

Dissecting Polo kinase recruitment to the centrosome



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Abstract

Dissecting Polo kinase recruitment to the centrosome

Ines Alvarez Rodrigo, St Catherine's College

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Polo kinase is a major regulator of the cell cycle. Polo is present at many different subcellular structures, including centrosomes. Centrosomes are formed when mother centrioles recruit pericentriolar material (PCM) around themselves. The PCM expands dramatically in preparation for mitosis, in a process that relies on Polo phosphorylation of the PCM protein Cnn. Polo is also known to regulate centriole disengagement, and Polo binding to the centriolar protein Sas-4 is crucial for Asl recruitment to daughter centrioles during their conversion into fully functional mothers.

The primary aim of my thesis was to fully characterise how Polo is recruited to centrosomes to perform its different functions. As a model, I use syncytial fly embryos. In addition to the previously known Polo binding site in Sas-4, I found that the PCM protein Spd-2 directly interacts with the PBD domain of Polo. While Spd-2 is not required for Polo recruitment to the centriole, it is crucial to recruit Polo to the PCM. I propose that this interaction is part of a Spd-2/Polo/Cnn positive feedback loop that drives mitotic PCM assembly. Furthermore, I found that a third protein (Ana1) is required for overall Polo recruitment to the centrosome. Ana1 has been previously linked to Asl recruitment and/or maintenance and centriole conversion. Losing Ana1-mediated Polo recruitment did not affect these processes, but it did severely hinder mitotic PCM expansion. I propose that, independently of its Asl-related role, Ana1 acts as a crucial reservoir of centriolar Polo. Thus, centriolar Polo recruited via Ana1 can phosphorylate Spd-2 to allow it to bind the PBD, in an example of Polo “self-priming”.

Publications resulting from this thesis

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Abbreviations

3D-SIM	3D structured illumination microscopy	DEST	Destination vector
a.u.	Arbitrary unit	DNA	Deoxyribonucleic acid
aa	Amino acid	DTT	Dithiothreitol
ACF	Autocorrelation function	ECT2	Epithelial cell-transforming sequence 2 oncogene
ALL	All potential Polo binding sites mutated	EDTA	Ethylenediaminetetraacetic acid
Ana	Anastral spindle	EGTA	Ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid
APC/C	Anaphase promoting complex/cyclosome	EM	Electron microscopy
Asl	Asterless	Emi	Early mitotic inhibitor
Asp	Abnormal spindle protein	ESCRT	Endosomal sorting complexes required for transport
ATP	Adenosine triphosphate	FCS	Fluorescence correlation spectroscopy
AUC	Area under the curve	FRAP	Fluorescence recovery after photobleaching
AurA	Aurora A kinase	Fzr	Fizzy-related protein
BLAST	Basic local alignment search tool	GaAsP	Gallium arsenide phosphide
BS ³	Bis(sulfosuccinimidyl)suberate	GBP	GFP-binding protein
BSA	Bovine serum albumin	GFP	Green fluorescent protein
BUB	Budding uninhibited by benzimidazoles	Grip	Gamma-tubulin ring complex
BUBR	BUB1-related protein	GST	Glutathione-S-transferase
CA	Centrosome amplification	GTP	Guanosine triphosphate
CDC	Cell division cycle	γ -TuRC	γ -tubulin ring complex
CDK	Cyclin-dependent kinase	HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Cdk5Rap	Cdk5 regulatory subunit associated protein	HF	High fidelity
Cep	Centromal protein	HRP	Horseradish peroxidase
CHK	Check-point kinase	Hsp90	Heat shock protein 90
CI	Confidence interval	IDL	Interdomain linker
CM	Cnn motif	IF	Immunofluorescence
Cnn	Centrosomin	INCEP	Inner centromere protein
CONS	Conserved potential Polo binding sites mutated	IP	Immunoprecipitation
CPAP	Centromal P4.1-associated protein	IPTG	Isopropyl b-D-1-thiogalactopyranoside
CPC	Chromosomal passenger complex	KD	Kinase domain
CPM	Photon count-rate per molecule	Klp	Kinesin-like protein
CS	Centrosome separation	KT	Kinetochore
CT	Carboxyl-terminus/terminal	LB	Luria broth
Cyk	Cytokinesis defect	LZ	Leucine zipper
DAPI	4',6-diamidino-2-phenylindole	M	Molar
		Map	Microtubule associated protein
		MBP	Maltose binding protein

MBS	Multi-track beam splitter	ptPBS	Potential Polo binding site
MCNR	Modulation contrast noise ratio	RacGAP	Rac GTPase-activating protein
MES	2-(N-morpholino)ethanesulfonic acid	RFP	Red fluorescent protein
MKlp	Mitotic kinesin-like protein	Rho	Ras homologous
MPS	Monopolar spindle	RNAi	RNA interference
mRNA	Messenger ribonucleic acid	ROI	Region of interest
MS	Mass spectrometry	RT	Room temperature
MT	Microtubule	SAC	Spindle assembly checkpoint
MTOC	Microtubule organising centre	SAP	Sas-6/Ana2 particle
MW	Molecular weight	SAS	Spindle assembly abnormal
MYPT	Myosin phosphatase target subunit	SB	Laemmli sample buffer
MYT1	Membrane-associated tyrosine- and threonine-specific cdc2-inhibitory kinase	Scc	Sister chromatid cohesion
NEB	Nuclear envelope breakdown	SCF- β TrCP	Skp, Cullin, F-box, β -transducing repeat-containing protein complex
NEDD	Neural precursor cell expressed, developmentally down-regulated	SD	Standard deviation
NHK	Nucleosomal histone kinase	SDS	Sodium dodecyl sulphate
NLS	Nuclear localisation sequence	SEC	Size exclusion chromatography
NT	Amino-terminus/terminal	SEM	Standard error of the mean
NudC	Nuclear distribution protein C	Ser	Serine
PAGE	Polyacrylamide gel electrophoresis	Sgt1	Suppressor of G2 allele of SKP1
PB	Polo box	SPB	Spindle pole body
PBD	Polo box domain	Spd	Spindle defective
PBS	Phosphate buffered saline	STAN	STIL/Ana2 domain
PBT	PBS-0.2% triton x-100	STIL	SCL/TAL1 interrupting locus
PCM	Pericentriolar material	SVIL	Supervillin
PCR	Polymerase chain reaction	TAE	Tris-acetate-EDTA
Plk	Polo-like kinase	Thr	Threonine
Plp	Pericentrin-like protein	TOG	Transporter-opsin-G protein-coupled receptor
Plx1	<i>Xenopus</i> Polo-like kinase	TPX	Targeting Protein for <i>Xenopus</i> Klp2
PP	Protein phosphatase	TPXL	TPX-like
PP1C	PP1 catalytic subunit	Ubq	Ubiquitin
PPB	Protein preparation buffer	unc	Uncoordinated
PRC	Protein regulator of cytokinesis	WB	Western blotting
PRem	Phospho-regulated multimerization domain	WT	Wild type
		Y2H	Yeast- two-hybrid
		YT	Yeast extract - tryptone buffer
		Zeo	Zeocin
		ZYG	Zygotic defective

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Chapter 1 - Introduction

1.1. Centrioles, centrosomes and cilia

Centrioles are protein-based structures with 9-fold radial symmetry that can organise two different cellular structures: centrosomes and cilia. Centrioles and centriole-derived structures are present in all major eukaryotic groups (Carvalho-Santos et al., 2011; Cavalier-Smith, 2002). Within these, even lineages that have completely lost centriole-derived structures (e.g. higher fungi) still assemble organelles that share some functions and components with animal centrosomes (e.g. spindle pole bodies (SPBs)) (reviewed in Azimzadeh, 2014; Cavanaugh and Jaspersen, 2017). This suggests that the last eukaryotic common ancestor had a single centriole, and that at multiple points in the evolution of eukaryotes these structures were either retained or lost as closely related species adapted to different niches (Carvalho-Santos et al., 2011; Cavalier-Smith, 2002). Thus, it has been proposed that certain proteins involved in centriole and centrosome biogenesis would be part of an ancestral, evolutionarily conserved module, whereas other key proteins would have emerged later in specific eukaryotic groups (Carvalho-Santos et al., 2010; Hodges et al., 2010).

1.1.1. Centrioles and the centriole duplication pathway

The centriolar structure itself is highly conserved (Carvalho-Santos et al., 2011). Centrioles are cylindrical structures, 200-250 nm in diameter, characterised by the presence of stable peripheral microtubules (MTs) arranged with 9-fold radial symmetry (Figure 1.1Ai). Their length, however, differs significantly between species, and even different cell types of the same species. In *Drosophila melanogaster*, for example, centrioles are typically 180-200 nm long (Moritz et al., 1995a), but they are much larger in spermatocytes (Gonzalez et al., 1998). In vertebrate cells, they typically

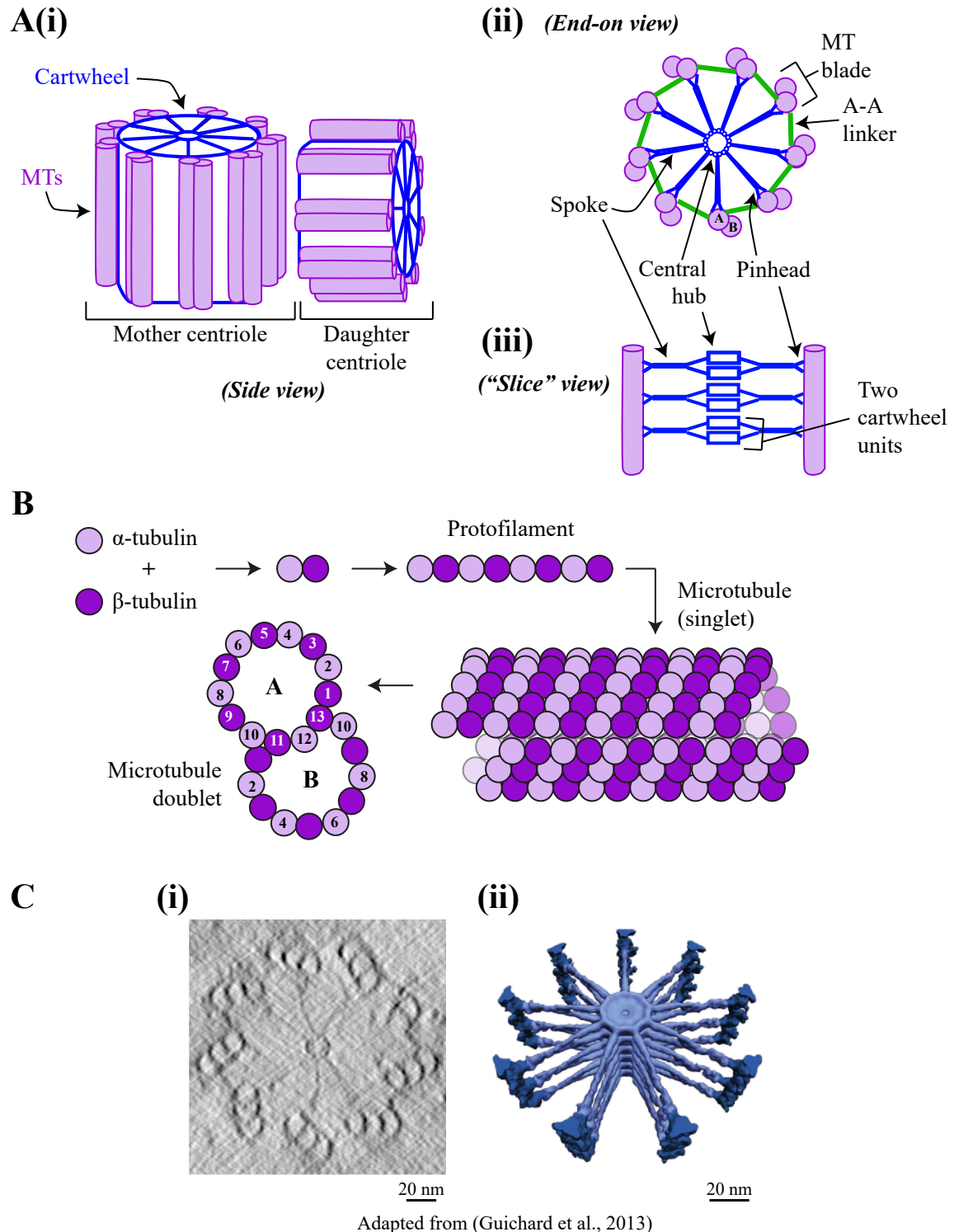


Figure 1.1 – Centrioles are made of a central cartwheel and peripheral microtubules.

(A) Schematic diagrams of typical fly centrioles. (i) Side-on view of a mother centriole (vertical) with an associated (engaged) daughter centriole perpendicular to the mother's vertical axis. (ii) End-on view of a centriole. (iii) View slicing through the cartwheel, showing that multiple units (Sas-6 rings) stack-up to form a cartwheel. (B) Dimers of α - and β -tubulin assemble into protofilaments, microtubules (MTs) and MT doublets (or triplets). (C) Images adapted from (Guichard et al., 2013). (i) Transverse section of a *Trichonympha* centriole cryotomogram. (ii) 3D representation of six stacked-up Sas-6 rings, which come together at their ends forming 3 pairs of rings. The Pinheads are indicated in *dark blue*, the MTs are not shown.

range from 150 to 500 nm (reviewed in Winey and O'Toole, 2014), but in some species, like *Trichonympha* they can reach 1.5 μm (Guichard et al., 2012).

The centriolar MTs (also known as MT blades) can be present as singlets: one hollow tubule created by the lateral association of 13 protofilaments made of α - and β -tubulin heterodimers. However, centriolar MTs often appear as doublets or triplets, in which case the innermost A-tubule is built from a complete ring of 13 protofilaments, with an incomplete B-tubule attached to the wall of the A-tubule, and an incomplete C-tubule (if present) attached to the B-tubule wall (Tilney et al., 1973) (Figure 1.1B). Each blade is linked to those adjacent via a proteinaceous linker, which might vary between species, and between different regions of the same centriole. For example, at proximal ends of mammalian centrioles each A-tubule is connected to the C-tubule of the next blade (clockwise), but at distal ends additional appendages extend from B-tubules (Greenan et al., 2018). Conversely, *Drosophila* centrioles present A-A linkers (Greenan et al., 2018) (Figure 1.1Aii). Once assembled, MT blades are stabilised partly due to extensive post-translational modification of their tubulin subunits (Janke and Bulinski, 2011).

At the centre of the centriole there is a cartwheel structure made out of a central hub approximately 22 nm in diameter from which nine radial spokes emanate (Guichard et al., 2012, 2013). This structure comprises a stack of ring-like units. Spokes from consecutive rings come together at their ends, forming junctions which join via a “Pinhead” to the MT blades (Figure 1.1A,C). The cartwheel may be present permanently throughout the length of the centriole, as in *Drosophila* (Moritz et al., 1995a); only present in one section of the centriole, as in *Chlamydomonas* (Guichard et al., 2013; O'Toole et al., 2003); or disappear from the assembling daughter centriole

once it matures into a mother, as in vertebrate cells (Vorobjev and Chentsov, 1982). The cartwheel is key in the establishment of the 9-fold symmetry of centrioles. This is most clear from the highly detailed map of the *Trichonympha* cartwheel obtained with cryo-electron tomography (Guichard et al., 2013), but it also appears to be the case in other species (Nakazawa et al., 2007).

The main structural component of the 9-fold symmetric cartwheel rings is **Sas-6** (van Breugel et al., 2011; Guichard et al., 2012; Kitagawa et al., 2011), which is one of the most ancestral centriolar proteins (Carvalho-Santos et al., 2010; Hodges et al., 2010), and also one of the first proteins recruited to the site of centriole assembly (Leidel et al., 2005; Rodrigues-Martins et al., 2007a). Nine Sas-6 homodimers can self-assemble into symmetric, cartwheel unit-like structures *in vitro*: the cartwheel hub is formed of the N-terminal globular heads, while the C-terminal extended coiled-coils form each of the spokes (van Breugel et al., 2011; Guichard et al., 2017; Kitagawa et al., 2011). However, other proteins (such as Bld10/Cep135 and Ana2) are necessary to efficiently assemble and maintain these structures (Cottee et al., 2015; Guichard et al., 2017).

Centrioles can assemble *de novo* (La Terra et al., 2005; Rodrigues-Martins et al., 2007b) or through the canonical centriole duplication pathway. Many aspects are similar, but this thesis will discuss only the latter. Ground-breaking work in *Caenorhabditis elegans* first showed that centriole assembly requires only a few essential proteins, which are recruited to the centriole assembly site sequentially (Delattre et al., 2004, 2006; Kemp et al., 2004; Kirkham et al., 2003; Leidel and Gönczy, 2003; Leidel et al., 2005; O'Connell et al., 2001; Pelletier et al., 2004, 2006). Genome-wide RNAi screens in *Drosophila* S2 cells have since identified a similar set of proteins (Dobbelaere et al., 2008; Goshima et al., 2007). Even if the amino acid

sequence of some these proteins has diverged significantly between species, functional homology remains between “core” components in flies, worms and vertebrates (Figure 1.2). These proteins are briefly discuss below (fly/human/worm nomenclature where required).

Centriole assembly starts with the recruitment of Polo-like kinase **Sak/PLK4/ZYG-1** to a pre-existing centriole, the “mother” (Bettencourt-Dias et al., 2005; Habedanck et al., 2005). Note that the fly homologue is now often referred to as PLK4. PLK4 is initially recruited as a ring around the centriole, but the ring eventually becomes a single dot on the side of the mother, which marks where the new centriole (“daughter”) will be assembled. This ring-to-dot transition is most likely determined, at least in part, by the interaction of PLK4 with Ana2/STIL (Dzhindzhev et al., 2017; Leda et al., 2018; Ohta et al., 2018; Sonnen et al., 2012) (see below). The cytoplasmic levels of PLK4 are tightly regulated, as PLK4 overexpression leads to centriole overduplication (Basto et al., 2008; Habedanck et al., 2005; Kleylein-Sohn et al., 2007; Rodrigues-Martins et al., 2007b). This is partly achieved by PLK4 being a “suicide kinase”, able to homodimerise via its cryptic Polo box domain. PLK4 *trans*-phosphorylates an internal degron motif, allowing the E3 ubiquitin ligase SCF^{Slimb/βTrCP} to mark PLK4 for degradation by the proteasome (Cunha-Ferreira et al., 2009; Guderian et al., 2010; Holland et al., 2012; Rogers et al., 2009; Sillibourne et al., 2009). Interestingly, centriolar levels of PLK4 oscillate, and this oscillation determines the rate and period of daughter centriole growth (Aydogan et al., 2018, 2019),

PLK4 presumably binds and sequentially phosphorylates various residues on **Ana2/STIL/SAS-5**, and this interaction also promotes PLK4 activity (Arquint et al., 2015; Dzhindzhev et al., 2014, 2017; Kratz et al., 2015; McLamarrah et al., 2018;

Centriole Duplication Pathway

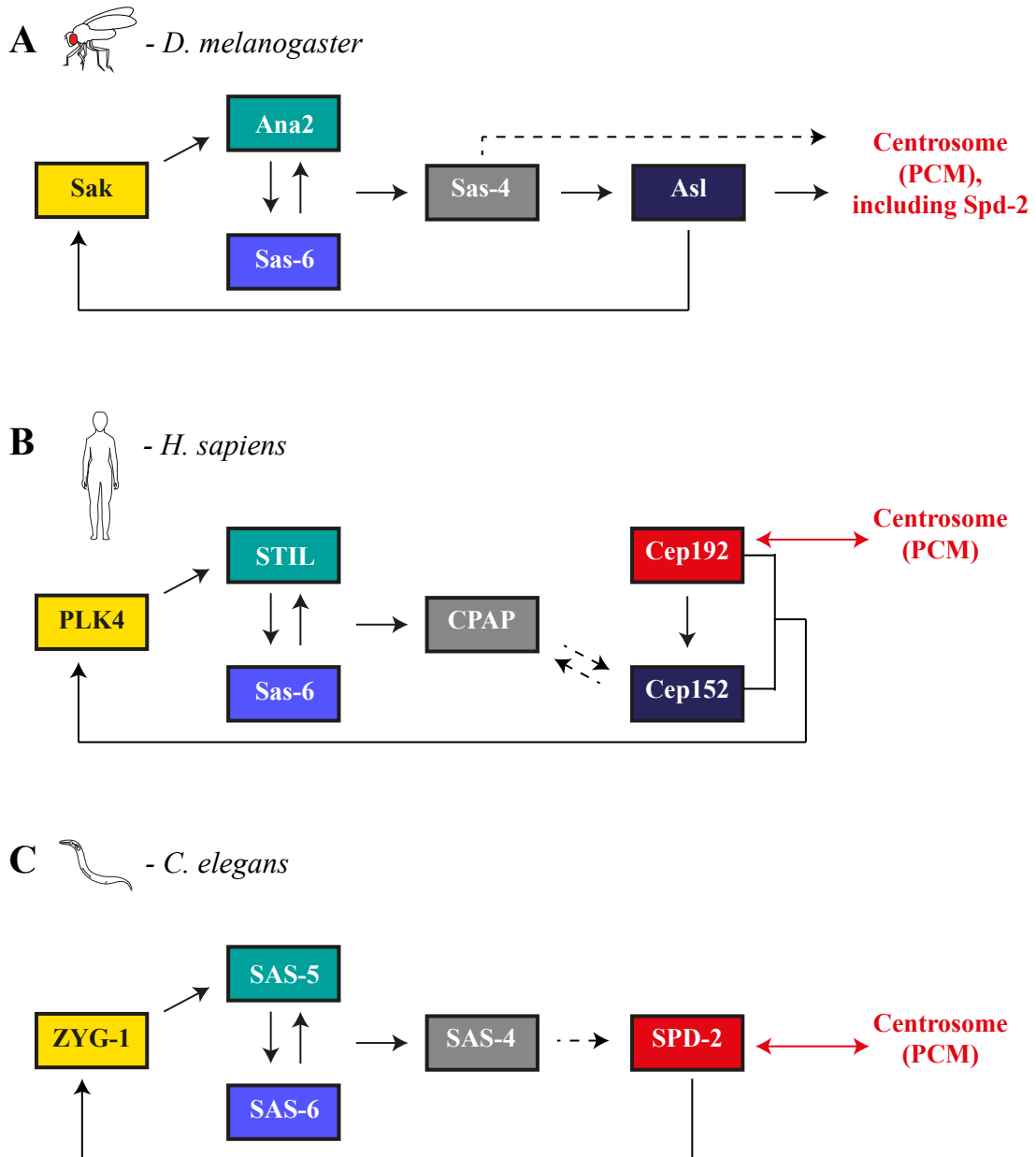


Figure 1.2 – The centriole duplication pathway is highly conserved.

Schematic showing the small number of proteins that are involved in the centriole duplication pathway in *D. melanogaster* (A), *H. sapiens* (B) and *C. elegans* (C). Boxes with the same colour indicate orthologues or functional homologues. Solid arrows indicate that one protein is required for the localisation of the other, dashed arrows indicate inferred localisation relationships. In flies, Asl is required for recruitment of Sak/PLK4 but also of the proteins that organise the pericentriolar material (PCM) around the mother centriole to form the centrosome. One such PCM protein is Spd-2 (indicated in red). However, in human cells, both Cep192 (the Spd-2 homologue, red) and Cep152 contribute to PLK4 recruitment. In *C. elegans* the ZYG-1 recruitment role is fulfilled by SPD-2, and there is no Asl homologue. Adapted from (Conduit et al., 2015a).

Moyer et al., 2015; Ohta et al., 2014). While Ana2, STIL and SAS-5 are only functional homologues, they share two conserved domains which are essential for centriole assembly: a central coiled-coil that drives homodimerization, and the STIL/Ana2 Domain (STAN), which is phosphorylated by PLK4 to promote Ana2/STIL binding to **Sas-6** (the main component of the cartwheel, see above) (Cottee et al., 2015; Dzhindzhev et al., 2014; Goshima et al., 2007; Moyer et al., 2015; Ohta et al., 2014; Stevens et al., 2010a). However, while crucial for cartwheel formation, the exact nature of the Sas-6/Ana2 interaction is still unclear.

Sas-6 or Ana2/STIL overexpression can also lead to centriole overduplication, and the co-overexpression of both proteins results in the formation of Sas-6-Ana2-Particles (SAPs) (Stevens et al., 2010b). These large macromolecular structures cannot divide or duplicate like centrioles, but they recruit many centriolar and centrosomal proteins in a similar manner to centrosomes (Costa Vicente, 2013; Gartenmann, 2018). Thus, the levels of Sas-6 and Ana2/STIL must also be tightly regulated. In vertebrate cells, both proteins are marked by the APC/C^{Cdh1} for degradation during late mitosis and early G1, ensuring that centriole duplication cannot take place before S-phase (Arquint et al., 2012; Strnad et al., 2007). Additionally, STIL and Sas-6 are SCF ligase targets, but can be protected from it by CDK2 and PLK4 activity, respectively (Arquint et al., 2018; Puklowski et al., 2011). Conversely, CDK1 phosphorylation of Ana2 appears to hinder centriole assembly, but the mechanism by which this happens is unclear and could differ between flies and vertebrates (Gartenmann, 2018; Zitouni et al., 2016).

Another factor essential for centriole assembly is **Sas-4/CPAP/SAS-4** (Basto et al., 2006; Kleylein-Sohn et al., 2007). Unlike Sak, Ana2 and Sas-6, overexpression of Sas-4 does not trigger centriole overduplication (Peel et al., 2007), but Sas-4/CPAP loss

can suppress centriole overduplication caused by overexpression of one of these factors (Basto et al., 2008; Kleylein-Sohn et al., 2007). Thus, as in *C. elegans* (Delattre et al., 2006; Pelletier et al., 2006), Sas-4/CPAP most likely functions downstream of PLK4, Ana2/STIL and Sas-6. During centriole assembly, Sas-4/CPAP may promote centriole MT nucleation and/or facilitate the Sas-6/Ana2 interaction (Schmidt et al., 2009; Tang et al., 2009, 2011). Additionally, once daughter centrioles have been built, Sas-4/CPAP binds to, and may recruit, Asl (Cizmecioglu et al., 2010; Dzhindzhev et al., 2010; Novak et al., 2014, 2016) (see 1.3.3).

Asl/Cep152 is the only protein essential for centriole duplication without a clear *C. elegans* homologue. In *C. elegans* it is SPD-2 that is involved in ZYG-1 recruitment (Delattre et al., 2006; Kemp et al., 2004; Pelletier et al., 2004, 2006). However, in flies, Asl is required to recruit PLK4 to mother centrioles (Cizmecioglu et al., 2010; Dzhindzhev et al., 2010; Hatch et al., 2010), as well as to recruit Spd-2 (Blachon et al., 2008; Novak et al., 2014, 2016), which is not involved in centriole duplication, but plays an important role in centrosome maturation (see below) (Dix and Raff, 2007). It has been proposed that Asl binding to PLK4 promotes PLK4 activation, which can then drive centriole assembly (see above). PLK4 can also phosphorylate Asl, potentially triggering its own release and subsequent degradation; thus, Asl would be key to generate the PLK4 oscillations that drive centriole duplication (Aydogan et al., 2019; Boese et al., 2018). In vertebrates both Cep192 (homologue of Spd-2) and Cep152 cooperate to recruit PLK4 for centriole duplication (Sonnen et al., 2013).

1.1.2. Centrosomes and the centrosome cycle

Centrosomes are membrane-free organelles that consist of a pair of centrioles (mother and daughter) surrounded by an electron-dense matrix known as pericentriolar material

(PCM). Experiments using mitotic centrosomes purified from the surf clam *Spisula solidissima* showed that, while most PCM proteins were lost if the centrosomes were extracted using high salt concentrations, a complex lattice built from 12- to 15-nm thick fibres remained (Schnackenberg et al., 1998). Thus, while centrosomes contain hundreds of proteins (Alves-Cruzeiro et al., 2014; Andersen et al., 2003; Jakobsen et al., 2011; Müller et al., 2010), it has long been proposed that the PCM is organised by an underlying, extended scaffold which (directly or indirectly) recruits the rest of the proteins that comprise the PCM.

Centrosomes have multiple functions in animal cells (reviewed in Conduit et al., 2015a) (see 1.1.3). While their best known role is MT nucleation and mitotic spindle organisation, it has been suggested that this is a later specialisation, perhaps to ensure equal centriole inheritance between daughter cells by positioning centrioles at the spindle poles (Basto et al., 2006; Carvalho-Santos et al., 2011; Hodges et al., 2010; Pickett-Heaps, 1974, 1975). Evidence for this is the lack of a strict correlation between the presence of centrioles and centrosomes in eukaryotes (reviewed in Azimzadeh, 2014). An interesting example is planarians, which have lost several genes crucial for centrosome formation (including Spd-2/Cep192 and Cnn/Cep215, two proteins that will be discussed frequently in this thesis). As a result, centrioles are only present in these organisms in non-proliferative cells, and they need to be built *de novo* (Azimzadeh et al., 2012).

Centrosome biogenesis is indeed strictly regulated to ensure that centrioles are duplicated once per cell cycle and that each daughter cell inherits one centrosome. This process is often referred to as “the centrosome cycle”, and it is tightly linked to the cell cycle itself (Robbins et al., 1968; Vorobjev and Chentsov, 1982) (Figure 1.3). After

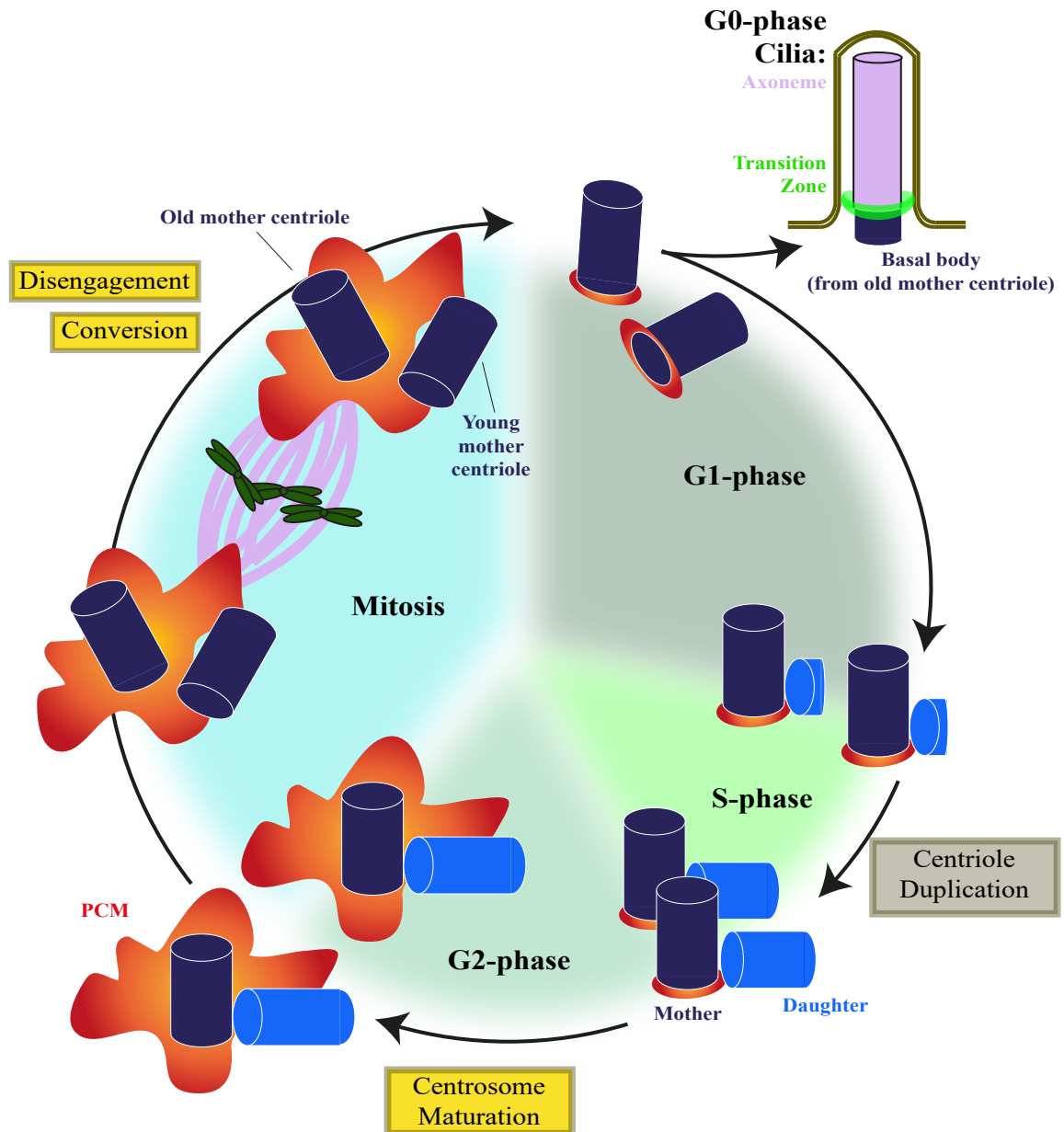


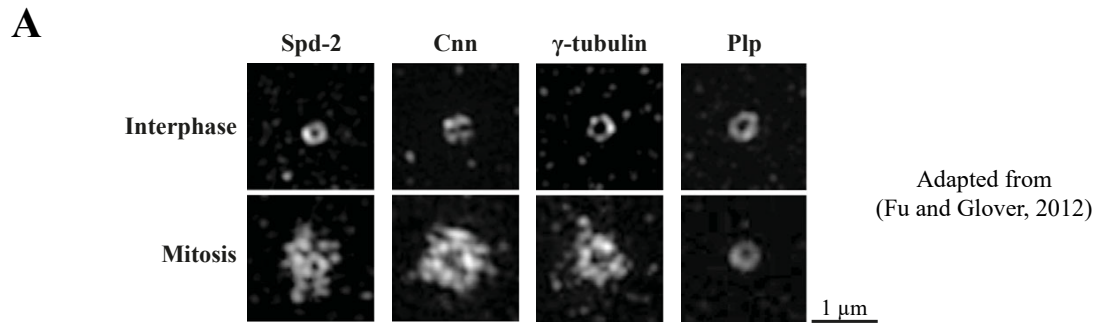
Figure 1.3 – The centrosome cycle.

Schematic of the centrosome cycle, highlighting in *yellow* the events which are regulated by Polo kinase. In G1-phase, a cell contains two mature centrioles which organise minimal amounts of PCM. At the beginning of S-phase, each centriole nucleates a new daughter centriole, which grows until the end of S-phase. In G2-phase, the two centrosomes migrate to opposite sides of the nucleus and their PCM expands dramatically in preparation for mitosis (**centrosome maturation**). During mitosis the centrosomes help organise the bipolar spindle. Within each centrosome, the mother and daughter lose their perpendicular arrangement (**centriole disengagement**) and a number of proteins are recruited to the newly formed daughter centriole to allow it to become a fully-functional mother, able to nucleate a new daughter centriole and organise PCM in the following cycle (**centriole conversion**). As a result, each daughter cell inherits one centrosome containing two mature centrioles (the old mother and the newly-converted young mother) and the cycle can re-start. However, if the cell enters G0-phase, the centriole pair usually migrates to the surface and the old mother centriole initiates ciliogenesis, becoming the basal body of the cilia.

mitosis, each daughter cell contains one centrosome with a pair of centrioles. During S-phase, each centriole assembles a new daughter centriole arranged perpendicularly to the mother centriole axis (orthogonal arrangement). As detailed above, **centriole duplication** is tightly regulated. At the start of G2-phase, the two centrosomes migrate to opposite sides of the nucleus. Three key processes happen during G2 and mitosis: **centrosome maturation**, which involves major changes in the organisation of the PCM; and **centriole disengagement** and **centriole conversion**, two licensing events that ensure that centriole duplication can take place in the following S-phase. These three processes are controlled by Polo kinase: a general overview is provided below, and further details specific to Polo regulation are discussed in 1.3.3. Towards the end of mitosis, each centrosome is segregated into a daughter cell and the cycle restarts, unless cells enter G0-phase, when centrioles generally form cilia.

Centrosome maturation, also known as PCM expansion or mitotic PCM assembly (these terms are used interchangeably in the literature and this thesis) is the process by which the PCM, which is highly organised and concentrated around the mother centriole in interphase, expands dramatically in preparation for mitosis (Fu and Glover, 2012; Lawo et al., 2012; Mennella et al., 2012; Sonnen et al., 2012) (Figure 1.4A). The specifics of PCM assembly can differ between different organisms and cell types (Conduit and Raff, 2015; see also Conduit et al., 2015a), but three fundamental aspects have been recognised.

First, PCM assembly nucleation requires the mother centriole (Figure 1.4Bi). If centrioles are physically eliminated (e.g. using laser ablation or injecting an antibody that causes centriole dissolution) or their assembly is disturbed using genetic tools, any existing PCM will become unstable, and new PCM assembly is impaired until



B

Centrosome Maturation

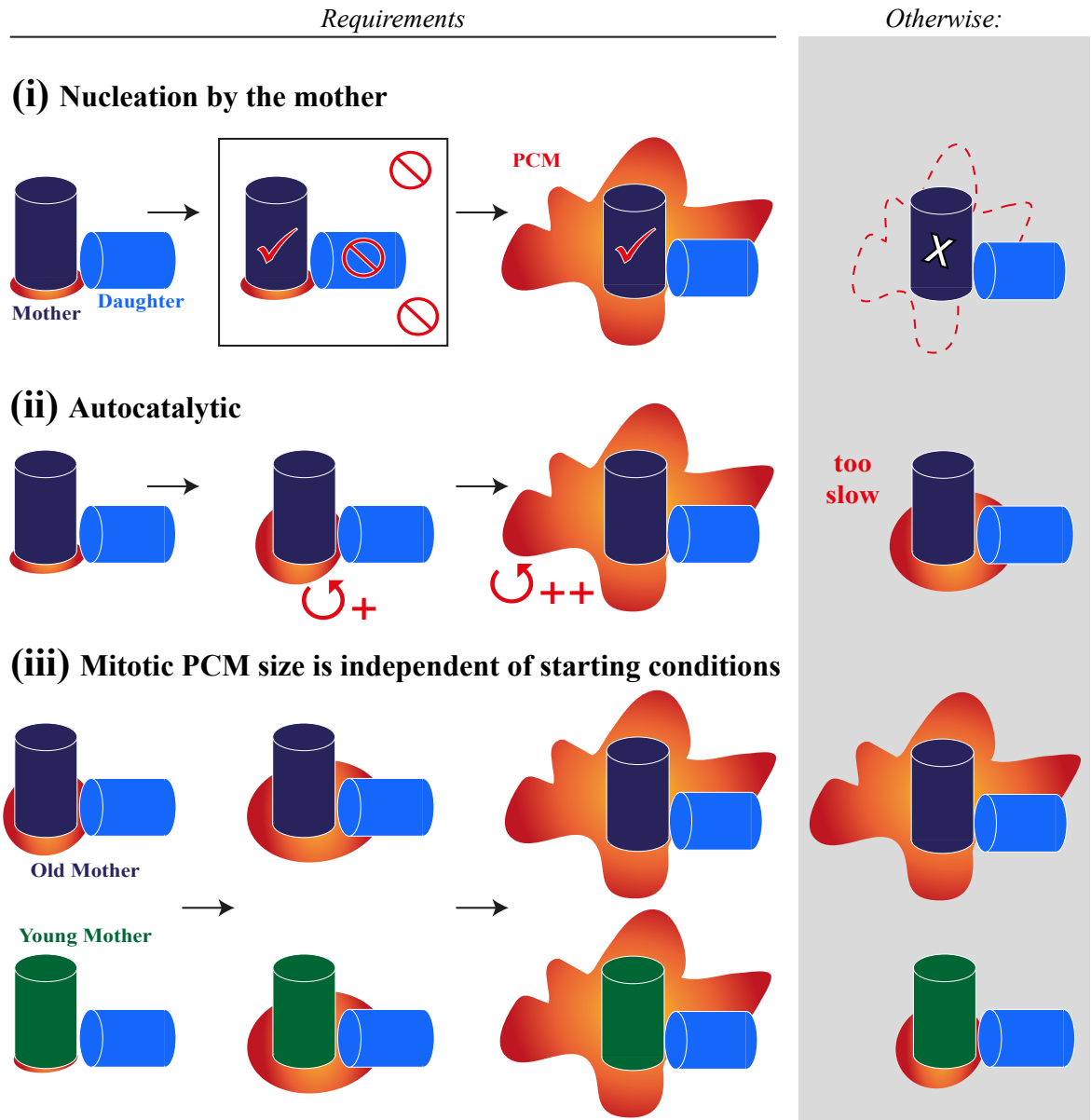


Figure 1.4 – Centrosome maturation in preparation for mitosis.

(A) 3D-SIM images of centrosomes from *Drosophila* S2 cells in interphase or mitosis, stained to show the distribution of PCM proteins Spd-2, Cnn, γ -tubulin and pericentrin-like protein (Plp). The distribution of all but Plp increases dramatically in preparation for mitosis. Adapted from (Fu and Glover, 2012). (B) Schematics of three fundamental requirements of mitotic PCM expansion: (i) New PCM material is recruited only to the mother centriole (red checkmark), and not to the daughter centriole or elsewhere in the cytoplasm; (ii) PCM expansion is autocatalytic; and (iii) centrosomes with different starting amounts of PCM must be able to reach similar sizes. The “Otherwise” grey box includes schematics representing what happens/would happen if one of these requirements is not fulfilled.

centrioles can reform (Basto et al., 2006; Bobinnec et al., 1998; Cabral et al., 2019; Kirkham et al., 2003; Wang et al., 2011b) (Figure 1.4Bi, *grey box*). Second, mathematical modelling has shown that, in order to account for the high rates of mitotic PCM assembly measured experimentally, this process must somehow be “autocatalytic”: i.e. the centriole initially recruits the key component(s) necessary for PCM assembly, and this event promotes further recruitment/PCM assembly (Zwicker et al., 2014) (Figure 1.4Bii). Third, during mitosis there are two spatially separated centrosomes (one at each mitotic pole) with a PCM of similar size. How this equalizing is achieved is not obvious, as one of these centrosomes will be organised by a young mother centriole that was nucleated in the previous cell cycle by the older mother centriole present in the other centrosome. The “younger” centrosome is often associated with less PCM at the start of the cycle; thus, centrosomes that differ in age and PCM content must somehow assemble a mitotic PCM of similar size (Conduit et al., 2010, 2015a; Raff, 2019) (Figure 1.4Biii).

A model that aims to satisfactorily explain mitotic PCM assembly must be able to address all three fundamental aspects of this process. For example, PCM assembly could rely on a single scaffold protein forming autocatalytic assemblies. However, such assemblies might then form everywhere in the cytoplasm, and not just around the mother centriole. Furthermore, under such model, if the older centrosome were even slightly enriched in such scaffold protein, it would more efficiently compete for scaffolding subunits. This would create an asymmetry in PCM assembly potential between the two centrosomes, resulting in a PCM size difference by the start of mitosis (Conduit et al., 2015a; Raff, 2019; Zwicker et al., 2014).

In fly embryos, two structural centrosomal proteins have key roles in PCM assembly: Spd-2 and Cnn (Conduit et al., 2010, 2014a, 2015a; Dix and Raff, 2007; Dobbelaere et al., 2008; Feng et al., 2017; Goshima et al., 2007; Lucas and Raff, 2007). Spd-2 and Cnn are recruited to the centriole wall by Asl (and perhaps Sas-4) and then “flux” outwards, forming co-dependent scaffolds which allow the recruitment of other components (e.g. γ -tubulin) to the expanding PCM (Conduit et al., 2010, 2014b; Gartenmann, 2018). This “inside-out” model can explain the requirement for PCM nucleation by a centriole, and also how centrosomes that initially differ in size may attain a consistent steady-state size, determined by the rate of recruitment to the mother centriole wall and the loss of scaffold at the centrosome periphery. However, as the rate of centrosome growth is limited by the very small size of the centriole wall, this model cannot easily account for the high rates of mitotic PCM assembly. However, it has been proposed that Spd-2 and Cnn could form a positive feedback loop that makes the overall PCM assembly process autocatalytic: Spd-2 recruits Cnn into the PCM, and Cnn helps maintain Spd-2 at the PCM, so more Spd-2 accumulates, which drives higher rates of Cnn recruitment (Conduit et al., 2014b, 2015a; Raff, 2019).

Centriole duplication is numerically restricted so that each mother centriole only generates one daughter centriole in any one cell cycle (Wong and Stearns, 2003). This is partly achieved by keeping the mother and daughter centrioles tightly engaged in their orthogonal arrangement from the start of centriole duplication in S-phase until G2/mitosis, when **centriole disengagement** ensures that this block is removed from the mother centriole. Thus, mother and daughter centriole become able to separate, and in the following S-phase the mother centriole can nucleate a new daughter centriole. If this block is prematurely removed (for example, destroying the daughter centriole of an S/G2 centrosome), the mother centriole regains its ability to generate a new

daughter in the same cycle (Loncarek et al., 2008, 2010). However, the molecular mechanism behind this block is still unclear.

In parallel with, or soon after, centriole disengagement, several proteins are recruited to the daughter centriole to allow it to become a fully-functional mother, able to nucleate its own daughter and to recruit PCM in the following cell cycle (Chang et al., 2016; Fu et al., 2016; Izquierdo et al., 2014; Novak et al., 2014, 2016; Tsuchiya et al., 2016). This process is known as (mitotic) **centriole conversion** (Wang et al., 2011b). Lack of centriole conversion ensures that prior to mitosis, even in conditions that lead to the assembly of multiple daughter centrioles from the same mother, the daughter centrioles are unable to nucleate additional (granddaughter) centrioles (Cunha-Ferreira et al., 2009; Habedanck et al., 2005; Kleylein-Sohn et al., 2007; Loncarek et al., 2008).

In flies, a hallmark of centriole conversion is Asl recruitment to daughter centrioles (Novak et al., 2014). This allows the converted centriole to recruit Sak/PLK4, necessary for the nucleation of a new daughter centriole cartwheel (Cizmecioglu et al., 2010; Dzhindzhev et al., 2010; Hatch et al., 2010), and Spd-2 and Cnn, necessary for mitotic PCM assembly (Conduit et al., 2014b). It has been reported that Asl recruitment is driven by the sequential recruitment of Cep135, Ana1 and then Asl to the maturing centriole—forming a “protein bridge” that extends outwards from the inner centriole (Fu et al., 2016) (Figure 1.5A). However, while Cep295 (human homologue of fly Ana1) seems to be involved in centriole conversion (Chang et al., 2016; Fu et al., 2016; Izquierdo et al., 2014; Tsuchiya et al., 2016), it is unclear whether Ana1 is involved in the initial recruitment of Asl during centriole conversion or just in the subsequent maintenance of Asl at mother centrioles in flies (Fu et al., 2016; Saurya et al., 2016) (see 5.1.1). Furthermore, while Cep135 has been shown to

Asl Recruitment (Centriole Conversion)

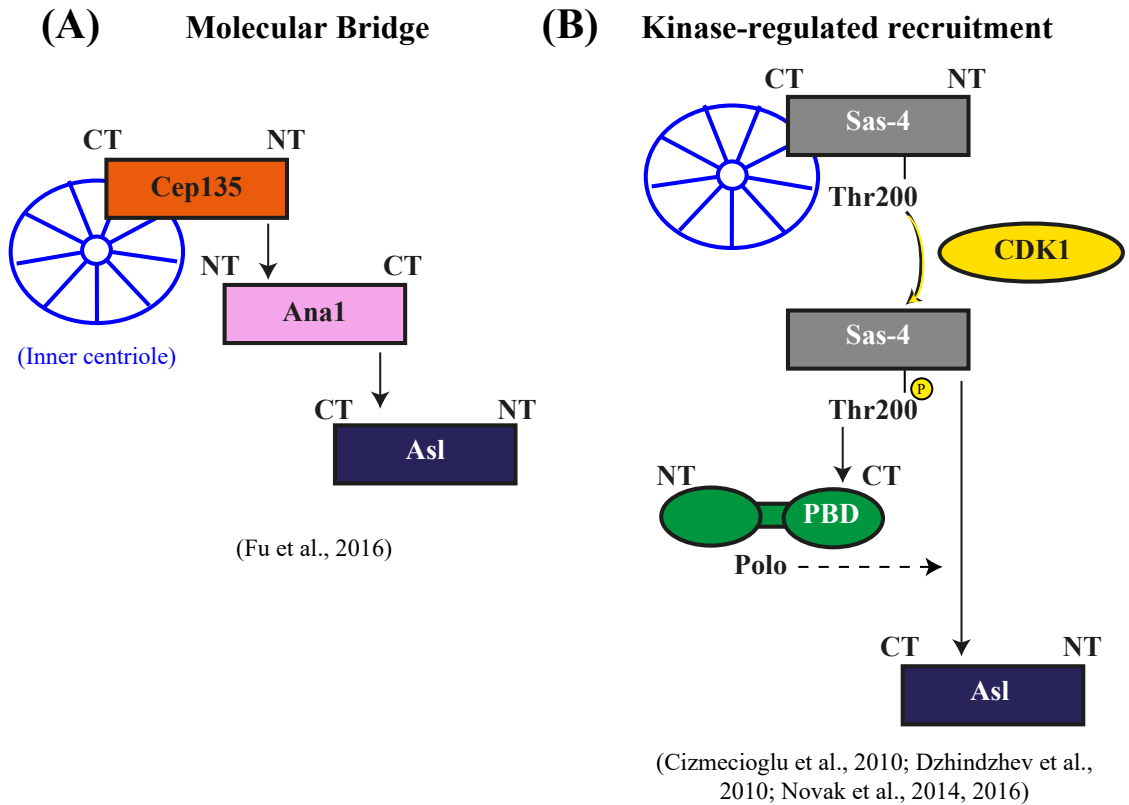


Figure 1.5 – Asl recruitment during centriole conversion.

Schematic models representing how Asl may be recruited to the daughter centriole for its conversion into a fully functional mother centriole. Note that these models represent alternative views of how centriole conversion might take place, but they are not necessarily incompatible. **(A)** The N-terminal domain (NT) of Cep135 can interact with the NT of Ana1, and the C-terminal domain (CT) of Ana1 can interact with the CT of Asl. Thus, these binding events result in the formation of a molecular bridge Cep135-Ana1-Asl, spanning from the inner area of the centriole (Cep135 CT) to the outer centriole (Asl NT). Recreating this bridge with alternative binding interactions (e.g. substituting the Cep135-NT/NT-Ana1 interaction by a Cep135-GFP/GFP-binding-protein-Ana1 interaction) can sustain Asl recruitment to centrioles (Fu et al., 2016). However, it is unclear that Cep135 and Ana1 are essential for centriole duplication in flies. **(B)** Asl recruitment depends on Sas-4, and the NT of Sas-4 also binds to the CT of Asl (Cizmecioglu et al., 2010; Dzhindzhev et al., 2010; Novak et al., 2014). Additionally, Sas-4 is phosphorylated by CDK1 at Thr200, and this phosphorylation event allows Polo to bind to Sas-4 via its PBD. This binding event is required for Polo as well as Asl recruitment to new centrioles (Novak et al., 2016). The exact mechanism by which Polo binding to Sas-4 promotes Asl recruitment is unclear (*dashed arrow*).

crosslink/bundle microtubule assemblies (hence contributing to centriole stability), it is uncertain that Cep135 is required for centriole assembly, as would be expected if it were essential for Asl recruitment (Fu et al., 2016; Kraatz et al., 2016; Roque et al., 2012). Conversely, it seems clear that there is a direct interaction between Sas-4 and Asl (human CPAP and Cep152, respectively) (Cizmecioglu et al., 2010; Dzhindzhev et al., 2010), and that Asl recruitment depends, at least in part, on Sas-4 and Polo kinase (Novak et al., 2014, 2016) (see 1.3.3) (Figure 1.5B).

1.1.3. MTOC, ciliogenesis and other functions

Microtubule-organising centres (MTOCs) nucleate and stabilise MTs when the concentration of tubulin is too low to support spontaneous MT polymerisation (Mitchison and Kirschner, 1984). In animal cells, **the PCM is a major MTOC** (Gould and Borisy, 1977; Robbins et al., 1968; Snyder and McIntosh, 1975). During interphase, centrosome-nucleated MTs help position organelles and determine cell polarity, whereas during cell division they are required for the efficient assembly of bipolar spindles during cell division (reviewed in Wu and Akhmanova, 2017).

Classical studies in various organisms (including *Drosophila*) have shown that centrosomal MT nucleation is highly dependent on γ -tubulin, that γ -tubulin localises to the PCM and forms part of ring-like complexes with other proteins, and that these γ -tubulin ring complexes (γ -TuRCs) can nucleate MTs *in vitro* and *in vivo* (Félix et al., 1994; Joshi et al., 1992; Moritz et al., 1995a, 1995b; Oakley et al., 1990; Raff et al., 1993; Stearns and Kirschner, 1994; Stearns et al., 1991; Sunkel et al., 1995; Zheng et al., 1995). The loss of γ -TuRCs from high-salt extracted centrosomes leads to loss of their MT nucleation potential, but this potential is restored upon treatment with high speed extract containing γ -TuRCs (Moritz et al., 1998; Schnackenberg et al., 1998).

However, in some cases (e.g. fly spermatocytes and worm embryos) some level of centrosomal MT nucleation can be maintained in the absence of γ -tubulin (Sampaio et al., 2001; Strome et al., 2001). Furthermore, it has been recently shown that aggregates of the worm PCM protein SPD-5 are able to concentrate tubulin and nucleate MTs *in vitro* upon addition of just two non- γ -TuRC proteins: TPXL-1 and ZYG-9 (part of the TPX2 and TOG domain families, respectively) (Woodruff et al., 2017).

Just as γ -tubulin is not always required for MT nucleation, centrosomes are not essential for cell division either (Basto et al., 2006). There are other, non-centrosomal pathways that can contribute to the formation of stable, bipolar mitotic spindles, even when centrosomes are present (reviewed in Conduit et al., 2015a; Wu and Akhmanova, 2017). Evidence of this is that *sas-4^{-/-}* mutant flies lose all their centrosomes by the third-instar larval stage, and yet they develop into morphologically normal adults (Basto et al., 2006). In mammalian cells, centrosomes can be destroyed by laser ablation or microsurgery, but bipolar spindles still form (Hinchcliffe et al., 2001; Khodjakov et al., 2000). Similarly, in many animals, oocytes naturally lack centrosomes but robust, bipolar meiotic spindles still form (McKim and Hawley, 1995). In *Drosophila* oocytes, this is thanks to two complementary pathways (Subito/MKlp2 and Augmin) that recruit γ -TuRCs to the spindle equator and the poles (Colombié et al., 2013; Giunta et al., 2002; Jang et al., 2005, 2007; Meireles et al., 2009; Reschen et al., 2012; Romé and Ohkura, 2018).

However, centrosomes are key for efficient assembly and orientation of the mitotic spindle. For example, centrosomes are very important in *Drosophila* neuroblasts, which divide asymmetrically to generate another neuroblast (self-renewing stem cell) and a differentiating daughter cell. In neuroblasts, centrosomes separate immediately

after cytokinesis but migrate together to the apical cortex. The centrosome containing the older centriole then loses its PCM due to decreased Cnn incorporation, and migrates basally (possibly due to downregulation of Spd-2-mediated Fzr recruitment), where it accumulates PCM again. Conversely, the apical centriole remains with the apical membrane. Thus, when the spindle assembles it is already in alignment with the polarity axis of the cell. The differentiating daughter inherits the basal centrosome, and the apical centrosome remains in the neuroblast (Conduit and Raff, 2010; Gambarotto et al., 2019; Januschke et al., 2011; Rebollo et al., 2007; Rusan and Peifer, 2007).

Similarly, Cdk5Rap2 (vertebrate homologue of Cnn) is necessary for correct glial spindle orientation in human cerebral organoids, and loss-of-function mutations result in abnormal, premature neurogenic non-proliferative divisions and loss of neural stem cells (Lancaster et al., 2013). Moreover, while mitotic spindles can form in vertebrate cells without centrosomes, bipolar spindle assembly and anaphase onset are delayed, and the frequency of lagging chromatids increases, leading to chromosome instability and aneuploidy (Sir et al., 2013). Thus, centrosomes are particularly important MTOCs in organisms with complex development or high chromosome numbers.

Additionally, **centrosomes are major signalling hubs** (reviewed in Arquint et al., 2014). Centrosomes can integrate and respond to signals from the cell cycle, development and the DNA damage response. Signalling molecules can be concentrated at the centrosome, thus enhancing the kinetics for specific biochemical reactions or creating local signalling gradients. Furthermore, centrosomes can act as a platform where proteins from different signalling pathways can come into contact, promoting signalling coordination and crosstalk (Arquint et al., 2014). For example, in fly embryos, DNA damage triggers DmCHK2 accumulation at centrosomes, which

leads to centrosome inactivation (including loss of γ -tubulin). This results in mitotic delay and the detachment of the damaged nuclei, preventing their incorporation into the blastoderm (Sibon et al., 2000; Takada et al., 2003). In fly embryos, centrosomes are also involved in the degradation of cyclinB, which starts at the spindle poles towards the end of metaphase and spreads to the rest of the spindle (Huang and Raff, 1999). If centrosomes detach from mitotic spindles, cyclinB disappears from centrosomes but not from the detached spindles, which remain arrested (Wakefield et al., 2000). In vertebrate cells, centrosomes have even been implicated in the pro-inflammatory innate immune response and cytokine production (Vertii et al., 2016).

Finally, centrioles are required to build **cilia**. Cilia are long, thin appendage-like organelles that protrude from the surface of many non-dividing or terminally differentiated cells. In vertebrates, almost all cell types present a single primary cilium, and cilia have many different functions (reviewed in Fliegauf et al., 2007; Ishikawa, 2017; Wheway et al., 2018). However, in *Drosophila* there are only two main types of cells that have cilia or flagella: type I mechanosensory neurons and sperm. Thus, *sas-4*^{-/-} mutant flies without centrosomes eclose, but they lack cilia in their mechanosensory neurons, so they are uncoordinated (they cannot hold their wings and legs normally) and they die shortly after eclosion (Basto et al., 2006). This uncoordinated phenotype is also seen in other fly strains with mutations in key players involved in centriole and cilia assembly (Bettencourt-Dias et al., 2005; Blachon et al., 2008, 2009; Martinez-Campos et al., 2004; Peel et al., 2007; Stevens et al., 2009; Wang et al., 2011a).

1.1.4. Centrioles, centrosomes and cilia in human disease

Given the many functions of centrioles and centriole-derived structures, it is not surprising that they are linked to a wide range of developmental and adult diseases.

These can be broadly classified into three categories depending on the underlying defect: abnormal ciliogenesis, which leads to cilia-related disorders with very diverse phenotypes (reviewed in Braun and Hildebrandt, 2017; Fliegauf et al., 2007); abnormal numbers of centrosomes; or abnormal centrosome structure, function and positioning. However, the molecular mechanisms by which these abnormalities lead to disease are still unclear (Nigg and Raff, 2009).

For example, it has long been proposed that centrosomal aberrations could contribute to tumorigenesis (Boveri, 1914, 2008). Centrosome amplification (CA) is a hallmark of cancer, often linked to chromosomal aberrations and poor disease prognosis (Chan, 2011; Lingle et al., 2002; Pihan et al., 2003). However, flies with extra centrosomes do not show signs of increased tumorigenesis (Basto et al., 2008). Transplantation of larval neuroblasts with either extra or no centrosomes into the abdomen of normal adults can lead to tumours, but this could be due to the specific role of centrosomes in neuroblast asymmetric divisions (see 1.1.3), rather than an increase in genome instability and/or chromosomal aberrations (Basto et al., 2008; Castellanos et al., 2008). Conversely, CA can drive metastatic behaviour in affected human epithelial cells and their normal neighbours (Arnandis et al., 2018; Godinho et al., 2014); and it may also increase the number of errors in chromosome segregation due to the formation of transient multipolar spindles (Ganem et al., 2009; Rhys et al., 2018). Nevertheless, CA can only trigger tumorigenesis in some mouse models (Levine et al., 2017) and not others (Kulukian et al., 2015; Marthiens et al., 2013; Vitre et al., 2015), unless p53 activity is already deficient (Coelho et al., 2015; Serçin et al., 2016). Thus, whether CA can truly drive tumorigenesis is still unclear, and might be tissue specific.

Additionally, recent studies have shown that structural centrosome aberrations may also promote tumour formation, either by triggering CA (Marteil et al., 2018), or by directly disrupting MT organisation and cell-to-cell contacts, altering cell polarity and epithelial integrity (Ganier et al., 2018a, 2018b; Schnerch and Nigg, 2016).

It appears that p53 signalling is crucial to prevent the proliferation of mammalian cells with extra centrosomes (Ganem et al., 2014), as well as to trigger apoptosis in those without centrosomes (Lambrus et al., 2015; Wong et al., 2015). p53-mediated apoptosis is the reason why mouse embryos without centrosomes die during midgestation (Bazzi and Anderson, 2014); but also why CA in mouse brains causes microcephaly (Marthiens et al., 2013). In humans, mutations that cause abnormal centrosomal numbers, structure and/or function have also been linked to primary recessive microcephaly, as well as other developmental disorders such as primordial dwarfism (Arquint and Nigg, 2014; Boonsawat et al., 2019; Chavali et al., 2014; Lancaster et al., 2013; Marthiens et al., 2013).

1.2. Polo kinase, a master regulator of the cell cycle

Polo-like kinases (Plks) are serine/threonine protein kinases characterised by the presence of a C-terminal Polo box domain (PBD) containing two ~70-80 aa segments called Polo boxes (Elia et al., 2003a) (Figure 1.6A). Their name derives from *polo*¹ mutants identified in *Drosophila*, which often presented spindle pole abnormalities (Llamazares et al., 1991; Sunkel and Glover, 1988). Since then, Plks have been shown to be highly conserved from yeast to humans, although absent in plants (Golsteyn et al., 1994; Hartwell et al., 1973; Karpov et al., 2010; Ohkura et al., 1995). In humans there are five Plks, although PLK5 is inactive (Pintard and Archambault, 2018). PLK1 (the best characterised), PLK2 (possibly implicated in S-phase checkpoint, (Matthew

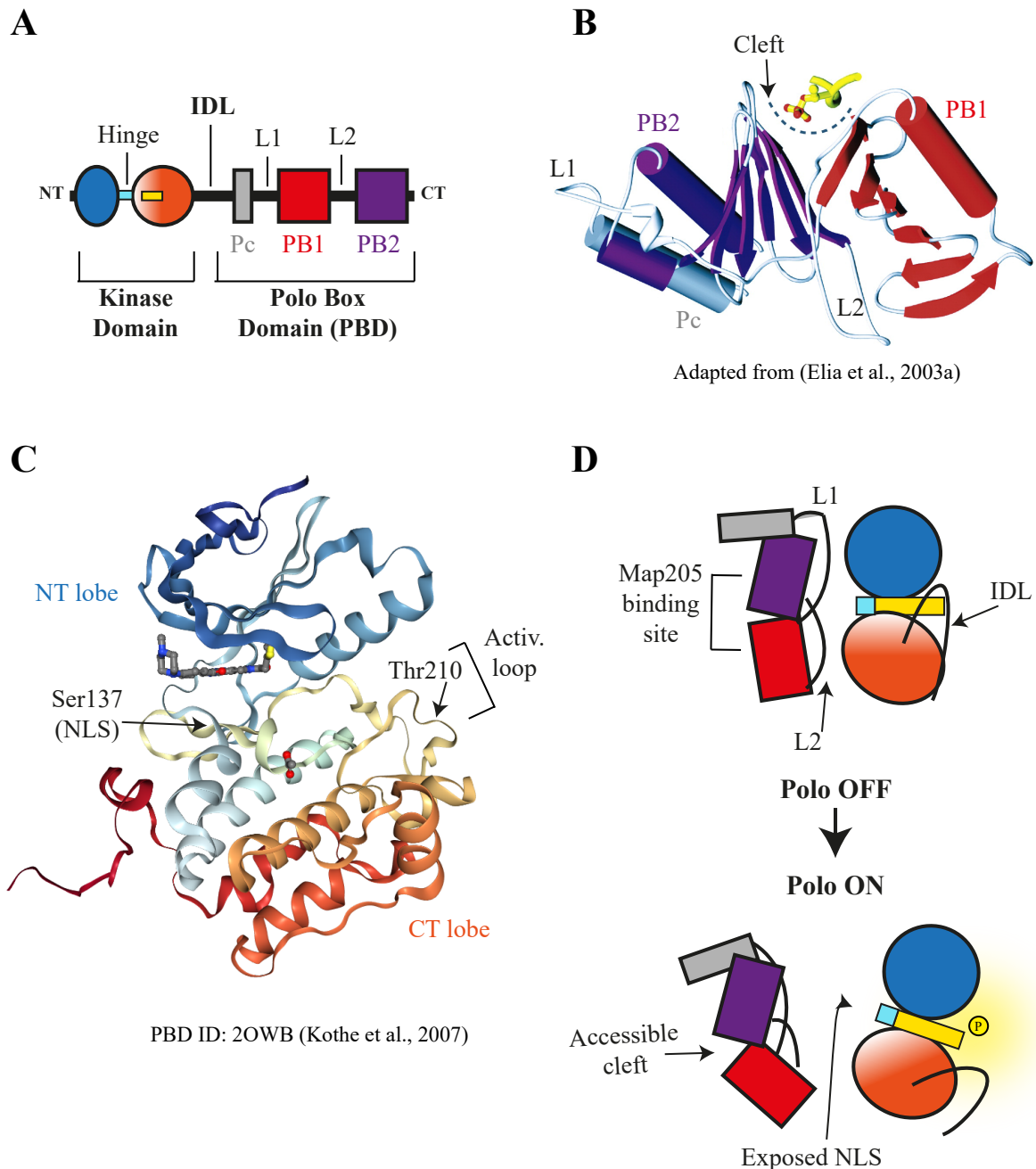


Figure 1.6 – The structure of Polo kinase.

(A) Schematic representation of the different domains of Polo kinase (not to scale). The yellow box represents the active site within the C-terminal lobe of the kinase domain (KD) (grey/orange). (B) Crystal structure of the PLK1 Polo box domain (PBD) (coloured as in (A)) with a pThr-containing peptide (yellow) occupying the PBD shallow cleft. Adapted from (Elia et al., 2003a). (C) Crystal structure of PLK1 KD (coloured as in (A)) complexed with the pyrrolo-pyrazole inhibitor PHA-680626 (dark grey). The hinge region (light blue) connects the N-terminal and C-terminal lobes (dark blue and grey/yellow/orange, respectively) and contains Ser137, which is part of an NLS. The cleft between the two lobes contains the active site, including the activation loop with Thr210 (in this structure, mutated to Val to facilitate protein crystallisation) (yellow). Image created using the RCSB PDB website <http://www.rcsb.org/> (Berman et al., 2000), PBD ID: 2OWB (Kothe et al., 2007). (D) Schematic representation of Polo autoinhibition due to KD/PBD interactions which may be facilitated by Map205 binding, based on a crystal structure from (Xu et al., 2013). The different domains are depicted as in (A), but for ease of visualisation the complete interdomain linker (IDL) connecting the C-terminal lobe of the KD and the Pc is not shown. The two linker regions of the PBD (L1 and L2) make multiple contacts with the KD, and the IDL occludes the activation loop. The L1, L2 and IDL re-arrange upon canonical PBD binding to a substrate and/or phosphorylation of Ser137 or Thr210, making these contacts no longer possible.

et al., 2007)) and PLK3 (required for G1-S phase transition and stress response (Helmke et al., 2016)) have very similar structure and are homologous to *Drosophila* Polo. Conversely, PLK4, which specifically regulates centriole cartwheel assembly (see 1.1.1), is characterised by having a “cryptic Polo box domain” (made of two Polo boxes in tandem) and an additional Polo box at its C-terminus, instead of a regular PBD (Slevin et al., 2012).

In the following sections I provide a brief overview of the structure and function of Polo, focused mostly on the fly and vertebrate proteins. As they are clear orthologues, they are generally referred to as “Polo”, but since most structural data available is based on human PLK1, amino acid residues are numbered based on the hPLK1 sequence. While observations relating to one species are assumed to be applicable to others, special care has been taken to highlight any known significant differences.

1.2.1. Polo localisation, structure and regulation

PLK1 localises to a variety of subcellular structures throughout the cell cycle, with changing kinetics: from G2 to metaphase it is found at centrosomes, where it is highly dynamic; it is also at the nucleus during prophase; after NEB it is also at kinetochores (KTs); upon anaphase entry it re-localises to the central spindle; and finally during telophase/cytokinesis it is at the midbody, where it displays a slower turnover (Arnaud et al., 1998; Colicino et al., 2017; Golsteyn et al., 1994, 1995; Kishi et al., 2009). In early fly embryos, which have no Gap phases (Foe and Alberts, 1983), Polo presents a similar localisation, although it remains at centrosomes throughout the cell cycle (Llamazares et al., 1991; Moutinho-Santos et al., 1999).

The **PBD** is responsible for Polo recruitment to many of these structures, including the centrosome (Elia et al., 2003a, 2003b; Hanisch et al., 2006; Jang et al., 2002a;

Kachaner et al., 2017; Lee et al., 1998; Reynolds and Ohkura, 2003; Seong et al., 2002; Song et al., 2000). It allows Polo to bind to phosphorylated proteins displaying a very simple, preferred motif (Ser-[pSer/pThr]-X/Pro) (Elia et al., 2003a, 2003b). Initial X-ray structures of the PLK1 PBD bound to a phosphopeptide (Cheng et al., 2003; Elia et al., 2003a) showed that, despite the Polo boxes (PBs) sharing only ~20-25% sequence similarity, they form very similar structures ($\beta_6\alpha$ motifs) (Figure 1.6B). The PBs come together as a 12-stranded β sandwich flanked by three α -helices, which the Polo cap (Pc, region immediately upstream of PB1) wraps around, keeping the structure together. The binding site is formed by a shallow cleft between the two PBs. This region is highly conserved amongst Polo kinases in different species and it is the only positively charged exposed surface.

The PBD binds the phosphopeptide by forming a complex network of hydrogen bonds, as well as direct contacts between the phosphate group and a PB2 “pincer” made of residues His-538 and Lys-540, which are essential for the centrosomal localisation of the PLK1 PBD. In addition, the extremely high selectivity for Ser at position -1 of the optimal PBD binding motif is due to the side chain of Ser-1 being oriented into a groove on the PBD surface. There it makes three hydrogen bonds and it is further stabilised by van der Waals interactions with Trp-414, which is highly conserved. Ser-1 cannot be substituted by Thr without disturbing PBD binding, as the γ -methyl group of Thr causes a steric clash with Trp-414 (Cheng et al., 2003; Elia et al., 2003a).

Rarely, Polo may exhibit non-canonical binding to certain proteins, often Polo regulators. For example, Polo can be inhibited by the MT-associated protein Map205 (Archambault et al., 2008; Xu et al., 2013). This binding does not involve a Ser-Ser/Thr motif nor does it require Map205 to be phosphorylated, although it requires

both the PBD and the kinase domain (KD). Conversely, Polo binding to Bora (a partner of Aurora A) promotes Polo activation by Aurora A (see below) and Polo regulation of Aurora A localisation. CDK1 phosphorylation drives canonical Bora binding to the PBD (Chan et al., 2008; Macûrek et al., 2008; Vigneron et al., 2018), but at least one study found that non-phosphorylated Bora could still bind Polo, and perhaps interact with its KD (Seki et al., 2008). Another example is PLK1 recruitment to the central spindle, where PLK1 phosphorylates and binds MKlp2 kinesin and PRC1. This binding involves the PBD, and PRC1 presents canonical Ser-Thr binding sites (Neef et al., 2003, 2007). However, while there are many Ser-Ser/Thr sites in the MKlp2 sequence, none of the phosphorylated residues identified via mass spectrometry fit this motif. Indeed, Ser528, which was shown to be phosphorylated and required for strong PBD binding, is part of a His-pSer-Leu motif (Neef et al., 2003). Finally, the “Matrimony protein” also binds non-canonically to Polo, sequestering it in fly oocytes arrested during meiosis (Bonner et al., 2013; Xiang et al., 2007).

The structure of the hPlk1 **KD** is similar to that of other kinases, with two lobes forming a cleft where the active site is found (Kothe et al., 2007) (Figure 1.6C). The N-terminal lobe comprises mostly antiparallel β -sheets. It is connected to the primarily alpha-helical C-terminal lobe by a short hinge region. The C-terminal lobe includes an activation loop with a DFG motif, where Asp194 is positioned to interact with Mg^{2+} and the phosphate of the nucleotide substrate. However, unlike most other kinases, the hinge region has Val114 and Leu132 at positions normally occupied by bulkier amino acids, creating a pocket that allows the binding of BI2536, a highly specific PLK1 inhibitor (Kothe et al., 2007). The hinge contains a nuclear localisation sequence (NLS) that helps PLK1 access the nucleus before nuclear envelope breakdown (Taniguchi et al., 2002).

Polo kinase is thought to preferentially phosphorylate the consensus motif Asp/Glu-X-Ser/Thr- ϕ , where Ser/Thr is the phosphoacceptor residue, X is any amino acid and ϕ is a hydrophobic amino acid (Nakajima et al., 2003). However, a study mapping the PLK1-dependent phosphoproteome of isolated human mitotic spindles found several alternative variations of this motif. Additional *in vitro* experiments showed that the originally described motif is only a rough approximation and, depending on the context, Polo can be tolerant of a variety of amino acids at certain positions (Santamaria et al., 2011). For example, PLK1 can phosphorylate six sites in p31^{comet}, none of which fit the traditional consensus well (Kaisari et al., 2019).

Polo kinase activity is promoted by KD phosphorylation at two regulatory amino acids: Thr210 in the activation loop, which is the major *in vivo* phosphorylation site, and Ser137 (Jang et al., 2002b; Kelm et al., 2002; Qian et al., 1999). Thr210 fits the Aurora phosphorylation consensus motif, and Aurora A and Aurora B have both been implicated in Polo activation (reviewed in Archambault et al., 2015). Phospho-mimicking mutations at either one or both sites have a similar effect on hPLK1 activity (Jang et al., 2002b), suggesting that phosphorylation of either residue results in the KD adopting an active conformation. Conversely, for *Xenopus* Plx1, the double phospho-mimicking mutant had higher activity than either single mutant (Qian et al., 1999). Ser137 is part of the NLS, and is not as easily accessible as Thr210 (Figure 1.6C), so it is unclear how efficiently it is phosphorylated *in vivo*, and how this event could have the same effect as phosphorylating Thr210 (Kothe et al., 2007). However, Ser137 phosphorylation may disrupt the KD/PBD interaction (Xu et al., 2013) (see below). Other phosphorylation sites in the KD have been identified but their function is less clear, and they have so far only been described in vertebrates or mammals (Kasahara et al., 2013; Kelm et al., 2002; Maroto et al., 2008).

Polo KD activity is downregulated if the PBD is not bound to a phosphorylated protein (Elia et al., 2003a; Xu et al., 2013). X-ray structures of a zebrafish PLK1 KD and PBD complex stabilised by Map205 showed that the KD can bind the PBD of the same molecule at a site far from the PBD binding cleft. In doing so the PBD linker regions (between Pc and PB1, and PB1 and PB2) undergo a significant conformational change and bind the KD at the hinge between the two KD lobes, burying the NLS. Thus, the kinase active site is not occluded, but the hinge region is less flexible, resulting in autoinhibition. In this conformation, the interdomain linker (IDL) between the KD and PBD may also contribute to KD inhibition by occluding Thr210, making it less accessible to other kinases. Conversely, if Thr210 is phosphorylated, the KD/PBD inhibitory interaction is less likely (Kachaner et al., 2017; Xu et al., 2013) (Figure 1.6D).

Polo kinase activity is also regulated by PP1 phosphatases (reviewed in Barr et al., 2011). For example, the catalytic subunit PP1C binds to MYPT1, a regulatory subunit which can bind the PBD upon phosphorylation by CDK1. Thus, phosphorylated MYPT1 can recruit Polo to PP1C, and PP1C dephosphorylates Thr210 (Yamashiro et al., 2008). Polo activity is further regulated by the molecular chaperone Hsp90 and its co-chaperone Sgt1, which probably help to stabilise it (Cárcer, 2004; Cárcer et al., 2001; Martins et al., 2009). Other regulatory mechanisms include mono-ubiquitination and SUMOylation at Lys492 of the PBD, which lead to PLK1 dissociation from KTs (Beck et al., 2013) and promote Polo stability and correct localization (Li et al., 2017a; Wen et al., 2017), respectively. Finally, poly-ubiquitination of the IDL by APC/C^{Cdh1} triggers Polo degradation until the start of G1 (Lindon and Pines, 2004), although Polo is not completely degraded, as it is an important regulator of cytokinesis (see below).

1.2.2. Polo has many functions

Polo has many functions in the cell (reviewed in Archambault and Glover, 2009; Barr et al., 2004; Zitouni et al., 2014). Low levels of Polo activity appear to be enough to drive early mitotic events such as spindle assembly, while higher levels are required for later mitotic events such as cytokinesis (Lera and Burkard, 2012). This fits well with the idea that PLK1 binding to (pro)metaphase-partners is generally reliant on CDK1 priming, whereas in anaphase PLK1 must self-prime its own binding sites (Neef et al., 2007). Figure 1.7B summarises many of Polo's known roles, some of which are further discussed below; Polo centrosomal functions are detailed elsewhere (1.3).

Entry into mitosis (and meiosis) is marked by the activation of CDK1-cyclinB, which is regulated by a variety of factors, including kinases WEE1 and MYT1 (which inhibit it) and phosphatase CDC25/String (which activates it). During mitotic entry, Polo is imported into the nucleus via its NLS, which becomes exposed upon Thr210 phosphorylation (Bruinsma et al., 2015; Kachaner et al., 2017; Taniguchi et al., 2002). In the nucleus, Polo binds and activates CDC25 and triggers changes in its localisation (Elia et al., 2003a; Kachaner et al., 2017; Kumagai and Dunphy, 1996; Qian et al., 2001). Phospho-String is exported into the cytoplasm in fly embryos (Kachaner et al., 2017), whereas phospho-CDC25 appears to accumulate at the nucleus in human cells (Lobjois et al., 2009; Toyoshima-Morimoto et al., 2002). However, further work is required to assess if phospho-CDC25/String is truly transported in opposite directions in different species (Pintard and Archambault, 2018). Polo also inactivates MYT1 (Nakajima et al., 2003), promotes WEE1 degradation (Watanabe et al., 2005) and phosphorylates cyclinB at centrosomes, triggering its accumulation at the nucleus (Toyoshima-Morimoto et al., 2001). Once active, CDK1-cyclinB further phosphorylates its regulators CDC25 and WEE1. Thus, complete Polo inhibition

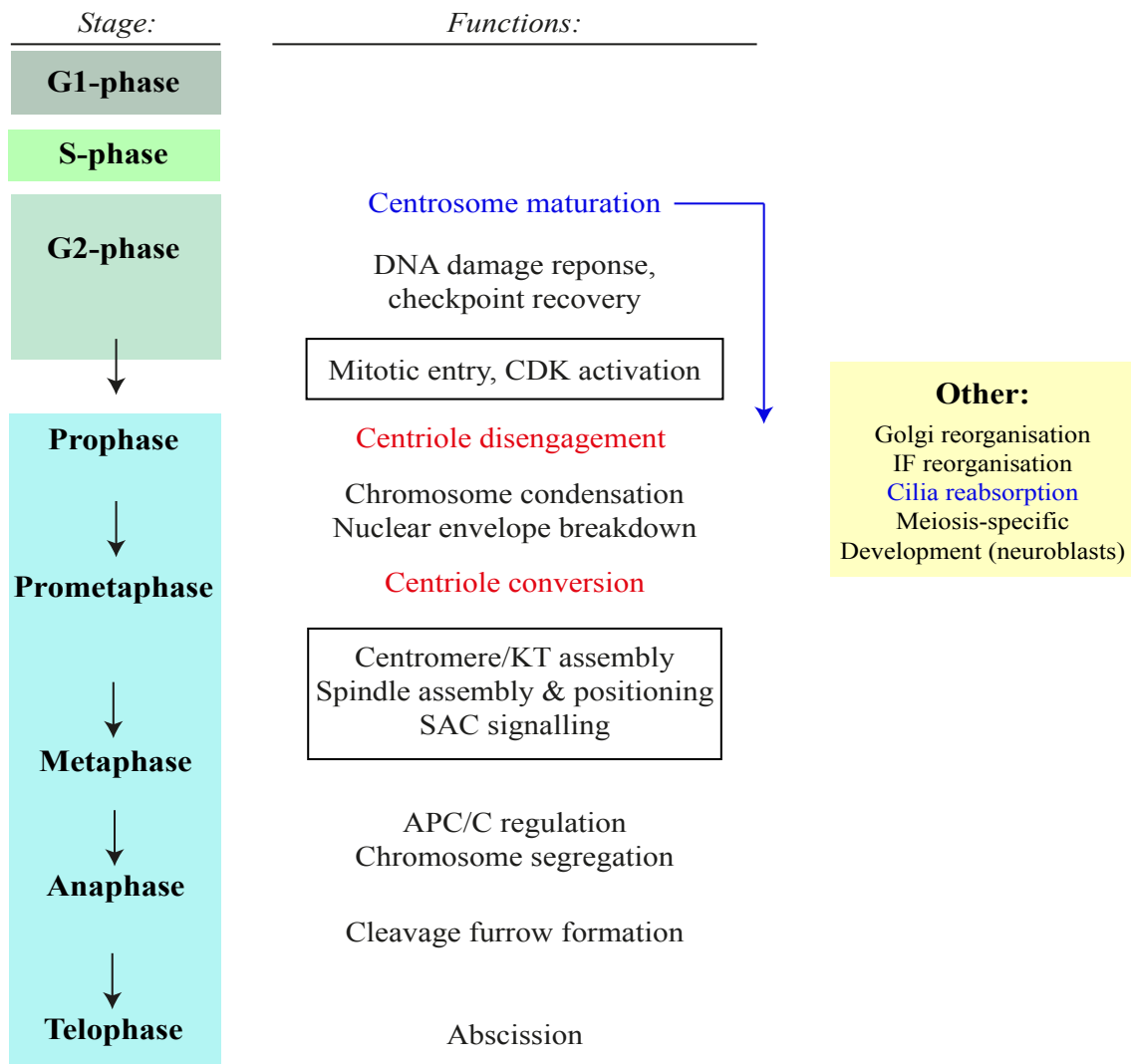
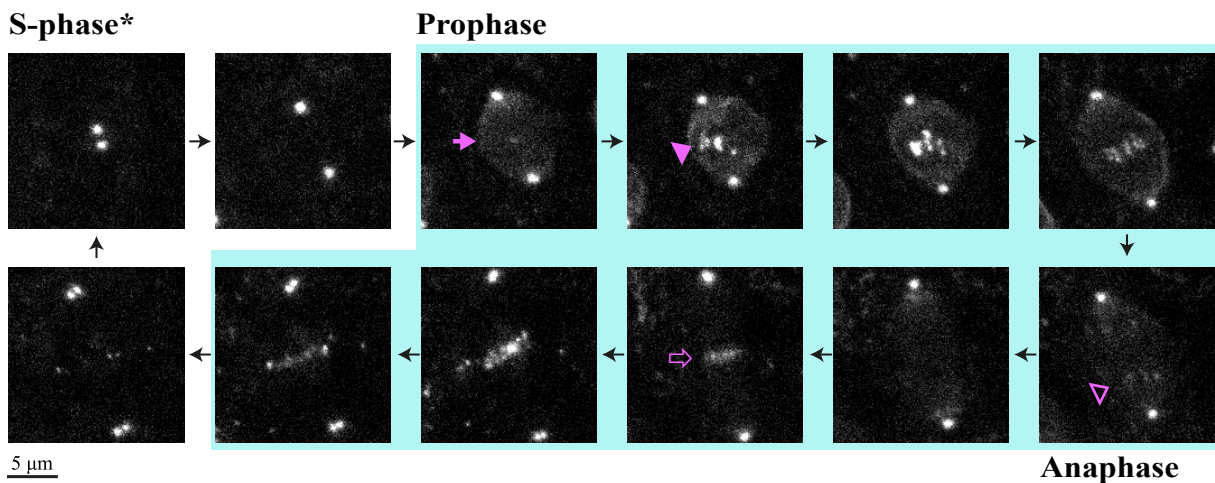
A**B**

Figure 1.7 – Polo functions and localisation throughout the cell cycle.

(A) (Left) Schematic showing an approximate timeline of major G2-phase and mitotic events known to be regulated by Polo. (Right) The yellow box lists other Polo functions. Non-centrosomal Polo functions in the cell are indicated in black. Most studies have focused on Polo functions prior to anaphase, of which some key ones are highlighted (boxes). Polo functions at centrosomes/cilia are indicated in blue if the specific cell cycle stage at which the event takes place is well known, e.g. in cells with Gap phases centrosome maturation starts at G2-phase and continues until mitosis (arrow). However, centriole disengagement is thought to start in G2 or early mitosis, although centriole separation is only visible at the end of mitosis; and centriole conversion presumably takes place in early mitosis, in parallel with or soon after centriole disengagement and culminating with Asl recruitment, which requires CDK1 activity. Hence, these events are indicated in red. (B) PoloGFP distribution throughout the cell cycle in syncytial fly embryos, which lack Gap phases (Foe and Alberts, 1983). Only one nucleus associated with a pair of centrosomes is shown for simplicity. PoloGFP localises to centrosomes and, from prophase, to the nuclear envelope (arrow) and KTs (arrowhead). From anaphase PoloGFP is lost from KTs (empty arrowhead) and re-localises to the midzone (empty arrow).

results in G2 arrest (Aspinall et al., 2015; Gheghiani et al., 2017), and Polo activation by Aurora A/Bora (which is phosphorylated by CDK1-cyclinA) triggers a CDK1-cyclinB positive feedback loop that drives mitotic entry (Vigneron et al., 2018).

Polo-dependent degradation of WEE1 is particularly important for mitotic entry after G2 arrest triggered by **DNA damage** checkpoint activation (van Vugt et al., 2004).

Polo has been traditionally linked to DNA damage repair silencing (Li et al., 2017b; Mailand et al., 2006; Mamely et al., 2006; van Vugt et al., 2010; Yoo et al., 2004), but recent work in human cells suggests that in some cases PLK1 may promote DNA repair via homologous recombination (Yata et al., 2012). During prophase, Polo also promotes bulk cohesin removal from **chromosome** arms (Godinho and Tavares, 2008; Lénárt et al., 2007; Sumara et al., 2002) and chromosome condensation (Abe et al., 2011; Kagami et al., 2017); and **nuclear envelope breakdown** (NEB) (Chase et al., 2000; Linder et al., 2017; Martino et al., 2017).

Polo also has many different functions at different subcompartments of **centromeres and kinetochores** (KTs), often in collaboration with other kinases (Lera et al., 2016; Liu et al., 2012). KTs need to assemble correctly, bind chromosomes to MTs, and stop premature chromosome segregation until all chromosomes have correctly attached to MTs. It has been proposed that the bulk of Polo at centromeres/KT localises to inner KTs/close to the inner centromere, independent of MT attachment status (Kang et al., 2006; Lera et al., 2016; Park et al., 2015). Prior to anaphase, inner centromeres also present Aurora B, INCEP, Survivin and Borealin, which form the Chromosomal Passenger Complex (CPC) and are necessary to destabilise erroneous KT-MT attachments, amongst many other important functions during mitosis (Hauf et al., 2003; Klein et al., 2006; Lampson et al., 2004). INCEP binds Polo and allows Aurora

B to phosphorylate and activate it (Carmena et al., 2012; Goto et al., 2006). At the same time, Polo promotes CPC activity and recruitment to centromeres (Carmena et al., 2014; Chu et al., 2011; Moutinho-Santos et al., 2012). Furthermore, Polo regulates the presence of centromere-specific histone marks via Haspin and NHK-1 kinases (Brittle et al., 2007; Ghenoïu et al., 2013; Zhou et al., 2014). Thus, Polo is important for correct centromere organisation.

At outer KT, Polo contributes to the stabilisation of KT-MT attachments, **bipolar spindle formation** and chromosome congression (Lénárt et al., 2007; Li et al., 2010; Maia et al., 2012; McInnes et al., 2006; Nishino et al., 2006; Peters et al., 2006; Santamaria et al., 2007; Seong et al., 2002). Polo at the spindle poles also contributes to pole focusing, centrosome-spindle coupling and spindle positioning by promoting the activity of the minus end MT-binding protein Asp/ASPM (Bosveld et al., 2017; Riparbelli et al., 2002; Schoborg et al., 2015; Wakefield et al., 2001), and downregulating cortical dynein-dynactin localisation if a spindle pole comes in too close proximity to a specific region of the cortex (Kiyomitsu and Cheeseman, 2012; Kschonsak and Hoffmann, 2018; Zhu et al., 2013).

But most importantly, Polo (in collaboration with CDK1) binds and phosphorylates MAD3/BUBR1, a member of the **spindle assembly checkpoint** (SAC). The SAC is a surveillance mechanism that inhibits the APC/C and stops the metaphase-to-anaphase transition if there are unattached and/or untensed KTs (reviewed in Hayward et al., 2019; Musacchio, 2015). Polo being involved in SAC and tension sensing has long been suspected (Ahonen et al., 2005), and indeed, Polo phosphorylation of BUBR1 (at least at Ser676) is tension-dependent, so it disappears once chromosomes congress (Elowe et al., 2007). Phospho-BUBR1 then recruits the phosphatase PP2A-B56 α to

counteract Aurora B-mediated KT-MT attachment destabilisation and, potentially, Polo itself (Elowe et al., 2007; Foley et al., 2011; Liu et al., 2012; Matsumura et al., 2007; Suijkerbuijk et al., 2012). Polo also regulates other SAC components, including MPS1 kinase (Combes et al., 2017; Conde et al., 2013), BUB1 (Qi et al., 2006), and CDC20 (Jia et al., 2016). Thus, while high Polo activity is required during prometaphase, Polo is part of a complex feedback loop that controls SAC signalling (Combes et al., 2017; Nijenhuis et al., 2014), and a decrease in Polo activity at metaphase KTs is crucial for SAC silencing and cycle progression (Liu et al., 2012).

Despite its role in SAC signalling, Polo may also promote **APC/C activation**. Polo triggers the degradation of the APC/C inhibitor Emi1 during prophase (Hansen et al., 2004; Moshe et al., 2004). Additionally, Plo1 (fission yeast homologue of Polo) binds the APC/C subunit Cut23 and promotes APC/C activity (May et al., 2002), and in other species Polo can phosphorylate different APC/C subunits and partially contribute to APC/C activation (Golan et al., 2002). Expression of the activating mutant PLK1-S137D triggers premature cyclinB degradation and cytokinesis (signs of premature APC/C activation), although expression of PLK1-T210D does not (van de Weerd et al., 2005). In yeasts and vertebrates, Polo further facilitates **chromosome segregation** by phosphorylating the cohesin subunit Scc1, promoting its cleavage by the protease separase (Alexandru et al., 2001; Hauf et al., 2005; Sumara et al., 2002)

When Plo1 was identified, it was observed that it was required for septation as well as bipolar spindle formation (Ohkura et al., 1995). Indeed, analysis of *polo*¹, the first *Drosophila* hypomorph identified, showed that Polo is required for localisation of certain proteins (e.g. CPC components and pavarotti, the fly homologue of MKlp1) to the spindle midzone and for contractile ring formation during **cytokinesis** (Carmena et

al., 1998, 2014). In flies, Polo binds pavarotti, and this is likely required for its localisation to the central spindle (Adams et al., 1998), although some have since reported that Polo recruitment relies instead on Subito/MKlp2 (perhaps indirectly) (Cesario et al., 2006) and/or direct binding to a complex made of Fascetto (fly homologue of PRC1) and the kinesin Klp3A (D'Avino et al., 2007). In human cells PLK1 self-primes its own binding to MKlp2 and PRC1-isoform2, and regulates MKlp2 function at the central spindle (Neef et al., 2003, 2007). PRC1 and MT presence facilitate PLK1 binding and phosphorylating the Cyk-4/RacGAP subunit of centralspindlin, which allows Cyk-4 to recruit the Rho-GTPase exchange factor ECT2 (Burkard et al., 2009; Wolfe et al., 2009).

If Polo activity is inhibited after metaphase, or Polo is lost from the central spindle, cells often fail cytokinesis and present cleavage furrow defects. ECT2 is lost from the central spindle, resulting in loss of RhoA from the equatorial cell cortex and loss of contractile ring assembly; MKlp2 is also mislocalised and, as a result, so is a fraction of Aurora B bound to MKlp2 (Brennan et al., 2007; Burkard et al., 2009; Gruneberg et al., 2004; Petronczki et al., 2007; Santamaria et al., 2007; Wolfe et al., 2009). Polo also phosphorylates supervillin (SVIL), an actin/myosin II-binding protein, thus facilitating SVIL binding to PRC1, SVIL localisation to the central spindle, and myosin II activation at the equatorial cortex (Hasegawa et al., 2013).

The last step of cytokinesis is abscission (i.e. cutting the thin membrane connection between daughter cells). It is mediated by the ESCRT-III complex, which is recruited to the midbody by Cep55 (Carlton and Martin-Serrano, 2007). Polo phosphorylates Cep55 to prevent its premature binding to MKlp1 and abscission failure (Bastos and Barr, 2010; Fabbro et al., 2005; Seong et al., 2002). Interestingly, both Polo and

Aurora B bind and phosphorylate NudC, another protein required for abscission, but while Polo phosphorylation would promote NudC function, Aurora B phosphorylation opposes it (Aumais et al., 2003; Weiderhold et al., 2016; Zhou et al., 2003); another example of the complex functional relationship between these two kinases.

Other Polo roles include **Golgi fragmentation** (Lin et al., 2000; Preisinger et al., 2005; Sütterlin et al., 2001) and **intermediate filament reorganisation** (Yamaguchi et al., 2005). During **meiosis**, Polo also regulates the pachytene checkpoint (Pek et al., 2012) and the disassembly of the central element of the synaptonemal complex (Jordan et al., 2012), based on observations from fly oocytes and mouse spermatocytes, respectively. Furthermore, Polo's many mitotic roles make it absolutely required for early embryo **development** (Jeong et al., 2010; Lu et al., 2008; Sunkel and Glover, 1988). Beyond this, cell-specific Polo deletions have shown that Polo is crucial for correct stem cell divisions, for example those of muscle stem cells during embryonic myogenesis and muscle regeneration in mice (Jia et al., 2019). In flies, Polo is particularly required for correct spindle orientation during asymmetric division in **neuroblasts** (Lee et al., 2006; Wang et al., 2006, 2007) and eye development (Kachaner et al., 2017).

1.3. The centrosome cycle is tightly regulated by Polo

Polo/PLK1 is thought to regulate three key aspects of the centrosome cycle: centrosome maturation, centriole disengagement and centriole conversion.

1.3.1. Centrosome maturation

A series of classical experiments showed that Polo is key in the regulation of mitotic PCM assembly. For example, injection of anti-PLK1 antibodies into HeLa cells not only inhibited mitotic progression but also resulted in smaller than normal centrosomes with very reduced levels of γ -tubulin (Lane and Nigg, 1996). Since then, similar

results have been obtained knocking down Polo with RNAi (Dobbelaere et al., 2008; Hanisch et al., 2006; Sumara et al., 2004) or using PLK1 inhibitors (Lénárt et al., 2007; McInnes et al., 2006; Peters et al., 2006). However, the molecular mechanisms behind Polo's regulation of centrosome maturation are still being elucidated.

In flies, Polo collaborates with the key PCM proteins Cnn and Spd-2 to drive centrosome maturation (Conduit et al., 2014a, 2015a; Dobbelaere et al., 2008; Feng et al., 2017; Goshima et al., 2007). The homologues of Polo, Spd-2 and Cnn also appear to play an important role in centrosome maturation in worms (Cabral et al., 2019; Decker et al., 2011; Hamill et al., 2002; Woodruff et al., 2017; Wueseke et al., 2016) and vertebrates (Barr et al., 2010; Fong et al., 2008; Gomez-Ferreria et al., 2007; Haren et al., 2009; Joukov et al., 2010, 2014; Lee and Rhee, 2010, 2011; Santamaria et al., 2011; Zhu et al., 2008). For example, it has been proposed that SPD-2/Cep192 recruit Polo to centrosomes in worms and vertebrate cells (Decker et al., 2011; Joukov et al., 2014; Meng et al., 2015). Additionally, *in vitro* experiments using *Xenopus* egg extracts showed that Cep192 phosphorylation by Plx1 allows Cep192 recruitment of γ -TuRC (and, possibly, other spindle assembly factors like XMAP215), promoting MT nucleation (Joukov et al., 2014). However, it is possible that this mechanism is exclusive to *Xenopus*, as the five γ -TuRC-binding serines of *Xenopus* Cep192 are not conserved in human Cep192 (Joukov et al., 2014), and Cep192 and γ -TuRC do not extensively colocalise in the PCM in human cells (Sonnen et al., 2012).

In vertebrates, pericentrin/Kendrin is also key for centrosome maturation. Like Cep192 and Cep215/Cdk5Rap2 (vertebrate homologue of Cnn), its distribution expands massively upon mitotic entry (Lawo et al., 2012; Lee and Rhee, 2011; Sonnen et al., 2012), allowing it to form a lattice that interacts with γ -tubulin (Dictenberg et al.,

1998; Zimmerman et al., 2004). The three proteins were suggested to be PLK1 substrates (Santamaria et al., 2011), which has since been confirmed for Cep192 (Joukov et al., 2014) and pericentrin (Lee and Rhee, 2011). In the latter case, PLK1 phosphorylates Ser1235 and Ser1241 as the cells transition into mitosis, and this phosphorylation event is essential for recruitment of other PCM proteins, including Cep192 and PLK1 themselves (Lee and Rhee, 2011), but not Cep215 (Kim and Rhee, 2014). It has also been proposed that other components (such as hCenexin1) are able to bind PLK1 and trigger pericentrin accumulation ectopically in the cytoplasm, but it is unclear that this pathway contributes to PCM assembly *in vivo* (Soung et al., 2009).

Nevertheless, in flies, Pericentrin-like protein (Plp) remains mostly concentrated at the centriole upon mitotic entry, whereas the distribution of Cnn and Spd-2 in the PCM increases dramatically (Figure 1.4A) (Conduit et al., 2010; Fu and Glover, 2012). Plp appears faintly at the outer regions of the PCM, where it helps stabilise the outer Cnn scaffold, but overall it does not seem to be essential for mitotic PCM expansion (Lerit et al., 2015; Martinez-Campos et al., 2004; Mennella et al., 2012; Richens et al., 2015). Thus, it is unlikely that Polo regulates centrosome maturation mainly via Plp in flies.

Instead, recent structural studies have shed some light on how Polo may control Cnn scaffold formation and, thus, centrosome maturation (Figure 1.8). Centrosomal Cnn is specifically phosphorylated during mitosis by Polo at a phospho-regulated multimerization (PReM) domain (Conduit et al., 2014a) which contains a previously identified leucine zipper motif (LZ) (Heuer et al., 1995) followed by a series of highly conserved Ser/Thr residues, including the key residue Ser567. Phospho-resistant mutations in this region block Cnn scaffold formation, whereas phospho-mimicking mutations lead to spontaneous Cnn aggregates forming everywhere in the embryo

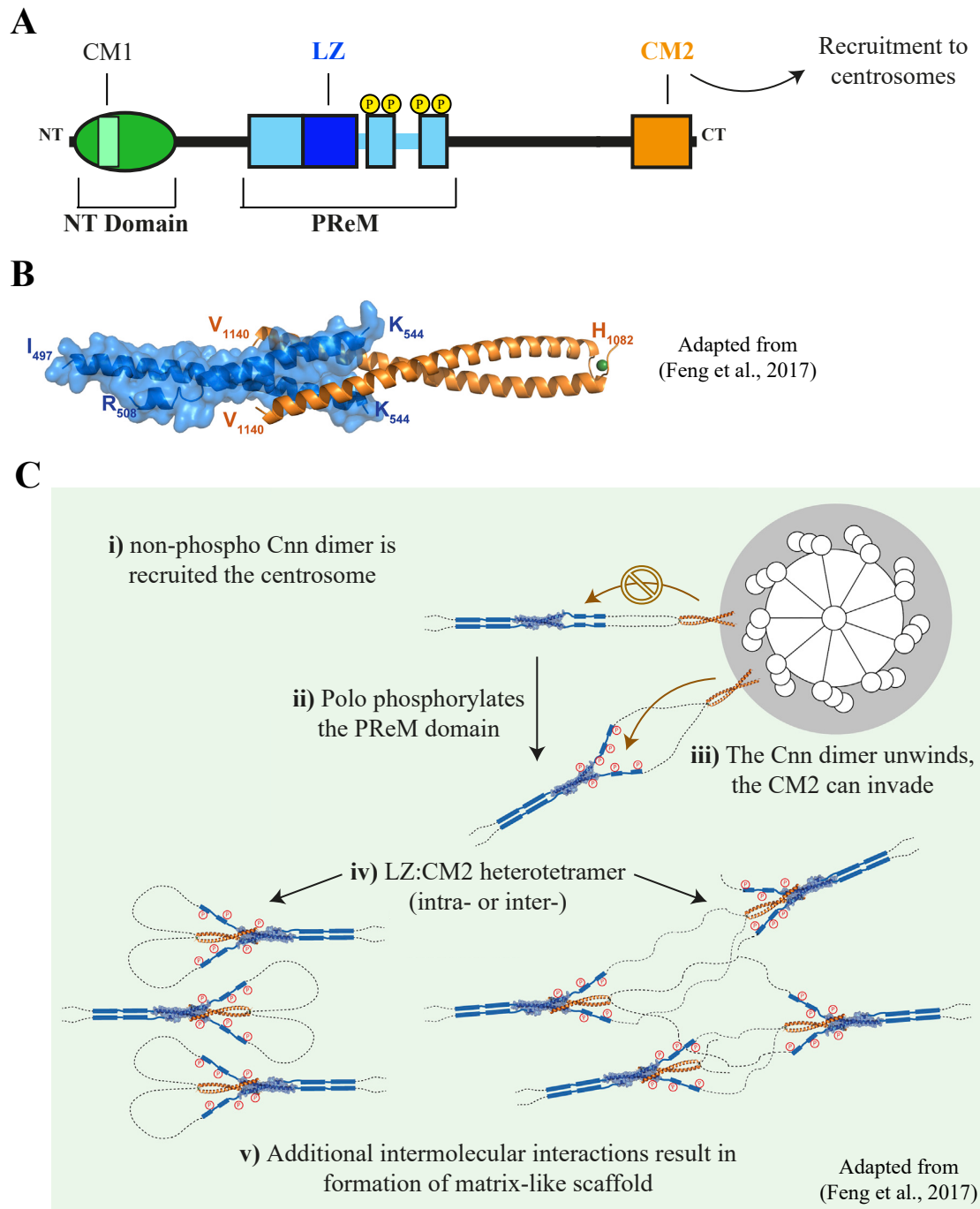


Figure 1.8 – Polo regulates Cnn scaffold formation.

(A) Schematic representation of a Cnn monomer, not to scale. The N-terminal domain contains the CM1, which is involved in γ -tubulin recruitment. Next there is the PReM domain, which contains a leucine zipper motif (LZ, dark blue) followed by several highly conserved Ser/Thr residues which can be phosphorylated by Polo (yellow circles). At the C-terminus there is the CM2 (orange), which is important for Cnn recruitment to the centrosome and Cnn scaffold formation. The regions between the PReM and the CM2 are disordered and flexible. **(B)** Crystal structure of the LZ:CM2 heterotetramer. Note that only the LZ is shown, and not the rest of the PReM. Two LZ molecules form a parallel α -helical homodimer (coiled-coil), and a similar structure is formed by two CM2 molecules, although it is slightly “splayed” at its C-terminus. This allows the two homodimers to slot into each other in an antiparallel fashion. Adapted from (Feng et al., 2017). **(C)** Schematic model of how centrosomal Polo regulates Cnn scaffold formation. **(i)** Cnn dimers are recruited to the centrosome via their CM2. The PReM is not phosphorylated so the LZ:CM2 heterotetramer cannot form. **(ii)** Once at the centrosome, the PReM is phosphorylated by Polo. **(iii)** This destabilises the dimer, allowing it to open up, and a CM2 coiled-coil can now invade. **(iv)** The resulting heterotetramer can form from the interaction of CM2 and LZ from the same Cnn dimer (intra-molecular) or from different Cnn dimers (inter-molecular). **(v)** Inter-molecular LZ:CM2 interactions and other inter-molecular interactions made possible by the unwinding of the PReM and the formation of the heterotetramer result in the assembly of matrix-like scaffolds. Adapted from (Feng et al., 2017).

cytoplasm (Conduit et al., 2014a). It appears that the LZ included within the PReM forms an antiparallel 2:2 heterotetramer with the CM2 domain, a conserved motif within Cnn which is important for both Cnn recruitment to the centrosome and scaffold formation (Barr et al., 2010; Citron et al., 2018; Feng et al., 2017; Wang et al., 2010). This interaction is facilitated by Polo phosphorylation of the Cnn PReM, which unwinds as a result, allowing the CM2 helix to invade. The formation of the heterotetramer allows Cnn to form inter-molecular interactions that result in solid-like aggregates *in vitro* and Cnn scaffold formation *in vivo* (Feng et al., 2017; A. Haensele, unpublished). Similarly, PLK-1 phosphorylation promotes the formation of aggregates (also known as condensates) of the worm Cnn homologue SPD-5 *in vitro* (Woodruff et al., 2015, 2017; further discussed in 4.1.2). Furthermore, in vertebrate cells, the scaffold protein Gravin has been reported to downregulate PLK1 centrosomal activity and Cep215 phosphorylation (Colicino et al., 2017). While the role (if any) of Gravin in centrosome assembly is still unclear, this is consistent with Cep215 being a true PLK1 substrate, as previously suggested (Santamaria et al., 2011).

If Polo phosphorylation is required for PCM assembly, it would be expected that dephosphorylation results in PCM disassembly. Indeed, the phosphatase PP2A was identified in a RNAi screen as an important regulator of centrosome maturation in S2 cells (Dobbelaere et al., 2008). In combination with its regulatory subunit SUR-6, PP2A can dephosphorylate SPD-5 *in vitro*, and a combination of PP2A activity and microtubule-depending pulling forces results in SPD-5 scaffold breakdown and PCM disassembly in worm embryos during mitotic exit (Enos et al., 2018).

Finally, in addition to regulating the formation of the scaffold that supports PCM expansion, PLK1 has also been shown to regulate the centrosomal accumulation of γ -

TuRCs in vertebrates. At mitotic onset, PLK1 phosphorylates Ninein-like protein, an interphase-specific γ -TuRC interacting protein, triggering its substitution for mitosis-specific adaptors (Casenghi et al., 2003). PLK1 also activates Nek9, another kinase which then controls the levels of γ -tubulin and the adaptor protein NEDD1 (also known as GCP-WD) at the centrosome (Haren et al., 2009; Sdelci et al., 2012). Furthermore, PLK1 can phosphorylate and bind directly to NEDD1, which is important for centrosomal and non-centrosomal based MT nucleation (Gomez-Ferreria et al., 2012; Johmura et al., 2011; Zhang et al., 2009). However, in flies, Grip71 (the *Drosophila* homologue of NEDD1) is not so important for centrosomal accumulation of γ -TuRCs, although it is key in other cellular and developmental contexts, such as the formation of the Meiosis I spindle (Dobbelaere et al., 2008; Liu and Wiese, 2008; Reschen et al., 2012; Vérollet et al., 2006). Instead, γ -tubulin is mainly recruited to centrosomes via the CM1 domain in Cnn (Chen et al., 2017; Zhang and Megraw, 2007). It has been proposed that phosphorylation, perhaps by Polo, could help expose the CM1 domain to allow Cnn binding to γ -TuRCs (Tovey and Conduit, 2018), but testing this formally will be challenging, as any mutants must be carefully designed to not disturb Polo-mediated Cnn scaffold formation.

1.3.2. Centriole disengagement and separation

It is now clear that centriole disengagement is regulated by Polo. However, this field of study has struggled with confounding observations and with some studies assuming that centriole disengagement and centriole separation are one and the same. Centrioles must be disengaged in order to visibly move away, but lack of visible centriole separation might not necessarily mean that the mother and daughter centrioles are still engaged and that the mother is blocked from duplicating a new daughter centriole.

The protease separase is a key regulator of centriole disengagement (Tsou and Stearns, 2006). When this was first observed, it was proposed that centrioles were held together by cohesin rings, as are chromosomes. Polo can phosphorylate cohesin to trigger its dissociation from chromosomes in prophase (Sumara et al., 2002), so it was not surprising to discover that centriole disengagement was also dependent on Polo activity (Kong et al., 2014; Loncarek et al., 2010; Schöckel et al., 2011; Tsou et al., 2009). However, while centriole separation could be artificially triggered in *Xenopus* egg extracts containing cohesin rings that could be cleaved independently of separase (Schöckel et al., 2011), experiments using fly embryos arrested in metaphase showed that this could be due to the centrosomes going through mitotic exit, rather than the opening of cohesin rings themselves (Oliveira and Nasmyth, 2013). Thus, the role of separase-mediated cohesin removal in centriole disengagement was called into question, outside of a limited number of developmental contexts (Cabral et al., 2013; Prosser et al., 2012).

In parallel, pericentrin was identified as a novel target for separase, and it was proposed that it was through pericentrin cleavage that separase regulates centriole disengagement (Lee and Rhee, 2012; Matsuo et al., 2012). PLK1 would control this process by phosphorylating pericentrin at a subset of sites different from those required for centrosome maturation in vertebrates (see 1.3.1), priming it for cleavage during mitotic exit, and thus triggering PCM disassembly and centrosome separation (Kim et al., 2015). However, other data from the Rhee laboratory has since questioned the role of separase in this process (Seo et al., 2015). While pericentrin/PCM stability is clearly required to keep overduplicated centrioles from separating (Karki et al., 2017; Kim et al., 2019), it is unclear whether pericentrin cleavage/PCM disassembly is required for centriole disengagement itself or just centriole separation. Similarly, PLK1

phosphorylation also triggers the degradation of Cep68, a component of the linker between vertebrate centrosomes, but this event is required for centrosome separation rather than disengagement (Pagan et al., 2015).

Perhaps the most direct evidence of Polo-dependent disengagement comes from observations using correlative live/electron microscopy. Engagement was maintained as long as the mother and daughter centriole remained in extreme physical proximity (<80 nm distance between their centriole walls). Sustained PLK1 activity would lead to this distance increasing and the mother centriole becoming able to nucleate a new daughter (Shukla et al., 2015). Such short-range distancing is not to be confused with the separation of centrosomes that occurs at the end of mitosis. Indeed, in conventional light microscopy assays used previously in the field, the mother and old daughter centrioles might still appear to be engaged, not least because the older (disengaged) daughter centriole can maintain its perpendicular orientation with respect to the mother centriole (Shukla et al., 2015).

While recruitment of CPAP (vertebrate homologue of Sas-4) to the daughter centriole was required for disengagement, no linker or structure was distinguished that could be driving centriole wall distancing in response to PLK1 activity (Shukla et al., 2015) – but this does not mean that such structure does not exist. The simplest distance-only sensitive engagement model is if a diffusible signal from the daughter centriole stops the mother from generating a new daughter. It would then be expected that as soon as the daughter moves away, nucleation of the new daughter centriole occurs at the furthest site of the mother centriole (i.e. 180° from the old daughter engagement site). However, new daughters are often nucleated 90° from the old daughter site. Interestingly, in *Drosophila* sensory organ precursor cells, electron tomogram images

show a protein “cloud” surrounding the mother centriole that connects it to the daughter centriole. Loss of Plp led to loss of these clouds, as well as premature centriole disengagement (Roque et al., 2018). These observations suggest that pericentrin/Plp might truly be involved in centriole disengagement, and that (at least in some cell types or organisms) there is a physical connection between centrioles, most likely regulated by Polo, that could stop the daughter from “drifting” away, and the mother from nucleating a new daughter centriole.

1.3.3. Centriole conversion

As introduced in 1.1, Asl is a centriolar protein incorporated during mitosis which marks the conversion of daughter centrioles into fully functional mothers. This process requires Sas-4, which is incorporated into daughter centrioles during S-phase, and Polo (Conduit et al., 2015b; Novak et al., 2014, 2016).

During mitosis, Sas-4 is phosphorylated by CDK1 at Thr200 (Novak et al., 2016). This amino acid is part of an “STP” motif, which makes it both a CDK1 phosphorylation target (Songyang et al., 1994) and a binding site for Polo via its PBD (Elia et al., 2003a, 2003b). Polo binding to this site is crucial for Asl recruitment to the daughter centriole, although the mechanism by which Polo promotes Asl recruitment is still unclear (Figure 1.5B). If Polo binding is abrogated, the daughter centriole cannot be efficiently converted into a fully functional mother: in embryos expressing Sas-4-T200A-GFP, new centrioles cannot efficiently nucleate MTs and form spindle poles (signs of a defective PCM) (Novak et al., 2016); and as these unconverted centrioles lack Asl, they will also be unable to duplicate (Novak et al., 2014). However, Polo binding to Sas-4-Thr200 is not required for disengagement – the unconverted daughter

centriole still separates from the mother, and the mother is able to nucleate a new daughter centriole in the following cycle (Novak et al., 2016).

In vertebrate cells, this process appears to be more complex. Both Cep152 (homologue of Asl) and Cep192 (homologue of Spd-2) cooperate to recruit PLK4 to the converted centriole (Cizmecioglu et al., 2010; Dzhindzhev et al., 2010; Hatch et al., 2010; Sonnen et al., 2013); and Cep152 also affects recruitment of CPAP (homologue of Sas-4) (Cizmecioglu et al., 2010). Nevertheless, even abnormally amplified, Cep192-positive daughter centrioles rely on mitotic exit for Cep152 recruitment, highlighting that this recruitment event is tightly regulated (Kim et al., 2019). Although CPAP knockdown did not appear to affect Cep152 levels (Sonnen et al., 2013), in agreement with the model proposed in (Novak et al., 2016), Cep152 recruitment and centriole conversion require PLK1 activity (Kong et al., 2014; Wang et al., 2011b).

Furthermore, unlike in flies, mature vertebrate centrosomes present a collection of appendages which are needed for MT anchoring during interphase and for ciliogenesis, and which are only formed after centrioles complete their second mitosis (Chrétien et al., 1997; Graser et al., 2007; Ishikawa et al., 2005; Lange and Gull, 1995; Paintrand et al., 1992; Piel et al., 2000; Tanos et al., 2013; Vorobjev and Chentsov, 1982). The initial recruitment of appendage proteins is also dependent on PLK1 activity, and PLK1 overexpression can lead to their premature accumulation on daughter centrioles (Kong et al., 2014; Loncarek et al., 2010). PLK1 has also been shown to phosphorylate and/or bind to several appendage proteins (Guarguaglini et al., 2005; Soung et al., 2009), although this could partly be due to an additional PLK1 role in distal appendage re-arrangement and cilia reabsorption (Bowler et al., 2019; Wang et al., 2013).

1.4. Thesis outline

In this thesis, I investigate how Polo is recruited and maintained at the centrosome to regulate the centrosome cycle. As a model, I use the syncytial *Drosophila melanogaster* embryo, which is ideal for live imaging of large numbers of centrosomes due to its rapid, synchronous mitotic cycles. First, I identified and mutated multiple potential Polo binding sites present in the main proteins involved in centrosome biogenesis. I carried out a screen using mRNA injection into embryos to test which of these mutations disturbed Polo levels and distribution at the centrosome (**Chapter 3**). This screen revealed two positive hits (Spd-2 and Ana1). I then proceeded to characterise the effect of losing Polo recruitment mediated by either Spd-2 (**Chapter 4**) or Ana1 (**Chapter 5**) in transgenic fly lines. I tested the effect of the mutations introduced on the protein's known functions, on the localisation of other centrosomal proteins, and on embryo development as a whole. My results show that Spd-2 binds directly to the PBD, and that this is important for Polo recruitment to the PCM, but not for Polo recruitment to the centriole wall. Conversely, Ana1 is required for overall Polo recruitment to the centrosome. In both cases, losing Polo recruitment resulted in impaired mitotic PCM assembly and accumulation of mitotic defects, leading to embryo lethality. However, other stages of the centrosome biogenesis (e.g. centriole conversion) were unaffected. Finally, in **Chapter 6** I summarise my main findings and propose a model that integrates them with previously reported observations.

Chapter 2 - Materials and Methods

2.1. Fly husbandry

2.1.1. Fly handling

Flies were kept at 25 °C or 18 °C on *Drosophila* culture medium (0.77% agar, 6.9% maize, 0.8% soya, 1.4% yeast, 6.9% malt, 1.9% molasses, 0.5% propionic acid, 0.03% ortho-phosphoric acid and 0.3% nipagin). Stocks were kept in 8 cm x 2.5 cm plastic vials or 0.25-pint plastic bottles. Embryos were collected on cranberry-raspberry juice plates (25% cranberry-raspberry juice, 2% sucrose and 1.8% agar) supplemented with fresh yeast. Standard fly handling techniques were employed (Roberts, 1998).

Drosophila melanogaster Oregon-R flies were used as a WT stock where indicated.

Balancer chromosomes and markers used have been described previously (Lindsley and Zimm, 1992). Table 2.1 lists all of the fly lines that I used in this thesis.

Table 2.1. *Drosophila* lines used in this thesis

Chapter	Genotype	Description	Reference
3/4/5	Polo::GFP	GFP-protein trap insertion into the endogenous <i>polo</i> gene (referred to as PoloGFP)	(Buszczak et al., 2007)
3/5	eAsl-mCherry	Line expressing Asl-mCherry under an endogenous promoter	(Conduit et al., 2015b)
3/4	Ubq-Spd-2-mCherry	Line expressing WT Spd-2-mCherry under the ubiquitin promoter	This thesis
3/5	Ubq-GFP-Cnn	Line expressing GFP-Cnn under the ubiquitin promoter	(Conduit et al., 2010)
4/5	Ubq-Spd-2-GFP	Line expressing WT Spd-2-GFP under the ubiquitin promoter	(Dix and Raff, 2007)
4	Ubq-Spd-2-ALL-GFP	Line expressing Spd-2-GFP with the all ptPBSs mutated (Ser to Thr) under the ubiquitin promoter	This thesis
4	Ubq-Spd-2-CONS-GFP	Line expressing Spd-2-GFP with the conserved ptPBSs mutated (Ser to Thr) under the ubiquitin promoter	This thesis

4	Ubq-Spd-2-ALL-mCherry	Line expressing Spd-2-mCherry with the all ptPBSs mutated (Ser to Thr) under the ubiquitin promoter	This thesis
4	Ubq-Spd-2-CONS-mCherry	Line expressing Spd-2-mCherry with the conserved ptPBSs mutated (Ser to Thr) under the ubiquitin promoter	This thesis
4	Ubq-Spd-2-11A-GFP	Line expressing Spd-2-GFP 11 phosphosites (including two ptPBSs) mutated to Ala under the ubiquitin promoter	(Baumbach, 2014)
4	<i>spd-2^{z35711}</i>	Line with one <i>spd-2</i> allele with R57term point-nonsense mutation (mutagen: ethyl-methanesulfonate)	(Giansanti et al., 2008)
4	<i>spd-2^{G20143}</i>	Line with one <i>spd-2</i> mutant allele (from transposable element insertion)	(Dix and Raff, 2007)
4	Jupiter::mCherry, <i>spd-2^{z3571}</i>	mCherry-protein trap insertion into the endogenous <i>Jupiter</i> gene, recombinant with the <i>spd-2^{z35711}</i> allele	Original line: gift from St Johnston lab, (Conduit et al., 2014a). Recombinant: P. Conduit
4	Ubq-Spd-2-mCherry, <i>spd-2^{G2014}</i>	Line expressing WT Spd-2-mCherry under the ubiquitin promoter, recombinant with the <i>spd-2^{G2014}</i> allele	Recombinant: this thesis
4	Ubq-Spd-2-ALL-mCherry, <i>spd-2^{G20143}</i>	Line expressing Spd-2-mCherry with the all ptPBSs mutated (Ser to Thr) under the ubiquitin promoter, recombinant with the <i>spd-2^{G2014}</i> allele	Recombinant: this thesis
4	Ubq-Spd-2-CONS-mCherry, <i>spd-2^{G20143}</i>	Line expressing Spd-2-mCherry with the conserved ptPBSs mutated (Ser to Thr) under the ubiquitin promoter, recombinant with the <i>spd-2^{G2014}</i> allele	Recombinant: this thesis
4	Polo::GFP, <i>spd-2^{z3571}</i>	PoloGFP line, recombinant with the <i>spd-2^{z35711}</i> allele	Recombinant: J. Raff
4	Ubq-RFP-Cnn	Line expressing RFP-Cnn under the ubiquitin promoter	(Conduit et al., 2010)
4	Ubq-Aur-A-GFP, <i>spd-2^{z35711}</i>	Line expressing Aurora A-GFP under the ubiquitin promoter, recombinant with the <i>spd-2^{z35711}</i> allele	Original line: (Lucas and Raff, 2007) Recombinant: P. Conduit
5	Ubq-Ana1-GFP	Line expressing WT Ana1-GFP under the ubiquitin promoter	This thesis

5	Ubq-Ana1-ALL-GFP	Line expressing Ana1-GFP under the ubiquitin promoter with the all ptPBSs mutated (Ser to Thr) under the ubiquitin promoter	This thesis
5	Ubq-Ana1-mCherry	Line expressing WT Ana1-mCherry under the ubiquitin promoter	This thesis
5	Ubq-Ana1-ALL-mCherry	Line expressing Ana1-mCherry under the ubiquitin promoter with the all ptPBSs mutated (Ser to Thr) under the ubiquitin promoter	This thesis
5	<i>ana1^{mecB}</i>	Line with one <i>ana1</i> allele with Q1120term point-nonsense mutation	(Blachon et al., 2009), (Avidor-Reiss et al., 2004) collection
5	<i>Df(3R)Exel7357</i>	Deficiency line covering the <i>ana1</i> gene	Bloomington <i>Drosophila</i> Stock Center
5	Polo::GFP, <i>Df(3R)Exel7357</i>	PoloGFP line, recombinant with the <i>Df(3R)Exel7357</i> deficiency	Recombinant: this thesis

Throughout this thesis, flies said to be “*spd-2^{-/-}* mutant” have the genotype *spd-2^{G20143/spd-2^{z35711}}* and flies said to be “*ana1^{-/-}* mutant” have the genotype *ana1^{mecB}/Df(3R)Exel7357*.

2.1.2. Generation of transgenic lines

Stable transgenic fly lines were generated via random *P*-element insertion of the construct of choice, containing a *w⁺* gene for selection, in to a *w¹¹⁸* background. Plasmid containing constructs were generated as described (see 2.3). The injection, selection of transformants, generation of transgenic stocks and mapping of the chromosome on which the transgene inserted was performed by the Cambridge Fly Facility service at the Department of Genetics, University of Cambridge (UK).

Recombinant lines were derived via the appropriate crossing scheme, exposing the doubly heterozygous female larvae to 30 °C for 48 h to increase the frequency of

recombination (Plough, 1921). Recombinant events were scored based on eye colour and/or via a PCR screen (see 2.3.6).

2.1.3. Assessing the *unc* phenotype

To assess whether *ana1*^{-/-} flies expressing fluorescently-tagged Ana1 (WT or ALL) under the ubiquitin promoter were uncoordinated, pupae were picked out of the vials prior to eclosion, so that any uncoordinated flies would not get stuck in the food. Female flies were observed under a dissection microscope to analyse if they could walk and the position of their wings. As an additional control, these flies were compared to female siblings that had not inherited the corresponding Ubq-Ana1 construct.

2.1.4. Embryo handling for live analysis

Embryos were collected and aged as required, dechorionated by using double-sided tape onto a slide and mounted on a strip of glue onto a 35 mm glass bottom petri dish with a 14 mm micro-well (MatTek). After desiccation for 1 min (non-injection experiments) or 3 min (pre-mRNA injection) at 25°C, embryos were covered in Voltalef oil (ARKEMA). Live microscopy experiments were performed using 1.5-2 h-old embryos (syncytial blastoderm stage).

For mRNA injection, the embryos were collected for 20 min. The injection was performed on a Nikon TMS inverted microscope mounted with a manual injection apparatus (Micro Instruments LTD). The in vitro transcribed mRNA was pipetted in to 0.5 mm diameter glass needles and then injected into the live embryos. The embryos were imaged after 120 min at 21°C (but always within the syncytial blastoderm stage of development), unless otherwise stated.

For hatching assays, embryos were collected for approximately 4 h at 25°C and aged for 24 – 36 h at 25°C.

2.2. Bioinformatics

2.2.1. Identification of potential Polo binding sites (ptPBSs)

The amino acid sequences of the *Drosophila melanogaster* proteins of interest were obtained from FlyBase. Amino acid sequences from homologues in other *Drosophila* species were obtained by performing a FlyBase BLAST search, selecting the genus *Drosophila* and the “Annotated proteins database”. The resulting species used are: *D. melanogaster*, *D. simulans*, *D. sechellia*, *D. yakuba*, *D. erecta*, *D. ananassae*, *D. pseudoobscura*, *D. persimilis*, *D. willistoni*, *D. mojavensis*, *D. virilis* and *D. grimshawi*. The amino acid sequences of the top hit for each *Drosophila* species were downloaded from the NCBI Protein database. For the Spd-2 and Cnn ptPBS comparisons, I downloaded from the NCBI Protein database the amino acid sequences for the known homologues in *Homo sapiens* and *Caenorhabditis elegans*. Additional mammalian homologues were identified via a NCBI BLAST search, whereas additional nematode homologues were obtained from WormBase.

The protein sequences were uploaded into Jalview (Waterhouse et al., 2009), which was used for protein sequence visualisation and alignment (using the ClustalO algorithm). ptPBSs along the different amino acid sequences were identified by searching for the consensus Polo binding motif Ser-Thr/Ser. Conserved ptPBSs were classified as such if they were present in at least all but 1 of the species analysed for each conservation group (*Drosophila* genus, mammals or *Caenorhabditis* genus).

2.2.2. Identification of potential phosphorylation target sites

Protein sequences were analysed using Group-based Prediction System (Xue et al., 2011). Initially version 3.0 was used, but since then a new version (5.0) has been released. Threshold was set at medium (cutoff = 3.096).

2.3. Molecular biology

2.3.1. Primer design, PCR amplification, DNA gel extraction

All plasmid and primer *in silico* design and DNA sequencing data analysis was performed using SnapGene (GSL Biotech). DNA sequencing was performed by Source BioScience. All the primers used in this thesis are listed in Table 2.2 and were synthesised by Invitrogen (Thermo Fisher). Phusion PCR Master Mix with HF Buffer (NEB, M0531S) was used (following manufacturer's instructions) to amplify DNA fragments which were then cloned into the appropriate plasmids via Gateway cloning or HiFi assembly (see sections 2.3.2, 2.3.4 and 2.3.5). For Gateway cloning, primers were designed to incorporate *attB* sites into the PCR product, whereas HiFi assembly primers were designed to incorporate >20 nt overlapping regions.

Linearised DNA fragments were run on 0.8% agarose gels (TAE, 0.8% agarose) containing SYBR Safe stain (Thermo Fisher, S33102). The DNA was purified using the Monarch DNA Gel Extraction Kit (NEB, T1020L), following manufacturer's instructions. All plasmids were extracted using Qiagen kits and protocols (QIAprep Spin Miniprep Kit (27106), QIAfilter Plasmid Midi Kit (12243)).

Table 2.2. List of primers used in this thesis

Gene	Primer	Sequence	Use
Sas-4	Attb_Sas-4_FW	ggggacaagtttgtaaaaaagcaggcttcacgcag gaggctggcgaaagtc	Gateway

	Attb_Sas-4_RV	ggggaccactttgtacaagaaagctgggtcatactgg catagtctgtgtcc	
Asl	58_Saroj_atb1	ggggacaagttgtacaaaaagcaggcttc	Gateway
	59_Saroj_atb2	ggggaccactttgtacaagaaagctgggtc	
	oZN187_Asl_cDNA_ FW	gcaggcttcataaacacgccagggtataagcc	
	oZN2_Asl_RV	agctgggtcgctgtgaccattgccttgggct	Mutagenesis
	AslS372T_FW	tcgcgccttgttggtaccacaccgctg	
	AslS372T_RV	cagcggtgtggtaccaacaaggcgca	
Cep135	71Saroj_Cep135F1	atgaatatcaacgatgggtgacttta	Gateway
	72Saroj_Cep135_CT_ R1	aagagtcttcgatggcaccgggggcta	
ana1	Attb_Ana1_FW	ggggacaagttgtacaaaaagcaggcttcaggctc tcagctaacagtaaag	Gateway
	Attb_Ana1_RV	ggggaccactttgtacaagaaagctgggtcttttcggg gagactttcgatg	
	Ana1_Insert1_FW	tgtacaaaaaagcaggcttcaggcttcgagctaaca gtaaag	HiFi
	Ana1_Insert1729_RV	tgtacaagaaagctgggtcttttcggggcgactttcgat g	
	Ana1_Insert756_FW	gtgagtcagggtgcatcaaagggtgacaag	
	Ana1_Insert756_RV	cattgtcacctttgatgcacctgactcac	
	Ana1_Insert935_FW	cccatggtggttcaatatataaatcgctctgtagg	
	Ana1_Insert935_RV	cctagcagacgatttatattgaaccacctgagg	
spd-2	Attb_FP1_Mut	ggggacaagttgtacaaaaagcaggcttcacaagtt tgtacaaaaaagcaggcttcaggactaccagtgg agcc	Gateway
	Attb_FP1	ggggacaagttgtacaaaaaagcaggcttcacaagtt tgtacaaaaaagcaggcttcaggactaccagtgg aagcc	
	Attb_RP1147	ggggaccactttgtacaagaaagctgggtcaaatttaa aactaatcgggacactgatgc	
	Spd2_Insert1m_FW	tgtacaaaaaagcaggcttcaggactaccagtgg ag	HiFi
	Spd2_Insert1146_RV	tgtacaagaaagctgggtcaaatttaaaactaatcggg acactgatgc	
	Spd2_Insert1wt_FW	tgtacaaaaaagcaggcttcaggactaccagtgg aag	
	Spd2_Insert552_FW	ctgcaccgaggatgaaacgatgaggagga	
	Spd2_Insert552_RV	tcctcctcatcgttttcacctcggtgcag	

	oZN246_Spd2S625A	cctgtcctcgccaagaagctgtctctcggcgccgctgc tggacagcacaaccagttcc	
	oZN247_Spd2S625A	ggaactgggttgctgtccagcagcggcgccgagag acagcttcttggcgaggacagg	
	oZN248_Spd2T516A	cagaagccgagggcgacatcgatgagtgccctcgg cgccggtaaaggagcctagccgcagagtgagacg	Mutagenesis
	oZN249_Spd2T516A	cgtctcactctgcggctaggctcctttaccggcgccga gggccactcatcgatgtccgcc	
	Andi_HiFi-DSpd- 2(Mid)_FW	aggaaaacctgtacttccagggtactggctcgaatctc ggacg	HiFi
	Andi_HiFi-DSpd- 2(Mid)_RV	tcggatccgggtacctcattacatggcttgagtgatc g	(in vitro)
Plp	Plp_Insert_FW	tgtacaaaaaagcaggcttcatgaatctgtacactatat acgattggatcacatcattg	HiFi
	Plp_Insert_RV	tgtacaagaaagctgggtcatgatgccgcgcatgc	
	pRNA_PCR_RV	gaagcctgctttttgtacaaacttg	HiFi
	pRNA_PCR_FW	gaccagctttctgtacaaagtg	
	Kate_reset_FW	ccctagcaaactggggcacagatagactagtaagcg	
	Kate_reset_RV	cgcttaactagtctatctgtgcccagtttgtaggg	Mutagenesis
	Katelink_reset_RV	ctcgctcaccatgctagctcgaaccactttgtaca	
	Katelink_reset_FW	tgtacaaagtgggttcgagctagcatgggtgagcgag	
Other	pETM-44_TEV_RV	accctggaagtacaggttttccttgctacccggagtctg cgcgtc	HiFi
	pETM-44_FW	taatgaggtaccggatccgaattcgagctc	(in vitro)
	Pry4	caatcatatcgctgtctcactca	<i>spd-2</i> ^{G20143}
	Spd2gRV2	gactgtagggaatgaggattacttgctg	screening
	Exelixis_XP5'	aatgattcgagtggaaggct	<i>Df(3R)Exel73</i>
	Ana1_Exel7357_RV	tgtgtgtgcaaatctgaaactacgatccgcattg	57 screening

2.3.2. Bacterial transformation

All bacterial transformations were carried out following the heat shock protocol. 1 μ L of $\sim$ 100 ng/ μ L DNA was added to 50 μ L chemically competent *E. coli* cells, and were subsequently incubated on ice for 30 min. Cells were heat shocked at 42 °C for 35 s, before a 2 min incubation on ice. 300 μ L 2xYT medium was added, and the cells were allowed to recover at 37 °C with shaking for 1 h. Cells were then plated onto LB agar

plates (LB medium, 16.7 g/L agar) containing the appropriate antibiotic (50 µg/mL kanamycin for pUbq and pETM44 plasmids, 50 µg/mL ampicillin for pRNA plasmids, 50 µg/mL Zeocin (Thermo Fisher) for pDONR plasmids) for overnight growth. For Zeocin-resistant plasmids, low salt 5 g/L LB medium was used to make the agar plates.

2.3.3. Construction of plasmids for *in vitro* mRNA production

The 10-S/T, ALL, and CONS mutant DNA fragments were synthesised externally by GENEWIZ Co. Ltd. (Suzhou, China). Most WT and mutant constructs were derived via Gateway cloning (Thermo Fisher, 11789100 and 11791100). Constructs were cloned into the pDONR/Zeo vector (Gateway, Thermo Fisher) and from the resulting entry clone, the DNA fragments were then cloned into the appropriate destination vector, containing a T3 promoter compatible with the mMESSAGE mMACHINE T3 Transcription Kit (Thermo Fisher, AM1348): pRNA-mKate2-CT-DEST (Novak et al., 2016) or pRNA-GFP-CT-DEST (Cottee et al. 2015). However, the pRNA-Asl-mKate2 and pRNA-Spd-2-mKate2 plasmids were derived from previous versions with a C-terminal GFP tag in collaboration with Z. Novak via NEBuilder HiFi assembly (NEB, E2621S). Additionally, the plasmids pRNA-Sas-4-mKate2 and pRNA-Asl-mKate2 had accumulated point mutations in the pRNA backbone which were reverted to the original via site-directed mutagenesis using the Quikchange II XL Site-directed Mutagenesis Kit (Agilent, 200522), following manufacturer's instructions.

NEBuilder HiFi assembly was also used to obtain pRNA-Plp-mKate2 and pRNA-Plp-ALL-mKate2, and to derive pRNA-mKate2 plasmids encoding Spd-2 and Ana1 “partial mutants” (i.e. versions of these proteins with large sets of ptPBSs mutated) from a series of PCR-amplified fragments obtained from the corresponding WT and ALL plasmids. Finally, the single- and double-point mutants (pRNA-Asl-S372T-

mKate2 and pRNA-Spd-2-AA-mKate2) were generated via site-directed mutagenesis from the corresponding WT plasmids.

All the plasmids used and generated in the process are listed in Table 2.3.

Table 2.3. List of plasmids used for in vitro mRNA production

Gene	Plasmid	Reference
Sas-4	pDONR-Sas-4-10S/T	This thesis
	pRNA-Sas-4-mKate2	(Novak et al., 2016)
	pRNA-Sas-4-mKate2	This thesis (reverted)
	pRNA-Sas-4-10S/T-mKate2	This thesis
Asl	pDONR-Asl-ALL	This thesis
	pRNA-Asl-mKate2	Collaboration with Z. Novak
	pRNA-Asl- S372T-mKate2	This thesis
	pRNA-Asl-mKate2	This thesis (reverted)
	pRNA-Asl-ALL-mKate2	This thesis
Cep135	pDONR-Cep135	(Roque et al., 2012)
	pDONR-Cep135-ALL	This thesis
	pRNA-Cep135-mKate2	This thesis
	pRNA-Cep135-ALL-mKate2	This thesis
ana1	pDONR-Ana1	This thesis, from pDONR-ANA1-STOP (Saurya et al., 2016)
	pDONR-Ana1-ALL	This thesis
	pRNA-Ana1-mKate2	This thesis
	pRNA-Ana1-ALL-mKate2	This thesis
	pRNA-Ana1-NT-mKate2	This thesis, partial mutant
	pRNA-Ana1-Mid-mKate2	This thesis, partial mutant
	pRNA-Ana1-CT-mKate2	This thesis, partial mutant
	pRNA-Ana1-GFP	This thesis
spd-2	pRNA-Ana1-ALL-GFP	This thesis
	pDONR-Spd-2-ALL	This thesis
	pDONR-Spd-2-CONS	This thesis
	pRNA-Spd-2-mKate2	Collaboration with Z. Novak
	pRNA-Spd-2-AA-mKate2	This thesis
	pRNA-Spd-2-ALL-mKate2	This thesis

	pRNA-Spd-2-CONS-mKate2	This thesis
	pRNA-Spd-2-NT-mKate2	This thesis, partial mutant
	pRNA-Spd-2-CT-mKate2	This thesis, partial mutant
Plp	pDONR-Plp-WT	Kind gift from A. Caballe
	pRNA-Plp-WT	This thesis
	pRNA-Plp-ALL	This thesis

2.3.4. Construction of plasmids to generate transgenic fly lines

Gateway cloning (Thermo Fisher) was used to generate all the vectors required for *P*-element mediated transformation in flies. I used the corresponding pDONR plasmids made for pRNA plasmid generation, except for pDONR-Spd-2 (WT), which was already available in the lab (kind gift from J. Baumbach). I used pre-existing *attR* destination vectors which contained the ubiquitin promoter (for moderate levels of expression of the protein of interest in all tissues (Lee et al., 1988)) and a C-terminal GFP or mCherry fluorescent tag (Basto et al., 2008). The recombination was done via the Gateway LR reaction and performed according to the manufacturer's instructions. The final plasmids generated are listed in Table 2.4.

Table 2.4. List of plasmids used to generate transgenic fly lines

Gene	Plasmid
spd-2	Ubq-Spd-2-ALL-GFP
	Ubq-Spd-2-CONS-GFP
	Ubq-Spd-2-mCherry
	Ubq-Spd-2-ALL-mCherry
	Ubq-Spd-2-CONS-mCherry
ana1	Ubq-Ana1-GFP
	Ubq-Ana1-ALL-GFP
	Ubq-Ana1-mCherry
	Ubq-Ana1-ALL-mCherry

2.3.5. Construction of plasmids for *in vitro* production of Spd-2 fragments

The plasmids pRNA-Spd-2-mKate2 and pRNA-Spd-2-ALL-mKate2 were used as templates to amplify DNA fragments corresponding to Spd-2 amino acids 352 – 758. These fragments were introduced via HiFi assembly into a modified pETM-44 plasmid for bacterial expression of the protein of interest with an N-terminal His6-MBP tag.

2.3.6. Diagnostic PCRs for genetic recombination events

Anesthetised, possibly recombinant male flies were put into single wells of a 96-well plate and squashed in “squishing buffer” (10mM Tris-HCl, 1 mM EDTA, 25 mM NaCl, 200 µm/mL Proteinase K) on ice. Subsequently, the sealed 96 well plate was treated at 55 °C for 30 min and then at 95 °C for 10 min. PCR reactions for the screening of genetic recombination events were performed using the OneTaq Quick-Load 2xMM (NEB, M0486S) according to the manufacturer’s protocol, using the primers indicated in Table 2.2. The final PCR products were run on a 1% agarose-ethidium bromide gel (1x TAE, 1% agarose, 0.005% ethidium bromide) and visualised with UV light.

2.3.7. *In vitro* mRNA transcription

To generate mRNA for microinjection into embryos, 4 µg of DNA (pRNA plasmids) was linearised in 80 µL using the restriction enzyme AscI (NEB, R0558S) for 8 h at 37 °C. The enzyme was then inactivated at 80 °C for 20 min. The linearised DNA was precipitated in 250 µL of precipitation buffer (4mM EDTA, 96 mM Na-Acetate and 70% ethanol) at -20 °C overnight, pelleted at 4 °C for 15 min at 21.1 g, washed twice with 1 mL of -20 °C 70% ethanol, air-dried and resuspended in 10 µL of DEPC-treated ddH₂O. The transcription reaction was performed using the mMACHINE mMESSAGE mMACHINE T3 Transcription Kit according to the manufacturer’s instructions. The

mRNA was purified using the RNeasy Kit (Qiagen, 74106) according to the manufacturer's instructions, diluted to 2 µg/µL aliquots and stored at -80 °C.

2.4. Biochemistry and Cell biology

2.4.1. *Drosophila* embryo collection and fixation

For immunoprecipitations using embryos extracts, 0-3 h *Drosophila* embryos were collected and washed thrice in washing buffer (PBS-0.0005% Tween-20), dechorionated in 60% bleach for 2 min and washed thrice in washing buffer. The embryos were directly collected into a pre-weighted tube using a brush, weighted, snap frozen in liquid nitrogen and stored at -80 °C.

To test protein levels in the embryos via western blotting, 0-2 h *Drosophila* embryos were collected in a similar way, but after being dechorionated they were washed into a glass vial containing 2 mL of methanol-EGTA (15mM) with 2 ml of heptane and vigorously shaken for 30 s. Embryos were allowed to collect at the bottom of the vial, removing the excess liquid and those embryos that remained floating at the liquid-liquid interface. The remaining embryos were washed thrice with a total of 6 mL of methanol. The embryos were stored in methanol at 4 °C for at least 24 h before use.

For immunofluorescence, *Drosophila* embryos were collected for 1 h and aged for 1 h at 25 °C. The embryos were collected and fixed following the same protocol as for western blotting, but including 15mM EGTA in the 6 mL of methanol used for washes after fixation.

2.4.2. Protein production and purification

Spd-2 352 – 758 aa fragments (WT and 19T) were obtained in collaboration with S. Saurya. S. Saurya did the expression and purification of the proteins, while I tested the

different samples at each point of the protocol via SDS-PAGE (see 2.4.4). The corresponding pETM-44 constructs were transformed into *E.coli* BL21 cells. All the colonies were scrapped off and resuspended in LB. 1L cultures (with 50 µg/mL kanamycin) were grown per construct and cell type at 30 °C, until they reached OD₆₀₀ of 0.6. IPTG was then added to a final concentration of 100 µM, and the cultures were incubated for a further 4 h at 25°C. To judge expression of the protein of interest, 500 µL of pre- and post-induction samples were pelleted and re-suspended to the same density in Laemmli sample buffer (SB; contains 120 mM Tris pH 6.8, 4% SDS, 10% 2-β-mercaptoethanol, 20% glycerol, 0.04% bromophenol blue). The samples were boiled at 95 °C for 10 min and 5 µL of each sample were loaded per well of a SDS-PAGE gel. Once we were certain that the protein was being expressed, the cells were pelleted by centrifugation at 4,000 rpm for 20 min at 4 °C. The cells were resuspended to a total of 50 mL with ice cold PBS. Cells were centrifuged again at 4,000 rpm for 20 minutes at 4°C. After discarding the supernatant, cell pellets were flash frozen in liquid nitrogen and stored at -80 °C until protein purification.

For protein purification, pellets were resuspended in protein preparation buffer (PPB, 50 mM Tris pH 8.0, 1 mM EDTA, 100 mM KCl). A 10 µL sample was removed for later analysis. Lysozyme was added to each resuspended pellet (final concentration of 100 µg/mL). Samples were spun at 4 °C for 30 min and lysed using an Emulsiflex C5 homogeniser (Avestin). A 10 µL sample was removed for later analysis. The rest of the samples were spun at 15,000 rpm for 20 min at 4 °C. A 10 µL sample was removed for later analysis. The rest of the supernatant was poured onto a pre-poured amylose column containing 4 mL amylose resin (NEB E8021L). A 10 µL sample was taken from the flowthrough. Once the supernatant had flowed through completely, the column was washed twice with PPB-0.05 % Tween-20 and twice again with PPB. The

fusion protein was eluted by gently adding 1 mL aliquots of 10 mM maltose in PPB to the column. Each 1 mL sample was collected in a separate tube, until 10 different fractions had passed through the column and had been collected. 10 μ L from each fraction were taken to test protein concentration using the Bradford assay. This allowed identification of the peak (most concentrated three fractions) and side fractions (three fractions on each side of the peak). 10 μ L of PEAK and SIDE were taken for further analysis, the rest of the protein was flash frozen. 10 μ L of 2X SB were added to each of the 10 μ L samples, which were boiled at 95 °C for 10 min.

A. Haensele further purified 5 mL of the resulting products (3 mL of PEAK and 2 mL of SIDE samples) via size exclusion chromatography (SEC), using an AKTA pure chromatography system with a HiLoad 16/600 Superdex 200 column attached (GE Healthcare). As a result, the protein was resuspended in protein suspension buffer (20 mM Tris pH 8.0, 150 mM NaCl, 0.5 mM TCEP). I took 10 μ L aliquots from the isolated 1 mL fractions corresponding to the protein peak of the expected size, sides of the peak, and peaks of other sizes (presumed contaminants). Aliquots were mixed with 10 μ L of 2X SB and boiled at 95 °C for 10 min and analysed by SDS-PAGE. After confirming the quality of the purified protein, the samples were concentrated by S. Saurya using a 30,000 MW concentrator, spinning at 4,000 rpm for 5 min at 4 °C.

2.4.3. Immunoprecipitation and *in vitro* interaction assays

PoloGFP immunoprecipitation and the initial Spd-2-GFP immunoprecipitation experiments were performed using anti-GFP antibodies (Table 2.5) coupled to protein A conjugated magnetic Dynabeads (Thermo Fisher, 10002D). Beads (30 μ L of slurry/reaction) were washed twice (PBS-0.1% Triton X-100) and incubated with 7.5 μ g/reaction of antibody in PBS for 30 min at RT, spinning at 15 rpm on a rotator (SB3

Rotator, Stuart, UK). The beads were then washed twice with equal volumes of conjugation buffer (20 mM sodium phosphate pH 7.9, 150 mM NaCl) and incubated for 30 min at RT with 250 μ L/reaction 5 mM BS³ (Thermo Fisher, 21580) diluted in conjugation buffer. The crosslinking reaction was quenched by adding 12.5 μ L/reaction of 1M Tris pH 7.5 to the bead suspension. The beads were washed thrice with PBS-0.1% Triton X-100 and stored at 4 °C before use. BS³ creates irreversible amide bonds between primary amines (e.g. lysines and arginines between the antibody and the protein A) but it can also destroy the epitope-recognising surface of the antibody. Thus, during the optimisation of the immunoprecipitation-interaction assay for this thesis, I decided to switch to covalently coupling 7.5 μ g of primary antibody (anti-GFP or anti-MBP) to 1 mg of Dynabeads M-270 Epoxy beads using the Dynabeads Antibody Coupling Kit (Thermo Fisher, 14311D), following manufacturer's instructions. Beads prepared in this way were stored at 4 °C for 1-2 weeks. Before use, the required amount of beads was incubated in PBS-0.1%BSA for 15 min at RT while rotating.

Embryo extracts were prepared in the cold room. Snap frozen embryos (see 2.4.1) were homogenised on ice using a disposable pestle, adding 100 μ L of homogenisation buffer (50 mM HEPES pH 7.6, 1 mM MgCl₂, 1 mM EGTA, 1X phosphatase inhibitor cocktail II and III (Sigma, P5726 and P0044), 1X protease inhibitor cocktail) per 100 mg of embryos (minimal amount of embryos homogenised at once). The 1X protease inhibitor cocktail had been prepared in advance and was made of 300 mM benzamidine (Sigma, B6506), 200 mM phenanthroline (Sigma, P9375), 10 mg/mL aprotinin (Sigma, A6103), 50 mg/mL leupeptin (Sigma, L2884) and 1 mg/mL pepstatin A (Sigma, P5318). 2 μ L of 1 M DTT per 100 mg of embryos were added to the homogenised extract, which was then centrifuged twice at 13,000 g for 15 min at

4°C. In every case the clear supernatant was kept and the pellet was discarded, if the pellet was disturbed in the process the sample was centrifuged again for 10 min. Additional homogenisation buffer (half of the volume of supernatant obtained) was added to dilute the samples. For the PoloGFP immunoprecipitation, all of the extract obtained from 100 mg of embryos was used per experiment (taking a 10 µL aliquot taken as input). For the Spd-2-GFP immunoprecipitation, 12.75 µL of extract was taken into 300 µL of homogenisation buffer, and then the 10 µL input aliquot was taken. Any remaining embryo extract was aliquoted and snap frozen in liquid nitrogen for storage at -80 °C.

For immunoprecipitation, beads were aliquoted into protein LoBind Eppendorf tubes (Thermo Fisher, 10708704), the supernatant removed and replaced with embryo extract. The beads were incubated with the extract overnight at 4 °C while rotating. The following day, a 10 µL aliquot of the supernatant was taken (unbound) and the beads were washed (PBS-0.1% Triton X-100 with 1X phosphatase inhibitor cocktail II and III) rotating for 10 min at 4 °C, 5 times. For simple immunoprecipitation, the supernatant was removed and the protein was eluted from the beads (this section, below). For *in vitro* interaction assay, the beads in the last volume of washing buffer were transferred to a clean LoBind tube.

In vitro interaction assays were performed with two different types of samples: anti-GFP-beads treated with extract from Spd-2-GFP embryos (as described above), or anti-MBP-beads bound to *in vitro* produced WT and 19T MBP-Spd-2(352–758) (see 2.4.2). In the latter case, each sample of BSA-blocked anti-MBP-beads were incubated with 32.2 µg of the appropriate protein in protein suspension buffer (see 2.4.2) for 30 min while rotating at RT (taking input and unbound aliquots of supernatant before and

after bead incubation). The rest of the *in vitro* interaction assay protocol was similar for all types of samples. The beads were rinsed twice with kinase buffer (CST, 9802) and resuspended in 60 μ L of kinase buffer containing 200 μ M of ATP (CST, 9804) and either kinase storage buffer (50 mM HEPES pH 7.6, 100 mM NaCl, 5mM DTT, 20% glycerol, 15 mM reduced glutathione), for non-phosphorylated “blank” controls; or commercial PLK1 kinase (ProQinase, 0183-0000-1, 30 ng/ μ L for assays using embryo extracts and 8.8 ng/ μ L for assays using *in vitro* protein) for phosphorylated samples. The samples were rinsed thrice with binding buffer, of final composition after optimisation: 50 mM Tris pH 8.0, 200 mM NaCl, 1 mM DTT, 0.1% Tween-20, 10 mg/mL BSA, 1X phosphatase inhibitor cocktail II and III, 1X SIGMAFAST EDTA-free protease inhibitor cocktail (Sigma, S8830). The beads were then resuspended in 0.3 mL of binding buffer with GST-PLK1-PBD (Sigma, SRP0360), with final concentration of 0.015 μ M in assays using embryo extracts, and 0.2 μ M in assays using *in vitro* protein (as the latter are expected to have more Spd-2 fragments bound per bead). 10 μ L aliquots of beads resuspended in PBD-binding buffer were taken (PBDin). Samples were incubated rotating for 2.5-3 h at 4 °C. The supernatant was then discarded, but keeping an aliquot (PBDout). Beads were rinsed with 500 μ L of bead wash buffer B (50 mM Tris pH 8.0, 200 mM NaCl, 1 mM DTT, 0.1% Tween-20, 1 mg/mL BSA, 1X phosphatase inhibitor cocktail II and III, 1X SIGMAFAST EDTA-free protease inhibitor cocktail) thrice, transferred to a clean tube, and rinsed once with bead wash buffer HA (50 mM Tris pH 8.0, 150 mM NaCl, 1 mM DTT, 0.1% Tween-20, 1X phosphatase inhibitor cocktail II and III, 1X SIGMAFAST EDTA-free protease inhibitor cocktail) before protein elution.

For protein elution, the supernatant was removed from the beads. The beads were resuspended in 50 μ L of 1X Laemmli SB (see 2.4.2). Samples were boiled at 95 °C for

10 min, spun 4-5 s and the supernatant was removed from the beads and stored at -20 °C. For all the other samples collected during the immunoprecipitation and *in vitro* interaction assays, an equal volume of 2X SB was added, and the samples were boiled, spun and stored as above.

All the above steps that required separating the magnetic beads from the supernatant were performed on a magnetic rack.

2.4.4. SDS-PAGE and western blotting

To test protein levels in the embryos, collected and fixed embryos (see 2.4.1) were washed for 15 min with PBS-0.2% Triton X-100 thrice while rotating. The embryos were transferred to a clean watch glass to select those that were well-fixed and pre-cellularised. 20 embryos selected in 20 µL of PBS-0.2% Triton X-100 were mixed with 20 µL of 2X SB, boiled at 95 °C for 10 min, and stored at -20 °C.

Pre-boiled samples were run on 3-8% NuPAGE Tris-Acetate acrylamide gels (Thermo Fisher EA03785BOX) with the appropriate NuPAGE Tris-Acetate SDS running buffer (Thermo Fisher LA0041) at 150 V in a XCell SureLock Mini-Cell. The only exceptions were the SEC samples obtained during protein purification, which were run on 4-12% NuPAGE Bis-Tris gels (Thermo Fisher, NP0322BOX) with MES buffer, to ensure that, if there were any small size contaminants, they would not be run off the gel. For analysis of protein purification samples, gels were rinsed in ddH₂O and incubated with InstantBlue dye (Expedeon, ISB1L) for 30 min – overnight, depending on sample concentration, and rinsed again in ddH₂O before imaging. Otherwise, samples were transferred onto a nitrocellulose membrane (BIO-RAD, 0.2µm 162-0112) in a BIO-RAD Mini Trans-Blot system using transfer buffer (25 mM Tris pH 7.4, 192 mM glycine, 25% methanol, 1% SDS) at 100 V for 1.5 h.

After transfer, membranes were incubated in Ponceau staining (Sigma, P7170), washed with ddH₂O and blocked for 1 h with blocking buffer (PBS, 4% milk powder, 0.1% Tween-20). Membranes were then incubated for 1 h with primary antibody solution (see Table 2.5) at RT and washed thrice for 1 min in blot washing buffer (TBS, 0.1% Tween-20). Membranes were then incubated for 45 min with HRP conjugated secondary antibodies (see Table 2.6). Membranes were finally washed thrice for 15 min in washing buffer and incubated in HRP-substrate (Thermo Scientific SuperSignal West Femto Maximum Sensitivity Substrate, 34095; different dilutions deviating from manufactures instructions) for 1 min. The membranes were exposed on film and/or on the Amersham Imager 600 (GE Healthcare) for a digital image. When necessary, membranes were stripped for re-blotting by incubating with Restore Western Blot Stripping Buffer (Thermo Fisher, 21059) for 15 min at 37 °C.

2.4.5. *Drosophila* embryo staining for immunofluorescence

Methanol was removed from the embryos before washing them for 15 min with PBS-0.2% Triton X-100 (PBT) rotating at 15 rpm, thrice. They were blocked by incubating them twice in PBT-5% BSA for 30 min. Embryos were then incubated overnight at 4 °C with primary antibody (see Table 2.5) in PBT-5% BSA while rotating at 15 rpm. The antibody was removed and the embryos were washed with PBT for 20 min at RT, thrice. The samples were then incubated for 2 h at RT with fluorescently conjugated secondary antibodies (see Table 2.6). Finally, the samples were washed in PBT for 15 min thrice, and the embryos were mounted in 20 µL of Vectashield (Vector Laboratories, Vectashield Antifade Mounting Medium H-1000 or H-1200 with DAPI), on slides with 1.5H high-precision coverslips (CellPath) and sealed with nail polish. Slides were stored at 4 °C.

2.4.6. Antibodies used in this study

The following tables list all the antibodies used for immunoprecipitation and *in vitro* interaction assays (IP), western blotting (WB) and immunofluorescence (IF).

Table 2.5. Primary antibodies used in this study

Lab ID	Protein	Species	Application	Dilution	Source/Reference
221	Actin	Mouse	WB	1:500	Sigma, A3853
3	Ana1	Rabbit	IF, WB	1:500	Eurogentec, animal #SK4818, (Stevens et al., 2010a)
200	Asl	Guinea pig	IF	1:500	Eurogentec, animal #SKC124 (Roque et al., 2012)
55	Cnn	Guinea pig	IF	1:1000	Eurogentec, animal #SK42, (Basto et al., 2008)
191	Cnn	Rat	IF	1:500	Eurogentec, animal #SKR104, like #55 but raised in rat
236	Cnn pSer567	Rabbit	IF	1:500	Pocono Rabbit Farm & Laboratory, animal #30129, (Feng et al., 2017)
144	GAGA factor	Rabbit	WB	1:500	Berkeley Antibody Company, Z13-3, (Raff et al., 1994)
118	GFP	Rabbit	IP WB	N/A 1:500	Eurogentec, animal #SK495. (Huang and Raff, 1999)
166	GFP Booster Atto488	Llama	IF	1:500	Chromotek, gba488
264	GST	Mouse	WB	1:500	Thermo Fisher, MA4-004
MBP	MBP	Rabbit	IP	N/A	Eurogentec, animal #SK2860, (Barros et al., 2005), initially raised against MBP-AurA
107	Polo	Rabbit	IF	1:500	Eurogentec, animal #SK365
230	PLK1 (pT210)	Rabbit	IF, WB	1:500	Abcam, ab12157
251	Polo (MA294)	Mouse	IF	1:100, 1:15	Raised against fusion protein missing 1-28 aa. Kind gift from C. Sunkel, (Logarinho and Sunkel, 1998)
57	Spd-2	Rabbit	IF, WB	1:500	Eurogentec, animal #SK3428, (Dix and Raff, 2007)
141	α -Tubulin	Mouse	IF	1:1000	Sigma, DM1a, T9026
142	γ -tubulin	Mouse	IF	1:500	Sigma, GTU-88*, T6557

Table 2.6. Secondary antibodies used in this study

Species	Reacts with	Application	Dilution	Source/Reference
Sheep	Mouse	WB (HRP)	1:3000	Sigma, NA934V
Donkey	Rabbit	WB (HRP)	1:3000	Sigma, NA934V
Swine	Rabbit	WB (HRP)	1:3000 (normally) 1:15,000 (*)	Dako, P0399
Chicken	Rat	IF (Alexa Fluor 647)	1:500	Thermo Fisher, A21472
Goat	Mouse	IF (Alexa Fluor 647)	1:500	Thermo Fisher, A21236
Goat	Mouse	IF (Alexa Fluor 594)	1:500	Thermo Fisher, A11032
Goat	Rabbit	IF (Alexa Fluor 594)	1:500	Thermo Fisher, A11012
Donkey	Rabbit	IF (Alexa Fluor 488)	1:500	Thermo Fisher, A21206
Donkey	Guinea pig	IF (CF405S)	1:500	Biotium, 20356

* For blotting against rabbit primary antibodies as a loading control in blots for interaction assays.

2.5. Microscopy and image analysis

2.5.1. Perkin Elmer ERS Spinning Disk microscope

Embryos were imaged at RT on a Perkin Elmer ERS spinning disk (VLOCITY software) mounted on a Zeiss Axiovert 200M microscope using a 63X/1.4NA oil immersion objective and an Orca ER CCD camera (Hamamatsu Photonics, Japan). An immersion oil with a refractive index of 1.518 (ImmersolT 518 F, Zeiss International) was used to minimise spherical aberration. A 488 nm Argon gas laser and a 561 nm diode pumped solid-state laser were used to excite GFP and RFP/mCherry/mKate2, respectively. All

red/green images were taken in the UltraVIEW ERS ‘emission discrimination’ mode with these filter specifications: green emission filter (527/55 nm band pass centre filter) and blue/red emission filter (445/60 and 615/70 nm band pass centre filter). Full frame images (672x512 pixels) were collected with 1x1 binning. Confocal sections of 13 z-slices with 0.5- μ m-thick intervals were collected every 30 s (17 slices for the analysis of protein dynamics throughout the cell cycle). The size of the z-stack was chosen to minimise the number of z-slices captured, while including all centrosomes over the duration of the movie. Focus was occasionally readjusted in between intervals, due to centrosomes moving in z during the cell cycle. The exposure times, gain and laser power settings were determined for each fluorescent fusion-protein individually. All image analysis was done using ImageJ (NIH, version 2.0.0).

2.5.2. OMX V3 Blaze 3D-Structured Illumination (3D-SIM) microscope

3D-sim microscopy was performed at RT on an OMX V3 Blaze microscope (GE Healthcare) with a 60x/1.42-NA oil UPlanSApo objective (Olympus); 405-, 488-, 593- and 633-nm diode lasers, and four different Edge 5.5 sCMOS cameras (PCO). A 1.514 immersion oil was used for refractive index matching to reduce spherical aberration (Demmerle et al., 2017). The “standard drawer” set of emission filters was used (band pass filters 442/46 for 405 nm track, 525/50 for 488 nm track, 615/24 for 593 nm track, and 684/40 for the 633 nm track), except for live two-colour imaging of samples where the signal intensity of the 488- or 593-nm channels was expected to be low, in which case I uses the “red/green drawer” set (band pass filter 540/80 for 488 nm track, long pass filter 605 for 593 nm track). 512x512-pixel images were acquired using the sequential SIM mode and 1x1 binning. Stacks of 6 z-slices were acquired for live imaging, whereas stacks of 13 z-slices were acquired for fixed samples, always at 0.125- μ m intervals. For each plane, five phases and three angles were acquired. The

exposure times and laser power setting were determined individually for each experiment. The raw acquisition was reconstructed using softWoRx 6.1 (GE Healthcare) with a Wiener filter setting of 0.006 and channel-specific optical transfer functions. For multi-colour 3D-SIM, images from the different colour channels were registered with alignment parameters obtained from calibration measurements using 1 μm to 0.2 μm TetraSpeck Microspheres (Thermo Fisher) using OMX Editor (Micron Oxford) and Chromagon (Matsuda et al., 2018) alignment software. The SIM-Check plug-in in ImageJ (NIH) was used to assess the quality of the SIM reconstructions (Ball et al., 2015).

2.5.3. LSM 880 with Airyscan detector microscope

Samples from certain immunostaining experiments (γ -tubulin staining and examination of mitotic spindles in rescued *ana1*^{-/-} mutant embryos) were imaged using an inverted Axio Observer.Z1 Zeiss 880 Laser Scanning Microscope fitted with an Airyscan detector (Zeiss International, Micron Oxford). The microscope was equipped with a Plan-Apochromat 63x/1.4 NA oil objective and immersion oil of refractive index of 1.518 was used. The microscope was additionally equipped with an Airyscan module containing a 32-channel GaAsP detector. The images were taken at 16-bit depth using a 488 nm Argon laser and 405 nm, 561 nm and 633 nm solid-state lasers. All experiments were set up using the 'Smart Setup' function, which allows for an optimal imaging with respect to dichroic mirror and emission discrimination, depending on the fluorophores used. The emission discrimination settings used were the MBS -405 (405 nm track), and MBS 488/561 or MBS 488/561/633 with band pass filters 420-480 + 495-550 nm (488 nm track) and 420-480 + 495-620 nm (561 nm track, in experiments that also use the 633 nm track), for the Airyscan mode. The exposure times, zoom, and the laser power settings were determined specifically for

each experiment, keeping the detector gain in the 700-800 range and the amplification gain at 1.0. Stacks of 25 slices with 0.14- μm -thick intervals were collected with pixel size (xy) of 0.035 μm , using a piezo-driven z -positioner stage. Images were Airy-processed in 3D with a strength value of “auto” (~ 6). The software used to acquire images and process the images taken in super-resolution Airyscan mode was ZEN (Zeiss International, black edition).

2.5.4. Radial profiles

I used ImageJ to calculate an average “radial profile” of the distribution of the different centriolar and PCM proteins around the mother centriole. For embryos imaged live, I selected the z -slice where the most centrosomes were in focus and I analysed the five brightest centrosomes per embryo (the number of individual embryos analysed per genotype or type of mRNA injected is indicated in the corresponding Figure). For the analysis of γ -tubulin in fixed embryos, I analysed one pair of centrosomes per embryo, five embryos per technical replicate (embryos collected and processed independently), and three technical replicates in total per condition (total $n = 15$).

I first scaled the images so that each pixel was split into 25 (5×5) pixels in order to increase the resolution of the radial profile. For each individual centrosome I found its centre of mass by thresholding the image and running the “analyse particles” (centre of mass) macro on the most central z plane. I then centred concentric rings spaced at 0.021 μm and spanning across 2.09 μm on this centre (0.007 μm and spanning across 1.41 μm for the fixed γ -tubulin images) and measured the average fluorescence around each ring (radial profiling). After subtracting the average cytosolic signal, each profile was normalised to the average peak intensity of the control condition (embryos expressing WT version of the protein of interest). Each profile was then mirrored to

produce a full centrosome profile. This results in “absolute” radial profiles, which reflect changes in signal intensity and distribution between conditions. Additionally, “normalised” radial profiles were calculated by normalising each profile to the average peak intensity of its own condition. Thus, the peak intensity of all profiles is equal to 1 and any difference in curve width will reflect changes in protein distribution from the centre of the centrosome.

For statistical analysis, I calculated the area under the curve (AUC) per individual centrosome profile. Prism computes AUC using the trapezoid rule. For each profile, the output includes mean total AUC and mean peak Y value (i.e. maximum intensity of the peak). I then compared the mean peak values obtained from absolute radial profiles, and the mean total AUC values obtained from normalised radial profiles, using the appropriate statistical test (see 2.5.13).

2.5.5. Quantifying the effect of Ana1-mKate2 injection into PoloGFP embryos

The images obtained from embryos expressing PoloGFP injected with Ana1-ALL-mKate2 or Ana1-CT-mKate2 could not be quantified using radial profiles because these injections result in complete loss of PoloGFP from certain centrosomes (see 3.2.4, 5.2.7). Instead, all the images from embryos injected with these mRNAs (or their control counterparts) were analysed as follows: I selected embryos that developed normally and had similar Ana1-mKate2 levels, I prepared a two-colour z-slice with the most centrosomes in focus per embryo, the images from all the different conditions tested were blinded and mixed together, and one independent scorer was asked to determine for each image whether all the centrosomes visible in the red channel (i.e. those that had incorporated WT or mutant Ana1-mKate2) were also visible in the green channel (i.e. had visible amount of PoloGFP).

2.5.6. Quantification of successful completion of pronuclear fusion

Embryos were fixed and stained using a mouse anti- α -tubulin, followed by Alexa 594 anti-mouse and GFP Booster Atto488. Samples were mounted in Vectashield medium with DAPI (see 2.4). Embryos were counted using a Zeiss Axioskop 2 microscope (Zeiss International) with a 10x/0.30-NA and a 40x/0.75-NA objectives. Embryos were counted as developing beyond pronuclear fusion if they had clearly reached syncytial/gastrulation stages, or clear signs of mitosis (e.g. mitotic spindles), or more than 16 nuclei (independent DAPI dots). Non-developing embryos often had only one or two bright dots of α -tubulin and DNA. More than two DAPI/MT clusters were possible if the pronuclei had started to break apart, but they were never >15. For each of the four conditions (non-rescue, WT-rescue, CONS-rescue and ALL-rescue) I preformed two biological replicates (embryos from separate sets of mothers), each with three technical replicates (embryos collected and processed independently); >50 embryos were counted per sample.

2.5.7. Analysis of centriole, centrosome and microtubule fluorescent intensities

For quantification of Spd-2-GFP and Jupiter::mCherry intensities, I used ImageJ to calculate the maximum intensity projection of z-stacks of movies taken from the Perkin Elmer spinning disk system. The time frame chosen for analysis corresponded to 1 min before nuclear envelope breakdown (NEB). The five brightest centrosomes per embryo were identified via manual thresholding and analysed (the number of embryos analysed is indicated in the corresponding Figure). For both the green and red channels I measured the mean intensity within a square of fixed size (5.04 μm x 5.04 μm) centred manually on each individual centrosome. Similarly, I measured the mean intensity of the background near each centrosome. I calculated the average centrosome intensity and subtracted the average background intensity per embryo.

For quantification of centrosomal protein levels in *ana1*^{-/-} embryos expressing Ana1 transgenes I followed a similar protocol, with a few changes to make it more comparable to previously reported data (Saurya et al., 2016). Embryos were analysed at mid/late S-phase, unless otherwise indicated (see below). I analysed 10 pairs of centrosomes, randomly chosen, using a square of fixed size (2.74 µm x 2.74 µm), unless otherwise indicated (see below).

For embryos expressing only Ana1-GFP, I used the maximum intensity projection of the z-stack and I classified the data into two subsets: BRIGHT (data from the brightest centrosome from each pair) and FAINT (data from the other centrosomes). I then calculated average intensities at centrosome separation (beginning of S-phase) and late S-phase, and compared differences between WT and ALL Ana1-GFP within each subset.

For embryos co-expressing Ana1-GFP and Asl-mCherry (either from mRNA injection or transgenic experiments), due to too much abnormal movement of centrosomes in the mutant condition I could not use the maximum intensity projection of the z-stack. Instead, I selected the z-slice where the most centrosomes were in focus. The data was blinded before quantification to remove any unconscious bias. Each measurement was classified into the subsets “OLD MOTHERS” and “YOUNG MOTHERS”, as young mother centrioles only start to recruit Asl after separation from the old mother centrioles (Novak et al., 2014) and, therefore, within each pair of centrosomes, Asl-mCherry localises preferentially to, and is marker of, the older mother centriole. Asl maintenance was scored by calculating the mean OLD and mean YOUNG Asl-mCherry intensity per embryo, calculating the ratio between these values per embryo,

and then calculating the average embryo Asl OLD/YOUNG intensity ratio, as in (Saurya et al., 2016).

For embryos co-expressing Ana1-mCherry and PoloGFP I also used a single z-slice image due to abnormal movement in the mutant embryos. Because there were not enough centrioles in the embryos rescued with Ana1-ALL-mCherry to analyse 10 random centrosome pairs, I instead quantified the mean intensity of every single Ana1-ALL-mCherry spot that I could image, without distinguishing between old and young mother centrioles and under the assumption that they would correspond to separate centrosomes. I then quantified the mean intensity of the corresponding area in the 488 nm channel, and the corresponding nearby background for both channels. A total of 24 Ana1-ALL-mCherry foci from six different embryos were analysed in this way. This was on average four centrosomes/embryo, so from every WT control embryo I analysed two random pairs of centrosomes. As it was not possible to distinguish embryo age or cell cycle stage in the mutant rescued embryos, I analysed a panel of WT control embryos as diverse as possible: embryos at nuclear cycle 10 to 14, and at early S-phase, late S-phase and metaphase/anaphase.

2.5.8. Analysis of GFP-Cnn and Spd-2-GFP levels in tracked centrosomes

For quantification of GFP-Cnn and Spd-2-GFP centrosomal levels in *ana1*^{-/-} embryos co-expressing Ana1-mCherry transgenes, I used the maximum intensity projection of the z-stack, and I adapted the protocol described in the previous section (2.5.7).

The embryos were ideally imaged through two complete nuclear divisions (with first division being nuclear division 10, 11 or 12). At the beginning of the first nuclear division imaged, I selected a random pair of separating centrosomes (1st generation centrosomes) based solely on the Ana1-mCherry image (to avoid selection bias). I then

manually tracked the pair until the following S-phase. If the tracking was successful, both pairs of 2nd generation centrosomes were visible and at least one of the two pairs visibly separated, that group of six centrosomes was included in the analysis. Note however that for three (of eight) embryos co-expressing GFP-Cnn and WT Ana1-mCherry there were three recordings where no centrosomes could be tracked into the 2nd generation due to technical difficulties, so only the 1st generation centrosomes were included in the analysis.

I then measured the intensity of the GFP-Cnn or Spd-2-GFP signal from each 1st generation pair. The centrosome with the highest value was labelled “B” (for “Big scaffold”) and the other was labelled “S” (for “Small scaffold”). The 2nd generation centrosome pairs were then labelled BX or SX, depending on whether they were derived from the B or S centrosome of the 1st generation. I then measured the intensity of the 2nd generation centrosomes and for each pair I similarly identified the centrosome with the highest value. Thus, I identified BB and BS centrosomes from pair BX, and SB and SS centrosomes from pair SX (see also schematic in Figure 5.10C). Thus, six different centrosome “types” were defined per 1st generation pair.

The measured centrosomal intensities were then plotted in three different ways: a) pooling all centrosomal values per condition together (Figure 5.10D and Figure 5.11C), b) calculating the average cumulative intensity (Figure 5.10E and Figure 5.11D), or c) separating each subset by centrosome type (Figure 5.10F and Figure 5.11E). The cumulative centrosomal intensity is described as the sum of GFP-Cnn or Spd-2-GFP intensity values of all centrosomes from each pair for a specific generation: i.e. the 1st generation cumulative is the sum of B and S values, and the 2nd generation cumulative is the sum of BB, BS, SB and S values (all together). Because there are too

many possible comparisons between the 12 different centrosomal types (six WT and six ALL), only the *p*-values for the most informative statistical comparisons have been reported.

2.5.9. Blind analysis of scaffold formation from 3D-SIM images

Centrosome images were selected based on quality of the reconstruction as assessed by the SIM-Check plug-in and the presence of a visible, well-formed ring corresponding to the presence of protein at the mother centriole wall. Each individual centrosome image was saved as a separate file, renamed and randomised post acquisition. The entire dataset for each experiment was scored blindly. Scorers were asked to rate whether a centrosome of any given image presented a large PCM, little PCM or no PCM (scorers were given examples of each phenotype). Depending on the number and quality of the reconstruction of the images in each dataset, and the difficulty to score each phenotype; the datasets were scored once (Spd-2-GFP in mutant background and Spd-2-mKate2 injections into PoloGFP datasets) or thrice, by different, independent scorers (Spd-2-GFP/Spd2-mCherry and Spd-2-mCherry/PoloGFP datasets), in which case an average score was calculated. Table 2.7 lists the number of centrosomes and images analysed by each independent scorer.

Table 2.7. Blind scoring of 3D-SIM images

Scorers	Dataset	Number of conditions /dataset	Number of centrosomes /condition	Total images scored
1	Spd-2-GFP (mutant background)	3 (WT, CONS, ALL)	36	108
	Spd-2-mKate2 injections into PoloGFP	2 (WT, CONS)	10 (WT), 11 (CONS)	42
3	Spd-2-GFP/Spd2-mCherry	3 (WT, CONS, ALL)	16	96
	Spd-2-mCherry/PoloGFP	2 (WT, CONS)	15	60

2.5.10. Analysis of Ana1 3D-SIM images

To study the localisation of Ana1-GFP at the centriole using superresolution microscopy, I imaged live Ana1-ALL-GFP and WT Ana1-GFP embryos in mid/late S-phase with 3D-SIM. Of all the images acquired, I selected those that reconstructed without any warnings. The selected images were then analysed using SIM-Check (Ball et al., 2015), and I removed from the analysis any image that failed the reconstruction reliability checks. I then selected the two best images from every embryo analysed (when possible) and manually identified well-oriented mother centrioles (i.e. showing a clear Ana1-GFP ring). The selected centrioles were then classified based on whether they only presented a mother-ring or they also presented a dot corresponding to the daughter centriole.

2.5.11. Fluorescence Recovery After Photobleaching (FRAP)

FRAP experiments using Spd-2-GFP embryos were done using the Perkin Elmer system (see above). I optimised the settings to try to obtain as large z-stacks as possible with the shortest time interval (2.5 s). A region of interest (ROI) was drawn around separate centrosomes. The regions were photobleached with a solid-state 440 nm laser at 100% laser power with 10 iterations lasting 1 ms each. Embryos were imaged approximately 3 min after centrosome separation (which I take to represent the start of S-phase) of nuclear cycles 11 or 12, for at least 35 s after photobleaching.

For each embryo, I analysed three photobleached and three control centrosomes. I did a maximum intensity projection of the z-stack and I quantified the mean intensity at the centre of the centrosome by manually zooming in and drawing a square ROI encompassing the four brightest pixels in the area of the centrosome. The 4-pixel ROI was moved accordingly by manually tracking each centrosome across the time frames.

However, for the first time point after photobleaching, when no centrosomal signal could be detected, the ROI was kept in the same xy coordinates chosen to measure the previous time frame (pre-bleach control). I also measured the mean background intensity for the whole embryo (total area avoiding all centrosomes) at each time frame and subtracted the corresponding values according to the formula:

$$\text{Signal} = 4 \times \text{Mean Centrosomal Intensity}_t - 4 \times \text{Mean Background Intensity}_t$$

To analyse if the initial recovery rates were statistically different between WT and CONS Spd-2-GFP, I used Prism to fit a linear regression curve to the data from the earliest time points. However, because the initial recover after photobleaching is not necessarily linear, I also considered each embryo as one group with three technical replicates (photobleached centrosomes). I then plotted the data and calculated the area under the curve (AUC) per embryo for the earliest time frames (0 - 5 s). I then calculated the mean AUC for WT and CONS embryos, doing an unpaired t-test to assess if the differences were statistically significant.

2.5.12. Cell cycle dynamics analysis and modelling

Spd-2-GFP dynamics throughout the cell cycle were analysed as described (Aydogan et al., 2018). Briefly, I used ImageJ to obtain maximum-intensity projections of the z-stack. The background was subtracted using the subtract background function with a rolling ball radius of 10 pixels. Centrosomes (Spd-2-GFP foci) were tracked using TrackMate plug-in (Tinevez et al., 2017), with the following analysis settings: track spot diameter size of 2.1 μm , initial threshold of 0, and quality of >0.03 . The output was the mean intensity of every centrosome, for every time point. Prism was used to plot this data as the average centrosomal intensity per embryo at every time point.

Prism was also used for the mathematical modelling of these curves with the nonlinear regression (curve fit) analysis function, excluding the last three points (90 s) of the cycle, as the data was very variable towards the end of mitosis.

The data for WT Spd-2-GFP was fitted against four different functions to assess the most suitable model (Figure 4.20A): (1) linear growth followed by linear decrease; (2) linear growth followed by plateau followed by linear decrease; (3) Gaussian function; (4) Lorentzian function. Functions (1) and (2) are bespoke algorithms, with the following equations for X amount of time:

(1)

$$Y_1 = \text{intercept}_1 + \text{slope}_1 * X$$

$$Y_{X_0} = \text{slope}_1 * X_0 + \text{intercept}_1$$

$$Y_2 = Y_{X_0} + \text{slope}_2 * (X - X_0)$$

$$Y = IF(X < X_0, Y_1, Y_2)$$

(2)

$$Y_1 = \text{intercept}_1 + \text{slope}_1 * X$$

$$Y_{X_0} = \text{slope}_1 * X_0 + \text{intercept}_1$$

$$Y_2 = Y_{X_0} + \text{slope}_2 * (X - X_0)$$

$$\text{slope}_2 = 0$$

$$Y_{X_1} = Y_{X_0} + \text{slope}_2 * (X_1 - X_0)$$

$$Y_3 = Y_{X1} + slope_3 * (X - X_1)$$

$$Y = IF(X < X_0, Y_1, IF(X < X_1, Y_2, Y_3))$$

The only constraints applied to these equations were the requirements for $slope_1$ and inflection points (X_0, X_1) to be greater than 0, and $slope_3$ to be less than 0. Centrosomes that come from a single embryo were treated as internal replicates, and thus the fitting used only the mean Y value of each time point. To judge and control the quality and precision of regression (goodness-of-fit), I used the R^2 , adjusted R^2 , and absolute sum-of-square values. To compare the fits, the extra sum-of-squares F test was applied, and the appropriate fit was chosen by selecting the simpler model unless p -value < 0.05. The “linear growth followed by plateau followed by linear decrease” model best fit the data (Figure 4.20A), but it is likely a simplification of a more complex model, so individual curves are also shown for each embryo without any model fitted (average of >44 centrosomes per embryo) (Figure 4.18B and Figure 4.19).

The data for Spd-2-CONS-GFP was fitted against the “linear growth followed by plateau followed by linear decrease” model, as this was the preferred model for the WT data. As this data seemed to better be described as a straight line, this model was also compared to a standard straight line model (with no slope constraints)—function (5); or a user-defined constant line function (6) (Figure 4.20B):

(6)

$$Y = intercept_1 + 0 * X$$

The preferred model was the straight line—function (5) (Figure 4.20B)—although the average slope value was nearly zero (-0.0005 ± 0.0054 ; mean $\pm$ SD), indicating that the appropriate model in practice would be a constant line.

2.5.13. Fluorescence Correlation Spectroscopy (FCS)

FCS measurements were performed by T. Steinacker, following the protocol described in (Aydogan et al., 2019). For all measurements, the laser power was kept constant at $6.31 \mu\text{W}$, and the heating unit of the microscope at 25°C . Purified monomeric Spycatcher-GFP (kind gift from A. van der Merwe and colleagues) was measured *in vitro* in PBS-0.05 % Tween-20 at a similar concentration as Spd2-GFP *in vivo*. Embryos from mothers expressing one copy of WT Spd-2-GFP in a full *spd-2^{-/-}* mutant background or in a *spd-2^{+/-}* heterozygous background were measured at the centrosomal plane and in nuclear cycles 11-14 at the beginning of S-phase. All autocorrelation functions (ACFs) were fitted with the eight previously described models, and the best model was chosen based on the Bayesian information criterion. All fitting parameters and chosen diffusion models are stated in Table 2.8.

Table 2.8. FCS fitting parameters and models

Protein	Fitting boundaries (ms)	Anomalous subdiffusion parameter	Structural parameter	Model
mGFP	$4 \times 10^{-4} - 1 \times 10^2$	1.00	5	1 ds 1 ts
Spd-2-GFP	$4 \times 10^{-4} - 1 \times 10^3$	0.65		1 ds 1 ts
mNeonGreen (pPlk4)	$4 \times 10^{-4} - 2 \times 10^2$	0.70		1 ds 1 ts
mNeonGreen (pSas-6)	$4 \times 10^{-4} - 2 \times 10^2$	0.75		1 ds 1 ts
dNeonGreen	$4 \times 10^{-4} - 4 \times 10^2$	0.85		1 ds 1 bs 1 ts

*Where ds = diffusing species, bs = blinking state of the fluorophore, ts = triplet state of the fluorophore.

The molecular brightness of the measured fluorophore (presented as photon count-rate per molecule (CPM)) was used to identify the oligomerization state of cytoplasmic Spd2-GFP (in comparison to monomeric GFP). The CPM measurements were

corrected for background fluorescence (with 10 control measurements of empty buffer for *in vitro* experiments, and ~20 recordings from embryos for *in vivo* experiments) using the following equation:

$$CPM = \frac{(\textit{Photon count rate}^{SAMPLE} - \textit{Photon count rate}^{CONTROL})}{\textit{Number of particles in observation spot}}$$

2.5.14. Statistics

As indicated, Prism 7 (GraphPad Software) was used for all statistical analyses, including column statistics and Gaussian distribution of the data (using the D’Agostino–Pearson omnibus normality test). Unless otherwise specified, to assess if differences between the means of two different groups were statistically significant, I used the unpaired t-test with Welch correction, as the variance was not necessarily expected to be similar. For comparisons of three or more groups I used the one-way ANOVA with Tukey’s multiple comparison test. If the data was not normally distributed and the sample size was small, I instead used the corresponding non-parametric tests. Significance was defined as $p\text{-value} < 0.05$, unless otherwise indicated. To assess results from statistical analysis of radial profiles in Chapter 3, I also report the 95% confidence interval (CI) of the difference between means.

Chapter 3 - An mRNA injection-based screen for proteins that recruit Polo to the centrosome

3.1. Introduction

3.1.1. Polo relies on Ser-pSer/pThr for recruitment to the centrosome

Polo kinase is recruited to many locations of the cell, including the centrosome, via its Polo box domain (PBD) (Elia et al., 2003a, 2003b; Hanisch et al., 2006; Jang et al., 2002a; Lee et al., 1998; Reynolds and Ohkura, 2003; Seong et al., 2002; Song et al., 2000). For example, overexpression of a Polo-GFP mutant form with a defective PBD results in much reduced localisation to the centrosome (Kachaner et al., 2017). *In vitro* and structural studies have shown that the optimal sequence bound by the PBD is a very simple motif: Ser-[pSer/pThr]-X/Pro. Phosphorylation of the amino acid at position 0 is essential, as is the presence of Ser at position -1 (Elia et al., 2003a, 2003b) (see 1.2.1). A Pro at position +1 makes the motif a potential CDK1 phosphorylation target (Songyang et al., 1994)—and CDK1 often appears to prime proteins for Polo binding (Barr et al., 2004; Decker et al., 2011; Elia et al., 2003a; Elowe et al., 2007; Linder et al., 2017; Novak et al., 2016; Preisinger et al., 2005; Qi et al., 2006; Soung et al., 2009; Wang et al., 2013; Watanabe et al., 2005; Yamaguchi et al., 2005; Zhang et al., 2009)—but it is not absolutely required for PBD binding (Elia et al., 2003a, 2003b; Yun et al., 2009).

3.1.2. Rationale behind this screen

It follows that any protein that contributes to Polo kinase recruitment to the centrosome is likely to have at least one site that fits the canonical PBD binding motif. In an effort to identify potential Polo recruiters to the centrosome, I searched through

the amino acid sequences of candidate centrosomal proteins looking for the simplest version of this motif: Ser-Ser/Thr. Any such site identified was considered a potential Polo binding site (referred to as “ptPBS” in this thesis), even if its specific phosphorylation state at the centrosome was unknown. A similar approach has been previously used in a large-scale bioinformatic search for PBD interactors in the human proteome, although this previous effort pre-selected hits based on the incidence of the PLK1 phosphorylation consensus motif, was restricted to *in silico* analysis and modelling simulations, and did not identify any centrosomal proteins (Bibi et al., 2013).

While I did test the effect on Polo recruitment of mutating a single ptPBS (Asl-Thr373 STP motif), in most cases I mutated a combination of ptPBSs at once, including a mutant version of each candidate protein in which I mutated all the existing ptPBSs. Many point mutations are required for this, so there is a risk of disturbing the structure of the candidate proteins and rendering them non-functional and/or unable to localise to the centrosome. To avoid this, I disrupted ptPBSs by mutating the Ser at position -1 into Thr, so Ser-Ser/Thr motifs become Thr-Ser/Thr motifs. Ser and Thr are chemically very similar so this is a very conservative substitution (French and Robson, 1983), but this replacement is enough to completely abolish PLK1 PBD-phosphopeptide binding *in vitro* (Elia et al., 2003a). There was only one exception (Spd-2-AA-mKate2) where I disturbed two ptPBSs by mutating the Ser/Thr at position 0 to Ala.

The importance of the different candidate proteins for Polo recruitment was tested via mRNA injection into *Drosophila* embryos, a technique that has been routinely used in the Raff laboratory to test the effects of different mutations on centrosomal proteins.

For example, it helped confirm *in vivo* the effects of certain Cnn mutations which, based on structural evidence, were predicted to disturb the interaction interface between the LZ motif of the Cnn PReM and the Cnn CM2 domain (Feng et al., 2017). Here, I employed a very similar approach to that used to identify Sas-4 Thr200 as a binding site for Polo kinase that is required for Asl recruitment during mitosis (Novak et al., 2016). Briefly, I made *in vitro* mRNA encoding WT or mutant versions of the candidate centrosomal proteins followed by a C-terminal red fluorescent tag (mKate2). The mRNA was injected into young (<20 min old) embryos from a Polo::GFP protein trap line. The fusion protein (from here on referred to as “PoloGFP”) is expressed from the endogenous *polo* promoter (Buszczak et al., 2007), and it is brighter than a commonly used Polo-GFP transgenic line (Moutinho-Santos et al., 1999; Singh et al., 2014). The embryos were imaged 2 h after injection to allow the injected mRNA to be translated, the protein to incorporate into centrosomes, and the fluorescent tag to mature.

Therefore, although in this assay endogenous WT copies of the candidate centrosomal proteins are still present in the embryo, they are expected to have been mostly outcompeted by the more abundant, fluorescently-tagged proteins by the time of imaging. Thus, unlike approaches based on the generation of transgenic lines, there is no need to cross the PoloGFP allele into a genetic background mutant for the candidate of interest. This is key because fully mutant flies for most of the candidate proteins included in this study are uncoordinated and/or infertile (Basto et al., 2006; Blachon et al., 2008, 2009; Dix and Raff, 2007). This method is also much faster than generating transgenic lines expressing every single mutant.

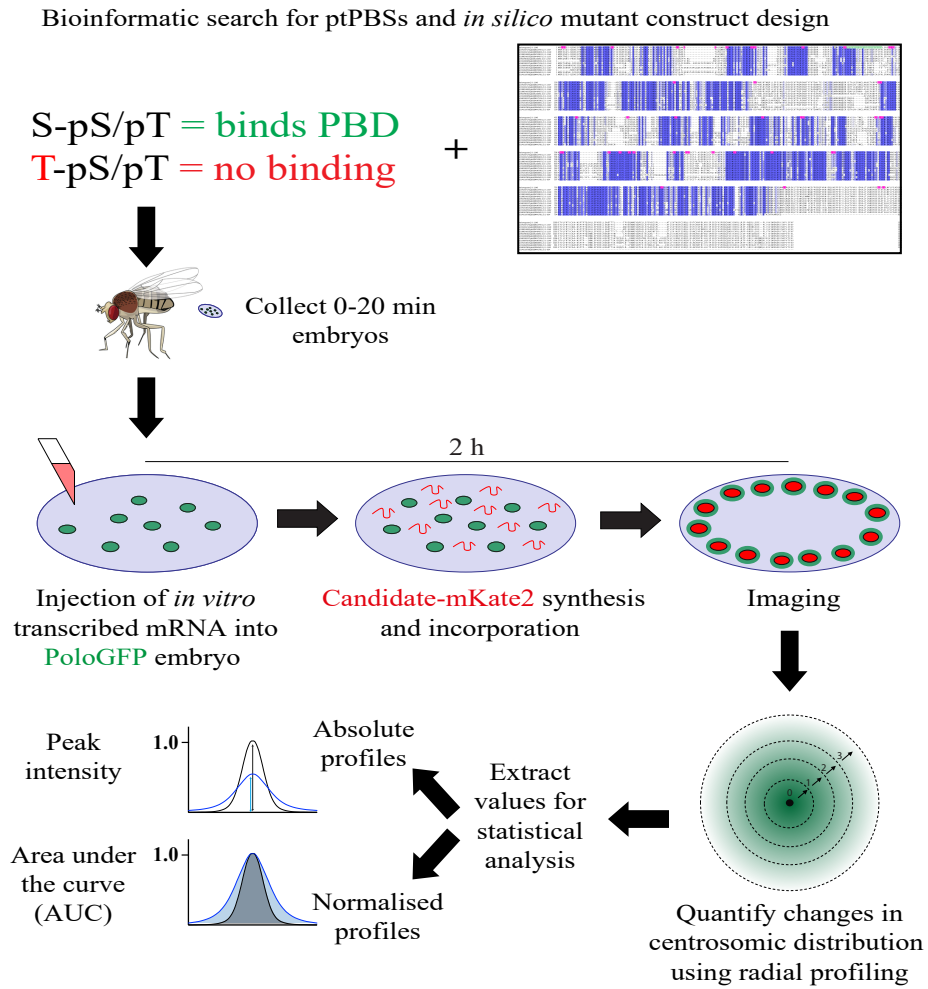
The complete screen strategy is summarised in Figure 3.1A. For quantification of the effects, I generated radial profiles of the different proteins imaged. Radial profiles have been used extensively by the Raff laboratory (Conduit and Raff, 2015; Conduit et al., 2014b, 2014a, 2015b; Feng et al., 2017; Novak et al., 2014; Richens et al., 2015) and others (Cabral et al., 2019; Laos et al., 2015; Vora and Phillips, 2015), and they are particularly useful for visualising the centrosomal localisation of proteins in a quantitative manner. Using radial profiles, we can distinguish differences in absolute centrosomal intensity levels (a proxy for amount of protein at the centrosome), but also differences in distribution at the centrosome. For example, centriolar proteins like Asl have a narrower radial profile than PCM proteins like Spd-2 (Figure 3.1B).

3.1.3. Comparison to previous large scale studies

Although this mRNA-based approach is more limited in scope than several previously published, large scale protein-protein interaction studies in the *Drosophila* and centrosome fields, it has several advantages. For example, affinity purification/mass spectrometry (MS) experiments (Hein et al., 2015; Lowery et al., 2007) can identify strong interactions between proteins in the “general” context of the cell. However, these studies struggle to identify interactions that are tightly regulated and might only happen at the centrosome at a specific point of the cell cycle (e.g. Polo and Sas-4 at the end of mitosis, (Novak et al., 2016)). This type of approach also works best for highly expressed proteins. Neither of these studies found a statistically significant interaction between PLK1 and any centrosomal protein.

Alternatively, a *Drosophila* centrosome interactome has been described using a directed yeast two-hybrid (Y2H) screen (Galletta et al., 2016). This type of approach relies on expression of “bait” and “prey” versions of the proteins of interest in yeast

A



B

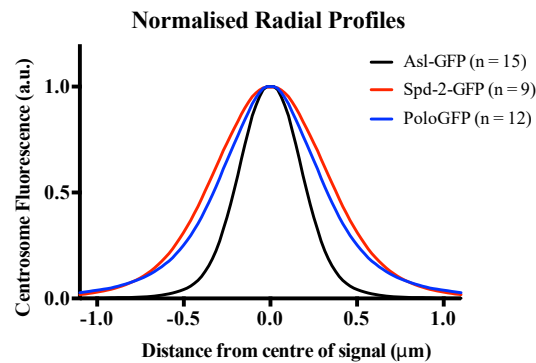


Figure 3.1 – An mRNA injection-based screen for proteins that recruit Polo to the centrosome. (A) Rationale behind the screen, including identification of ptPBSs, mutant design *in silico*, injection of mRNA into embryos, imaging, and quantification of changes in protein levels and distribution. (B) Example of three radial profiles that have been normalised to bring their three peaks to the same level (1.0). The radial profile corresponding to the centriolar protein Asl is narrower than those of Spd-2 and Polo, which are present at the centriole wall and the PCM.

cells, and activation of a reporter gene upon physical interaction between the two proteins. However, because the proteins are expressed in yeast cells, it may not identify any interaction that relies on their specific, *in vivo* cellular context; or that requires post-translational modifications that do not happen in yeast cells. As Polo binding via its PBD requires the docking site to be phosphorylated (Elia et al., 2003a, 2003b), this is potentially a significant drawback for studying interactions between Polo and other centrosomal proteins. Indeed, although many potential interactions have been identified between centrosomal proteins, very few of them (four) involved Polo, and only two of them (Sas-4 and Sak/PLK4) scored highly (Galletta et al., 2016).

Another possibility is BioID, a method based on proximity biotin labelling. Like the mRNA injection approach, proteins can be studied in their original cellular context, provided that tagging the protein of interest with the BirA biotin ligase does not disturb protein localisation. Two different studies have used BioID to build interactosomes for human centrosomal proteins (Firat-Karalar et al., 2014; Gupta et al., 2015). However, BioID has the limitation of only highlighting proximity interactions, not direct protein-protein interactions, and this can be misleading in the very restricted context of the centriole wall. Furthermore, one of the studies did not include PLK1 in their screen (Gupta et al., 2015), and the other only identified one interaction between PLK1 and a centriolar protein (CPAP, the human homologue of Sas-4) (Firat-Karalar et al., 2014).

3.1.4. Candidate proteins included in this screen

Although hundreds of centrosomal proteins have been previously identified (Alves-Cruzeiro et al., 2014; Andersen et al., 2003; Jakobsen et al., 2011; Müller et al., 2010), I focused my screen on the relatively small number of proteins with known, important roles in centriole or PCM assembly. Of these proteins, I selected those that evidence

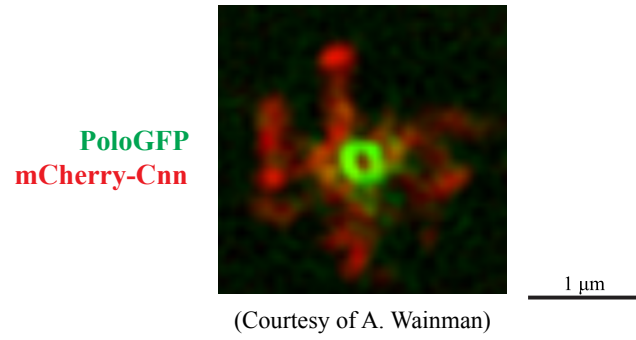
suggested might be involved in recruiting Polo to centrioles and/or the mitotic PCM (as detailed in their respective sections), and other proteins that localise to regions of the centrosome where Polo is found. I excluded the centriole cartwheel proteins (Sas-6 and Ana2) as Polo is clearly localised to the outer wall of the centrioles (Conduit et al., 2014b; Fu and Glover, 2012). I also excluded Cnn as, although Polo phosphorylates Cnn (Conduit et al., 2014a), the interaction between the two proteins must be very transient, as Polo localises only to the PCM closest to the centriole, whereas Cnn spreads out much further (Conduit et al., 2014b; Raff, 2019) (Figure 3.2). Plp was initially included in this screen, but it is such a large protein (2895 amino acids) that *in vitro* production of Plp mRNA was extremely challenging, and the protein was not visibly expressed 2 h after injection of the mRNA into PoloGFP embryos. The final list of candidate centrosomal proteins analysed includes four centriolar proteins (Sas-4, Asl, Cep135, Ana1), two of which are essential for centriole assembly, and the PCM protein Spd-2.

3.1.5. Key findings

In this Chapter, I have used an mRNA injection-based approach to test whether disrupting the ptPBSs present in five key centriolar and centrosomal proteins (Sas-4, Asl, Cep135, Ana1 and Spd-2) results in changes in the amount or distribution of Polo at the centrosome. Two of the proteins tested were positive hits:

1. Disrupting ptPBSs in Spd-2 resulted in a major reduction (although not the complete loss) of Polo at the centrosome.
2. Disrupting ptPBSs in Ana1 resulted in complete loss of Polo at most of the centrosomes in embryos.

A



B

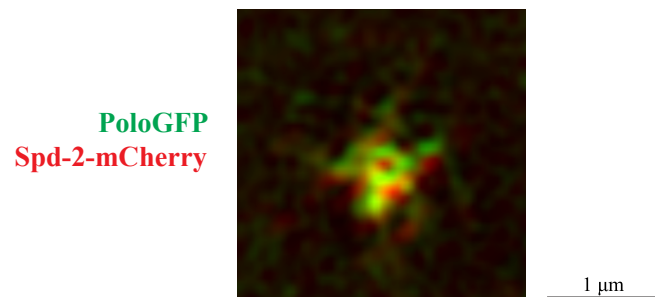


Figure 3.2 – Polo, Cnn and Spd-2 at the centrosome.

Micrographs show 3D-structured illumination (3D-SIM) images of centrosomes in living *Drosophila* embryos expressing PoloGFP and either mCherry-Cnn (courtesy of A. Wainman) (**A**) or Spd-2-mCherry (**B**).

In both cases, loss of Polo was linked to a potential PCM assembly defect, rather than defects in centriole conversion or centriole disengagement. These results are further interrogated in Chapters 4 and 5 of this thesis.

3.2. Results

3.2.1. Quantification of PoloGFP distribution in uninjected embryos using radial profiles

First, I set out to quantify the levels of Polo and its distribution at the centrosome in uninjected embryos expressing PoloGFP (Figure 3.3). Embryos injected with mRNA encoding mKate2-tagged proteins had to be imaged at nuclear cycles 12 – 13 because expression and maturation of the mKate2 tag requires imaging to happen at least 2 h after injection, so obtaining younger embryos (cycles 10 – 11) would be technically challenging. However, for this first control I decided to test whether PoloGFP levels or distribution changed significantly during embryo development. Radial profiling shows that the levels of PoloGFP at centrosomes in mid-to-late S-phase are very similar between embryos at cycles 10 – 11 (pooled together) and cycles 12 – 13 (pooled together), with only a small, but significant, drop in intensity in very late embryos (nuclear cycle 14) (Figure 3.3A,Dii). When the radial profiles were normalised so that the peak intensity for all of them = 1 (to account for differences in peak intensity), I observed that the centrosomal distribution of PoloGFP in mid-to-late S-phase centrosomes was similar across the different nuclear cycles (Figure 3.3B).

When comparing the means between different groups, there is a possibility that any observed difference will appear to be statistically significant if the sample size is large enough, even if the difference is biologically insignificant (Sullivan and Feinn, 2012). As large numbers of centrosomes tend to be analysed for radial profiling, this is an

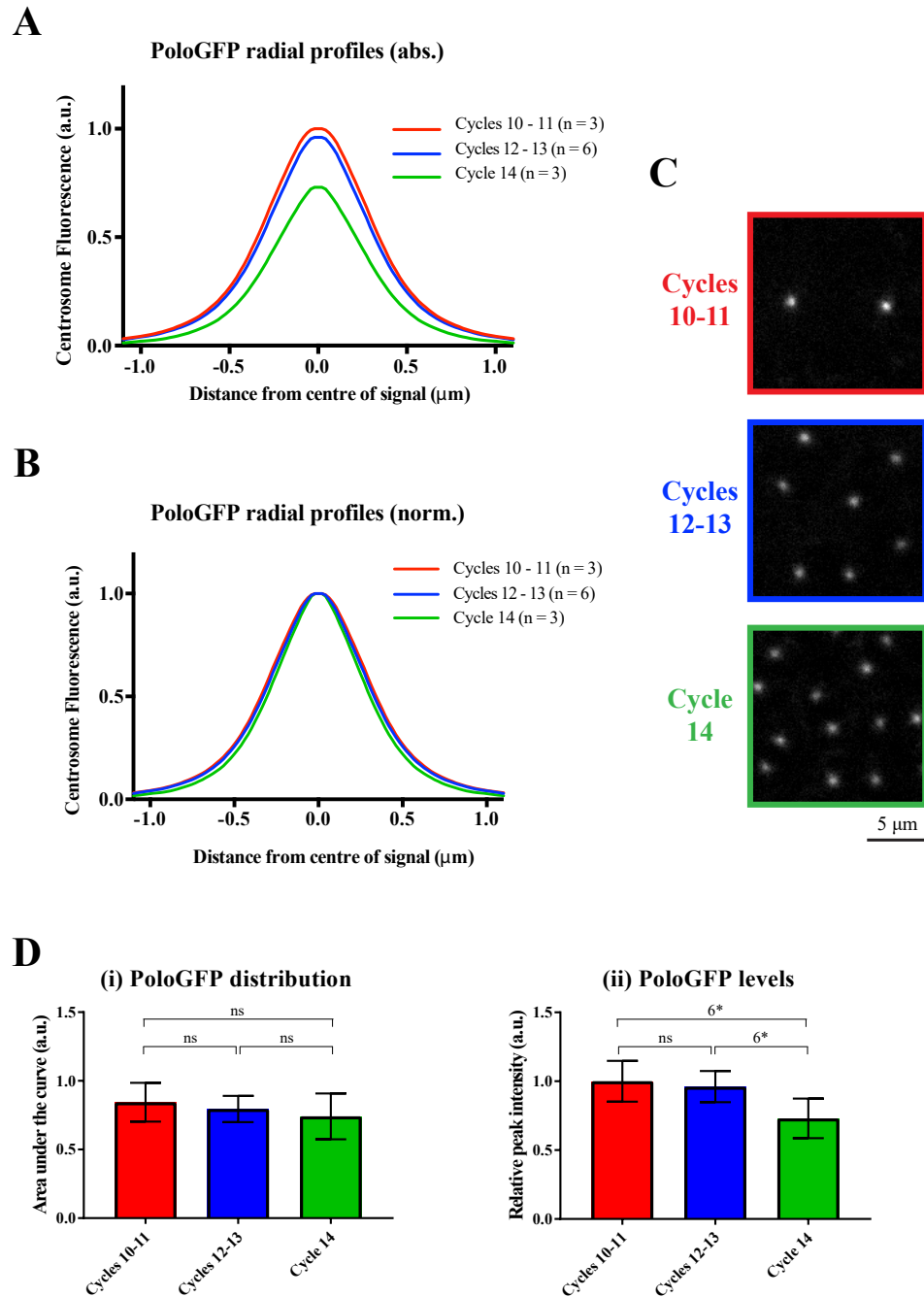


Figure 3.3 – Quantification of PoloGFP distribution in the syncytial *Drosophila* embryo.

Differences in PoloGFP levels and distribution between embryos at different stages of development were quantified using radial profiles. **(A)** “Absolute” radial profiles (“abs.”), which reflect differences in protein distribution (profile width) and intensity (profile height). **(B)** Normalised radial profiles (“norm.”), where the radial profile peaks have all been normalised to 1.0 to reflect only differences in profile width. **(C)** Micrographs showing examples of centrosomes in embryos in different nuclear cycles, labels follow the same colour scheme as indicated in (A,B). **(D)** Parameters extracted from the radial profiles: area under the curve of the normalised profiles **(i)**, to reflect changes in protein distribution; and average peak intensity from absolute profiles **(ii)**, to reflect changes in protein levels. ns = non-statistically significant difference, p -value > 0.05 ; 6* = p -value in 10^{-6} (E-6) range. 5 centrosomes analysed per embryo, n = number of embryos. Error bars represent SD.

important consideration. In these circumstances, one should take into account both the size of the effect between the different treatments and the *p*-value. In this thesis, the size of the effect is reported as the 95% confidence interval (CI) for the difference between two means. For example, the relative mean PoloGFP peak intensity for cycles 10-11, 12-13 and 14 were 1.0, 0.96 and 0.73, respectively. Therefore, the difference between cycles 10-11 and cycles 12-13 is 0.04, and between cycles 10-11 and cycle 14 it is 0.27. However, the 95% CI for the difference between cycles 10-11 and cycles 12-13 ranges from -0.06 to 0.14, and the difference in mean PoloGFP peak intensity between these two groups of embryos is not statistically significant. Conversely, the 95% CI of difference between cycles 10-11 and cycle 14 PoloGFP peak intensity means is 0.14 to 0.36, and the obtained *p*-value was extremely small ($1.75\text{E-}6$), suggesting that the difference between these two groups is statistically significant and biologically relevant.

I decided to use this as a baseline to judge the significance of any “small effect” differences in mean peak intensity arising from the mRNA injection assay. If the *p*-value was equal or below the 10^{-6} range (i.e. $\text{E-}6$, or 6^* in Figures) I would conclude that the difference was statistically significant and potentially biologically relevant. Note that this issue does not appear to be as important for the statistical analysis of differences in PoloGFP distribution, as although cycle 14 centrosomes appeared to have a slightly narrower PoloGFP distribution, this difference was not deemed statistically significant (Figure 3.3B,Di).

3.2.2. PoloGFP recruitment to the centrosome is strongly reduced if several Spd-2 ptPBSs are mutated

Spd-2 was the first candidate to be tested, as its distribution appears to be quite similar to that of Polo (Figure 3.1B, Figure 3.2B), a single phosphorylated Ser-pThr motif in SPD-2 is required to efficiently recruit PLK-1 to centrosomes in worms (Decker et al., 2011), and there is *in vitro* evidence of an interaction between the mammalian homologues (Joukov et al., 2014; Meng et al., 2015). Because the single site in *C. elegans* SPD-2 that recruits PLK-1 is also a potential CDK1 site (Thr233-STP motif), I first generated a form of Spd-2 in which I had mutated the amino acid at position 0 of the only two conserved ptPBSs that are also potential CDK1 phosphorylation targets (T516A and S625A) (Spd-2-AA-mKate2). Although slightly less Spd-2-AA-mKate2 localised to centrosomes compared to WT, PoloGFP levels and distribution appeared unaffected (Figure 3.4).

I then tested the effects of mutating either all the ptPBSs present in Spd-2 (ALL mutant, 34 sites) or only those ptPBSs conserved in at least 11/12 *Drosophila* species included in my analysis (CONS mutant, 16 sites). After injection with the mutant mRNAs, PoloGFP distribution became narrower (although the difference was only statistically significant for Spd-2-ALL-mKate2, p -value = 0.036) and there was a major, highly statistically significant drop in PoloGFP peak intensity (p -value = $4\text{E-}14$ for both mutants) (Figure 3.5)). This is despite a large, and very surprising, increase in mutant Spd-2-mKate2 levels at the centrosome (Figure 3.5D,Eiv). This suggests that the ptPBSs in Spd-2 play a major part in recruiting Polo to the centrosome.

To address this further, I quantified PoloGFP levels only 1 h after mRNA injection, when Spd-2-mKate2 has started to be incorporated into centrosomes, but the mKate2

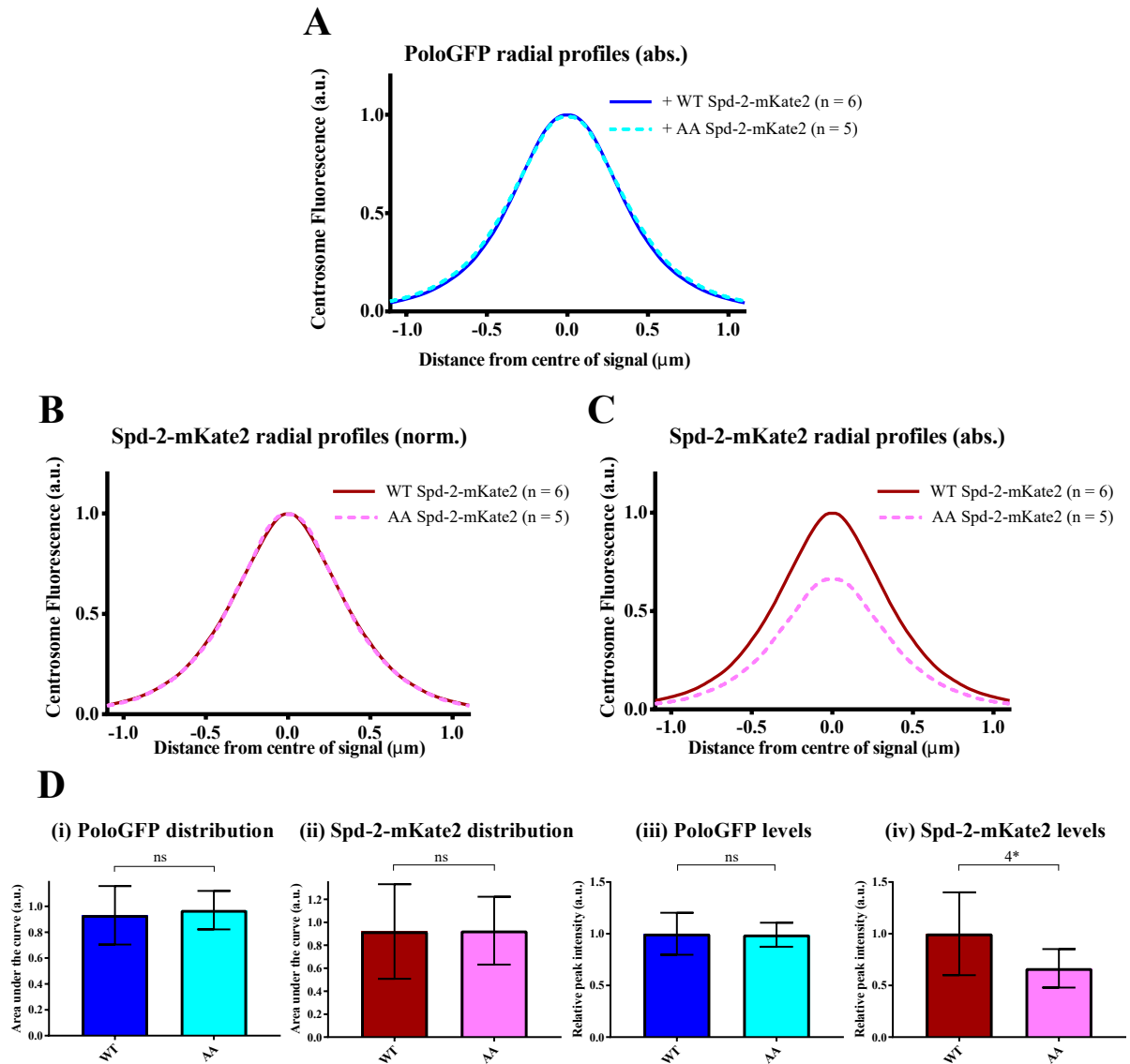


Figure 3.4 – Mutation of two conserved Spd-2 ptPBSs that are potential CDK1 phosphorylation targets does not affect PoloGFP recruitment.

(A) PoloGFP absolute radial profiles after expression of WT or AA mutant Spd-2-mKate2. The normalised profiles are not shown because peak intensities are very similar for both conditions. (B) Normalised radial profiles for WT and AA Spd-2-mKate2. (C) Absolute radial profiles of the different Spd-2-mKate2 proteins. (D) Parameters extracted from the radial profiles: area under the curve from PoloGFP (i) and Spd-2-mKate2 (ii) normalised profiles, average peak intensity from PoloGFP (iii) and Spd-2-mKate2 (iv) absolute profiles. ns = non-statistically significant difference, p -value > 0.05 ; 4* = p -value in 10^{-4} (E-4) range. 5 centrosomes analysed per embryo, n = number of embryos. Error bars represent SD.

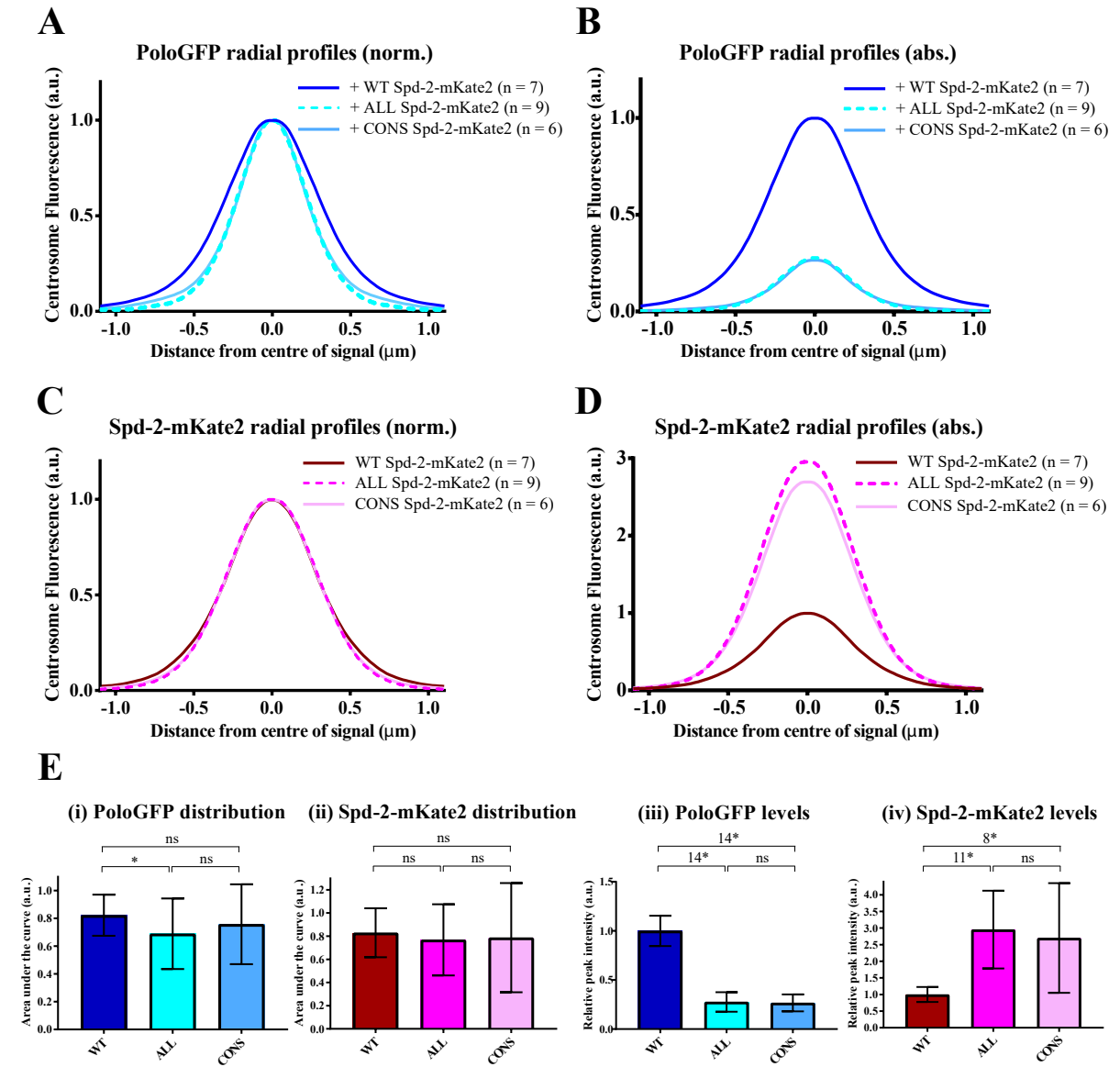


Figure 3.5 - PoloGFP recruitment to the centrosome is strongly reduced if several Spd-2 ptPBSs are mutated.

(A) Normalised PoloGFP radial profiles after expression of WT or mutant Spd-2-mKate2 (ALL or CONS). (B) PoloGFP absolute radial profiles. (C) Normalised radial profiles for WT and mutant Spd-2-mKate2 proteins. (D) Absolute radial profiles of the different Spd-2-mKate2 proteins. (E) Parameters extracted from the radial profiles: area under the curve from PoloGFP (i) and Spd-2-mKate2 (ii) normalised profiles, average peak intensity from PoloGFP (iii) and Spd-2-mKate2 (iv) absolute profiles. ns = non-statistically significant difference, p -value > 0.05 ; * = $0.05 > p$ -value > 0.01 ; 8* = p -value in 10^{-8} (E-8) range; 11* = p -value in E-11 range; 14* = p -value in E-14 range. 5 centrosomes analysed per embryo, n = number of embryos. Error bars represent SD.

tag is immature and so not fluorescent yet. Under these conditions, Spd-2-ALL-mKate2 appears to have an additional negative effect on the peak PoloGFP levels compared to Spd-2-CONS-mKate2. This difference was statistically significant, although the *p*-value did not fulfil my “biological relevance” criterion and the effect size was small (0.090 to 0.32 95% CI, *p*-value = 1.5E-4) (Figure 3.6). This suggests that, although the conserved ptPBSs in Spd-2 have an important role in Polo recruitment, other ptPBSs might be able to take over if those sites are mutated (hence the stronger effect of injecting Spd-2-ALL-mKate2 compared to Spd-2-CONS-mKate2).

The centrosomes injected with mutant mRNA often failed to separate, and they moved irregularly (known as “centrosome rocketing”, data not shown). This behaviour is characteristic of centrosomes with defective PCM, like those lacking Cnn (Lucas and Raff, 2007). Given the importance of Polo phosphorylation of Cnn for correct PCM organisation (Conduit et al., 2014a), loss of Spd-2-mediated Polo recruitment should hinder PCM assembly. To test this hypothesis I injected Spd-2-mKate2 (WT, CONS or ALL) mRNA into embryos expressing GFP-Cnn. The observed phenotypes can be classified into two groups (Figure 3.7). Phenotype 1 was characterised by apparently unaffected GFP-Cnn recruitment and distribution, but appearance of faint Spd-2-mKate2 flares spreading outwards from the centrosome. Spd-2 flares have been previously described in embryos lacking Cnn, when Spd-2 is tightly associated with the centrioles, but moves rapidly away through the remains of the PCM (Conduit et al., 2014b). Conversely, Phenotype 2 was characterised by a “collapsed” GFP-Cnn distribution: GFP-Cnn was still recruited to the centrosome, but it was concentrated at its centre (as was Spd-2-mKate2), showing barely any outwards dispersion. Again, I observed an additive effect comparing Spd-2-ALL-mKate2 (3/8 injected embryos

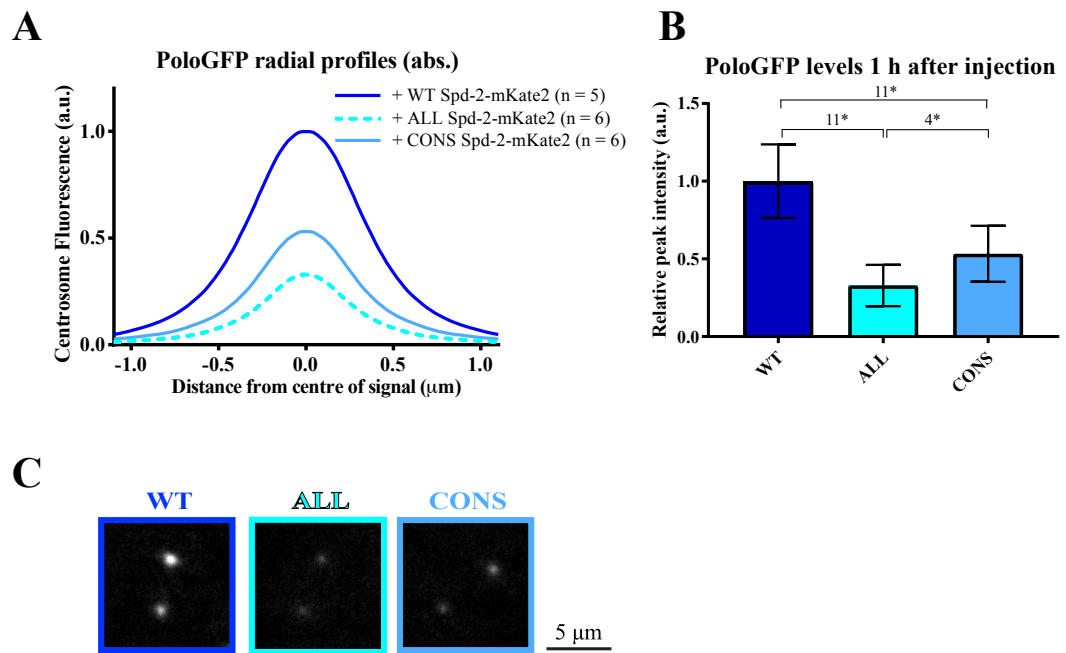


Figure 3.6 – PoloGFP distribution 1h after injection of Spd-2-mKate2 mRNA.

(A) PoloGFP absolute radial profiles after expression of WT, ALL or CONS Spd-2-mKate2. (B) Average peak levels of the PoloGFP radial profiles. (C) Micrographs showing examples of centrosomes in embryos injected with the different mRNAs. The loss of PoloGFP is most obvious in embryos injected with Spd-2-ALL-mKate2 mRNA. 4* = p -value in 10^{-4} (E-4) range; 11* = p -value in E-11 range. 5 centrosomes analysed per embryo, n = number of embryos. Error bars represent SD.

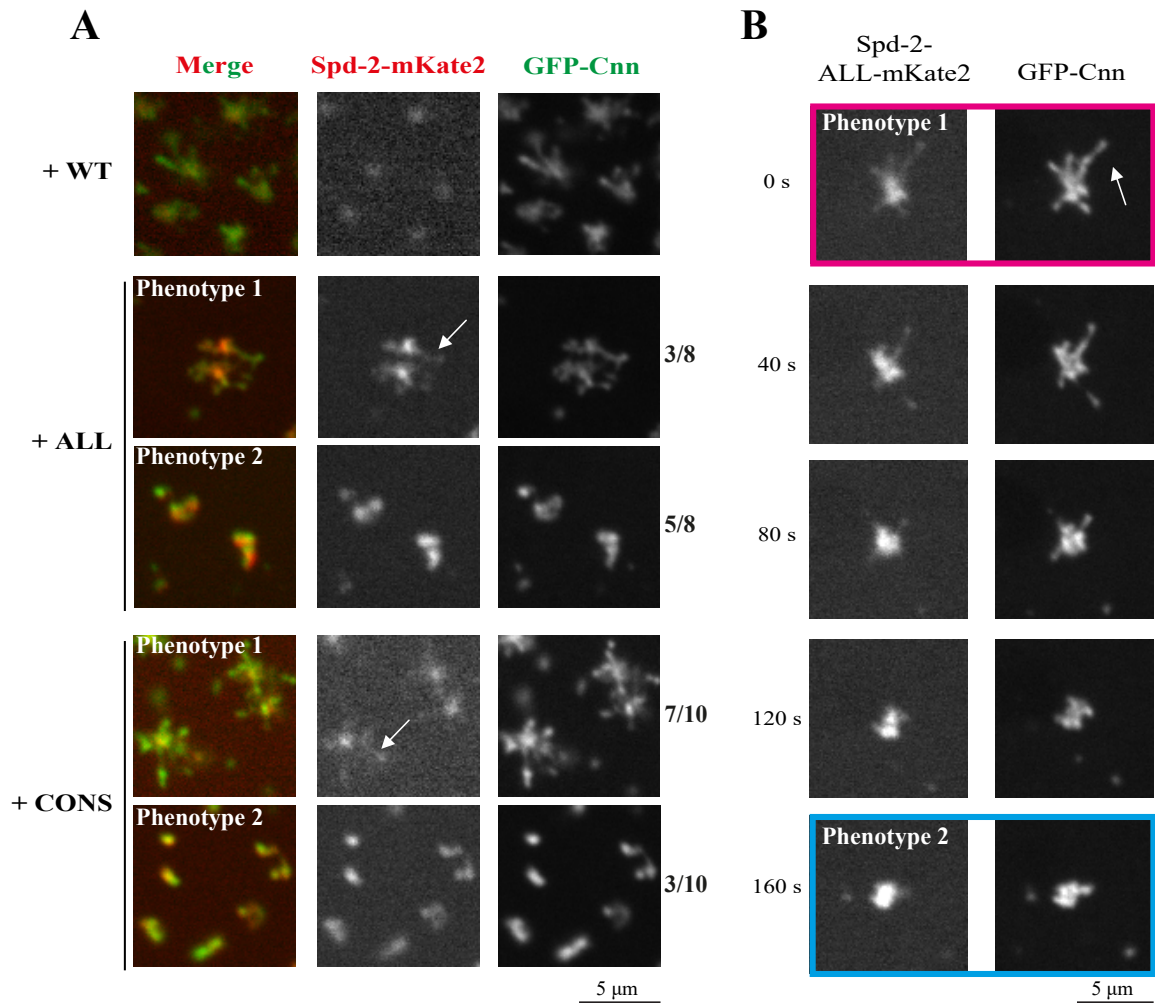


Figure 3.7 – Cnn distribution is dramatically affected if several Spd-2 ptPBSs are mutated.

(A) Representative images of GFP-Cnn embryos injected with WT, ALL or CONS Spd-2-mKate2 mRNA. Expression of the mutant proteins resulted in two different phenotypes. Phenotype 1 is characterised by abnormal Spd-2 flaring, whereas Phenotype 2 is characterised by Spd-2 and Cnn scaffold “collapse” and centrosome clustering. (B) Micrographs show the same centrosome imaged over 160 s, in an embryo expressing GFP-Cnn and injected with Spd-2-ALL-mKate2 mRNA. The distribution of these proteins at time point = 0 s is a good example of Phenotype 1, whereas at time point = 160 s both Spd-2-mKate2 and GFP-Cnn scaffolds appear to have collapsed (Phenotype 2). Arrows indicate Spd-2 flares.

show Phenotype 1 and 5/8 present Phenotype 2) and Spd-2-CONS-mKate2 (7/10 and 3/10, respectively). Figure 3.7B shows an example of an embryo expressing Spd-2-ALL-mKate2 imaged while transitioning from Phenotype 1 to Phenotype 2, suggesting that the effect of expressing the Spd-2 mutants on Cnn worsens with time as endogenous WT Spd-2 is outcompeted by mutant Spd-2-mKate2 and Polo is lost from centrosomes. Overall, these experiments suggest that the ptPBSs in Spd-2 have an important role in Polo recruitment and PCM assembly.

3.2.3. The Thr200-STP motif is the only Sas-4 site crucial for PoloGFP recruitment

The next candidate I tested was Sas-4. As the Thr200-STP motif had already been confirmed to be a Polo binding site (Novak et al., 2016), I mutated the 10 other ptPBSs I identified in Sas-4, leaving Ser199 WT. Both PoloGFP and Sas-4-10S/T-mKate2 localised normally to the centrosome in embryos injected with the mutant mRNA, with no changes in distribution compared to WT (Figure 3.8A,C) and only a small drop in the peak intensity levels (Figure 3.8B,D). While the relatively small difference in PoloGFP levels was statistically significant, its biological relevance is questionable based on my defined threshold (0.076 to 0.22 95% CI, p -value = 8.2E-5) and the effect was much weaker than the near-complete loss of Polo observed when the Thr200-STP motif is mutated ((Novak et al., 2016), also Figure 3.9B). Thus, I conclude that none of the additional ptPBSs in Sas-4 play a major part in recruiting Polo to centrosomes.

3.2.4. PoloGFP is undetectable at centrosomes if all Ana1 ptPBSs are mutated

I next tested the effect of mutating all 34 ptPBSs present in Ana1. Ana1 is an interesting candidate because a low-scoring interaction between the Ana1 C-terminus and Polo has been reported in a Y2H screen (Galletta et al., 2016) and because Ana1 is

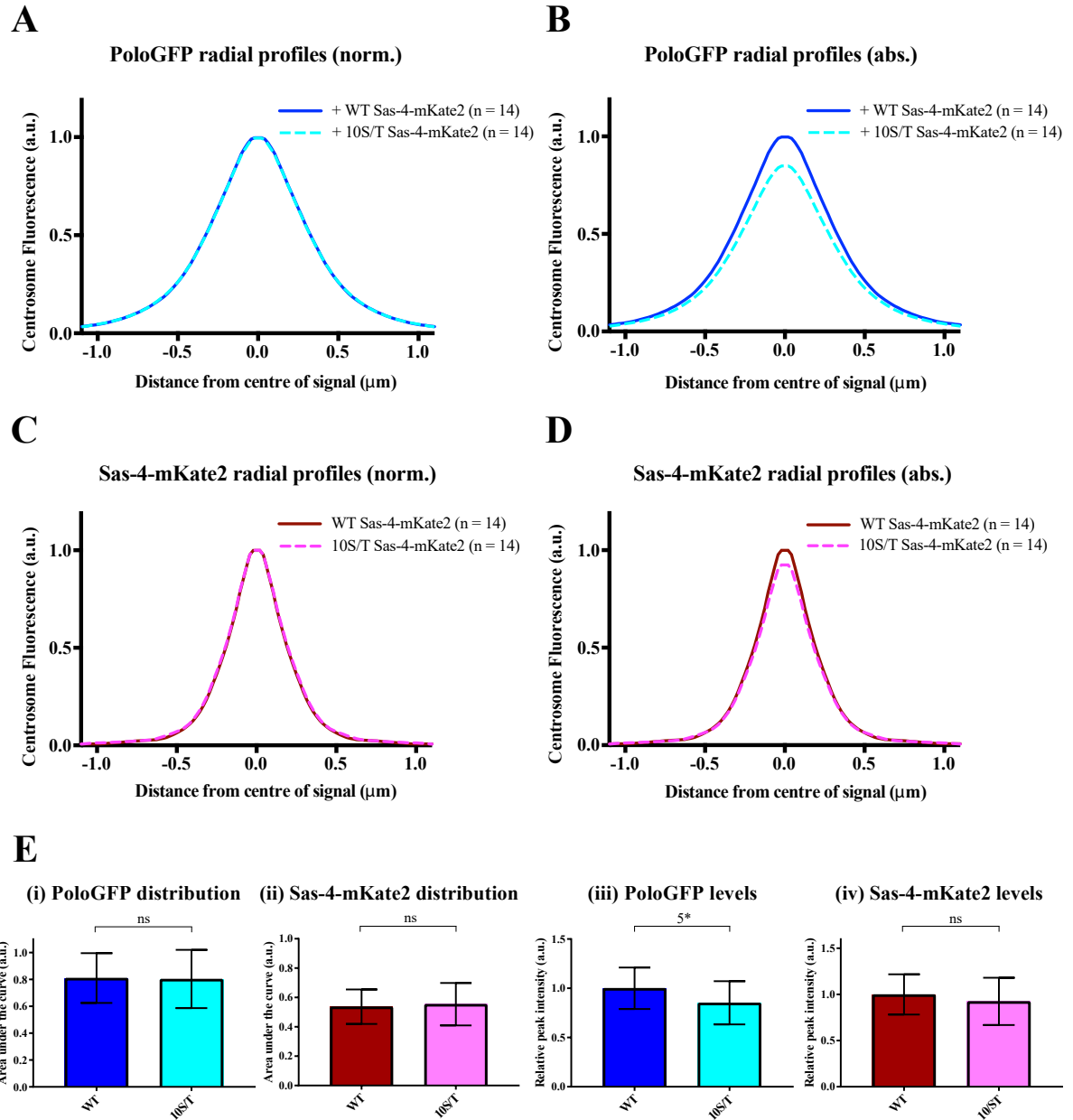


Figure 3.8 – The Thr200-STP motif is the only Sas-4 site crucial for PoloGFP recruitment.

(A) Normalised PoloGFP radial profiles after expression of WT or 10S/T Sas-4-mKate2. (B) PoloGFP absolute radial profiles. (C) Normalised radial profiles for WT and 10S/T Sas-4-mKate2 proteins. (D) Absolute radial profiles of the different Sas-4-mKate2 proteins. (E) Parameters extracted from the radial profiles: area under the curve from PoloGFP (i) and Sas-4-mKate2 (ii) normalised profiles, average peak intensity from PoloGFP (iii) and Sas-4-mKate2 (iv) absolute profiles. ns = non-statistically significant difference, p -value > 0.05 ; 5* = p -value in 10^{-5} (E-5) range. 5 centrosomes analysed per embryo, n = number of embryos. Error bars represent SD.

potentially involved in centriole-to-centrosome conversion (further discussed in 5.1.1). Injection of mRNA encoding Ana1-ALL-mKate2 resulted in strong PoloGFP loss from the centrosome but, interestingly, this loss was often more prevalent at one of the two separating centrosomes. Because of the near-complete loss of PoloGFP, it was not possible to quantify this effect using radial profiling, but this phenotype was observed in 7/7 embryos injected with Ana1-ALL-mKate2 mRNA, compared to 0/9 embryos injected with WT mRNA (scored blindly by Z. Novak) (Figure 3.9A). A similar phenomenon was observed when the Polo-recruiting T200 site was mutated in Sas-4 ((Novak et al., 2016); Figure 3.9B). This striking phenomenon is almost certainly due to Sas-4 and Ana1 being irreversibly incorporated into the centriole structure (Fu et al., 2016; Novak et al., 2014) (see 3.3 Discussion).

Ana1 has been previously implicated in Asl recruitment and/or maintenance (Fu et al., 2016; Saurya et al., 2016). Asl is preferentially enriched in old mother centrosomes (Novak et al., 2014), but expression of certain Ana1 mutants can disturb Asl maintenance at old mother centrosomes, reducing the ratio of enrichment of Asl in old mothers compared to young mothers (Saurya et al., 2016). I injected Ana1-GFP mRNA (WT or ALL) into embryos expressing Asl-mCherry to test if the mutations in Ana1-ALL-GFP affected Asl recruitment and/or maintenance. Surprisingly, this was not the case: Asl-mCherry levels in old and young mother centrosomes were similar after WT and ALL injections, suggesting that Asl recruitment was unaffected (Figure 3.10A). Furthermore, there was a slight decrease in the Asl-mCherry old mother/young mother ratio in embryos injected with Ana1-ALL-GFP, but the difference was not statistically significant (Figure 3.10B), suggesting that Asl level maintenance was unaffected in embryos expressing Ana1-ALL-GFP.

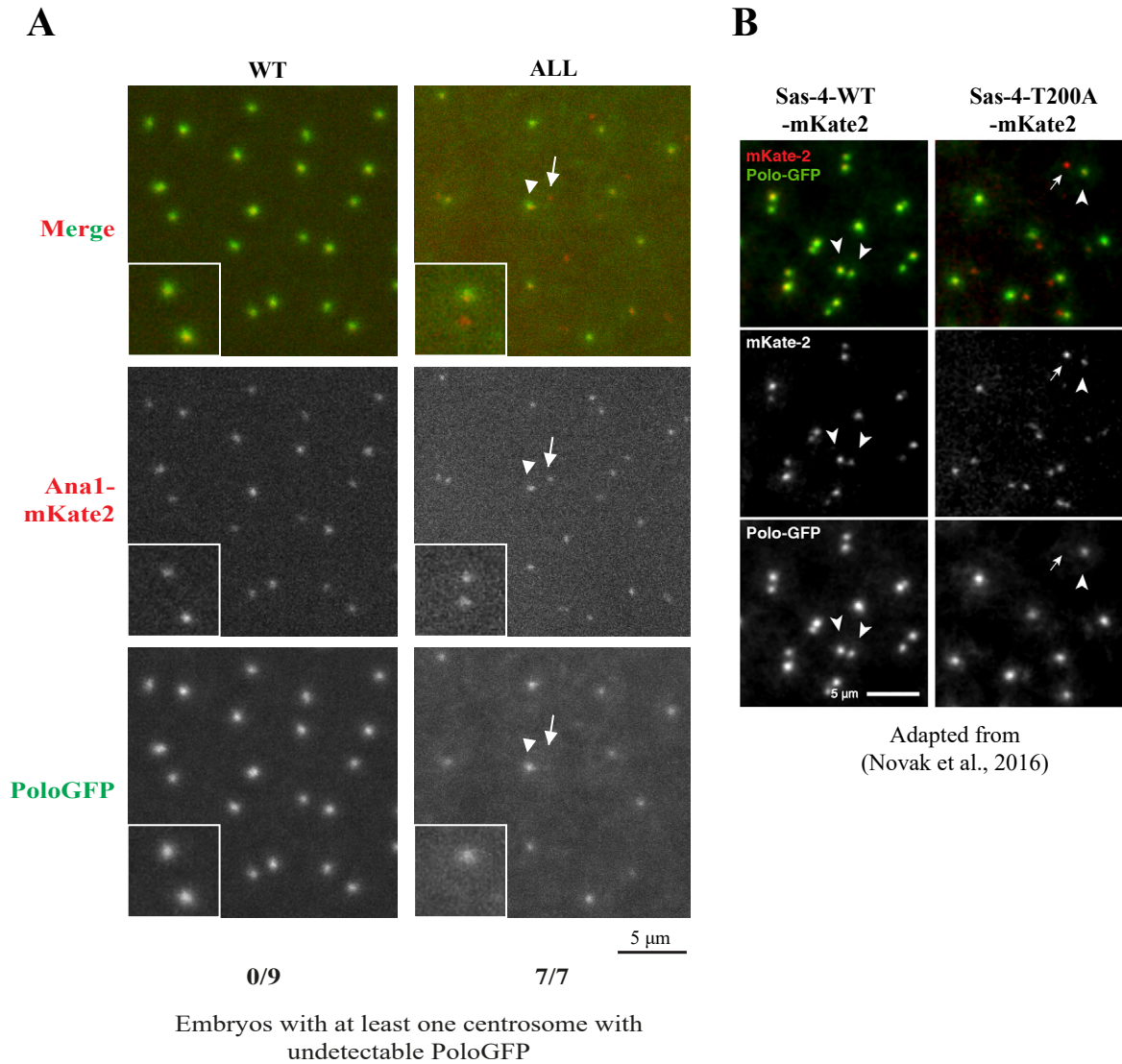


Figure 3.9 – PoloGFP is undetectable at centrosomes if all Ana1 ptPBSs are mutated.

(A) Micrographs of embryos expressing PoloGFP (*green* in the merge channel) injected with WT or ALL Ana1-mKate2-mRNA (*red* in the merge channel). Insets at the bottom left corner highlight a pair of centrosomes as example. In the Ana1-ALL example one centrosome still has PoloGFP and the other has completely lost it.

Embryos were scored blindly for loss of PoloGFP from at least one visible centrosome, the results for each injected construct are indicated as affected embryos/total injected embryos analysed below the corresponding column of panels. (B) Adapted from (Novak et al., 2016): micrographs of embryos expressing PoloGFP (*green* in the merge channel) injected with WT or T200A Sas-4-mKate2-mRNA (*red* in the merge channel). The phenotype of Sas-4-T200A-mKate2 is similar to that of Ana1-ALL-mKate2 on PoloGFP.

Arrowheads indicate centrosomes that have both PoloGFP and the corresponding mKate2 fusion protein. *Arrows* indicate centrosomes that are only visible in the red channel (i.e. have lost PoloGFP).

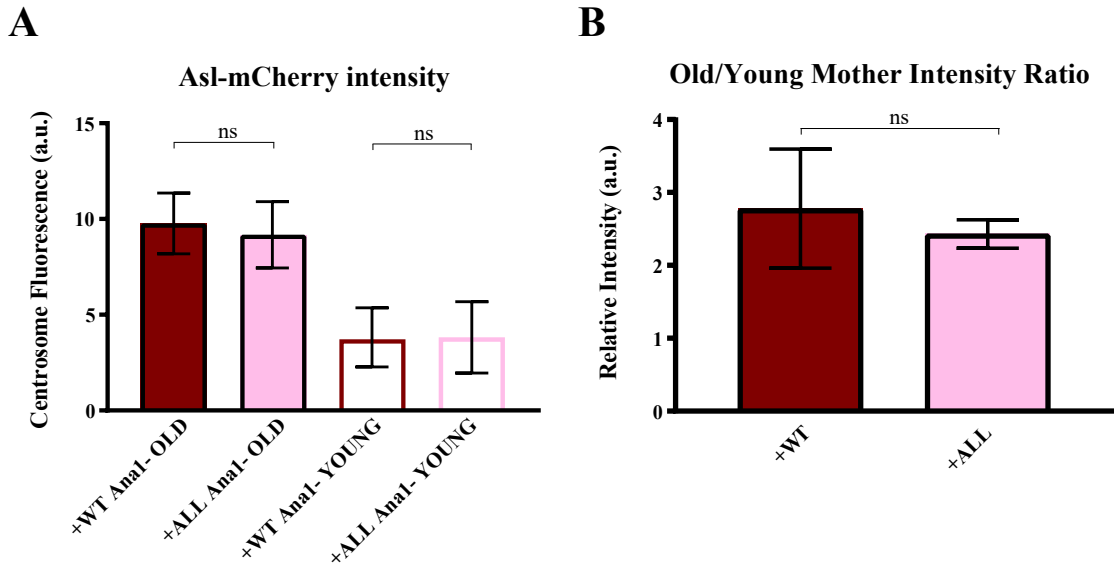


Figure 3.10 – Asl-mCherry is unaffected if all Ana1 ptPBSs are mutated.

(A) Graph shows mean Asl-mCherry intensity above background for embryos injected with WT or ALL Ana1-GFP mRNA. Five embryos were analysed per construct injected, and 10 pairs of centrosomes were analysed per embryo. For each pair, the centrosome with the highest mean Asl-mCherry intensity was considered to be the old mother, and the other centrosome was considered to be the young mother, because Asl-mCherry is known to preferentially associate with older centrosomes (Novak et al., 2014). Therefore, for statistical analysis I compared the mean intensity of Asl-mCherry at old mother centrosomes (WT vs ALL, *filled* boxes) and young mother centrosomes (WT vs ALL, *white* boxes) separately. (B) For every embryo analysed I calculated the old/young mother ratio: average intensity of the Asl-mCherry signal from old mother centrosomes divided by the average intensity of the signal from young mother centrosomes. The resulting values were averaged to calculate a mean per condition (injected construct), and the unpaired t-test was used to assess if the differences are statistically significant. ns = non-statistically significant difference, p -value > 0.05. Error bars represent SD.

Knowing how important Polo is for correct PCM assembly, I then tested the effects of mutating all the ptPBSs in Ana1 on Spd-2-mCherry distribution at the centrosome.

Unlike previous examples of radial profiling, I analysed the five brightest centrosomes and their corresponding, fainter, centrosomes in the same pair. This was done to account for the difference in effect observed within centrosome pairs in some of the PoloGFP embryos injected with Ana1-ALL-mKate2. However, both “bright” and “faint” centrosomes that incorporated Ana1-ALL-GFP presented similar, major contractions of the Spd-2-mCherry scaffold and a decrease in peak Spd-2-mCherry levels, all differences being highly statistically significant (Figure 3.11). This suggests that PoloGFP recruitment, Spd-2 scaffold formation and (potentially) mitotic PCM expansion are impaired in embryos injected with Ana1-ALL-GFP mRNA.

3.2.5. PoloGFP recruitment to the centrosome is unaffected if all Asl ptPBSs are mutated

Next, I tested Polo recruitment by Asl, which is specifically phosphorylated at Thr373 at the centrosome and not in the cytoplasm (Baumbach, 2014). This site is part of an STP motif, so it is both a ptPBS and a potential CDK1 phosphorylation target. It is also somewhat well conserved amongst *Drosophila* species, making it a prime candidate for Polo recruitment. However, expression of a S372T Asl mutant had no effect on PoloGFP recruitment (Figure 3.12A,Di,Diii).

I then mutated all six ptPBSs present in Asl, but neither PoloGFP nor Asl-mKate2 distribution were affected (Figure 3.13A,C,Ei,Eii). Although there was a decrease in the peak Asl-mKate2 levels, which had also been observed for the S372T mutant (Figure 3.12C,Div and Figure 3.13D,Eiv); peak PoloGFP levels appeared to increase

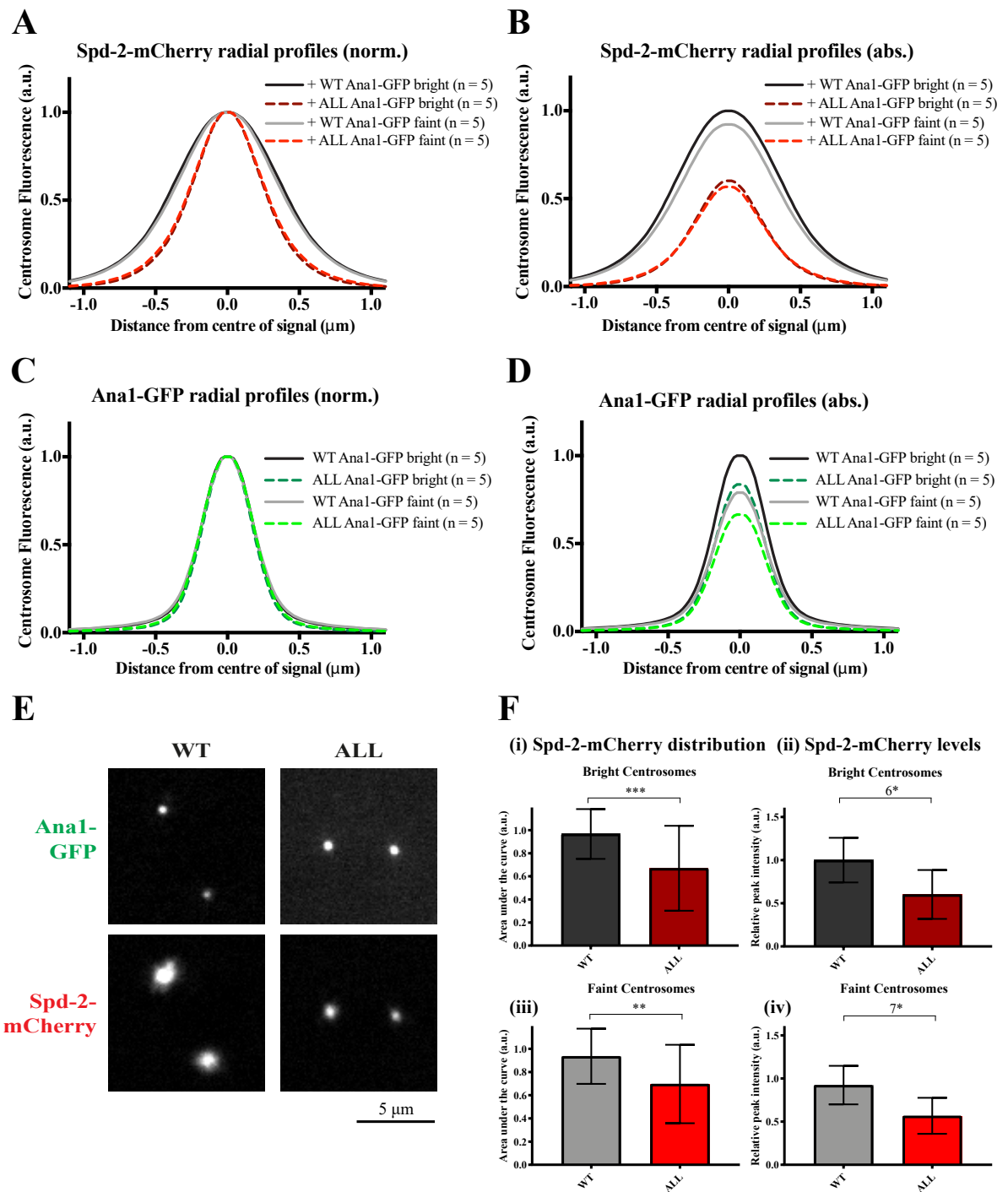


Figure 3.11 – Spd-2-mCherry distribution at the centrosome is strongly disturbed if all Ana1 ptPBSs are mutated.

(A) Normalised Spd-2-mCherry radial profiles after expression of WT or ALL Ana1-GFP. Unlike other instances of analysis using radial profiling, I analysed 5 pairs of centrosomes per embryo. The data was divided into 2 subsets per construct injected: “bright” (brightest centrosomes from each pair, as judged from the corresponding Ana1-GFP signal) and “faint”. (B) Spd-2-mCherry absolute radial profiles. (C) Normalised radial profiles for WT and ALL Ana1-GFP proteins. (D) Absolute radial profiles of the different Ana1-GFP proteins. (E) Micrographs show a representative centrosome pair per construct injected; the collapse of the Spd-2-mCherry signal to the centre of the centrosome is clear in the embryos expressing Ana1-ALL-GFP. (F) Parameters extracted from the normalised and absolute Spd-2-mCherry radial profiles: area under the curve (i) and average peak intensity (ii) from “bright” centrosomes, and area under the curve (iii) and average peak intensity (iv) from “faint” centrosomes. ** = 0.01 > p -value > 0.005; *** = 0.005 > p -value > 0.001; 6* = p -value in 10^{-6} (E-6) range; 7* = p -value in E-7 range. n = number of embryos. Error bars represent SD.

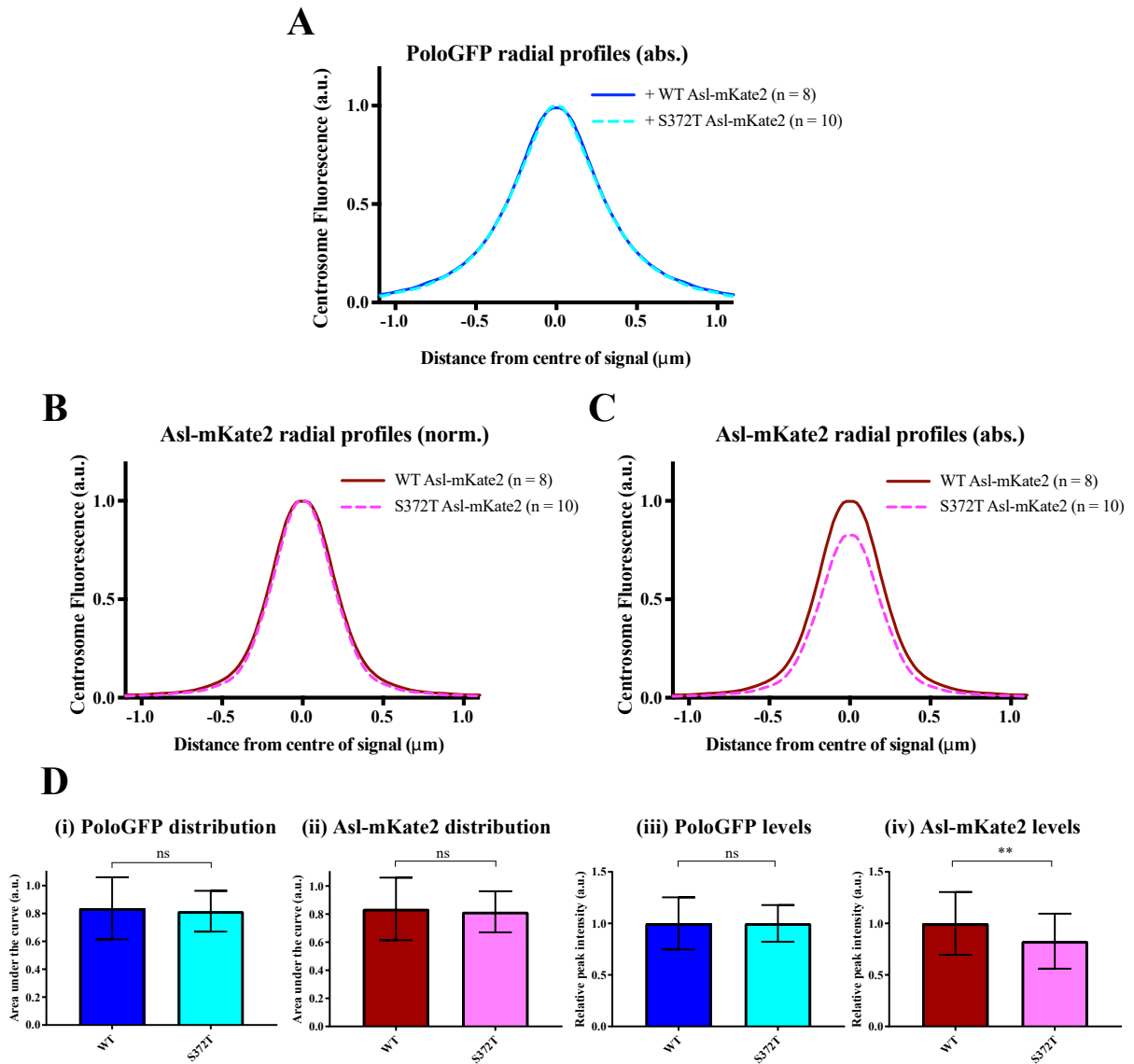


Figure 3.12 – Asl Thr373-STP motif is not necessary for PoloGFP recruitment.

(A) PoloGFP absolute radial profiles after expression of WT or mutant Asl-mKate2. The normalised profiles are not shown because peak intensities are very similar for both conditions. (B) Normalised radial profiles for WT and mutant Asl-mKate2 proteins. (C) Absolute radial profiles of the different Asl-mKate2 proteins. (D) Parameters extracted from the radial profiles: area under the curve from PoloGFP (i) and Asl-mKate2 (ii) normalised profiles, average peak intensity from PoloGFP (iii) and Asl-mKate2 (iv) absolute profiles. ns = non-statistically significant difference, p -value > 0.05 ; ** = $0.01 > p$ -value > 0.005 . 5 centrosomes analysed per embryo, n = number of embryos. Error bars represent SD.

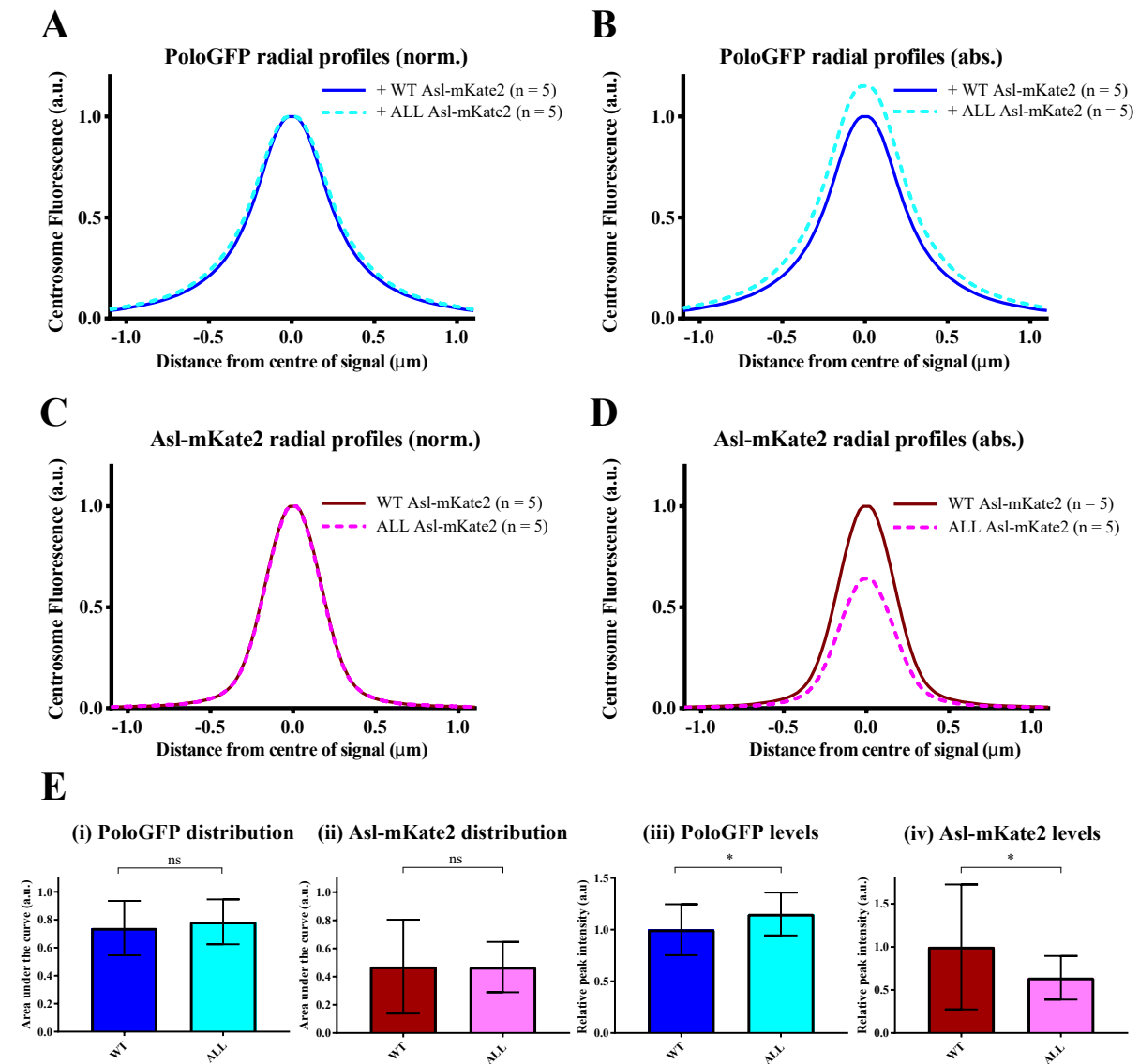


Figure 3.13 – PoloGFP recruitment is unaffected if all Asl ptPBSs are mutated.

(A) Normalised PoloGFP radial profiles after expression of WT or ALL Asl-mKate2. (B) PoloGFP absolute radial profiles. (C) Normalised radial profiles for WT and ALL Asl-mKate2 proteins. (D) Absolute radial profiles of the different Asl-mKate2 proteins. (E) Parameters extracted from the radial profiles: area under the curve from PoloGFP (i) and Asl-mKate2 (ii) normalised profiles, average peak intensity from PoloGFP (iii) and Asl-mKate2 (iv) absolute profiles. ns = non-statistically significant difference, p -value > 0.05 ; * = $0.05 > p$ -value > 0.01 . 5 centrosomes analysed per embryo, n = number of embryos. Error bars represent SD.

after expression of Asl-ALL-mKate2 (Figure 3.13B,Eiii). However, in all cases the difference in peak levels was barely statistically significant (p -value ≥ 0.02) and the effect sizes were small, so these changes might not be biologically relevant. Thus, it seems that the ptPBSs in Asl are not required for Polo recruitment to the centrosome.

3.2.6. PoloGFP recruitment to the centrosome is unaffected if all Cep135 ptPBSs are mutated

Finally, I tested Cep135, mutating all 10 ptPBSs present in this protein. I observed no changes in PoloGFP or Cep135-ALL-mKate2 distribution. There was a very small, non-statistically significant decrease in Cep135-ALL-mKate2 peak levels, and a very small decrease in PoloGFP levels. The latter was statistically significant but did not clear the “biological relevance” criteria (0.032 to 0.24 95% CI, p -value = 0.011) (Figure 3.14), so I conclude that Cep135 does not have a major role in directly recruiting Polo to centrosomes.

3.2.7. The presence of ptPBSs is potentially an evolutionarily conserved feature of centrosomal proteins

For the *in silico* design of the mutants used in the mRNA assay I had to search the amino acid sequences of my candidate centrosomal proteins looking for ptPBSs. I decided that this bioinformatics analysis could be exploited to complement the results for the mRNA assay. Using what is thought to be the major expressed isoform of each protein from *Drosophila melanogaster*, I quantified the total number of ptPBSs and the number of conserved ptPBSs (here defined as being present in at least 11 of the 12 *Drosophila* species whose sequence I analysed) for every candidate protein I screened, as well as Cnn and Plp—two large PCM proteins implicated in Polo-regulated aspects of centrosome biogenesis (Conduit et al., 2014a; Feng et al., 2017; Lee and Rhee,

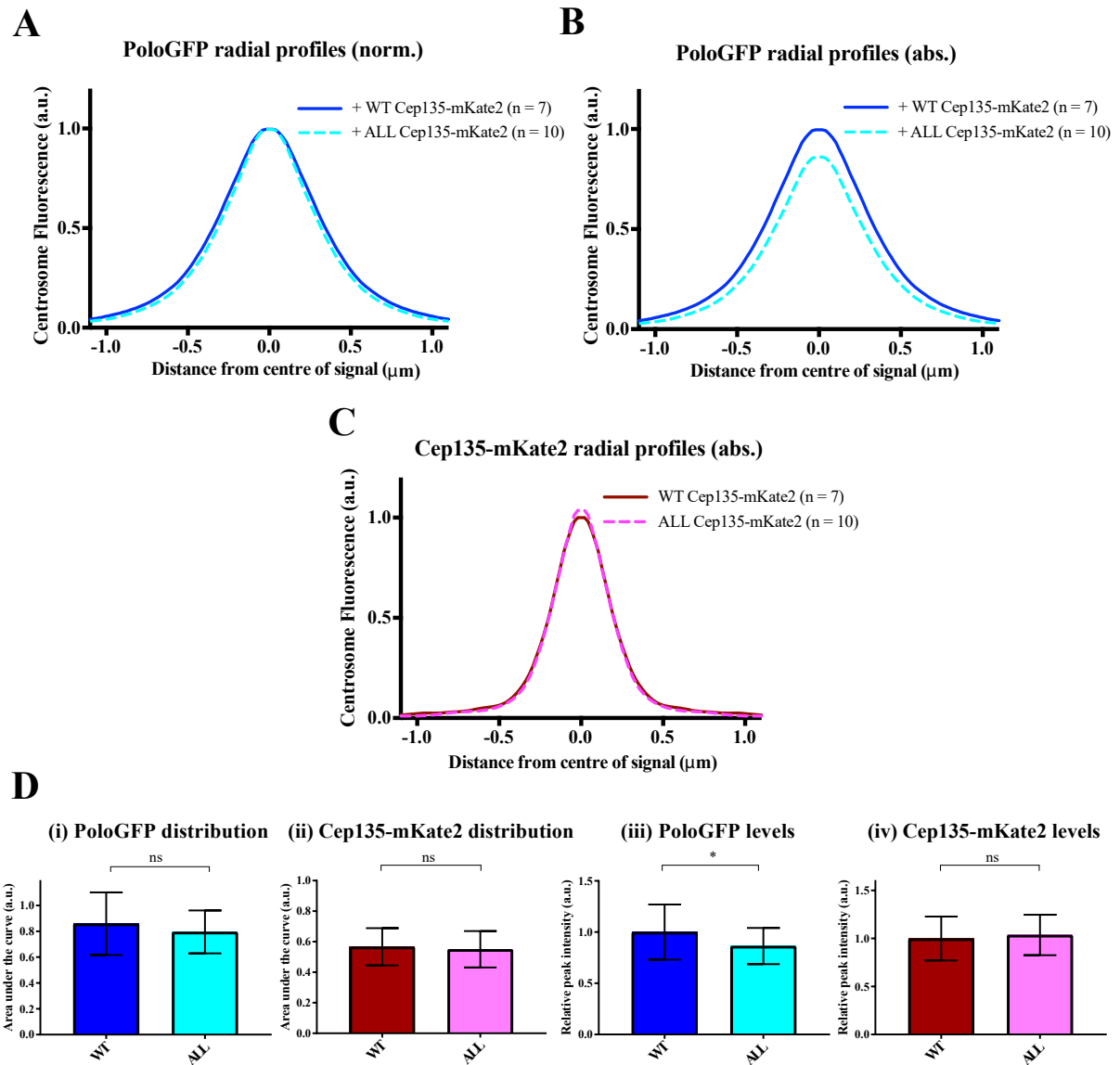


Figure 3.14 – PoloGFP recruitment is unaffected if all Cep135 ptPBSs are mutated.

(A) Normalised PoloGFP radial profiles after expression of WT or ALL Cep135-mKate2. (B) PoloGFP absolute radial profiles. (C) Absolute radial profiles for WT and ALL Cep135-mKate2 proteins, normalised profiles are not shown because WT and ALL peak intensity levels are very similar. (D) Parameters extracted from the radial profiles: area under the curve from PoloGFP (i) and Cep135-mKate2 (ii) normalised profiles, average peak intensity from PoloGFP (iii) and Cep135-mKate2 (iv) absolute profiles. ns = non-statistically significant difference, p -value > 0.05; * = $0.05 > p$ -value > 0.01. 5 centrosomes analysed per embryo, n = number of embryos. Error bars represent SD.

2011; Roque et al., 2018) (see 1.3). I then “normalised” these results to the total number of amino acids in each protein. The final readout is referred to as a ptPBSs/aa ratio and indicates how many total ptPBSs or conserved ptPBSs are found on average in a 100 amino acid stretch of each protein. Interestingly, the two positive hits from the mRNA injection assay (Spd-2 and Ana1) also have the highest total and conserved ptPBSs/aa ratios (Figure 3.15). I reasoned, therefore, that Spd-2 and Ana1 may function as important scaffolds to recruit Polo to centrosomes via their ptPBSs, thus providing an evolutionary pressure for these proteins to maintain a large number of these motifs. I further investigate the possibility that these proteins serve as important scaffolds for Polo recruitment in Chapters 4 and 5. Plp and Sas-4 take the third and fourth positions, with Plp having a larger total ptPBSs/aa ratio but Sas-4 having a larger proportion of conserved ptPBSs.

Although Spd-2 and Cnn have similar number of amino acids (1146 aa and 1148 aa, respectively), similar localisation at the centrosome (the PCM) and presumably similar functions (scaffold formation), Spd-2 has >5 times more conserved ptPBSs than Cnn. I was struck by this difference, so I decided to look at whether the difference in the number and conservation of ptPBSs between Spd-2 and Cnn is true also for the *C. elegans* and human homologues of these two proteins (Figure 3.16). Human Cep192 has two isoforms (one of which includes an N-terminal section that is not present in *D. melanogaster* Spd-2), and overall the human and fly homologues of both proteins share limited amino acid identity. However, the difference in conserved ptPBSs/aa ratio between human Cep192 (1941aa) and Cep215/Cdk5Rap2 (1893aa) is strikingly similar to that between Spd-2 and Cnn. Conversely, *C. elegans* SPD-5 presents more conserved ptPBSs per 100 amino acids, and the difference in total and conserved ptPBSs/aa between SPD-2 and SPD-5 is much lower than for the fly and

A

Protein	Length (aa)	Number of ptPBSs	Number of conserved ptPBSs	Total (ptPBS/aa)*100 ratio	Conserved (ptPBS/aa)*100 ratio
Sas-4	901	11	7	1.220865705	0.776914539
Asl	994	6	0	0.60362173	0
Cep135	1059	10	2	0.944287063	0.188857413
Ana1	1729	34	14	1.966454598	0.809716599
Spd-2	1146	34	16	2.966841187	1.396160558
Cnn	1148	14	3	1.219512195	0.261324042
Plp	2895	41	14	1.416234888	0.483592401

B

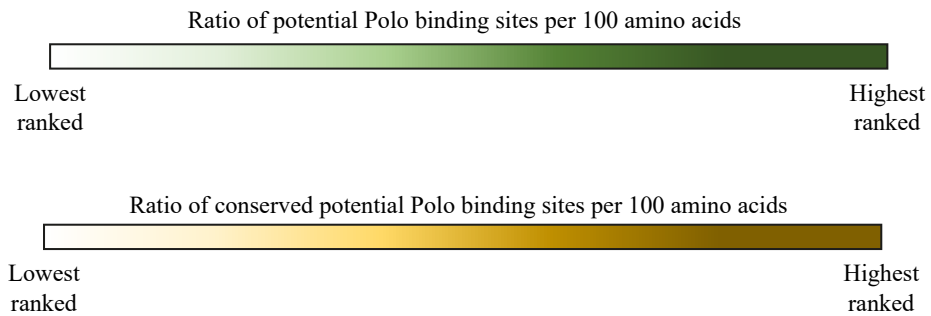


Figure 3.15 – Spd-2 and Ana1 have many ptPBSs and conserved ptPBSs compared to other centrosomal proteins.

(A) Table comparing several different centrosomal proteins on the basis of their protein sequence length (expressed as number of amino acids, aa), total number of potential Polo binding sites (ptPBSs) found in their sequence (i.e. any Ser-Ser/Thr motif), and number of ptPBSs which are conserved amongst at least 11 of 12 *Drosophila* species included in this analysis. The number of total or conserved ptPBSs was divided by the total number of amino acids of the protein and multiplied by 100 to calculate the ratio of ptPBSs per 100 aa (*green* column) and the ratio of conserved ptPBSs per 100 aa (*yellow* column). (B) Colour schemes used to highlight the highest four ratios for each column in (A).

A

Protein	Species	Length (aa)	Number of ptPBSs	Number of conserved ptPBSs	Total (ptPBS/aa)*100 ratio	Conserved (ptPBS/aa)*100 ratio
Spd-2	<i>H. sapiens</i>	1941	37	16	1.9062339	0.824317362
	<i>H. sapiens</i> (i.3)	2537	46	19.5	1.813165156	0.768624359
	<i>D. melanogaster</i>	1146	34	16	2.966841187	1.396160558
	<i>C. elegans</i>	824	27	11	3.276699029	1.334951456
Cnn	<i>H. sapiens</i>	1893	18	3	0.950871632	0.158478605
	<i>D. melanogaster</i>	1148	14	3	1.219512195	0.261324042
	<i>C. elegans</i>	1198	22	7	1.83639399	0.584307179

Spd-2/Cnn	Total ptPBSs ratio	Conserved ptPBSs ratio
<i>H. sapiens</i>	2.004722652	5.201442555
<i>H. sapiens</i> (i.3)	1.906845355	4.850019708
<i>D. melanogaster</i>	2.432809773	5.333333333
<i>C. elegans</i>	1.784311562	2.284674064

B

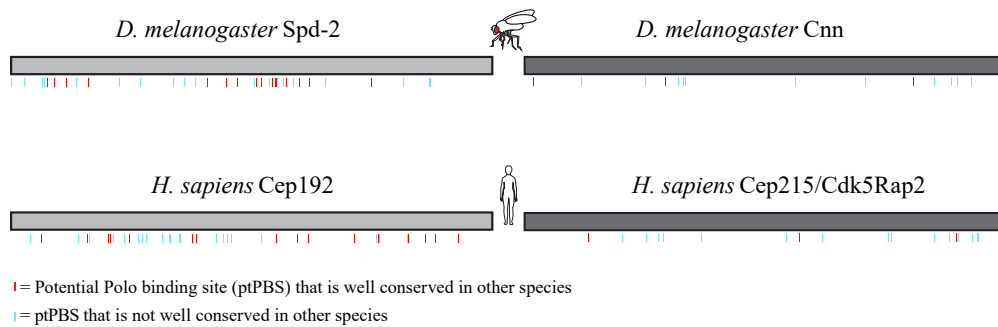


Figure 3.16 – The number and conservation of ptPBSs in Spd-2 and Cnn is potentially an evolutionarily conserved feature.

(A) Tables comparing Spd-2, Cnn, and their corresponding homologues in *H. sapiens* and *C. elegans* on the basis of their protein sequence length (expressed as number of amino acids, aa), total number of ptPBSs found in their sequence, and number of ptPBSs which are conserved amongst at least all but one of the species included in each subset analysed (*Drosophila* species for *D. melanogaster* sequences, mammalian species for *H. sapiens* sequences, worm species for *C. elegans* sequences). The number of total or conserved ptPBSs was divided by the total number of amino acids of the protein and multiplied by 100 to calculate the ratio of total or conserved ptPBSs per 100 aa, as required. In the case of the human Spd-2 homologue (Cep192), I included both isoforms 1 and 3 (referred to as i.3) in the analysis. The total or conserved ptPBSs/aa ratio for Spd-2 (or its homologues) is then divided by the corresponding ratio for Cnn (or its homologues) to calculate a final ratio which indicates how many more ptPBSs or conserved ptPBSs there are in Spd-2 compared to Cnn. The red arrows indicate an example: *D. melanogaster* Spd-2 and Cnn have a conserved ptPBSs/aa ratio of 1.396 and 0.261, respectively. Hence, *D. melanogaster* Spd-2 has more than 5 times more conserved ptPBSs than Cnn ($1.396/0.261=5.334$).

(B) Schematic representation of the amino acid sequences of *D. melanogaster* Spd-2 and *H. sapiens* Cep192 (isoform 1) (light grey), and *D. melanogaster* Cnn and *H. sapiens* Cep215/Cdk5Rap2 (dark grey). Because these proteins have different numbers of amino acids, the cartoons have been normalised to the same length for ease of comparison. The position of each ptPBS is indicated with lines underneath each cartoon: red lines indicate ptPBSs that are conserved in at least 11/12 *Drosophila* species or 8/9 mammalian species; light blue lines indicate sites that are less well conserved. Note that the fly and human homologues of Spd-2/Cep192 and Cnn/Cep215 share little sequence identity (~23% and ~24%, respectively), yet the number and overall distribution of ptPBSs is quite similar.

human proteins. This could help explain some differences in behaviour between SPD-5 and Cnn (see 3.3 Discussion and Chapter 4).

3.3. Discussion

In this Chapter I have presented the results of an mRNA-based screen for proteins that have a role in recruiting Polo to centrosomes. Two proteins clearly stand out from my screen: Spd-2 and Ana1. The role of these proteins in Polo recruitment will be further interrogated in Chapters 4 (Spd-2) and 5 (Ana1) of this thesis.

In the case of Spd-2, PoloGFP levels were significantly reduced at centrosomes, and its radial profile narrowed, becoming more similar to the radial profile of centriolar proteins like Asl, than that of PCM proteins like Spd-2. It seems likely that several ptPBSs within Spd-2 are required for Polo recruitment, as I observed some evidence of an additive effect of mutating all the ptPBSs compared to mutating only those sites conserved amongst *Drosophila* species. It is therefore not surprising that mutating only Thr516 and Ser625 had no effect on PoloGFP distribution. Moreover, as these two sites are the only conserved ptPBSs that are also potential CDK1 phosphorylation targets, this observation suggests that it is not essential that Spd-2 is “primed” to recruit Polo by CDK1 phosphorylation—although such priming by CDK1 has been observed for several other proteins that recruit PLK1 (Barr et al., 2004; Decker et al., 2011; Elia et al., 2003a; Elowe et al., 2007; Linder et al., 2017; Novak et al., 2016; Preisinger et al., 2005; Qi et al., 2006; Soung et al., 2009; Wang et al., 2013; Watanabe et al., 2005; Yamaguchi et al., 2005; Zhang et al., 2009).

A surprising observation from the injection of Spd-2-ALL-mKate2 and Spd-2-CONS-mKate2 mRNA is that the mutant proteins are recruited to the centrosome at much higher levels than WT Spd-2-mKate2. If the Spd-2 mutants were misfolded, it would

be expected that their recruitment to the centrosome is impaired, rather than they are overly recruited. Indeed, none of the mutations in any of the candidate proteins tested in this assay resulted in changes in protein distribution at the centrosome, and only a few resulted in a small decrease in overall recruitment to the centrosome. Another possibility is that multiple centrosomes might have been measured at once in the Spd-2 mutant-expressing embryos. Although centrosomes still disengage in embryos expressing the Spd-2 mutants, they often fail to move away from one another and remain clustered, possibly due to PCM defects and lack of MT nucleation. I tried to avoid including any clusters in my analyses, but perhaps some clusters were compact enough to appear as a single spot when imaged. In that case, because multiple centrosomes would have been measured together, peak intensity measurements would have been overestimated. However, this should also have resulted in a broader distribution profile, which was not observed. Alternatively, it has previously been suggested that the protein levels of SPD-2 are tightly regulated in worms embryo (Decker et al., 2011), so perhaps this control is lost in the mutant proteins and they are simply over-recruited relative to the WT protein. However, my subsequent experiments with transgenic lines expressing these proteins suggest that this is not the case, and these experiments did not recapitulate the excessive recruitment of the mutant proteins to the centrosome (Chapter 4). Thus, this phenomenon currently remains unexplained.

In the case of Ana1, mutation of all the ptPBSs resulted in the near complete loss of PoloGFP, but usually only from one centrosome in each separating pair. This asymmetry of effect, which leads to some centrosomes being a lot more affected than others, was also observed in the embryos injected with Sas-4-T200A-mKate2 mRNA (Novak et al., 2016), and it is probably due to the incorporation dynamics of these two

proteins. Sas-4 is irreversibly incorporated into daughter centrioles as they are built, although some “excess” is lost during mitosis (Novak et al., 2014). Similarly, FRAP experiments have shown that the incorporation profile of Ana1-GFP is very similar between bleached and non-bleached centrosomes, suggesting that Ana1 is also irreversibly incorporated into centrioles (Saurya et al., 2016). Thus, in the mRNA injection experiments, older centrosomes would have incorporated and retained more of the endogenous (WT) Ana1, and would therefore be less affected than young centrosomes, which would have incorporated mostly Ana1-ALL-mKate2 and are more likely to exhibit any mutant phenotype. However, if this were the full picture, one would expect to find a few, very old centrosomes (marked by PoloGFP) with no or minimal Ana1-mKate2 (WT or ALL). Furthermore, there were some embryos injected with Ana1-ALL-mKate2 where all the visible centrosomes had lost PoloGFP, not just one from each pair. This could be due to injecting into very young embryos a slightly variable amount of mRNA, with embryos receiving more mRNA showing the stronger effects. However, it could also be that some fraction of incorporated Ana1 can be exchanged, either between the mother and daughter centrioles before disengagement, or between a mature centriole and the cytoplasm if the concentration of cytoplasmic Ana1 is particularly high (as it might be in mRNA injection experiments).

The total loss-of-Polo phenotype observed with Ana1-ALL-mKate2 is similar to that reported in (Novak et al., 2016) when injecting Sas-4-T200A-mKate2 (Figure 3.9). However, whereas loss of Polo recruitment by Sas-4-T200A lead to loss of Asl recruitment during centriole-to-centrosome conversion, Asl recruitment and maintenance are seemingly unaffected in embryos injected with Ana1-ALL-mKate2 mRNA. Indeed, both Spd-2- and Ana1 appear to be involved only in the Polo-mediated regulation of PCM assembly, and none of the mutants tested in this screen

disturbed the other two main Polo-regulated events at the centrosome: centriole conversion and disengagement.

This negative result is in itself interesting because one of the mutants tested was Sas-4-10S/T, which has all ptPBSs mutated except for the Thr200-STP site. Expression of this mutant resulted in a small, but statistically significant, decrease in peak PoloGFP intensity. However, given the small effect size and the large sample number, it is possible that this difference appears to be statistically significant but it is not really biologically relevant. Independently of whether the resulting *p*-value clears the chosen threshold or not, centrosomes that incorporated Sas-4-10S/T-mKate2 appear to duplicate correctly and none showed signs of PCM defects. Thus, I conclude that only the Thr200-STP motif is crucial for Sas-4-mediated recruitment of Polo during centriole conversion. The small loss of PoloGFP observed in embryos injected with Sas-4-10S/T-mKate2 mRNA could be due to experimental variability, or due to a slight deleterious effect of the mutations on Sas-4 function. Similarly, I excluded Cep135 and Asl as key Polo recruiters, and the role (if any) of potential CDK1-mediated phosphorylation of the Asl Thr373-STP motif remains a mystery.

As for centriole disengagement, a clear candidate to recruit Polo is Plp (Pericentrin-like protein). In *Drosophila* sensory organ precursor cells, loss of Plp leads to premature centriole disengagement (Roque et al., 2018), and in human cultured cells, pericentrin phosphorylation by PLK1 has been proposed to promote centriole disengagement (Kim et al., 2015; Lee and Rhee, 2012). Additionally, in a bioinformatic analysis performed in parallel to the mRNA injection screen, Plp appears to have a moderately high ratio of total and conserved ptPBSs, similar to that of Sas-4 (Figure 3.15). While this bioinformatic analysis is quite simplistic and does not

account for differences in evolutionary constraints between the different proteins, it is in very good agreement with the results from the mRNA injection assay, with Spd-2 and Ana1 scoring the highest ratios. It is also in agreement with previous reports that PLK1 PBD ligands identified via MS are enriched in Ser-Ser/Thr-Pro motifs (Lowery et al., 2007). Plp is such a large protein (2895 amino acids) that mRNA injection could not be used to test if its ptPBSs are important for centriole disengagement and/or Polo recruitment. However, this is an important question that should be addressed in the future.

A final observation to arise from the combination of the mRNA injection assay and the bioinformatics analysis is that Spd-2 is a crucial Polo recruiter with many ptPBSs, whereas Cnn has remarkably fewer ptPBSs. Not only do Spd-2 and Cnn differ in their number of ptPBSs and the conservation of such sites across closely related species, but this difference appears to be a conserved feature between mammalian and *Drosophila* species, even if the proteins themselves are not very well conserved. While it is not definitive evidence, this would suggest that there is a “division of labour” between these two proteins, with Spd-2 acting as an intermediate between Polo and Cnn to allow for extra regulation of Cnn scaffold formation. This idea is further explored in Chapter 4 of this thesis. Moreover, it is potentially interesting that the asymmetry in number and conservation of ptPBSs is not so clear between the *C. elegans* homologues SPD-2 and SPD-5. The PCM presents slightly different behaviours in flies and worms (reviewed in (Woodruff, 2018)) and mathematical modelling has described *C. elegans* centrosomes as “autocatalytic” (Zwicker et al., 2014), a property that is now attributed to SPD-5 because this protein can self-aggregate into condensates *in vitro* (Woodruff et al., 2015), but such a property has not yet been observed for Cnn (further discussed

in Chapter 4). In the future, it would be interesting to test if expression of a Cnn mutant with extra ptPBSs can bypass the need for Spd-2-mediated Polo recruitment.

While this study has been limited in scope due to time constraints, it could be expanded in the future to include other proteins in centrosomes, such as Cnn, cartwheel proteins (Sas-6, Ana2), proteins whose role is still unclear (e.g. Ana3), and/or other centrosomal proteins with a high ptPBSs/aa ratio. It would be particularly interesting to test the effects of mutating ptPBSs in proteins that locate to the distal end of the centrosome, such as Cep97, CP110 and Klp10A. These proteins have recently been identified in a PLK1 immunoprecipitation/MS experiment (K. Legg and F. Esashi, personal communication), and they show a complex oscillatory behaviour which interacts with the PLK4 homeostatic clock, although the mechanism by which this happens is still unclear (L. Hankins and M. Aydogan, unpublished).

Finally, a similar approach could also be used to study Polo recruitment to other subcellular structures. A concern when devising this strategy was that, although Ser-Thr is a conservative substitution, making so many mutations might simply lead to protein misfolding. However, my results suggest that misfolding is not a major problem as almost all the mutant proteins localised to the centrosome in a manner that was very similar to the WT (indicating that they are sufficiently folded that they can bind to the interacting partners that recruit and maintain these proteins at centrosomes). Since Polo is recruited to a large number of sites during mitosis, a “scorched earth” approach similar to the one taken here (mutating all ptPBSs so that the candidate proteins cannot recruit Polo via canonical PBD binding) may be a very effective way of testing the relative roles of different proteins in recruiting Polo to particular sites.

Chapter 4 - Spd-2 recruitment of Polo kinase drives centrosome maturation in fly embryos

4.1. Introduction

4.1.1. Spd-2 as a potential Polo recruiter

In Chapter 3, I observed a strong loss of PoloGFP from centrosomes in embryos expressing two different mutant forms of Spd-2 in which binding to Polo should be impaired (ALL and CONS), suggesting that Spd-2 is involved in Polo recruitment to the centrosome in fly embryos.

As mentioned previously, an interaction had already been reported between the homologues of these two proteins in worms and vertebrates (Decker et al., 2011; Joukov et al., 2014; Meng et al., 2015). Joukov et al. reported that Cep192 and Plx1 (homologues of Spd-2 and Polo, respectively) co-immunoprecipitate in experiments with *Xenopus* egg extracts. While an indirect interaction cannot be excluded (as other centrosomal proteins are also bound to the beads), mutation of a specific ptPBS in Cep192 (T46A) resulted in less Plx1 being co-eluted. An interaction between recombinant Plx1 and Cep192 was also observed *in vitro* upon addition of recombinant Aurora A, but this was not disrupted by the T46A mutation. While Joukov et al. did not report whether expression of Cep192-T46A affected Plx1 recruitment to *Xenopus* centrosomes, they did observe a partial reduction in PLK1 localisation to centrosomes in HeLa cells expressing Cep192-T46A upon depletion of endogenous Cep192 (Joukov et al., 2014). Additionally, it was found that a second site in human Cep192 (Ser994-Ser995) which could bind PLK1 in immunoprecipitation and *in vitro* assays,

but mutation of both sites still resulted in only partial loss of PLK1 from centrosomes *in vivo* (Meng et al., 2015).

Finally, the *C. elegans* homologues SPD-2 also has one site (Thr233-STP motif) which binds the PBD *in vitro*. Importantly, expression of a SPD-2 S232T/T233A mutant results in strong loss of centrosomal PLK-1 and centrosome growth defects *in vivo*, as well as loss of other pericentriolar proteins such as SPD-5 (Decker et al., 2011).

4.1.2. Different models to explain mitotic PCM expansion

The above data strongly suggests that both PLK-1 and SPD-2 are key factors for mitotic PCM assembly in worm embryos (Decker et al., 2011). This is in good agreement with data from fly embryos and S2 cells (Conduit et al., 2014a, 2014b, 2015a; Dobbelaere et al., 2008; Feng et al., 2017). For example, the amount of centrosomal Cnn is reduced by 80% in fly eggs that lack Spd-2. At the same time, Spd-2 recovery after photobleaching was unaffected in embryos lacking Cnn (so Cnn might not be required for Spd-2 recruitment); but overall centrosomal Spd-2 levels were lower in these embryos, suggesting that Cnn is required for maintenance of Spd-2 at the PCM (Conduit et al., 2014b). As described in Chapter 1 (1.3.1), Cnn scaffold formation *in vivo* is also absolutely dependent on phosphorylation by Polo (Conduit et al., 2014a). Thus, it has been proposed that Polo, Spd-2 and Cnn cooperate to drive mitotic PCM expansion (Conduit et al., 2014b, 2014a, 2015a; Raff, 2019).

However, recent studies have proposed alternative models in which, although SPD-2 and PLK-1 are essential for PCM expansion *in vivo*, Cnn/SPD-5 is the main PCM organiser, at least in worm embryos (reviewed in Woodruff, 2018; Raff, 2019).

Purified full-length SPD-5 can aggregate *in vitro* into large assemblies (referred to as “condensates”) that appear to have transient liquid-like properties, although they

rapidly harden into gel-like structures. While PLK-1 and SPD-2 enhance SPD-5 condensate formation (Woodruff et al., 2015, 2017), PLK-1 localisation to SPD-5 condensates does not require SPD-2, and *vice versa* (Woodruff et al., 2015). In this model, SPD-5 would serve as the true scaffold and PLK-1 and SPD-2 would be merely “PCM clients” (Woodruff et al., 2017).

During the writing of this thesis, a third model has been put forward based on observations in worm embryos in which centrioles, SPD-2, PLK-1 or AIR-1 (*C. elegans* homologue of Aurora A) were eliminated at different points of the cell cycle using laser ablation and genetic tools. This model proposes that centrioles are not required for SPD-5 scaffold (proxy for PCM) organisation *per se*, but allow the PCM to resist pulling forces from MTs. SPD-2 would be important to trigger initial PCM growth by recruiting PLK-1 to centrioles, but only PLK-1 would be needed for PCM maintenance during mitosis (Cabral et al., 2019).

4.1.3. Spd-2 is phosphorylated specifically at the centrosome

The Spd-2 mutants tested in Chapter 3 were designed based on identification of potential Polo binding sites (ptPBSs) across their sequence: Ser-Ser/Thr motifs. For Polo to bind canonically to a protein via its PBD, this site must be phosphorylated at position 0 (Elia et al., 2003a, 2003b). Indeed, several MS studies have identified 17 different ptPBSs in Spd-2 that appear to be phosphorylated (with different degrees of confidence) (Table 4.1, (Bodenmiller et al., 2007; Habermann et al., 2012; Hu et al., 2018; Zhai et al., 2008)). Furthermore, previous work from P. Conduit has shown that Spd-2 is phosphorylated specifically at centrosomes (Alvarez-Rodrigo et al., 2019). Centrosomal fractions from fly embryo extracts were partially purified by sucrose step-gradient centrifugation and subject to SDS-PAGE and western blotting. As

reported for Cnn (Conduit et al., 2014a), the centrosomal fractions have a slow migrating form of Spd-2 which the cytoplasmic fractions lack, and which disappears upon treatment with phosphatase. However, unlike Cnn, centrosomal fractions also contain a faster migrating form of Spd-2 that appears to be the non-phosphorylated protein that is also present in the cytoplasm (Figure 4.1).

Table 4.1. Potential Polo binding sites in Spd-2 reported to be phosphorylated

Peptide sequence	Phospho sites	Ref.
VFGDL SS FSKGRR	S34, S35	(Zhai et al., 2008)
ALETLEKPRPSRS S QAK	S76	(Bodenmiller et al., 2007)
EKPSSLVAEILK S FVE K	(S156)	(Bodenmiller et al., 2007; Zhai et al., 2008)
SSSS	(S185)	(Hu et al., 2018)
SENIWNIVSN SS PNRSR	S310, S311	(Bodenmiller et al., 2007; Zhai et al., 2008; Hu et al., 2018)
RPP SSS EILSLSAIDK	(S389), (S390), S391	(Bodenmiller et al., 2007; Zhai et al., 2008)
DIDLNSDT ST VEVVNH LWEHGR	(S413), (T414)	(Zhai et al., 2008)
ADTDPVETEAEADIDE WP ST PVKEPSRR	(S515), (T516)	
AASP SSS SDGVRPLTCTE DENDEEDEDKTPVNKK	S538, S539, (S540)	
KA SS LSSTRLDGCDVA VASSTER	S581, S582	
NLSPL SS PR	S617, S618	(Bodenmiller et al., 2007; Zhai et al., 2008; Hu et al., 2018)
SCL SS PLLD ST TSSDRR	S624, S625, (S630), (T631)	(Zhai et al., 2008; Hu et al., 2018)
SCLSSPLLDSTT S DRR	S634	(Habermann et al., 2012)
AN SS PAGSEASSTSGFT ASGR	S650	(Bodenmiller et al., 2007)
KANSSPAGSEAS ST SGF TASGR	(S658)	(Zhai et al., 2008)
RGLGT SS VAVPR	S673, S674	(Zhai et al., 2008; Habermann et al., 2012; Baumbach, 2014)

This table lists several previously identified Spd-2 phospho-peptides that include potential PBD binding sites (ptPBSs, Ser-Ser/Thr), indicating the phospho-proteomic screens in which these peptides were identified. Definitively identified phosphorylated ptPBSs are shown in *red*. Sites listed in brackets appear to be phosphorylated but the scores were low (*blue*); or have not been identified in *Drosophila melanogaster*, but

have been definitively identified in closely related *Drosophila* species (*green*).

4.1.4. Key findings

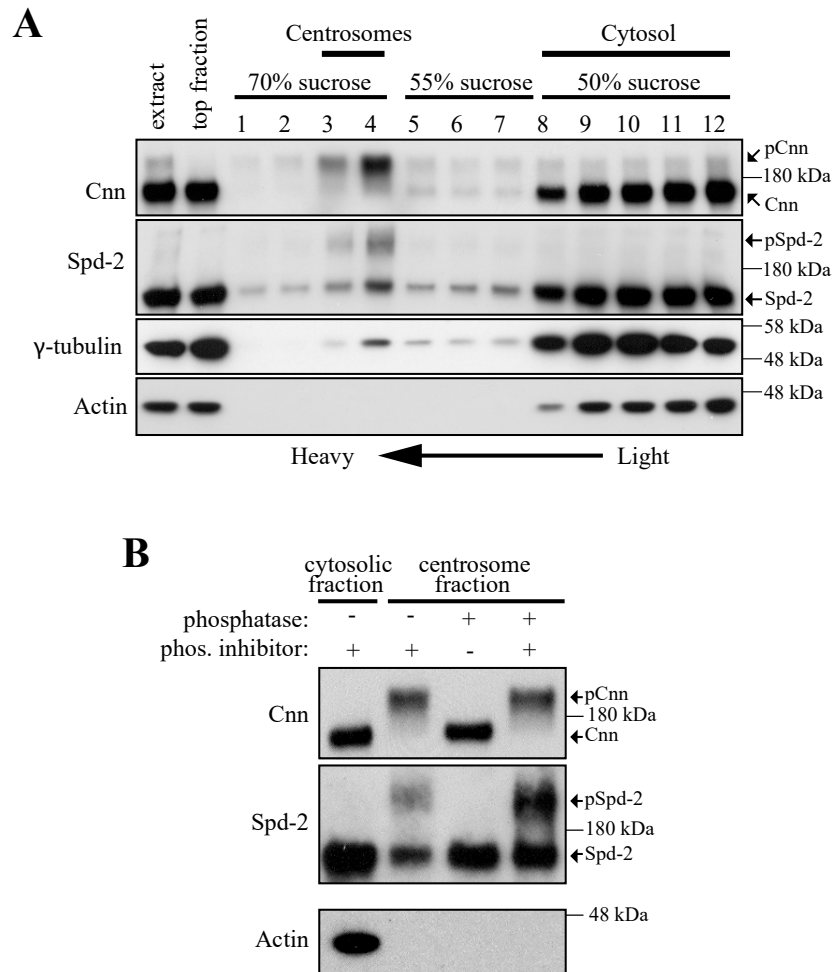
In this Chapter, I have explored in-depth the role of Spd-2 in Polo recruitment to the centrosome. Here I show that the Spd-2 mutants tested in Chapter 3 are largely functional proteins whose binding to the PBD appears to be strongly perturbed. I show that Spd-2 is required to recruit Polo specifically to the PCM. Intriguingly, in the presence of these mutant proteins the Cnn and Spd-2 scaffolds are initially assembled around the mother centriole, but the PCM specifically fails to expand outwards, and centrosome maturation fails. These findings are consistent with the proposal that (at least in fly embryos) Spd-2, Polo and Cnn collaborate to form a positive feedback loop that drives the dramatic expansion of the mitotic PCM. Much of the work presented in this chapter was recently published (Alvarez-Rodrigo et al., 2019).

4.2. Results

4.2.1. Generation and characterization of fly lines expressing Spd-2 mutants

unable to recruit Polo

The results from Chapter 3 revealed a potential role of Spd-2 in recruiting Polo to the centrosome, either directly (via an interaction with the PBD) or indirectly. To confirm and further explore this result, I made transgenic lines expressing either of the two Spd-2 mutant constructs which gave a large loss of Polo from centrosomes: Spd-2-ALL has all 34 ptPBSs present in *D. melanogaster* Spd-2 with the Ser at position -1 mutated to Thr, whereas Spd-2-CONS only has mutated the 16 ptPBSs in Spd-2 which are highly conserved amongst *Drosophila* homologues (Figure 4.2A). The constructs were expressed with a C-terminal fluorescent tag (GFP or mCherry) under the control



(Courtesy of P. Conduit)

Figure 4.1 – Spd-2 is phosphorylated at centrosomes.

(A) Western blot of a sucrose step-gradient purification of centrosomes from embryo extracts blotted against Cnn, Spd-2, γ -tubulin and Actin (as indicated). The step-gradient fractions are labelled 1 (heaviest) to 12 (lightest), and the cytosolic and centrosomal-peak fractions are indicated above the blot. Centrosomal fractions are enriched in Cnn (slow migrating species), Spd-2 (slow and fast migrating species) and γ -tubulin. **(B)** Western blot of centrosomal fractions from the step-gradient that were treated with phosphatase or phosphatase inhibitor (as indicated), and probed with the indicated antibodies; the cytosolic fraction is also shown for reference. Treatment with active phosphatase eliminated the slower migrating species of Cnn and Spd-2. The blots shown here are representative of at least two technical replicates for each of two biological repeats. Data courtesy of P. Conduit. The Cnn and Actin blots have been previously published (Conduit et al., 2014a).

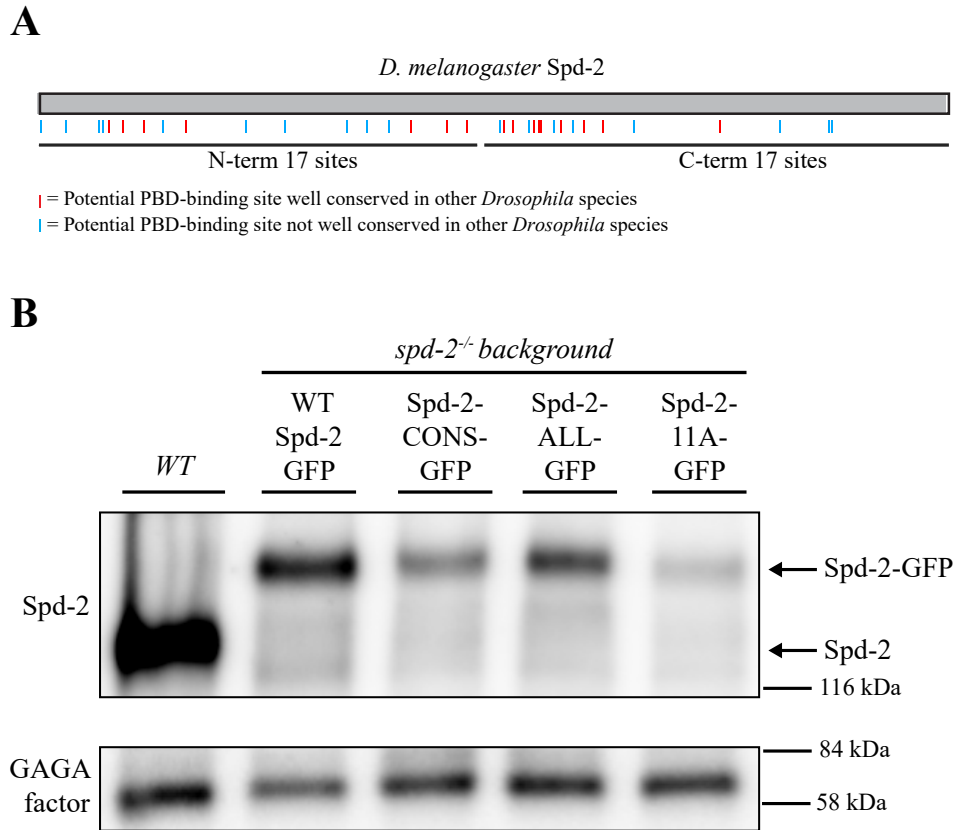


Figure 4.2 – Generating transgenic fly lines expressing Spd-2 that should be unable to bind the PBD.

(A) Schematic representation of *Drosophila melanogaster* Spd-2 indicating ptPBSs (Ser-Ser/Thr) that are either conserved (present in at least 11/12 *Drosophila* species analysed, red lines) or not conserved (blue lines). (B) Western blot of OregonR (*WT*) embryos and *spd-2^{-/-}* embryos expressing either WT Spd-2-GFP, Spd-2-CONS-GFP, Spd-2-ALL-GFP or Spd-2-11A-GFP. The GAGA transcription factor (Raff et al., 1994) was used as a loading control. The WT-ALL-CONS blot is representative of four technical repeats and two different biological repeats. Analysis of Spd-2-11A-GFP protein levels had been previously done by J. Baumbach (Baumbach, 2014), so it was only included in one technical repeat of this blot.

of the *ubiquitin* promoter. A transgenic line expressing WT Spd-2-GFP under the *ubiquitin* promoter already existed in the lab and has been extensively characterised (Dix and Raff, 2007), and I also made a transgenic line expressing WT Spd-2-mCherry under the control of the *ubiquitin* promoter as control. Western blotting revealed that the mutant Spd-2-GFP fusions were expressed at similar, albeit slightly lower, levels than WT Spd-2-GFP in fly embryos (Figure 4.2B).

Spd-2^{-/-} mutant flies are viable and are not uncoordinated, as they have regular numbers of centrioles and cilia. However, mutant females are infertile: embryos laid by these females fail to complete pronuclear fusion because they lack maternally contributed Spd-2 which is required to recruit PCM to the sperm-contributed centriole. Thus, embryos lacking Spd-2 (referred to as *spd-2^{-/-}* embryos) arrest prior to the first mitotic division (Dix and Raff, 2007). To test whether ALL or CONS Spd-2 transgenes could rescue this *spd-2^{-/-}* phenotype, I collected 1-2 h old fertilised embryos from *spd-2^{-/-}* mutant females expressing either WT or mutant Spd-2-GFP transgenes, and stained them to visualise MTs and nuclei. Almost 100% of the mutant embryos rescued with WT Spd-2-GFP developed beyond the first mitotic division. Importantly, both Spd-2-ALL-GFP and Spd-2-CONS-GFP also rescued the defect in pronuclear fusion, although to a slightly lesser extent than the WT fusion protein (Figure 4.3). Thus, Spd-2-ALL-GFP and Spd-2-CONS-GFP retain at least some function and are not simply misfolded (see also below).

However, while *spd-2^{-/-}* embryos expressing either Spd-2-CONS-GFP (hereafter **Spd-2-CONS-GFP embryos**) or Spd-2-ALL-GFP (hereafter **Spd-2-ALL-GFP embryos**) were able to initiate development, they died early in development and almost never hatched as larvae (<1/500 embryos hatching; n > 2000 embryos scored). Interestingly,

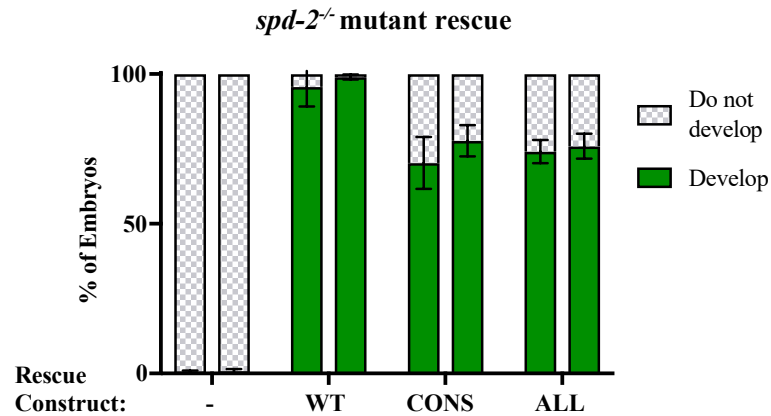


Figure 4.3 – The Spd-2 ptPBS mutants can rescue the initial *spd-2*^{-/-} mutant phenotype.

Bar charts quantify the percentage of *spd-2*^{-/-} embryos that had initiated development after expression of either WT Spd-2-GFP, Spd-2-CONS-GFP or Spd-2-ALL-GFP, as indicated. Development was judged based on number of visible nuclei (stained using DAPI) and MT organisation (stained using an anti- α -tubulin antibody). The chart shows the data from two independent biological repeats (two independent sets of *spd-2*^{-/-} mothers). For each biological repeat, 3 lots of >50 embryos were collected and scored independently; error bars represent the standard deviation (SD).

this resembles the phenotype observed in *cnm*^{-/-} mutant embryos, which commence development but accumulate progressively worse division defects during the syncytial blastoderm stage and eventually fail to cellularise and die (Megraw et al., 1999).

4.2.2. Centrosomes with Spd-2-CONS-GFP or Spd-2-ALL-GFP organise less robust asters of MTs

To investigate if Spd-2-CONS-GFP and Spd-2-ALL-GFP embryos have microtubule (MT) organization defects, I followed the behaviour of MTs using the MT-associated protein Jupiter fused with mCherry, an excellent marker for following MT dynamics *in vivo* (Karpova et al., 2006). As expected, *spd-2*^{-/-} embryos rescued with WT Spd-2-GFP presented robust astral and spindle MT arrays (Figure 4.4A, left panels). Conversely, Spd-2-CONS-GFP and Spd-2-ALL-GFP embryos presented weaker astral MT arrays (Figure 4.4A,B) and centrosomes were often detached from the spindle poles (*arrowheads*, Figure 4.4A). Mitotic spindle abnormalities (*arrows*, Figure 4.4A) were frequent and qualitatively, but reproducibly, more pronounced in Spd-2-ALL-GFP embryos compared to Spd-2-CONS-GFP embryos.

These observation match the results from Chapter 3 that expression of these Spd-2 mutant proteins leads to PCM defects, and that mutating all the ptPBSs present in Spd-2 has a worse phenotype than mutating just the most evolutionarily conserved ptPBSs. However, whereas injection of Spd-2-CONS-mKate2 and Spd-2-ALL-mKate2 mRNA lead to higher centrosomal levels of these proteins compared to WT Spd-2-mKate2 (for unknown reasons), in the transgenic fly lines the mutant proteins were less strongly recruited to centrosomes than WT (Figure 4.4A,C). Note that the reduction in Spd-2-CONS-GFP and Spd-2-ALL-GFP centrosomal levels is unlikely to be due to their slightly lower expression levels compared to WT Spd-2-GFP, as a different Spd-2

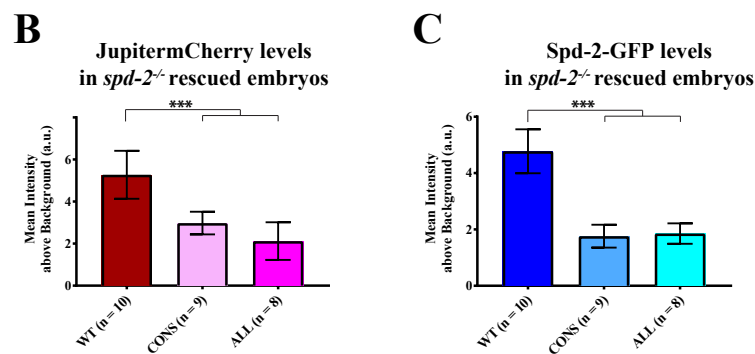
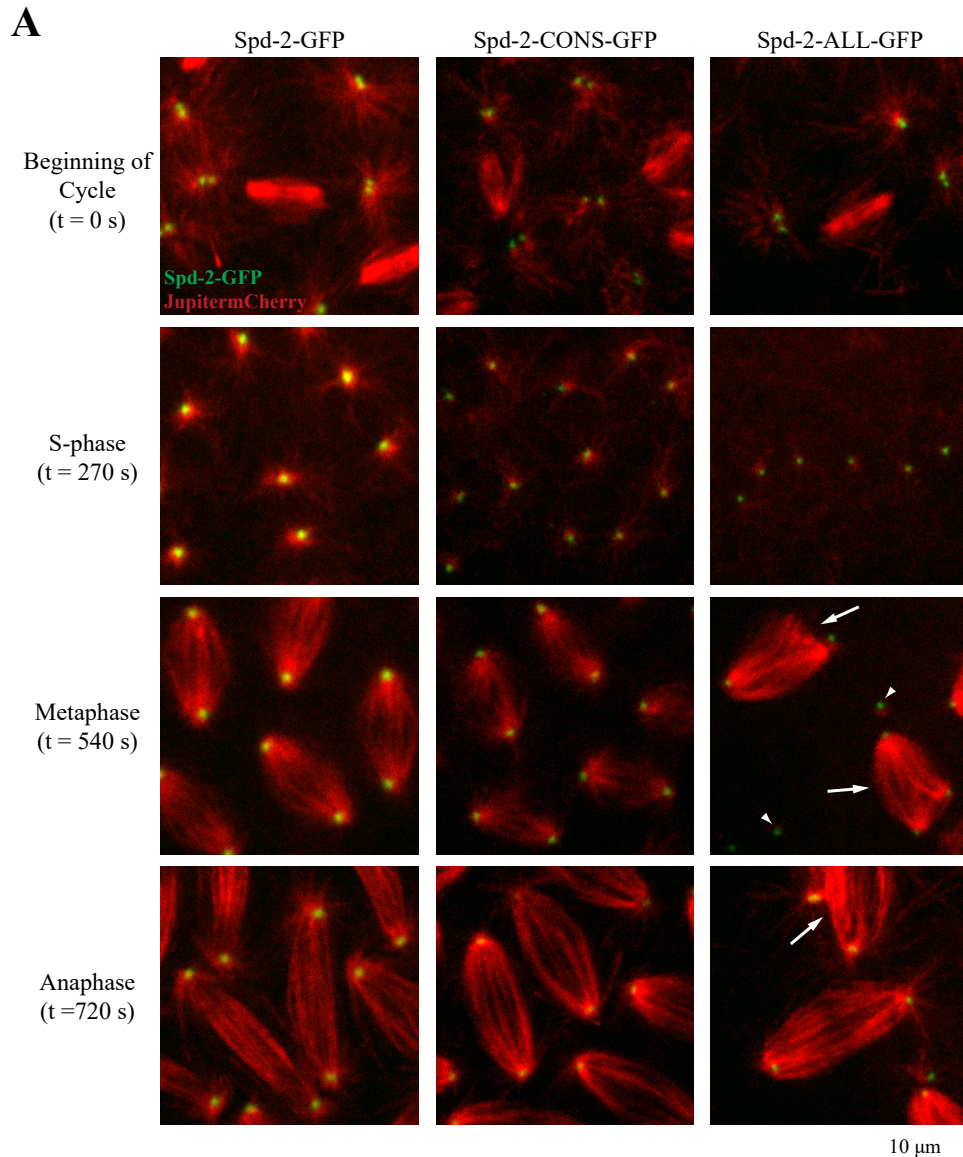


Figure 4.4 - Centrosomes in Spd-2-CONS and Spd-2-ALL embryos recruit less Spd-2 and organise fewer MTs.

(A) Micrographs of *spd-2*^{-/-} embryos expressing the MT-marker Jupiter with an mCherry fluorescent tag (red) and either WT Spd-2-GFP, Spd-2-CONS-GFP or Spd-2-ALL-GFP (green, as indicated). Time (in seconds) as the embryos progress from the beginning of the cycle (t = 0 s, judged by centrosome separation) to anaphase (t = 720s) is indicated. Embryos expressing the mutant proteins exhibited a range of mitotic defects, such as detached spindle poles (arrowheads) and abnormal mitotic spindles (arrows). (B,C) Graphs quantify the centrosomal MT intensity (B) or the centrosomal intensity of the various Spd-2-GFP fusion proteins (C) during late S-phase (t = 270s). n = number of embryos analysed, 5 centrosomes analysed per embryo. Error bars indicate SD. *** = *p*-value < 0.001.

mutant construct (Spd-2-11A-GFP), which has 11 potential phosphosites (including two ptPBSs) mutated to Ala, is expressed to lower levels than WT, CONS or ALL Spd-2-GFP (Figure 4.2B), and yet localises nearly normally to centrosomes and it can strongly support the full development of *spd-2^{-/-}* embryos without causing mitotic spindle or developmental defects (Baumbach, 2014).

4.2.3. Spd-2-CONS-GFP and Spd-2-ALL-GFP are still recruited to centrioles, but cannot efficiently form an extended scaffold

The reduction in the centrosomal levels of Spd-2-CONS-GFP and Spd-2-ALL-GFP could be due to loss of recruitment of these proteins to the centriole and/or to the Spd-2 mutants being unable to localise to the PCM properly. To distinguish between these possibilities, I imaged live *spd-2^{-/-}* embryos expressing WT, CONS or ALL Spd-2-GFP using 3D-structured illumination super-resolution microscopy (3D-SIM). As shown previously (Conduit et al., 2014b), WT Spd-2-GFP localised to the mother centriole wall and to the PCM nearby, forming a fibrous scaffold-like structure. However, Spd-2-CONS-GFP and Spd-2-ALL-GFP localised strongly to the mother centriole but not to the PCM, with most centrosomes having a much reduced scaffold or none at all (Figure 4.5) (note that these images were scored blind by my colleagues).

The lack of Spd-2 scaffold could be a direct consequence of the mutations rendering the protein unable to form such structures, but it could also be that mutating the ptPBSs in Spd-2 alters the PCM assembly process in a way that indirectly de-stabilises the Spd-2 scaffold. Distinguishing between these two possibilities is difficult because PCM assembly defects will impact Spd-2 scaffold formation and *vice versa*. To tackle this question, I imaged *spd-2^{-/-}* embryos co-expressing one copy of a WT Spd-2-mCherry transgene and one copy of either WT, CONS or ALL Spd-2-GFP. Under

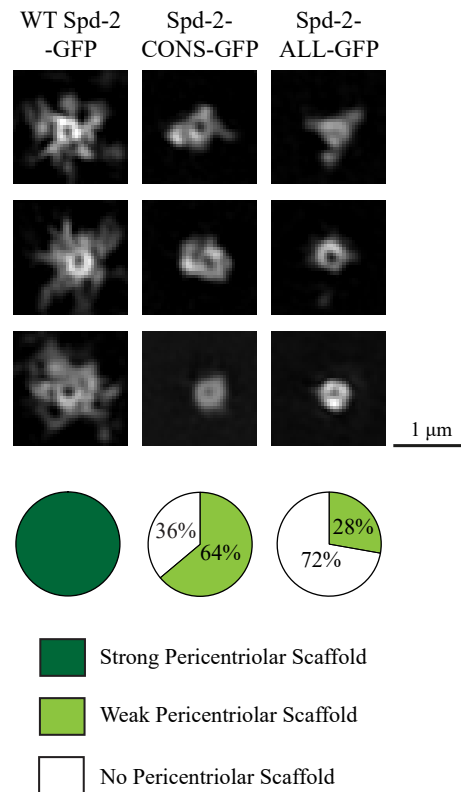


Figure 4.5 - Spd-2-CONS and Spd-2-ALL do not efficiently form a PCM scaffold.

Micrographs show 3D-SIM images of individual centrosomes from *spd-2*^{-/-} embryos expressing WT Spd-2-GFP, Spd-2-CONS-GFP or Spd-2-ALL-GFP (as indicated). Pie charts quantify the percentage of centrosomes that qualitatively showed a strong (*dark green*), weak (*light green*) or no (*white*) pericentriolar scaffold ($n = 36$, for each genotype, respectively, scored blindly). In this, and all other SIM experiments, images were only included in the analysis if the reconstruction was deemed of sufficient quality by SIM-Check (Ball et al., 2015) (see 2.5.9 for a full explanation of image quality control). All centrosomes were imaged in mid-late S-phase when the centrosomal levels of Spd-2 are maximal (see Figure 4.18B).

these conditions, Spd-2-CONS-GFP and Spd-2-ALL-GFP incorporated into the PCM scaffold organised by WT Spd-2-mCherry in a manner very similar to WT Spd-2-GFP (Figure 4.6). These observations further indicate that Spd-2-CONS-GFP and Spd-2-ALL-GFP are not simply misfolded, as the mutations introduced in Spd-2-ALL-GFP and Spd-2-CONS-GFP do not affect the ability of these proteins to form a scaffold *per se* or to interact with the proteins that normally recruit and maintain Spd-2 at centrioles and within the mitotic PCM. Instead, the lack of a Spd-2 scaffold in embryos that express only Spd-2-CONS-GFP or Spd-2-ALL-GFP would be due to a larger PCM assembly defect that destabilises the Spd-2 scaffold.

A possible criticism of this interpretation is that, in embryos expressing WT and mutant forms of Spd-2, the mutant proteins could form homo-oligomers with WT partners. Thus, a largely misfolded mutant protein could potentially be dragged to the centrosome by its WT partner. However, it has been previously shown using Fluorescence Correlation Spectroscopy (FCS) that SPD-2 is monomeric in worm embryos (Wueseke et al., 2014). To test whether this is also the case in fly embryos, I collaborated with T. Steinacker, another PhD student in the lab, who has developed an FCS-based assay to determine the oligomerization state of centrosomal proteins in the cytoplasm. FCS allows one to measure “molecular brightness” as a fluorescent count-rate per molecule (CPM). This can be used to distinguish between monomeric and dimeric proteins within living *Drosophila* embryos (e.g. monomeric and dimeric NeonGreen, Figure 4.7A).

As a baseline control, we measured the CPM of monomeric GFP (kind gift from the van der Merwe lab) *in vitro*. We then measured the CPM of WT Spd-2-GFP in *spd-2^{-/-}* embryos expressing only one copy of the transgene (Spd-2-GFP embryos), and

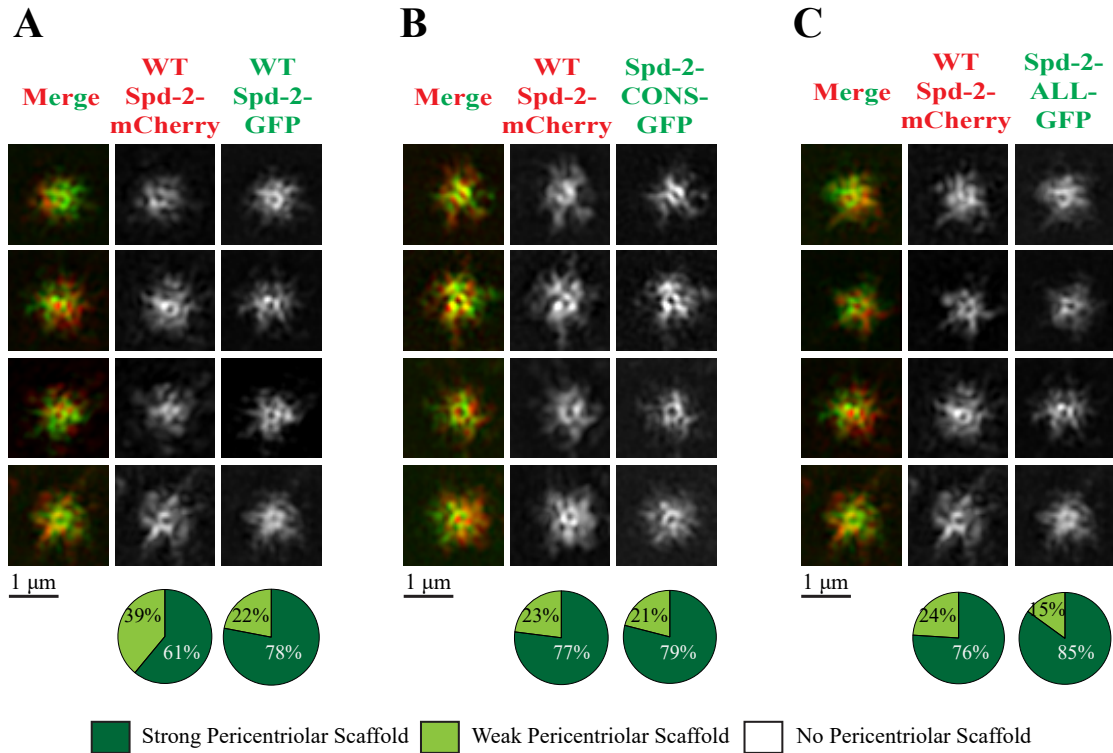
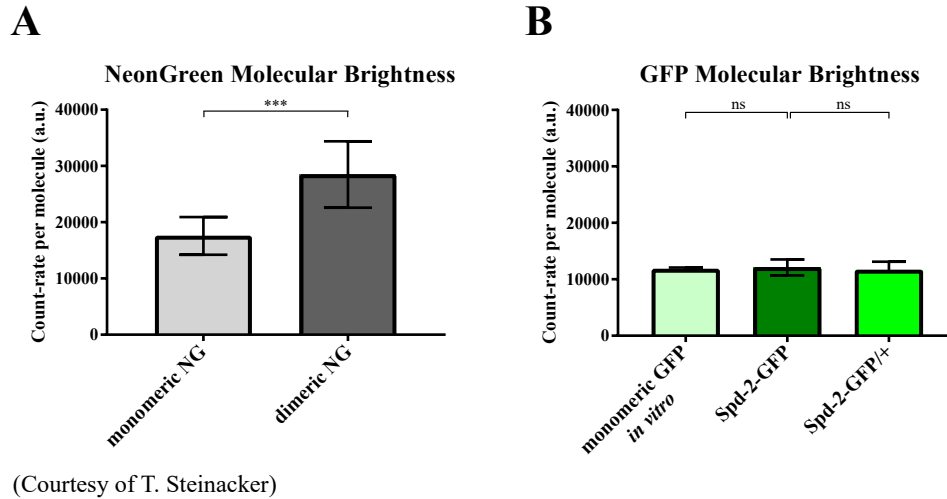


Figure 4.6 – Spd-2-CONS and Spd-2-ALL can be part of a scaffold in the presence of WT Spd-2-mCherry.

(A-C) Micrographs show 3D-SIM images of individual centrosomes from *spd-2*^{-/-} embryos expressing WT Spd-2-mCherry and one copy of either WT Spd-2-GFP, Spd-2-CONS-GFP or Spd-2-ALL-GFP (as indicated). Pie charts quantify the percentage of centrosomes that were scored qualitatively as having a strong (*dark green*), weak (*light green*) or no (*white*) pericentriolar scaffold (n = 16 individual centrosomes, two images (channels) per centrosome, for each genotype, respectively). All centrosomes were imaged in mid-late S-phase when the centrosomal levels of Spd-2 are maximal (see Figure 4.18B) and scored blindly.



(Courtesy of T. Steinacker)

Figure 4.7 - Spd-2-GFP diffuses as a monomer in the cytoplasm.

(A) Fluorescence Correlation Spectroscopy (FCS) can be used to calculate a fluorescent count-rate per molecule (CPM). Measurements from embryos expressing monomeric or dimeric NeonGreen illustrate how the CPM can clearly distinguish monomeric and dimeric proteins within living *Drosophila* embryos. Data courtesy of T. Steinacker. **(B)** Collaboration with T. Steinacker to measure the CPM of Spd-2-GFP in *spd-2^{-/-}* embryos (Spd-2-GFP), which was basically identical to the CPM of monomeric GFP *in vitro*, and to that of Spd-2-GFP in *spd-2^{+/-}* (heterozygous) embryos expressing one copy of Spd-2-GFP and one copy of the endogenous unlabelled *spd-2* gene (Spd-2-GFP/+). This indicates that Spd-2 is monomeric in the cytoplasm of *Drosophila* embryos. The Y axis of both graphs is set to the same scale, but note that mNeonGreen is much brighter than GFP, hence the higher CPM. Bars represent mean CPM, error bars represent SEM. ns = non-statistically significant difference, *p*-value > 0.05; *** = *p*-value < 0.001.

compared it to the CPM of WT Spd-2-GFP in *spd-2^{+/-}* embryos expressing one copy of the GFP-tagged transgene and one copy of endogenous, untagged *spd-2* (Spd-2-GFP/+ embryos). If Spd-2 were a monomer, all the Spd-2-GFP molecules measured would be expected to have the same number of fluorophores (and molecular brightness), irrespective of whether endogenous, untagged copies of the protein were expressed. However, if Spd-2 were a dimer, three different species would exist in Spd-2-GFP/+ embryos (untagged-untagged, GFP-untagged and GFP-GFP), and this would result in a decrease in the average brightness of the diffusing GFP-tagged molecules compared to the CPM in Spd-2-GFP embryos. The first scenario seems to be the case: the CPM of Spd-2-GFP was extremely similar in Spd-2-GFP and Spd-2-GFP/+ embryos, and it was also nearly identical to the CPM of monomeric GFP (Figure 4.7B). Thus, the mutant Spd-2 proteins are also likely to be recruited to the centriole wall as monomers.

4.2.4. Measuring the dynamics of Spd-2-GFP recruitment to the centriole

Following on the above results, I tried to directly measure the rate of recruitment of WT and mutant Spd-2-GFP fusions to centrosomes. Spd-2 is first recruited to centrioles and then spreads out (“fluxes”) into the PCM. Thus, the rate of Spd-2 incorporation to centrosomes can be estimated from the initial rate of recovery of Spd-2-GFP signal in fluorescence recovery after photobleaching (FRAP) experiments (Conduit et al., 2014b). Fluorescence recovery after photobleaching will occur when bleached Spd-2-GFP units are exchanged with unbleached Spd-2-GFP units from the cytoplasm. Because Spd-2-GFP is incorporated at the centriole wall and then moves outwards into the PCM, the initial Spd-2-GFP recovery after photobleaching will correspond to the first units of unbleached Spd-2-GFP being incorporated at the centriole wall. However, after some time, unbleached Spd-2-GFP will move into the PCM, while incorporation at the centriole wall continues (Figure 4.8A). We do not

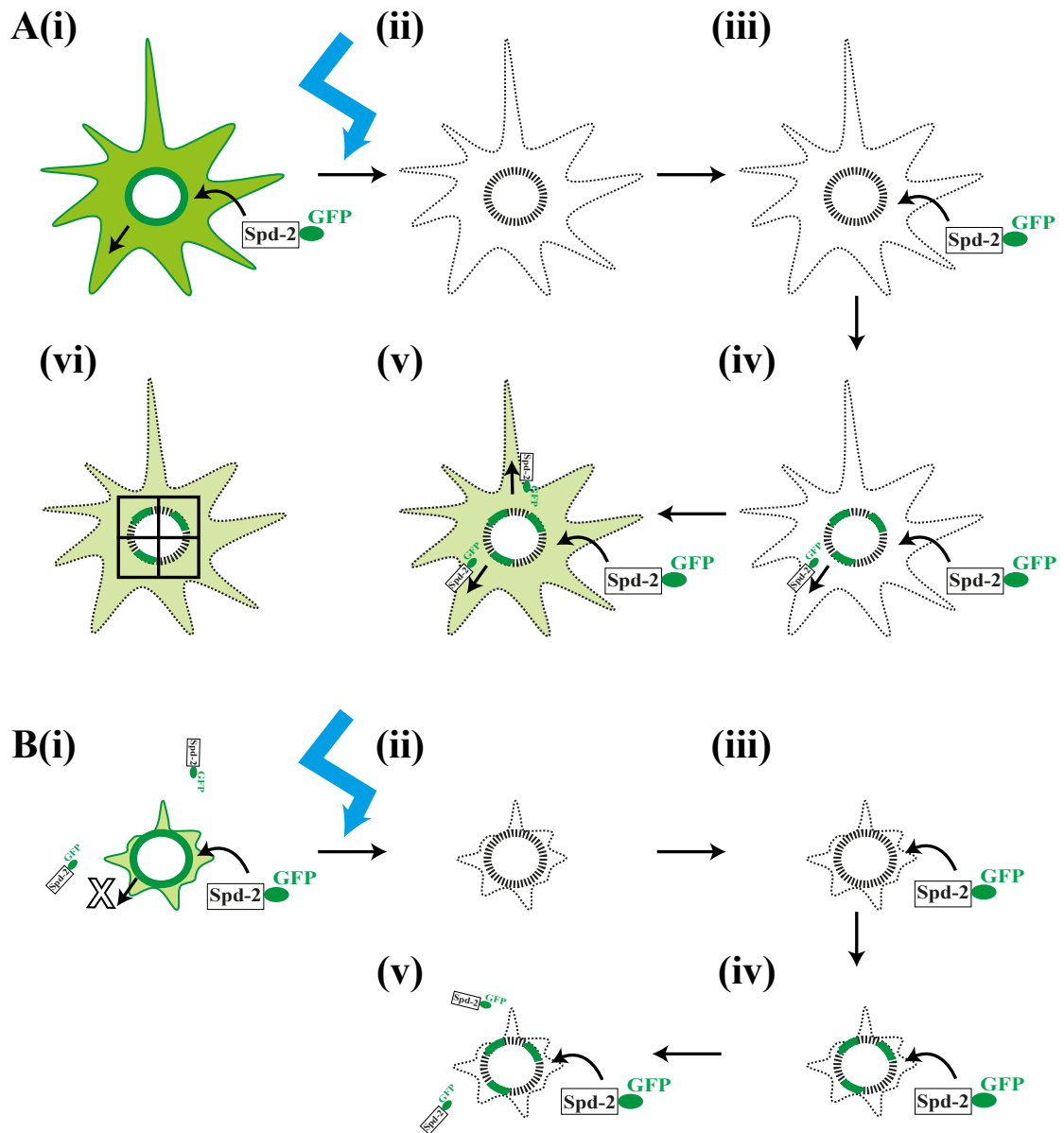


Figure 4.8 – Schematic of Spd-2-GFP dynamics and FRAP analysis.

(A) Schematic of WT Spd-2-GFP recovery after photobleaching. **i)** WT Spd-2-GFP is incorporated into centrosomes via recruitment to the centriole wall (dark green circle at the centre of the centrosome), from where it spreads outwards into the PCM (Conduit et al., 2014b). **(ii)** During FRAP experiments, all the Spd-2-GFP proteins at the centrosome are bleached using a 440 nm laser. **(iii)** Spd-2-GFP intensity at the centrosome starts to recover when unbleached Spd-2-GFP from the cytoplasm is recruited to the centriole wall. **(iv)** While unbleached Spd-2-GFP is recruited to the centriole wall, after some time unbleached Spd-2-GFP molecules will spread outwards into the PCM. **(v)** After this point, centrosomal Spd-2-GFP intensity depends on both the rate of Spd-2-GFP recruitment to the centriole wall and its dynamics (e.g. persistence) at the PCM. **(vi)** To minimise the PCM dynamics component of the Spd-2-GFP recovery rate, only the centre-most four pixels of Spd-2-GFP labelled centrosomes were measured for my FRAP analysis, however, some of the signal analysed might still correspond to Spd-2-GFP in the PCM. (B) Schematic of Spd-2-CONS-GFP recovery after photobleaching. **(i)** Spd-2-CONS-GFP is also incorporated into the centrosomes via recruitment to the centriole wall, but very little or no PCM scaffold can form in the absence of WT Spd-2 units. **(ii-iii)** Similar to WT Spd-2-GFP analysis, after centrosomes are completely bleached, new unbleached Spd-2-CONS-GFP molecules can be recruited to the centriole wall. **(iv)** Thus, the intensity of the Spd-2-CONS-GFP signal starts to recover. **(v)** However, unlike WT Spd-2-GFP, Spd-2-CONS-GFP cannot be maintained at the PCM near the centriole. Even if a minimal scaffold can eventually be formed, the dynamics of Spd-2-CONS-GFP at the PCM will be very different from those of WT Spd-2-GFP, so from this point onwards the recovery rates of WT Spd-2-GFP and Spd-2-CONS-GFP are expected to be very different.

perfectly understand the dynamics of Spd-2-GFP residency at the PCM, but presumably there might be some variability in how fast Spd-2-GFP proteins move outwards and dissociate from the scaffold at different points of the cell cycle or between different developmental stages.

First, I decided to test the above assumption by comparing the recovery of WT Spd-2-GFP across nuclear cycles 11 and 12. To obtain the most accurate estimate of the initial recovery rate, I analysed only the earliest time points after total centrosome photobleaching, acquiring images as fast as possible to increase time resolution (one frame every 2.5 s), and measuring centrosome intensity only at the very centre (brightest four pixels) of the centrosome (Figure 4.8A_{vi}). Additionally, to minimise experimental variability, centrosomes were imaged at a comparable point of the cell cycle (S-phase, 3 min after centrosome separation) of a specific nuclear cycle (11 or 12, as indicated).

Unbleached centrosomes (CONTROL) from embryos in cycle 11 had a higher average centrosome fluorescence of the four brightest pixels compared to those from cycle 12 embryos (Figure 4.9A). This means that in the same central area of the centrosome there were more Spd-2-GFP molecules in cycle 11 centrosomes compared to cycle 12 centrosomes, already suggesting that the parameters that determine Spd-2-GFP PCM dynamics change slightly from one nuclear cycle to the next. When comparing WT Spd-2-GFP recovery after photobleaching between nuclear cycles 11 and 12, I observed a similar intensity recovery until 7.5 s after bleaching (Figure 4.9B). However, after this point, recovery increased faster in cycle 11 than in cycle 12 embryos. This could represent a change in the recruitment rate of Spd-2 to the centriole wall, but it is difficult to understand why Spd-2 recruitment would suddenly

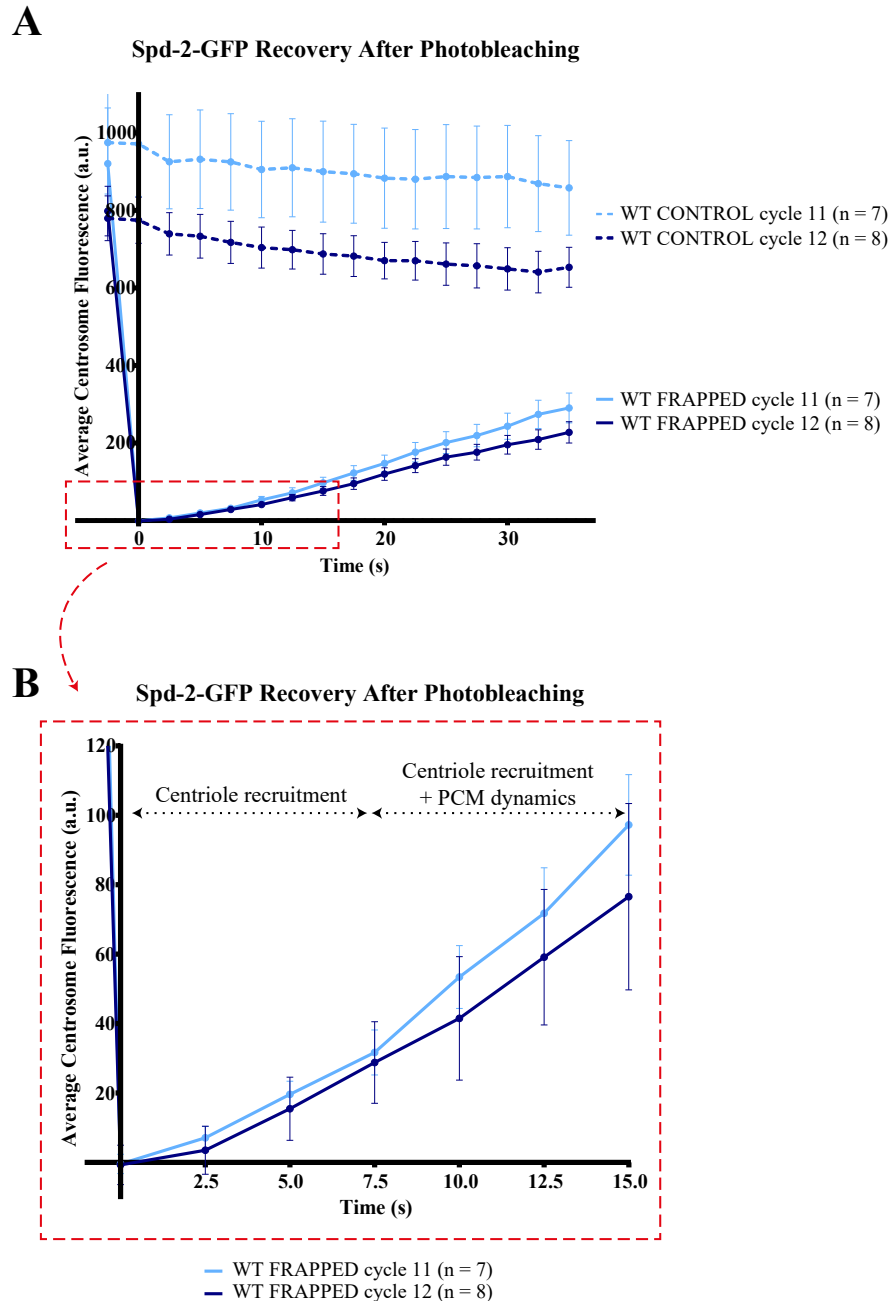
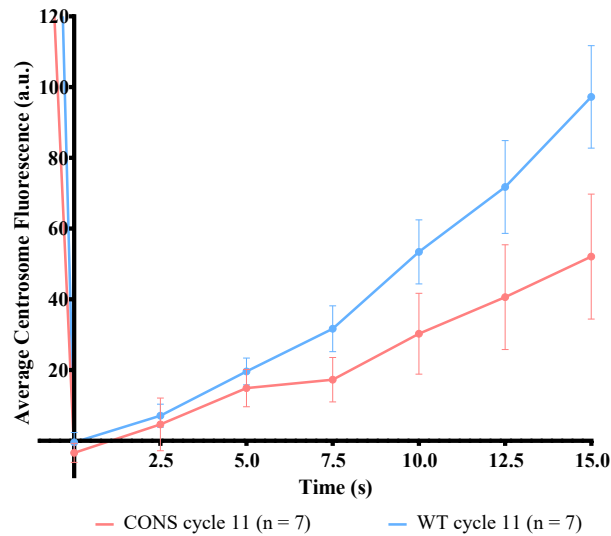
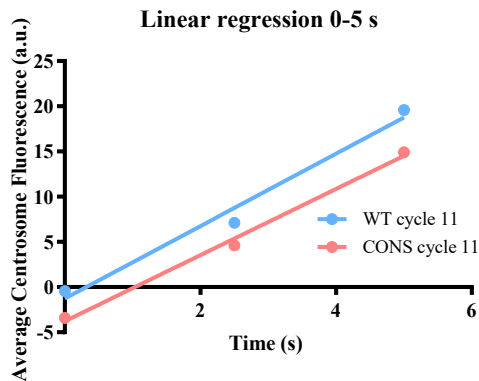
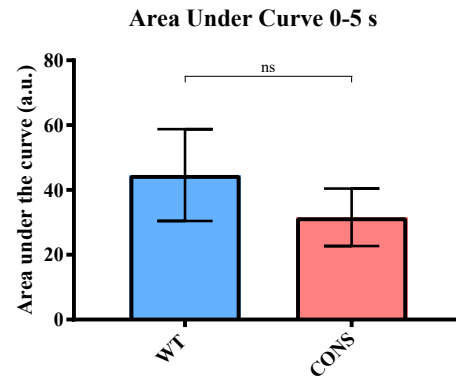


Figure 4.9 – Spd-2-GFP dynamics change with embryo age.

(A) Changes in (WT) Spd-2-GFP intensity of the four most central pixels of centrosomes with time. Each data point corresponds to the mean intensity of three centrosomes per embryo, n = number of embryos analysed per condition. For easier visualisation of the intensity changes over time, the data points are joined by lines, but note that these lines do not correspond to any fitted regression models. At time = 0 s, some centrosomes were completely bleached using a 440 nm laser (“FRAPPED” conditions, *continuous* lines). Unbleached centrosomes from the same embryos were also analysed (“CONTROL” conditions, *dashed* lines). To minimise sample variability, embryos were analysed at a specific point of the cell cycle (3 min after centrosome separation) and only embryos at two different developmental stages were included in the analysis: nuclear cycle 11 embryos (*light* lines) presented higher Spd-2-GFP intensity at the central four pixels than nuclear cycle 12 embryos (*dark* lines). Embryos were imaged as frequently as the system would allow with the required settings (i.e. one frame every 2.5 s) to obtain the best possible time resolution. Because of this, embryos were imaged for a short period of time rather than until full recovery of the Spd-2-GFP signal in bleached centrosomes, and even CONTROL unbleached centrosomes experienced minor photobleaching over time. (B) Expansion of the section of the graph in (A) in the *red dashed* box, to focus on the initial time points after photobleaching. 7.5 s after bleaching, Spd-2-GFP recovery speeded up in cycle 11 compared to cycle 12 embryos. Error bars indicate 95% CI of the mean.

speed up specifically in cycle 11 embryos at a given time after bleaching. It is more likely that the Spd-2-GFP recovery rate beyond the 7.5 s time point becomes a composite of the Spd-2 recruitment rate and Spd-2 dynamics in the PCM, with the latter factor being different between cycles 11 and 12. While not conclusive evidence, these observations support the hypothesis that WT Spd-2 PCM dynamics change with subsequent nuclear cycles and are in agreement with observations from other members of the Raff laboratory (S.S. Wong, unpublished).

Based on the results above, I decided to compare WT and CONS Spd-2-GFP recovery in centrosomes of cycle 11 embryos, at the earliest time points. I excluded Spd-2-ALL-GFP embryos from this analysis because they presented more obvious developmental defects earlier on (data not shown). In the absence of endogenous, untagged Spd-2 protein, WT and CONS Spd-2-GFP recovery was similar for the very early time points (0 - 5 s) (Figure 4.10). There was no statistically significant difference in the slopes of the rate of recovery when a linear regression curve is fitted to the data (WT 95% CI slope = 3.1 to 4.9 a.u., CONS 95% CI slope = 3.0 to 4.4, p -value = 0.54) (Figure 4.10B). Because this method assumes that the rate of recovery would necessarily be linear, I also calculated the area under the curve for the WT and CONS data, and found that although there was a slight decrease in CONS Spd-2-GFP recovery, the difference was not statistically significant (Figure 4.10C). However, 7.5 s after photobleaching the pattern of WT and CONS Spd-2-GFP recovery changed drastically (Figure 4.10A), as one might expect if beyond this point the recovery rate is partially dependent on dynamics in the PCM (Figure 4.8Av,Bv). Due to experimental constraints, this analysis relies on data from very few time points, but these observations are in agreement with a model in which Spd-2-CONS-GFP (and Spd-2-ALL-GFP) are recruited to the centriole wall with relatively normal kinetics (Figure 4.8Biii), but

A**Spd-2-GFP Recovery After Photobleaching****B****C****Figure 4.10 – CONS and WT Spd-2-GFP initial recovery after photobleaching.**

(A) Changes in WT Spd-2-GFP (*blue*) and Spd-2-CONS-GFP (*red*) intensity of the four most central pixels of photobleached centrosomes with time. Each data point corresponds to the mean intensity of three centrosomes per embryo, n = number of embryos analysed per condition. For easier visualisation of the intensity changes over time, the data points are joined by lines, but note that these lines do not correspond to any fitted regression models. At time = 0 s, centrosomes were completely bleached using a 440 nm laser. To minimise sample variability, embryos at the same developmental stage (nuclear cycle 11) were analysed at a specific point of the cell cycle (3 min after centrosome separation). Embryos were imaged as frequently as the system would allow with the required settings (i.e. one frame every 2.5 s) to obtain the best possible time resolution. Only the earliest time points are shown in this graph. Error bars indicate 95% CI of the mean. (B) A linear regression model (*lines*) was fitted to the data from (A) corresponding to the first three time points after photobleaching (0 – 5 s). There was no statistically significant difference between WT and CONS slopes, indicating that initial Spd-2-CONS-GFP recovery is similar to that of WT Spd-2-GFP. (C) Differences in Spd-2-GFP recovery were also quantified by calculating the area under the curve (AUC) for the data from (A) corresponding to the first three time points after photobleaching (0 – 5 s). While Spd-2-CONS-GFP had a slightly smaller AUC than WT Spd-2-GFP, the difference was not statistically significant (ns = p -value > 0.05).

subsequently fail to assemble into a stable PCM scaffold (Figure 4.8Biv-v).

4.2.5. Polo is lost from the PCM if it cannot be recruited by Spd-2

Next, I tested whether Polo was still present in the PCM despite the lack of a stable Spd-2 scaffold. To do this, I used the same PoloGFP protein trap line employed for the mRNA injection assay, but in a *spd-2*^{-/-} background expressing either WT Spd-2-mCherry, Spd-2-CONS-mCherry or Spd-2-ALL-mCherry. Expressing PoloGFP with the mutant Spd-2-mCherry proteins led to embryos with even worse mitotic defects than those observed when mutant GFP- or mCherry-fusions were expressed in the absence of the PoloGFP allele, suggesting that the GFP-tagged Polo sensitises the embryos to the expression of the mutant Spd-2-fusions (Figure 4.11). These defects were particularly severe in embryos expressing Spd-2-ALL-mCherry, such that they could not be reliably staged and they were excluded from further analysis. Note however that embryos expressing WT Spd-2-mCherry and PoloGFP developed normally.

As shown in Figure 4.12A (and also Figure 3.2B), WT Spd-2-mCherry and PoloGFP co-localised at the mother centriole wall and spread outwards together into the PCM scaffold, supporting the idea that Polo is maintained at the PCM by binding to Spd-2. In contrast, although Spd-2-CONS-mCherry and PoloGFP still co-localised at the centriole wall, they were largely undetectable in the PCM (Figure 4.12B). This suggests that the ptPBSs in Spd-2 are not required to recruit Polo to the centriole wall, but are necessary to recruit Polo to the PCM scaffold. However, just like in the case of the Spd-2 scaffold formation, an alternative explanation would be that the mutations introduced in Spd-2-CONS-mCherry have an indirect effect on mitotic PCM assembly,

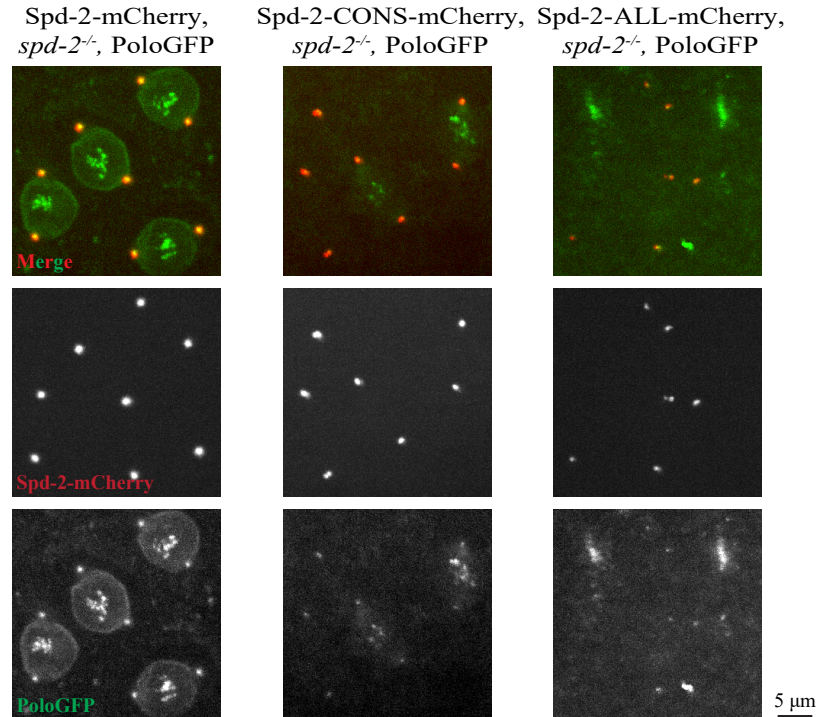


Figure 4.11 – Abnormal PoloGFP and Spd-2-mCherry distribution and mitotic defects in *spd-2*^{-/-} embryos rescued with Spd-2-CONS-mCherry or Spd-2-ALL-mCherry.

Micrographs show maximum intensity projections of standard spinning disk confocal images of *spd-2*^{-/-} embryos expressing PoloGFP (*green*) and either WT Spd-2-GFP, Spd-2-CONS-GFP or Spd-2-ALL-GFP (*red*, as indicated). Most embryos rescued with Spd-2-ALL-mCherry did not develop to syncytial blastoderm stages and the few that did rapidly accumulated severe defects (as in the example shown here), making it difficult to accurately stage or analyse the embryos. Thus, Spd-2-ALL embryos were excluded from further analysis. While it is difficult to determine the primary cause of these defects, my data is consistent with the defects being due to centrosome dysfunction. These defects are much worse than any defects observed with *spd-2*^{-/-} embryos expressing mutant Spd-2 fusion proteins (GFP or mCherry) only, which suggests that the worsening of the phenotype is due to a genetic interaction between PoloGFP and ptPBS mutant Spd-2-mCherry.

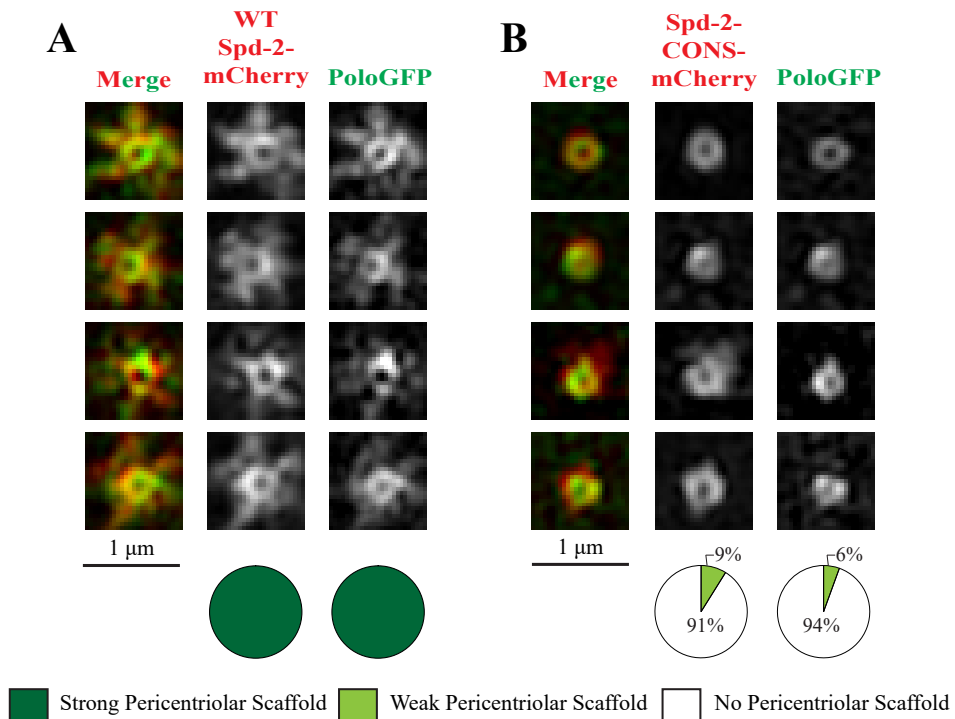


Figure 4.12 - Polo is recruited to centrioles but not to the PCM in Spd-2-CONS and Spd-2-ALL embryos. (A,B) Micrographs show 3D-SIM images of individual centrosomes from *spd-2*^{-/-} embryos expressing PoloGFP (green in merge images) and either WT Spd-2-mCherry or Spd-2-CONS-mCherry (as indicated, red in merge images). Pie charts quantify the percentage of centrosomes that were scored qualitatively as having a strong (dark green), weak (light green) or no (white) pericentriolar scaffold/protein present in the PCM (n = 15 individual centrosomes, two images (channels) per centrosome, for each genotype, respectively). All centrosomes were imaged in mid-late S-phase when the centrosomal levels of Spd-2 are maximal (see Figure 4.18B) and scored blindly. The defect in Spd-2 scaffold formation was worse in these embryos than in those expressing Spd-2 mutant fusion proteins and no PoloGFP (see Figure 4.5 and Figure 4.6). This is consistent with a worsening of the phenotype observed due to a genetic interaction between PoloGFP and Spd-2-CONS-mCherry (see Figure 4.11).

so PoloGFP cannot be recruited to the mitotic PCM because the expanded mitotic PCM simply does not exist in these embryos.

To distinguish between these two possibilities I returned to the mRNA injections into embryos. In 3.2.2, I observed that PoloGFP distribution (as measured by radial profiling) became narrower (i.e. more “centriole-like”) upon expressing Spd-2 proteins with mutated ptPBSs. This would be consistent with PoloGFP being lost specifically from the PCM. Indeed, in PoloGFP embryos injected with Spd-2-CONS-mKate2 mRNA and imaged with 3D-SIM I observed that PoloGFP was often severely reduced or completely lost from the PCM, whereas Spd-2-CONS-mKate2 could often still be detected as an expanded PCM scaffold (Figure 4.13). Presumably, this phenotype corresponds to an intermediate state, where a Spd-2 scaffold is made out of mostly Spd-2-CONS-mKate2 with some endogenous, unlabelled Spd-2 proteins. As Spd-2-CONS-mKate2 molecules outcompete the endogenous Spd-2, the scaffold becomes less able to maintain recruitment of PoloGFP to the PCM, because the Spd-2-CONS-mKate2 in the scaffold cannot recruit PoloGFP efficiently. Thus, pericentriolar PoloGFP is lost prior to the disassembly of the Spd-2 scaffold and other PCM defects, rather than as a consequence of them. These data supports my initial hypothesis that the ptPBSs in Spd-2 are necessary to efficiently recruit Polo to the PCM scaffold. Once PoloGFP is completely lost from the PCM, the consequences for mitotic PCM assembly are such that the Spd-2 scaffold becomes unstable and disassembles.

4.2.6. If Spd-2 cannot recruit Polo, the entire pericentriolar scaffold fails to expand outwards efficiently

This model relies on the idea that losing Polo recruitment would have serious deleterious effects on mitotic PCM assembly. This is a reasonable assumption based

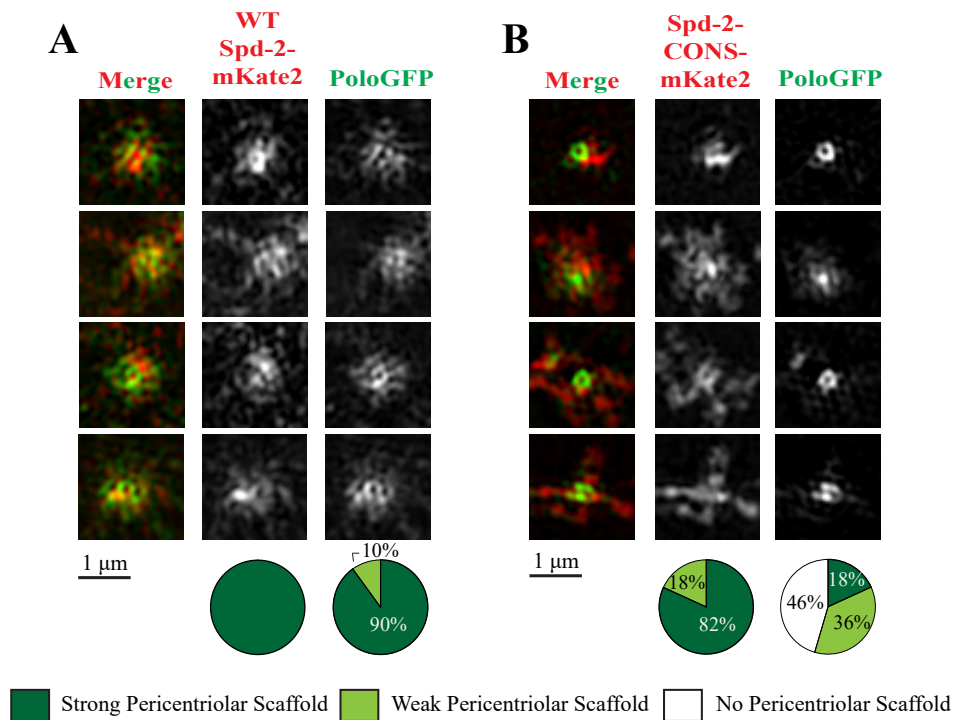


Figure 4.13 – When Spd-2 is unable to bind Polo, Polo is lost from the PCM prior to Spd-2 scaffold disassembly.

Micrographs show 3D-SIM images of individual centrosomes from embryos expressing PoloGFP (*green* in merge images) injected with either WT Spd-2-mKate2 (**A**) or Spd-2-CONS-mKate2 mRNA (**B**) (*red* in merge images). As in the experiments detailed in Chapter 3, embryos were imaged 2 h after mRNA injection. Pie charts quantify the percentage of centrosomes that were scored qualitatively as having a strong (*dark green*), weak (*light green*) or no (*white*) pericentriolar scaffold/protein present in the PCM ($n = 10$ and 11 individual centrosomes, two images (channels) per centrosome, for WT and CONS injections, respectively). All centrosomes were imaged in mid-late S-phase when the centrosomal levels of Spd-2 are maximal (see Figure 4.18B) and scored blindly. Note that mKate2 is relatively slow folding, and the fusion proteins are just expressed from the injected mRNA, so the signal-to-noise ratio is low and the 3D-SIM images reconstruct relatively poorly. Nevertheless the presence of a Spd-2-CONS-mKate2 scaffold in these centrosomes is clear, even when the PoloGFP signal in the scaffold is very weak (B).

on previous reports that Spd-2 cooperates with Polo and Cnn to form the mitotic PCM scaffold in fly embryos, and that Polo is required for Cnn scaffold formation (Conduit et al., 2014a, 2014b, 2015a; Feng et al., 2017). To confirm this, I examined the distribution of RFP-Cnn in living *spd-2^{-/-}* embryos expressing WT Spd-2-GFP or Spd-2-CONS-GFP using 3D-SIM. In the embryos expressing WT Spd-2-GFP, RFP-Cnn formed a large, robust scaffold which spread outwards beyond the Spd-2-GFP scaffold (Figure 4.14A). Conversely, in the Spd-2-CONS-GFP embryos the RFP-Cnn scaffold was minimal. Both Spd-2-CONS-GFP and RFP-Cnn localised mostly to the centriole wall and to the occasional flare that tried to spread into the PCM but was unstable (*arrowheads*, Figure 4.14B). I also imaged these embryos on a standard spinning disk confocal system and obtained RFP-Cnn radial profiles under these conditions. This confirmed that in the Spd-2-CONS-GFP embryos there is a major drop in centrosomal RFP-Cnn levels, and that RFP-Cnn distribution becomes a lot narrower and similar to that of Spd-2-CONS-GFP (Figure 4.14C,D). This is also similar to the Cnn scaffold “collapse” observed in some of the embryos injected with Spd-2-CONS-mKate2 and Spd-2-ALL-mKate2 (see 3.2.2). Therefore, if Polo cannot bind Spd-2, Polo is lost from the PCM, and the Cnn and Spd-2 scaffolds cannot spread outwards for proper PCM assembly.

This result is surprising because Polo is still recruited to the centriole wall, so it should still be able to phosphorylate Cnn and promote Cnn scaffold formation (Conduit et al., 2014a; Feng et al., 2017). To test whether Cnn recruited to the centriole wall in Spd-2-CONS-GFP embryos is still phosphorylated, I imaged fixed embryos stained with two different antibodies: an anti-Cnn antibody, and an antibody that specifically recognises a phospho-epitope in Cnn (pSer567-Cnn) that is phosphorylated by Polo (Feng et al., 2017). In embryos rescued with WT Spd-2-GFP the central section of the Cnn scaffold

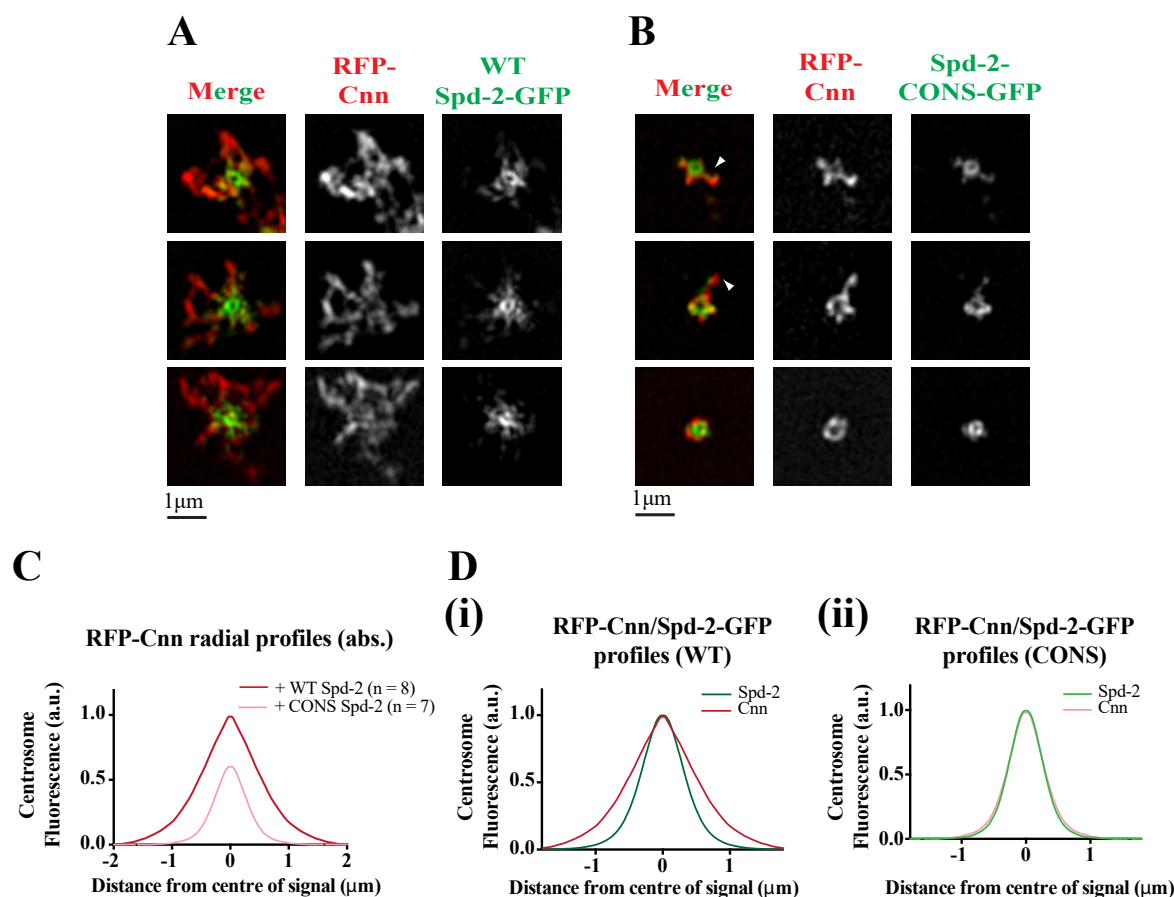


Figure 4.14 – The Cnn scaffold cannot spread outwards into the PCM if Spd-2 cannot bind Polo.

(A,B) Micrographs show 3D-SIM images of individual centrosomes from *spd-2*^{-/-} embryos expressing RFP-Cnn (red in merge images) and either WT Spd-2-GFP (A) or Spd-2-CONS-GFP (B) (green in merge images). Cnn is recruited to the centriole wall in Spd-2-CONS-GFP embryos, but it cannot form a stable PCM scaffold. Occasionally, outward protrusions containing both RFP-Cnn and Spd-2-CONS-GFP could be seen (arrowheads). (C) Absolute radial profiles of RFP-Cnn in embryos expressing WT Spd-2-GFP or Spd-2-CONS-GFP imaged using a standard spinning disk confocal system (5 centrosomes per embryo, n = number of embryos analysed). The shape of the absolute radial profile is determined by the levels of RFP-Cnn at the centrosome and its distribution. Similar radial profiles were obtained for WT Spd-2-GFP and Spd-2-CONS-GFP from the corresponding embryos (data not shown). (D) Overlay of Spd-2-GFP and RFP-Cnn profiles, normalised so that peak intensity = 1.0. (i) Profiles obtained from embryos rescued with WT Spd-2-GFP: the centrosomal distribution of RFP-Cnn is wider than that of Spd-2-GFP. (ii) Profiles obtained from embryos rescued with Spd-2-CONS-GFP: the profiles of RFP-Cnn and Spd-2-GFP are markedly narrower (i.e. more “centriole-like” and basically overlap).

is recognised by the anti-pCnn antibody, but Cnn appears to be de-phosphorylated at the edges of the scaffold. Conversely, in Spd-2-CONS-GFP embryos, Cnn forms a minimal scaffold around the centriole wall but it cannot spread outwards, despite the fact that it is phosphorylated at Ser567 (Figure 4.15). Therefore, Cnn scaffold formation requires the presence of Spd-2 and Polo in the PCM, such that even if Cnn is phosphorylated, the Cnn scaffold cannot spread outwards into the PCM on its own.

Since Polo, Spd-2 and Cnn are essential for mitotic PCM assembly (Conduit et al., 2014b; Dobbelaere et al., 2008; Feng et al., 2017), the defects in scaffold formation observed in Spd-2-CONS-GFP embryos should lead to other PCM proteins not being properly recruited to centrosomes. To test this I looked at Aurora A kinase in live embryos (Figure 4.16), and γ -tubulin in fixed embryos (Figure 4.17). Their distribution at the centrosome was statistically significantly narrower and more “centriole-like” in CONS-rescued embryos. The centrosomal levels for both proteins were also markedly decreased. In the case of Aurora A-GFP this difference was highly statistically significant and cleared the “biological relevance” criteria established in Chapter 3 (0.43 to 0.60 95% CI of difference to WT, p -value = $4E-13$). In the case of γ -tubulin, the signal was more variable, but the difference was still statistically significant and the effect size was large (0.26 to 0.84 95% CI of difference to WT, p -value = $5E-4$); with some (but not all) centrosomes having completely lost γ -tubulin (Figure 4.17E). Therefore, loss of Spd-2-mediated Polo recruitment to the PCM results in general centrosome maturation failure.

4.2.7. Centrosome maturation fails in Spd-2-CONS-GFP embryos

Syncytial fly embryos have a shortened cell cycle with no Gap phases (Foe and Alberts, 1983), so when they exit mitosis they immediately enter S-phase and the

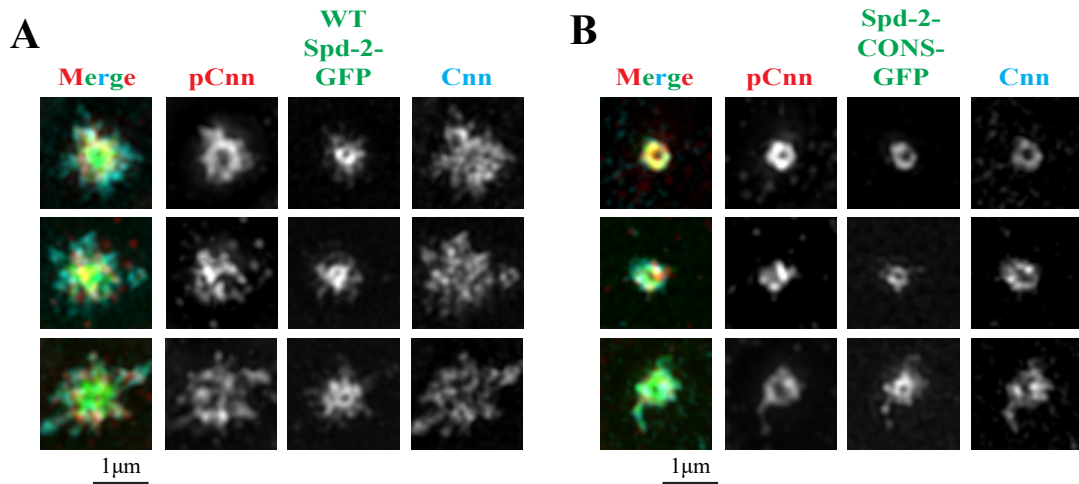


Figure 4.15 – Cnn is still phosphorylated at centrioles in Spd-2-CONS embryos, but it does not efficiently assemble into an extended scaffold.

Micrographs show 3D-SIM images of individual centrosomes from *spd-2*^{-/-} embryos expressing WT Spd-2-GFP (A) or Spd-2-CONS-GFP (B). The embryos were fixed and stained with antibodies that recognise a specific Polo-dependent phospho-epitope (pSer567) in Cnn (“pCnn”, red), GFP (green), or Cnn independent of its phosphorylation state (“Cnn”, cyan). In WT-rescued embryos, most of the Cnn scaffold appeared to be phosphorylated at Ser567, except at the outermost edges of the scaffold. This matches previous reports (Feng et al., 2017). As expected from the live imaging results, in Spd-2-CONS-GFP embryos the Cnn scaffold could not spread outwards into the PCM. However, in these embryos Cnn was still phosphorylated by Polo at Ser567.

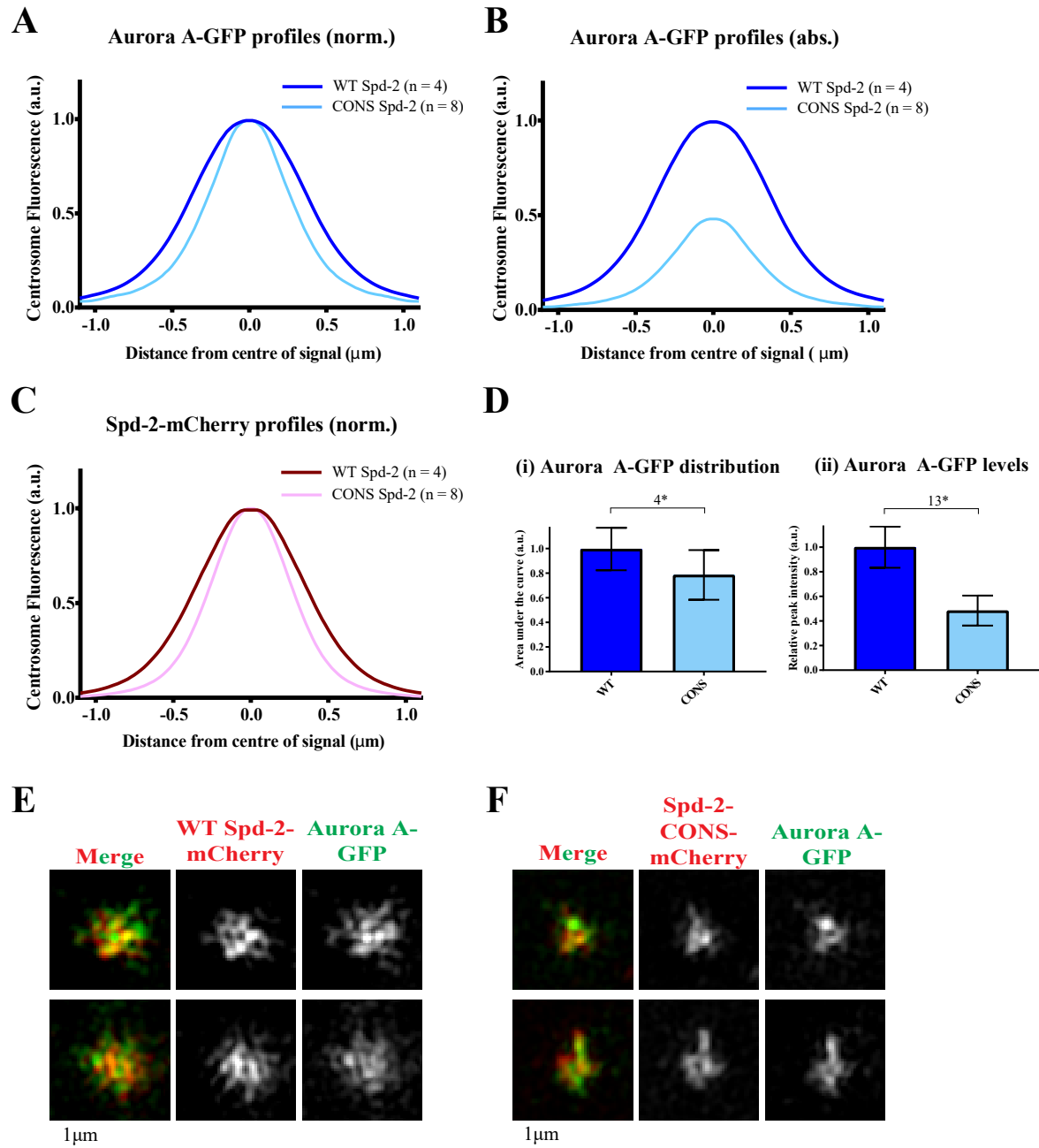


Figure 4.16 – Loss of centrosomal Aurora A (a mitotic PCM client) in Spd-2-CONS embryos.

(A) Normalised Aurora A-GFP radial profiles from *spd-2*^{-/-} embryos rescued with WT Spd-2-mCherry or Spd-2-CONS-mCherry (as indicated). (B) Aurora A-GFP absolute radial profiles. (C) Normalised radial profiles for WT Spd-2-mCherry and Spd-2-CONS-mCherry proteins, for reference. As expected from Figure 4.5 and Figure 4.12-Figure 4.15, the distribution of the CONS mutant protein was narrower (more “centriole-like”) than that of the WT. (D) Parameters extracted from the Aurora A-GFP radial profiles: area under the curve (i), from the normalised profiles; and average peak intensity (ii), from the absolute profiles. Compared to Aurora A-GFP in WT-rescued embryos, Spd-2-CONS-mCherry embryos had less centrosomal Aurora A-GFP and its distribution was narrower. (E,F) Micrographs show 3D-SIM images of individual centrosomes from *spd-2*^{-/-} embryos expressing Aurora A-GFP (green in merge images) and either WT Spd-2-mCherry or Spd-2-CONS-mCherry (as indicated, red in merge images).

4* = *p*-value in 10⁻⁴ (E-4) range; 13* = *p*-value in E-13 range. 5 centrosomes analysed per embryo, n = number of embryos. Error bars represent SD.

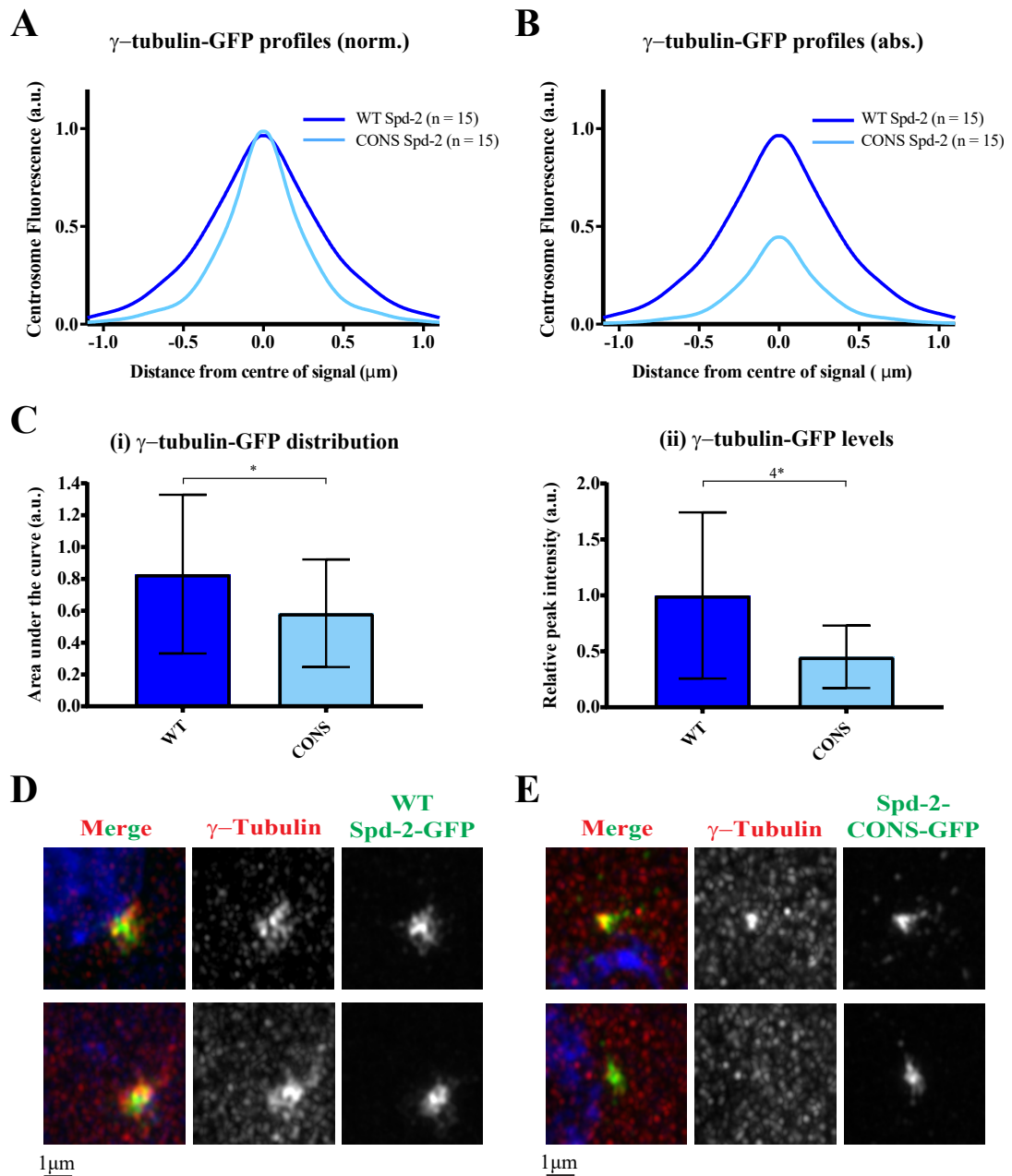


Figure 4.17 – Loss of centrosomal γ -tubulin (a mitotic PCM client) in Spd-2-CONS embryos. (A) Normalised γ -tubulin radial profiles from *spd-2*^{-/-} embryos rescued with WT Spd-2-GFP or Spd-2-CONS-GFP (as indicated). Unlike previous examples of radial profiling, the embryos analysed were fixed and stained for γ -tubulin and GFP rather than imaged live. Thus, instead of analysing the five brightest centrosomes per embryo, I analysed one pair of centrosomes per embryo, five embryos per technical replicate (embryos collected and processed independently), and three technical replicates, total n = 15 embryos per condition. (B) γ -tubulin absolute radial profiles. (C) Parameters extracted from the γ -tubulin radial profiles: area under the curve (i), from the normalised profiles; and average peak intensity (ii), from the absolute profiles. Compared to γ -tubulin in WT-rescued embryos, Spd-2-CONS-GFP embryos had less centrosomal γ -tubulin and its distribution was narrower; however the variability within the samples was quite large. (D,E) Micrographs show Airyscan images of individual centrosomes from *spd-2*^{-/-} embryos expressing either WT Spd-2-GFP or Spd-2-CONS-GFP (as indicated, *green* in merge images), fixed and stained with antibodies against γ -tubulin (*red*) and GFP. Some of the centrosomes in Spd-2-CONS-GFP embryos still had some visible γ -tubulin, whereas others had completely lost it; representative images of both phenotypes are shown. * = 0.05 > *p*-value > 0.01, 4* = *p*-value in 10⁻⁴ (E-4) range. Error bars represent SD.

centrosomes start to grow in size in preparation for mitosis. For example, the amount of GFP-Cnn at the centrosome increases linearly throughout S-phase and then plateaus as embryos enter mitosis ((Conduit et al., 2010; Megraw et al., 2002); Figure 4.18A). Our lab has recently develop a semi-automated imaging and analysis protocol to quantify the dynamics of centriolar proteins (Aydogan et al., 2018, 2019; see 2.5.12), so I decided to apply these tools to describe the behaviour of WT Spd-2-GFP during centrosome maturation, and to study how this process is disturbed if Spd-2 cannot recruit Polo to the PCM.

WT Spd-2-GFP levels appeared to increase during S-phase, reaching a maximum soon before nuclear envelope breakdown (NEB). Some (but not all) embryos were able to maintain maximum Spd-2-GFP levels for a variable period of time before Spd-2-GFP started to decline as the embryos entered mitosis (*blue* line, Figure 4.18B; Figure 4.19). I tried to model this behaviour with the help of M. Aydogan, but the best fit for the Spd-2-GFP “wave” was not a simple Gaussian or Lorentzian curve. Instead, the preferred model was our bespoke “linear growth followed by plateau followed by linear decrease” equation, but this must surely be a simplification of the underlying biology (Figure 4.20). Nevertheless, in Spd-2-CONS-GFP embryos this wave-like behaviour was never observed, instead Spd-2-CONS-GFP remained at a constant low level throughout the cycle (*red* line, Figure 4.18B; Figure 4.21). This is further evidence that centrosome maturation fails if Spd-2 cannot recruit Polo.

4.2.8. Multiple sites in Spd-2 contribute to Polo recruitment

The results presented here are in agreement with previous reports of an interaction between the Spd-2 and Polo homologues in worms, frogs and human cells (Decker et al., 2011; Joukov et al., 2014; Meng et al., 2015). However, the previously reported

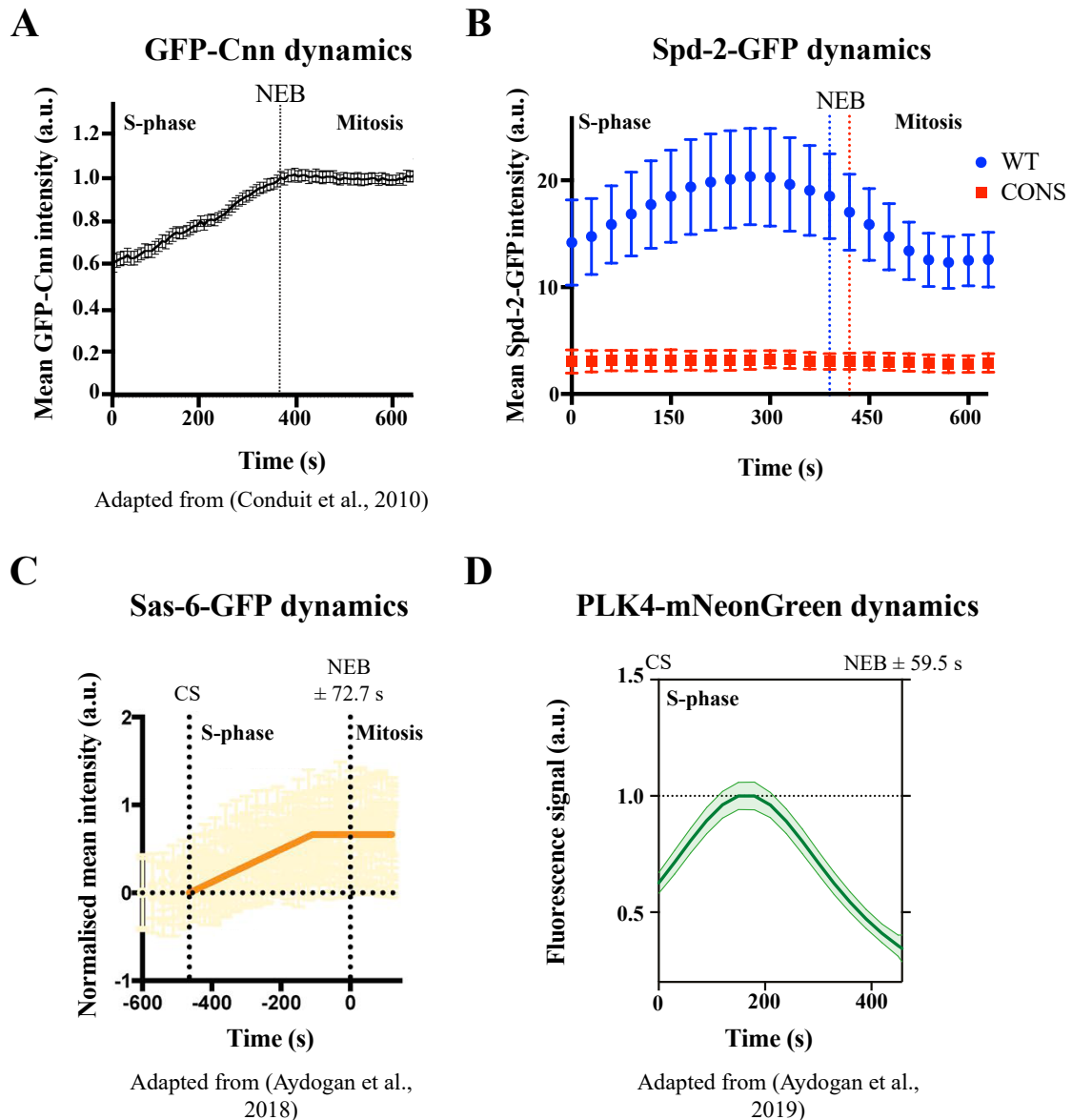


Figure 4.18 – Following the dynamics of Spd-2-GFP and other centrosomal proteins throughout the cell cycle.

(A) Average GFP-Cnn intensity of 20 centrosomes, error bars denote SEM. The amount of GFP-Cnn at the centrosome increases during S-phase and then remains constant as embryos enter mitosis. Adapted from (Conduit et al., 2010). (B) Graph compares the mean centrosomal Spd-2-GFP intensity through an entire embryonic cell cycle (nuclear cycle 12) in *spd-2*^{-/-} embryos expressing either WT Spd-2-GFP (blue) or Spd-2-CONS-GFP (red); $n = 155$ and 75 centrosomes analysed, respectively. The length of S-phase varies in individual embryos: $384 \text{ s} \pm 46 \text{ s}$ or $369 \text{ s} \pm 30 \text{ s}$ (mean $\pm$ SD) for WT Spd-2-GFP and Spd-2-GFP-CONS, respectively. Thus, we cannot simply average the data at each time point from multiple embryos, so one representative embryo per condition is shown here. The analysis of all 14 embryos per condition that were monitored in this way is shown in Figure 4.19 and Figure 4.21. Error bars indicate SD. (C) Centriolar Sas-6-GFP incorporation profile (orange line) calculated from modelling and averaging Sas-6-GFP intensity changes over time (underlying data from ≥ 15 embryos at nuclear cycle 12 is shown in *partial opacity*, error bars indicate SD). Adapted from (Aydogan et al., 2018). (D) Graphs show the mathematical regression of centriolar Plk4-NeonGreen dynamics during S-phase of cycle 12 (Regression Mean $\pm$ SEM). The underlying data was obtained from 37 centrosomes per embryo (on average), from ≥ 15 embryos. Adapted from (Aydogan et al., 2019). NEB = time of nuclear envelope break down, marks entry into mitosis, CS = time of centrosome separation, marks the beginning of a new nuclear cycle.

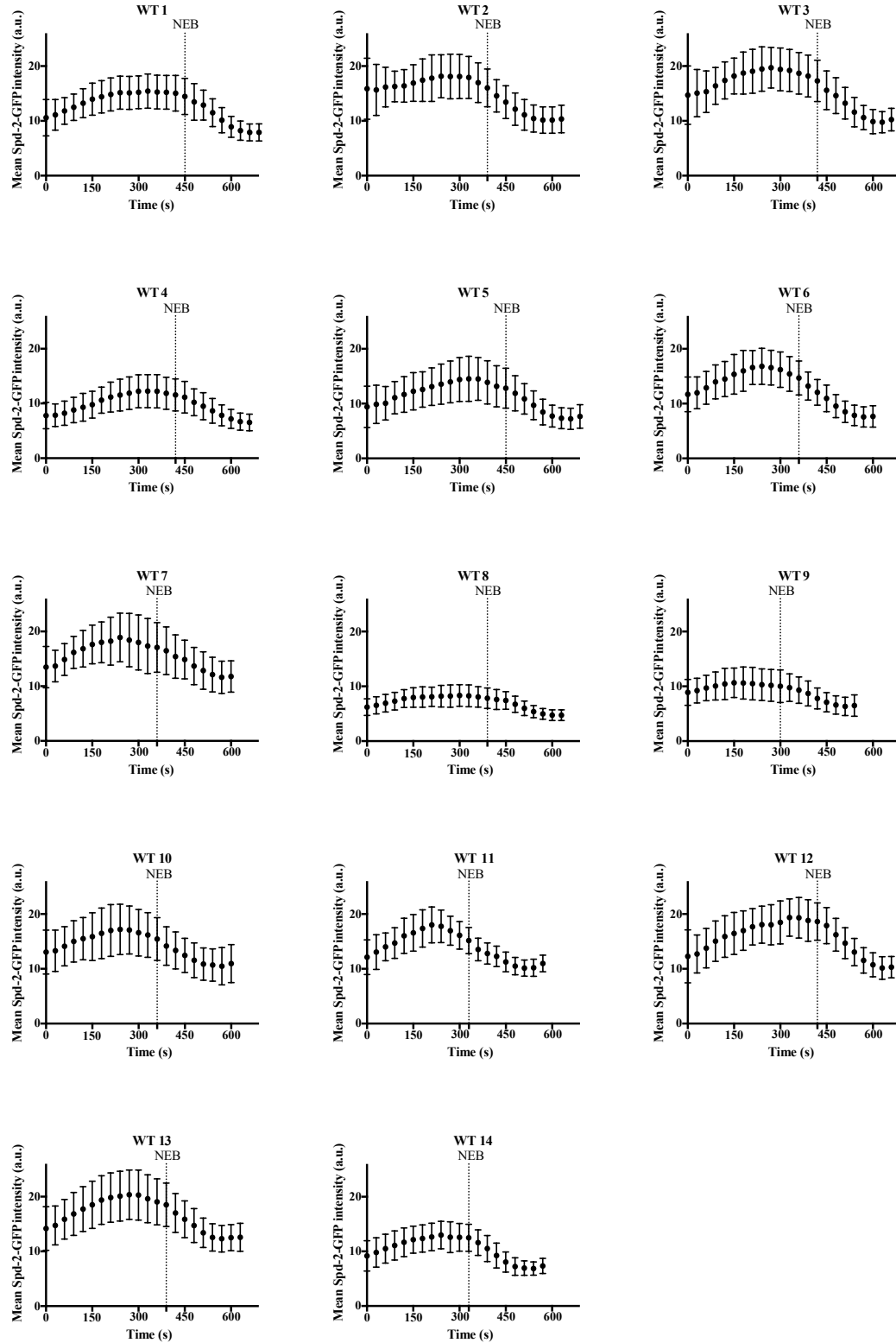
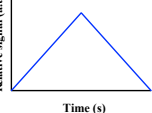
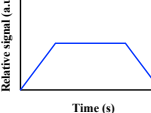
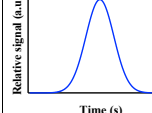
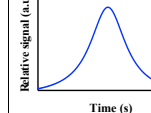


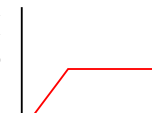
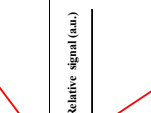
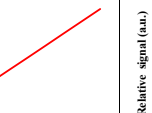
Figure 4.19 - Centrosome maturation dynamics in *spd-2*^{-/-} embryos rescued by WT Spd-2-GFP.

Graphs show the mean $\pm$ SD centrosomal WT Spd-2-GFP through an entire embryonic cell cycle (nuclear cycle 12) for 14 different embryos. Number of centrosomes analysed per embryo = 52-171. Time (s) is indicated; $t = 0$ was set as the time that centrosomes first separated at the start of S-phase. Dashed lines indicate nuclear envelope breakdown (NEB).

A

WT Spd-2-GFP data set	Increase - Decrease (1)	Increase - Plateau - Decrease (2)	Gaussian (3)	Lorentzian (4)
N = 14 embryos				
R ² value	0.96 ± 0.03	0.99 ± 0.01	0.96 ± 0.02	0.96 ± 0.02
R ² value (adjusted)	0.96 ± 0.03	0.98 ± 0.02	0.96 ± 0.02	0.96 ± 0.03
SS _{Absolute}	2.23 ± 1.62	0.87 ± 0.61	2.91 ± 2.40	3.16 ± 2.92
% of fit preference	14.3%	64.3%	7.1%	14.3%

B

Spd-2-CONS-GFP data set	Increase - Plateau - Decrease (2)	Straight line (5)	Constant (6)
N = 14 embryos*			
R ² value	0.70 ± 0.29	0.51 ± 0.37	NA
R ² value (adjusted)	0.62 ± 0.38	0.48 ± 0.39	NA
SS _{Absolute}	0.50 ± 0.60	0.69 ± 0.58	2.43 ± 1.71
% of fit preference	14.3%	57.1%	28.6%

* Data from 2 embryos did not converge for Increase – Plateau – Decrease model

Figure 4.20 – Summary of the models tested for the regression analysis of Spd-2-GFP dynamics during centrosome maturation.

The Spd-2-GFP dynamic data for each individual embryo (Figure 4.18, Figure 4.19 and Figure 4.21) was assessed using the nonlinear regression (curve fit) analysis function in GraphPad Prism 7. The functions tested are illustrated schematically in the graphs. R², adjusted R², and absolute sum-of- square values are used as a measure for goodness-of-fit. **(A)** The data for WT Spd-2-GFP was fitted against four different functions to assess the most suitable model: (1) user-defined linear growth followed by linear decrease; (2) user-defined linear growth followed by plateau followed by linear decrease; (3) Gaussian function; (4) Lorentzian function. The preferred model was (2). **(B)** The data for Spd-2-CONS-GFP was fitted against the preferred model for the WT data (2), but the data appeared to be described better as a straight line, so it was also fitted against two additional models: (5) a standard straight line; (6) a user-defined linear constant line. The preferred model was (5), although the average slope value was nearly zero (-0.0005 ± 0.0054 ; mean $\pm$ SD), indicating that the appropriate model in practice would be (6), a constant line.

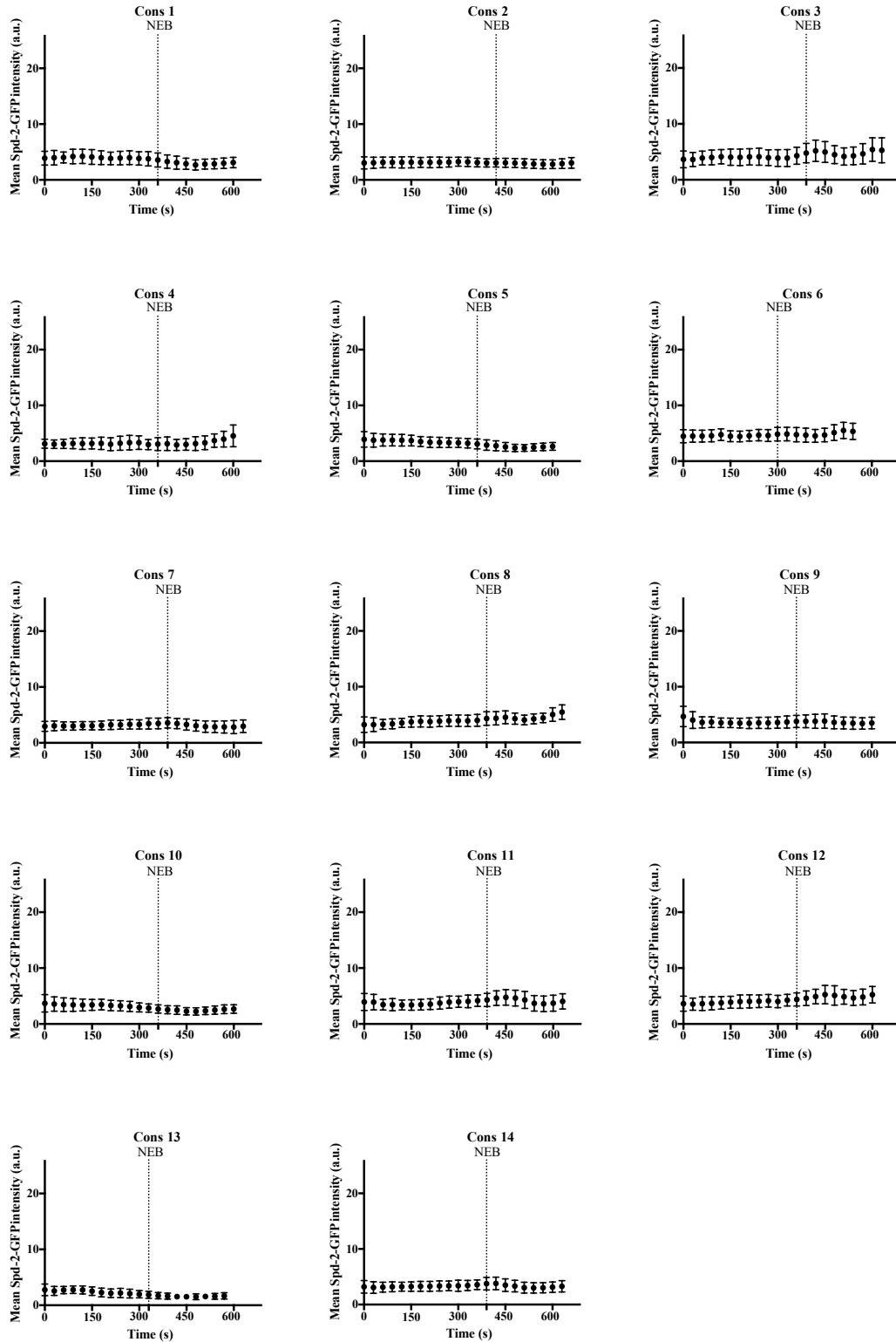


Figure 4.21 – Centrosome maturation dynamics in Spd-2-CONS-GFP embryos.

Graphs show the mean $\pm$ SD centrosomal Spd-2-CONS-GFP through an entire embryonic cell cycle (nuclear cycle 12) for 14 different embryos. Number of centrosomes analysed per embryo = 44-124. Time (s) is indicated; $t = 0$ was set as the time that centrosomes first separated at the start of S-phase. Dashed lines indicate nuclear envelope breakdown (NEB).

interactions relied only on one or two Ser-pSer/pThr sites. Conversely, in fly embryos it would appear that multiple sites in Spd-2 can be involved in Polo recruitment, hence the additive effect of mutating all ptPBSs compared to only a subset of them.

There are no obvious *Drosophila* ptPBSs that could be the equivalent to the sites that allow human Cep192 to interact with PLK1, but multiple sequence alignments show that, should they exist, they would localise to the N-terminus of Spd-2 (*red* boxes, Figure 4.22). Conversely, there is a clear *Drosophila* equivalent to the *C. elegans* site (Ser515-Thr516, *blue* box, Figure 4.22), which also localises to the N-terminal half of the protein. However, this site was one of the two ptPBSs mutated in the Spd-2-AA-mKate2 construct, which had no effect on PoloGFP distribution in the mRNA injection assay (see 3.2.2).

Thus, to test if the Polo recruitment role could be delimited to a specific region of Spd-2, I classified the 34 ptPBSs present along the sequence into two groups and I made two new mutant constructs: Spd-2-NT-mKate2, which has the 17 ptPBSs closest to the N-terminus of the protein mutated; and Spd-2-CT-mKate2, which has the other 17 ptPBSs mutated (Figure 4.2A and Figure 4.22). In all cases, ptPBSs were disrupted by mutating the Ser at position -1 to Thr. The mutants were tested via mRNA injection into PoloGFP embryos and radial profiling, as described in Chapter 3. Based on the previous studies, Spd-2-NT-mKate2 would be expected to alter PoloGFP recruitment to the centrosome, but not so Spd-2-CT-mKate2. Indeed, PoloGFP distribution was unaffected by Spd-2-CT-mKate2, whereas it became more centriole-like upon Spd-2-NT-mKate2 expression (Figure 4.23A,C[i]). However, this does not mean that centrosomal PoloGFP was unaffected in embryo expressing Spd-2-CT-mKate2: both Spd-2-NT-mKate2 and Spd-2-CT-mKate2 caused a highly statistically significant drop



Figure 4.22 – A multiple sequence alignment (MSA) of Spd-2 protein sequences from different species highlighting previously reported Polo binding sites.

MSA showing two isoforms of human Cep192, *C. elegans* SPD-2, and 13 different Spd-2 homologues from various *Drosophila* species. The previously identified Polo binding sites in human Cep192 and *C. elegans* SPD-2 are indicated in boxes (*red* and *blue*, respectively), and the potential corresponding *Drosophila* sequences, if any, are highlighted in the larger surrounding boxes. No obvious *Drosophila* motifs are found at equivalent positions for the human Cep192 sites (*red* boxes), but these regions are not well conserved between Cep192 and Spd-2 so equivalent positions are difficult to infer. There is a ptPBS conserved amongst *Drosophila* Spd-2 sequences at an equivalent position to the single *C. elegans* motif (*blue* box). This motif is potentially phosphorylated by Cdk1, so I also mutated the other ptPBS that is conserved amongst *Drosophila* species and that could be phosphorylated by Cdk1 (*yellow* box) to generate the Spd-2-AA-mKate2 construct. All the *Drosophila* sites that could be the equivalent of any of the previously identified sites (*red* and *blue* highlight boxes) are located within the group of 17 ptPBSs closest to the N-terminus of Spd-2 (NT group). The border between the NT and CT groups is indicated with the *black dashed* box.

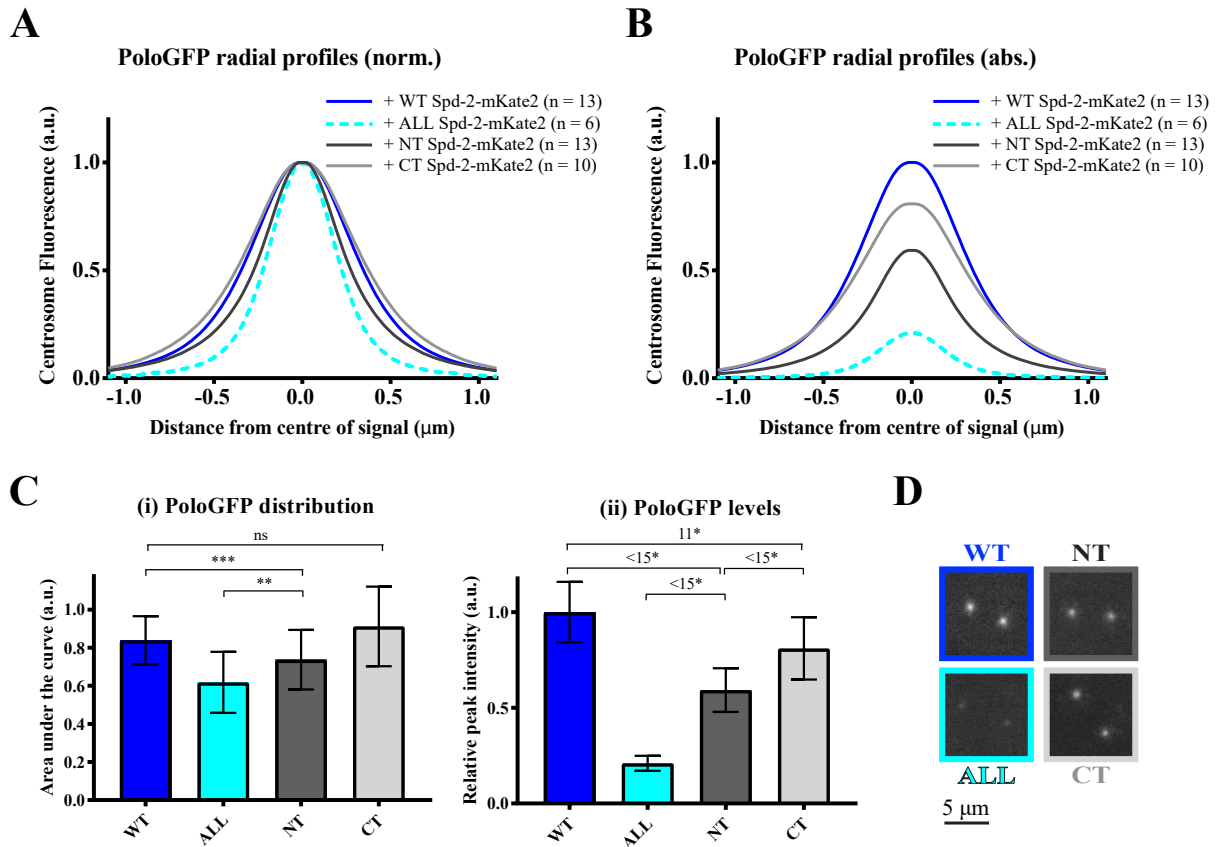


Figure 4.23 – Multiple sites throughout Spd-2 are involved in Polo recruitment.

(A) Normalised PoloGFP radial profiles after expression of WT or mutant Spd-2-mKate2 (ALL, NT or CT). (B) PoloGFP absolute radial profiles. (C) Parameters extracted from the PoloGFP radial profiles: area under the curve (i) from normalised profiles; and average peak intensity (ii), from the absolute profiles. (D) Micrographs showing examples of centrosomes in embryos injected with the different mRNAs. The loss of PoloGFP is most obvious in embryos injected with Spd-2-ALL-mKate2 mRNA. ns = non-statistically significant difference, p -value > 0.05 ; $** = 0.01 > p\text{-value} > 0.005$; $*** = 0.005 > p\text{-value} > 0.001$; $11^* = p\text{-value in } 10^{-11} \text{ (E-11) range}$; $15^* = p\text{-value in E-15 range}$. 5 centrosomes analysed per embryo, n = number of embryos. Error bars represent SD.

in centrosomic PoloGFP levels which is very likely biologically relevant (0.35 to 0.47 95% CI of difference NT to WT, p -value $<1E-15$; 0.12 to 0.26 CI of difference CT to WT, p -value = $1.4E-11$) (Figure 4.23B,C[ii]). Furthermore, embryos injected with Spd-2-CT-mKate2 mRNA showed more obvious signs of PCM defects (e.g. centrosome rocketing, nuclei dropping back into the interior of the embryo; data not shown) than those injected with Spd-2-NT-mKate2 mRNA. Neither Spd-2-NT-mKate2 nor Spd-2-CT-mKate2 had as strong effect on PoloGFP centrosomal distribution and levels as Spd-2-ALL-mKate2. Overall, this indicates that multiple sites in both the N- and C-terminal regions of Spd-2 play a role in Polo recruitment.

4.2.9. Spd-2 and the Polo Box Domain (PBD) directly interact *in vitro*

My conclusions from Chapters 3 and 4 rely on two key assumptions which are well supported in the literature: Polo relies on its PBD to localise to the centrosome (Elia et al., 2003a, 2003b; Jang et al., 2002a; Kachaner et al., 2017; Lee et al., 1998; Reynolds and Ohkura, 2003; Seong et al., 2002; Song et al., 2000), and mutating the Ser at position -1 into Thr would abolish canonical PBD binding to the site of interest (Elia et al., 2003a, 2003b). As PBD binding requires the binding site to be phosphorylated (Elia et al., 2003a, 2003b), and since Spd-2 appears to be phosphorylated only at the centrosome (P. Conduit, (Alvarez-Rodrigo et al., 2019); see 4.1.3), one would expect that Spd-2 and Polo only interact at the centrosome. Indeed, FCS experiments in worms have suggested that SPD-2 and PLK-1 do not form a stable complex in the cytoplasm (Wueseke et al., 2014), and I found that the same was true in fly embryos: when PoloGFP was immunoprecipitated from *Drosophila* embryo extracts (which had been centrifuged to remove centrosomes), cytoplasmic Spd-2 did not co-elute with it (Figure 4.24).

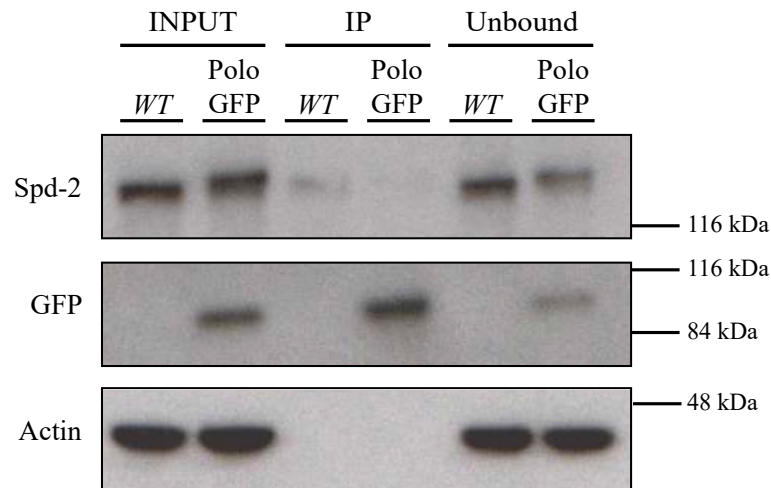


Figure 4.24 – Polo and Spd-2 do not interact in the cytoplasm.

Western blot of an immunoprecipitation experiment using extracts from PoloGFP and OregonR (*WT*) embryos (one technical repeat). An aliquot of each extract was taken prior to incubating the extracts with magnetic beads covalently bound to GFP antibodies (*INPUT* samples). The extracts and beads were incubated overnight. The extract was then removed (taking an aliquot for analysis, *Unbound* samples) and the beads were washed repeatedly before elution of the protein bound to the beads (*IP* samples). The samples were analysed by western blotting using antibodies against Spd-2, GFP and Actin, as indicated. As expected, most of the PoloGFP present in the PoloGFP extracts (*INPUT*) was bound by the antibody-coupled beads (*IP*). Conversely, *INPUT* and *Unbound* samples were enriched in Actin, which did not bind to the beads. Similarly, endogenous Spd-2 is present in the *INPUT* and *Unbound* samples and was not co-immunoprecipitated with the PoloGFP in the *IP* samples, suggesting that Polo and Spd-2 do not interact in the cytoplasm.

To test the two key assumptions, I wanted to recapitulate *in vitro* the interaction between Spd-2 and Polo. However, Spd-2 and its homologues in other species have been notoriously difficult to work with *in vitro*, and neither our lab nor collaborators have been able to produce functional, full-length *Drosophila* Spd-2 so far (A. Haensele, M. Ruer and M. van Breugel; personal communication). Thus, I started off by using extracts from embryos expressing WT or ALL Spd-2-GFP, from which I could isolate Spd-2-GFP proteins on beads. Because PBD binding requires the target site to be phosphorylated, the proteins on the beads were *in vitro* phosphorylated with commercial kinase. PLK1 was chosen as kinase because it is the one for which the most total/conserved ptPBSs are predicted phosphorylation targets (13/6, compared to 5/2 for CDK1 and 3/1 Aurora A), and because mutating the two conserved ptPBSs which are predicted CDK1 phosphorylation targets had no effect on PoloGFP distribution (Spd-2-AA-mKate2, see 3.2.2). The beads were then incubated with commercial human GST-PLK1-PBD (Sigma, SRP0360) and the samples were washed, eluted and analysed via SDS-PAGE and Western blot (Figure 4.25A).

After much optimisation of the buffers and conditions used, I could see some enrichment for GST-PBD in the WT Spd-2-GFP samples compared to those from Spd-2-ALL-GFP or Oregon-R WT embryos (negative control), or WT Spd-2-GFP samples treated with kinase storage buffer instead of PLK1 (non-phosphorylated control) (Figure 4.25B,C). This trend was most obvious when the signal was quantified and normalised for the amount of sample loaded in the gel (Figure 4.25D), but the effect was quite small and quite variable. The signal from non-phosphorylated WT Spd-2-GFP samples appeared lower than the negative control equivalents, which suggested that other proteins present in the extract (e.g. endogenous Spd-2) might be binding non-specifically to the beads, despite the extensive washing steps. Even if this included

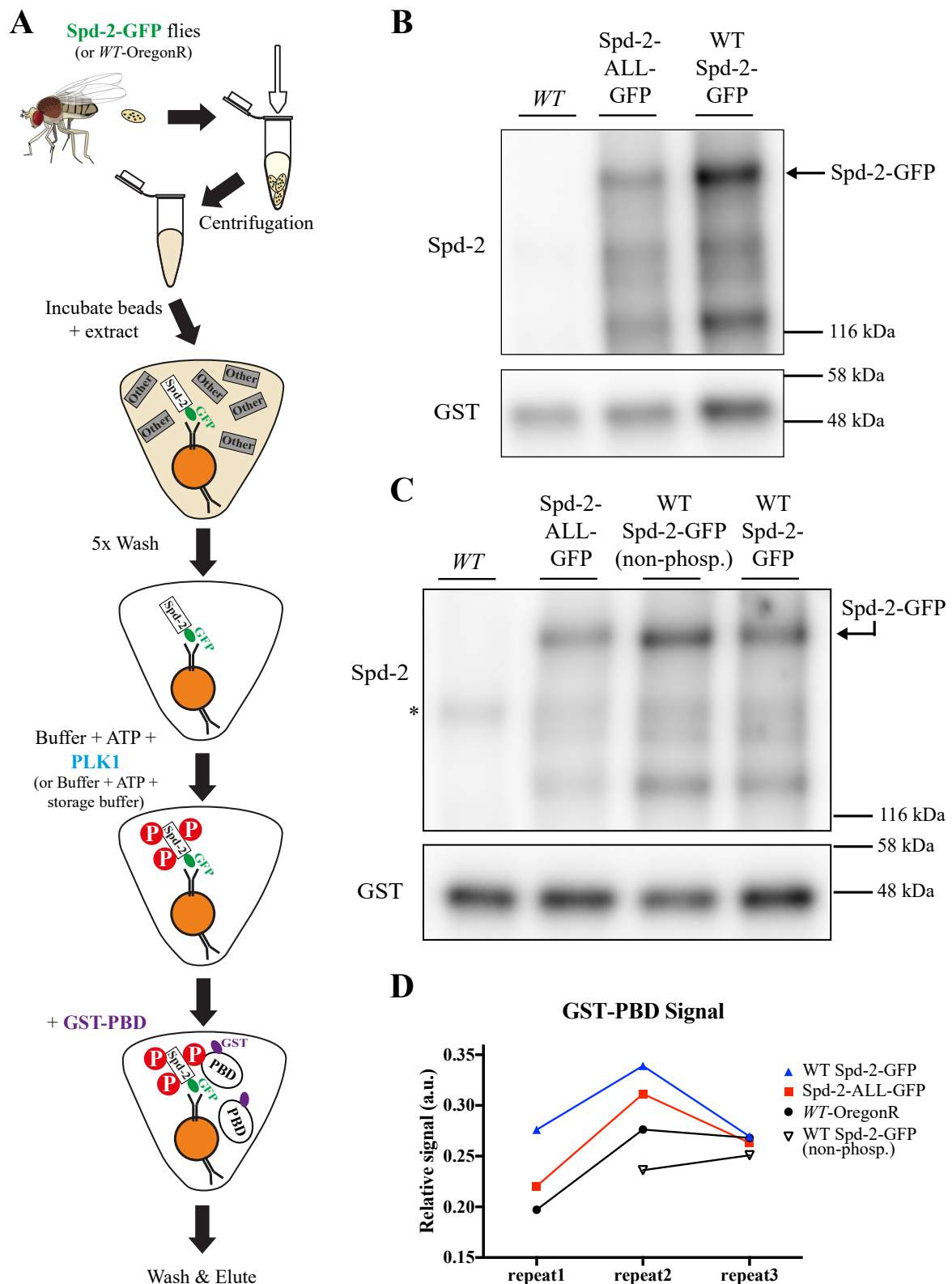


Figure 4.25 – Extract-based *in vitro* interaction assay to test Spd-2 binding to the PBD.

(A) Schematic of the interaction assay using embryo extract detailed in 2.4.3. (B,C) Results from two technical repeats. In (B), GST-PBD is enriched in the WT Spd-2-GFP sample compared to *WT* and Spd-2-ALL-GFP samples, but there is also more WT Spd-2-GFP compared to Spd-2-ALL-GFP. In (C), GST-PBD levels appear more similar between WT Spd-2-GFP, *WT* and Spd-2-ALL-GFP; with only a clear enrichment compared to a WT Spd-2-GFP treated with buffer instead of PLK1 (non-phosphorylated control, included in repeats #2 and #3). Some endogenous unlabelled Spd-2 in the *WT* control appeared to bind unspecifically to the beads (*). (D) Quantification of the GST-PBD signal, normalising for sample loading on gel. For all repeats, GST-PBD is most enriched in WT Spd-2-GFP samples and least enriched in non-phosphorylated controls.

small quantities of Polo binding proteins, upon *in vitro* phosphorylation they would be able to bind to GST-PBD and increase the background signal.

Since using embryo extracts was challenging, and recombinant full-length Spd-2 is not stable *in vitro*, I decided to produce *in vitro* a Spd-2 fragment. As Polo recruitment could not be linked to a specific site or region of the protein (see 4.2.8), I decided to focus on the middle 1/3 of Spd-2 (352-758 aa), a fragment which had been previously produced to raise antibodies against (Dix and Raff, 2007). This region contains 19 ptPBSs, 11 of which were conserved amongst *Drosophila* species. Protein production and purification was done in collaboration with S. Saurya and A. Haensele. Briefly, *E. coli* cells were induced to express a MBP-fusion protein including amino acids 352-758 from either WT or ALL Spd-2 (producing **MBP-Spd2-WT** and **MBP-Spd-2-19T**, respectively) (Figure 4.26A). Cells were homogenised and the MBP-fusion proteins were purified using an amylose column. We obtained nine 1-mL elution fractions containing a total of approximately 17 mg of protein (i.e. fragment of interest with co-eluted contaminants) per sample. Although MBP-Spd-2-19T was originally induced more weakly, it was more stable and easy to purify than MBP-Spd-2-WT. 5 mL of fractions for each construct were pooled for size exclusion chromatography (SEC; HiLoad 16/60 Superdex 200 column (GE Healthcare)). Gel analysis showed that fractions corresponding to the fastest eluting SEC peak contained protein of the expected size, clear from contaminants and/or degradation products (Figure 4.26B). For the full protocol, see 2.4.2.

I then repeated the *in vitro* phosphorylation and interaction assay, but using beads bound to MBP-Spd2-WT and MBP-Spd-2-19T instead of embryo extract (Figure 4.26C). As control, I compared MBP-Spd2-WT and MBP-Spd-2-19T samples treated

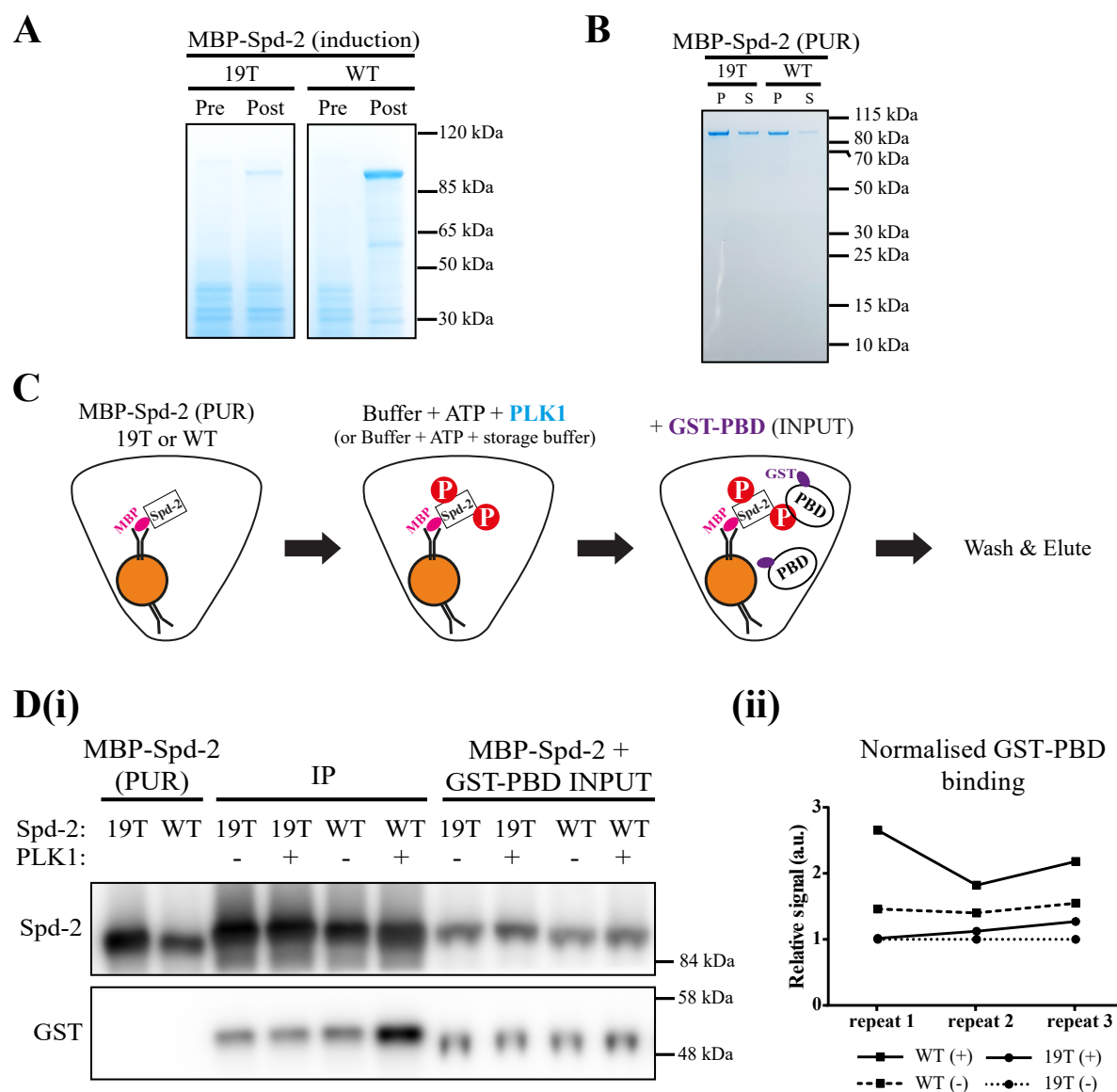


Figure 4.26 – Spd-2 and the PBD directly interact *in vitro*.

An MBP-tagged fragment of Spd-2 (352-758 aa) containing 19 ptPBSs, either WT or all of them mutated (Ser to Thr) was produced and purified *in vitro*. **(A)** SDS-PAGE analysis of bacterial pellets pre- and post-induction of protein expression with IPTG. MBP-Spd-2-19T was not as strongly induced as MBP-Spd-2-WT. **(B)** SDS-PAGE analysis of samples from purification (PUR) of MBP-Spd-2-19T and MBP-Spd-2-WT using an amylose column followed by size exclusion chromatography (SEC). Peak (P) and side (S) samples shown per construct. **(C)** Schematic of the interaction assay using recombinant protein detailed in 2.4.3. The MBP-tagged protein was incubated with beads covalently bound to anti-MBP antibody. The beads were then treated with kinase buffer containing ATP and either PLK1 or an equivalent volume of PLK1 kinase storage buffer (non-phosphorylated negative control). GST-PBD was then added to the beads (INPUT samples). After a 3 h incubation at 4 °C, the beads were washed to remove any unbound GST-PBD. Finally, the protein that remained bound to the beads was eluted (IP samples). **(D) (i)** Western blot of the samples obtained during the *in vitro* interaction assay, using antibodies against Spd-2 (*top* panel) or GST (*bottom* panel). **(ii)** A quantification of the level of GST-PBD enrichment in each sample. The amount of GST-PBD bound to the indicated beads was normalised to the amount of Spd-2 in each fraction. To facilitate comparisons between repeats, the data is shown normalised to the signal from each 19T(-) sample. Enhanced binding of GST-PBD to the phosphorylated WT protein was observed in all three technical repeats, but was somewhat variable. Due to the variation and the small sample size (n = 3), I could not determine whether the data was normally distributed, so I could not apply parametric tests for statistical significance. Non-parametric tests did not indicate a significant increase in binding to the phosphorylated protein (p < 0.05), but these tests do not work well with such a low number of repeats.

with PLK1 to MBP-Spd2-WT and MBP-Spd-2-19T samples treated with kinase storage buffer instead of PLK1. When treated with PLK1, MBP-Spd2-WT exhibited an enhanced ability to co-immunoprecipitate (IP) with recombinant GST-PBD. This phosphorylation-enhanced binding to GST-PBD was abolished in the MBP-Spd-2-19T samples (Figure 4.26D). Therefore, a fragment of Spd-2 can bind directly to the PBD *in vitro*. As expected from previous literature, this binding is prevented when the Ser-Ser/Thr motifs are mutated to Thr-Ser/Thr. The *in vitro* interaction is promoted by PLK1 treatment, which further suggests that the Spd-2/Polo interaction *in vivo* might rely on Polo self-priming.

4.3. Discussion

In this Chapter I have confirmed that Spd-2 is required for recruitment of Polo to the centrosome, specifically to the PCM. I have presented evidence for an interaction between these two proteins both *in vivo* and *in vitro*. If Spd-2 cannot interact with the PBD (or its capacity to do so is greatly diminished), Spd-2, Polo, Cnn and other PCM proteins are still recruited to the centriole wall, but the PCM fails to expand in preparation for mitosis. However, if Spd-2 can recruit Polo to the PCM, this promotes Cnn scaffold formation, which will further stabilise the Spd-2 scaffold (Conduit et al., 2014b), thus allowing more Spd-2, Polo and Cnn to accumulate in the PCM. Thus, mitotic PCM expansion would be driven by a positive feedback loop formed by the three proteins. While it cannot be completely ruled out that the Spd-2/Polo interaction is partly controlled by key cell cycle regulators such as CDK1 or Aurora A, *in vitro* evidence suggests that Polo is sufficient to phosphorylate some of the ptPBSs present in Spd-2 to allow PBD binding (i.e. self-priming). This model is discussed in further detail in Chapter 6.

Rather than mitotic PCM expansion relying mostly on an independent or autocatalytic Cnn scaffold (as has been suggested for the worm homologue SPD-5, (Woodruff et al., 2015)), my results are in agreement with previous reports that Spd-2, Cnn and Polo are all essential for centrosome maturation in fly (Conduit et al., 2014b; Dobbelaere et al., 2008; Feng et al., 2017) and worm embryos (Cabral et al., 2019; Decker et al., 2011). However, Cabral et al. suggest that SPD-2 does this by recruiting PLK-1 to centrioles or the PCM closest to centrioles, and that PLK-1 is maintained in the PCM via slowed diffusion (Cabral et al., 2019); whereas my data show that Spd-2 is needed to recruit Polo to the PCM and not to the centriole. Perhaps these contrasting observations are due to differences in how these protein are distributed throughout the PCM. In worms, the SPD-2 and PLK-1 signals appear as a bright “spot” at the centre of the PCM (near the centriole), but also as a faint “halo” of protein throughout the PCM, as extended as the SPD-5 scaffold (Cabral et al., 2019). However, in fly embryos Spd-2 and Polo do not spread out into the PCM as far as the Cnn scaffold (Figure 3.2, Figure 4.14).

The mutations introduced to generate Spd-2 proteins unable to bind the PBD were conservative (Ser to Thr), but many sites had to be mutated (Figure 4.2A). Thus, it was important to check that the mutant proteins were otherwise functional and not simply misfolded. To this end, I have presented several different pieces of evidence. First, the mutant proteins are stably expressed at levels very similar to those of WT Spd-2 fusion proteins. Second, the mutant proteins rescued the initial *spd-2*^{-/-} phenotype, allowing the embryo to develop beyond fertilization. Third, Spd-2-ALL and Spd-2-CONS can be recruited to the centriole wall. Based on the FRAP experiments, the initial recruitment rates of WT and CONS Spd-2-GFP appear to be similar, although it is challenging to distinguish between Spd-2-GFP recruitment to the centriole and maintenance at the PCM. These FRAP experiments would ideally be repeated in the

future using a system that allows for better temporal resolution. Finally, Spd-2-ALL and Spd-2-CONS can form part of the PCM scaffold as long as there is a minimal amount of WT Spd-2 there to recruit Polo and prevent the mitotic PCM from disassembling.

While Spd-2 and Cnn are not thought to incorporate as a complex into the PCM, Spd-2 is the most likely candidate for Cnn recruitment to the centrosome in fly embryos (Conduit et al., 2014b). Since Cnn is still recruited to the centriole wall in embryos expressing only Spd-2-CONS, it would appear that the mutations introduced do not disturb Cnn recruitment either. Y2H screens have revealed strong interactions between Spd-2 and Cnn (Boxem et al., 2008; Conduit et al., 2014b; Galletta et al., 2016); but these have not yet been recapitulated *in vitro*, probably because producing recombinant Spd-2 is very challenging. Furthermore, it has been suggested that the interaction between the *C. elegans* homologues SPD-2 and SPD-5 only happens in the context of the scaffold, and not between unassembled molecules (Woodruff et al., 2015; Wueseke et al., 2014). Thus, while it would be ideal to be able to study the Cnn/Spd-2 interaction *in vitro* and directly confirm that it is not disturbed by the ptPBS mutations, this might prove difficult in the near future.

Characterising the Spd-2 mutants was further complicated by the fact that there is much that we still do not understand about WT Spd-2 regulation and dynamics at the centrosome. For example, previous work in the lab has shown that Spd-2 is specifically phosphorylated at the centrosome (Figure 4.1). However, we lack an antibody that specifically recognises a phospho-epitope in Spd-2 that would allow us to distinguish between total and phosphorylated Spd-2 at the centrosome (as we can for Cnn), or to track changes in the phosphorylation state of centrosomal Spd-2

throughout the cell cycle. During the course of my studies, we raised a rabbit antibody against the phospho-peptide S-N-S-pS-E-F-S-H-R-D-G-K-L-L-C, where pS represents a phosphorylated Ser residue corresponding to Ser688, which is a predicted PLK1 phosphorylation target and part of a conserved ptPBS in *Drosophila*. However, when I tested this antibody using immunofluorescence, it failed to recognise anything (data not shown). It would be interesting to repeat the experiments in Figure 4.1 to test if the mutant forms of Spd-2 are still phosphorylated at the centrosome. However, collecting enough mutant material to do the biochemistry could be very time-consuming, and the results would probably not be easily comparable to any WT controls, as we would expect a generalised loss of Spd-2 compared to WT samples due to the PCM expansion defect.

With regards to Spd-2 dynamics, during my studies I have observed that WT Spd-2 levels at the centrosome present a wave-like behaviour (*blue*, Figure 4.18B; Figure 4.19), suggesting that Spd-2 oscillates with the cell cycle. It is not unexpected that centrosomal Spd-2 levels would increase during S-phase in preparation to mitosis, as this has also been reported for Cnn (Conduit et al., 2010; Figure 4.18A). However, it is striking that they would reach their peak before NEB and then start decreasing as the embryo enters mitosis, rather than be maintained at a maximum, like Cnn is (Conduit et al., 2010).

Interestingly, our lab has recently shown that Sas-6-GFP is incorporated linearly during S-phase as the daughter centriole cartwheel is built (Aydogan et al., 2018; Figure 4.18C). Centriole growth is controlled by Sak/PLK4, which also appears to oscillate throughout the cell cycle (Aydogan et al., 2019; Figure 4.18D). The idea that Spd-2 could be a regulatory oscillator that controls mitotic PCM assembly like the

PLK4 oscillator controls cartwheel growth is a very attractive hypothesis, however these two systems are very different in nature. First, Spd-2 is not an enzyme, although its coupling with Polo could make up for this. Second, PLK4 dynamics fit simple Lorentzian and Gaussian regression models reasonably well and have been successfully described by mathematical modelling (Aydogan et al., 2019), but this was not the case for Spd-2 dynamics (see 4.2.7). Finally, the centriole cartwheel grows stably and unidirectionally, whereas the PCM is an extremely complex, variable structure that grows outwards in all directions. In addition to changes in volume, the density of material within the PCM might also change in response to centrosome age, cell cycle progression, or as embryos develop (see 4.2.3). Clearly a lot more work is required to characterise WT Cnn and Spd-2 dynamics in the developing *Drosophila* embryo.

Chapter 5 - Polo recruitment to the centriole as a novel

function of Ana1

5.1. Introduction

5.1.1. Ana1, a mysterious centriolar protein

Ana1 was first identified as an important player in centriole assembly in two RNAi screens (Dobbelaere et al., 2008; Goshima et al., 2007). Depletion of Ana1 leads to mitotic spindles forming without centrosomes at their poles (i.e. *anastral* spindles, hence the name). Furthermore, flies that are *ana1*^{-/-} mutant are uncoordinated due to the absence of basal bodies, which leads to loss of cilia in mechanosensory neurons (Blachon et al., 2009), similar to other fly strains with centriole assembly defects (see 1.1.3).

Initially, it was suggested that Ana1 might be involved in Sas-6 incorporation and/or centriole formation (Goshima et al., 2007). However, it is now clear that *Drosophila* Ana1 and its human homologue Cep295 (also known as KIAA1731) play a key role in the conversion of daughter centrioles into fully functional mother centrioles able to duplicate and recruit PCM, rather than centriole duplication *per se* (Chang et al., 2016; Fu et al., 2016; Izquierdo et al., 2014; Knorz et al., 2010; Saurya et al., 2016; Tsuchiya et al., 2016). For example, while CEP295 knockdown or deletion leads to a reduction in the number of centrosomes, within each centrosome there are two centrioles (mother and daughter) rather than a singlet (Izquierdo et al., 2014). Independent of its role in centriole conversion, Cep295 has also been shown to interact with tubulin and could potentially regulate centriole length (Chang et al., 2016).

How Ana1 contributes to centriole conversion is a point of some contention. One study (Fu et al., 2016) reported that in *Drosophila* S2 cells, the N-terminal region (1-935 aa) interacts with Cep135, and that this recruits Ana1 to centrioles and helps maintain Cep135 at the centriole wall, while the C-terminal region (756-1729 aa) recruited Asl to centrioles. Asl was not recruited to daughter centrioles if endogenous Ana1 was depleted using RNAi, and only a form of Ana1 containing the C-terminus of the protein was able to rescue centriole conversion. Thus, Cep135, Ana1 and Asl would be sequentially recruited and form a “protein bridge” essential for centriole conversion, in a manner that did not require Sas-4 (Figure 1.5A).

However, soon after it was reported that CDK1 phosphorylation of Sas-4 and recruitment of Polo to the centriole were crucial for Asl recruitment during centriole conversion in fly embryos (Novak et al., 2016). Furthermore, a different study into Ana1 function in fly embryos (Saurya et al., 2016) found that expression of GFP-Ana1delCT, an Ana1 mutant with the C-terminal deletion (756-1729 aa) did not result in complete loss of Asl, unlike what would be expected from the observations by Fu et al. Instead, although the Cep135-interacting region was intact in this mutant, the protein localised more weakly to the centrosomes. The lower levels of GFP-Ana1delCT compared to WT were linked to a decrease in Asl levels, but Asl was preferentially lost from the older mother centrioles within each pair, rather than from the centrioles that had just been converted into young mothers. Thus, Saurya et al. proposed that Ana1 was required for Asl maintenance at mother centrioles, whereas Asl initial recruitment during centriole conversion would be dependent on Sas-4, as proposed previously (Novak et al., 2016).

5.1.2. Key findings

In this Chapter, I have explored in-depth the role of Ana1 in Polo recruitment to the centrosome. Here I show that the Ana1 mutant tested in Chapter 3 is a functional protein able to perform Ana1's best known role (Asl recruitment and/or maintenance). However, expression of this mutant leads to a generalised loss of Polo from both the centriole and the PCM. Centrioles expressing this mutant are still able to duplicate, suggesting that centriole conversion and disengagement are mostly unaffected. Conversely, PCM assembly is majorly impaired, leading to accumulation of spindle abnormalities and aberrant divisions. This suggests that Ana1 is (directly or indirectly) involved in the recruitment of most centrosomal Polo, thus being a crucial regulator of centrosome maturation.

5.2. Results

5.2.1. Generation and characterization of fly lines expressing Ana1-ALL, a mutant protein unable to recruit Polo

The results from Chapter 3 revealed a potential novel role of Ana1 in recruiting Polo to the centrosome, either directly (e.g. interacting with the PBD) or indirectly. To confirm and further explore this result, I made transgenic lines expressing either WT Ana1 (control) or Ana1-ALL, which has all 34 ptPBSs present in *D. melanogaster* Ana1 with the Ser at position -1 mutated to Thr. Following the same strategy used to characterise the Spd-2 ptPBS mutants, the constructs were expressed with a C-terminal fluorescent tag (GFP or mCherry) under the control of the *ubiquitin* promoter. An Ubq-GFP-Ana1 transgenic line has been previously characterised in the Raff laboratory and shown to fully rescue the mutant phenotype, although the fluorescently-

tagged protein was 3-5X overexpressed compared to endogenous Ana1 and centriole length was slightly increased (Saurya et al., 2016).

The uncoordinated phenotype was rescued in *ana1*^{-/-} mutant flies expressing WT Ana1-GFP or Ana1-mCherry control transgenes, and these females were viable and fertile. Strikingly, Ana1-ALL-GFP and Ana1-ALL-mCherry also rescued the uncoordinated phenotype in *ana1*^{-/-} mutant females (Figure 5.1A). This is strong evidence that, despite the many mutations introduced, Ana1-ALL-GFP and Ana1-ALL-mCherry retain at least some functionality and are not simply misfolded (see also below). Western blotting revealed that the fluorescently-tagged proteins were somewhat overexpressed (2.5-3X) compared to endogenous Ana1 levels. However, Ana1-ALL and WT Ana1 were expressed to similar levels in fly embryos (Figure 5.1B,C), suggesting that the mutant proteins are not unstable in the cytoplasm. Nevertheless, unlike the WT-rescued females, *ana1*^{-/-} mutant females expressing Ana1-ALL transgenes were sterile. Even *ana1*^{+/-} heterozygous females expressing Ana1-ALL transgenes had their fertility compromised (data not shown), presumably due to the large amount of Ana1-ALL competing out the endogenous unlabelled protein. Overall, these results suggests that Ana1-ALL can be incorporated into centrioles, outcompete the endogenous protein, and contribute to centriole assembly enough to rescue the main aspect of the *ana1*^{-/-} phenotype (uncoordination), but it is not completely functional.

To further explore the Ana1-ALL phenotype, I collected 1-2 h old fertilised embryos from *ana1*^{-/-} mutant females and stained them to visualise MTs and nuclei. 92.5% and 83.2% of *ana1*^{-/-} embryos expressing WT Ana1-mCherry or Ana1-ALL-mCherry (respectively) developed beyond the first mitotic division (n > 50 embryos scored per

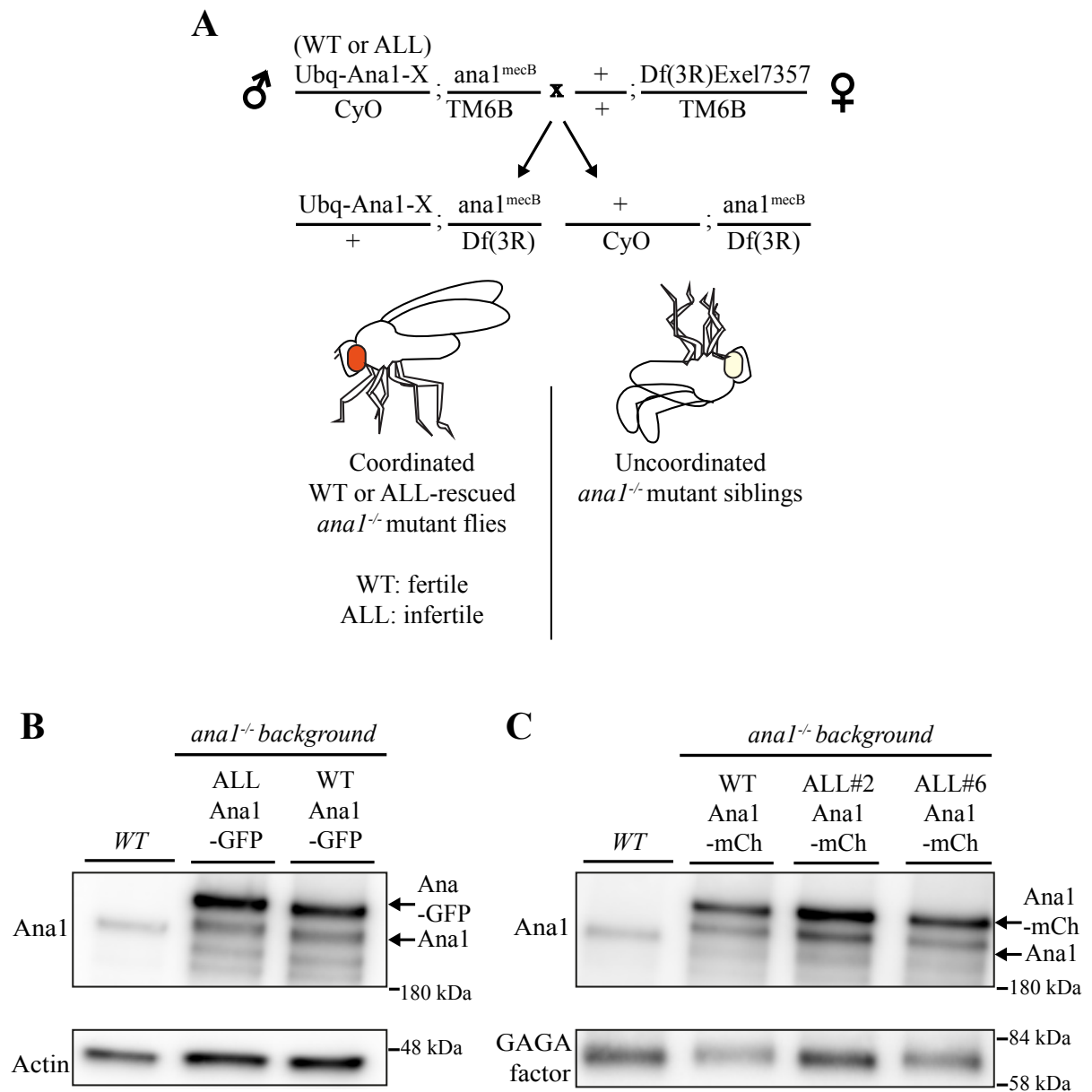


Figure 5.1 – Characterisation of Ana1 transgenic fly lines.

(A) Schematic of the crossing strategy to generate *ana1*^{-/-} flies (*ana1*^{mecB}/*Df(3R)Exel7357*) expressing an Ana1 transgene (WT or ALL mutant) with a C-terminal fluorescent tag X (GFP or mCherry), under the control of the *ubiquitin* promoter. The transgene is associated to a copy of the *w*⁺ gene to track its inheritance. This cross generated two different types of *ana1*^{-/-} flies: those that inherited the Ana1 transgene had orange eyes and were coordinated, whereas their siblings that inherited instead the *Cyo* chromosome had curly wings, white eyes and were uncoordinated. (B,C) Western blot of total Ana1 levels in OregonR (*WT*) embryos compared to *ana1*^{-/-} embryos expressing: Ana1-ALL-GFP or WT Ana1-GFP transgenes under the *ubiquitin* promoter (B); or WT Ana1-mCherry or Ana1-ALL-mCherry transgenes under the *ubiquitin* promoter (C). Two different Ana1-ALL-mCherry fly lines were tested, Ana1-ALL-mCherry #6 was selected for all following experiments. Actin and GAGA transcription factor (Raff et al., 1994) were used as loading controls. Each blot represents one technical repeat.

condition, one technical replicate). However, although *ana1*^{-/-} embryos expressing either Ana1-ALL-GFP or Ana1-ALL-mCherry (hereafter **Ana1-ALL-GFP embryos** and **Ana1-ALL-mCherry embryos**, respectively) were able to start developing, they died early in embryonic development and almost never hatched as larvae (~1/500 embryos hatching; n > 2000 embryos scored), whereas 76-80% of WT-rescued embryos hatched as larvae. This resembles the phenotype of Spd-2-CONS-GFP and Spd-2-ALL-GFP embryos (see Chapter 4), and that of *cnn*^{-/-} mutant embryos (Megraw et al., 1999).

5.2.2. Expression of Ana1-ALL leads to severe mitotic abnormalities

To investigate if Ana1-ALL-GFP embryos die before cellularisation due to accumulation of mitotic abnormalities, I looked again at 1-2 h old embryos stained for MT and DNA, focusing on those at the syncytial blastoderm stage where at least one mitotic spindle was visible. The nuclei in WT-rescued embryos appeared to divide synchronously, with well-formed mitotic spindles (~ 96% of spindles had one centrosome at each pole) that were evenly distributed. Detached centrosomes were rarely visible in these embryos (Figure 5.2A).

Conversely, Ana1-ALL-GFP embryos presented giant, deformed nuclei (*arrowheads*), large barrel-shaped spindles (*empty arrowhead*) and linked spindles sharing the same pole (*asterisks*, 8% of all centrosomes scored, 16% of all poles scored). Additionally, I found spindles with no centrosomes (*empty circles*, 17% of all spindle poles scored), spindles with too many centrosomes (*arrows*, 4% of all spindle poles scored), and detached centrosomes (*empty arrows*, 26% of all centrosomes scored) (Figure 5.2B). Only one out of ten Ana1-ALL-GFP embryos analysed did not show any defects whatsoever, but this embryo also appeared to be the youngest of the set, and would

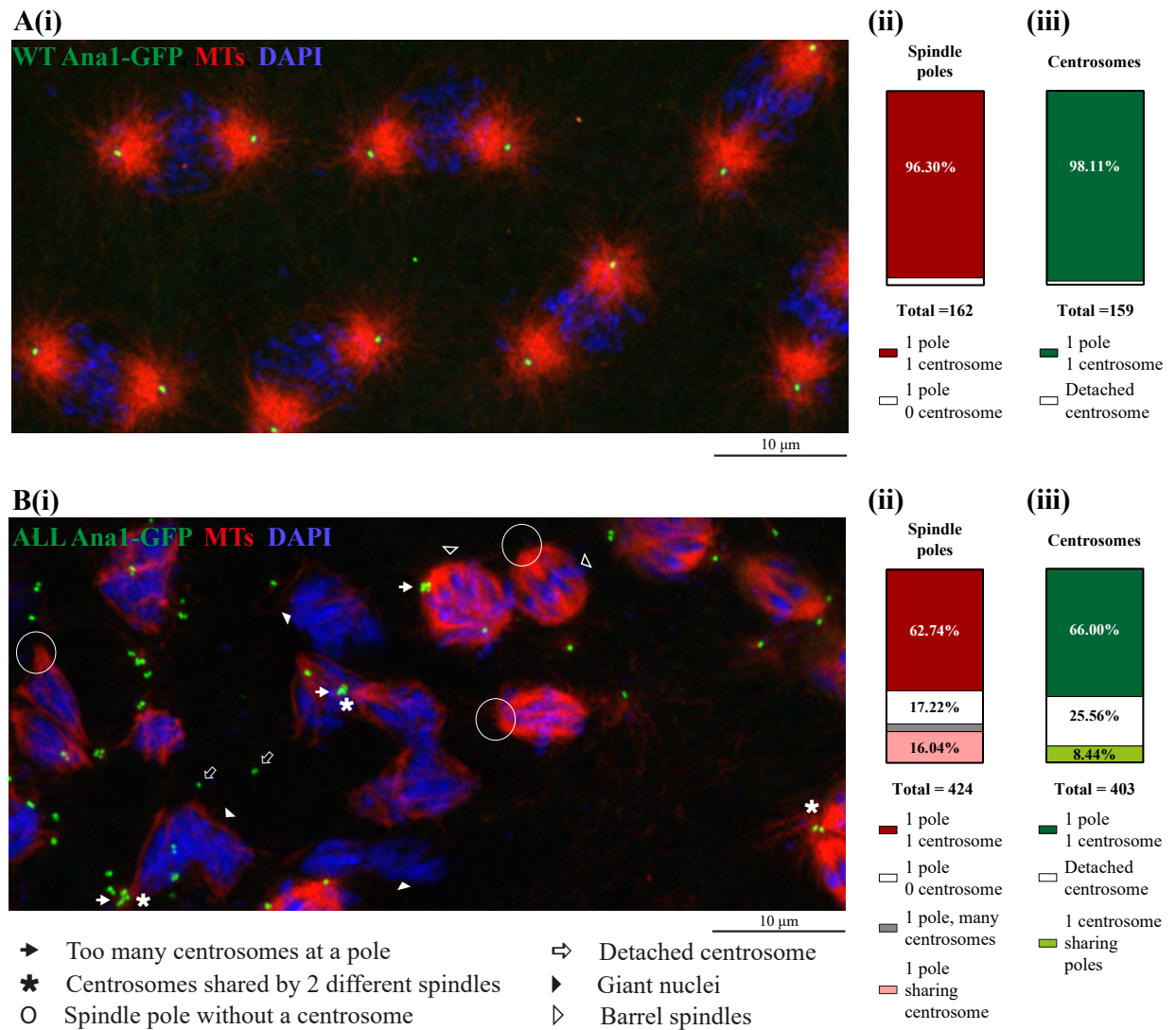


Figure 5.2 – Ana1-ALL-GFP accumulate severe mitotic abnormalities.

Fixed *ana1*^{-/-} embryos expressing WT Ana1-GFP (**A**) or Ana1-ALL-GFP (**B**) (green) were stained using an anti- α -tubulin antibody (to visualise MTs, red) and DAPI (to visualise nuclei, blue). While mitotic abnormalities were rare in WT-rescued embryos, they were very frequent in Ana1-ALL-GFP embryos. **(i)** Example micrographs. The different types of defects observed are indicated in the figure legend. Note that only a few examples of each defect are marked in the Ana1-ALL-GFP embryo image in order to facilitate its visualisation. **(ii-iii)** Quantification of some of the mitotic defects observed in fixed WT and ALL Ana1-GFP rescued embryos undergoing mitosis ($n = 8$ and 10 , respectively, from one technical repeat). **(Aii)** A total of 162 individual spindle poles from WT-rescued embryos were scored, 96.3% of which were “normal” (i.e. associated with one centrosome that was not linked to another spindle). **(Aiii)** Similarly, 98.11% of 159 centrosomes in WT-rescued embryos were “normal” (associated with a single spindle pole). Conversely, only 62.74% of 424 individual spindle poles and 66% of 403 centrosomes in Ana1-ALL-GFP embryos could be classified as “normal” (**Bii** and **Biii**, respectively).

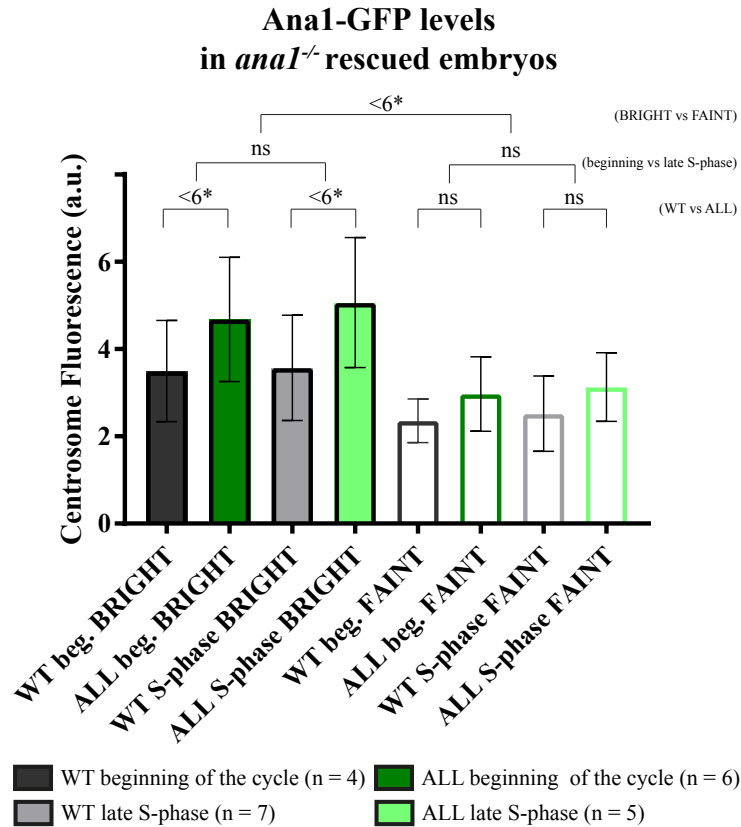
therefore not be expected to have accumulated as many abnormalities. These defects are similar to those described in *cnn*^{-/-} embryos (Megraw et al., 1999), and overall this phenotype is consistent with the observations in Chapter 3 that ptPBSs in Ana1 might be required for correct mitotic PCM assembly (see 3.2.4).

5.2.3. WT and ALL Ana1 are similarly recruited to mother and daughter centrioles

The rescue of the uncoordinated phenotype in *ana1*^{-/-} flies suggests that Ana1-ALL-GFP is not misfolded and can be recruited to the centrosome similarly to WT Ana1-GFP. However, I tested this directly by measuring the intensity of the Ana1-GFP signal in centrosomes of Ana1-ALL-GFP and WT-rescued embryos (Figure 5.3A).

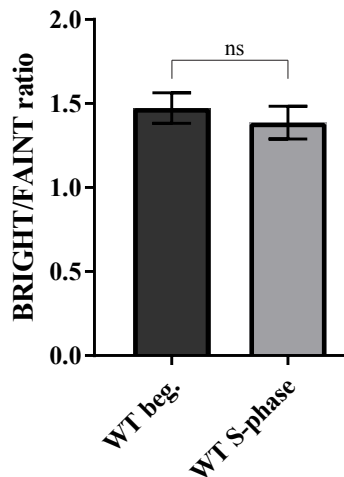
As has been previously reported using Ubq-GFP-Ana1 flies (Saurya et al., 2016), WT Ana1-GFP and Ana1-ALL-GFP exhibited a slight asymmetry, being enriched in one centrosome of every pair (presumably the older mother). I analysed embryos at two different points of the cell cycle, and divided each dataset into two subgroups, with the brighter centrosomes from each pair being referred to as the “BRIGHT” subset, in contrast to the “FAINT” subset. Strikingly, the levels of Ana1-ALL-GFP appeared to be statistically higher than those of WT Ana1-GFP in the “BRIGHT” subset. There was also a slight increase in Ana1-ALL-GFP levels compared to WT in the “FAINT” subset, but the difference was not statistically significant (Figure 5.3A). Therefore, the mutations introduced do not stop Ana1-ALL-GFP from being recruited to the centrosome. A possible explanation for why the difference between WT and ALL is significant for the “BRIGHT” subset but not for the “FAINT” subset is that, when centrosomes first start incorporating Ana1, the WT and ALL Ana1-GFP are recruited to very similar degrees. Over time Ana1-GFP accumulates at older centrosomes

A



B

BRIGHT/FAINT intensity ratio



C

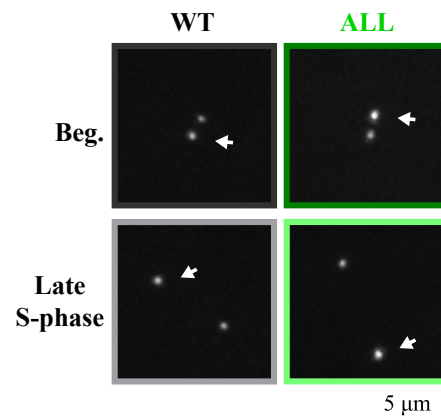


Figure 5.3 – WT and ALL Ana1-GFP are similarly recruited to centrioles.

(A) Graph showing mean centrosomal Ana1-GFP intensity at the beginning of the cycle (*dark fill/lines*) and at the end of S-phase (*light fill/lines*) from WT-rescued and Ana1-ALL-GFP embryos (n = number of embryos analysed per category, 10 pairs of centrosomes analysed per embryo). For each centrosome pair, the centrosome with the highest value was classified as “BRIGHT” (*filled boxes*), and the other was classified as “FAINT” (*white boxes*). For all the conditions analysed, the difference between the “BRIGHT” and “FAINT” subsets was highly statistically significant. (B) For every WT-rescued embryo analysed in (A) I calculated the BRIGHT/FAINT ratio: relative intensity of the Ana1-GFP signal from the BRIGHT centrosomes divided by the relative intensity of the signal from the FAINT centrosomes. The resulting values were averaged to calculate a mean per condition (beginning or end of S-phase). (C) Example micrographs, indicating the “BRIGHT” centrosome from each pair (*arrow*). Error bars indicate SD. ns = non-statistically significant difference, *p*-value > 0.05; <6* = *p*-value < 10⁻⁶ (E-6).

(“BRIGHT” subset), with slightly more protein accumulating in Ana1-ALL-GFP embryos.

It has been proposed that Ana1 is irreversibly incorporated into daughter centrioles from the beginning of S-phase (in parallel to the growth of the centriole cartwheel), and then continuing during mitosis (unlike Sas-6 incorporation, which stops before mitosis, Figure 4.18C) (Saurya et al., 2016). This is consistent with reports that human CEP295 appears enriched at the nascent daughter centriole in immunogold electron microscopy experiments (Chang et al., 2016) and that it can localise to the extra daughter centrioles induced by PLK4 overexpression (Izquierdo et al., 2014).

Conversely, in interphase *Drosophila* S2 cells Ana1 was mainly associated with mother centrioles, and it was rarely seen associated with daughter centrioles until prophase; unlike cartwheel proteins such as Sas-6 which were seen associated with both mother and daughter centrioles at interphase (Fu et al., 2016; Figure 5.4A). This would suggest that Ana1 is not recruited to daughter centrioles until after the cartwheel has been built and the centriole maturation process has started. Furthermore, another member of the Raff laboratory has observed that Ana1, Asl and Cep135 are all incorporated (in this order) into the new centriole at about the time that it separates from its mother (A. Wainman, unpublished).

Interested by this diversity of observations, I decided to study Ana1-GFP distribution at the centrosome with 3D-SIM. At the beginning of the cell cycle (determined by centrosome separation), WT Ana1-GFP localised at the mother centriole wall (appearing as a ring, Figure 5.4B). If Ana1-GFP were incorporated during S-phase to the daughter centriole, one would expect Ana1-GFP in most centrosomes of embryos in mid/late S-phase to present as a ring (corresponding to the mother centriole) and a

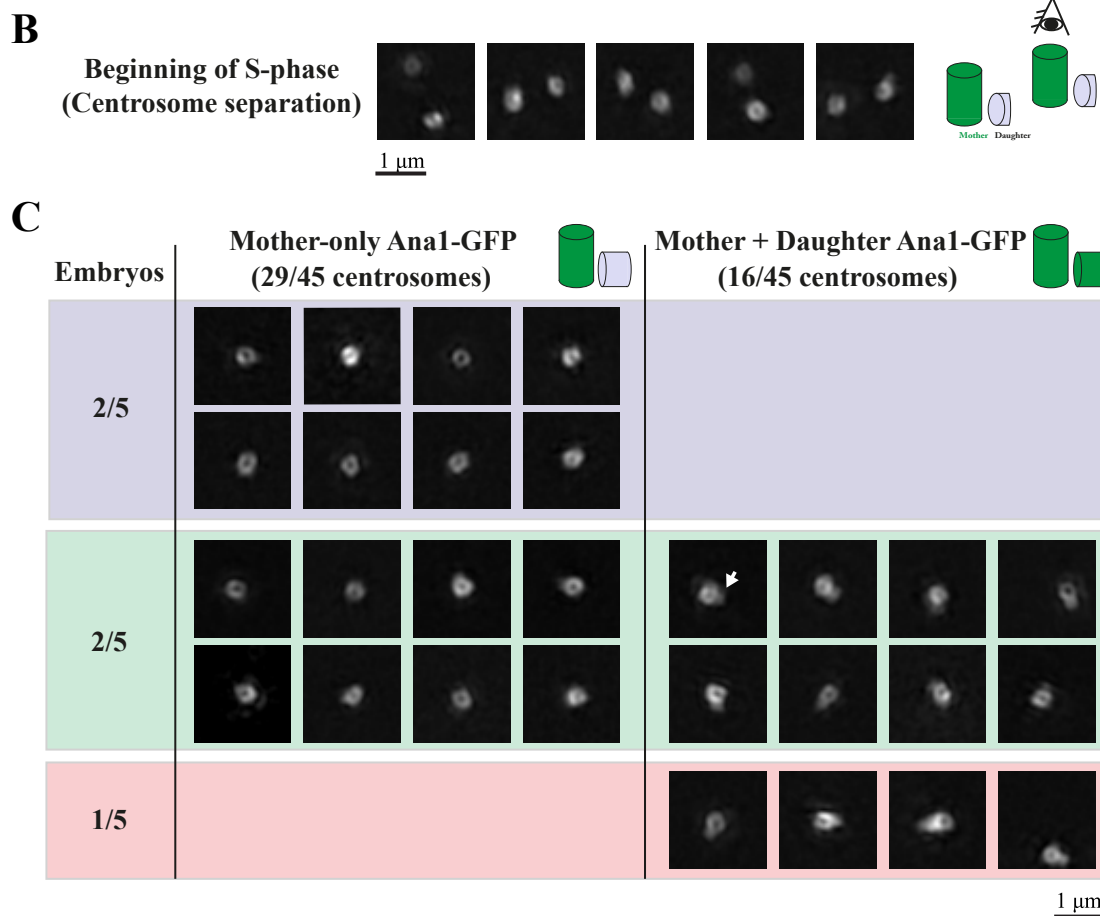
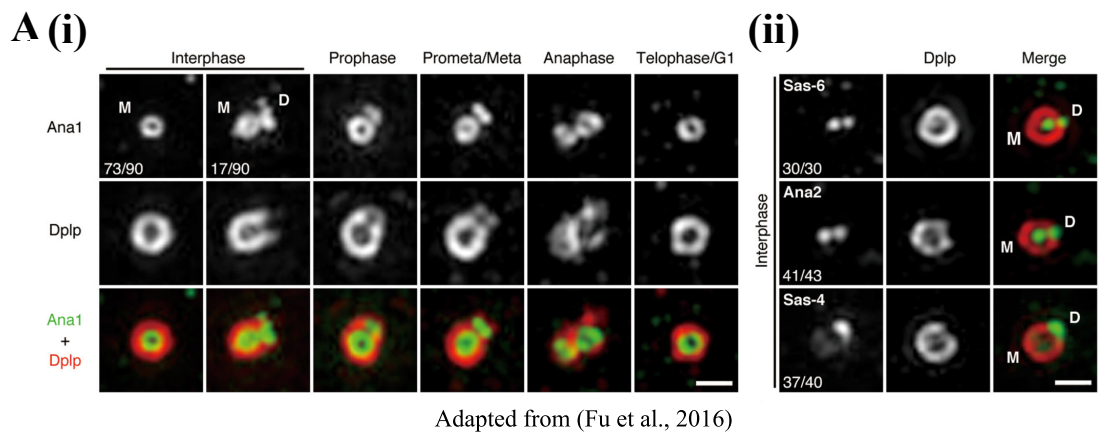


Figure 5.4 – WT Ana1-GFP localises preferentially to mother centrioles during S-phase.

(A) 3D-SIM images of *Drosophila* S2 cells immunostained to reveal the localisation of endogenous Ana1 throughout the cell cycle (i) and compare it to that of other proteins (Sas-6, Ana2 and Sas-4) that localise to the daughter centriole in interphase (ii). Scale bars: 500 nm. Adapted from (Fu et al., 2016). (B) Live 3D-SIM images of WT Ana1-GFP rescued embryos. At the beginning of S-phase Ana1-GFP localises to the separating mother centrioles (see diagram). Mother centrioles tend to be oriented perpendicularly to the plane of imaging, so Ana1-GFP appears as a ring. (C) Examples of live 3D-SIM images of WT Ana1-GFP rescued embryos in mid/late S-phase. Two out of five analysed embryos presented Ana1-GFP only at the mother centriole (blue box), two other embryos (green box) had centrosomes with Ana1-GFP only at the mother centriole and centrosomes with Ana1-GFP present in both mother and daughter centrioles, and only one of the five embryos analysed presented only centrosomes with Ana1-GFP localisation to both centrioles (red box). An example of Ana1-GFP at a daughter centriole is indicated with an arrow. 45 individual centrosomes were analysed in total, selected if the reconstruction was deemed of sufficient quality by SIM-Check (Ball et al., 2015) and if the mother centriole ring appeared well-oriented.

dot (corresponding to the growing daughter). For my analysis, I focused on embryos in mid/late S-phase and selected specific centrosomes based on reconstruction reliability (as judged using SIM-Check (Ball et al., 2015)) and clear reconstruction of the mother-ring (to control for centriole orientation).

In total, I analysed five embryos and 45 centrosomes, only 16 of which showed Ana1-GFP at the mother centriole and also at the daughter (*arrow*, Figure 5.4C).

Furthermore, in 2/5 of these embryos all of the Ana1-GFP was localised in a ring around the mother centriole, and I could detect no hint of signal associated with the nearby daughter centriole; in 2/5 embryos I detected two classes of centrioles, with and without Ana1-GFP detectable at the daughter (10 centrioles each per class), and only in 1/5 embryo all centrioles that I examined exhibited a ring-like distribution with a clear hint of localisation at the nearby daughter centriole (Figure 5.4C). While a larger sample size and more stringent (blind) analysis are required in the future, my preliminary data are consistent with the observations of (Fu et al., 2016) that Ana1 is first loaded onto daughter centrioles towards the end of S-phase/beginning of mitosis. Incidentally, it is at this stage of the cycle when the highest Ana1 recruitment rate and recovery after photobleaching have been observed (Saurya et al., 2016).

In Ana1-ALL-GFP embryos I also observed some centrosomes where Ana1-ALL-GFP localised only to the mother centriole (Figure 5.5A), and others where it was present at both the mother and daughter centrioles (Figure 5.5B). It is not possible to do a more in-depth analysis because even early syncytial embryos showed some degree of asynchrony, so these embryos were difficult to stage. Additionally, centrosomes often failed to separate and formed clusters (Figure 5.5C shows reconstructions from 3D-SIM

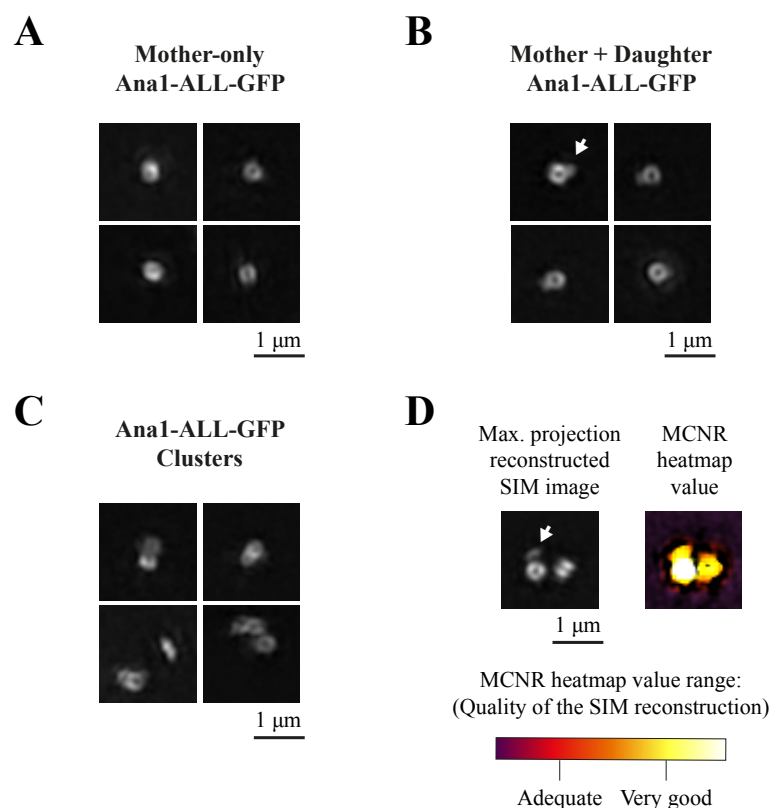


Figure 5.5 – Ana1-ALL-GFP also localises to mother and daughter centrioles.

Examples of live 3D-SIM images of Ana1-ALL-GFP embryos. Because of the mitotic defects suffered by these embryos it was not possible to stage them accurately. **(A)** Examples of centrosomes where Ana1-ALL-GFP localised only to the mother centriole. **(B)** Examples of centrosomes where Ana1-ALL-GFP localised to both the mother and daughter centrioles. An example of Ana1-GFP at a daughter centriole is indicated with an *arrow*. **(C)** Clusters of mother centrioles in Ana1-ALL-GFP embryos. **(D)** Example of two disengaged mother centrioles in close proximity. One of them is tilted (*right*), but the other appears perpendicular to the imaging plane, with the Ana1-ALL-GFP ring clearly visible, and it is associated with a third, presumably younger centriole (*arrow*). In this case, in addition to the 3D-SIM *z*-projected image (*left*), the Modulation Contrast Noise Ratio (MCNR) heatmap corresponding to a single image of the *z*-stack is shown (*right*). The MCNR is one of the outputs from SIM-Check (Ball et al., 2015). It is the ratio of the SIM illumination pattern strength to the noise strength, and it indicates the reliability of the SIM reconstruction (i.e. what is the likelihood that a certain object in the reconstructed SIM image is not a reconstruction artefact). The MCNR heatmap colour scheme (*bottom*) ranges from purple (inadequate reconstruction), to red (adequate), orange (good), yellow (very good) and white (excellent).

imaging; but see also Figure 5.2B, *arrows*). Thus, a pair of mother centrioles in the 3D-SIM images which are in close proximity and present no visible daughter centrioles could correspond to a centrosome pair soon after centrosome separation, a pair of centrosomes that failed to disengage, or a pair of centrosomes that disengaged but failed to move apart. I was able to find at least one example of a well-reconstructed centrosome cluster made of two mothers, one of which had nucleated a daughter centriole (Figure 5.5D), which would suggest that the third scenario (disengagement without proper separation) is true for some of the centrosomes in Ana1-ALL-GFP embryos. This is consistent with previous reports that Ana1 is not required for disengagement (Fu et al., 2016; Izquierdo et al., 2014).

Overall, the results in this section show that, despite the many mitotic defects observed in Ana1-ALL-GFP embryos, Ana1-ALL-GFP is recruited to centrioles to a similar (if not slightly higher) level as WT Ana1-GFP. Similar to WT Ana1-GFP, there are examples of both Ana1-ALL-GFP “mother-only” localisation and “mother and daughter” localisation, suggesting that the mutations introduced do not stop protein localisation to either centrosomal compartment. Finally, as expected from the rescue of the uncoordinated phenotype, centrioles in Ana1-ALL-GFP embryos can complete disengagement and conversion into functional mothers able to duplicate new centrioles, however some appear to remain in close proximity forming clusters.

5.2.4. Polo is undetectable at the centriole wall and the pericentriolar scaffold in Ana1-ALL-mCherry embryos

Next, I tested whether expression of Ana1-ALL resulted in the reduction of centrosomal Polo levels, as suggested from the results of the mRNA injection assay (3.2.4). To do this, I used the same PoloGFP protein trap line employed for the

mRNA injection assay, but in an *ana1*^{-/-} background rescued with WT Ana1-mCherry or Ana1-ALL-mCherry. Similarly to the sensitisation effect observed when combining PoloGFP and Spd-2-ALL-mCherry (see 4.2.5), expressing PoloGFP with Ana1-ALL-mCherry led to embryos exhibiting worse mitotic defects than those observed when mutant GFP- or mCherry-fusions were expressed in the absence of the PoloGFP allele. While *ana1*^{-/-} females expressing PoloGFP and Ana1-ALL-mCherry were not uncoordinated, very few of their embryos developed, and the ones that did had very few centrosomes (n = 24 visible centrosomes in total, from six developing embryos). However, *ana1*^{-/-} embryos co-expressing WT Ana1-mCherry and PoloGFP appeared to develop normally (data not shown).

It was impossible to accurately stage PoloGFP embryos expressing Ana1-ALL-mCherry, so I quantified the levels of PoloGFP in all the centrosomes obtained from Ana1-ALL-mCherry embryos and compared them to those from a panel including WT-rescued embryos at three different points of their cell cycle (beginning of S-phase, late S-phase and mitosis) and of different ages (cycles 10-11 (pooled together), 12, 13 and 14). Figure 5.6A shows the comparison between Ana1-ALL-mCherry and all the WT-rescued embryos pooled together, and Figure 5.6C,E show the comparison breaking down the WT group in subsets by embryo age or cycle stage, respectively. As expected, centrosomal PoloGFP levels were very low in Ana1-ALL-mCherry embryos, sometimes indistinguishable from background levels, and the difference was statistically significant for all comparisons to WT-rescued embryos (p -value < 10⁻⁶). Similar to Ana1-ALL-GFP, the levels of Ana1-ALL-mCherry were slightly higher than WT Ana1-ALL-mCherry, but the variability within the mutant sample was very high and this difference was not always statistically significant (Figure 5.6B,D,F).

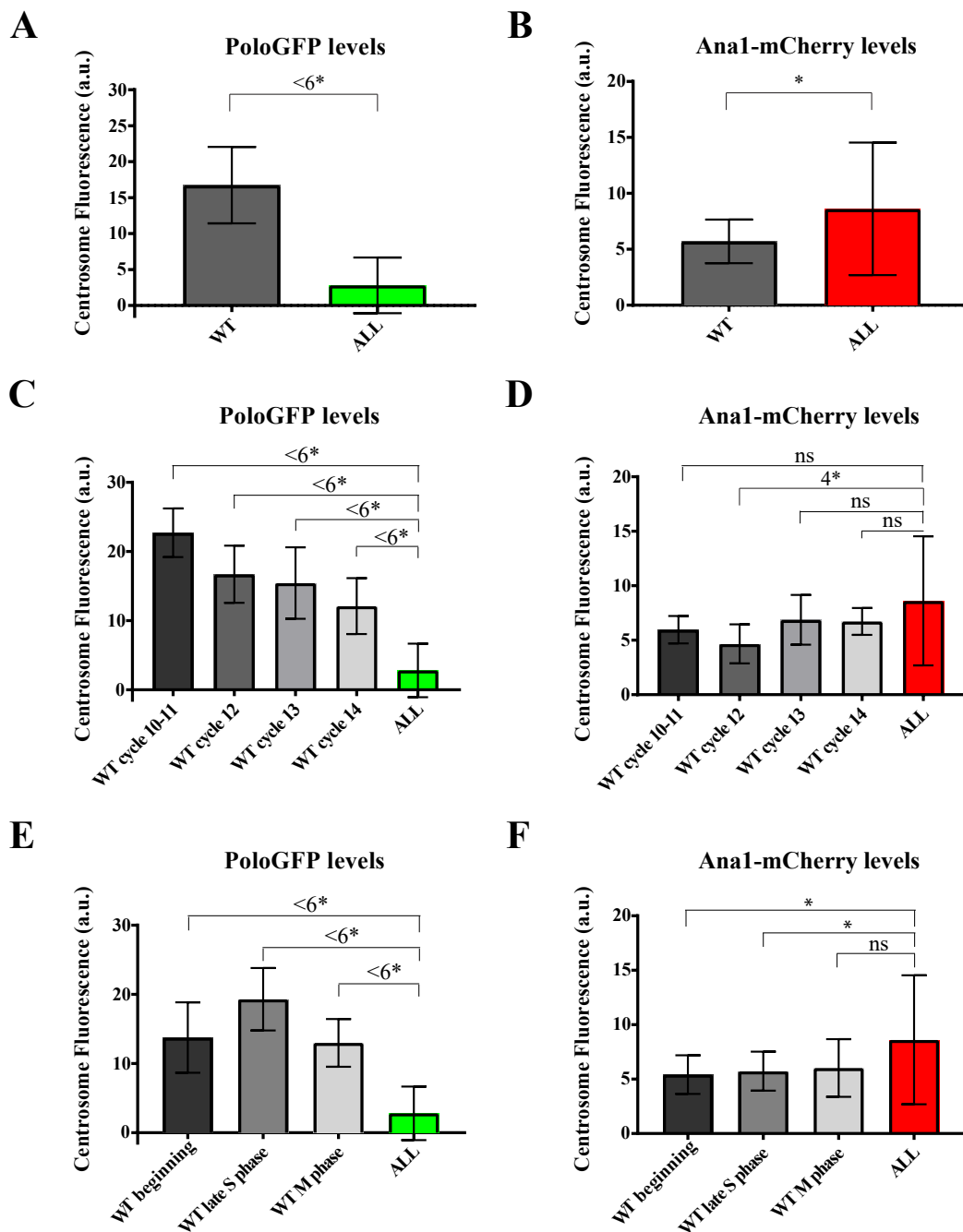


Figure 5.6 – PoloGFP levels are extremely low in Ana1-ALL-mCherry embryos.

Graphs show the mean centrosomal PoloGFP (A,C,E) and Ana1-mCherry (B,D,F) intensities in WT-rescued embryos (grey) and Ana1-ALL-mCherry embryos (green and red, respectively). In total, 23 centrosomes from six different embryos co-expressing PoloGFP and Ana1-ALL-mCherry were analysed. As it was not possible to accurately stage these embryos, they were compared to a panel of WT-rescued embryos at different points of their cell cycle/development. In total, 10 different WT-rescued embryos were analysed, although not all were analysed at every cell cycle/developmental stage. Since four centrosomes were analysed on average per Ana1-ALL-mCherry embryo, two pairs of centrosomes were analysed per WT-rescued embryo at any specific stage of cell cycle and development. (A,B) Average centrosomal intensities of all analysed centrosomes per genotype (n = 64 and 23 centrosomes for WT and ALL, respectively). (C,D) Average centrosomal intensities of WT centrosomes classified by developmental stage, compared to ALL (n = 12, 28, 12, 12 and 23 centrosomes for cycles 10-11 (pooled together), 12, 13, 14 and ALL, respectively). (E,F) Average centrosomal intensities of WT centrosomes classified by cell cycle stage, compared to ALL (n = 16, 36, 12 and 23 centrosomes for beginning of S-phase, late S-phase, mitosis and ALL, respectively). Error bars indicate SD. ns = non-statistically significant difference, p -value > 0.05 ; * = $0.05 > p$ -value > 0.01 ; 4* = p -value $< 10^{-4}$ (E-4); <6* = p -value $< E-6$.

Together, these data suggest that centrosomal PoloGFP levels are drastically reduced in embryos expressing the Ana1-ALL protein (Figure 5.7A,B).

I wanted to directly test whether PoloGFP was lost from the centriole wall, the PCM, or both. However, in order to distinguish this, I needed to use 3D-SIM, and *ana1*^{-/-} embryos co-expressing PoloGFP and Ana1-ALL-mCherry could not be imaged live with 3D-SIM. Therefore, I shifted to 3D-SIM imaging of fixed embryos. Ideally, I would have used *ana1*^{-/-} embryos expressing only Ana1-ALL-GFP (or Ana1-ALL-mCherry) stained with an antibody against Polo. However, I tested three different antibodies against Polo (see Table 2.5), but none appeared to work. Thus, I was restricted to 3D-SIM imaging of fixed *ana1*^{-/-} embryos co-expressing PoloGFP and Ana1-mCherry (WT or ALL). The embryos were stained for Ana1 and two additional centrosomal markers (Cnn and Asl) to facilitate the identification of centrosomes. I was only able to find three centrioles in fixed Ana1-ALL-mCherry embryos, but all of these lacked detectable PoloGFP and Asl, and they had only a minimal Cnn scaffold (Figure 5.7C,D). Although these analyses are not ideal, together they strongly support the conclusion that the recruitment of Polo to both centrioles and the PCM is drastically impaired in embryos expressing Ana1-ALL protein.

5.2.5. Asl recruitment and maintenance are unaffected if Ana1 cannot recruit

Polo

The images from fixed *ana1*^{-/-} embryos co-expressing PoloGFP and Ana1-ALL-mCherry suggest that mutating the ptPBSs in Ana1 leads to a drastic loss of Polo which affects both daughter centriole conversion into a mature mother (hence the loss of Asl) and PCM formation (causing a reduction of the Cnn scaffold). A defect in centriole conversion would be consistent with the reduced number of centrosomes in

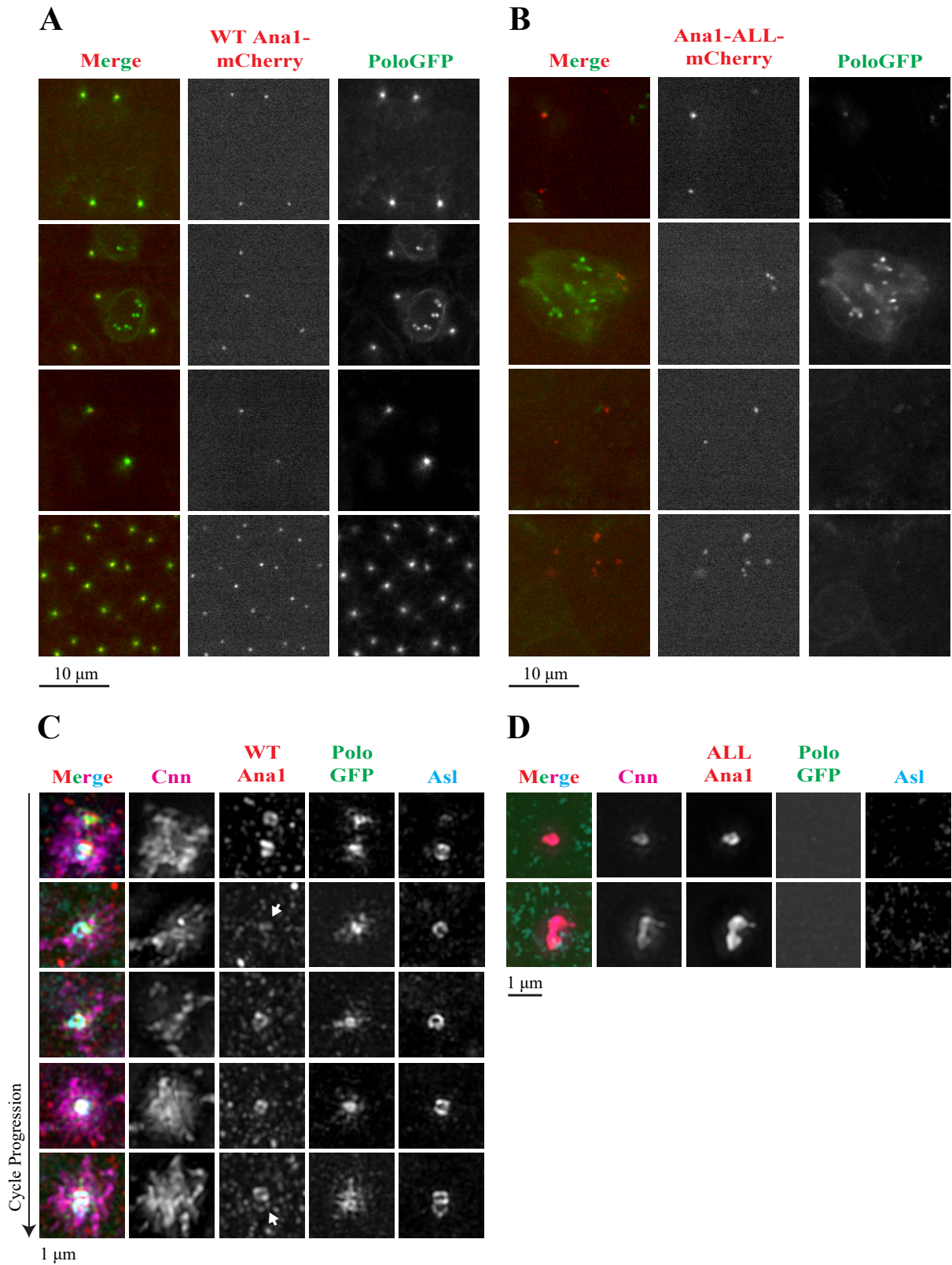


Figure 5.7 – PoloGFP is undetectable in the centrosomes and PCM of Ana1-ALL-mCherry embryos. (A,B) Examples of conventional spinning disk confocal images used for the quantification in Figure 5.6. (C,D) 3D-SIM images from fixed WT (C) and ALL (D) Ana1-mCherry rescued embryos co-expressing PoloGFP. The mCherry fluorophore is not visible after embryo fixation, so embryos were stained for Ana1 (red) as well as Cnn (far-red channel, shown as magenta) and Asl (cyan), and the GFP signal was boosted with an anti-GFP alpaca nanobody (green). PoloGFP localised strongly to centrosomes in WT Ana1-mCherry embryos (A,C) but it was often missing from centrosomes in Ana1-ALL-mCherry embryos (B,D), although it was visible elsewhere in the embryo (e.g. kinetochores, 2nd row of panels in (B)). Note that the anti-Ana1 antibody used in (C,D) recognised centrosomes strongly in embryos at the end of mitosis/beginning of S-phase (centrosome separation), but the signal was very low in centrosomes from S-phase embryos and newly converted mothers (arrows), and it was often undistinguishable in the pre-reconstruction images (data not shown).

these embryos. However, it could be that the loss of Asl is a consequence of the genetic interaction between the PoloGFP allele and the Ana1-ALL-mCherry transgene. Indeed, from my analysis so far it is clear that centrioles can duplicate, at least to some extent, in *ana1*^{-/-} embryos expressing only Ana1-ALL-GFP or Ana1-ALL-mCherry, although they often appeared to have problems properly separating afterwards (see 5.2.2 and 5.2.3), which would indicate that Asl recruitment and centriole conversion are unaffected in these embryos. Furthermore, injection of Ana1-ALL-GFP mRNA had no effect in Asl-mCherry recruitment or maintenance (see 3.2.4).

To address this further I quantified Asl-mCherry levels in *ana1*^{-/-} embryos rescued with WT Ana1-GFP or Ana1-ALL-GFP, following the same analysis protocol as in 3.2.4 and (Saurya et al., 2016). There was no statistically significant difference in the levels of Asl-mCherry in either older or younger mothers between WT and ALL Ana1-GFP embryos (Figure 5.8A), suggesting that the ptPBS mutations introduced in Ana1-ALL do not affect Asl recruitment. I then calculated the ratio of Asl-mCherry between old and young mothers. If the mutations introduced in Ana1-ALL affected Asl level maintenance, the preferential accumulation of Asl-mCherry at old mothers would be lost and this ratio would decrease. However, the old/young mother ratio is actually slightly increased in Ana1-ALL-GFP embryos with respect to WT-rescued embryos (Figure 5.8B), indicating that Asl level maintenance is unaffected.

The asymmetry in WT Ana1-GFP within each centrosomal pair was not as clear in this experiment (Figure 5.8C) as it was in 5.2.3 (Figure 5.3). A possible reason for this discrepancy is that previously I classified the centrosomes into subsets based solely on their Ana1-GFP levels (hence distinguishing between “BRIGHT” and “FAINT”). Conversely, in this experiment I classified them based on their Asl-mCherry signal,

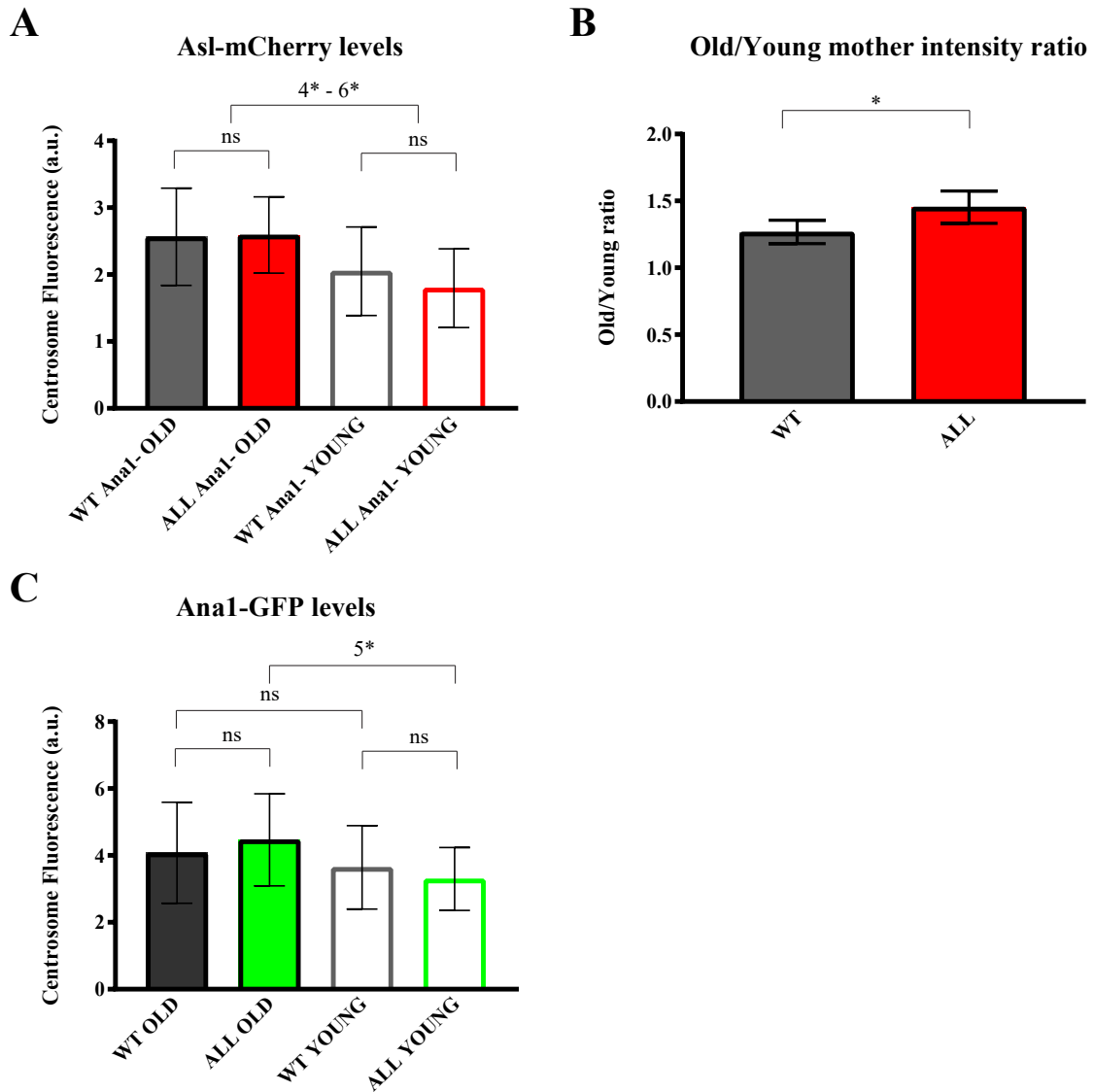


Figure 5.8 – Asl-mCherry is unaffected in Ana1-ALL-GFP embryos.

(A) Graph shows mean Asl-mCherry intensity above background for Ana1-ALL-GFP and WT-rescued embryos. Five embryos were analysed per genotype, and 10 pairs of centrosomes were analysed per embryo. For each pair, the centrosome with the highest mean Asl-mCherry intensity was considered to be the old mother (“OLD”), and the other centrosome was considered to be the young mother (“YOUNG”), because Asl-mCherry is known to preferentially associate with older centrosomes (Novak et al., 2014). Therefore, for statistical analysis I compared the mean intensity of Asl-mCherry at old mother centrosomes (WT vs ALL, *filled* boxes) and young mother centrosomes (WT vs ALL, *white* boxes) separately. (B) For every embryo analysed I calculated the old/young mother ratio: average intensity of the Asl-mCherry signal from old mother centrosomes divided by the average intensity of the signal from young mother centrosomes. The resulting values were averaged to calculate a mean per genotype, and the unpaired t-test was used to assess if the differences were statistically significant. (C) Graph showing mean Ana1-GFP intensity from the corresponding centrosomes analysed in (A,B). Centrosomes from each pair were classified in “OLD” or “YOUNG” subsets based on their Asl-mCherry levels. ns = non-statistically significant difference, p -value > 0.05 ; * = $0.05 > p$ -value > 0.01 ; 5* = p -value in 10^{-5} (E-5) range; 4* - 6* = average p -value in E-4 to E-6 range. Error bars represent SD.

which allows the unambiguous identification of the older and younger mother within each pair. Interestingly, I also did not observe a statistically significant difference between WT Ana1-GFP and Ana1-ALL-GFP levels for either older or younger mothers, which further suggests that the mutations introduced in Ana1-ALL-GFP have very little effect on its recruitment to the centrosome. However, there is a statistically significant enrichment in Ana1-ALL-GFP in older mothers compared to younger mothers. Since Ana1 is implicated in Asl recruitment and/or maintenance (Fu et al., 2016; Saurya et al., 2016), and since the mutations introduced in Ana1-ALL-GFP do not appear to disturb this function of the protein, a slight increase in Ana1-ALL-GFP levels in old mothers could explain the slight increase in the Asl-mCherry old/young ratio in Ana1-ALL-GFP embryos.

5.2.6. Mitotic PCM expansion is impaired if Ana1 cannot recruit Polo

The similarities between the phenotypes of *cnn*^{-/-} and Ana1-ALL-GFP embryos (see 5.2.2), and the reduction in Spd-2 distribution after Ana1-ALL-mKate2 mRNA injection (see 3.2.4) suggest that PCM expansion might be compromised if Ana1 cannot recruit Polo. Based on previous reports that Spd-2, Polo and Cnn cooperate to build the mitotic PCM (Conduit et al., 2014a, 2014b, 2015a; Feng et al., 2017), and following the results from Chapter 4 of this thesis, it was reasonable to speculate that the dramatic loss of centrosomal Polo observed in Ana1-ALL-mCherry embryos might have catastrophic consequences for mitotic PCM assembly.

I had already observed that fixed *ana1*^{-/-} embryos co-expressing PoloGFP and Ana1-ALL-mCherry have a much reduced Cnn scaffold. However, I had been able to obtain very few images from these embryos, and this phenotype could be due to the sensitisation effect of the PoloGFP allele. Therefore, I decided to test this formally by

fixing Ana1-ALL-mCherry embryos (without the PoloGFP allele), and staining them to visualise both total Cnn, Cnn phosphorylated at Ser567 ((Feng et al., 2017), see also 4.2.6), and MTs. As expected, I observed a major reduction in total Cnn scaffold formation in most centrosomes (Figure 5.9), similar to that observed in Spd-2-CONS-GFP embryos (see 4.2.6). However, unlike in in Spd-2-CONS-GFP embryos, some of the centrosomes in Ana1-ALL-mCherry embryos were completely devoid of the pCnn epitope (*arrow*, Figure 5.9B), which would be consistent with total loss of Polo from both the PCM and the centriole wall. Interestingly, in centrosomes where pCnn was not detectable, the total Cnn appeared to be localised to a small region directly around the centriole wall, and minimal MT nucleation was detected. Conversely, there were some centrosomes that still retained detectable pCnn (*arrowhead*, Figure 5.9B), their total Cnn scaffold spread out further (although not as much as in WT-rescued embryos) and some MT nucleation was occasionally detectable. These latter centrosomes were often shared by different poles to create trains of linked mitotic spindles (*asterisks*, Figure 5.9C; see also 5.2.2). This variability is surprising, as all centrosomes in Spd-2-CONS-GFP embryos were affected to a similar extent.

To investigate the reason for this variability I turned to live imaging of *ana1*^{-/-} embryos co-expressing GFP-Cnn and either WT Ana1-mCherry or Ana1-ALL-mCherry. WT-rescued embryos presented extended GFP-Cnn scaffolds (Figure 5.10A) which at the beginning of S-phase were slightly larger for one centrosome from each separating pair. This asymmetry is consistent with previous reports that soon after centrosome separation old mother centrioles are associated with more GFP-Cnn than young mother centrioles (Conduit et al., 2010). Conversely, most centrosomes in Ana1-ALL-mCherry embryos had a much reduced GFP-Cnn signal (Figure 5.10B), consistent with the little or no Cnn scaffold formation observed with 3D-SIM in fixed samples

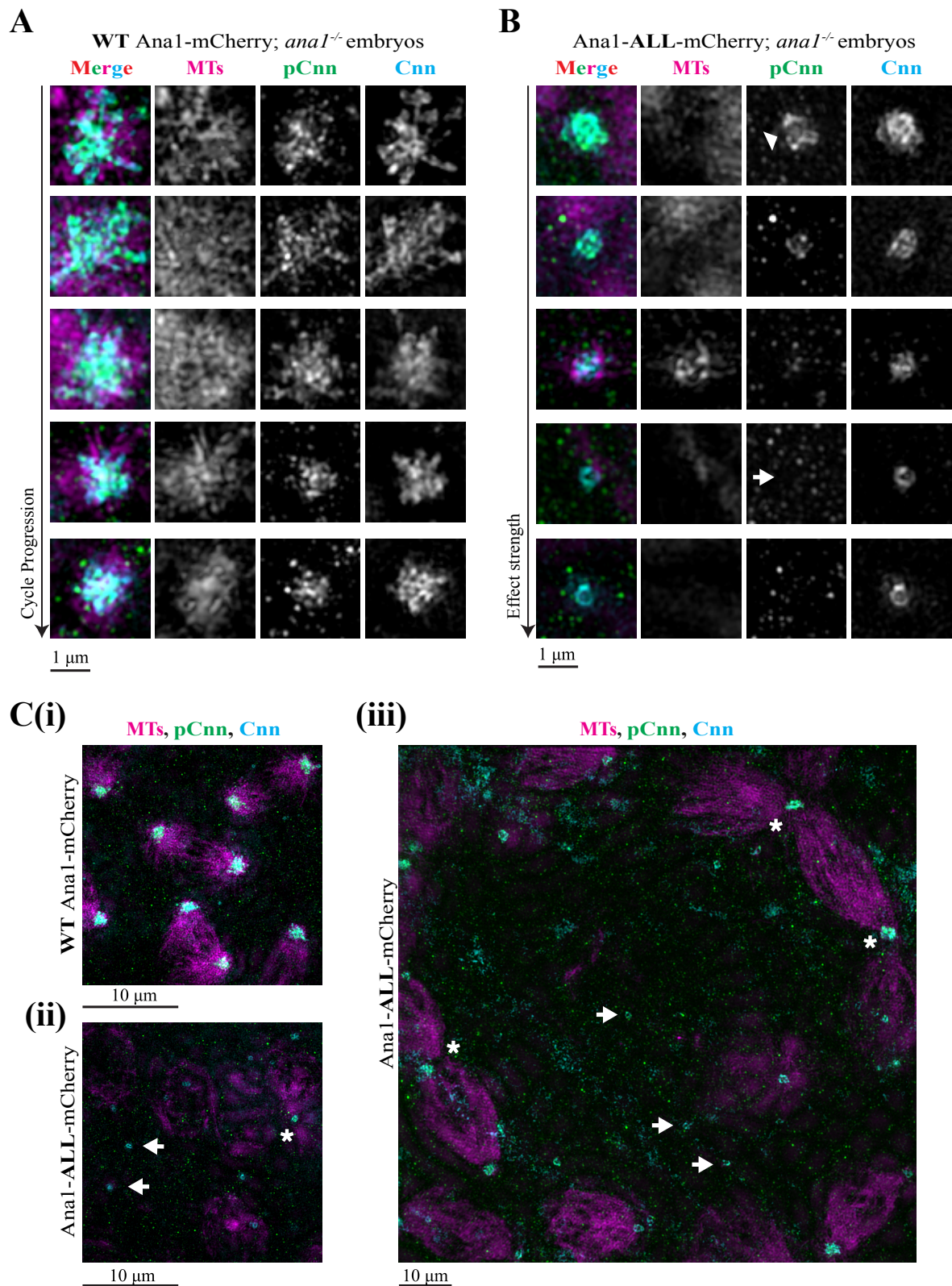


Figure 5.9 – Cnn scaffold formation is impaired if Ana1 cannot recruit Polo.

3D-SIM images from WT (A,C(i)) and ALL (B,C(ii)-(iii)) Ana1-mCherry rescued embryos. The embryos were fixed and stained for α -tubulin (to visualise MTs, far-red, shown as *magenta*), a specific Polo-dependent phospho-epitope (pSer567) in Cnn (“pCnn”, *green*), or Cnn independent of its phosphorylation state (“Cnn”, *cyan*). Ana1 itself is not visible because the embryos could not be stained with anti-Ana1 antibodies, and the mCherry fluorophore is not visible after embryo fixation. **(A)** In WT-rescued embryos, most of the Cnn scaffold was phosphorylated at Ser567, except at the edges of the scaffold (particularly noticeable during mitosis, *bottom* panels). **(B)** Cnn was present at the centriole wall but did not spread outwards into the PCM in most centrosomes of Ana1-ALL-mCherry embryos, similar to the phenotype observed in Spd-2-CONS-GFP embryos (Figure 4.15). However, in Ana1-ALL-mCherry embryos, phosphorylated Cnn was present in some centrosomes (*arrowhead*), but undetected in others (*arrow*). **(C)** Zoomed-out images showing strong MT organisation in WT-rescued embryos **(i)**. This was impaired in Ana1-ALL-mCherry embryos **(ii)**, although some mitotic spindles were still organised and shared the least affected centrosomes (*asterisk*, **(ii-iii)**), forming “trains” spindles (e.g. large circular chain in **iii**). As in B, *arrows* indicate centrosomes without visible pCnn.

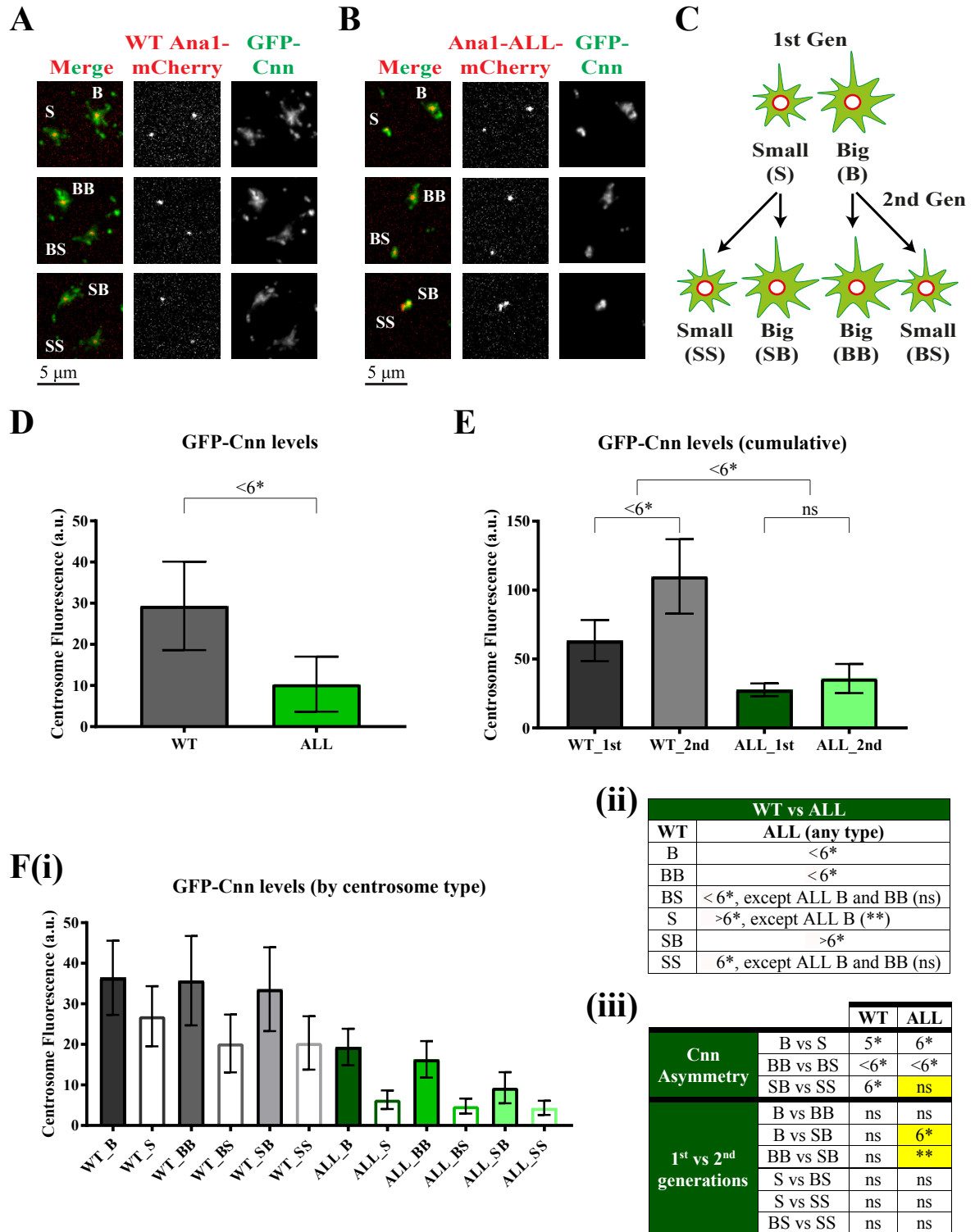


Figure 5.10 – Newly formed centrosomes are unable to accumulate Cnn if Ana1 cannot recruit Polo.

(A,B) Examples of conventional spinning disk confocal images used for the quantification in (D-F). The centrosomes analysed were classified into six different “types” (white labels) based on their GFP-Cnn levels and their genealogy, as explained by the schematic in (C). (D-F) Graphs showing mean GFP-Cnn intensity above background for Ana1-ALL-mCherry and WT-rescued embryos. Eight embryos were analysed per genotype, with three pairs of 1st generation centrosomes, and the corresponding four 2nd generation centrosomes per 1st generation pair, being analysed per embryo. However, for three WT-rescued embryos the 2nd generation centrosomes could not be tracked. (D) shows GFP-Cnn levels per genotype when the values from all centrosomes are averaged together, (E) compares average sum of GFP-Cnn levels for all 1st or 2nd generation centrosomes per genotype, and (F(i)) shows average GFP-Cnn levels per centrosome type per genotype. Note that three “S” centrosomes failed to separate in Ana1-ALL-mCherry embryos, in which case only an “SB”-type centrosome was scored. To facilitate visualisation, only the most informative statistical comparisons between WT and ALL centrosomal types are shown in tables (F(ii) and iii)). Key statistical comparisons in (iii) are highlighted in yellow. ns = non-statistically significant difference, p -value > 0.05 ; * = $0.01 > p$ -value > 0.005 ; 5* = p -value in 10^{-5} (E-5) range; 6* = p -value in E-6 range. Error bars represent SD.

(Figure 5.9B). However, there were still some centrosomes with more GFP-Cnn (Figure 5.10B, centrosomes marked “B” and “BB”) than others.

To investigate the reasons for this difference, I performed a pedigree analysis, following centrosomes through two cycles of division. As Ana1-ALL-mCherry embryos are very disorganised and difficult to stage, I analysed a group of young embryos ($n = 8$, from nuclear cycles 10-12 pooled together) which appeared to be the least affected (i.e. most centrosomes were still able to duplicate and separate somewhat synchronously). I also analysed a group of WT-rescued embryos of similar age. From each embryo, I selected three pairs of separating centrosomes and measured their mean GFP-Cnn signal intensity early in S-phase (“1st generation”). From each pair, the centrosome with the highest value was labelled “B” (“Big scaffold”), and the other was labelled “S” (“Small scaffold”). I then manually tracked each centrosome until they separated into an old and young mother at the beginning of the following cycle. I again measured the mean GFP-Cnn signal intensity of the newly separating centrosomes (“2nd generation”), which were labelled based on which 1st generation centrosome they were derived from and the size of their GFP-Cnn scaffold (see Figure 5.10C for a schematic of the different “types” of centrosomes analysed).

As expected, when the mean intensity values for all centrosomes were pooled together, the average centrosomal GFP-Cnn intensity was markedly higher in WT-rescued compared to Ana1-ALL-mCherry-rescued embryos (Figure 5.10D). I then calculated the average “cumulative” GFP-Cnn signal (i.e. the sum of intensities for the two “1st generation” centrosomes or for the four “2nd generation” centrosomes from each initial pair). In WT-rescued embryos, the number of centrosomes doubled from one generation to the next, and so did the cumulative GFP-Cnn signal (Figure 5.10E).

Conversely, in Ana1-ALL-mCherry embryos the cumulative GFP-Cnn signal in the 2nd generation was only slightly higher than that of the 1st generation, and this difference was not statistically significant. This result suggests that the duplicating centrosomes in Ana1-ALL-mCherry embryos cannot efficiently accumulate new GFP-Cnn.

The defects in Ana1-ALL-mCherry embryos were even more clear when GFP-Cnn levels were analysed separating the data by centrosome type (Figure 5.10F). The centrosomes with the bigger GFP-Cnn scaffolds (“B”, presumed to be the older mothers of each pair) were able to maintain a similar-sized scaffold as they entered the next cycle: there was a small decrease in GFP-Cnn levels in “BB” compared to “B” centrosomes, but the difference was not statistically significant. However, their GFP-Cnn scaffolds were still much smaller than their WT counterparts, suggesting that Cnn scaffold assembly may still be compromised in these centrosomes. Similarly, Ana1-ALL-mCherry centrosomes with low GFP-Cnn levels (presumed to be young mothers) also had a lot less GFP-Cnn than their WT counterparts. Crucially, the young centrosomes in WT-rescued embryos were able to recruit GFP-Cnn such that by the next cycle their levels were comparable to those of older centrosomes (i.e. there was no statistically significant difference comparing WT “SB” to WT “B” and “BB”). Conversely, in Ana1-ALL-mCherry embryos the levels of GFP-Cnn in young centrosomes could not reach those of older mothers (there is a statistically significant difference in GFP-Cnn in ALL “SB” compared to ALL “B” and “BB”), and remained very low (no statistically significant difference between ALL “SB” compared to ALL “S”, “BS” and “SS”).

These results are consistent with a model in which young centrosomes containing Ana1-ALL can recruit Cnn to the centriole wall but are unable to start forming an

expanded Cnn scaffold because Polo cannot bind Ana1 via its PBD and thus, not enough Polo is recruited to the centriole wall and Cnn is not phosphorylated. However, this does not account for older centrosomes being able to maintain at least a minimal Cnn (and pCnn) scaffold. A possible explanation is that older centrosomes may have enough time during their maturation (or enjoy less competition from other centrosomes) to accumulate enough Spd-2 at their centriole wall. Because Polo can be first recruited to the centriole wall by Sas-4 during daughter-to-mother conversion (Novak et al., 2016) and Spd-2 can bind Polo (see Chapter 4), this might be enough to trigger Cnn scaffold formation, which in turn would help maintain Spd-2, in a positive feedback loop (see 4.3 Discussion and Chapter 6).

If this were the case, Ana1-ALL-mCherry embryos should also present asymmetric Spd-2 distribution, with most centrosomes having a minimal or no Spd-2 scaffold but some centrosomes having bigger Spd-2 scaffolds. However, when I injected Ana1-ALL-GFP mRNA into Spd-2-mCherry embryos I observed a generalised reduction in centrosomal Spd-2 levels and distribution, but no clear asymmetry (see 3.2.4). To test this further, I examined *ana1*^{-/-} embryos co-expressing Spd-2-GFP and either WT Ana1-mCherry or Ana1-ALL-mCherry. Similarly to the GFP-Cnn analysis, I manually tracked three centrosomal pairs per embryo through two consecutive cycles. Similar to GFP-Cnn, there was a large, statistically significant drop in centrosomal Spd-2-GFP levels in Ana1-ALL-mCherry embryos compared to WT-rescued controls (Figure 5.11A-C). I also observed that the cumulative Spd-2-GFP levels almost doubled from the 1st to the 2nd generation in WT-rescued embryos, whereas in Ana1-ALL-mCherry embryos there was no statistically significant increase. This suggests that, like GFP-Cnn, centrosomes in Ana1-ALL-mCherry embryos cannot efficiently accumulate Spd-2-GFP (Figure 5.11D).

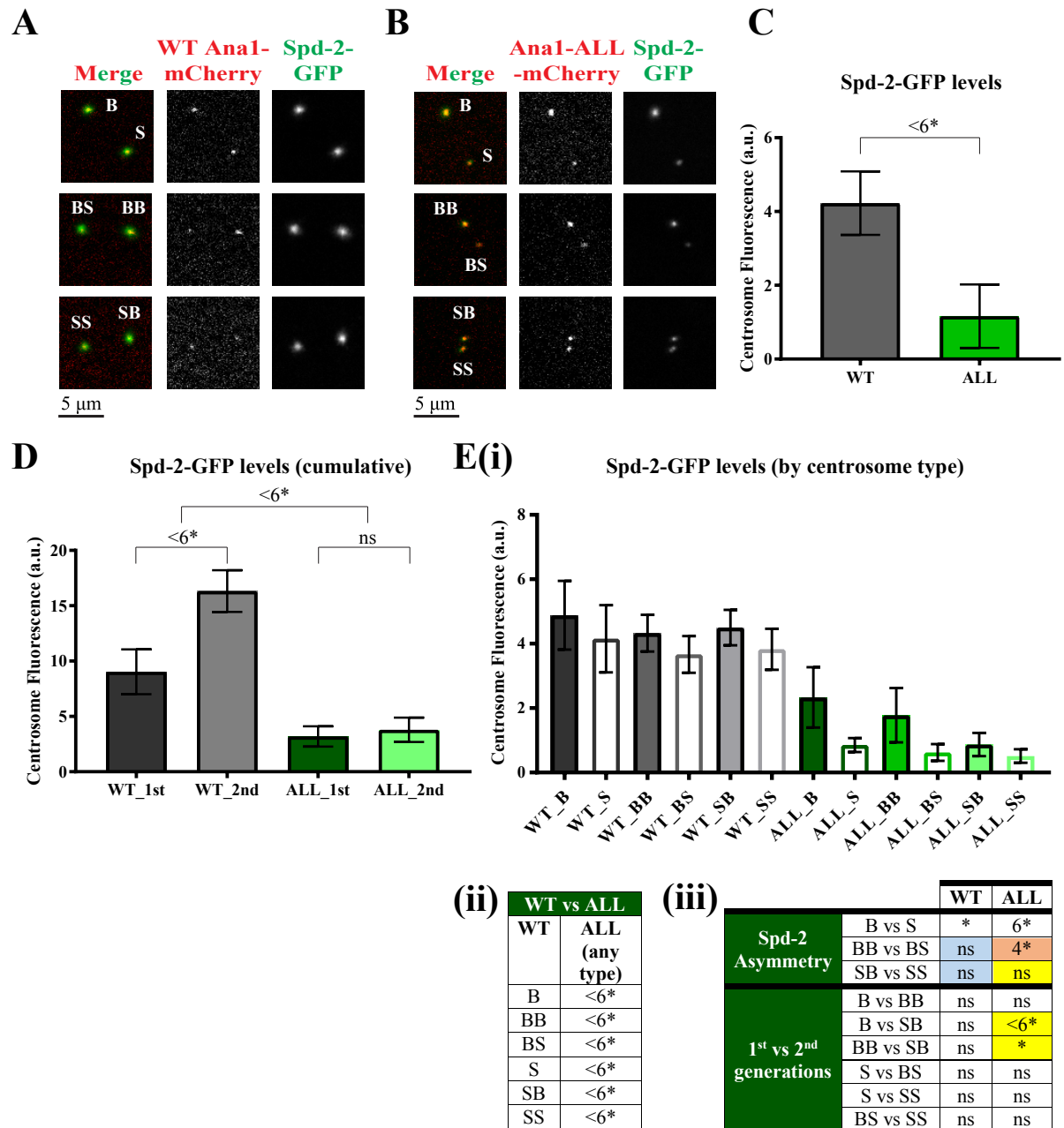


Figure 5.11 – Newly formed centrosomes are unable to accumulate Spd-2 if Ana1 cannot recruit Polo. (A,B) Examples of conventional spinning disk confocal images used for the quantification in (C-E). The centrosomes analysed were classified into six different “types” (white labels) based on their Spd-2-GFP levels and their genealogy, as explained by the schematic in (Figure 5.10C). (C-E) Graphs showing mean Spd-2-GFP intensity above background for Ana1-ALL-mCherry and WT-rescued embryos (n = 5 and 7 embryos, respectively, with three pairs of 1st generation centrosomes and the corresponding four 2nd generation centrosomes per 1st generation pair being analysed per embryo). (C) shows Spd-2-GFP levels per genotype when the values from all centrosomes are averaged together and (D) compares average sum of Spd-2-GFP levels for all 1st or 2nd generation centrosomes per genotype. (E(i)) shows average Spd-2-GFP levels per centrosome type per genotype. To facilitate visualisation, only the most informative statistical comparisons between WT and ALL centrosomal types are shown in tables (ii-iii). Key statistical comparisons in (iii) are highlighted in blue, red and yellow. Unlike for GFP-Cnn, the Spd-2-GFP asymmetry was not as clear in WT-rescued embryos, with no statistically significant differences in Spd-2-GFP levels between 2nd generation centrosomes. However, in Ana1-ALL-mCherry embryos the asymmetry between BB and BS centrosomes was very stark, but it was still not significant between SB and SS centrosomes, and SB centrosomes had statistically less Spd-2-GFP than B and BB centrosomes. ns = non-statistically significant difference, p -value > 0.05; * = 0.05 > p -value > 0.01; 4* = p -value in 10⁻⁴ (E-4) range; 6* = p -value in E-6 range. Error bars represent SD.

Unlike Cnn, Spd-2 distribution is only slightly asymmetrical between old and young centrosomes (P. Conduit and S.S. Wong, unpublished). Indeed, if the data was subdivided by centrosome type (Figure 5.11E), I observed that in WT-rescued embryos the levels of Spd-2-GFP were statistically significantly higher in “B” centrosomes compared to “S” centrosomes, but due to sample variation this increase was not statistically significant in the 2nd generation centrosomes. However, in Ana1-ALL-mCherry embryos this asymmetry was highly statistically significant between “B” and “S” and also “BB” and “BS” centrosomes, highlighting that although there is a generalised loss of centrosomal Spd-2-GFP in these embryos, older centrosomes tend to maintain some Spd-2-GFP, whereas younger centrosomes have very little Spd-2 (presumably only that recruited to the centriole). As for GFP-Cnn, the levels of Spd-2-GFP of “SB” centrosomes in Ana1-ALL-mCherry embryos were statistically lower than those of “B” and “BB” centrosomes, and no different than those of “S”, “BS” and “SS” centrosomes. This shows that in Ana1-ALL-mCherry embryos the levels of Spd-2-GFP in young centrosomes could not grow to reach those in older centrosomes.

To confirm that Spd-2-GFP was preferentially lost from the PCM (rather than from the centrioles), I imaged live Ana1-ALL-mCherry embryos and WT-rescued controls with 3D-SIM. While the Ana1-mCherry signal was difficult to reconstruct, Spd-2-GFP was clearly localised to the centriole wall and the PCM in WT Ana1-mCherry embryos (Figure 5.12A). Conversely, in Ana1-ALL-mCherry embryos some centrosomes still had an expanded Spd-2-GFP scaffold, whereas others had completely lost it and presented Spd-2-GFP only at the centriole wall (Figure 5.12B).

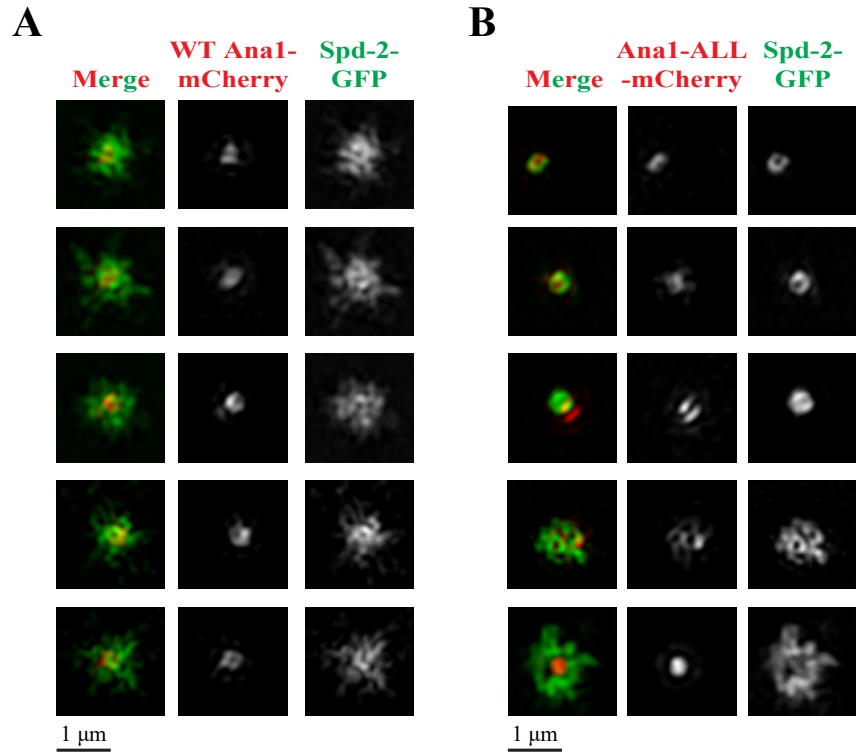


Figure 5.12 – Spd-2 scaffold formation is impaired if Ana1 cannot recruit Polo.

Micrographs show 3D-SIM images of individual centrosomes from *anal*^{-/-} embryos expressing Spd-2-GFP (green in merge images) and either WT Ana1-mCherry (A) or Ana1-ALL-mCherry (B) (red in merge images). While Ana1-mCherry images are shown for references, reliably reconstructing the Ana1-mCherry signal is difficult due to its low levels and fast bleaching of the fluorophore. Thus, images were selected based only on whether the Spd-2-GFP reconstructed image was deemed of sufficient quality by SIM-Check (Ball et al., 2015). Centrosomes in WT-rescued embryos presented well-spread out Spd-2-GFP PCM scaffolds. However, in Ana1-ALL-mCherry embryos many centrosomes presented Spd-2-GFP only at the centriole wall, although some centrosomes were still able to assemble large Spd-2-GFP PCM scaffolds.

5.2.7. The C-terminal region of Ana1 is responsible for the Polo recruitment function

Finally, I decided to test if a specific region of Ana1 is involved in Polo recruitment. I divided the 34 ptPBSs present in Ana1 into three groups based on whether they were present in the N-terminal region defined in (Fu et al., 2016), which interacts with Cep135 (1-756 aa, including 10 ptPBSs); the C-terminal region, which interacts with Asl (935-1729 aa, including 20 ptPBSs), or the middle section where the two constructs made by Fu et al. overlap (756-935 aa, 4 ptPBSs) (Figure 5.13A). Since neither Asl recruitment nor its maintenance are affected by Ana1-ALL-GFP, perhaps it would be expected that the Polo recruitment function is localised to the N-terminus of Ana1. However, the C-terminal region has the highest ratio of total and conserved ptPBSs per 100 amino acids (2.5 and 1.1 respectively, compared to 1.32 and 0.39 for the N-terminus and 2.2 and 1.0 for the middle section).

I mutated all the ptPBSs within each group, introducing the conservative mutation Ser (at position -1) to Thr. The three different mutants were tested by mRNA injection, comparing their effect on PoloGFP distribution to that of WT and ALL Ana1-mKate2 fusions (Figure 5.13B). As predicted based on the ptPBS ratios, Ana1-CT-mKate2 mRNA injection resulted in PoloGFP being lost from at least one of the centrosomes within each pair for 11/11 embryos injected, similar to Ana1-ALL-mKate2 mRNA injection (arrows, Figure 5.13B). Conversely, WT Ana1-mKate2, Ana1-NT-mKate2 and Ana1-Mid-mKate2 had little effect on PoloGFP distribution. Thus, the C-terminus of Ana1 appears to play the largest part in Polo recruitment to the centrosome.

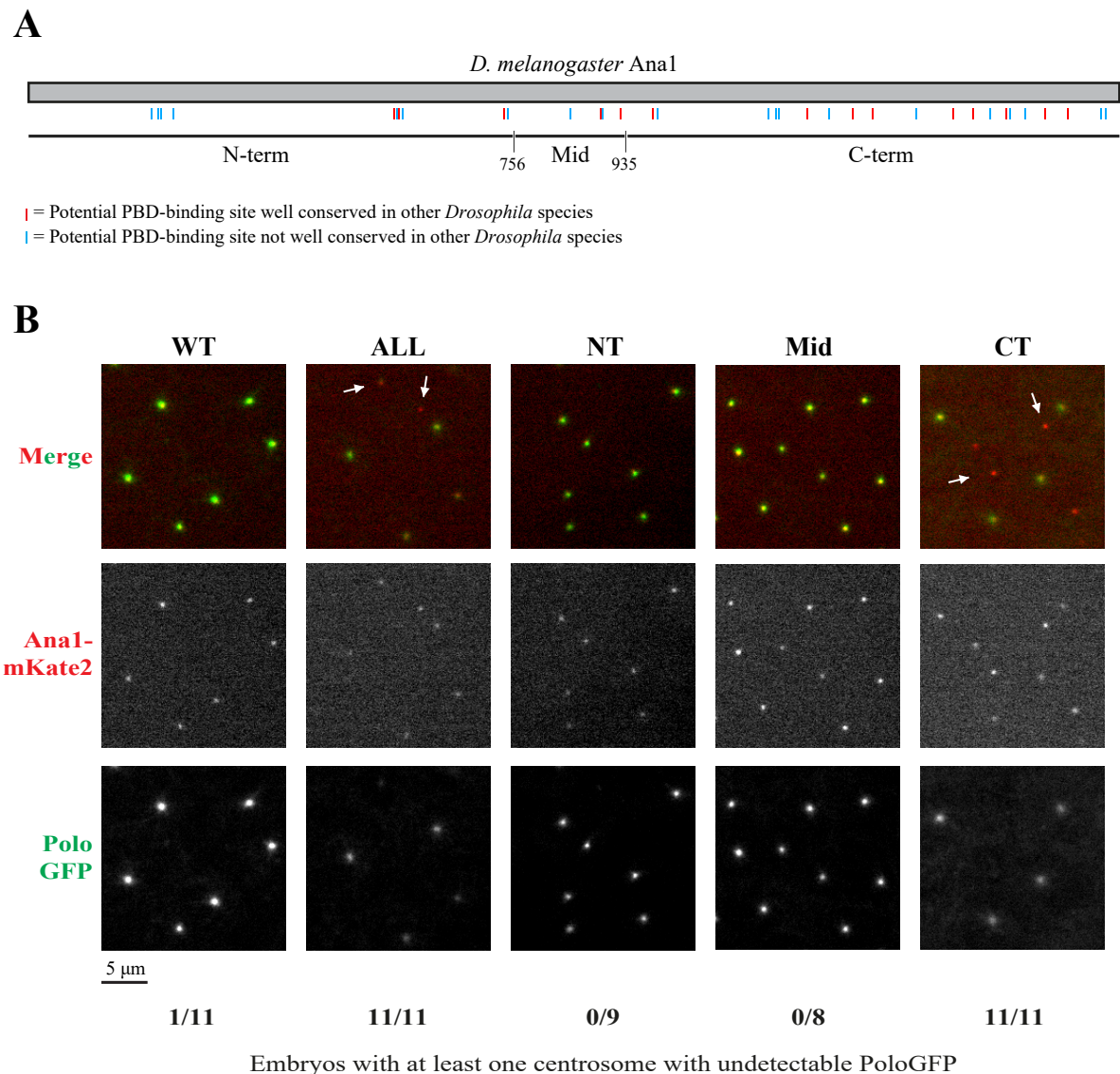


Figure 5.13 – The C-terminal region of Ana1 is involved in PoloGFP recruitment to the centrosome.
(A) Schematic representation of *Drosophila melanogaster* Ana1 indicating ptPBSs (Ser-Ser/Thr) that are either conserved (present in at least 11/12 *Drosophila* species analysed, red lines) or not conserved (blue lines). The boundaries of the Ana1 regions defined in a previous study (Fu et al., 2016) are also indicated. **(B)** Micrographs of embryos expressing PoloGFP (green in the merge channel) injected with WT, ALL, NT, Mid or CT Ana1-mKate2-mRNA (red in the merge channel). Arrows indicate centrosomes that are only visible in the red channel (i.e. have lost PoloGFP). Embryos were scored blindly for loss of PoloGFP from at least one visible centrosome, the results for each injected construct are indicated as affected embryos/total injected embryos analysed, below the corresponding column of panels.

5.3. Discussion

In this Chapter I have confirmed that Ana1 has an important role in recruiting Polo to the centrosome in fly embryos. Losing Ana1-mediated Polo recruitment strongly perturbs PCM assembly in newly formed centrosomes at the syncytial blastoderm stage, resulting in the embryo accumulating mitotic defects and dying prior to gastrulation. PCM assembly is somewhat impaired but still possible in older centrosomes, presumably because they are able to recruit enough Polo via the Thr200-STP motif of Sas-4 and accumulated Spd-2 to trigger the Spd-2/Polo/Cnn positive feedback loop and so partially bypass the need for Polo recruitment via Ana1. This idea is further explored in Chapter 6 of this thesis.

The simplest explanation for my observations is that there is a direct interaction between the PBD and ptPBSs in the C-terminal region of Ana1. Indeed, a low-scoring interaction between the Ana1 C-terminus and Polo was detected in a Y2H screen (Galletta et al., 2016). While I did not have time to test this formally during the course of my studies, future work will involve purifying a recombinant C-terminal fragment of Ana1 and testing its binding to commercial GST-PBD using the same *in vitro* assay I optimised for Spd-2 (see 4.2.9). I would also like to confirm that other PCM proteins (e.g. γ -tubulin) are mislocalised in *ana1*^{-/-} embryos rescued with Ana1-ALL.

Additionally, during my studies I have produced a *Jupiter::mCherry, Df(3R)Exel7357* recombinant fly line that could be used to study MT dynamics in these embryos.

A significant aspect of this model is that canonical PBD binding to Ana1 would require centriolar Ana1 being phosphorylated. In the Ana1 C-terminus there are four ptPBSs that are also predicted to be likely Polo phosphorylation targets and two ptPBSs which are predicted Aurora A targets. Furthermore, three ptPBSs in the Ana1 C-terminus

have been shown to be phosphorylated in fly embryos (*D. melanogaster* or related species) via MS (Bodenmiller et al., 2007; Hu et al., 2018; Zhai et al., 2008). While the low endogenous levels of Ana1 make any biochemical studies challenging, the transgenic lines generated for this thesis expressing the Ana1-fusion proteins under the control of the *ubiquitin* promoter could be useful for co-immunoprecipitation or MS experiments. I would also like to repeat the centrosome purification experiments to test if phosphorylated Ana1 can be detected specifically at centrosomes (similarly to Cnn and Spd-2, Figure 4.1). Additionally, during my studies I identified an anti-Ana1 antibody which labels centrosomes clearly during mitosis but poorly in S-phase (arrows, Figure 5.7). It would be interesting to test whether this difference is due to differential phosphorylation states of Ana1 during the cell cycle, or occlusion of the antibody epitope in some other way.

An important observation is that, despite the many ptPBSs mutated, Ana1-ALL does not appear to be misfolded. Ana1-ALL was recruited to the centriole similarly to WT Ana1, and it is functional enough to rescue the uncoordinated phenotype of *ana1*^{-/-} flies. Furthermore, Asl recruitment and maintenance were unaffected by the mutations introduced in Ana1-ALL, suggesting that the C-terminal region of the protein performs at least two different roles: the previously known Asl-related function, and the novel role in Polo recruitment identified in this study.

Newly formed centrioles incorporating Ana1-ALL could disengage from their older mothers and be converted into mothers able to nucleate new daughter centrioles. They did so despite sometimes remaining in physical proximity to other centrosomes (forming clusters) and not being able to recruit an extensive PCM scaffold. Thus, most of my data is consistent with the possibility that Ana1-mediated Polo recruitment is

crucial for mitotic PCM assembly but not for centriole disengagement or conversion into a mature mother. However, co-expression of PoloGFP and Ana1-ALL-mCherry did result in a decrease in the number of centrosomes present in embryos, although it is very likely that this was due to a genetic interaction between the two alleles. It would be ideal to find alternative fluorescently-tagged Polo fly lines or antibodies to test Polo recruitment in the Ana1-ALL-rescued embryos that did not suffer from this sensitisation effect. In the future, I would also like to test whether the recruitment of other proteins involved in centriole conversion (e.g. Sas-4 or Cep135) is unaffected in Ana1-ALL-rescued embryos. While this study has been focused on the developing fly embryo, its scope could be expanded to include other *Drosophila* tissues such as third-instar larval brains, which have often been used to study defects in centriole assembly and conversion (Basto et al., 2006; Bettencourt-Dias et al., 2005; Blachon et al., 2008, 2009; Martinez-Campos et al., 2004; Peel et al., 2007; Rodrigues-Martins et al., 2007a; Stevens et al., 2009; Wang et al., 2011a).

It has been previously suggested that Ana1 is incorporated into daughter centrioles as they grow during S-phase, with incorporation continuing into mitosis before plateauing (Saurya et al., 2016) (Figure 5.14A,B). However, my preliminary data shows that during S-phase, many centrosomes present Ana1-GFP only at the wall of the mother centriole, with no detectable Ana1-GFP at the daughter centriole. The main caveat of using the Ana1-GFP transgenic fly line generated during my studies is that the fluorescently-tagged protein is 2.5-3X overexpressed compared to endogenous Ana1 (Figure 5.1B), and thus, could potentially localise to additional places in the centrosome: i.e. that endogenous Ana1 might localise only to the mother centriole, but the overexpressed protein might localise to both mother and daughter centrioles.

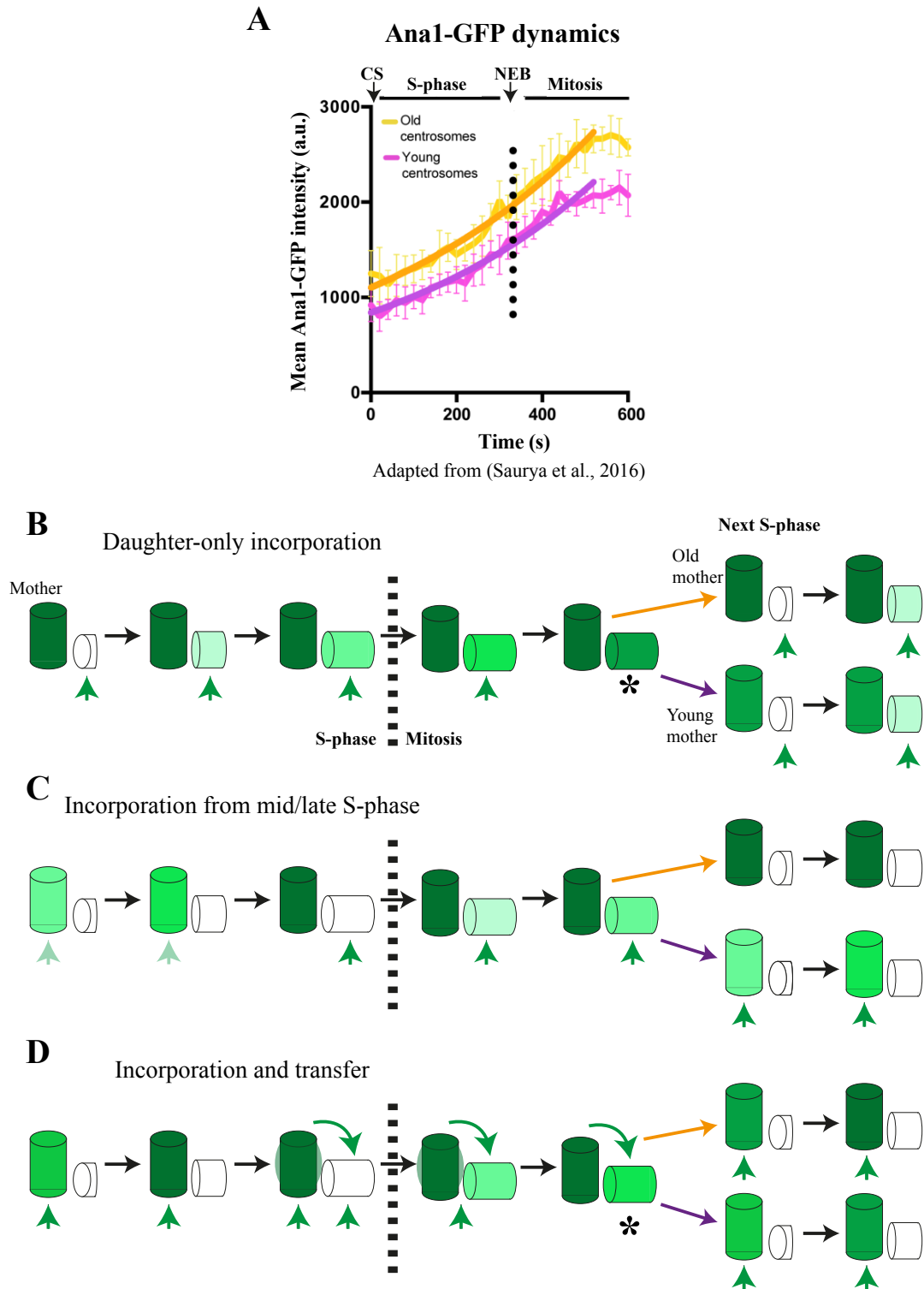


Figure 5.14 – Different models to explain Ana1 recruitment.

(A) Mean centrosomal Ana1-GFP intensity increases exponentially from centrosome separation (CS), until it reaches a plateau during mitosis. Adapted from (Saurya et al., 2016). (B) Ana1 incorporation model proposed in (Saurya et al., 2016). Green arrows indicate net Ana1 incorporation. Ana1 is recruited to growing daughter centrioles with similar rates in old and young centrosomes, from CS until mitosis. The levels in young mother centrioles never reach those of old mothers, creating a small asymmetry (*asterisk*). (C) Alternative model, where Ana1 incorporation at daughter centrioles starts once they have been built. Young mothers continue incorporating Ana1 throughout mitosis and into the next S-phase. This model explains why centrosomes appear to incorporate Ana1 from the start of S-phase, but Ana1-GFP may often not be detected at daughter centrioles. However, old mothers should stop incorporating Ana1 at some point, in which case the *orange* and *purple* lines in (A) should be different. (D) Complex model, where mother centrioles recruit Ana1 from CS. Once built, daughters can also incorporate Ana1 from the cytoplasm (until mitosis) or receive it from their mothers. At CS, the old mother is still slightly enriched compared to the young mother, creating a slight asymmetry (*asterisk*).

However, since a majority of centrosomes and embryos show that overexpressed Ana1-GFP does not localise to daughter centrioles in mid/late S-phase, it is very likely that the endogenous protein does not either.

Thus, my data suggests that Ana1 is not incorporated into daughter centrioles from the beginning of S-phase. Instead, Ana1 recruitment to the daughter centriole would start in mid/late S-phase, once cartwheel growth has been completed and as part of daughter centriole conversion into a fully functional mother. Ana1 incorporation would continue throughout mitosis and into the beginning of the following S-phase, and be completed only after the centriole (now a young mother) has started to duplicate a new daughter. This fits with previous reports from S2 cells (Fu et al., 2016) and unpublished data from other members of the Raff laboratory (A. Wainman, personal communication) (Figure 5.14C). As Ana1-GFP incorporation would be completed during the first S-phase following centriole maturation, this model assumes that centrosomes containing older mother centrioles (*orange* arrow) should present none or little Ana1-GFP incorporation during early S-phase.

However, it has been previously reported that Ana1-GFP incorporation dynamics are very similar between centrosomes with old and young mother centrioles (Figure 5.14A), and that this incorporation is irreversible (Saurya et al., 2016). This creates quite a conundrum, as Ana1 would have to be recruited to all mother centrioles, accumulating with every cycle. Thus, very old mothers should present very large amounts of Ana1, with a strong Ana1 asymmetry compared to young mother centrioles derived from them. However, GFP-Ana1 is only slightly enriched on old mothers (Saurya et al., 2016), and in my studies the asymmetry is also very small (Figure 5.3B). It is also unclear what molecular mechanism could explain such sustained

accumulation. Thus, it is necessary to revisit the evidence presented in (Saurya et al., 2016). If these results can be validated, a third, more complex model would be required, with some Ana1 being recruited to mother centrioles early in S-phase and then transferred to their daughters once their cartwheels have been built (Figure 5.14D). This potential Ana1 exchange could also explain why in mRNA injection experiments all centrioles appear to incorporate some level of Ana1-mKate2 (see 3.2.4 and 5.2.7), and why some embryos (presumably injected with large amount of mutant mRNA) had completely lost PoloGFP from all centrosomes, and not just from young centrosomes (data not shown).

It must also be noted that in previous reports centriolar Ana1-GFP appeared to increase exponentially during S-phase (Saurya et al., 2016) (Figure 5.14A). However, I did not observe any statistically significant difference in Ana1-GFP centriolar levels between embryos at the beginning and end of S-phase (Figure 5.3A). As the transgene in the fly line I used is overexpressed (Figure 5.1B), perhaps having more cytoplasmic Ana1-GFP accelerates its incorporation into centrioles. Thus, all the Ana1-GFP subunits that would be incorporated in any one cycle do so soon after centrosome separation, rather than requiring all of S-phase to do so. Incidentally, this idea is incompatible with the daughter-only incorporation model (Figure 5.14B). I did not directly test this, but it is also possible that more Ana1-GFP is recruited to centrioles overall. In any case, Ana1-GFP overexpression must not lead to saturation of all the Ana1 binding sites at centrioles in these embryos during one cycle. Otherwise, there would be no asymmetry within each centrosome pair (i.e. no statistically significant difference in average Ana1-GFP levels between the “BRIGHT” and “FAINT” centrosome subsets) or at least this asymmetry should decrease as S-phase progresses. In actuality, there was no statistically significant difference in the WT Ana1-GFP BRIGHT/FAINT ratio

between early and late S-phase centrosomes, indicating that this is not the case (Figure 5.3B). Nevertheless, further insight into the dynamics of Ana1 incorporation is required, taking into account any potential effects from changes in cytoplasmic Ana1 levels.

Chapter 6 - General discussion and conclusion

6.1. Summary

This thesis has focused on how Polo kinase is recruited to different compartments within the centrosome, and how these recruitment events impact centrosome biogenesis. Polo kinase localises to the centriole wall and to the nearby PCM. Of all the centrosomal proteins which have similar localisations and are crucial for centrosome biogenesis, only three appear to have a major role in Polo recruitment: Sas-4 (via the single Thr200-STP motif, (Novak et al., 2016)), Ana1 (via ptPBSs in its C-terminal region) and Spd-2 (via multiple ptPBSs spread throughout the protein). Spd-2 can directly interact with the PBD to recruit Polo to the expanding PCM, but it is not required for Polo recruitment to the centriole wall. Conversely, Ana1 is required for overall Polo recruitment (directly or indirectly) to the centriole wall and the PCM. In addition, I have shown that having a high frequency of ptPBSs (Ser-Ser/Thr) is a conserved feature of these proteins. It appears that the frequency and conservation of ptPBSs in a protein could potentially be a strong predictor of whether a protein is involved in canonical Polo binding. Thus, while the results presented in this thesis are of particular interest to the centrosome field, the strategy employed here could be adapted to study Polo/PLK1 recruitment to other subcellular structures.

Some of the results and areas for future work have already been discussed at the end of the corresponding Chapters. Therefore, in the following sections I focus on my main observations, discussing them in the wider context of previously available literature, and proposing a unified model of Polo recruitment to the centrosome.

6.2. A positive feedback loop drives centrosome maturation in fly embryos

The PCM expands dramatically around the mother centriole in preparation for mitosis (Palazzo et al., 1999). Any model that attempts to explain such complex process has to account for several fundamental aspects that have been recognised over the years: nucleation by a mother centriole (Basto et al., 2006; Bobinnec et al., 1998; Cabral et al., 2019; Kirkham et al., 2003; Wang et al., 2011b), autocatalysis (Zwicker et al., 2014), and mitotic PCM size being independent from starting conditions (Conduit et al., 2010, 2015a; Raff, 2019). It has also been previously proposed that mitotic PCM scaffold formation in fly and worm embryos relies on the cooperation between three proteins: Spd-2, Polo and Cnn (Cabral et al., 2019; Conduit et al., 2014a, 2014b, 2015a; Decker et al., 2011; Dobbelaere et al., 2008; Feng et al., 2017; Hamill et al., 2002; Kemp et al., 2004; Pelletier et al., 2004; Wueseke et al., 2016). I have shown that Spd-2 recruits Polo to the PCM, and I propose that this interaction is part of a Spd-2/Polo/Cnn positive feedback loop (Figure 6.1A) that can account for the fundamental aspects of centrosome maturation described above.

The model is summarised in Figure 6.1B. During interphase, Spd-2, Polo and Cnn are recruited to the centriole wall (Fu and Glover, 2012) but Polo is inactive and Spd-2 and Cnn are not phosphorylated and so do not form scaffolds (Figure 6.1Bi). As cells prepare for mitosis, Spd-2 becomes phosphorylated, and thus able to directly bind to the PBD of Polo kinase (Figure 6.1Bii). Indeed, a fraction of Spd-2 appears to be specifically phosphorylated at centrosomes (P. Conduit and J. Baumbach, (Alvarez-Rodrigo et al., 2019)). My *in vitro* and bioinformatics data suggest that Polo is involved in this phosphorylation. Polo self-priming its own binding has been

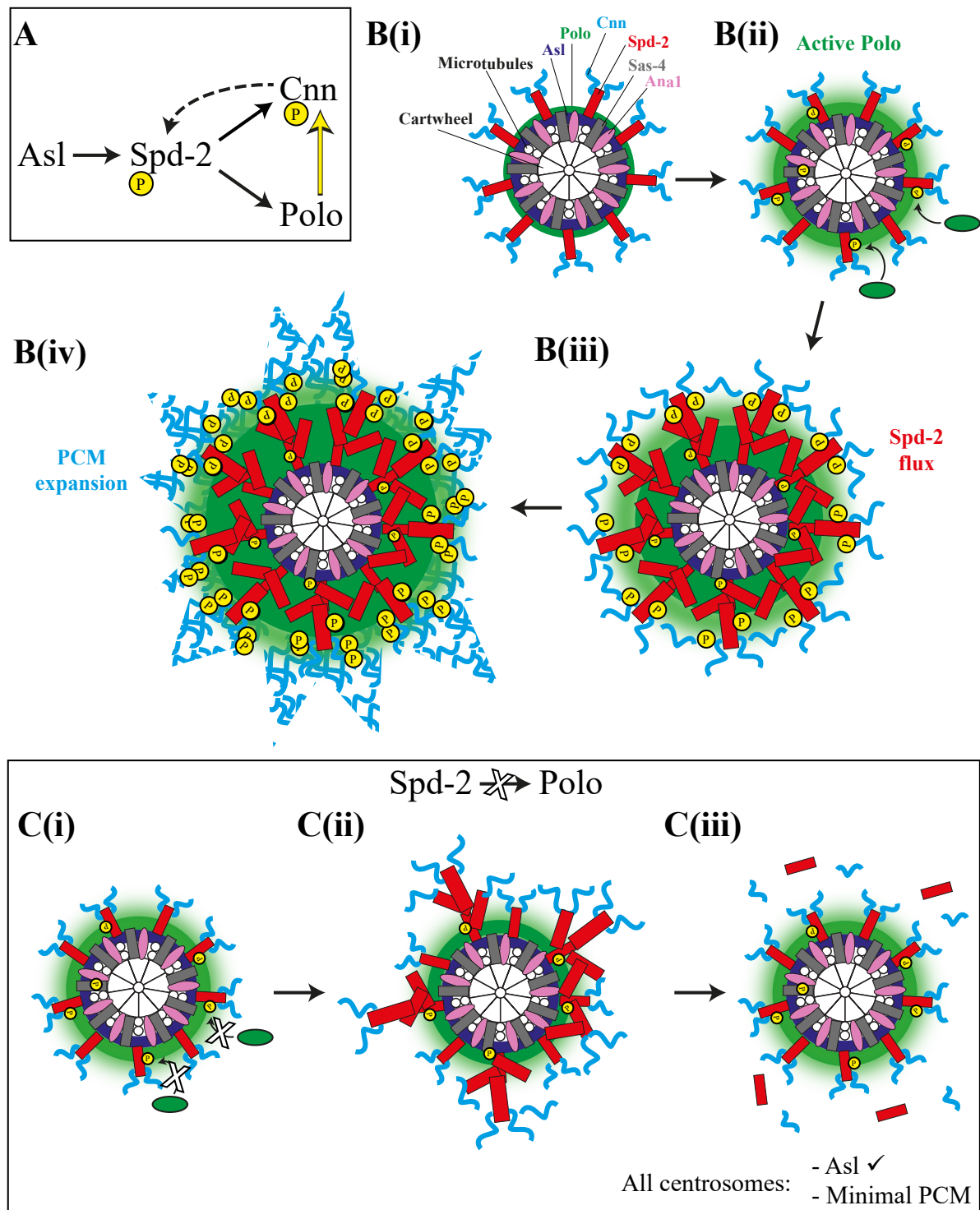


Figure 6.1 - Spd-2, Polo and Cnn appear to form a positive feedback loop that drives the expansion of the mitotic PCM scaffold.

(A) A schematic summary of the proposed positive feedback loop that drives the expansion of the mitotic PCM scaffold in *Drosophila* embryos. It has been previously proposed that Asl recruits Spd-2 and Spd-2 recruits Cnn (solid black lines), and that Cnn helps stabilise the Spd-2 scaffold (dashed black line, Conduit et al., 2014b). It has also been shown that Polo phosphorylates Cnn (yellow line) (Conduit et al., 2014a). In this thesis, I have shown that, in addition to these interactions, Spd-2 also recruits Polo. (B) Schematic of the process of mitotic PCM expansion in WT conditions. (i) Approximate localisation of the main centriolar and centrosomal players discussed in this thesis. (ii) New units of Polo bind to phosphorylated Spd-2 via their PBD. (iii) Phosphorylated Spd-2 fluxes outwards and accumulates, bringing Polo and Cnn with it, thus initiating the formation of a PCM scaffold. (iv) The Cnn scaffold cannot recruit more Spd-2 or Polo, but it stabilises the expanding Spd-2 scaffold, creating a positive feedback loop that drives mitotic PCM expansion around the mother centriole. (C(i)) If Polo cannot bind to Spd-2, Spd-2 and Cnn are still phosphorylated at the centriole wall. (ii) Spd-2 fluxes outwards, bringing Cnn with it, but not Polo. (iii) Due to the absence of Polo from the PCM, Cnn cannot be efficiently phosphorylated and so it cannot form scaffold. Thus, the Spd-2 scaffold cannot be maintained, the positive feedback loop is broken and PCM expansion fails.

previously reported in other cellular contexts (Kang et al., 2006; Neef et al., 2003, 2007), but other mitotic kinases could also be involved. The Spd-2 scaffold starts fluxing outwards, recruiting both active Polo and Cnn to the PCM nearest to the centriole wall (Figure 6.1Biii); and active Polo phosphorylates Cnn, allowing it to form its own scaffold (Conduit et al., 2014a). Note that Spd-2 scaffold formation may also require phosphorylation by Polo, but this is yet to be determined and not essential for this model. Crucially, it has been reported that the presence of the Cnn scaffold helps stabilise and maintain the Spd-2 in the PCM around the centriole (Conduit et al., 2014b). Thus, the Spd-2 scaffold spreads further, recruiting more Polo and Cnn, allowing the Cnn scaffold to spread further as well, and so on. This results in the dramatic expansion of mitotic PCM (Figure 6.1Biv).

This model is a classical positive feedback loop in which the Output (the PCM scaffold *in toto*) directly increases the Input (the Spd-2 scaffold). Furthermore, my observations suggests that, although Polo recruitment might rely mostly on conserved ptPBSs, other ptPBSs in Spd-2 may be able to contribute to this process (hence the stronger phenotype of Spd-2-ALL mutant compared to Spd-2-CONS mutant). Having multiple sites that can be phosphorylated and recruit Polo could further contribute to signal amplification and establishment of the positive feedback loop.

If Spd-2 cannot efficiently bind to the PBD (as appears to be the case for the Spd-2-ALL and Spd-2-CONS mutants), Polo is still recruited to the centriole wall (see below) (Figure 6.1Ci). Additionally, Spd-2 still recruits Cnn to the centriole wall. Thus, centriolar Polo is still able to phosphorylate Spd-2 and Cnn, and these proteins start forming mini-scaffolds (Figure 6.1Cii). However, if Polo does not spread outwards into the PCM with the Spd-2 scaffold, the Cnn scaffold cannot be maintained. The

existing Spd-2 scaffold becomes unstable and the little PCM that had been organised around the centriole collapses (Figure 6.1Ciii).

This mechanism still relies on Spd-2 being continuously recruited and phosphorylated the centriole wall, fulfilling the “nucleation by a mother centriole” aspect of centrosome maturation. Although it relies on three proteins interacting rather than one, the positive feedback loop makes PCM expansion autocatalytic: the accumulation of Spd-2, Cnn and Polo at the PCM acts as a catalyst for further accumulation. However, while the different PCM components help stabilise each other, PCM expansion is ultimately limited by how much Spd-2 can be recruited to the centriole, rather than by how much PCM already exists around the centriole. Thus, although younger mother centrioles might start with less assembled PCM than older mother centrioles, by the time they reach mitosis all centrosomes will have been able to build similar-sized PCM, fulfilling the “mitotic size is independent from starting conditions” requirement.

A key aspect of this model is that Spd-2 and Cnn are recruited to the centriole wall and then “flux” outwards into the PCM. While this phenomenon is clear in fly embryos, Cnn does not flux outwards in fly somatic cells, although Spd-2 does (Conduit and Raff, 2015; Conduit et al., 2010, 2014b). It is unclear why this fluxing would be present in some cell types but not in others. Furthermore, the *C. elegans* homologues (SPD-2 and SPD-5) do not appear to be recruited to the centre of the centrosome and then flux outwards, but (at least in the worm embryo) they are recruited isotropically throughout the volume of the PCM (Cabral et al., 2019; Laos et al., 2015). Indeed, unlike in fly embryos, SPD-2 and PLK-1 are present throughout the worm PCM, although they are mostly concentrated at the centre of the centrosome. Interestingly, my bioinformatics analysis showed that fly Spd-2 and human Cep192 are clearly

enriched in ptPBSs compared to fly Cnn and human Cep215/Cdk5Rap2 (respectively), but this difference is not so clear between worm SPD-2 and SPD-5. This suggests that these two protein families may have generally evolved to ensure that phosphorylated Cnn cannot efficiently recruit Polo on its own, requiring Spd-2 to act as an intermediary between these two proteins; but perhaps this pathway has not been as strongly conserved in worms. Worm embryos present very large mitotic centrosomes, so perhaps the process of PCM assembly has evolved to accommodate this. It will be important to determine whether Spd-2 and Cnn homologues flux in other species, and if this fluxing is important for mitotic PCM growth in these species.

Finally, the idea that many non-membrane bound organelles like the centrosome may assemble via liquid-liquid phase separation has gained traction in recent years (Banani et al., 2017; Boeynaems et al., 2018; Raff, 2019). One of the first landmark discoveries of the phase separation field was the observation that P granules in the germ cells of *C. elegans* presented liquid-like behaviour (Brangwynne et al., 2009). The strongest evidence supporting the idea that PCM assembly occurs via phase separation also comes from *C. elegans* studies (reviewed in Woodruff, 2018). Specifically, purified recombinant SPD-5 can form large aggregates *in vitro* which have transient liquid-like properties, although they rapidly harden into gel-like structures (Woodruff et al., 2015, 2017). Additionally, the mathematical model that first proposed the importance of autocatalysis in mitotic PCM expansion was based on worm embryo data and treated centrosomes as liquids (Zwicker et al., 2014). However, others have reported that SPD-5 does not undergo internal rearrangements within the worm PCM (Laos et al., 2015), unlike what would be expected of liquid-like droplets. In flies, both Cnn *in vivo* and Cnn fragments *in vitro* exhibited solid-like behaviour (Conduit et al., 2010, 2014b; Feng et al., 2017). Indeed, while the idea of a single protein species (Cnn/SPD-5)

triggering PCM assembly via phase separation is attractive, this model struggles to explain several of the fundamental aspects of centrosome maturation, such as the need for nucleation by a mother centriole (as Cnn could catalyse its own recruitment), or how two centrosomes of different ages can reach similar sizes (as the larger Cnn assembly would be expected to outcompete the smaller aggregate). Conversely, the model proposed in this thesis, where Spd-2 emanates from the centriole wall to the PCM, bringing Polo along, functions within the conceptual framework of a non-liquid-like scaffold that requires the mother centriole, but the amplification implicit in the Spd-2/Polo/Cnn positive feedback loop can still provide an “autocatalytic” mechanism.

6.3. Ana1 is a crucial reservoir of centriolar Polo

The model of Polo recruitment to the PCM described above requires Spd-2 phosphorylation, most likely mediated by Polo itself, but it does not explain how Polo is initially recruited to the centriole wall. Previous work in the Raff laboratory has shown that the Thr200-STP motif in Sas-4 is involved in Polo recruitment to young centrioles as they are converted into fully functional mothers. Thr200-mediated Polo recruitment is also essential for Asl recruitment to young mothers. However, it is not required for Polo or Asl maintenance at older mothers (Novak et al., 2016). Thus, it was proposed that other proteins at the centriole might be involved in “bulk” Polo recruitment/storage at the centriole wall.

My mRNA injection-based screen has shown that Polo recruitment does not rely on any other ptPBS in Sas-4, nor in other proteins that were promising candidates (Asl and Cep135). While this does not rule out that sites in these proteins could inefficiently contribute to maintaining Polo at the centrosome, it appears that most centriolar Polo relies on Ana1-mediated recruitment.

I propose the following model to bring together previous observations (Novak et al., 2016) and my results (Figure 6.2A,B). Sas-4 is incorporated into daughter centrioles during S-phase, with Ana1 incorporation occurring sometime later. Once in mitosis, Sas-4 Thr200 is phosphorylated by CDK1, which allows a few Polo molecules to bind to it, triggering Asl recruitment and centriole conversion into a fully functional mother (Novak et al., 2016) (Figure 6.2Bi-iii). The Ana1 C-terminal region becomes phosphorylated (Figure 6.2Biv) (see below) and additional Polo molecules bind to it via their PBD (Figure 6.2Bv), amplifying the signal initially triggered by CDK1. Both centrosomes are now fully functional (Figure 6.2Bvi). In preparation for a new round of mitosis, Spd-2 starts accumulating at the centriole and is phosphorylated by Polo recruited by Sas-4 and Ana1, triggering the positive feedback loop discussed above.

If Polo binding to the Sas-4 Thr200 STP-motif is disturbed in fly embryos that already have some matured centrosomes (e.g. injecting an antibody that occludes this site, Novak et al., 2016) (Figure 6.2C), new centrioles cannot recruit the initial Polo molecules, so Ana1 is not phosphorylated and cannot recruit Polo, and both Asl recruitment/centriole conversion and mitotic PCM expansion are impaired. However, older centrosomes are unaffected because they already contain a pool of Polo bound to Ana1 (at the centriole) and Spd-2 (at the PCM).

Conversely, if Ana1-mediated Polo recruitment is abolished (Figure 6.2D), the Sas-4 Thr200-STP motif can still recruit Polo, and daughter centrioles are converted into young mothers. These centrioles lack the Polo “reservoir” provided by Ana1, so not enough Polo is recruited to efficiently phosphorylate Spd-2 and trigger the Spd-2/Polo/Cnn positive feedback loop. Nevertheless, some (presumably very old) centrosomes in fly embryos are still able to organise a somewhat extended mitotic

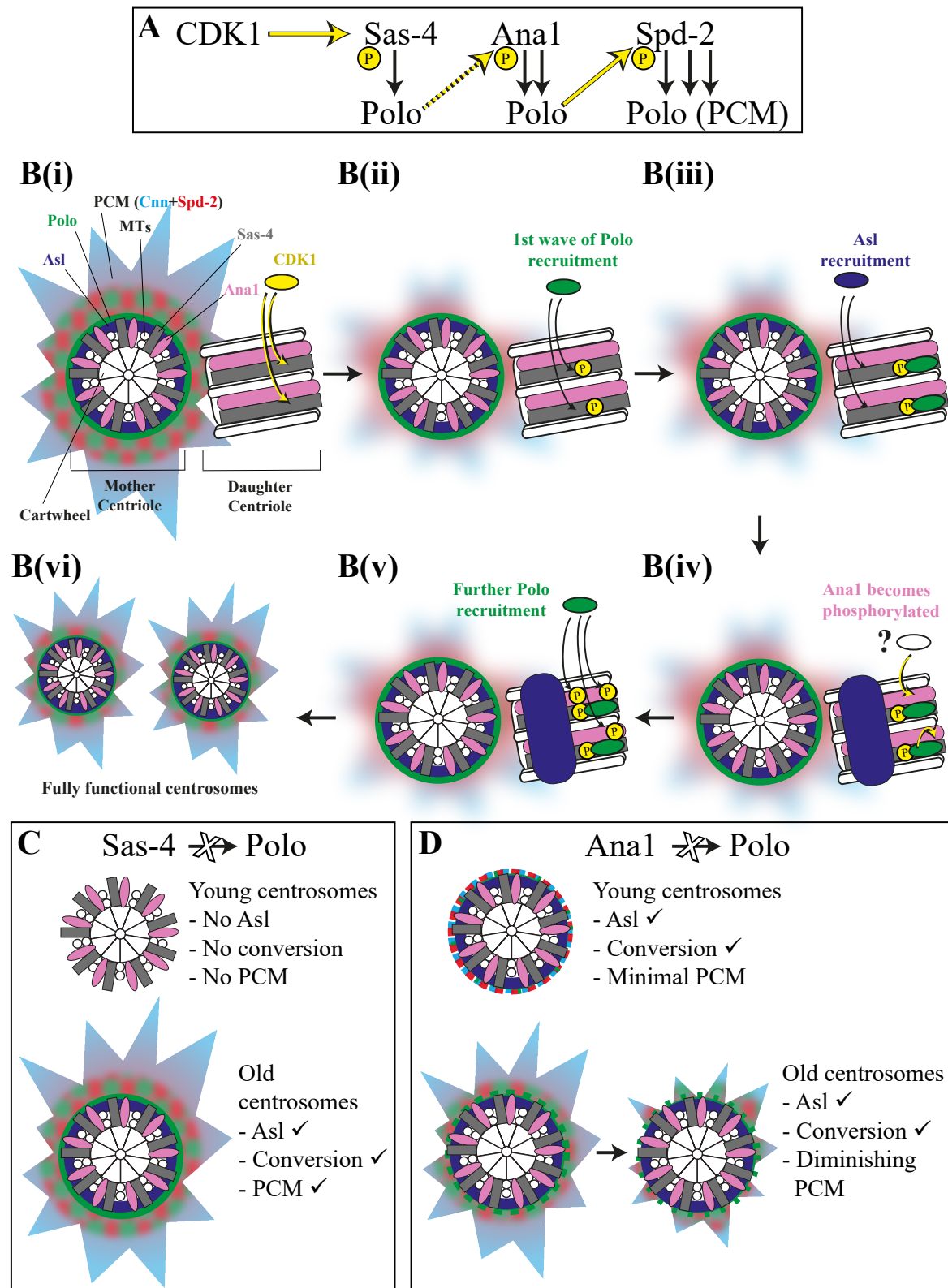


Figure 6.2 – Sas-4 and Ana1 recruit Polo to the centrosome.

(A) A schematic summary of different Polo recruitment events at the centrosome. CDK1 phosphorylates Sas-4 at Thr200 to allow PBD binding (Novak et al., 2016). I propose that Polo then phosphorylates Ana1, triggering further Polo accumulation. Centriolar Polo then phosphorylates Spd-2, which recruits more Polo to the PCM.

(B) Schematic of Polo recruitment events in the context of the centrosome cycle. For ease of visualisation, the PCM is depicted as a homogeneous “cloud” of proteins, and blurred out during the steps depicting the daughter centriole conversion. **(i-iii)** Polo recruitment by Sas-4, required for Asl recruitment (Novak et al., 2016). **(iv-vi)** Ana1 mediates a 2nd wave of Polo recruitment, most likely by being phosphorylated (by Polo or another kinase) and directly binding the PBD. This process generates two fully functional mother centrioles able to assemble mitotic PCM.

(C) Consequences of losing Polo binding to Sas-4 (Novak et al., 2016). **(D)** Consequences of losing Ana1-mediated Polo recruitment. New centrioles still recruit Asl, Spd-2 and Cnn, but do not have enough Polo to trigger PCM assembly. Old centrosomes may be able to recruit enough Polo through Sas-4 and Spd-2 (and potentially others) to inefficiently trigger PCM assembly.

PCM. This could be because very early in embryo development there are other factors that contribute to Polo recruitment, or it could be that these centrosomes are formed at a time when there are very few centrosomes, so there might be less competition to recruit Polo and/or Spd-2, and thus they can trigger the PCM positive feedback loop without requiring the Ana1 “reservoir”. Polo is maintained at these older centrosomes by Sas-4 and the Spd-2 scaffold, but PCM assembly is inefficient. After PCM disassembly at the end of mitosis not enough Spd-2 scaffold can be re-built, and so their PCM is smaller than that of WT controls.

What happens if Polo kinase activity is reduced? Throughout my studies I have observed a consistent sensitization effect when a PoloGFP protein trap allele was co-expressed with Spd-2 or Ana1 ptPBS mutants. A potential explanation is that the PoloGFP fusion protein is not as active as its untagged counterpart. Thus, in addition to suffering a significant reduction in centrosomal Polo levels, some of the Polo molecules that are recruited (by Sas-4 Thr200-STP and by Ana1 or Spd-2, respectively) are not as functional, so it is more difficult for these centrosomes to present enough Polo activity to drive mitotic PCM assembly or to recruit Asl normally during centriole conversion.

On this note, while it seems clear that Asl and Ana1 interact and that Ana1 contributes to Asl maintenance at the centrosome (Fu et al., 2016; Galletta et al., 2016; Saurya et al., 2016), there is some disagreement on whether Ana1 is required for the initial recruitment of Asl. The key piece of evidence supporting Ana1-mediated Asl recruitment comes from observations in S2 cells where Ana1 depletion (via RNAi) was rescued by artificially tethering the Ana1 C-terminus to centrosomes (Fu et al., 2016). In light of my observations, it would be interesting to investigate whether this

reported Asl loss is due to losing the Ana1-Asl interaction and/or the centriolar Polo reservoir recruited by Ana1. It could be that in S2 cells both the Sas-4 Thr200-STP motif and the Ana1 C-terminus are required to recruit enough Polo to trigger Asl recruitment during centriole conversion, or that in S2 cells initial Polo recruitment during centriole conversion occurs via Ana1 without requiring the Sas-4 Thr200-STP motif.

The model presented here makes several assumptions. The simplest explanation to my observations is that Polo binds via its PBD to ptPBSs in the Ana1 C-terminal region, but I have not tested this formally yet. I am also assuming that Ana1-mediated Polo recruitment occurs downstream of Sas-4-mediated Polo recruitment, on the basis that Asl recruitment and centriole conversion require Sas-4-mediated Polo recruitment but are unaffected in Ana1-ALL-GFP embryos. Similarly to Spd-2-mediated Polo recruitment, I am also proposing that Polo self-primes the binding of the PBD to Ana1, but other regulators could contribute to Ana1 phosphorylation.

Based on my bioinformatic analysis of Ana1, the best alternative candidate to phosphorylate ptPBSs is Aurora A, a mitotic kinase present on centrosomes from late S-phase that plays an important role in centrosome maturation in many species (Barr and Gergely, 2007). In vertebrates, Cep192 serves as a scaffold for PLK1 and Aurora A, and the two kinases influence each other's activity and centrosomal localisation (Joukov et al., 2010, 2014; Meng et al., 2015). In *Drosophila*, Spd-2 plays an important role in recruiting Aurora A to centrosomes (Dobbelaere et al., 2008; Conduit et al., 2014b), although it is unclear if this is through direct binding. In worms, the Aurora A homologue AIR-1 is required to trigger mitotic PCM expansion, but unlike SPD-2, it is not required for subsequent PCM growth (Cabral et al., 2019). Clearly,

Aurora A must be able to influence the Spd-2/Polo/Cnn feedback loop, but currently it is unclear how. Surprisingly, a strong interaction was detected between the Ana1 C-terminal region and Aurora A in a yeast two-hybrid screen (Galletta et al., 2016). Thus, an interesting possibility is that Aurora A could contribute to PCM assembly regulation by promoting Polo recruitment and activation at the centriole wall.

In summary, this model attempts to integrate previous knowledge and the results presented in this thesis to generate an overview of how Polo co-ordinates two key events of centrosome biogenesis: centriole conversion into a fully functional mother and mitotic PCM assembly.

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