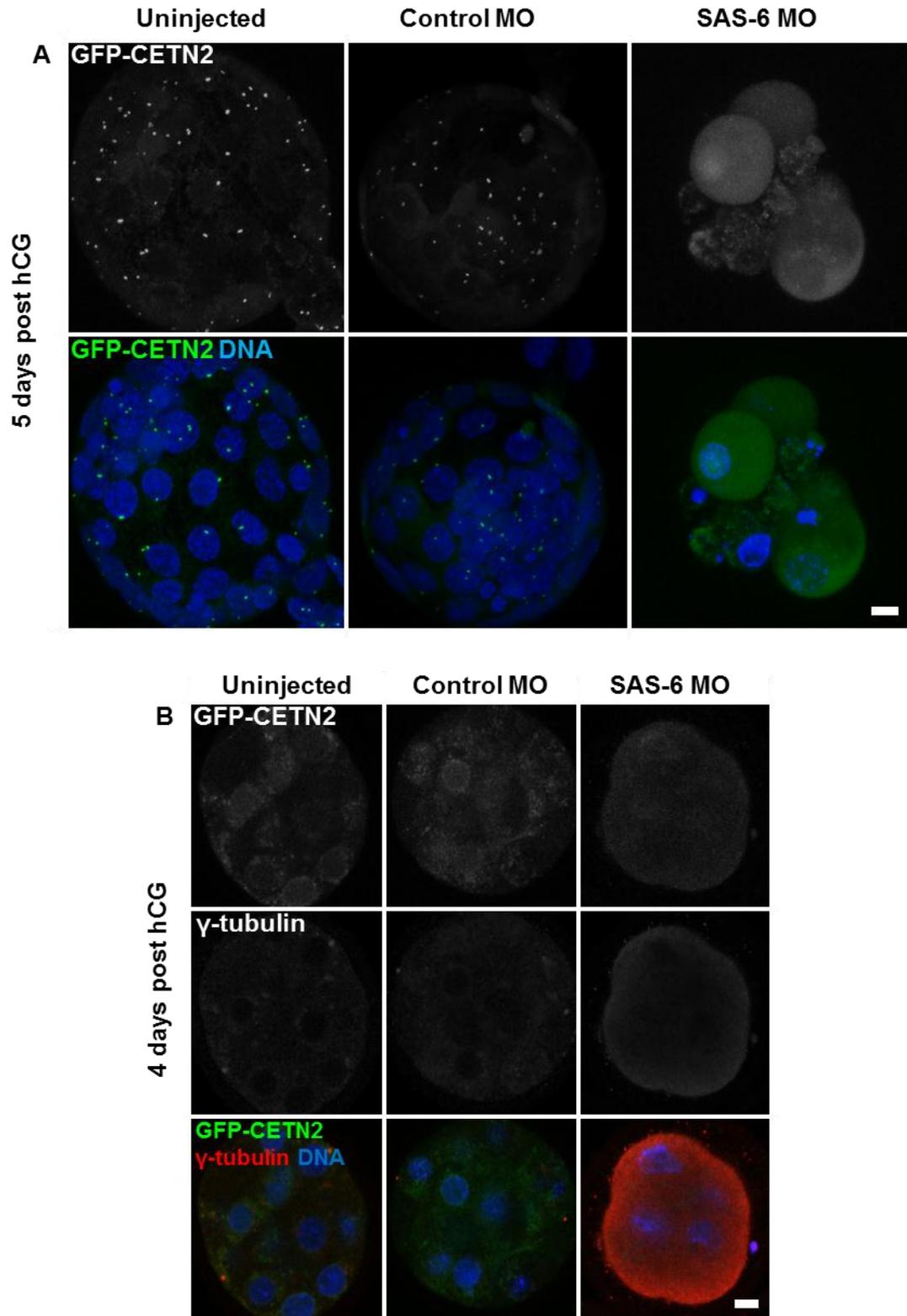
therefore typically fail to divide beyond the 4-8 cell stage and then undergo compaction. This result is unexpected as centrioles normally appear 4-5 cell divisions later at the blastocyst stage and therefore raises the possibility that SAS-6 could be playing a role in acentriolar cell divisions earlier in embryogenesis.

#### **5.2.11. SAS-6-depleted embryos lack centrioles and centrosomes**

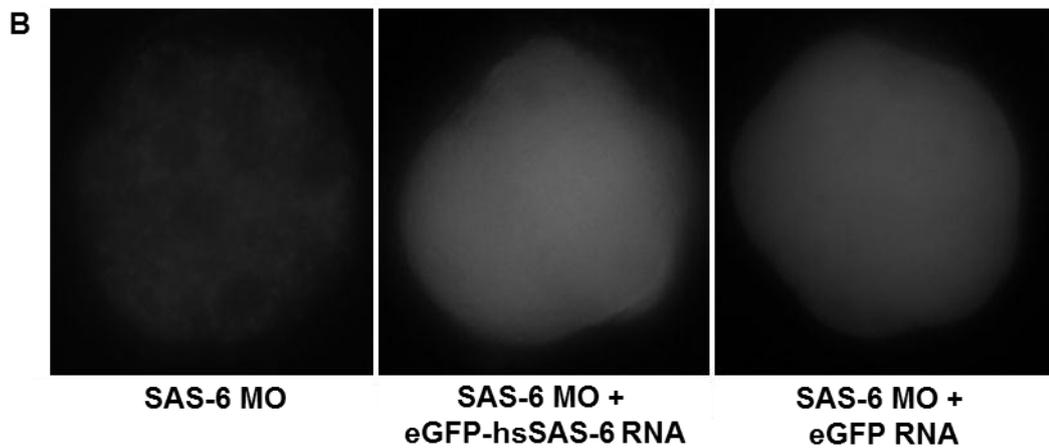
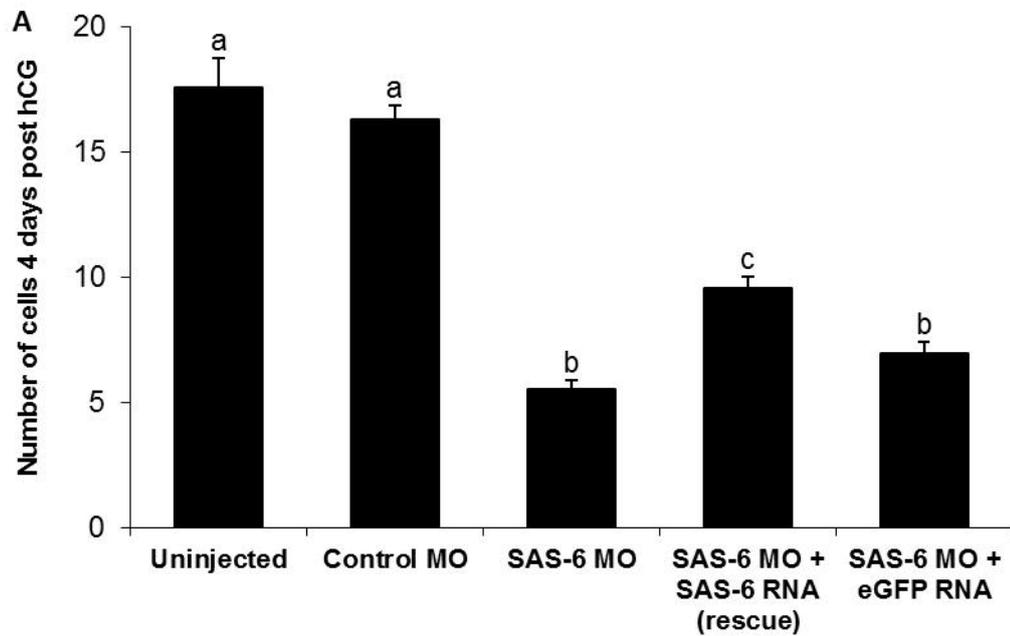
SAS-6-MO-injected embryos fail to develop beyond the 4-8 cell stage and therefore fail to reach the stage at which centrosomes/centrioles are expected to appear. Nevertheless, it remained plausible that centriole emergence itself might be a timed process rather than being linked to the number of cell divisions. Thus, SAS-6-depleted embryos were examined for the presence of centrioles/centrosomes. As previously, a SAS-6 MO was used to knockdown SAS-6 in GFP-CETN2-expressing embryos. 4 days post hCG (i.e. normally morula stage) embryos were imaged for the presence  $\gamma$ -tubulin-containing centrosomes. 5 days post hCG the presence of GFP-CETN2-labelled centrioles was examined. Whereas control MO-injected and uninjected embryos had normal centrioles and centrosomes, SAS-6 MO-injected embryos lacked both centrioles and centrosomes (Fig 5.13). Therefore, SAS-6 MO causes an early embryonic failure phenotype and these embryos lack centrioles and centrosomes.

#### **5.2.12. SAS-6 knockdown-induced embryonic failure can be partially rescued by hsSAS-6 RNA**

The early arrest of SAS-6 MO-injected embryos was unexpected since this embryonic failure occurs at the 4-8-cell stage (which is several cell cycles before the appearance of centrioles). Therefore, it was necessary to confirm the specificity of the knockdown. Since Western blots of centriole proteins are infeasible in mouse oocytes/embryos (see section 4.3.2), morpholino specificity was confirmed by attempting to rescue the phenotype with fluorescently-tagged wild type RNA. To this end, eGFP-tagged hsSAS-6 RNA was co-injected with the SAS-6 MO into 2-cell embryos in order to replace the endogenous SAS-6 protein. The cell number 4 days post hCG administration was found to partially recover after injection of the rescue RNA (SAS-6 MO: 5.52 $\pm$ 0.39 cells; SAS-6 MO + eGFP-hsSAS-6 RNA: 9.57 $\pm$ 0.42 cells;  $p < 0.01$ , ANOVA + Tukey-Kramers; Fig 5.14). Furthermore, injection of SAS-6 MO concomitant with eGFP RNA fails to recover the cell number phenotype observed in uninjected and control morpholino injected embryos (SAS-6 MO + eGFP RNA: 6.93 $\pm$ 0.49 cells; SAS-6 MO: 5.52 $\pm$



**Figure 5.13. SAS-6 morpholino-injected embryos lack centrosomes and centrioles.** (A) Confocal Z-projections of embryos 5 days post hCG. Note that control MO-injected and uninjected embryos possess centrioles (as labelled by GFP-CETN2) whereas SAS-6 MO-injected embryos lack centrioles. (B) Single confocal sections of embryos 4 days post hCG. Note that SAS-6 MO-injected embryos lack centrosomes (as labelled by  $\gamma$ -tubulin) Scale bar 10  $\mu$ m.



**Figure 5.14. *hsSAS-6* RNA partially rescues the *SAS-6* knockdown phenotype.** (A) Cell number at morula is significantly reduced in *SAS-6* morpholino-injected embryos. Embryos co-injected with *SAS-6* morpholino and *hsSAS-6* RNA have a partially recovered cell number. All comparisons significant ( $p < 0.05$ ) by ANOVA and Tukey-Kramers post-hoc test (excluding uninjected vs. control morpholino and *SAS-6* MO vs. *SAS-6* MO + eGFP RNA). (B) Representative examples of epifluorescence in morulae. Repeated on 4 experimental days.

0.39 cells,  $p > 0.05$ , ANOVA + Tukey-Kramers). This partial recovery effect may be due to proteasomal degradation of the exogenous SAS-6. In agreement with this theory, evidence has suggested that eGFP-hsSAS-6 is subject to degradation in mouse embryos (Fig 5.9;5.10). Nonetheless, the partial recovery was significant ( $p < 0.01$ , ANOVA + Tukey-Kramers) and repeatable (N=4) implying that the embryonic failure is in fact a result of SAS-6 depletion as opposed to unspecific knockdown of unrelated proteins.

### 5.3. Discussion

The experiments presented in this chapter investigate the role of SAS-6 in the control of centriole number in mouse embryos using knockdown and overexpression approaches. Unexpectedly, knockdown of SAS-6 produced an early embryonic failure phenotype as a result of which it was not possible to directly test the requirement for SAS-6 in centriole assembly in mouse blastocysts. Also unexpectedly, SAS-6 overexpression was able to form discrete foci in embryos, but not in immature or mature oocytes (Fig 5.4), thus alluding to a shift in the ability to form new centrioles at fertilisation. This shift was found to be independent of sperm contribution (Fig 5.7) and MAP kinase activity levels (Fig 5.8). The discussion which follows will focus on three main points. Firstly, the ability of SAS-6 to form centriole-like foci in mouse embryos will be addressed. Secondly, the unexpected outcome of SAS-6 morpholino experiments will be discussed and the implications of these results will be examined. Finally, the molecular basis of the shift in the cytoplasmic “sensitivity” to exogenous SAS-6 at the oocyte-embryo transition will be considered as a possible mechanism for maintaining the acentriolar status of oocytes.

#### 5.3.1. SAS-6 overexpression causes the formation of centriole-like foci in embryos but not in oocytes

In this chapter, immunofluorescence experiments show that endogenous SAS-6 relocates to become coincident with  $\gamma$ -tubulin at the time of centriole formation providing the first indication of a possible role for SAS-6 in *de novo* centriole formation in mouse blastocysts (Fig 5.1). SAS-6 has previously been shown to be a key determinant of daughter centriole number in canonical centriole replication (Strnad et al., 2007), as well as *de novo* centriole formation in *Drosophila* oocytes (Peel et al., 2007; Rodrigues-Martins et al., 2007b; Strnad et al., 2007). In agreement with this, overexpression of SAS-6 can cause the premature formation of centriole-like structures in normally acentriolar 2-cell mouse embryos (Fig 5.2). Since SAS-6 possesses a coiled-coil domain, SAS-6 overexpression may cause aggregation of the exogenous protein via coiled-coil domain interactions (Dammermann et al., 2004; Leidel et al., 2005). It would be interesting to establish whether SAS-6-induced foci possess the classical symmetric structure of normal centrioles (Azimzadeh and Marshall, 2010) or a tube-like centriole precursor similar to that observed upon overexpression of SAS-6 in *Drosophila* oocytes (Rodrigues-Martins et al., 2007a).

The formation of SAS-6-induced foci in 2-cell embryos is able to proceed in the absence of a MT network (Fig 5.2). Previous data showed that extra centrosome-like foci formed in the presence of MT depolymerisers can organise centrosomal proteins but do not contain a bonafide centriole (Collins et al., 2010; Khodjakov et al., 2002). As such, EM analysis will be useful to reveal any differences in the composition of centriole-like foci formed upon SAS-6 overexpression in embryos with or without an intact MT network.

Some SAS-6-induced foci are able to colocalise with the centriole replication factor Plk4 (Fig 5.3C). Plk4 is considered the major regulator of centriole replication and is involved upstream of SAS-6 in the centriole replication pathway (Bettencourt-Dias et al., 2005; Habedanck et al., 2005; Kleylein-Sohn et al., 2007). The finding that SAS-6 accumulations can recruit other centriole replication factors lends further support to the idea that these foci possess centriole-like function. Furthermore, the maintenance of SAS-6 at nascent procentrioles has found to be dependent on Plk4 (Strnad et al., 2007) consistent with the requirement of Plk4 for centriole replication (Habedanck et al., 2005; Kitagawa et al., 2009). It would therefore be interesting to test the ability of SAS-6 to induce cytoplasmic accumulations in Plk4-depleted embryos.

### **5.3.2. Morpholino experiments suggest an unexpected role for SAS-6 in acentriolar cells**

Having found that SAS-6 overexpression can induce the precocious formation of centriole-like foci (Fig 5.2-5.4), SAS-6 was then depleted using a morpholino oligonucleotide in order to elucidate whether SAS-6 is necessary for the physiological *de novo* centriole formation process occurring at the blastocyst stage in mouse embryos.

The data in this chapter suggest that SAS-6 is degraded in mouse embryos (Fig 5.9;5.10) and therefore represented a good target for knockdown using a morpholino. Knockdown of SAS-6 was found to prevent development to the morula and blastocyst stage (Fig 5.11), with SAS-6 morpholino-injected embryos compacting with an average of only 6.25 $\pm$ 0.45 cells (Fig 5.12). Compaction usually occurs at the 8-cell cleavage completion (Johnson et al., 1986) but cell cycle arrest has been shown to induce compaction of mouse embryos with fewer cells (Samake and Smith, 1996). These SAS-6-depleted embryos do not divide beyond the 4-8-cell stage and consequently fail to reach the stage at which  $\gamma$ -tubulin and centrioles

begin to emerge (morula and blastocyst stage respectively (Abumuslimov et al., 1994; Gueth-Hallonet et al., 1993; Szollosi et al., 1972)). Since centrioles appear 4-5 cell divisions after this embryonic failure phenotype is observed, it was possible that SAS-6 is playing a role in acentriolar cell divisions. SAS-6 is a substrate of the APC<sup>Cdh1</sup> (Strnad et al., 2007) and therefore knockdown of SAS-6 could promote degradation of other Cdh1 substrates essential for cell cycle progression. This substrate competition effect could explain the unexpected phenotype observed after knockdown of SAS-6. Alternatively SAS-6 may be involved in acentriolar cell divisions prior to centriole formation. However, this possibility was not investigated further. Unfortunately, the early embryonic failure observed upon injection of a SAS-6 morpholino meant that the requirement for SAS-6 in centriole assembly in mouse blastocysts could not be directly examined here.

If a lack of SAS-6 was responsible for the absence of centrioles until the blastocyst stage of mouse development, overexpression of SAS-6 in earlier stages may be expected to overcome this block to centriole formation. However, supplementation of SAS-6 alone was not sufficient for the formation of new centrioles since overexpression of SAS-6 in immature or mature oocytes was unable to trigger the formation of characteristic foci as seen in embryos (Fig 5.4). Moreover, SAS-6 accumulations were observed at the morula stage suggesting that acentriolar stages of development do possess SAS-6 protein despite being incorrectly localised (Fig 5.1). These findings imply that an absence of SAS-6 is unlikely to be the sole cause of the absence of centrioles in oocytes and early embryos. However, it remains possible that the absence of another centriole replication protein(s) may be responsible for the lack of centrioles in earlier stages of development.

In this chapter, evidence is found for degradation of SAS-6 in mouse embryos (Fig 5.9;5.10). Since APC<sup>Cdh1</sup> is active in 2-cell embryos (Li et al., 2007; Marangos et al., 2007; Reis et al., 2006), this lends support to the theory that SAS-6 is subject to proteasomal degradation in mouse early development. Proteasomal degradation pathways have now been found to be crucial for controlling the levels of many centriole replication proteins (Arquint et al., 2012; Cunha-Ferreira et al., 2009b; Guderian et al., 2010; Holland et al., 2010; Korzeniewski et al., 2009; Korzeniewski et al., 2010; Puklowski et al., 2011; Rogers et al., 2009; Strnad et al., 2007) and therefore the number of procentrioles produced in each round of centriole replication. Taken together, these data point to a potential role for an active proteasomal

degradation pathway targeting SAS-6 in mouse embryos and suggest that the regulation of SAS-6 levels, which is crucial for controlling centriole number in human cells (Strnad et al., 2007), may also be important for regulating centriole formation in mouse embryos.

### **5.3.3. The molecular basis of the shift in responsiveness to SAS-6 overexpression at the egg-embryo transition**

This work reveals that SAS-6 overexpression alone is not sufficient for the *de novo* formation of new centrioles in mouse oocytes and uncovers a shift in the ability to activate the *de novo* centriole formation pathway in response to SAS-6, wherein embryos, but not oocytes, are able to permit the formation of centriole-like structures in response to SAS-6 overexpression (Fig 5.4). On revealing this striking cytoplasmic shift between oocytes and embryos, further experiments were conducted in order to attempt to uncover the molecular basis for this switch. The transition was found to be independent of fertilising sperm since parthenogenetically-activated embryos also exhibited a similar number of SAS-6-induced foci (Fig 5.7). This suggests that the switch is under the control of a maternal developmental programme. Furthermore, ethanol activation induces a monotonic rise in intracellular  $Ca^{2+}$  concentration (Steinhardt et al., 1974; Swann and Yu, 2008), indicating that the repetitive  $Ca^{2+}$  oscillations which normally occur at fertilisation, are not necessary for sensitisation of the SAS-6-dependent *de novo* centriole formation pathway. Centrioles can also form *de novo* in parthenogenetically-activated rabbit blastocysts at approximately the same time as fertilised embryos (Szollosi and Ozil, 1991) which further supports the theory that a paternal contribution is not required for centriole formation. Szollosi and Ozil (1991) suggest that upon activation of the embryonic genome, a new gene encoding a master regulator is transcribed which initiates the formation of centrioles in the parthenogenetically-activated embryos. However, in mouse, the shift in the ability of centrioles to form *de novo* in response to SAS-6 overexpression occurs at the egg-embryo transition, rather than upon embryonic genome activation, suggesting that the shift from meiotic to mitotic cytoplasm permits the formation of SAS-6-induced foci. However, it remains possible that upon the egg-embryo transition in mouse, a centriole master regulator is activated which licenses the *de novo* formation of centrioles in response to SAS-6 overexpression.

Recently it was found that Plk4 expression in *Xenopus* leads to the *de novo* formation of foci in activated eggs but not in oocytes (Eckerdt et al., 2011). This is reminiscent of the cytoplasmic shift in the ability to

induce cytoplasmic foci which was observed upon overexpression of SAS-6 in mouse oocytes and embryos. Eckerdt and colleagues (2011) find that reactivation of MAP kinase by Mos supplementation is sufficient to block the formation of new centrioles in activated eggs expressing Plk4 and conversely, inhibition of the MAP kinase pathway was found to promote *de novo* centriole biogenesis in unfertilised eggs (Eckerdt et al., 2011). These data point to an additional cytoplasmic factor (potentially Mos-MAP kinase activity) as the inhibitor of premature centriole formation in *Xenopus*. However in mouse eggs, inhibition of MAP kinase activity in eggs did not permit the formation of SAS-6-induced MTOCs (Fig 5.8). In addition, in mouse, MAP kinase activity is initially low in GV oocytes and resumption of meiotic maturation is accompanied by a gradual increase in MAP kinase activity which then remains high throughout meiosis II (Verlhac et al., 1993; Verlhac et al., 1994). The experiments presented herein reveal an absence of SAS-6-inducible foci in both GV oocytes (where MAP kinase activity is low), but also in MII eggs which possess a high level of MAP kinase activity (Verlhac et al., 1993; Verlhac et al., 1994). Coupled with the finding that depletion of MAP kinase activity in SAS-6-expressing eggs fails to authorize the formation of MTOCs (Fig 5.8), these results indicate that downregulation of the SAS-6-inducible centriole formation pathway is not dependent on MAP kinase activity levels in mouse. Although the molecular basis of this switch is yet to be elucidated, it is interesting that both mice and *Xenopus* are able to repress the centriole biogenesis pathway in oocytes in response to the overexpression of a centriole replication protein.

Since some SAS-6-induced foci in mouse embryos are able to recruit Plk4 (Fig 5.3), this suggests that Plk4 protein is present in embryos and is supportive of a master role for Plk4 in centriole replication. However, a potential explanation for the lack of centriole-like foci formed in response to SAS-6 overexpression in oocytes could be a lack of upstream Plk4 kinase or Plk4 kinase activity in the earlier developmental stages. Recent work in *C. elegans* has revealed that phosphorylation of SAS-6 at a critical residue by the Plk4 homologue ZYG-1, is necessary for centriole formation (Kitagawa et al., 2009). Similarly, in human cells, Plk4 is vital for the function of SAS-6 at the centriole (Strnad et al., 2007). Finally, Plk4 has recently been shown to regulate the SCF ubiquitin ligase complex by phosphorylating the F-box protein FBXW5 (Puklowski et al., 2011). This phosphorylation was found to protect SAS-6 from degradation by inhibiting the ubiquitylating activity of the SCF, thereby controlling SAS-6 levels and regulating centriole replication (Puklowski et al., 2011). Together, these data suggest that the

interplay between SAS-6 and ZYG-1/Plk4 kinase is necessary for the promotion of new centriole formation and the regulation of centriole replication and therefore points to a possible absence of Plk4 as the limiting factor which prevents the formation of centriole-like foci in response to SAS-6 overexpression in oocytes. Thus, it would be interesting to examine Plk4 levels throughout mouse pre-implantation development. Western blots of centriole proteins in mouse oocytes are infeasible (see Chapter 4 discussion) and were therefore not attempted. Furthermore, the absence of a specific substrate for phosphorylation by Plk4 meant that direct determination of Plk4 activity levels was not possible. However, indirect evidence suggests that endogenous Plk4 activity is present in both mouse oocytes and embryos (Fig 4.5). Recent data has shown that expression of a kinase-dead form of Plk4 (D154A) in somatic cells can drive the formation of extra centrioles (Guderian et al., 2010). However, this overduplication effect is abrogated in the absence of endogenous Plk4. Thus, the ability of a Plk4 mutant (D154A) to induce foci indicates the presence of endogenous Plk4 activity (Guderian et al., 2010). In line with this, overexpression of Plk4 D154A was able to drive the formation of foci in GV oocytes as well as 2-cell embryos, suggesting that endogenous Plk4 activity is present in both stages. However, using this indirect approach it was not possible to quantify any small differences in Plk4 kinase activity so it remains possible that upregulation of Plk4 activity at the egg-embryo transition could permit the *de novo* formation of centriole-like foci in response to SAS-6 overexpression.

Another interesting possibility is that an additional cytoplasmic factor is acting to restrain centriole formation in response to SAS-6 in mouse oocytes. Recent data has revealed a role for Cep76 in restraining centriole duplication to once per cell cycle (Tsang et al., 2009). siRNA-mediated knockdown of this protein allows multiple rounds of centriole duplication in cancer cells and, intriguingly, the concomitant depletion of SAS-6 and Cep76 reverses this phenotype suggesting that these proteins may be acting antagonistically (Tsang et al., 2009). Therefore, a decrease in Cep76 levels could cause cells to become permissive for SAS-6-induced centriole amplification at the egg-embryo transition. Consistent with this hypothesis, the BioGPS portal ([www.biogps.org](http://www.biogps.org)) reveals a marked decrease in Cep76 gene expression between oocytes and blastocysts. Therefore, it would be interesting to investigate whether depletion of Cep76 licenses the formation of centrosome-like foci in response to SAS-6 overexpression in oocytes.

#### **5.3.4. Maintenance of the acentriolar status of the growing oocyte**

In order to ensure that the correct centriole complement is retained following fertilisation, most species employ differential contributions from each gamete (reviewed in (Manandhar et al., 2005; Schatten, 1994)). In most species, centrioles are eliminated in oogenesis and the sperm contributes the centrioles at fertilisation (Manandhar et al., 2000; Manandhar et al., 2005; Schatten, 1994). In mouse, both the oocyte and sperm lack centrioles (Manandhar et al., 1998; Manandhar et al., 1999; Szollosi et al., 1972) and therefore the first few mitotic divisions are acentriolar (Abumuslimov et al., 1994; Gueth-Hallonet et al., 1993). In order to obtain the correct number of centrioles at fertilisation, mouse oocytes must eliminate their centrioles in the pachytene stage of oogenesis which occurs during fetal development (Szollosi et al., 1972). Furthermore, the oocyte must then avoid manufacturing new centrioles *de novo* and instead remain acentriolar throughout the extended prophase I meiotic arrest period (up to 45 years in humans). Therefore, it is unsurprising that the oocyte employs additional means to prevent the appearance of supernumerary MTOCs in order to avoid the introduction of extra centrioles into the embryo at fertilisation. Whilst the molecular basis for the shift in permissiveness of the centriole biogenesis pathway in early murine development is still unclear, it is possible that suppression of the SAS-6-induced centriole biogenesis pathway and its activation at the egg-embryo transition may represent a common means for retaining the acentriolar status of the egg. However, evidence from *Xenopus* (Eckerdt et al., 2011) and mouse (this work) suggest that different species may employ diverse strategies to achieve this.

### **5.4. Summary**

In this chapter, the function of the structural replication protein SAS-6 in centriole manufacture in mouse pre-implantation development is investigated. The data presented in this chapter reveal that SAS-6 overexpression can trigger the formation of new centriole-like structures in mammalian cells.

Strikingly, this work also reveals a switch in the ability for SAS-6 overexpression to drive the formation of centriole-like structures at the egg-embryo transition. Although this shift was found to be independent of fertilisation or MAP kinase activity levels, the molecules underpinning this switch remain to be elucidated. It is well established that supernumerary centrioles can have devastating effects on cell division and chromosome segregation (Ganem et al., 2009; Nigg, 2006; Silkworth et al., 2009) and here I propose that this cytoplasmic shift represents a general strategy by which centriole biogenesis is

suppressed prior to fertilisation such that the oocyte is prevented from contributing dangerous extra centrioles to the zygote.

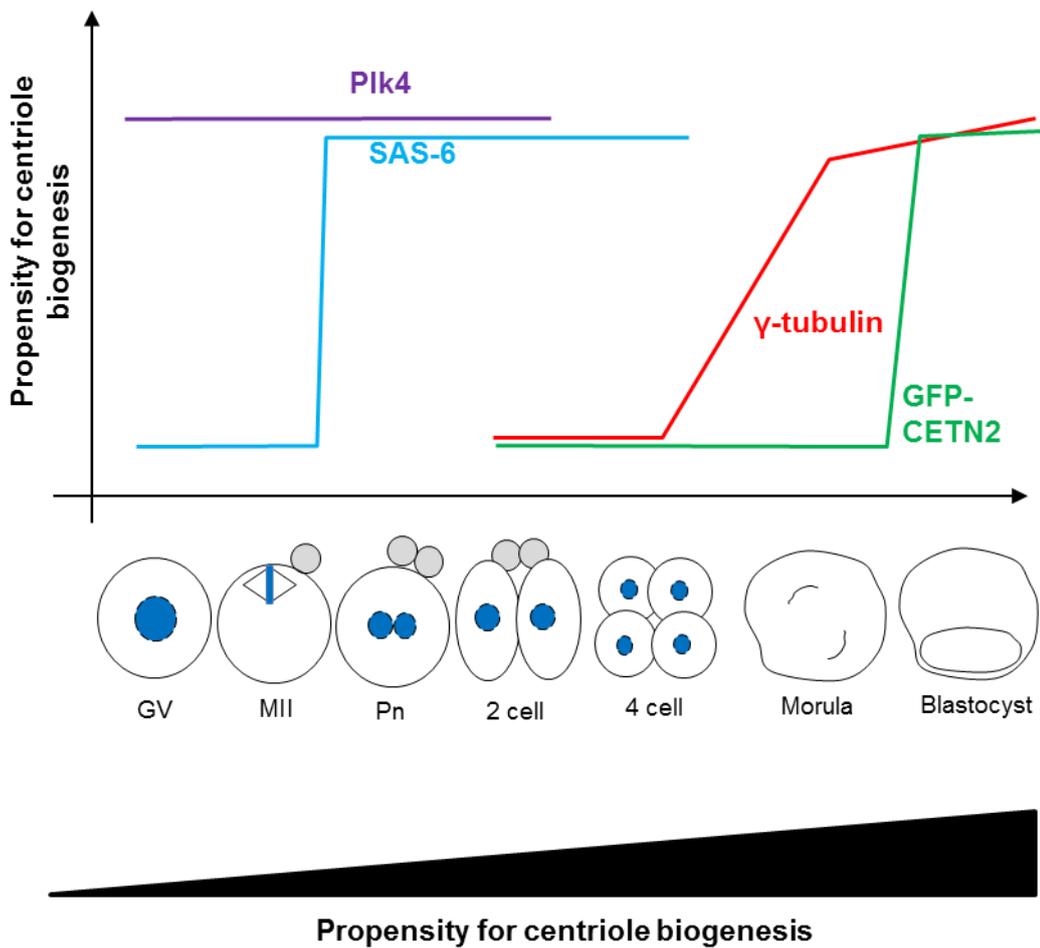
## **6. CONCLUSIONS**

This thesis has presented the results of experiments investigating the mechanisms of centriole biogenesis in early murine development. Firstly, in Chapter 3, tools were developed with which to examine centriole biogenesis in early mouse embryos. Using this GFP-CETN2 transgenic mouse model, previous work suggesting that centrioles are generated *de novo* at the blastocyst stage in mouse embryos was confirmed and extended to show that centrioles first appear preferentially in the TE lineage. This model was then used to examine the impact of centriole emergence upon MT organisation and dynamics in mouse embryos. In Chapter 4, overexpression of Plk4 was found to drive the *de novo* formation of centrosome-like structures in all developmental stages examined. These foci cause aberrant spindle formation. Interestingly, the proportion of multipolar spindles was decreased later in mitosis pointing to a mechanism to ensure bipolar mitoses in cells with *de novo*-formed extra centrosome-like structures. Furthermore, embryos can apparently tolerate these multipolar spindles since embryo development and chromosome segregation proceed normally. In Chapter 5, experiments show that SAS-6 overexpression is able to drive the formation of foci in embryos but, strikingly, is not able to do so in oocytes thus indicating a shift in the ability of the cytoplasm to support the presence of new centrioles at the egg-embryo transition. The main findings of the thesis are illustrated in the schematic diagram shown in figure 6.1.

The implications of each of the findings have been discussed in the individual chapters in which the experiments were performed. Therefore, in this final section I will elaborate on some topics of interest and highlight some of the remaining questions in this field. I will first focus on the finding that centrioles are manufactured at the blastocyst stage and address possible reasons as to why this occurs at this particular stage of development and how this process is controlled at a molecular level (6.1). Following this, some final remaining questions in the field of centriole biogenesis will be discussed (6.2).

### **6.1. On the timing of centriole emergence in mouse embryos**

An important outcome of this work is that a state-of-the-art approach has been developed for monitoring centriole emergence in mouse embryos. Using this model I provide important corroboration of the observation originally made by EM, that centrioles are first manufactured *de novo* in mouse blastocysts (Abumuslimov et al., 1994; Gueth-Hallonet et al., 1993; Szollosi et al., 1972). In this section I will



The establishment of polarity as a trigger for centriole formation? (6.1.1.)
The absence of a centriole replication factor in early development? (6.1.2.)
An inhibitory factor in oocytes and early embryos? (6.1.3.)
Increasing length of S-phase allows centriole formation? (6.1.4.)

**Figure 6.1.** Cartoon diagram to illustrate some of the findings of this thesis. Note that the propensity for the formation of centrioles/centrosome-like structures increases throughout pre-implantation development. The coloured boxes indicate possible explanations for the mechanism which may limit centriole formation in early mouse embryos. The section in which these explanations are discussed are indicated in brackets.

examine possible physiological explanations as to why centriole emergence occurs specifically at the blastocyst stage in mouse embryos and the molecular basis underpinning this. In this section various hypotheses pertaining to how centriole formation is controlled in mouse oocytes and early embryos will be discussed. These possible explanations are also shown on the schematic diagram in Figure 6.1.

#### **6.1.1. Is there a link between centriole emergence and the establishment of cellular polarity?**

Centrosomes are known to play an important role in cell symmetry breaking and the maintenance of cell polarity in many organisms (Bornens, 2012). In *C. elegans* embryos, the sperm centrosome triggers the asymmetric distribution of Par proteins and the establishment of the polarization axis in the zygote (Cowan and Hyman, 2004; Zonies et al., 2010). In addition, the centrosome has been proposed to be important in asymmetric cell divisions in larval neuroblasts as well as male germline stem cells in *Drosophila*. Here, the mother centrosome is always inherited by the stem cell and the daughter centrosome is distributed to the differentiating cell (Basto et al., 2008; Bettencourt-Dias et al., 2005; Januschke et al., 2011; Rodrigues-Martins et al., 2008; Rusan and Peifer, 2007; Stevens et al., 2007; Yamashita et al., 2007). In contrast, *Drosophila* female germline stem cells do not require centrioles (Stevens et al., 2007).

A possible reason why centrioles are not required until the blastocyst stage of mouse embryo development could be related to the establishment of polarity at this stage. Individual embryo blastomeres do not show any obvious inherent polarity until compaction when outward facing (apical) regions become distinct from inward facing (basolateral) regions. At this stage the membrane protein ezrin and the polarity proteins Par3 and Par6 accumulate at the apical domain (Louvvet et al., 1996; Plusa et al., 2005) and Par1 localises basolaterally (Vinot et al., 2005). Subsequent divisions give rise to two populations of cells (Johnson and Ziomek, 1981; Sutherland et al., 1990): outside polar cells which eventually become the trophoectoderm (TE) and inside, non-polar cells which produce the inner cell mass (ICM). At the 32-cell stage, a fluid-filled cavity known as the blastocoele forms and the embryo is then considered a blastocyst. At this point cells become committed to either the TE or ICM lineage.

Since centrosomes are crucial for the establishment and maintenance of polarity, it is possible that they only become necessary for cell divisions at the blastocyst stage as this is the stage at which the

intracellular polarity becomes evident. It is interesting that *de novo* centriole formation also occurs at the blastocyst stage in parthenogenetically-activated rabbit embryos (Szollosi and Ozil, 1991), as well as in fertilised mouse embryos. This suggests that the establishment of polarity which occurs at the blastocyst stage may represent the reason why centrioles are manufactured at this stage. To investigate this possibility further it would be interesting to expedite the formation of blastocysts, for example by removal of cytoplasm from the pronucleate embryo as demonstrated by Feng and Gordon (Feng and Gordon, 1997), and examine the effect of this precocious blastocyst formation on centriole emergence. Furthermore, regulators of polarity such as ezrin/E-cadherin (Aghion et al., 1994; Dard et al., 2001) or regulators of blastocoele cavity formation such as Na<sup>+</sup>/K<sup>+</sup>-ATPase (Madan et al., 2007) could be manipulated in early mouse embryos and the effect on centriole emergence examined.

### **6.1.2. Is there an absence of a key centriole replication factor in oocytes and early embryos?**

An attractive explanation for the lack of centrioles in mouse oocytes and early embryos could be that early developmental stages lack a crucial centriole replication protein which is then synthesized or activated at the blastocyst stage. A possible candidate for this missing factor could be one or more of the core centriole replication proteins as these have been shown to be important in controlling centriole number in many cell types (see Appendix 1). To this end it would be interesting to analyse the levels of centriole replication proteins throughout mouse early development. However, previous attempts at Western blots of SAS-6 in 40 *Drosophila* oocytes failed to detect any protein (Peel et al., 2007). Given that *Drosophila* oocytes are ~9000x larger in volume than mouse oocytes (~9nL and ~270pL respectively), Western blots are not feasible for centriole replication proteins in mouse oocytes or embryos. Thus, RT-PCR for detection of transcript levels of centriole replication proteins may provide an alternative approach.

Despite this appealing theory, individual overexpression of several different centriole replication proteins (SAK/SAS-6/SAS-4/Asterless) can result in *de novo* centriole assembly in *Drosophila* eggs (Dzhindzhev et al., 2010; Peel et al., 2007; Rodrigues-Martins et al., 2007a; Rodrigues-Martins et al., 2007b; Stevens et al., 2010a). This suggests that inhibition of the *de novo* formation pathway in *Drosophila* may not be due to a deficiency of one particular protein (Loncarek and Khodjakov, 2009). Similarly, individual overexpression of either SAS-6 or Plk4 can drive the *de novo* formation of centrosome-like foci in mouse

embryos (this work), indicating that a lack of one of these proteins is unlikely to be the limiting factor which prevents *de novo* centriole formation until the blastocyst stage. The finding that SAS-6 supplementation is not sufficient to drive the formation of foci in mouse oocytes also suggests that a lack of SAS-6 alone is not the cause of the absence of centrioles in early murine development. Moreover, *de novo* centriole formation is normally suppressed in unfertilised *Drosophila* eggs which possess sufficient subunit pools to make millions of centriole pairs (Peel et al., 2007; Rodrigues-Martins et al., 2008). Finally, physical removal of centrioles induces *de novo* centriole assembly despite endogenous levels of Plk4 and SAS-6 (Khodjakov et al., 2002; La et al., 2005; Uetake et al., 2007). Collectively, these data suggest that the absence of centrioles in mouse early development may not be simply due to the lack of a centriole replication protein in these early stages and points to an alternative mechanism to prevent the untimely appearance of centrioles until the blastocyst stage.

### **6.1.3. Is there an inhibitory factor in oocytes and early embryos?**

An alternative explanation for the absence of centrioles in mouse early development could be due to an additional inhibitory mechanism that specifically acts to prevent *de novo* centriole formation in acentriolar cells. One strategy for preventing centriole emergence in mouse oocytes and embryos could be the persistent activity of a factor which is also involved in centriole elimination in oogenesis.

Recently, the cyclin-dependent kinase (cdk) inhibitor cki-2 has been implicated in centriole elimination in *C. elegans* oocytes (Kim and Roy, 2006). In this study, centriole-like structures persisted into late stages of oogenesis in cki-2-depleted worms. Moreover, this defect can be suppressed by reducing cyclin E or cdk2 levels (Kim and Roy, 2006). This data implies that the regulation of cyclin E-cdk2 by cki-2 is critical for the appropriate elimination of maternal centrioles during oogenesis. However, EM analysis would be needed to confirm centrioles are indeed maintained in oocytes from cki-2-depleted worms. Since the mechanism of centriole formation in *C. elegans* has many parallels with other species, it is an interesting possibility that Cdk inhibitors may play a role in centrosome elimination in other species such as mouse. Extrapolating this idea, it is also possible that high levels of cki-2 activity could persist in mouse embryos until the blastocyst stage when the *de novo* formation of centrioles is permitted. Although, cyclin E1 and E2 were found to be ubiquitously expressed in embryo development, mice deficient in cyclin E or Cdk2 display limited phenotypes suggesting that cyclin E-cdk2 function could be

redundant in embryos or other cell cycle proteins could compensate for a lack of these proteins (Yu and Sicinski, 2004). This could point to activity of cki-2 in these early stages, which prevents precocious centriole emergence. More broadly, it is interesting to speculate that the mechanisms which destroy centrioles in oocytes might also be responsible for preventing the precocious appearance of centrioles until the blastocyst stage.

Another possible factor which could be inhibiting the formation of centrioles is Cep76. This protein has recently been found to specifically restrain centriole over-replication in cancer cells (Tsang et al., 2009). It is interestingly to speculate that a decrease in Cep76 levels (or some similar factor) could cause cells to become permissive for *de novo* centriole formation at the blastocyst stage. Since the *de novo* formation of centrioles in mouse blastocysts occurs in a tightly coordinated manner and thereafter centriole replication proceeds normally, it is doubtful that a complete depletion of Cep76 occurs at the blastocyst stage as this could have deleterious effects on centriole replication in later cycles. It is more likely that the expression or activity of Cep76 (or similar factor) might be reduced below a threshold level which permits *de novo* centriole formation but does not decrease enough to permit centriole over-replication in subsequent cycles of centriole replication. Therefore, it would be interesting to examine the role of Cep76 in *de novo* centriole formation in mouse embryos by investigating whether depletion of Cep76 in acentriolar mouse embryos can drive the precocious formation of centrioles.

#### **6.1.4. Is there a link between centriole emergence and the duration of S-phase?**

In the previous two sections, the possibility that a change in the levels of a particular factor (either an inhibitor or a promoter) at the blastocyst stage licenses the cell for *de novo* centriole formation was discussed. However, another feasible explanation for the absence of centrioles in earlier stages might be that the length of S-phase is not sufficiently long enough to permit *de novo* centriole formation until the blastocyst stage.

*De novo* centriole formation can be induced in S-phase-arrested CHO cells after removal of endogenous centrioles (Khodjakov et al., 2002). However, this process takes ~24 hours which is much greater than the duration of one complete cell cycle in this cell type (Khodjakov et al., 2002). This finding also explains why centrioles are not regenerated after centriole removal in cycling cells (Hinchcliffe et al., 2001;

Khodjakov and Rieder, 2001). Thus, *de novo* centriole formation requires a much longer S-phase than is physiological for CHO cells. Whereas the *de novo* formation of centrioles in cultured cells is a slow process (Khodjakov et al., 2002; La et al., 2005), *de novo* centriole formation in response to overexpression of centriole replication proteins is considerably faster (Peel et al., 2007; Rodrigues-Martins et al., 2007b). However, *de novo* centriole formation in *Drosophila* eggs still takes longer than canonical centriole replication in the same organism (Dzhinzhev et al., 2010; Peel et al., 2007; Rodrigues-Martins et al., 2007b), suggesting that centrioles take longer to assemble in the absence of a mother centriole. Rodrigues-Martin et al., (2007b) propose that assembly is faster in the presence of centrioles as the mother centriole provides a platform for regulatory molecules which catalyse the assembly of daughter centrioles. The establishment of this platform would therefore be less efficient without an existing mother centriole. This adds further weight to the notion that a shorter S-phase duration may be preventing premature *de novo* centriole formation in mouse blastocysts.

In order to investigate this hypothesis it would be necessary to examine the length of S-phase in progressive cell cycles in mouse early development by expression of an S-phase marker such as PCNA (proliferating cell nuclear antigen) which has previously been used to visualise S-phase in *C. elegans* and sea urchin embryos (Kisielewska et al., 2005). Alternatively, a fluorescence ubiquitination-based cell cycle indicator (termed Fucci) could be used. This probe exploits the cell cycle-regulated degradation of Cdt1 and geminin to label the nuclei of cells in G1 in red whilst those in S/G2/M-phase appear green (Newman and Zhang, 2008; Sakaue-Sawano et al., 2008). This tool therefore allows tracking of cell-cycle progression in a multicellular context and has been used to monitor cell-cycle progression in cultured cells (Kaida et al., 2011; Kaida and Miura, 2012; Malka and Eden, 2011), as well as in developing zebrafish embryos (Sugiyama et al., 2009).

## **6.2. Other outstanding questions concerning centriole biogenesis in early murine development**

In this section I will cover some remaining questions in this field and highlight some alternative strategies which could be used to further investigate the mechanism of *de novo* centriole biogenesis and how it is controlled in mouse oocytes and early embryos.

### **6.2.1. Is there a similar hierarchy of involvement of centriole replication proteins in *de novo* centriole formation as well as canonical centriole replication?**

Following overexpression of the centriole replication proteins SAK (the *Drosophila* Plk4 homologue), SAS-6 and SAS-4 in various *Drosophila* cell types, a clear hierarchy emerges regarding the potency of these three proteins at stimulating the formation of centriole-like structures (Peel et al., 2007). In *Drosophila* cells it appears that the further upstream in the replication pathway, the more potent the protein is at inducing the formation of centrioles following overexpression (Peel et al., 2007). In mouse a similar hierarchy was observed in which the master regulator of centriole replication, Plk4, can drive the formation of foci in all developmental stages but the downstream SAS-6 can only drive the *de novo* formation of centriole-like structures in later stages of development. It is therefore tempting to speculate that overexpression of CPAP (the mammalian homologue of SAS-4) in mouse embryos would be less potent than either SAS-6 or Plk4 and fail to drive *de novo* centriole formation, reflecting its position at a late stage of centriole biogenesis (Kohlmaier et al., 2009; Pelletier et al., 2006; Schmidt et al., 2009; Tang et al., 2009) and predicted inability to assemble the upstream factors into a functional and structurally recognisable centriole

### **6.2.2. What is the impact of preventing centriole emergence on early mouse development?**

An initial aim of this project was to investigate the impact of a lack of centrioles on development in the mouse model system. Seminal work by Raff and colleagues illustrated that flies were capable of developing to adulthood in the absence of centrioles (Basto et al., 2006). Although the centriole assembly protein SAS-4 was found to be crucial for centriole replication, SAS-4 mutant flies develop with normal timing into morphologically normal adults (Basto et al., 2006). Hence, I endeavored to knockdown another well characterised centriole replication protein SAS-6 in mouse embryos. However, SAS-6-depleted embryos undergo early developmental failure before the stage at which centrioles normally appear. Although the reason for this unexpected outcome was not elucidated, regrettably this meant that the impact of centriole absence on mouse embryo development was not examined here. Nevertheless, it may still be possible to knockdown another centriole replication protein and prevent the emergence of centrioles in mouse blastocysts. Although adult flies were able to develop in the absence of centrioles, these flies lack cilia in their sensory neurons and thus are highly uncoordinated. Therefore, it is likely that a lack of centrioles would have a more devastating effect on vertebrate cells, which often have primary

cilia in order to respond to extracellular signals and to facilitate the development of organs such as the kidney (Oh and Katsanis, 2012).

Alternative strategies to investigate the effect of depletion of centriole replication proteins on centriole emergence could include the use of dominant-negative proteins or morpholinos raised against other centriole replication proteins. Since work in human cells has shown that Plk4 and CPAP are both subject to proteasomal degradation to regulate their levels throughout the cell cycle (Cunha-Ferreira et al., 2009a; Cunha-Ferreira et al., 2009b; Korzeniewski et al., 2010; Rogers et al., 2009; Tang et al., 2009), this suggests that these proteins may be rapidly turnover in mouse embryos which would make them good targets for knockdown with morpholinos. Furthermore, use of the *ZP3-Cre-LoxP* system to specifically knockdown a centriole replication factor in mouse oocytes and resulting early embryos could prove informative by specifically allowing investigation of the effect of a lack of centriole replication protein on centriole emergence at the blastocyst stage. Interestingly, *SAK*<sup>-/-</sup> embryos arrest after gastrulation at E7.5 (Hudson et al., 2001). It would interesting to further examine these *SAK*<sup>-/-</sup> embryos to ascertain if centrioles still emerge in the absence of *SAK*.

### **6.2.3. What is the impact of exogenous centrioles in cloned embryos?**

Another important question in centrosome biology concerns the effect of the introduction of foreign centrioles into cells which normally lack centrioles and the fate of these exogenously-introduced centrioles. During the process of somatic cell nuclear transfer (SCNT) which is used in the production of cloned embryos, a recipient oocyte is enucleated and the nucleus of a donor somatic cell is then introduced into the anuclear recipient oocyte. Importantly, the donor cell's centrosome is introduced into the recipient oocyte along with the nuclear material. The exogenous centrosome must therefore perform all the functions of the embryonic centrosome and thus must be remodeled by the enucleated oocyte. However, the fate of the donor centrosome and its impact on spindle assembly has not been extensively studied. Since centrioles are normally eliminated in oogenesis (Delattre and Gonczy, 2004; Manandhar et al., 2005; Schatten, 1994), the deleterious effects of extra centrioles are avoided but as the oocyte usually retains some PCM proteins which are reconstituted into a functional centrosome when the sperm contributes the centrioles at fertilisation, it is possible that an imbalance of centrosome proteins could have deleterious effects on subsequent development. Indeed, only 1-5% of cloned embryos are viable and

centrosome dysfunctions have been identified as a possible cause of these low cloning efficiencies (Zhong et al., 2007). Following nuclear transfer into porcine, bovine, rat or horse oocytes, SCNT-reconstructed embryos display several centrosome and MT abnormalities (Dai et al., 2006; Li et al., 2006; Tomioka et al., 2007; Zhong et al., 2007). However, it is still unclear if this is due to perturbation of the normal balance of centrosomal proteins, the inefficient activation of the SCNT oocyte by electrical stimulation or to incomplete MT polymerization by the donor centrosome which normally organises MTs in much smaller somatic cells (Zhong et al., 2007).

Since early mouse embryos are normally acentriolar, in the production of cloned mouse embryos somatic cell centrosomes are introduced into normally acentriolar cells. However, it is unknown whether the donor centrosomes are degraded and the first few divisions are acentriolar as they would be in a physiological setting, or if the embryo can cope with exogenous centrioles. One study reported that the donor centrosome is degraded immediately in SCNT-reconstructed mouse embryos (Zhong et al., 2005). This conclusion was based on the absence of detectable staining by antibodies against centrin-2 in SCNT embryos. However, Zhong and colleagues (2005) failed to show a positive control confirming centrin-2 antibodies can label centrosomes in normal mouse blastocysts. Therefore, whether the experimenters can ultimately conclude that centrioles are degraded following SCNT is questionable. Moreover, in Chapter 3 of this thesis, two centrin antibodies were tested and failed to identify centrosomes in mouse blastocysts therefore questioning the validity of the claim that exogenous centrosomes are eliminated following SCNT. A more recent paper using  $\gamma$ -tubulin and NuMA as centrosome markers shows that the donor cell centrosome is not degraded after SCNT in mouse oocytes (Van et al., 2006). In fact, van Thuan and colleagues find that the first cleavage and pre-implantation development is impaired in cloned embryos produced from donor cells which had had their resident centrosome removed prior to transfer, suggesting that the donor cell centrosome is necessary for the normal development of the cloned embryo (Van et al., 2006). Despite this, to date there are no experiments which investigate the long-term fate of exogenous centrosomes in SCNT-reconstructed mouse embryos. It is possible that since mouse embryos do not normally require centrosomes until the blastocyst stage, that the exogenous centrosome will be destroyed. (This would point to an additional factor in oocytes/early embryos which inhibits precocious centriole assembly as discussed in 6.1.3). Conversely, if donor centrosomes are maintained, it would be interesting to examine the effect of this exogenous centrosome on subsequent cell divisions.

### 6.3. Concluding comment

Cancer cells are characterised by high incidences of centrosome abnormalities particularly aberrations in centrosome number (Lingle et al., 2005; Lingle and Salisbury, 1999; Nigg, 2002; Pihan et al., 2003; Salisbury et al., 2004). Therefore, understanding the mechanisms leading to centrosome amplification is a fundamental question in cancer biology. Extra centrosomes may arise from the aberrant activation of the *de novo* centriole formation pathway. Although the presence of resident centrioles normally precludes *de novo* centriole manufacture (Dzhinzhev et al., 2010; Khodjakov et al., 2002; La et al., 2005; Peel et al., 2007; Rodrigues-Martins et al., 2007b; Stevens et al., 2010a; Uetake et al., 2007), one recent study has shown that overexpression of the centriole replication protein SAS-6 can induce the *de novo* formation of centriole-like structures in the presence of an existing centriole (Rodrigues-Martins et al., 2007a). Thus, spontaneous *de novo* centriole formation may occur in tumour cells which already contain centrioles and could therefore represent a potential route to supernumerary centrioles in cancer cells. Since there are very few physiological examples of *de novo* centriole formation, this pathway has been poorly studied. However, the mouse embryo, being a rare example of *de novo* centriole formation in a natural setting, provides an excellent model system in which to investigate the mechanism of *de novo* centriole manufacture and how this may go awry in tumour cells.

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*Appendix I: Summary of proteins involved in centriole replication and effect of overexpression/disruption.*

*Abbreviations used: Hs: Homo sapiens, Ce: Caenorhabditis elegans, Dm: Drosophila melanogaster, Cr: Chlamydomonas reinhardtii. .XI: Xenopus laevis*

Protein	Functional homologue (Species)	Disruption	Overexpression
SPD-2	SPD-2 (Ce)	<p><b>Kemp et al., 2004:</b></p> <ul style="list-style-type: none"> <li>SPD-2 mutant/RNAi = abrogates centriole duplication, less PCM recruited</li> </ul> <p><b>Pelletier et al., 2004:</b></p> <ul style="list-style-type: none"> <li>SPD-2 RNAi = centriole assembly fails, spindle assembly defects, less PCM recruited</li> </ul>	
	D-Spd-2 (Dm)	<p><b>Giansanti et al., 2008:</b></p> <ul style="list-style-type: none"> <li>D-Spd2 null mutations in Dm neuroblasts/sperm = suppress astral MT nucleation, reduced PCM recruitment</li> </ul> <p><b>Dix and Raff 2007:</b></p> <ul style="list-style-type: none"> <li>D-Spd-2 mutant sperm – centriole over-replication, multipolar spindles</li> <li>D-Spd-2 mutant flies = have centrioles and cilia so not uncoordinated.</li> <li>D-Spd2 not essential for centriole replication in Dm but PCM recruitment is inefficient in Dm mutant sperm/embryos</li> </ul>	
ZYG-1	Cep192 (Hs)	<p><b>Zhu et al., 2008:</b></p> <ul style="list-style-type: none"> <li>siRNA Cep192 in HeLa = centriole duplication defects, aberrant spindles, impaired PCM recruitment and MT repolymerisation.</li> </ul> <p><b>Gomez-Ferreria et al., 2007:</b></p> <ul style="list-style-type: none"> <li>siRNA Cep192 in HeLa/U2OS = no impact on centriole duplication, disorganized spindles, impaired PCM recruitment and MT repolymerisation</li> </ul>	<p><b>Gomez-Ferreria et al., 2007</b></p> <ul style="list-style-type: none"> <li>overexpress Cep192 in HeLa cells = disorganised spindles and puncta in cytoplasm which can recruit PCM components but cannot organise MTs</li> </ul>
	ZYG-1 (Ce)	<p><b>O'Connell et al., 2001:</b></p> <ul style="list-style-type: none"> <li>siZYG-1 in Ce embryos = blocks centriole duplication</li> </ul>	
ZYG-1	SAK (Dm)	<p><b>Bettencourt-Dias et al., 2005:</b></p> <ul style="list-style-type: none"> <li>mutate/RNAi SAK Dm S2 cells = inhibits centriole duplication but cell cycle progression allowed with disorganised spindles</li> <li>mutate SAK in Dm sperm = inhibits centriole duplication, abnormal cell divisions, unable to make sperm axonemes</li> </ul>	<p><b>Cunha-Ferreria et al., 2009:</b></p> <ul style="list-style-type: none"> <li>RNAi Slimb (SCF component) = SAK at centrosome increases, multiple procentrioles per mother</li> </ul> <p><b>Basto et al., 2008:</b></p> <ul style="list-style-type: none"> <li>overexpress SAK in Dm cell lines = centriole amplification but extra centrosomes cluster to form bipolar spindles, asymmetric division of neural stem cells is compromised, transplantation of cells with extra centrioles into adult flies causes tumours.</li> </ul>

Protein	Functional homologue (Species)	Disruption	Overexpression
ZYG-1 cont...	SAK (Dm) cont ...	<p><b>Rodrigues-Martin et al., 2008:</b></p> <ul style="list-style-type: none"> <li>overexpress dominant negative SAK in Dm embryos (or SAK mutant embryos) = inhibits centriole duplication, aberrant spindle formation, embryos fail to develop = centrioles necessary for early embryonic divisions</li> <li>mutant SAK in Dm sperm = inhibits centriole duplication, abnormal spindles, chromosome segregation errors = centrioles necessary for male meiosis</li> </ul>	<p><b>Rodrigues-Martin et al., 2007b:</b></p> <ul style="list-style-type: none"> <li>overexpress SAK in Dm embryos = centriole amplification (multiple rounds of duplication), abnormal spindles did not develop.</li> <li>overexpress SAK in Dm unfertilized eggs = <i>de novo</i> formation (then duplicate by canonical pathway)</li> <li>overexpress SAK in SAK-depleted (acentriolar) cells = <i>de novo</i> formation</li> <li>Depletion of SAS-6/SAS-4 prevents SAK-mediated centriole biogenesis in eggs/embryos.</li> </ul> <p><b>Peel et al., 2007:</b></p> <ul style="list-style-type: none"> <li>overexpress SAK in Dm embryos = mitotic defects but could not visualise overduplication</li> <li>Overexpress SAK in Dm larval brains = extra centrioles (but not in DSAS-4 mutant so SAS-4 required)</li> <li>overexpress SAK in Dm spermatocytes = no effect</li> <li>overexpress SAK in Dm unfertilized eggs = <i>de novo</i> formation of functional centrioles (only with high level overexpression - UAS/Gal4 promoter)</li> <li>overexpression SAK in Dm oocytes = does not prevent loss of centrioles</li> </ul>
Plk-4 (Hs)		<p><b>Dzhinzhev et al., 2010:</b></p> <ul style="list-style-type: none"> <li>siRNA Plk4 = abrogates centriole over-replication in S phase arrested cells</li> </ul> <p><b>Kuriyama et al., 2009:</b></p> <ul style="list-style-type: none"> <li>abnormal centrioles in colorectal cancer cells due to insufficient Plk4</li> </ul> <p><b>Duensing et al., 2007:</b></p> <ul style="list-style-type: none"> <li>RNAi Plk4 in U2OS cells treated with proteasome inhibitor = reduces multiple procentrioles phenotype</li> </ul> <p><b>Habedanck et al., 2005:</b></p> <ul style="list-style-type: none"> <li>RNAi Plk4 in HeLa cells = inhibits centriole replication, spindle aberrations</li> <li>RNAi Plk4/anti-Plk4 antibodies in S-phase arrested HeLa cells = reduced centriole over-replication</li> </ul> <p><b>Bettencourt-Dias et al., 2005:</b></p> <ul style="list-style-type: none"> <li>RNAi Plk4 HeLa cells = inhibits centriole replication, abnormal spindles.</li> <li>RNAi Plk4 in S-phase arrested U2OS cells = reduced centriole over-replication</li> </ul>	<p><b>Dzhinzhev et al., 2010:</b></p> <ul style="list-style-type: none"> <li>overexpress Plk4 = increased centrosomes per cell</li> </ul> <p><b>Korzeniewski et al., 2009:</b></p> <ul style="list-style-type: none"> <li>dominant negative or siCUL1 (SCF component) = prevents degradation of Plk4 = multiple procentrioles per mother centriole</li> </ul> <p><b>Kuriyama et al., 2009:</b></p> <ul style="list-style-type: none"> <li>overexpress Plk4 in colorectal cancer cells = multiple centrioles and suppressed abnormal centrioles.</li> </ul> <p><b>Duensing et al., 2007:</b></p> <ul style="list-style-type: none"> <li>overexpress Plk4 in U2OS cells = multiple procentrioles per mother centriole but can still form bipolar spindles due to centriole clustering</li> </ul> <p><b>Kieylein-Sohn et al., 2007:</b></p> <ul style="list-style-type: none"> <li>overexpress Plk4 in U2OS cells = multiple procentrioles formed simultaneously per mother</li> <li>overexpress Plk4 in SAS-6<sup>-/-</sup>-tubulin/CPAP/Cep135/CP110 depleted cells = centriole biogenesis suppressed</li> </ul>

Protein	Functional homologue (Species)	Disruption	Overexpression
ZYG-1 cont...	Plk4 (Hs) cont...		<p><b>Habedanck et al., 2005:</b></p> <ul style="list-style-type: none"> <li>overexpress Plk4 in HeLa/U2OS cells = centriole amplification</li> <li>overexpress Plk4 in SAS-6/CP110 depleted cells = centriole amplification reduced</li> </ul>
	Plx4 (XI)		<p><b>EckerdT et al., 2011:</b></p> <ul style="list-style-type: none"> <li>overexpress Plx4 in Xenopus = de novo formation of centriole like foci in activated oocytes/egg extracts but not in immature oocytes.</li> </ul>
SAS-6	SAS-6 (Ce)	<p><b>Leidel et al., 2005:</b></p> <ul style="list-style-type: none"> <li>RNAi SAS-6 in Ce embryos = failed centriole duplication</li> </ul> <p><b>Dammermann et al., 2004:</b></p> <ul style="list-style-type: none"> <li>RNAi SAS-6 in Ce embryos = new centrioles fail to assemble</li> </ul>	
	DSAS-6 (Dm)	<p><b>Rodrigues-Martin et al., 2007a:</b></p> <ul style="list-style-type: none"> <li>RNAi D-SAS-6/D-SAS-6 null mutant = reduced centrioles in larval brains/S2 cells/spermatocytes and loss of 9 fold symmetry,</li> </ul> <p><b>Peel et al., 2007:</b></p> <ul style="list-style-type: none"> <li>D-SAS-6 mutant embryos = decreased centrioles, lethal as flies uncoordinated, centrioles necessary for early embryonic development</li> </ul>	<p><b>Peel et al., 2007:</b></p> <ul style="list-style-type: none"> <li>overexpress D-SAS-6 in Dm embryos = multiple rounds of duplication,</li> <li>overexpress D-SAS-6 in Dm brains = increased centrioles</li> <li>overexpress D-SAS-6 in Dm spermatocytes = no effect</li> <li>overexpress D-SAS-6 in Dm unfertilized eggs = <i>de novo</i> formation of functional centrioles</li> <li>overexpress D-SAS-6 in Dm oocytes = does not prevent centriole loss</li> </ul> <p><b>Rodrigues-Martin et al., 2007a:</b></p> <ul style="list-style-type: none"> <li>overexpress D-SAS-6 in female germ line = SAS-6 aggregates but centrioles still lost in oogenesis</li> <li>overexpress D-SAS-6 in Dm embryos/unfertilised eggs = <i>de novo</i> formation of a tube-like structure but no functional centrioles</li> </ul>
	HsSAS-6 (Hs)	<p><b>Leidel et al., 2005:</b></p> <ul style="list-style-type: none"> <li>RNAi HsSAS-6 in S-phase arrested U2OS cells = abrogated centriole overduplication</li> </ul> <p><b>Strnad et al., 2007:</b></p> <ul style="list-style-type: none"> <li>RNAi HsSAS-6 in HeLa/U2OS cells = increased monopolar spindles</li> <li>RNAi HsSAS-6 in HeLa/U2OS cells = inhibits centriole duplication, monopolar spindles</li> </ul>	<p><b>Leidel et al., 2005</b></p> <ul style="list-style-type: none"> <li>overexpress HsSAS-6 = multiple foci and increased multipolar spindles</li> </ul> <p><b>Strnad et al., 2007:</b></p> <ul style="list-style-type: none"> <li>overexpress non-degradable HsSAS-6 in HeLa cells = dose dependent centriole overduplication, multiple procentrioles formed per mother centriole</li> </ul>
Bld12p (Cr)	<p><b>Nakazawa et al., 2007</b></p> <ul style="list-style-type: none"> <li>Bld12p mutant = deficient in establishing 9-fold radial symmetry, lacks flagella,</li> </ul>		

Protein	Functional homologue (Species)	Disruption	Overexpression
	SAS-4 (Ce)	<p><b>Kirkham et al., 2003:</b></p> <ul style="list-style-type: none"> <li>RNAi SAS-4 in Ce embryos = failed centriole duplication,</li> <li>partial SAS-4 depletion = structurally defective centrioles, less PCM recruited to centrosome, centriolar SAS-4 levels correlate with centriole size</li> </ul> <p><b>Leidel and Gonczy 2003:</b></p> <ul style="list-style-type: none"> <li>RNAi SAS-4 in Ce embryos = impairs centriole duplication, monopolar spindles</li> </ul>	
SAS-4	DSAS-4 (Dm)	<p><b>Rodriguez-Martin et al., 2008:</b></p> <ul style="list-style-type: none"> <li>D-SAS-4 mutant sperm = inhibits centriole duplication, abnormal spindles, chromosome segregation defects = centrioles required for male meiosis</li> </ul> <p><b>Stevens et al., 2007:</b></p> <ul style="list-style-type: none"> <li>D-SAS-4 mutant female germline stem cells = lack centrioles and centrosomes but can still divide asymmetrically and spindles oriented correctly so centrioles not required for oogenesis.</li> <li>D-SAS-4 mutant embryos = lethal, centrioles essential for early embryonic development</li> </ul> <p><b>Basto et al., 2006:</b></p> <ul style="list-style-type: none"> <li>D-SAS-4 Ab injection in Dm embryos = inhibits centriole duplication, spindles still assemble,</li> <li>D-SAS-4 mutant flies = progressive reduction in centriole numbers, mitosis slowed, some defects in asymmetric division in larval neuroblasts, develop to adulthood but lack cilia so uncoordinated</li> </ul>	<p><b>Peel et al., 2007:</b></p> <ul style="list-style-type: none"> <li>overexpress D-SAS-4 in Dm larval brains = no effect</li> <li>overexpress D-SAS-4 in Dm spermatocytes = no effect</li> <li>overexpress D-SAS-4 in Dm unfertilised eggs = <i>de novo</i> formation of functional centrioles (only with high level expression UAS/Gal4 promoter)</li> </ul>
	CPAP (Hs)	<p><b>Kohlmaier et al., 2009:</b></p> <ul style="list-style-type: none"> <li>siRNA CPAP in U2OS = prevents centriole duplication (HsSAS-6 still recruited i.e. CPAP is downstream)</li> </ul> <p><b>Tang et al., 2009:</b></p> <ul style="list-style-type: none"> <li>siRNA in HeLa = suppressed centrosome amplification (in Plk4 activated and S-phase arrested cells)</li> </ul> <p><b>Kleyein Sohn et al., 2007:</b></p> <ul style="list-style-type: none"> <li>siRNA CPAP human cells = suppresses centriole amplification in Plk4-overexpressing cells</li> </ul>	<p><b>Schmidt et al., 2009:</b></p> <ul style="list-style-type: none"> <li>overexpress CPAP in U2OS/hTERT = elongated centrioles/procentrioles, no increase in centriole number or spindle defects</li> <li>overexpress CPAP + Plk4 = flower like structures with parental <i>and</i> procentrioles elongated, elongation does not affect positioning of appendages (nimein, cep164),</li> <li>overexpress CPAP and deplete CP110 = significant cell death, surviving cells had exceptionally long MT structures emanating from centrioles</li> <li>overexpress CPAP and CP110 = suppressed CPAP-induced centriole elongation</li> </ul>

Protein	Functional homologue (Species)	Disruption	Overexpression
SAS-4 cont...	CPAP (Hs)		<p><b>Tang et al., 2009:</b></p> <ul style="list-style-type: none"> <li>overexpress CPAP in human cells = long centrioles,</li> <li>overexpress mutant CPAP which cannot bind tubulin dimer = reduced long centrioles phenotype, abnormal mitotic spindles</li> <li>siRNA hsSAS-6 and overexpress CPAP = no long procentrioles (so CPAP is downstream of SAS-6)</li> </ul> <p><b>Kohlmaier et al., 2009:</b></p> <ul style="list-style-type: none"> <li>overexpress CPAP in U2OS = elongated centrioles <i>and</i> procentrioles, can recruit PCM which promotes more supernumerary procentrioles, multipolar spindles assemble</li> </ul>
CP110	CP110 (Hs)	<p><b>Kohlmaier et al., 2009:</b></p> <ul style="list-style-type: none"> <li>siRNA CP110 = overly long centrioles</li> </ul> <p><b>Schmidt et al., 2009:</b></p> <ul style="list-style-type: none"> <li>siRNA CP110 U2OS/hTERT = elongated centrioles, does not affect positioning of appendages, CP110 a distal end capping protein</li> <li>overexpress CPAP and deplete CP110 = significant cell death, surviving cells had exceptionally long MT structures emanating from centrioles</li> </ul> <p><b>Tang et al., 2009:</b></p> <ul style="list-style-type: none"> <li>siRNA CP110 in U2OS = overly long centrioles</li> </ul> <p><b>Chen et al., 2002:</b></p> <ul style="list-style-type: none"> <li>siCP110 in U2OS cells = prevents centriole overduplication in S-phase arrested cells</li> </ul>	<p><b>Schmidt et al., 2009:</b></p> <ul style="list-style-type: none"> <li>overexpress CPAP and CP110 = suppressed CPAP-induced centriole elongation</li> </ul>
SAS-5	SAS-5 (Ce)  Ana2 (Dm)	<p><b>Delattre et al., 2004:</b></p> <ul style="list-style-type: none"> <li>siRNA SAS5 in Ce embryos = abrogates centriole duplication</li> </ul>	<p><b>Stevens et al., 2010a:</b></p> <ul style="list-style-type: none"> <li>overexpress Ana2 in Dm eggs = <i>de novo</i> formation of centrioles</li> <li>overexpress Ana2 in Dm sperm = centriole overduplication, multipolar spindles</li> <li>overexpress Ana2 in Dm embryos/brains = no overduplication</li> <li>overexpress Ana2 and D-SAS-6 in Dm eggs = massively enhances Ana2-induced <i>de novo</i> formation of centrioles</li> <li>overexpress Ana2 and D-SAS-6 in Dm sperm = coassemble into tubules</li> <li>overexpress Ana2, SAK and SAS-6 in Dm sperm = centriole overduplication, extra SAK loads extra SAS-6 and Ana-2 onto centrioles</li> </ul>

Protein	Functional homologue (Species)	Disruption	Overexpression
SAS-5 cont...	STIL/SIL (Hs)	<p><b>Arquint et al., 2012:</b></p> <ul style="list-style-type: none"> <li>siRNA STIL in U2OS/HeLa cells = defective centriole duplication, monopolar spindles</li> </ul> <p><b>Tang et al., 2011:</b></p> <ul style="list-style-type: none"> <li>siRNA STIL in U2OS cells = suppresses centriole duplication, monopolar spindles, longer mitosis, inhibits Plk4-mediated centriole amplification and CPAP-induced centriole elongation</li> </ul> <p><b>Vulprecht 2012:</b></p> <ul style="list-style-type: none"> <li>siRNA STIL in U2OS cells = diminishes centrosomal SAS-6 (not vice versa so STIL recruited to centrosome before SAS-6)</li> <li>siRNA STIL = monopolar spindles, reduction in centriole numbers, abrogates Plk4-mediated centriole amplification</li> </ul>	<p><b>Arquint et al., 2012:</b></p> <ul style="list-style-type: none"> <li>overexpress STIL in U2OS cells = centriole amplification in rosette arrangement (multiple daughters)</li> </ul> <p><b>Tang et al., 2011:</b></p> <ul style="list-style-type: none"> <li>overexpress STIL in U2OS/HeLa cells = multiple procentrioles</li> <li>Overexpress STIL in CPAP-depleted cells = inhibits STIL-induced centriole amplification</li> <li>overexpress STIL in SAS-6 depleted cells = inhibits STIL-induced centriole amplification and perturbs STIL localisation (SAS-6 and STIL mutually dependent)</li> <li>overexpress STIL in Plk4-depleted cells = blocks STIL-induced centriole amplification and disrupted STIL targeting</li> </ul> <p><b>Vulprecht et al., 2012:</b></p> <ul style="list-style-type: none"> <li>overexpress STIL in U2OS = centriole overduplication</li> </ul>
Cep135	Cep135 (Hs)	<p><b>Ohta et al., 2002:</b></p> <ul style="list-style-type: none"> <li>siRNA Cep135 = disorganised spindle</li> </ul> <p><b>Kim et al., 2008:</b></p> <ul style="list-style-type: none"> <li>siRNA Cep135/overexpress mutant Cep135 = premature centriole splitting</li> </ul> <p><b>Hiraki et al., 2007:</b></p> <ul style="list-style-type: none"> <li>Bld10p mutants = cartwheel spokes shorter and loss of 9 fold radial symmetry</li> </ul> <p><b>Mottier-Pavie et al., 2009:</b></p> <ul style="list-style-type: none"> <li>Bld10 mutants = assemble short centrioles,</li> <li>mutant flies are viable but male sterile, immotile sperm with axonemes lacking central MT pair</li> </ul> <p><b>Tsang et al., 2009:</b></p> <ul style="list-style-type: none"> <li>siRNA Cep76 in U2OS = centriole amplification but no mitotic defects (NB only in cancer cells)</li> <li>siRNA SAS6 and Cep76 = abrogates siCep76-induced centriole amplification (antagonistic function)</li> <li>siCep76 and siCP110/Cep97 = suppresses siCep76-induced centriole amplification</li> </ul>	<p><b>Ohta et al., 2002:</b></p> <ul style="list-style-type: none"> <li>overexpress Cep135 = cytoplasmic foci, can organise MTs, EM similar to centrioles</li> </ul>
Cep135	Bld10p (Cr)	<p><b>Hiraki et al., 2007:</b></p> <ul style="list-style-type: none"> <li>Bld10p mutants = cartwheel spokes shorter and loss of 9 fold radial symmetry</li> </ul> <p><b>Mottier-Pavie et al., 2009:</b></p> <ul style="list-style-type: none"> <li>Bld10 mutants = assemble short centrioles,</li> <li>mutant flies are viable but male sterile, immotile sperm with axonemes lacking central MT pair</li> </ul> <p><b>Tsang et al., 2009:</b></p> <ul style="list-style-type: none"> <li>siRNA Cep76 in U2OS = centriole amplification but no mitotic defects (NB only in cancer cells)</li> <li>siRNA SAS6 and Cep76 = abrogates siCep76-induced centriole amplification (antagonistic function)</li> <li>siCep76 and siCP110/Cep97 = suppresses siCep76-induced centriole amplification</li> </ul>	
Cep76	Bld10 (Dm)	<p><b>Mottier-Pavie et al., 2009:</b></p> <ul style="list-style-type: none"> <li>Bld10 mutants = assemble short centrioles,</li> <li>mutant flies are viable but male sterile, immotile sperm with axonemes lacking central MT pair</li> </ul> <p><b>Tsang et al., 2009:</b></p> <ul style="list-style-type: none"> <li>siRNA Cep76 in U2OS = centriole amplification but no mitotic defects (NB only in cancer cells)</li> <li>siRNA SAS6 and Cep76 = abrogates siCep76-induced centriole amplification (antagonistic function)</li> <li>siCep76 and siCP110/Cep97 = suppresses siCep76-induced centriole amplification</li> </ul>	
Cep76	Cep76 (Hs)	<p><b>Tsang et al., 2009:</b></p> <ul style="list-style-type: none"> <li>overexpress Cep76 = suppresses S-phase arrest-mediated centriole amplification, (ie Cep76 can suppress multiple rounds of duplication),</li> <li>overexpress Cep76 and Plk4 = centriole amplification (ie Cep76 can't repress multiple daughters per cell)</li> <li>overexpress Cep76 in untreated cells = no effect (does not affect normal duplication (just reduplication))</li> </ul>	<p><b>Tsang et al., 2009:</b></p> <ul style="list-style-type: none"> <li>overexpress Cep76 = suppresses S-phase arrest-mediated centriole amplification, (ie Cep76 can suppress multiple rounds of duplication),</li> <li>overexpress Cep76 and Plk4 = centriole amplification (ie Cep76 can't repress multiple daughters per cell)</li> <li>overexpress Cep76 in untreated cells = no effect (does not affect normal duplication (just reduplication))</li> </ul>

Protein	Functional homologue (Species)	Disruption	Overexpression
<b>Cep152</b>	<b>Cep152 (Hs)</b>	<p><b>Dzhindzhev et al., 2010:</b></p> <ul style="list-style-type: none"> <li>▪ siRNA Cep152 = abrogates centriole amplification in S-phase arrested U2OS cells</li> </ul>	<p><b>Dzhindzhev et al., 2010:</b></p> <ul style="list-style-type: none"> <li>▪ Overexpress Cep152 = increased centrosomes per cell</li> </ul>
	<b>Asl (Dm)</b>	<p><b>Varmark et al., 2007:</b></p> <ul style="list-style-type: none"> <li>▪ Asl mutant Dm spermatocytes = reduced MT nucleation, meiotic spindle defects, aneuploidy</li> <li>▪ Asl mutant Dm embryos = non-functional MTOCs/spindles, terminal phenotype</li> </ul> <p><b>Dzhindzhev et al., 2010:</b></p> <ul style="list-style-type: none"> <li>▪ siRNA Asl = loss of centrosomes, Plk4 delocalised from centrosome, Asl stabilises and recruits Plk4</li> <li>▪ siRNA Asl = Plk4-induced biogenesis suppressed (i.e. Asl is upstream of Plk4)</li> </ul>	<p><b>Stevens et al., 2010a:</b></p> <ul style="list-style-type: none"> <li>▪ Overexpress Asl in Dm eggs = <i>de novo</i> formation of centrioles (mild phenotype compares to Ana-2)</li> </ul> <p><b>Dzhindzhev et al., 2010:</b></p> <ul style="list-style-type: none"> <li>▪ Overexpress Asl in Dm embryos = supernumerary centrosomes/increase in centrosome number in S-phase arrested cells =</li> <li>▪ Overexpress Asl in unfertilised eggs = supernumerary free MT asters, impairs spindle formation</li> </ul>

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