The Human Polo-like Kinase 4 is a Regulator of Centrosome Duplication.

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Hiermit erkläre ich, dass ich die vorliegende Dissertation selbständig und ohne unerlaubte Hilfe angefertigt habe. Sämtliche Experimente wurden von mir selbst durchgeführt,soweit nicht explizit auf Dritte verwiesen wird. Ich habe weder an anderer Stelle versucht, eine Dissertation oder Teile einer solchen einzureichen bzw. einer Prüfungskommission vorzulegen, noch eine Doktorprüfung zu absolvieren.

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SUMMARY

Polo-like kinase 4 (Plk4), also known as Snk/Fnk-akin kinase (Sak), is the most structurally divergent member of the Polo-like kinase family (Fode et al., 1994; Leung et al., 2002) and has been associated with cell proliferation (Fode et al., 1994). Most importantly, $Plk4^{-/-}$ mice were found to be embryonically lethal whereas heterozygous $Plk^{+/-}$ mice exhibited an increased incidence of tumorigenesis (Hudson et al., 2001; Ko et al., 2005). However, very little data has been reported on the function of human Plk4.

Here, we embarked on a functional characterisation of human Plk4 in cultured cells. This kinase localised to the centrosome throughout the cell cycle, and overexpression studies revealed that an excess of wild type Plk4 induced the overduplication of centrioles in cells. This process was dependent on the cyclin-dependent kinase (Cdk) 2 in addition to CP110 and human Sas-6, two proteins shown to be involved in centriole duplication (Chen et al., 2002b; Leidel et al., 2005). Conversely, depletion of endogenous Plk4 resulted in an inhibition of centriole duplication leading to a loss of centrioles from dividing cells. These data from gain-of-function and loss-of-function experiments demonstrated that Plk4 is an essential regulator of centriole duplication.

Further microscopic studies revealed that excess Plk4 activity resulted in the formation of rosette-like structures around centrioles. These structures recruited CPAP which is the human homologue of Sas-4, a protein required for centriole duplication in the nematode worm (*Caenorhabditis elegans*). We propose that these structures represent centriole precursors.

The study presented here is a starting point for further work. The identification of Plk4 as a central regulator of centriole duplication will allow the characterisation of upstream and downstream regulatory pathways, whereas a continued search for interaction partners should resolve the function of this kinase. In particular, the analysis of rosette-like centriole precursors is a promising avenue to provide insights into the mechanism of centriole duplication.

INTRODUCTION

Since Boveri's discovery of the centrosome more than a century ago, this cellular organelle has fascinated biologists with its intriguing and complex nature. One particular aspect that has recently garnered much attention is the centrosome's unusual duplication cycle, reminiscent in many respects to that of DNA. The interphase centriole must duplicate exactly once per cell cycle and this must be completed before mitosis. Furthermore, the regulation of this event involves several proteins, some located at the centrosome, others involved in mitotic processes not restricted to this organelle. At present it is clear that not all components and regulatory mechanisms of centriole duplication have been fully characterised.

Never-the-less, deregulation of centriole duplication is known to result in cells with multiple centrosomes and the formation of multipolar spindles, leading to chromosome instability (Brinkley, 2001; Nigg E.A., 2004; Goepfert and Brinkley, 2004). In fact, cells from several tumours are known to exhibit excess numbers of centrosomes (Lingle et al., 1998; Pihan et al., 1998; Carroll et al., 1999), and this situation cannot be remedied by the cell due to a lack of a specific checkpoint for multipolar spindles (Sluder et al., 1997). Centrosome duplication must therefore be tightly coordinated with other events during the cell cycle. Here, the basic structure and function of the centrosome will be presented, followed by a review of proteins involved in centrosome duplication. The causes and consequences of centrosome amplification will be discussed, and the members of the polo-like family of kinases will be introduced.

Structure of the centrosome

The centrosome is a small non-membraneous organelle occupying approximately $1\mu m^3$ of cell volume and is usually found in close proximity to the nucleus (Doxsey, 2001; Bornens, 2002; Gall, 2004; Azimzadeh and Bornens, 2004). It functions as the microtubule organising centre (MTOC) in interphase cells and is essential to the formation of cilia. These functions are conserved in evolution across a wide variety of exclusively eukaryotic organisms (Beisson and Wright, 2003), with the exception of higher plants and certain fungi that are thought to have lost the ability to form this

organelle. Specifically, retention of centrosomes may correlate with the maintenance of flagellated gametes or stages in an organism's reproductive cycle.

The mammalian centrosome comprises a pair of orthogonally arranged centrioles surrounded by the fibrous pericentriolar matrix (PCM), an electron dense structure containing proteins required for microtubule nucleation (Fig. 1) (Doxsey, 2001; Mority, 2004). The PCM thus provides docking sites for numerous proteins involved in this process, notably members of the γ-tubulin ring complex (γTuRC) but also larger coiled-coil proteins such as AKAP450, BBS4 and PCM-1 (Keryer et al., 2003a; Balczon et al., 1994)

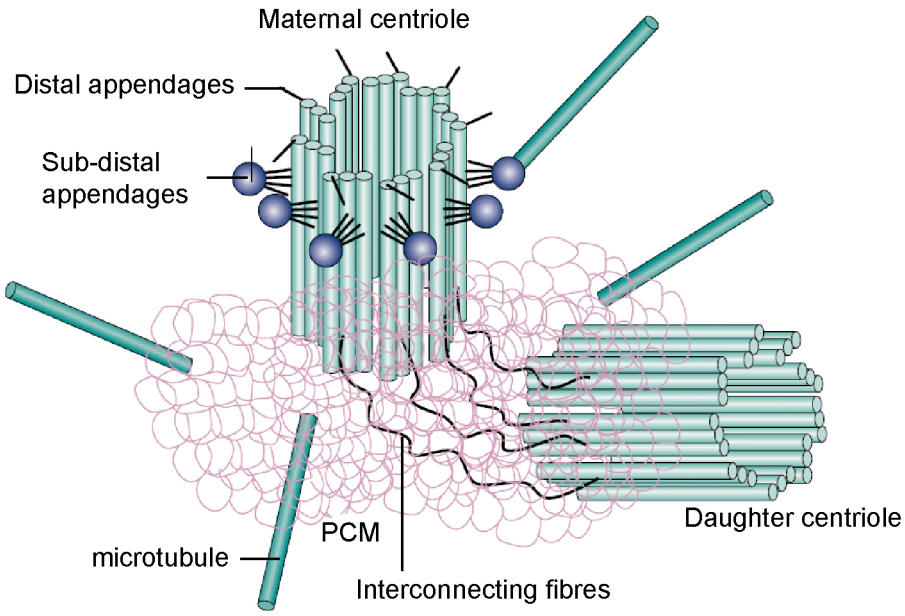

Figure 1. Structure of the centrosome. Schematic diagram depicting the centrosome consisting of the centrioles and the surrounding PCM. (Doxsey, 2001)

Embedded within the surrounding PCM, two centrioles are composed of a symmetrical barrel-shaped array of nine triplets of microtubulules generally 400 nm. Basal bodies found at the base of the cilium are identical in nature to centrioles (Beisson and Wright, 2003). Several proteins are found at centrioles, in particular δ-tubulin, centrins-2 and -3 and polyglutamylated β-tubulin (Dutcher, 2001; Salisbury et al., 2002; Azimzadeh and Bornens, 2004; Bobinnec et al., 1998). However, the two centrioles are structurally dissimilar. Several proteins are only found on the fully

matured centriole (or mother centriole) which is characterised by the presence of appendages where proteins such as ε-tubulin (Chang et al., 2003), ninein (Mogensen et al., 2000), odf2 (Ishikawa et al., 2005) or cep170 (Guarguaglini et al., 2005) are located.

Centrioles are integral to the whole structure of the centrosome. When antibodies against polyglutamylated tubulin are microinjected into cells, centrioles disassemble and the PCM is seen to disperse (Bobinnec et al., 1998). Centrioles therefore determine the site of PCM accumulation. Secondly, centrioles determine the reproductive capacity of the centrosome. When centrosomes are stripped of centrioles in sea urchin zygotes, the PCM is no longer capable of duplicating (Geissler et al., 1996; Salisbury, 2004)

Although the centriole is evolutionarily conserved across a wide variety of organisms, it has undergone structural alterations. In particular, the centriole in the fruit fly (*Drosophila melanogaster)* and the nematode (*Caenorhabditis elegans)* is characterised by a reduction in the number of microtubules, namely doublets and singlets, respectively (Raff, 2004; Delattre and Gonczy, 2004). Conversely, plants have generally lost the ability to form centrioles. Notable exceptions include several lower plants, such as bryophytes, cycads and *Ginkgo* which are able to form *de novo* centriole-like structures during gametogenesis. These sometimes accumulate forming giant agglomerations called blepharoplasts (Gall, 2004). In contrast, yeast have a functionally equivalent structure called the spindle-pole body (SPB), albeit of entirely different organisation (van Kreeveld Noane and Winey, 2004). Despite its divergent structure, some components of the SPB have distantly related proteins in the mammalian cell, thus revealing conserved roles for proteins in organelle structure and microtubule nucleation.

The centrosome cycle

The centrosome cycle is commonly classified as a sequence of four events: centriole disorientation, centriole duplication, centrosome disjunction and centrosome separation (Figure 2)(Sluder, 2004). The distinctions between each event are based on morphological changes that are observable by light and electron microscopy (Kuriyama and Borisy, 1981; Chretien et al., 1997). The individual steps of the

centrosome cycle occur during different stages of the cell cycle (summarised in Figure 2).

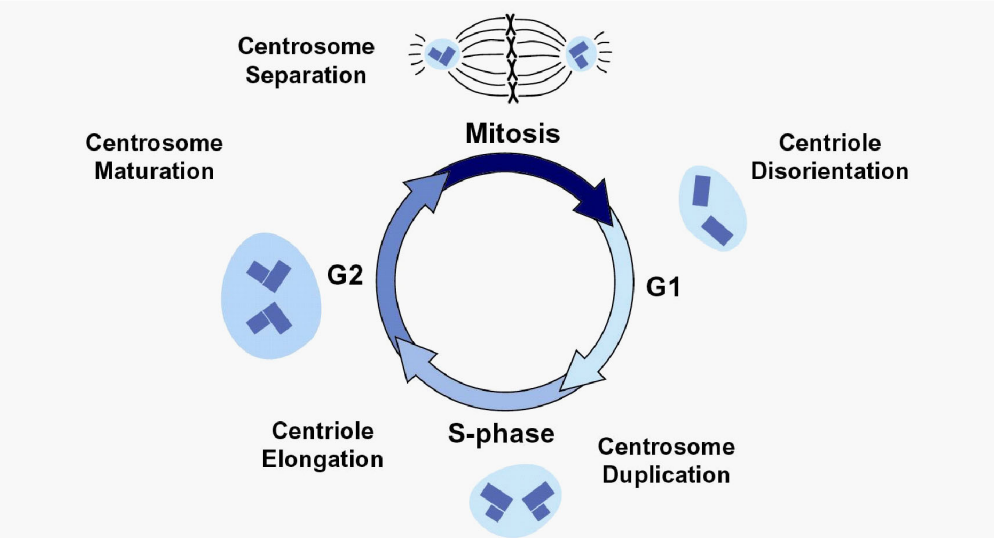

Figure 2. The centrosome cycle.

Schematic diagram showing the different stages of the centrosome cycle during the phases of cell division. Centrioles are in dark blue, the PCM in light blue.

The earliest event considered important for centriole duplication is centriole disorientation (Freed et al., 1999). This event takes place during late M- or G1 phase and involves the action of SCF ubiquitin ligase components and the proteasome (Freed et al., 1999). At this stage, centrioles lose their orthogonally paired relationship and in some cells are observed to separate widely, such as in HeLa cells (Piel et al., 2000). Although the significance of centriole disorientation for the process of centrosome reproduction remains unclear, centrioles have been observed to travel extensively, an action that has been proposed as essential for the completion of cytokinesis (Piel et al., 2001).

Centriole duplication begins with the appearance of short pro-centrioles at right angles to the proximal ends of the parental centrioles during G1/S or early S phase (Kuriyama and Borisy, 1981; Robbins et al., 1968; Vorobjev and Chentsov, 1982; Alvey, 1985; Kochanski and Borisy, 1990; Paintrand et al., 1992). Studies using

microinjection of biotinylated γ-tubulin have demonstrated that recruitment of labelled tubulin only takes place at the nascent centriole, revealing a conservative mode of centriole assembly (Kochanski and Borisy, 1990). Procentrioles elongate during the S-phase and attain their final length during G2. Although having then reached full size, the new daughter centrioles only mature by acquiring distal and sub-distal appendages in the following cell cycles. Although the description of centriole duplication steps are based on observational data, it has been put forward that the physical appearance of nascent daughter centrioles could follow on from earlier molecular events undetectable by present microscopic techniques (Sluder, 2004).

After centrioles have duplicated, a putative physical link between the two parent-daughter centriole pairs is cut, resulting in a semi-conservative distribution of centrioles to the two resulting centrosomes (Figure 2). At present, the composition of this linker is unknown. However, centrosome separation is thought to be regulated by the phosphorylation status of C-Nap1, a protein located at the proximal ends of the parental centriole and which may act as a docking site for other linker proteins (Fry et al., 1998; Mayor et al., 2000). The phosphorylation status of this protein is maintained in a balance by the centrosomal kinase Nek2 and its antagonistic protein phosphatase 1α (PP1α), which is inactivated at the beginning of mitosis (Meraldi and Nigg, 2001). Dephosphorylation of C-Nap1 leads to its displacement from the proximal centriole end during mitosis, thus allowing centriole separation to proceed through the action of plus and minus-end directed microtubule motor proteins. Additionally, the centrosomal protein rootletin, a component of the ciliary rootlet of retinal cells, has been recently described as an interactor of C-Nap1 and as a substrate of Nek2 (Bahe et al., 2005). Rootletin forms fibres emanating from the proximal ends of centrioles. Its depletion leads to precocious centriole splitting and accordingly, rootletin has been proposed to form part of the linker structure.

Other proteins are also involved in centrosome separation. A report suggests that the activity of the phosphatase Cdc14A may also be implicated (Mailand et al., 2002). Depletion of this phosphatase results in a lack of separation whereas an excess of Cdc14A leads to a loss of centrosome cohesion. Similarly, siRNA mediated depletion of dynamin 2, a large GTPase involved in vesicle formation and actin organisation, results in centrosome splitting (Thompson et al., 2004). Furthermore,

centrin phosphorylation has also been thought to play a role in this process (Lutz et al., 2001).

The centrosome cycle is completed in G2 by a maturation step characterized by a large increase in centrosome size caused by the recruitment of additional γ-tubulin ring complexes (Palazzo et al., 2000). Concomitantly, the levels of microtubule nucleation are drastically increased (Palazzo et al., 2000). Furthermore, maturation is distinguished by the recruitment of additional centrosomal proteins such as NuMA (Merdes et al., 1996) and Plk1 (Golsteyn et al., 1995; Lane and Nigg, 1996) while others are lost. Plk1 has been shown to regulate Nlp, a centrosomal protein that is involved in binding γ-tubulin ring complexes and nucleating microtubules (Casenghi et al., 2003; Casenghi et al., 2005). Furthermore, Aurora A (Berdnik and Knoblich, 2002; Hannak et al., 2001), Cdk11 (Petretti et al., 2006) and the posphatase PP4 (Helps et al., 1998; Sumiyoshi et al., 2002) have been implicated in centrosome maturation although the underlying mechanisms are still unknown.

Regulation of centriole duplication

How the parent centriole is able to control the assembly of a single daughter centriole perpendicularly to its surface remains a mystery. At present, a hypothesis has been proposed that a templating mechanism determines a docking site at the proximal end of the mother centriole to which centriolar proteins are recruited (Sluder, 2004; Delattre and Gonczy, 2004). Parallels are seen in yeast, where the half-bridge structure of the new spindle pole body defines the site of duplication in the next cell cycle (Francis and Davis, 2000; van Kreeveld Noane and Winey, 2004). A similar structure has not been observed in mammalian cells, thus the question remains whether a template exists and how it is restricted to one proximal site. Interestingly, cells from a temperature sensitive mutant of Cdk1 in *Drosophila* show prolonged S-phases and abnormal centriole duplication (Vidwans et al., 2003). Some of these cells exhibit daughter centrioles of increased length or parental centrioles with two or more daughter centrioles. Furthermore, centriole lengths vary in different tissues in *Drosophila* (Raff, 2004). The centrioles of the developing spermatocyte crypt are very long and are thus particularly suitable for microscopic analysis. Therefore, it seems plausible that centriole length and the number of assembly sites are not structurally restricted but instead are subject to regulatory mechanisms.

Uncoupled centriole propagation is thought to occur *de novo* in the absence of a putative templating mechanism (Beisson and Wright, 2003). In particular, differentiating ciliated epithelial cells generate multiple basal bodies from a putative structure called the deuterosome (Beisson and Wright, 2003). Similarly, cells whose centrioles have been removed by laser ablation are able to reform microtubule organizing centres containing centrioles, albeit at multiple copy numbers (Khodjakov et al., 2002). Furthermore, in the mouse zygote, centrioles are not detectable until several cell divisions after fertilization (Szollosi et al., 1972; Delattre and Gonczy, 2004), and are found in parthogenetically dividing sea urchin eggs (Kallenbach and Mazia, 1982). The number of centrioles and the mode of replication may therefore be variable and under developmental control.

For most cells, the rule that the centrosome must be restricted to a single duplication only once per cell cycle holds true. Earlier studies by Mazia and co-workers using sea-urchin zygotes revealed a regulatory mechanism that maintains the number of centrosomes to one, and which is dependent on the number of centriole pairs present in the cell (Sluder, 2004). Centrioles in these zygotic cells were artificially induced to split by a prolongued prometaphase delay, resulting in the formation of tetra-polar spindles. These poles contained only a single centriole which was incapable of splitting any further. The generated daughter cells therefore contained one centrosome with only one centriole. This single centriole was then seen to duplicate but not split during subsequent cell cycles, thus maintaining proper centrosome numbers. Consequently, the number of centriole pairs was found to determine the number of centrosomes under normal circumstances.

Cells do not have a checkpoint to stop the cell cycle in the presence of multiple centrosomes (Sluder et al., 1997). Instead, cells have a mechanism to prohibit excess centrosome duplication, as revealed by a study using mammalian cells (Wong and Stearns, 2003). Cells from different phases of the cell cycle were fused to assay whether previously duplicated centrosomes would reduplicate in an S-phase cytoplasm. Fusion of a G1 and a G2 cell revealed that the G1 centrosome was competent for duplication whereas the G2 was not, even though both were in an S-phase cytoplasm permissive for duplication. This intriguing result suggested that a duplication inhibitor must reside on the G2 centrosome itself. Conversely, several mammalian cell lines lack this control mechanism and are capable of breaking the 'once-per cell cycle' rule. In particular, CHO and U2OS cells subjected to a

prolonged S-phase arrest are able to overduplicate their centrosomes (Balczon et al., 1995). Taken together, cells have regulatory mechanisms to maintain centrosome numbers and limit their reproduction during the cell cycle.

Cdk2-cyclinA/E regulates centrosome duplication

A sensible manner to control timely centrosome reproduction would be to link this process to DNA replication. Indeed, evidence has accumulated supporting a role for the cyclin dependent kinase 2 (Cdk2) together with cyclin A/E in regulating centriole duplication (Sluder, 2004). Initial data was obtained from mammalian somatic cells competent for multiple rounds of centrosome duplication when arrested in S-phase. When Cdk2 activity in these cells was blocked by treatment with roscovitin, a potent inhibitor of Cdks, or by overexpression of the Cdk-inhibitor protein p21, centrosome reduplication was inhibited (Matsumoto et al., 1999b; Meraldi et al., 1999a). A similar result was obtained by transfecting cells with a phosphorylation site mutant of the Retinoblastoma protein, a tumour suppressor that must be phosphorylated to promote the expression of genes required for mitosis. This block could be relieved by contransfecting with Cdk2-cyclin A whereas cyclin E could not (Meraldi et al., 1999a). These studies therefore highlighted the role of Cdk2-cyclin A in mammalian centrosome reproduction.

In parallel, studies using S-phase arrested *Xenopus* egg extracts confirmed the requirement of Cdk2-cyclin E for centrosome duplication (Hinchcliffe et al., 1999b; Lacey et al., 1999). In these studies, egg extracts were arrested in S phase allowing multiple rounds of reduplication of exogenous sperm centrioles. Centriole duplication was inhibited when a truncated version of *Xenopus* p27, a Cdk-inhibitor protein related to p21, was added to the extract. Conversely, centrosome duplication was restored with an excess of purified Cdk2-cyclin E. Since Cdk2-cyclin A is not active in *Xenopus* zygotes early on (Rempel et al., 1995), these results suggest that only Cdk2 complexed with cyclin E was essential for centrosome duplication. However, differences may be explained by the assays used, pointing to an involvement of cyclin E early in zygotic development and cyclin A later on in somatic cells.

However, explanations involving these two cyclins become more complex when results from knockout mice are taken into consideration (Roberts and Sherr, 2003; Traganos, 2004; Aleem et al., 2004). Cdk2 knockout mice are largely viable and

show only minor defects in germ cell development (Berthet et al., 2003; Berthet et al., 2003), suggesting that Cdk2 is not strictly required for cell proliferation. Likewise, mice deficient in cyclin E1 and cyclin E2 as well as the double knockout mouse proceed with normal development until the 10th day of gestation (Geng et al., 2003), indicating that cell proliferation can occur in the absence of cyclin E. While cyclin A1 is not necessary for normal mouse development with the exception of gametes (Liu et al., 1998), a lack of cyclin A2 results in embryonic lethality (Murphy et al., 1997). It has therefore been proposed that cells are able to survive with only cyclins A2 and B complexed with Cdk1, and that these Cdk-cyclin complexes are sufficient to perform all necessary functions to drive the cell through S and M phases of the cell cycle (Roberts and Sherr, 2003). Taking this into consideration, it would appear likely that certain cyclins and Cdks are redundant or have particular developmental or tissue specific functions. However, how these data explain the requirement of Cdk2 in cultured mammalian cells, or whether a particular Cdk-cyclin complex is uniquely required for centriole duplication, remains unclear.

Other kinases required for centrosome duplication

Studies using the *Xenopus* egg extract system have revealed a role of Calcium/Calmodulin-dependent kinase II (CamKII) in centriole duplication (Matsumoto and Maller, 2002). Similar to the investigation of the role of Cdk2-cyclin E in this process, S-phase arrested egg extracts supportive of multiple cycles of centriole duplication were used. Centriole duplication was blocked when calcium signalling was inhibited by the use of chelating agents or an inhibitor of inositol 1,4,5 trisphosphate receptor mediated calcium oscillations. When CamKII was directly inhibited by the addition of a pseudosubstrate peptide to the egg extract, a similar result was observed. Furthermore, centriole duplication could be restored in these extracts by the addition of excess CamKII together with calmodulin. In contrast to the effects of Cdk2-cyclin E inhibition which led to a block after the first round of centriole duplication, inactivation of CamKII prevented centriole duplication right from the beginning. This intriguing result could point to a role of CamKII in kick-starting centriole duplication in response to early calcium signalling events such as those caused by fertilization (Sluder, 2004).

A screen for genes necessary for embryonic development of *C. elegans* uncovered Zyg-1, a kinase essential for all developmental stages (O'Connell et al., 1998). An elegant analysis of the earliest divisions of the *C. elegans* embryo using reciprocal crosses between wild type and mutant parents revealed the requirement of this kinase in centriole duplication (Figure 3) (O'Connell et al., 2001).

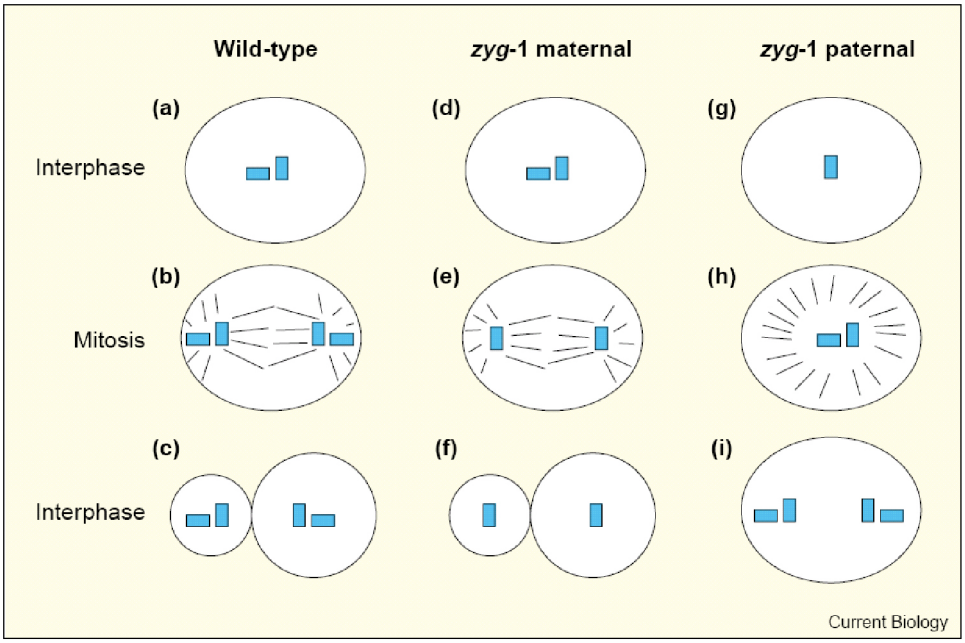

Figure 3. Zyg-1 is necessary for centriole duplication in *C. elegans*. Schematic diagram showing the two different phenotypes obtained for Zyg-1 mutants in early embryos. Normal embryos obtain a pair of centrioles from sperm and proceed through mitosis forming two daughter centrioles with normal centriole numbers (a,b,c). In a maternal Zyg-1mutant background, the wild type centrioles are unable to duplicate but split and form a bipolar spindle. The two resulting daughter cells only have one centriole each (d, e, f). Sperm centrioles do not duplicate in a paternal Zyg-1 mutant. Thus, the wild type oocyte is fertilised with only one centriole from mutant sperm (g), which then duplicates normally but only forms a monopolar spindle (h) and does not pass through cytokinesis (i). (Hinchcliffe and Sluder, 2001)

When mutant embryos were fertilised with wild type sperm, the parentally inherited centrioles split and formed a bipolar spindle during the first embryonic cleavage, but were unable to duplicate in subsequent cell divisions resulting in monopolar spindles (Figure 3). Zyg-1 is also required for centriole duplication during spermatogenesis, thus mutant sperm cells can only donate a single centriole to the wild type embryo upon fertilization. This single centriole was observed to duplicate correctly in the wild type cytoplasm containing maternal Zyg-1, but only formed a monopolar spindle. Immunofluorescence data places this kinase at the centrosome

during anaphase-telophase when centriole duplication begins in early embryos. Zyg-1 therefore is present at the centrosome at the right time. However, a direct substrate for this kinase has not been found nor have any homologues been identified in other organisms.

Reports have indicated a function in centriole duplication for human Mps1 (Fisk et al., 2003). Studies have focused on this kinase as the yeast homologue is necessary for duplication of the spindle pole body (Winey et al., 1991). However, other groups have disputed the the proposed centrosomal function of this kinase and instead provide data revealing a role for Mps1 in the spindle checkpoint in both mammalian cells and *Drosophila* (Stucke et al., 2002; Fischer et al., 2004).

The C. elegans *proteins Sas-4, Sas-5 and Sas-6*

The *C. elegans* centriole is different in many aspects to its mammalian counterpart. It consists of nine singlets of microtubules and lacks appendage structures (Delattre and Gonczy, 2004). Furthermore, important proteins involved in mammalian centriole duplication are missing, including ε-tubulin and centrin (see below). Although the underlying mechanisms of this process may therefore differ significantly in *C. elegans* when compared to other organisms, studies using this model organism may reveal functionally conserved mechanisms.

Large siRNA screens have identified Sas-4 and Sas-6 as two proteins essential for centriole duplication in *C. elegans* (Leidel and Gonczy, 2005). Although mutants of these proteins assemble a normal bipolar spindle during the first cleavage division, a monopolar spindle is formed in the two daughter cells due to reduced centriole numbers (Dammermann et al., 2004; Kirkham et al., 2003; Leidel et al., 2005; Leidel and Gonczy, 2003). Studies using fluorescence recovery after photobleaching (FRAP) demonstrate that Sas-4 and Sas-6 are recruited to the centriole at the onset of daughter centriole formation (Leidel et al., 2005; Leidel and Gonczy, 2003). However, Sas-4 is solely recruited to the nascent centriole whereas Sas-6 is present on both centrioles. Interestingly, Sas-6 is not found on centrioles in a Sas-4 depleted cytoplasm, revealing a protein recruitment function for Sas-4. Indeed, Sas-4 has been proposed to play a role in controlling PCM size (Kirkham et al., 2003) by regulating protein recruitment. Alternatively, Sas-4 has been proposed to control centriole length thus indirectly affecting the size of the PCM (Leidel and Gonczy, 2005).

Both Sas-4 and Sas-6 have mammalian homologues. The human counterpart of Sas-4 is the protein CPAP (centrosomal P4.1-associated protein) which has been shown to interact with γ-tubulin and was found in a mass-spectrometric analysis of the centrosome (Andersen et al., 2003; Hung et al., 2000). Unfortunately, it is not known whether CPAP is essential for mammalian centriole duplication. However, depletion of human Sas-6 from cell lines by siRNA inhibits centriole duplication and, intriguingly, a percentage of cells overexpressing human Sas-6 show excess centrioles, suggesting that a surfeit of this protein results in centriole overduplication (Leidel et al., 2005).

Like the two previous *C. elegans* proteins, Sas-5 has been identified by siRNA screens as a protein required for centriole formation (Delattre et al., 2004). This coiled-coil protein is dynamically recruited to the centriole throughout the cell cycle and has been shown to interact with Sas-6 in a yeast two-hybrid assay. Furthermore, the recruitment of both Sas-5 and Sas-6 to the centriole is dependent on the activity of Zyg-1 (Delattre et al., 2004), but neither protein has been shown to act as a substrate for this kinase. Taken together, these data suggest Zyg-1 is required for the targeting of Sas-5 and -6 to the centriole, allowing these proteins to recruit further proteins such as Sas-4 that are required for centriole assembly.

Finally, SPD-2 has the dual role of being required for PCM recruitment to the *C. elegans* centrosome and for centriole duplication (Pelletier et al., 2004; Kemp et al., 2004). Like the other centriole proteins discussed so far, SPD-2 is a large coiled-coil protein that has homologues in other organisms. In particular, a human homologue, Cep192, has been identified as a centrosomal protein (Andersen et al., 2003) but its function has not been elucidated.

In summary, there are five *C. elegans* proteins identified so far that are required for centriole duplication in the early embryo. These are Sas-4, -5 and -6 together with SPD-2 and the kinase Zyg-1. It is as yet unclear whether any more proteins remain undiscovered, or whether these five proteins are sufficient for centriole duplication in the nematode worm. However, since some of the homologues of these proteins are found in other organisms, it is likely that a similar core set of proteins could be functionally conserved in regulating human centriole duplication.

Other proteins required for centriole duplication

Other proteins have been identified in mammalian cells that are necessary for the duplication of centrioles (Table 1). Several of these contain extensive coiled-coil regions hinting at a structural role for these proteins. In particular, when the PCM is disturbed by the overexpression of a dominant negative form of the large coiled-coil protein AKAP450, centriole duplication is inhibited (Keryer et al., 2003b). Recently, the coiled-coil domain containing protein centrobin has been reported to function in centriole duplication (Zou et al., 2005). Interestingly, this protein localises to the newly forming daughter centriole and thus resembles Sas-4 in this manner.

Two proteins have been identified as substrates of Cdk2. CP110 is a large coiled-coil protein necessary for centriole reproduction (Chen et al., 2002a), as is nucleophosmin (Okuda et al., 2000; Tokuyama et al., 2001); reviewed in (Okuda, 2002). Overexpression of a non-phosphorylatable mutant of nucleophosmin was shown to block centriole duplication. Further work revealed that mice deficient of nucleophosmin died early in embryogenesis and MEFs exhibited centrosome amplification and genomic instability (Grisendi et al., 2005).

Members of the tubulin family of proteins have also been implicated in centriole duplication. γ-tubulin, an essential component of the γ-tubulin ring complexes involved in microtubule nucleation, is required for proper centrosome duplication in certain unicellular organisms (Ruiz et al., 1999; Shang et al., 2002). The related ε-tubulin, found to localise to centriole appendages, is also required (Chang et al., 2003). Moreover, two recent reports highlighted the role of NEDD1, another γ-tubulin ring complex component, in the maintenance of centriole numbers (Haren et al., 2006; Luders et al., 2006).

Some proteins essential to centriole duplication are conserved in evolutionarily distant organisms. The centriolar marker centrin-2 has been reported to function in this process and is an homologue of yeast Cdc31p (Salisbury et al., 2002; Middendorp et al., 2000; Baum et al., 1986), a protein that localises to the spindle pole body and is required for the duplication of this organelle (van Kreeveld Noane and Winey, 2004). Interestingly, centrins are Ca^{2+}-binding proteins related to the Calmodulin superfamily and are thought to form Ca^{2+} dependent fibres (Adams and Kilmartin, 2000). Likewise, the Cdc31p interactor Sfi1p is found in the spindle-pole

Table 1 Summary of proteins involved in centriole duplication.

Gene	Species	References
Kinases		
Zyg-1	C. elegans	(O'Connell et al., 2001)
Cdk2-cyclin E/A	Human, Xenopus	(Matsumoto et al., 1999a; Meraldi et al., 1999a; Lacey et al., 1999)
CamKII	Xenopus	(Matsumoto and Maller, 2002)
Plk2	Human	(Warnke et al., 2004)
Sas-4, Sas-6	*C. elegans*	(Leidel et al., 2005; Leidel and Gonczy, 2003; Dammermann et al., 2004)
Sas-5	*C. elegans*	(Delattre et al., 2004)
SPD-2	C. elegans	(Pelletier et al., 2004)
CP110	Human	(Chen et al., 2002a)
Centrin	Human	(Salisbury et al., 2002)
AKAP450	Human	(Keryer et al., 2003b)
ε-tubulin	*Xenopus*	(Chang et al., 2003)
γ-tubulin	Paramecium, tetrahymena	(Ruiz et al., 1999)
NEDD1	Human	(Haren et al., 2006; Luders et al., 2006)
centrobin	Human	(Zou et al., 2005)
PML3	Human	(Xu et al., 2005)
SHD1	Human	(Khuda et al., 2004)
ID1	Human	(Hasskarl et al., 2004)
STAT3	Human	(Metge et al., 2004)
E7	Human Papilloma Virus	(Duensing and Munger, 2003)
E2F3	Human	(Saavedra et al., 2003)
SCF components	*Xenopus, Drosophila, mouse*	(Freed et al., 1999; Guardavaccaro et al., 2003; Murphy, 2003; Wojcik et al., 2000)
nucleophosmin	Human	(Okuda et al., 2000; Tokuyama et al., 2001)
p53	Human	(Tarapore et al., 2001)
BRCA1, Gadd45	Human	(Tutt et al., 1999; Wang et al., 2004; Hut et al., 2005)

body half bridge structure and depletion of this protein results in defective spindle-pole body replication (Kilmartin, 2003). Sfi1p is thought to bind multiple copies of Cdc31p through its multiple coiled-coil repeats, thus potentially forming a fibrous linker structure required for the integrity of the spindle-pole body. However, it is not known whether the human homologue of Sfi1p has a similar function.

It is also apparent that regulation of centriole duplication requires several components of the SCF ubiquitin ligase complex and the proteasome (Freed et al., 1999). Mutations in the *Drosophila* skp1 homologue results in centriole overduplication (Murphy, 2003; Wojcik et al., 2000). Mice deficient for SCF-βTrcp1 were found to contain cells with excess centrosomes. This protein has therefore been proposed to degrade a licensing factor that normally restricts centriole duplication to one round per cell cycle (Guardavaccaro et al., 2003).

The centrosome cycle is also the target of viral oncogenic proteins. Specifically, the high-risk human papilloma virus protein E7 induces centriole overduplication when overexpressed in primary cells (Duensing et al., 2001); (Duensing and Munger, 2001). Infection with High-risk human papilloma viruses can result in the formation of malignant tumours. Accordingly, the centrosome overduplication phenotype resulting from overexpression of this viral protein is thought to contribute to the genomic instability and transformation of infected cells.

Origins of centrosome amplification and possible consequences

From observational studies of abnormal cell divisions in horse nematode eggs (*Ascaris megalocephala),* Boveri proposed a link between centrosome number, chromosome aneuploidy and cancer (Goepfert and Brinkley, 2004). This proposal has gained renewed interest with the discovery that several types of tumour cells exhibit centrosome amplification (Fukasawa et al., 1996; Lingle et al., 1998; Pihan et al., 1998; Ghadimi et al., 2000); reviewed in (Nigg, 2002; Nigg E.A., 2004). Boveri noticed that, in their role as MTOCs, supernumerary centrosomes usually lead to the formation of multipolar spindles and aberrant mitosis, thus providing a route to chromosome missegregation and aneuploidy (reviewed in (Goepfert and Brinkley, 2004). However, in tumour cells it is not clear whether deregulation of centrosome numbers precedes aneuploidy, or if centrosome amplification is a consequence of mitotic errors induced by transformation (Nigg, 2002).

Four possible mechanisms exist that allow cells to acquire additional centrosomes (Figure 4). Deregulated centriole duplication may occur by defects in the mechanism that ensures duplication is restricted to once per cell cycle (Wong and Stearns, 2003). As mentioned previously, primary cells tightly control centriole numbers whereas some transformed cell lines, such as CHO and U2OS cells, permit

reduplication during a prolonged S-phase (Meraldi et al., 1999a; Balczon et al., 1995). Experimentally, centriole overduplication can also be induced by overexpression of human Sas-6 (Leidel et al., 2005). Similarly, additional MTOCs may be obtained by splitting existing centriole pairs, or by overexpressing PCM components that lead to the formation of centrosome fragments with microtubule nucleating abilities. Regardless of the cause, all lead to the generation of diploid cells with abnormal numbers of centrosomes.

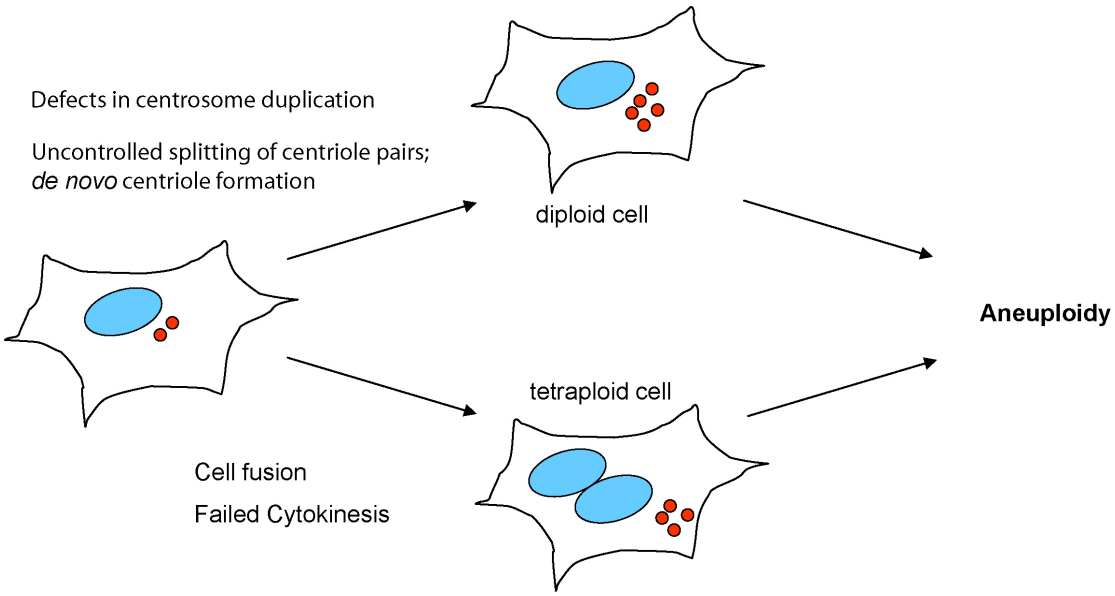

Figure 4. The origins of centrosome amplification. A schematic diagram showing four pathways for acquiring extra copies of centrosomes. (adapted from (Goepfert and Brinkley, 2004)

A failure of cell division may have several causes unrelated to centrosome biology but nevertheless lead to centrosome amplification and polyploidy, the latter a feature that has been shown to result in tumorigenesis (Fujiwara et al., 2005). Mutational activation or inactivation of genes involved in cell cycle events such as cytokinesis, DNA damage repair or the spindle-assembly checkpoint are possibly the most common causes resulting in cell division failure. In particular, p53, BRCA1 and some of their interactors were thought to be genuine regulators of the centrosome cycle (Tarapore et al., 2001); (Tutt et al., 1999; Wang et al., 2004; Hut et al., 2005). However, these findings have been questioned (Hut et al., 2005; Nigg, 2002). Similarly, overexpression of Aurora A, a kinase thought to regulate centrosome

numbers, has been observed to induce a cytokinesis failure in cells (Meraldi et al., 2002b). Therefore, several proteins presented in Table 1 may not be involved in centriole duplication, but actually achieve centrosome amplification indirectly by other means.

Several outcomes can befall cells with supernumerary centrosomes (Brinkley, 2001; Sluder and Nordberg, 2004). For example, $p53^{-/-}$ mouse embryonic fibroblasts (MEFs) with excess centrosomes usually formed multipolar spindles that mostly lead to cell death, presumably through loss of genetic material (Sluder and Nordberg, 2004). In rare cases when cells manage to survive aneuploidy and form progeny cells, excess centrosomes may coalesce into large MTOCs or have their microtubule nucleating capabilities silenced (Brinkley, 2001). Alternatively, cells with multiple centrosomes and aberrant spindles persist in tumour cell lines and are thought to be continually removed by apoptosis (Lingle and Salisbury, 1999). Surplus centrosomes may therefore cause initial events required for cancer formation, but are a disadvantage to dividing tumour cells.

Polo-like kinases

The polo-like kinases (Plks) are structurally related serine/threonine kinases that are essential regulators of mitotic and meiotic progression in diverse organisms ranging from yeast and humans (Figure 5) (Barr et al., 2004; Lee et al., 2005; Winkles and Alberts, 2005; Glover, 2005). Their name derives from a *Drosophila* mutant (Polo) that fails to complete normal mitosis, and which exhibited abnormal spindle and centrosome morphology (Sunkel and Glover, 1988; Llamazares et al., 1991), thus pointing to a role for Plks in mitotic events. In humans, four Plks have been identified and are known as Plk1, Plk2 (Snk), Plk3 (Fnk, Prk) and Plk4 (Sak) (Barr et al., 2004). Other organisms have reduced numbers of Plk-related genes; three are found in *C. elegans*, two in *Drosophila* (Polo and DmSak) and one in single cell eukaryotes (Cdc5, Plo1). The conservation of Plks in evolutionarily distant organisms indicates a function essential to life, and the increase in Plk orthologues may be related to the increased complexity of regulating cell division, polarity and checkpoints in multicellular organisms.

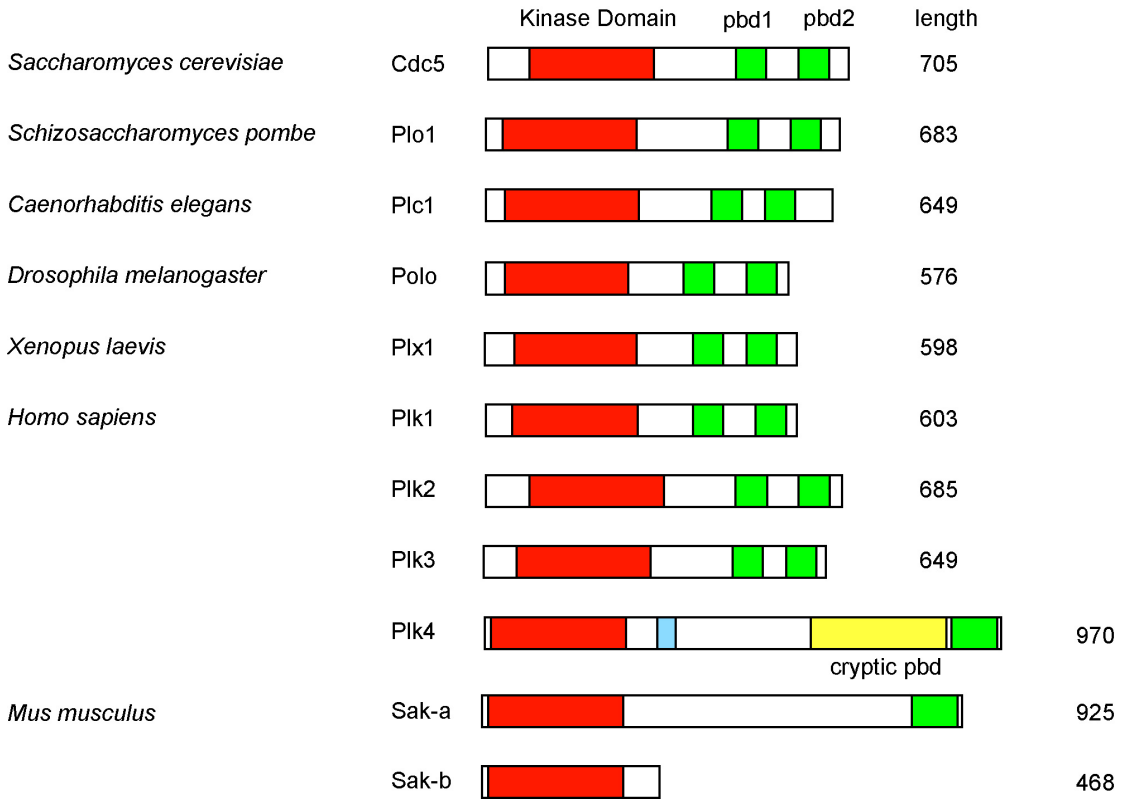

Figure 5. Members of the polo-like kinase family from different model organisms. All Plks share an N-terminal kinase domain (red) and two C-terminal polo-box domains (pbd, green), the exception being Plk4 with only one polo-box. The putative Plk4 cryptic polo-box, which is only loosely defined, is shown in yellow. For comparison, the mouse Plk4 sequences are included. The insertion found in human but not mouse Plk4 is shown in blue (adapted from (Dai, 2005).

Plks share an N-terminal kinase domain that is highly conserved and shows homology to other serine/threonine kinases (Figure 5)(Barr et al., 2004; Lowery et al., 2005; Dai, 2005). The catalytic activity of this domain is essential for Plks to fulfil their respective cellular functions. Most significantly, Plks are defined by their possession of the structural polo-box domain, located in the C-terminal region (Cheng et al., 2003; Elia et al., 2003b; Elia et al., 2003a). The Plk1 polo-box domain has been shown to bind phosphorylated serine/threonines found within a consensus peptide motif, and it is likely that the polo-box domains of other Plk family members behave similarly.

Plks regulate several events important for cell division such as centrosome maturation, onset of mitosis, bipolar spindle formation, chromosome segregation, Golgi fragmentation and cytokinesis (Barr et al., 2004). Plk2 and 3 also function in

events unrelated to the cell cycle. However, the similarity of human Plk homologues raises the question whether some of these, in particular Plk2 and Plk3, have redundant functions.

Plk1

Plk1 and its orthologues in other organisms are the best characterised members of the Polo-like kinase family (Barr et al., 2004);(Lowery et al., 2005; van Vugt and Medema, 2005) but a full treatise of the current knowledge would be beyond the bounds of this introduction. Only a short summary of the most salient features will therefore be given here.

Plk1 functions in centrosome maturation, spindle formation, chromosome cohesion and cytokinesis (Figure 6)(Barr et al., 2004) and has been implicated in tumorigenesis (Strebhardt and Ullrich, 2006). During the multiple stages of the mammalian cell cycle, Plk1 localises to various intracellular structures, appearing sequentially at the centrosome, the kinetochores and finally at the spindle mid-zone (Golsteyn et al., 1995).

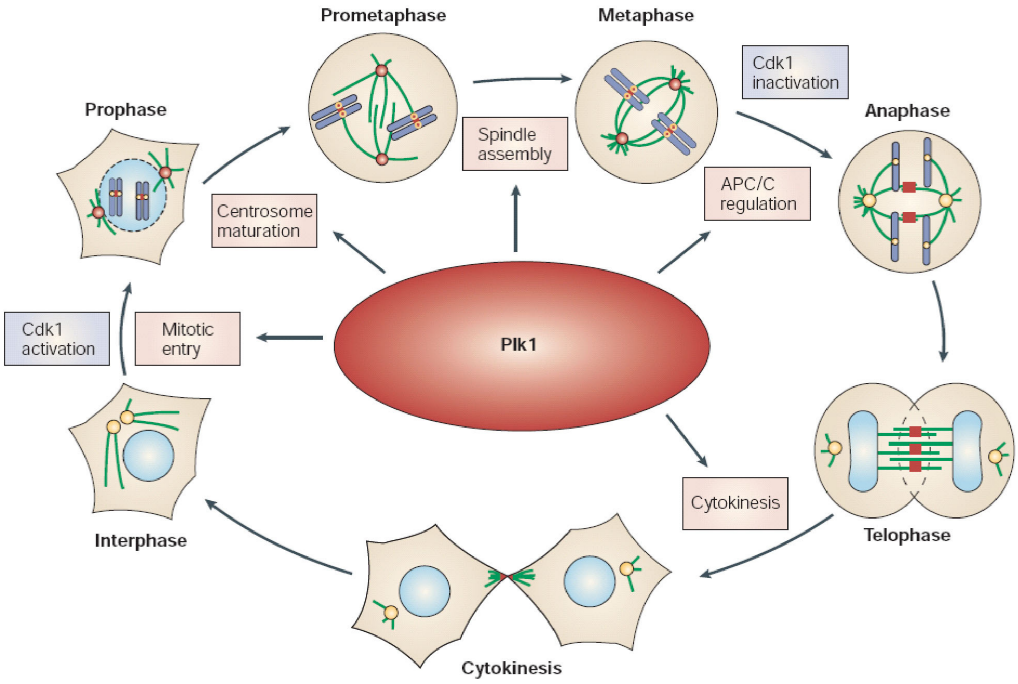

Figure 6. The multiple functions of Plk1. Schematic diagram showing the involvement of Plk1 in the various processes during the cell cycle. Structures to which Plk1 localises are in red (Barr et al., 2004).

The localisation of Plk1 and its interaction partners depends on a functional polo-box domain (Lee et al., 1998; Hanisch et al., 2006). As mentioned above, the polo-box domain has been identified as a phosphopeptide-binding motif (Elia et al., 2003a) and structural analysis of the crystallised domain revealed the molecular interactions between the polo-box interface and phosphorylated target sequences (Elia et al., 2003b; Cheng et al., 2003). Briefly, the human Plk1 polo-box domain contains the conserved residues His538 and Lys540 that directly interact with phospho- serine and threonine residues. This interaction is stabilised by van der Waals interactions and aided by hydrogen bonds involving water molecules and other conserved polo-box residues (Elia et al., 2003b; Cheng et al., 2003). Furthermore, the interacting residues sit in a cleft jointly formed by both polo-box motifs 1 and 2.

The optimal sequence motif with is recognised by the polo-box domain is S-pS/T-P/X and is reminiscent of Cdk and MAPK target phosphorylation sites (Elia et al., 2003a). Therefore, two models of how the Plk1 polo-box domain might function have been proposed (Figure 7) (Lowery et al., 2005).

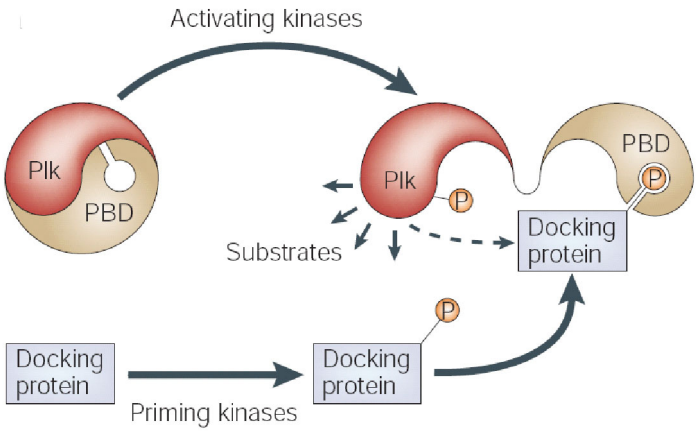

Figure 7. Activation of Plk1 and binding to primed substrates. Docking proteins are phosphorylated by upstream priming kinases allowing phospho-dependent binding of Plk1 via the polo-box domain. Together with kinases directly activating Plk1 by phosphorylating the T-loop of the catalytic domain, binding relieves polo-box mediated auto-inhibition of Plk1 (Barr et al., 2004).

In one model, the polo-box domain binds to a site on a docking protein that has been 'primed' by previous phosphorylation by an upstream kinase. The interaction

with the docking protein would target Plk1 in proximity to substrates and therefore introduce an important element of spatial control. The sequential localisation of Plk1 from centrosomes to kinetochores and spindle midzone as cells pass through mitosis is consistent with this model. Alternatively, Plk1 interactors would contain both polo-box binding sites and Plk1 phosphorylation motifs. Such a mechanism has recently been shown for the phospho-dependent binding of Plx1 to XErp-1, a factor whose phosphorylation by Plx1 and subsequent degradation is required for the release of *Xenopus* oocytes from meiosis II, and which depends on prior phosphorylation of a polo-box binding site by CamKII (Schmidt et al., 2005; Rauh et al., 2005).

In either case, Plk1 is thought to act as an integrator of upstream phosphorylation events by other mitotic kinases or by its own activity (Barr et al., 2004). Binding to phospho-serine and –threonine residues via the polo-box domain only takes place if upstream mitotic kinases have been active, thus ensuring a sequential activation of mitotic events by Plk1. Together with the polo-box domain mediated localisation of this kinase to distinct scaffolds, the function of Plk1 at discrete mitotic events is both temporally and spatially regulated.

In addition to the phosphorylation dependent regulation by the polo-box domain, Plk1 is activated by phosphorylation of the T-loop within the catalytic domain. This phosphorylation of a conserved threonine at position 210 in human Plk1 is essential for the tertiary structure of the kinase domain and can be mimicked by a mutation to aspartate, resulting in a constitutive activation (Qian et al., 1999; Lee and Erikson, 1997). In *Xenopus*, Plk kinase-1 and PKA have been identified as two candidate upstream activatory kinases (Qian et al., 1998; Kelm et al., 2002) but it is possible that other kinases are involved in activating Plk1. In contrast, activatory phosphorylation events are antagonised by the removal of human Plk1 from the cell by degradation (Barr et al., 2004). Degradation of Plk1 is relies on a destruction motif present in the linker region between the catalytic and regulatory domains, whereas the degradation mechanism depends on the anaphase-promoting complex/cyclosome APC/C that works together with the cofactors Cdc20 and Cdh1 (Lindon and Pines, 2004).

The passage of Plk1 from centrosomes to kinetochores and spindles points to a function of this kinase in relation to these structures. As previously mentioned, inactivation of Plk1 leads to the formation of aberrant monopolar spindles and a failure to recruit several proteins to the centrosome (Lane and Nigg, 1996; Gonzalez

et al., 1998; Donaldson et al., 2001). An important protein that fails to be enriched during centrosome maturation at the G2/M boundary when Plk1 function is impaired is γ-tubulin, essential for microtubule formation and proper spindle formation (Lane and Nigg, 1996). Moreover, a substrate of Plk1 at the centrosome, Nlp, interacts with γ-tubulin ring complexes (Casenghi et al., 2003). Nlp is phosphorylated by Plk1 at the beginning of mitosis and dissociates from the centrosome (Casenghi et al., 2005), probably allowing other γ-tubulin complex binding proteins to carry out their function during spindle formation. Additionally, several other potential Plk1 substrates are implicated in microtubule dynamics and reside at the centrosome (Barr et al., 2004), such as the microtubule severing protein Katanin (McNally et al., 2002).

The localisation of Plk1 at the centrosome gives it a 'pole position' to regulate the entry of mitosis. Evidence indicates that activation of the Cdk1-cyclin-B complex begins at the centrosome and depends on dephosphorylation of Cdk1 (Jackman et al., 2003) by Cdc25. This phosphatase in turn is activated by Plx1 in *Xenopus* egg extracts, and is bound by Plk1 via the polo-box domain in a phosphorylation-dependent manner (Kumagai and Dunphy, 1996; Elia et al., 2003a). Plk1 is also able to phosphorylate Myt1, the kinase antagonistic to Cdc25 activity (Nakajima et al., 2003). Finally, cyclin-B1 itself is a substrate of Plk1 (Jackman et al., 2003) but the consequences of this phosphorylation remain unclear. In summary, it seems that Plk1 may be involved in the initiation of the cdk1-cyclin B phosphorylation cascade at the centrosome.

Evidence suggests Plk1 and its homologues are involved in the regulation of the APC/C during mitosis and the onset of anaphase (Shirayama et al., 1998). This regulation is achieved by directly phosphorylating several of the APC/C components, or by indirectly influencing this complex through proteins such as Xerp1 (Rauh et al., 2005). Plk1 is also involved in sister chromatid cohesion, by phosphorylating and thereby activating the cleavage of cohesin subunits (Alexandru et al., 2001);(Sumara et al., 2002; Hauf et al., 2005) reviewed in (Watanabe, 2005). It is likely that Plk1 is recruited to kinetochores in close proximity to these substrates, similar to the localisation of Plk1 to the centrosome during G2/M.

Finally, towards the end of mitosis, Plk1 passes to the central spindle and is therefore appropriately positioned for its role in cytokinesis (van Vugt and Medema,

2005; Neef et al., 2006). Plk1 substrates at this stage include Mklp1 and Mklp2 (Lee et al., 1995; Neef et al., 2003).

Plk2

Plk2 was first identified as an early response gene upon mitogenic stimulation of cells with serum or with phorbol ester, (Simmons et al., 1992) and is structurally similar to Plk1. Analysis of protein stability revealed that Plk2 has a short half-time life during G1 and remains catalytically active during this stage of the cell cycle (Ma et al., 2003).

Several functions have been proposed for Plk2. In an siRNA screen for regulators of apoptosis and chemoresistence, a lack of Plk2 in cells treated with low doses of taxol correlated with increased levels of apoptosis (MacKeigan et al., 2005) suggesting that Plk2 may be necessary for stress response pathways. Similarly, Plk2 was identified as a transcriptional target of p53 by microarray analysis of diverse wild type and p53-null organisms, and silencing of Plk2 in tumour cells increased their sensitivity to microtubule destabilising and DNA damaging agents (Burns et al., 2003). Curiously, Plk2-null mice are viable indicating that this kinase is not essential for development or post-natal growth (Ma et al., 2003). Some retardation of growth and skeletal development was observed in these mice, and $Plk2^{-/-}$ embryo fibroblasts exhibited reduced proliferation and delayed entry into S-phase. Plk2 has therefore been proposed to play a dual role; involvement in stress response pathways and regulating cell cycle exit from G1 (Winkles and Alberts, 2005).

Plk2 may have other functions independent of the cell cycle. Plk2 was found to interact with the calcium-integrin binding protein CIB (Ma et al., 2003) and its gene transcripts increase after nerve cells are chemically stimulated (Kauselmann et al., 1999). In another study, Plk2 (and Plk3) was induced in neurones by synaptic activity (Pak and Sheng, 2003). Taken together, these data point to tissue specific functions for Plk2.

Most pertinently to this thesis, Plk2 has been described to play a role in mammalian centriole duplication (Warnke et al., 2004). When wt Plk2 was overexpressed in cultured cells, centrosome overduplication was observed. Conversely, overexpression of the catalytically inactive mutant, or depletion of the endogenous kinase by siRNA suppressed centrosome reduplication in S-phase arrested U2OS cells. It has therefore been proposed that Plk2 is an essential regulator

of centriole duplication (Warnke et al., 2004). However, it is difficult to reconcile these results with the finding that Plk2$^{-/-}$ mice are viable and only show minor defects in skeletal growth. Plk2 is therefore appears to be redundant in the whole organism, whereas it is essential in cultured mammalian cells. Clearly, Plk2 requires further research to elucidate its function as the centrosome.

Plk3

The third member of the polo-like kinase family is structurally similar to both Plk1 and 2 (Figure 5), and was originally identified as a fibroblast growth factor-inducible early response gene in NIH-3T3 cells (Donohue et al., 1995). Most confusingly, a whole plethora of functions have been ascribed to Plk3 in mammalian cells (Barr et al., 2004). Reports suggest an involvement in the response to DNA damage (Xie et al., 2002b; Bahassi et al., 2002), in the regulation of Cdc25C and mitotic entry (Bahassi et al., 2004), putatively via p53 (Xie et al., 2001a; Xie et al., 2001b) and chk2 (Xie et al., 2002a). Overexpression of Plk3 has been reported to inhibit cytokinesis (Conn et al., 2000) whereas microinjection of Plk3 transcripts into *Xenopus* oocytes resulted in accelerated maturation (Duncan et al., 2001). Furthermore, data indicates that Plk3 may have functions not related to the cell cycle. Plk3 interacts with Calcium-integrin-binding protein and may function in cell adhesion (Holtrich et al., 2000), synaptic plasticity and neuronal remodelling in mouse nerve cells (Kauselmann et al., 1999; Pak and Sheng, 2003). Finally, endogenous Plk3 has also been found at both the centrosome and the Golgi apparatus (Ruan et al., 2004; Jiang et al., 2006), and overexpression led to morphological changes in cells by affecting microtubules (Wang et al., 2002). In summary, much of the data on Plk3 appears contradictory and the function of this kinase in the cell remains unclear.

Plk4

Mouse Plk4 was first identified serendipitously in a screen to search for proteins regulating sialylation in mammalian cells using a murine cDNA library (Fode et al., 1994). The cDNA of the human homologue was separately isolated by a PCR-based search for novel kinases involved in cancer development (Karn et al., 1997). Both approaches yielded the primary sequence data and revealed that Plk4 is the

structurally most divergent member of the polo-like kinase family. However, neither of these studies provided any insights into the function of this kinase.

The Plk4 gene is located on chromosome 4q28 in humans and on chromosome 3 in mice (Hammond et al., 1999; Swallow et al., 2005). This region has been implicated in frequent rearrangements and loss in human tumour cells, particularly in hepatocellular carcinomas (Hammond et al., 1999). The murine Plk4 gene has been reported to consist of 15 exons stretching over 16 kb of genomic sequence (Hudson et al., 2000). Interestingly, in mouse two alternative spice variants are detectable and which differ in transcript length. The full length Sak-a transcript encodes all 15 exons whereas the shorter and rarer Sak-b transcript is derived from early termination and alternative splicing at exons 5 and 6 (Hudson et al., 2000). Sak-b is truncated and only comprises the complete kinase domain and a short C-terminal end, the latter encoded in 147 bp of exon 5. In human Plk4, a 102 bp sequence insertion results in the addition of 34 amino acids and in the production of a longer peptide of 970 residues, as opposed to the shorter mouse protein of 925 residues (Figure 5, (Hudson et al., 2000). However, the human equivalent of a truncated Sak-b isoform has not been found and only one major transcript corresponding to the full length protein is known for human Plk4

Like other polo-like kinase family members, the kinase domain of Plk4 resides in the N-terminus of the protein and spans residues 1 to 265 (Figure 5). The kinase domain is most closely related to those of other Plk-family members, in particular to Plk2 (Fode et al., 1994), and shares common ancestry with other serine/threonine kinases. However, unlike other Plk-family members, the kinase domain of Plk4 is followed by an extensive C-terminal region of >500 amino acids (Figure 5) and a single polo-box domain at the far C-terminal end. The Plk4 polo-box domain itself is equally related to both polo-box domains 1 and 2 of the canonical double fold of Plk1 (Fode et al., 1994) and, together with the loosely defined cryptic polo-box, was described to act as a dimerisation domain (Leung et al., 2002). The polo-box and the PEST sequences (mentioned below) aside, the purpose of the large remainder of the C-terminal region is completely mysterious as it shares no sequence homology to any known protein, has not been solved structurally nor have any functions been assigned to it.

X-ray crystallographic analyses of the polo-box domain structure of both Plk1 and 4 have revealed organisational differences despite apparently similar secondary

structures (Leung et al., 2002; Elia et al., 2003b; Cheng et al., 2003). Both are formed of six β-sheets and one α-helix when considered in isolation, yet form different overall structures. The Plk4 polo-box exists as a homodimer when crystallised, with a shared anti-parallel sheet being formed from β-strands from both subunits (Leung et al., 2002). Similarly, the Plk1- polo box domain forms a dimer when crystallised but achieves this intramolecularly from both sister polo-box domains (Elia et al., 2003b; Cheng et al., 2003). This difference affects which residues are placed in the structure, such that the Plk4 polo-box dimer structure contains internal amino acids that are located externally on the Plk1 polo-box. Furthermore, the Plk4 cleft was found to be shallower and broadened when compared to the Plk1 structure, and also lacks a clear positive charge on the surface. Additionally, residues directly responsible for the phospho-peptide binding function of the Plk1 polo-box are not conserved in the Plk4 primary sequence, suggesting that this functionality is not conserved (Figure 8). Indeed, no reports on a phospho-peptide binding ability of the Plk4 polo-box have been published up to the present.

The cryptic-polo-box has been reported to contain some similarity to the Plk4 polo-box domain itself (Leung et al., 2002), and functions as a homo-dimerisation domain. In mouse Plk4, it is located upstream and adjacent to the actual polo-box domain (Figure 5). When overexpressed, this domain has been reported to localise to the centrosome , nucleolus and the cleavage furrow (Hudson et al., 2001). The Plk4 cryptic-polo-box in conjunction with the polo-box domain could therefore form a structure that spatially regulates this kinase by binding to interaction partners or to putative phospho-peptides. Reports of two interactors, the Tec kinase and p53 (Yamashita et al., 2001; Swallow et al., 2005), which respectively bind to the cryptic- and the actual polo-box, could provide upstream inputs to the activity and localisation of Plk4 and thus reveal the existence of a regulatory mechanism as described for Plk1.

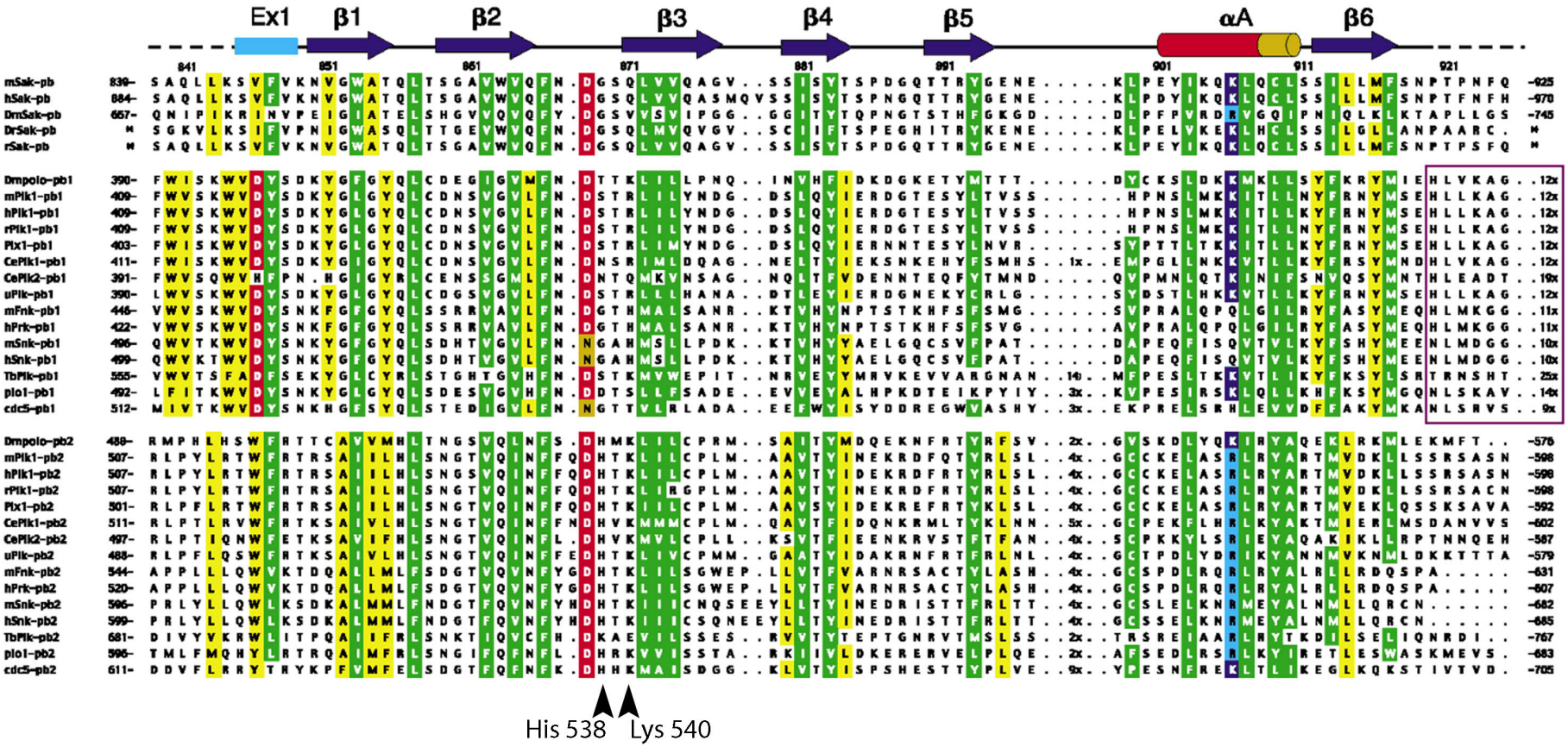

Figure 8. Alignment of polo-box sequences from various polo-like kinase homologues. Peptide sequences of polo-boxes from Plk4 (Sak) orthologues are aligned against the cognate motifs from other Plks. Arrows point to two residues not conserved in Plk4 but that are essential for the phosphopeptide binding ability of the Plk1 polo-box domain (Leung et al., 2002).

Three putative PEST motifs have been identified by sequence comparisons in the C-terminal region of Plk4, and are thought to be responsible for the short half-life of 2-3 hours of this kinase in asynchronous cells (Fode et al., 1996). One PEST motif is located proximal to the kinase domain whereas the other two are further downstream near the polo-box domain. Evidence supports the functionality of these PEST sequences in Plk4 turnover since deletion of these motifs increased the stability of overexpressed Plk4 (Yamashita et al., 2001). Furthermore, the involvement of ubiquitination and the anaphase-promoting complex (APC/C) in regulating ectopic Plk4 has been reported (Fode et al., 1996) and may indeed suggest a regulatory role for degradation in controlling Plk4 activity, similar in this aspect to Plk1 (Lindon and Pines, 2004).

Plk4 expression

Reports indicate that Plk4 is cell-cycle regulated at the transcript level in patterns comparable to other Plk family members (Fode et al., 1996). Studies using cultured cells and Northern blot analysis revealed that Plk4 mRNA is low in quiescent cells at G0 and early G1 (Fode et al., 1996). Transcript levels increase from late G1 through to S and G2, and reach a plateau in M phase. Likewise, when mice are subjected to partial hepatectomy, Plk4 transcripts in regenerating liver tissue increase from late G1 to S phase, reaching their highest level during G2/M (Ko et al., 2005). However, whether Plk4 is regulated through levels of mRNA, protein or catalytic activity is currently not known.

Similarly, Plk4 expression levels in adult tissues have only been analysed by Northern blotting (Fode et al., 1994; Karn et al., 1997). Plk4 transcripts were found in murine testis, thymus and spleen but were not detectable in brain, heart, kidney, liver or ovary tissue samples (Fode et al., 1994). In another study using human tissues, highest levels of Plk4 mRNA were found in thymus and testis (Karn et al., 1997), suggesting that tissues with actively dividing cells may contain the highest amounts of Plk4. This notion is supported by in situ hybridisation studies on whole sections of mouse embryos at various stages, which show that Plk4 mRNA is at first evenly distributed, but is then restricted to zones of proliferating cells (Fode et al., 1994). Conversely, overexpression of a Plk4 antisense fragment suppressed proliferation of

transfected CHO cells (Fode et al., 1994). Furthermore, a study on the levels of Plk4 in samples of colorectal cancer (Macmillan et al., 2001) revealed elevated levels of Plk4 in tumour tissue when compared to adjacent normal intestinal cells. The fragmentary evidence thus points to a possible link between Plk4 and cell proliferation.

Data on the Plk4 protein itself remains scarce. Overexpression studies using mouse epitope tagged Plk4 transfected into cells (Hudson et al., 2000) has placed this kinase in the nucleolus during G2, at the centrosomes at G2/M, to the cleavage furrow during cytokinesis and to the perinuclear region at G1. Deletion of the polo-box domain resulted in a mislocalisation of the overexpressed protein to the cytoplasm in all stages of the cell cycle. In a subsequent study, a fragment comprising the polo-box domain tagged with GFP was located at the centrosome and the cleavage furrow in cultured mammalian cells (Leung et al., 2002). Somewhat in contradiction to the earlier data, a Plk4 deletion mutant lacking the polo-box domain was still centrosomal. In summary, the above immunofluorescence data allows speculation on the involvement on Plk4 in diverse cellular processes such as in centrosome biology or cytokinesis. However, prior to the work described in this thesis, nothing was known about the localisation of the endogenous protein.

Plk4 knock-out mice

Hints as to the importance of Plk4 in cell proliferation have been gained from mouse knockout experiments (Hudson et al., 2001; Ko et al., 2005). $Plk4^{-/-}$ mouse embryos arrest shortly after gastrulation, with an increase in mitotic cells containing partially segregated chromosomes. Explants from these embryos also arrest in anaphase and telophase after a few cell divisions, with a concomitant 20 fold increase in apoptotic cells and consequently the cellular phenotype of these embryos has been described as a late mitotic delay (Hudson et al., 2001). A possible explanation for these observations is that Plk4 is partially redundant for cell division until gastrulation, since embryos are able to divide initially (Swallow et al., 2005; Hudson et al., 2001). Alternatively, Plk4 is supplied maternally and initial cell divisions run on pre-existing cellular stores of this kinase until they are depleted when gastrulation begins. Never-the-less, the lethality of Plk4 deficiency reveals that this unusual Polo-like kinase is

essential to the development of a complete organism, and stands in stark contrast to the viability of $Plk2^{-/-}$ mice.

Analysis of $Plk4^{+/-}$ mice has revealed a potential role for Plk4 in tumorigenesis (Ko et al., 2005). These mice grow normally to adulthood, but develop tumours over time, most notably in lung and liver tissue. When Plk4+/- mice were subjected to a partial hepatectomy to stimulate growth of tissue, increased numbers of liver cells with aberrant multipolar spindles were observed. Regenerating liver tissue showed altered regulation of cyclins D, E and B and reduced levels of p53 and p21, suggesting that cells are subject to a cell cycle delay. In particular, levels of cyclin B were flattened and extended during prolonged mitosis. Similarly, $Plk4^{+/-}$ MEFs displayed increased numbers of centrosomes, abnormal spindles as well as slower growth. Taken together, these data suggest that Plk4 haploinsufficiency results in abnormal mitotic progression and carcinogenesis.

AIMS OF THIS PROJECT

Early studies on Plk4 have uncovered a possible role of this kinase in cell proliferation and centrosome biology (Fode et al., 1994; Fode et al., 1996; Hudson et al., 2001; Leung et al., 2002). Moreover, the $Plk4^{-/-}$ mouse knockout is embryonic lethal, pointing to a non-redundant function of this Plk family member in cell division and early development (Hudson et al., 2001). Despite these initial reports, very little was known about the endogenous kinase and the precise function of this structurally divergent Plk-family member remained unclear. Therefore, the aim of this study was to characterise the function and regulation of human Plk4 in mammalian cells.

RESULTS

Sequence analysis of Plk4

Plk4 was discovered serendipitously in a screen for sialylated proteins, and by virtue of its homology to other polo-like kinases (Fode et al., 1994; Karn et al., 1997). These studies highlighted the presence of an N-terminal kinase domain and a C-terminal domain containing a polo-box motif which has been structurally solved by X-ray crystallography (Leung et al., 2002). However, very little detail has been obtained on the structure of the extensive linker region connecting these two easily identifiable domains. Simple predictive programs were therefore used to uncover potential motifs or secondary structures in this uncharacterised region of Plk4.

A motif scan of human Plk4 (ScanProsite, www.expasy.ch, not shown) confirmed the presence of an N-terminal dual specificity kinase domain, stretching from residues 12-265, and which is predicted to phosphorylate both serine/threonine and tyrosine residues. Whether the catalytic domain of Plk4 favours one or the other set of residues is at present unclear as no consensus substrate motifs have been reported. Additionally, the polo-box was identified at the far C-terminus, consistent with previous reports (Leung et al., 2002). However, no known domains were found in the linker region located between the catalytic and polo-box domains.

Scanning the primary sequence of Plk4 with a secondary structure prediction program (PsiPred, www.expasy.ch) revealed the presence of numerous α-helices upstream of the polo-box domain, in addition to some β-sheet structures (Figure 9). Whilst secondary structure programs are inherently limited in providing structural information, the program used here predicts five β-sheets and one α-helix for the region where the polo-box domain is found. This result recapitulates the reported crystal structure of the Plk4 polo-box (Leung et al., 2002) and, similarly, the extent of the kinase domain is faithfully reproduced. Interestingly, the previously uncharacterised linker now features numerous predicted secondary structures spanning the residues 610-810 upstream of the polo-box. These consist of several β-sheet structures interspersed with three α-helical domains which lie downstream of a predicted linker structure comprising approximately 350 residues. These structures most likely form part of the loosely defined cryptic polo-box and were not identified in the motif scan, thus representing novel domains if physiologically relevant *in vivo*.

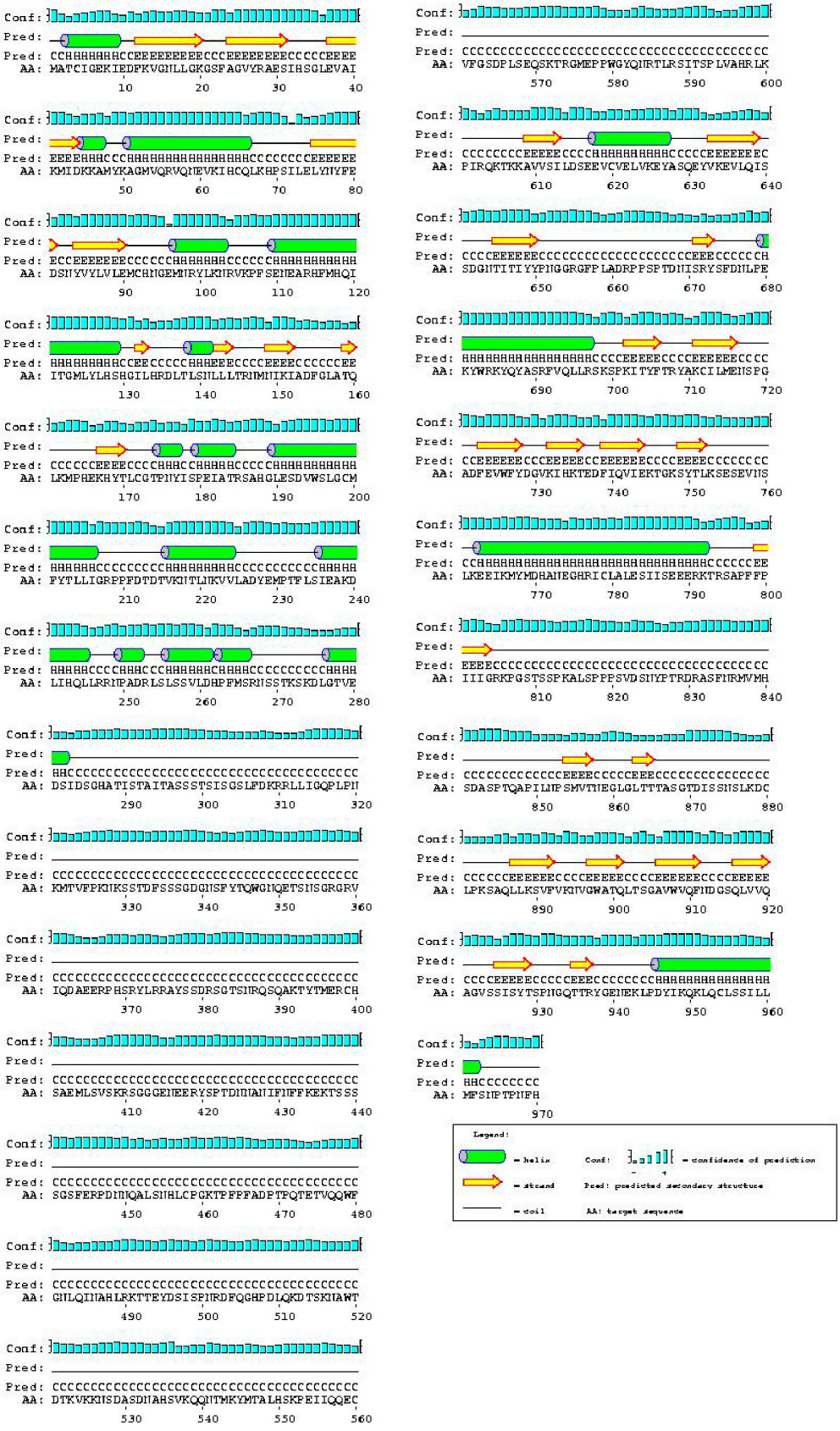

Figure 9. Plk4 contains additional structures outside of the kinase and polo-box domains. The peptide sequence of Plk4 was submitted to the PsiPred server (www.expasy.ch, (McGuffin et al., 2001). Predicted α-helices are depicted in green and β-sheet structures in yellow. The confidence of prediction is graphically represented in blue.

Numerous centrosomal proteins with structural roles such as AKAP450 or PCM-1 (Keryer et al., 2003b; Balczon et al., 1994) contain extensive coiled-coil regions. To reveal whether any of the predicted secondary structures form putative coiled-coil domains, the COILS program was used to scan the peptide sequence of Plk4 (COILS at www.expasy.ch; (Lupas et al., 1991). As seen in Figure 10, a short stretch located across residues 750-800 was predicted as a coiled-coil structure at significant values (Lupas et al., 1991). This putative coiled-coil domain coincides with an α-helical structure predicted by the previous secondary structure program and may therefore point out a domain important for a structural role of the C-terminus of Plk4. Whether the function of Plk4 depends on the presence of a coiled-coil structure is at present unclear.

In addition to the easily identified kinase and polo-box domains, Plk4 therefore contains several unknown domains that are revealed by secondary structure prediction programs. These domains are located upstream of the polo-box and consist of both β-sheets and α-helices, one of which is predicted to form a coiled-coil domain. If these structures exist *in vivo*, they could represent previously uncharacterised novel domains and thus deserve further investigation.

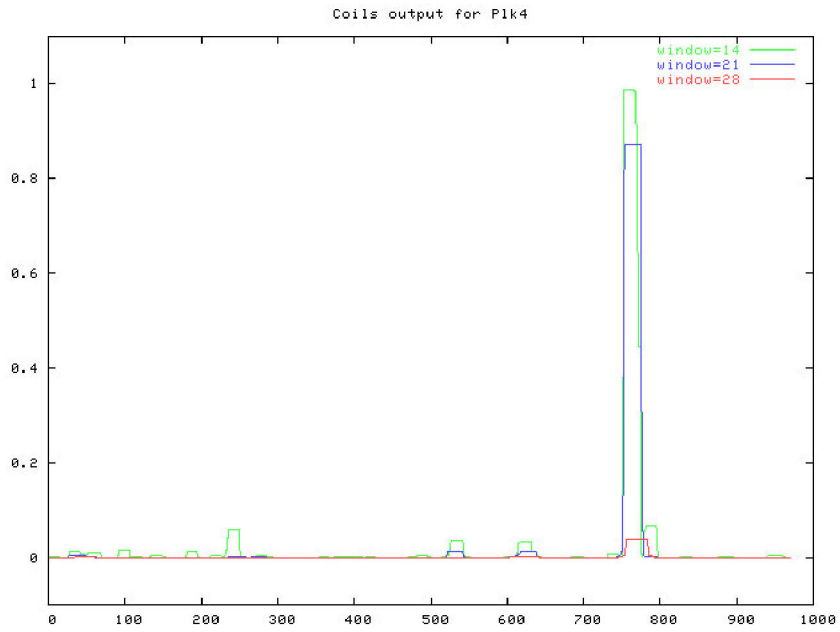

Figure 10. Plk4 contains a predicted coiled-coil domain. The primary sequence of Plk4 was analysed by the COILS program (**www.expasy.ch**,(Lupas et al., 1991). One short region of Plk4 (approximately residues 750-800) showed a significant prediction above a threshold value (>0.5).

Plk4 homologues identified in other organisms

The existence of several Plk4 orthologues in other organisms has been shown (Fode 1994, Karn 1994, Leung et al 2001). In particular, a report mentioned Plk4 homologues in Zebrafish (*Danio rerio)*, *Drosophila*, and rat (*Rattus norvegicus*), thus pointing to a conservation of this kinase in both vertebrates and invertebrates (Leung et al., 2002). However, few details on the degree of homology between related Plk4 proteins has been published.

A standard protein BLAST search was performed to uncover additional Plk4 homologues in other organisms (Altschul et al., 1990). When the human Plk4 protein sequence was submitted to the NCBI BLAST server and non-redundant protein databases were searched, several previously unreported homologues were found (summarised in Table 2 and Figure 11). Homologues were found in a range of both vertebrates and non-vertebrates, but no obvious fungal or plant Plk4 proteins were detected. Predictably, the closest homologues to human Plk4 were found in primates, followed by other mammals such as mouse, rat, cow and dog which overall showed more than 80% residue identity. However, despite this high degree of relatedness, some variability was found in the length of the Plk4 protein itself. For example, whereas the mouse Plk4 reference sequence comprises 925 residues, the rat Plk4 is 1247 amino acids long and contains N- and C-terminal sequences not found in human Plk4 (Figure 11). A motif scan of the rat N-terminal 200 residues did not find any known domains, and a BLAST-search did not detect any obvious homologues. The relevance of these rat sequences not found in the human Plk4 is unknown.

Plk4 homologues with 40-60% identity were found in fish species, *Xenopus* and chicken (Table 2). As with mammalian Plk4 homologues, all contained the kinase and the polo-box domains, with the exception of zebrafish for which only a partial sequence was available in the protein database. Similarly well conserved homologues were detected in five insect species, of which *Drosophila* Plk4 had been reported previously. Interestingly, all insect Plk4 proteins contain a central linker region that is not conserved in vertebrate Plk4s (Figure 11), an evolutionary development thus peculiar to this phylum.

	identity	similarity	residues with Plk4 homology	homology to Plk4 region	length of protein	Accession
Macaca fascicularis	96%	98%	1-575	396-970	575	BAE02419
Pongo pygmaeus	97%	98%	1-970	1-970	970	CAH91413
Chimpanzee (*Pan troglodytes)*	96%	97%	144-1094	3-970	1094	XP_517431
Dog (*Canis familiaris)*	88%	93%	49-1016	1-969	1017	XP_533295
Cow *(Bos taurus)*	87%	92%	58-1015	9-970	980	XP_592250
Rat *(Rattus norvegicus)*	79%	87%	200-1145	11-969	1247	XP_227064
Mouse *(Mus musculus)*	78%	86%	1-924	1-969	925	NP_035625
Chicken (*Gallus gallus)*	66%	79%	70-1026	9-966	1029	XP_420462
Xenopus laevis	59%	72%	1-940	1-964	944	AAH60363
Pufferfish *(Tetraodon nigroviridis)*	50%	64%	1-919	9-965	920	CAG10565
Zebrafish *(Danio rerio)*	42%	57%	1-707	230-965	711	AAH45434
Purple sea urchin *(Strongylocentrotus purpuratus)*	38%	54%	57-1092	7-955	1323	XP_793779
Honey Bee	63%	78%	9-289	6-286	754	XP_623133
(Apis mellifera)	38%	53%	403-748	592-965		
Red Flour Beetle*)*	62%	81%	1-261	5-267	720	XP_974451
(Tribolium castaneum	33%	50%	387-718	594-968		
Drosophila pseudoobscura	59%	82%	8-270	6-267	777	EAL29859
	34%	53%	382-736	588-952		
Fruit fly *(Drosophila melanogaster)*	57%	78%	8-291	6-268	769	AAF51737
	33%	53%	388-735	593-960		
Anopheles gambiae	58%	75%	1-279	11-286	496	EAA02947
	39%	65%	361-496	598-730		
C. elegans	26%	44%	7-351	6-371	706	Q9GT24

Table 2. Homologues of Plk4. Plk4 was submitted to the BLAST server as NCBI (www.ncbi.nlm.nih.gov (Altschul et al., 1990). Identity defines conserved residues whereas similarity refers to exchange of amino acids of similar properties. The regions of homology to human Plk4 are given together with the cognate residues in the human kinase.

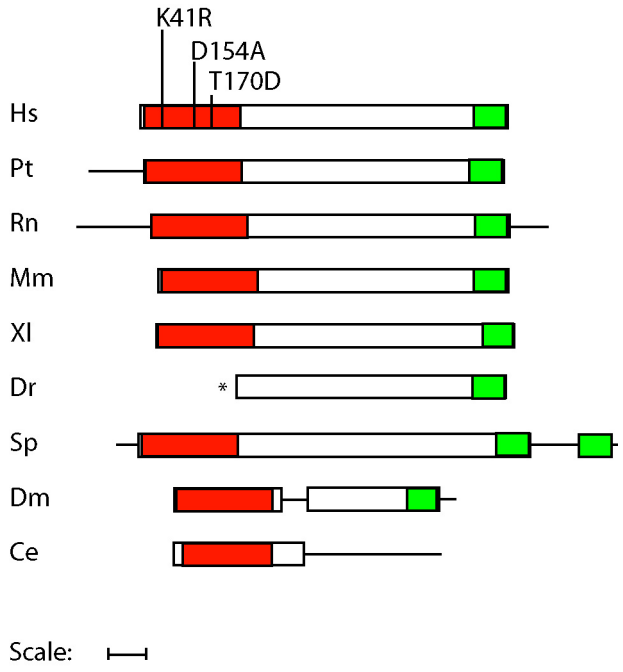

Figure 11. Plk4 homologues. Schematic representation of representative homologues of human Plk4 showing conserved domains, based on a BLAST search of a non-redundant protein database. The *C. elegans* sequence was aligned by pairwise-BLAST. Residues with significant homology are drawn as boxes, unrelated sequences as black lines. Kinase domains are in red, polo-boxes in green, linker regions are left white. Mutations introduced into the human Plk4 kinase domain are shown. Minor alignment gaps were omitted for clarity. Hs: human Plk4, Pt: *Pan troglodytes*, Rn: *Rattus norvegicus*, Mm: *Mus musculus*, Xl: *Xenopus* laevis, Dr: *Danio rerio*, Sp: *Strongylocentrotus purpuratus,* Dm: *Drosophila melanogaster*, Ce: *Caenorabditis elegans*. The asterisk denotes an incomplete Zebrafish sequence. Scale bar: 100 residues.

Curiously, this BLAST search also picked up a putative kinase from purple sea urchin (*Strongylocentrotus purpuratus,* Figure 11). This kinase has overall homology to Plk4, but contains an additional polo-box domain in the far C-terminus that is otherwise unrelated to any human Plk4 sequence. The presence of two polo-boxes would mark this kinase as an homologue of Plks 1, 2 or 3. However, the polo-boxes contained within this kinase resemble canonical Plk4 polo box motifs in their lack of conserved residues required for binding to phosphopeptides, generally found in other Plk-family members. Moreover, this kinase contains a large uncharacterised central region and widely spaced polo boxes, both features untypical of Plk1. Whether this sea urchin Polo-like kinase is a genuine homologue of Plk4 is however open to speculation.

Plk4 is not significantly related to Zyg-1

The Zyg-1 kinase has been identified as essential for centriole duplication in *C. elegans* (O'Connell et al., 2001). We hypothesised that Plk4 may regulate mammalian

centriole duplication and therefore asked whether this kinase is the closest human homologue of Zyg-1. H. Lutz and M. Vingron (Max-Planck-Institute for Molecular Genetics, Berlin) generously performed detailed comparative sequence analyses with Zyg-1 and Plk4, and a summary of their findings is recounted here.

The Zyg-1 kinase has an N-terminal catalytic domain, but otherwise no other domains are detected. A simple BLAST search of the kinase domain revealed a serine/threonine kinase in *Plasmodium falciparum* as the closest non-nematode homologue in addition to other related proteins including serine-threonine kinases in plants and several more distantly related vertebrate kinases. Among these various protein kinases, NimA related kinases and the polo-like kinase were found. However, when complete genome databases were searched for orthologues of Zyg-1 using tBLASTx, only one orthologue in the related nematode *Caenorhabditis briggsae* was detected. No obvious mammalian homologues were found using these methods.

When a PSI-BLAST search with three iterations was used to detect remote homologues of Zyg-1, multiple proteins were found that are related to the Zyg-1 C-terminus. Among these, the putative chicken Plk4 homologue was found, suggesting that a Plk4 homologue was indeed a related kinase. Unfortunately, no polo-box related sequences were found when Zyg-1 was aligned with the polo-box motif using the program Hmmalign. Furthermore, no RCC repeats typical of the NimA related kinase Nek8 (reviewed in (O'Connell et al., 2003) were found when Zyg-1 was scanned using Prosite (www.expasy.org/prosite), nor were any repeated structures detected. Homology of Zyg-1 to Plk4 therefore appears limited to N-terminal catalytic domains.

To clarify whether Plk-family members or NimA related kinases were more closely related to Zyg-1, a phylogenetic tree was assembled. A representative selection of 56 homologues from the Polo-like kinases, the Aurora/Ipl related kinases and the NimA related kinases in addition to the Zyg-1 kinases were included in this analysis. Tree construction was based on a multiple alignment of protein kinase domains only since the homology amongst these kinases was confined to this particular domain (Figure 12). The phylogenetic tree revealed that the two nematode Zyg-1 kinases were equally distant to Polo-like kinases and NimA related kinases. The hypothesis that Plk4 is the closest homologue of Zyg-1 was therefore not supported by these results.

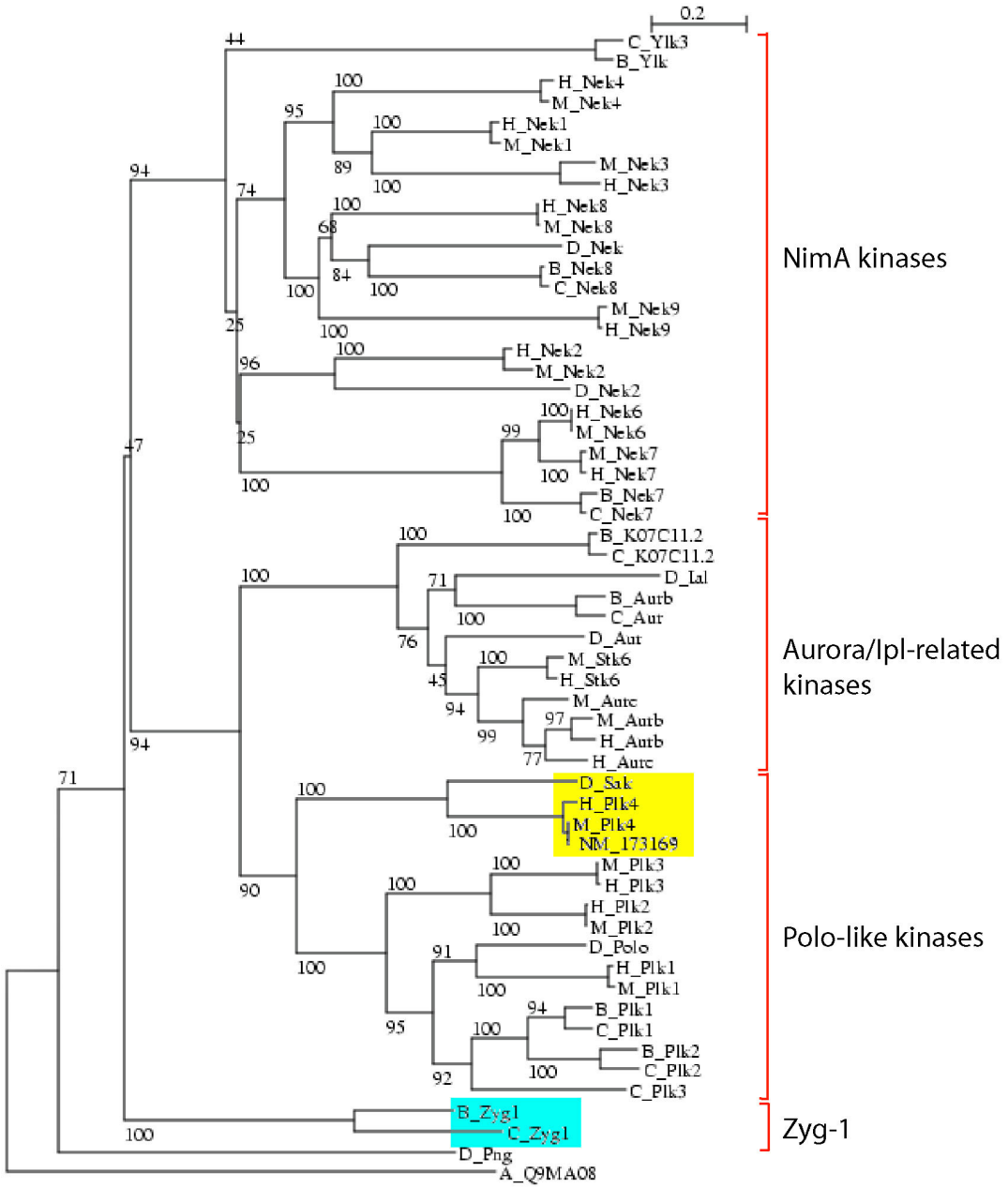

Figure 12. Phyolgenetic tree showing the relationship of Zyg-1 to Plks, Aurora- and NimA related kinases (Lutz & Vingron). Homology among sequences was restricted to the shared kinase domain. Tree assembly was based on an alignment of 56 sequences of 270 residues in length using the protein kinase HMM profile and the hmmalign program. Zyg-1 kinases are highlighted in blue and Plk4 homologues in yellow. The outgroup sequence Q9MA08 of an unrelated plant protein was used. Evolutionary distances were calculated by bootstrapping. B: *C. Briggsae*, C: *C. elegans*, D: *D. melanogaster*, H: *H. sapiens*, M: *M. musculus*. Note that the two Zyg-1 proteins group separately, and are equally distant from either NimA related kinases or Plks.

Recombinant human Plk4 is an active kinase

As an initial approach to the characterisation of Plk4 and its function in mammalian cells, the catalytic activity of Plk4 was investigated. The cDNA of Plk4 was obtained by purchasing an EST clone (AI561146) from the Image Consortium. After complete sequencing, this EST clone was found to contain the whole Plk4 coding sequence and additional 3´- and 5´- untranslated regions (UTRs). The Plk4 cDNA was subcloned into suitable mammalian and bacterial expression vectors, discarding UTRs in the process. As a control, residues located in the kinase domain that are critical for enzymatic activity were mutated (K41R, D154A, Figure 11) to generate catalytically inactive (dead) kinase. Moreover, a regulatory threonine residue that is usually phosphorylated for full catalytic activity was mutated to an aspartate (T170D) in an attempt to generate a constitutively active kinase.

Bacterial expression and purification of 6xHis-, GST- and maltose binding protein-tagged full length Plk4 was attempted to obtain soluble and active kinase. Unfortunately, tagged Plk4 fusions were either not expressed, or in the case of the maltose binding protein-Plk4 fusion protein, were completely insoluble (not shown). Similarly, 6xHis-tagged Plk4 from recombinant baculovirus infected Sf9 insect cells was also insoluble. Therefore, the only available method to obtain soluble recombinant full length Plk4 was to transiently transfect HEK293T cells with mammalian expression vectors and to immunoprecipitate myc-tagged kinase from total cell extracts (Figure 13).

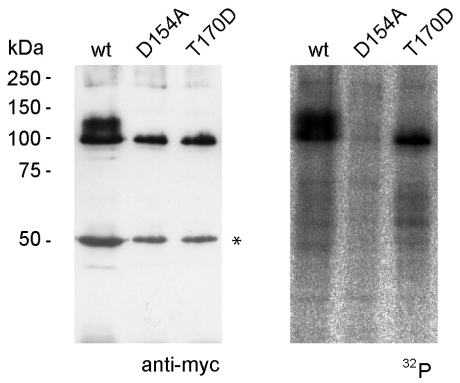

Figure 13. Full length wt Plk4 is an active enzyme. myc-tagged Plk4 was purified from overexpressing 293T cells, subjected to an *in vitro* phosphorylation assay, transferred to a nitrocellulose membrane and exposed to film. Left panel shows membrane probed with anti-myc antibody (IgG marked with an asterisk), right panel shows autoradiogram of kinase assay revealing autophosphorylation. Note upshift of wt, but not T170D mutant kinase.

To assay catalytic activity, purified wt and mutant Plk4 were used for *in vitro* kinase assays in the presence of [γ-^{32}P]ATP (Figure 13). Proteins were separated by SDS-PAGE, transferred to nitrocellulose membrane and exposed to film. Equal loading of wt and dead Plk4 was assayed by Western blot analysis using the same membrane. Unfortunately, when the commonly used exogenous substrates casein, myelin-basic protein, histone 1 and 3 were screened for suitability, no phosphorylation by Plk4 above background was detected in parallel kinase assays (not shown). As seen in Figure 13, the activity of wt Plk4 was therefore only detected by auto-phosphorylation, whereas dead Plk4 exhibited no signal. The wt kinase also showed some retarded electrophoretic mobility when compared to dead kinase, suggesting that phosphorylation was responsible for this characteristic. Surprisingly, although the T170D mutant was active, no upshift was detected. The phosphorylation of this particular threonine residue in the wt kinase may therefore be responsible for the retarded electrophoretic mobility of Plk4.

In an alternative approach, the GST-tagged kinase domain of Plk4 (residues 1-265) was bacterially expressed and purified. In contrast to full length Plk4, the kinase domain was readily expressed and also soluble, thus permitting the isolation of significant amounts of enzyme for further work. The recombinant Plk4 kinase domain was then used in kinase assays together with substrates in the presence of [γ-^{32}P]-ATP, and separated by SDS-PAGE. As seen in Figure 14, recombinant Plk4(1-265) readily phosphorylated the exogenous substrate casein and also showed prominent auto-phosphorylation, whereas catalytically inactive D154A-Plk4(1-265) used as control did not. This therefore confirms the specificity of the reaction. Furthermore, the β-subunit of casein was preferentially phosphorylated in contrast to Plk1 which phosphorylates the α-subunit (Golsteyn et al., 1995). This suggests that Plk4 favours different substrate motifs. Finally, autophosphorylation most likely accounted for the retarded electrophoretic mobility of Plk4(1-265) seen in Figure 14.

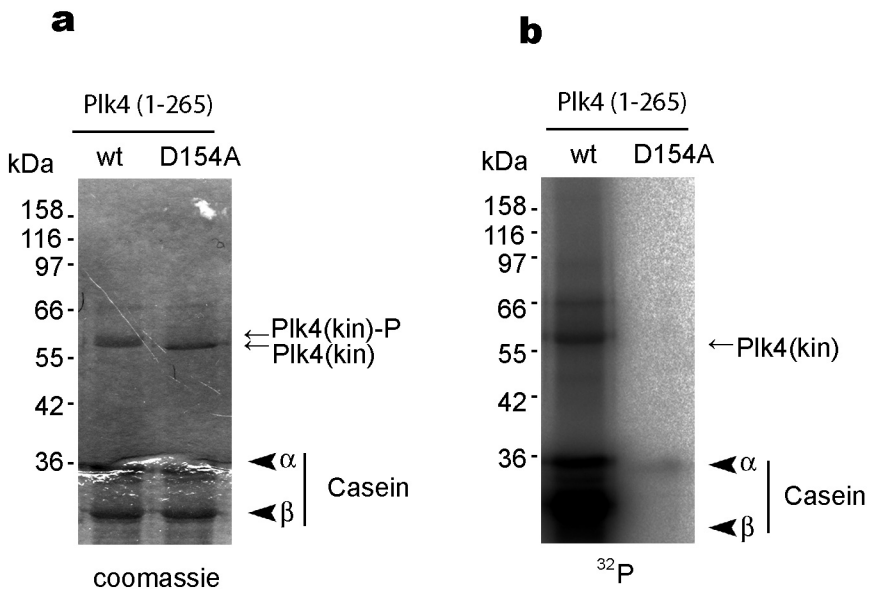

Figure 14. The kinase domain of Plk4 is an active enzyme. Equal amounts of purified wt and dead GST-Plk4(1-265) were subjected to an *in vitro* kinase assay in the presence of [γ-^{32}P]-ATP together with casein as substrate. **(a)** Kinase reactions were then separated by SDS-PAGE and stained with Coommassie Blue. Note retardation of wt Plk4(1-265). **(b)** Autoradiogram of kinase assay showing autophosphorylation and phosphorylation of substrate.

Taken together, these results demonstrate that Plk4 is an active protein kinase. In particular, autophosphorylation occurred on the kinase domain itself and was responsible for the retardation of electrophoretic mobility. Furthermore, casein may be used as an exogenous substrate for the recombinant kinase domain but is of limited use for full length Plk4 isolated from cell extracts.

Searching for interactors of Plk4

Although interacting partners of Plk4 have been reported (Yamashita et al., 2001; Swallow et al., 2005), no physiologically relevant interactor has yet been described. However, Plk4 has been reported as a centrosomal kinase (Leung et al., 2002) and plausible interactors may themselves be centrosomal. In an unbiased approach to find potential centrosomal binding partners, a human lymphocyte cDNA library was screened using yeast two-hybrid analysis. Full length dead Plk4 fused to the GAL4 DNA binding domain was used as bait and cotransformed with vectors containing library cDNA. Approximately 800,000 transformants were plated and screened, and a significant number of these were able to grow on selective drop-out medium. The library plasmids of 30 positive clones were isolated and co-transformed with the bait

to confirm a positive interaction. The plasmids scoring positive were sequenced (Table 3) but no known centrosomal components or immediately obvious interactors were found. Four plasmids were found to encode the protein MDS011 of unknown structure or function. Of more interest, a Guanine nucleotide binding protein subunit was found in addition to a 14-3-3 protein isoform, known to bind phosphopeptides (Mhawech, 2005).

Occurrence	Accession	
4x	AAG14960	MDS011
	AAH14256	Guanine nucleotide binding protein (G protein), beta polypeptide
	2C23_A	Chain A, 14-3-3 Protein Beta (Human)
	CAI23831	proto-oncogene tyrosine-protein kinase LCK
2x	AAH02323	ZYX protein, zyxin, actin cytoskeleton associated protein
	NP_002994	SEC14 (S. cerevisiae)-like 1 isoform a [Homo sapiens]
	CAH74116	glycine dehydrogenase
	NP_009152	polyamine-modulated factor 1
	NP_004387	DEAD (Asp-Glu-Ala-Asp) box polypeptide 5
	NM_001005271	CHD3 protein, chromodomain helicase DNA binding protein 3 isoform 3
	NP_699202	PDZ domain containing ring finger 1, interactor of Numb proteins
	AAA99715	putative tumour suppressor H37 RNA binding motif protein 5
	AAH30025	TIAL1 protein
	AAH19265	PKM2 protein, pyruvate kinase
	CAA71749	CAP-binding protein complex interacting protein 1
	BAD96885	leupaxin variant
2x	BAD96249	eukaryotic translation initiation factor 3 subunit 6 interacting protein variant
	AAH13311	Mitochondrial ribosomal protein L38
	NP_001025162	FLJ20859 protein isoform 1, predicted rRNA methylase

Table 3. List of identified yeast two-hybrid interactors of full-length Plk4. Full length Plk4 was used as bait to screen a human lymphocyte plasmid cDNA library. Listed are positive clones whose interaction was confirmed by retransformation with bait into host yeast. The number of each positive plasmid was one unless stated otherwise.

To reduce the number of positive clones, a truncated N-terminal fragment of Plk4 consisting of the kinase domain and part of the linker was used to screen a similar number of transformants of the same cDNA library. This screen resulted in a total of 11 positive interactors (Table 4). Again, no known centrosomal components were found, however a G-protein subunit as well as a G protein pathway suppressor

protein was identified. Furthermore, plasmids encoding an alpha-helical protein of mainly coiled-coil structure but of uncharacterised function were found in four positive clones.

Occurrence	Accession	
	NM_020482	FHL5 LIM protein Act
	NM_015338	ASXH1 human homologue of Dm 'additional sex-comb like 1', polycomb group protein
	NM_005648	Transcription elongation factor B
4x	AF216493	C6orf18 alpha-helical protein (HCR), mainly coiled-coil
	BC013652	GPS2 G protein pathway suppressor 2
	NM_031203	hnRPM heterogeneous nuclear ribonucleoprotein M
	BC029996	G protein beta-polypeptide-2
	AF018034	ECE-1 endothelin converting enzyme-1

Table 4. Yeast two-hybrid interactors of N-terminal Plk4. A truncated Plk4 bait consisting of residues 1-380 was used to screen the same human lymphocyte library. The number of each plasmid found was one unless stated otherwise.

Due to the inconclusive results obtained by yeast two-hybrid screening, an alternative approach to finding interactors of Plk4 was sought. A cell line that contains a stably integrated plasmid allowing inducible expression of Flag-tagged wt Plk4 under tetracycline control was used for immunoprecipitation of overexpressed kinase after 24 hours of induction. Immunocomplexes were pulled down using anti-Flag antibodies, competitively eluted with Flag peptide and separated by SDS-PAGE (Figure 15). After staining with Coomassie Blue, bands were excised and analysed by mass-spectrometry, kindly performed by X. Li (Max-Planck Institute of Biochemistry, Martinsried). Regions of unstained SDS-PAGE gel between prominently stained bands were also investigated since these were thought to contain low abundance proteins such as centrosomal components.

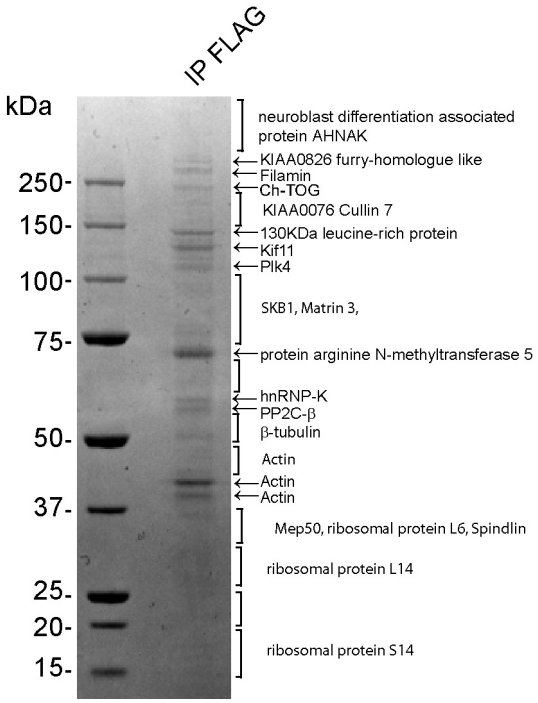

Figure 15. Mass-spectrometric analysis of Plk4 immunocomplexes. Flag-tagged Plk4 overexpressed in an inducible stable cell line was immunoprecipitated with anti-Flag antibodies and eluted with Flag-peptide. After separation by SDS-PAGE and staining with Coomassie Blue, the bands and regions indicated were excised and analysed by mass-spectrometry (X. Li, Max-Planck Institute for Biochemistry, Martinsried).

Using this approach, the known centrosomal component Ch-TOG was identified. Notably, Ch-TOG is the mammalian homologue of XMAP215, both being microtubule binding factors required for proper bipolar spindle formation in *Xenopus* egg extracts (Ohkura et al., 2001; Cassimeris and Morabito, 2004). Furthermore, the protein phosphatase 2C isoform β (PP2C-β, also known as PPM1B) was identified in this mass-spectrometric analysis of Plk4 immunocomplexes. This protein phosphatase is expressed as multiple isoforms and is the founder member of a monomeric group of phosphatases insensitive to commonly known phosphatase inhibitors such as calyculin A. Furthermore, PP2C-β has been implicated in the dephosphorylation of Cdks in mammalian cells, in particular Cdk2 (Kusuda et al., 1998; Cheng et al., 1999; Cheng et al., 2000; Seroussi et al., 2001). Revealingly, *Drosophila* mutant embryos defective for PP2C-β arrest early in development displaying a mitotic arrest, supernumerary centrosomes and defects in microtubule

attachment to centromeres (Snaith et al., 1996). PP2C-β may therefore act as the antagonistic protein phosphatase to Plk4.

Due to the relevance of PP2C-β, the cDNA of this phosphatase was acquired from the Image Consortium (BC064381) and subcloned into tagged mammalian expression vectors. U2OS cells transfected with Flag- or myc-tagged PP2C-β showed a diffuse staining distributed throughout the cell cytoplasm when analysed by immunofluorescence microscopy, but no overexpressed protein was enriched at the centrosome (not shown). Whether PP2C-β is therefore a cellular interactor of Plk4 remains unclear.

Taken together, the above results suggest that searching for Plk4 interactors using unbiased approaches is only moderately successful. Yeast two-hybrid screens using Plk4 as bait did not uncover any obvious interactors. Immunoprecipitation of overexpressed Plk4, however, allowed the identification of PP2C-β and Ch-TOG as two interesting candidate proteins, and further work will be required to confirm the physiological relevance of these proteins for the function of Plk4.

Overexpressed Plk4 localises to centrosomes

Previous reports have shown that overexpressed and GFP- tagged Plk4 localises to the centrosome, the perinuclear region, the nucleolus and the cleavage furrow in mammalian cells in a cell-cycle dependent manner (Hudson et al., 2001; Leung et al., 2002). U2OS cells were therefore transfected overnight with variously tagged Plk4 mammalian expression constructs and examined by immunofluorescence microscopy. Centrosomes were visualised by staining with anti-C-Nap1 antibodies.

As seen in Figure 16a, myc-tagged wt Plk4 localised to the centrosome but not to the nucleolus or perinuclear region in interphasic cells. Tagging Plk4 with FLAG or GFP at N- or C-terminal ends did not alter the centrosomal localisation of this kinase.

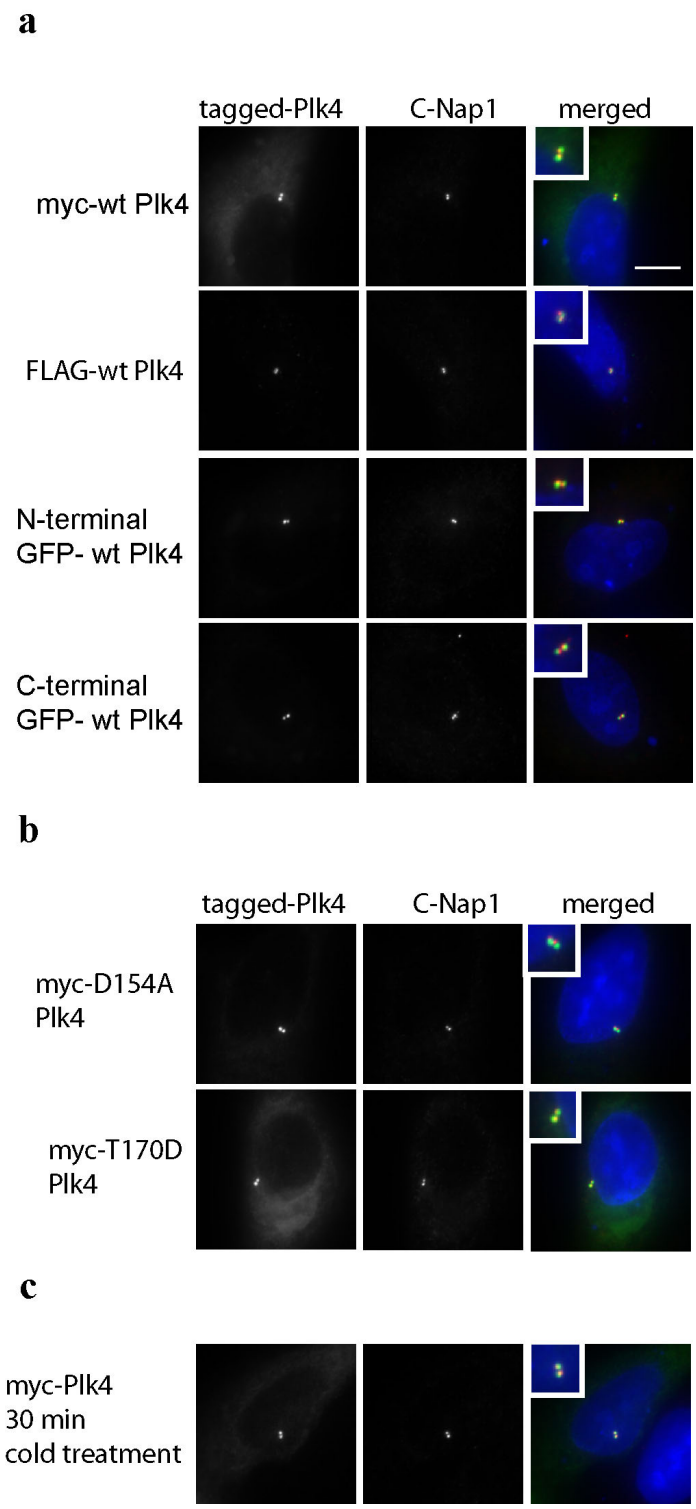

Figure 16. Overexpressed Plk4 localises to the centrosome in mammalian cells. (a) U2OS cells were transfected for 12 hours with myc-, Flag- or GFP-tagged wt Plk4 and prepared for immunofluorescence microscopy. Overexpressed kinase was detected with anti-myc or anti-Flag antibodies or by GFP fluorescence (green), centrosomes were detected by C-Nap1 antibodies (red). **(b)** Overexpressed myc-tagged dead (D154A) or T-loop mutant (T170D) Plk4 showed similar centrosomal staining. **(c)** Cells transfected with myc-tagged wt Plk4 were left on ice for 30 minutes to depolymerise the microtubule network and analysed by immunofluorescence microscopy. Insets show enlargements of the centrosome. Scale bar: 10μm

Likewise, the centrosomal association of Plk4 was not dependent on catalytic activity because tagged versions of dead or T170D mutant kinase were also found at this organelle (Figure 16b). Furthermore, the centrosomal localisation of Plk4 was maintained after cells were incubated on ice for 1 hour to depolymerise microtubules (Figure 16c). This result indicates that Plk4 is stably associated with the centrosome, and is not involved in a dynamic recruitment process dependent on an intact microtubule network.

In summary, these overexpression studies reveal that newly synthesised Plk4 is recruited to the centrosome independently of catalytic activity, of polymerised microtubles or the tag used. Whilst the previously reported centrosomal localisation of overexpressed Plk4 could be confirmed, no staining of other cellular structures was observed.

Endogenous Plk4 is a centrosomal kinase

To explore the localization and function of endogenous human Plk4, a rabbit polyclonal antibody was raised using a bacterially expressed fragment of Plk4 as antigen. The antigen consisted of the linker of Plk4, spanning residues 314 to 817 (Figure 17a), and was chosen due to its lack of homology with other polo-like kinases. The affinity purified antibody did not recognise a band of the correct size in Western blot analyses of lysates of asynchronously growing human cells. Since the antibody was unable to detect endogenous Plk4, U2OS cell lines expressing FLAG-tagged wild type and catalytically inactive (D154A) Plk4 under tetracycline control were established (Figure 17b). When used for Western blot analysis of lysates prepared from this cell line, the antibody readily recognized a protein of the expected size in cells induced to express Plk4, but did not reveal any specific bands in non-induced cells suggesting that endogenous Plk4 is of low abundance (Figure 17b). However, Western blot analysis confirmed the presence of endogenous Plk4 in purified centrosomes (Figure 17b). The antibody was also able to immunoprecipitate a protein of predicted size (Figure 17c).

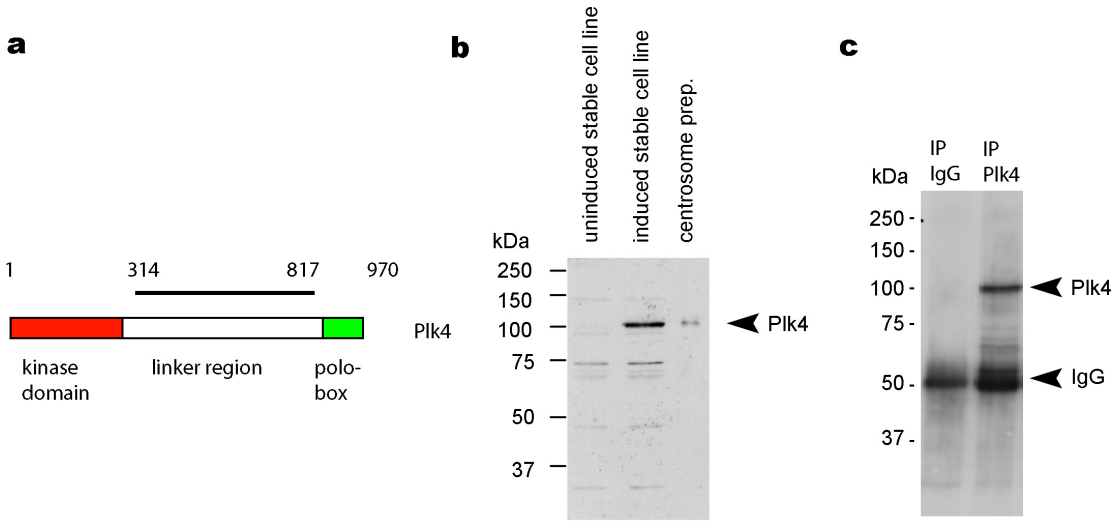

Figure 17. Antibody characterisation. **(a)** Schematic representation of Plk4, indicating the domain (residues 314-817) used for antibody production. **(b)** Extracts from cell lines carrying an inducible human wt Plk4 cDNA and purified centrosomes were probed by Western blotting with anti-Plk4 antibody. **(c)** Immunoprecipitation with purified anti-Plk4 antibody or non-immune rabbit IgG as control from equal amounts of total extracts prepared from asynchronous 293T cells. Immunocomplexes were probed with anti-Plk4 antibodies to detect kinase.

Immunofluorescence staining of asynchronously growing U2OS cells showed that endogenous Plk4 associates with centrosomes throughout the cell cycle, as indicated by its co-localization with γ-tubulin (Figure 18a). Similar results were obtained in HeLa and HCT116 cells (data not shown). Pre-incubation of antibodies with antigen abolished centrosome staining (Figure 18b). Interestingly, Plk4 staining of G1 phase cells generally revealed two closely spaced dots, whereas four dots could occasionally be seen in G2 phase cells. Invariably, these dots co-localized with centrin, a marker for centrioles (Figure 18c), when HeLa cells stably expressing centrin-1-GFP (Piel et al., 2001) were stained with the anti-Plk4 antibody. Endogenous Plk4 therefore associates with centrioles throughout the cell cycle. However, in contrast to the reported localization of EGFP-tagged murine Plk4, endogenous human Plk4 was not observed to localise to either nucleoli or cleavage furrows. In summary, these results thus confirm and extend previous data on EGFP-tagged murine Plk4 (Leung et al., 2002) and point to a role of this kinase in centrosome biology.

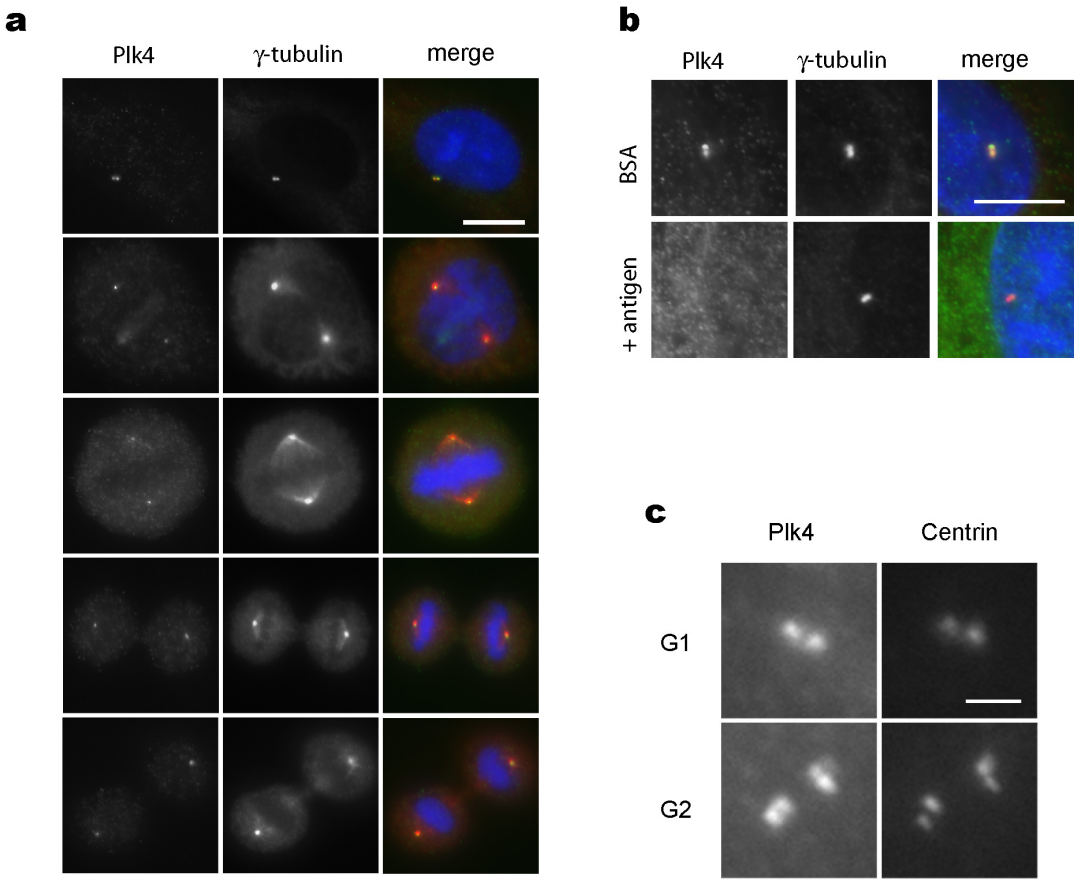

Figure 18. Endogenous localises to the centrosome throughout the cell cycle. (a) U2OS cells were co-stained with anti-Plk4 (green) and anti-γ-tubulin (red) antibodies and analyzed by immunofluorescence microscopy; DNA was stained with DAPI. Bar = 10μm. **(b)** Antigen competition. Plk4 antibodies were pre-incubated with antigen or BSA as control and used for immunofluorescence staining of U2OS cells. Bar = 10μm. **(c)** Co-localization of Plk4 with centrioles in G1 and G2 cells. Plk4 was stained by antibody and centrioles were visualized by EGFP-hCentrin. Bar = 1μm.

Plk4 is present in the cell throughout mitosis

The protein levels of Plk1 are subject to large changes over the course of the cell cycle (Golsteyn et al., 1995). Plk4 may be cell cycle regulated because levels of Plk4 mRNA have been reported to increase in cells released from a G_0 arrest (Fode et al., 1996), but no data on the protein levels of this kinase have been presented. However, the above immunofluorescence study on endogenous Plk4 in U2OS cells suggested that Plk4 is located at the centrosome throughout the cell cycle, thus arguing against drastic alterations in the protein levels of this kinase.

Unfortunately, the anti-Plk4 antibody did not detect the endogenous kinase by Western blot analysis, most likely due to the low abundance of Plk4. Therefore, immunoprecipitations from total extracts prepared from synchronised cells were

attempted. 293T cells were synchronised with either a double-aphidicolin block or by a nocodozale induced mitotic arrest to achieve S- and M-phases, respectively. G1 cells were obtained by releasing cells from a nocodazole induced mitotic arrest for two hours. As seen in Figure 19, Plk4 was immunoprecipitated at similar amounts from S-, M- or G1 phase cell extracts. Consequently, the levels of Plk4 do not vary greatly over the course of the cell cycle, and this result is consistent with the previous immunofluorescence data.

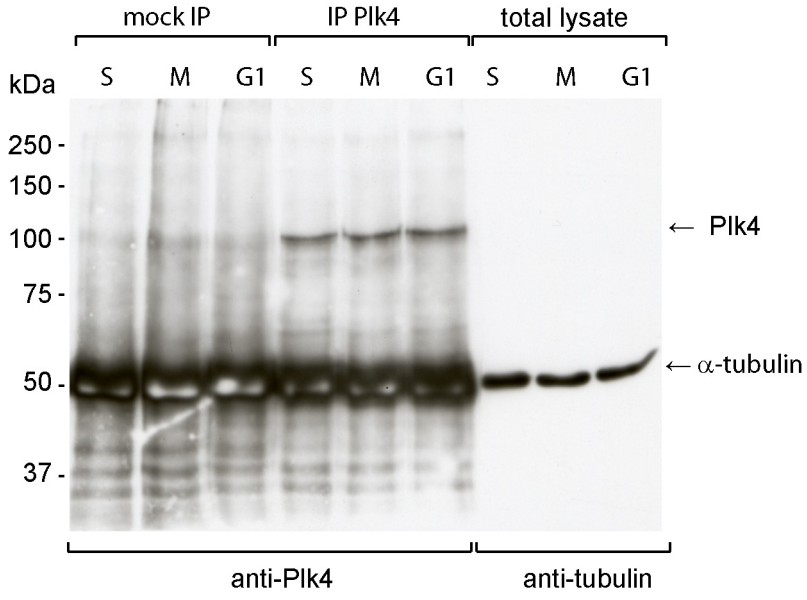

Figure 19. Plk4 is present in the cell throughout the cell cycle. 293T cells were synchronised in S-, M- or G1 phases as described in Methods. Immunoprecipitations were performed from similar amounts of cell lysate by incubation with anti-Plk4 antibody (IP Plk4) or control IgG (mock IP). Immunocomplexes were analysed by immunoblotting using anti-Plk4 antibodies. Protein amounts present in lysates used were verified by Bradford assay (not shown) or by probing with anti-tubulin antibodies. The strong band running at 50 KDa is rabbit IgG.

Overexpression of active Plk4 causes centrosome overduplication

The transient expression of epitope-tagged Plk4 in U2OS and HeLa cells confirmed the centrosomal localization of this kinase but did not reveal obvious associations with cleavage furrows or intranuclear structures, in line with the observations of the endogenous protein mentioned above. Most interestingly, overexpression of wild-type Plk4 in U2OS or HeLa cells for 48 hours resulted in a striking multiplication of Plk4-positive dots (Figure 20a), whereas most cells overexpressing catalytically inactive (D154A) kinase did not. These structures also formed asters in a microtubule

regrowth assay (Figure 20b). When overexpressed in cells, several centrosomal proteins are known to form aggregates seen as multiple punctate structures in the cell cytoplasm (Casenghi et al., 2003; Andersen et al., 2003), and these are most likely due to the presence of coiled-coil structures in the ovexpressed protein. However, the Plk4 induced structures stained positive not only for the overexpressed kinase but also for several known centrosomal and centriolar components (Figure 20c). In particular, the centriolar markers centrin-2, C-Nap1, γ-tubulin and polyglutamylated tubulin were located to these dots, as well as the known centrosomal proteins FOP, Cap350 and pericentrin (Figure 20c). The positive result obtained with the GT335 antibody directed against stabilised tubulin revealed that these dots contain organised microtubular structures. The staining of these punctuate structures with centrosomal markers therefore suggested that they constitute either centrioles or centriole precursors.

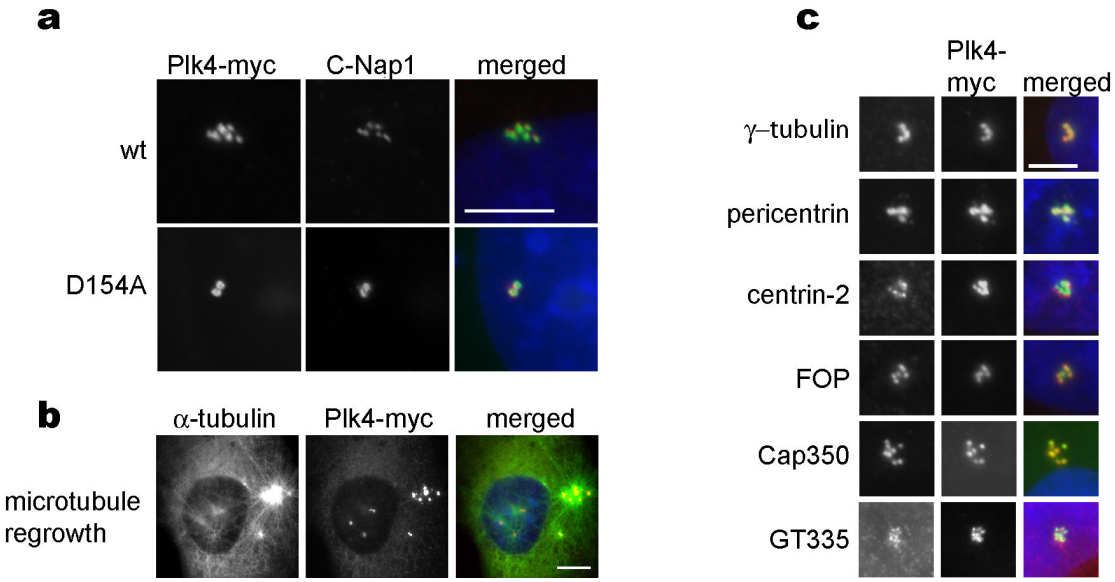

Figure 20. Overexpression of Plk4 leads to the overproduction of centrioles. **(a)** U2OS cells were transfected with myc-tagged wild-type (wt) or catalytically inactive (D154A) Plk4 for 48h. The overexpressed kinase was detected with anti-myc 9E10 antibody (green) and centrioles were stained with anti-C-Nap1 antibody (red). Bar = 10μm. **(b)** U2OS cells overexpressing myc-tagged wt Plk4 were incubated on ice for 30 min to depolymerise microtubules. Cells were then incubated in warm medium for 30 seconds to induce microtubule polymerisation, followed by immediate fixation and staining with antibodies against α-tubulin (green) and myc (red). Note formation of multiple asters **(c)** wt Plk4 overexpressing cells were probed with anti-myc antibodies (green) or antibodies against centrosomal proteins (red). Bars = 10μm

Confirming this conclusion, an electron microscopic analysis of cells from the aforementioned cell line induced to express wild-type Plk4 revealed multiple structures resembling canonical centriole cylinders (Figure 21). In contrast, the majority of cells expressing catalytically inactive Plk4 showed only two centrioles (Figure 21). Taken together, these data demonstrate that excess Plk4 activity results in centriole amplification.

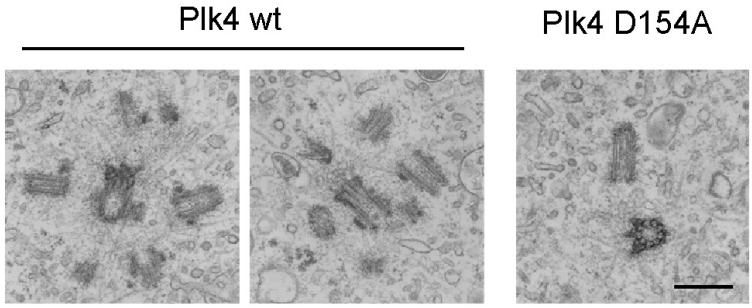

Figure 21. The structures formed by excess Plk4 are centrioles. Electron micrographs showing the presence of multiple centrioles in cell line induced for 48h to express wt Plk4 (left panels), whereas most cells expressing the D154A mutant show only two centrioles (right panel). Cells were fixed in glutaraldehyde and stained with uranyl-acetate and lead citrate. Bar = 500 nm. Electron microscopy kindly performed by Y-D Stierhof, ZMBP, University of Tübingen.

A priori, centriole amplification can arise through *bonafide* centriole overduplication during S phase or through cell division failure (Meraldi et al., 2002a; Nigg, 2002; Sluder, 2004). To distinguish between these possibilities, U2OS cells were transfected with Plk4 and then treated with or without aphidicolin. This inhibitor of replicative DNA polymerases causes cells to arrest in S phase and prevents passage through mitosis. Moreover, in aphidicolin-treated U2OS cells centrosomes are known to overduplicate (Balczon et al., 1995; Meraldi et al., 1999a). When compared to controls, wild-type Plk4 induced extensive centrosome amplification above background regardless of the presence or absence of aphidicolin (Figure 22a and b), indicating that it caused *bonafide* centrosome overduplication. Supporting this conclusion, active Plk4 also triggered centrosome amplification in aphidicolin-treated HeLa cells (Figure 22c), in which centrosomes do not spontaneously overduplicate during S phase arrest (Meraldi et al., 2002a). In contrast, catalytically inactive D154A Plk4 caused some centrosome amplification above background in untreated U2OS

cells (Figure 22a), but this was reduced to a level typical of aphidicolin-treated cells when cell division was suppressed (Figure 22b). These results indicate that only an excess levels of wild type kinase increased centrosome numbers whereas the catalytically inactive mutant kinase caused centrosome amplification through occasional division failure, presumably via a dominant-negative mode of action.

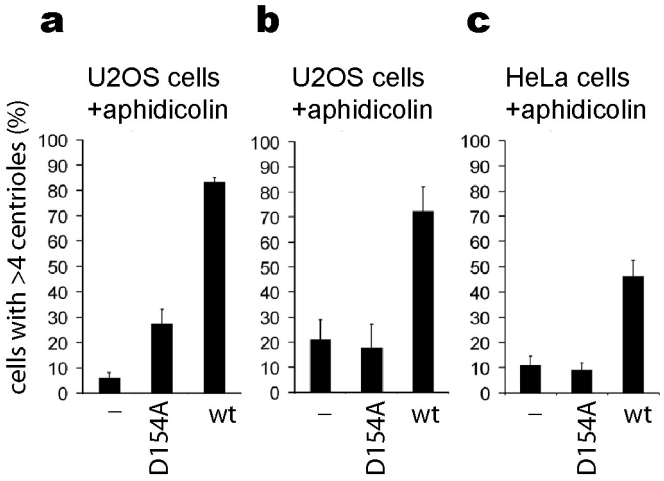

Figure 22. Excess wt Plk4 leads to centriole overduplication in arrested cells. Histograms show the percentages of cells with excess (>4) centrioles in U2OS **(a,b)** or HeLa **(c)** after incubation for 48 h with **(b,c)** or without **(a)** aphidicolin. Cells were either untransfected (-) or transfected with wt or D154A Plk4, as indicated. Data from three independent experiments, counting 150 cells each; error bars indicate standard deviation.

The evidence so far suggested that Plk4 activity was responsible for the overproliferation of centrosomes, however no evidence for a direct role of Plk4 at the centrosome had been obtained. We therefore set out to find Plk4 mutants that did not localise to centrosome, yet remained catalytically active.

A previous report had revealed the importance of the C-terminal sequences in localising Plk4 to the centrosome (Leung et al., 2002). In those studies, when a short section of the C-terminus including the polo-box had been deleted, the overexpressed truncation mutant no longer was recruited to the centrosome. Therefore, we generated several epitope-tagged Plk4 deletion constructs (Figure 23a) and examined their subcellular localization after overexpression in U2OS cells (Figure 23b). In particular, a close examination of the polo-box domain alignment in Figure 8

prompted us to delete the polo-box as defined by the previously published structure of the polo-box (Leung et al., 2002), thus generating the Plk4(1-888) mutant. Abolishing the Plk4 polo-box did not alter the localisation of this kinase (Figure 23b) whereas a myc-fusion fragment consisting of only the polo-box sequences (Plk4 888-970) was not recruited to the centrosome. These data suggest that the polo-box was not sufficient to confer localisation. Furthermore, they contrast with the role of the Plk1 polo-box in the recruitment of that kinase to intracellular structures (Lee et al., 1998). However, a mutant with a more extensive deletion of the C-terminus including most of the putative secondary structures found by predictive programs (Plk4 1-638, see Figure 9) was not found at the centrosome but in the cytoplasm instead (Figure 23b). The deleted fragment itself, however, was capable of localising to the centrosome (Plk4 638-970,). This result reveals that the sequences upstream of the polo-box are required for centrosome localisation.

Taken together, the recruitment of overexpressed fragments of Plk4 to the centrosome required sequences located in the C-terminal, non-catalytic domain, supporting the conclusion that both the C-terminal polo-box motif and additional upstream motifs (the so-called cryptic polo-box) contribute to confer correct localization (Leung et al., 2002).

Next, these various deletion mutants of Plk4 were assayed for their ability to induce centrosome overduplication in arrested cells (Figure 23a, c). As before, myc-tagged deletion mutants of Plk4 were transfected into U2OS cells in the presence of aphidicolin for 48 hours. As expected, none of the mutants lacking the catalytic domain were able to increase centriole numbers. The Plk4 mutant (1-888) lacking the polo-box motif, but still able to localise to centrosomes (Figure 23b), induced centriole overduplication, whereas the Plk4 (638-970) mutant lacked this ability (Figure 23c). A summary of these results is given in Figure 23a. Taken together, Plk4 deletion constructs able to localise to the centrosome and which contain a kinase domain are capable of inducing centriole overduplication.

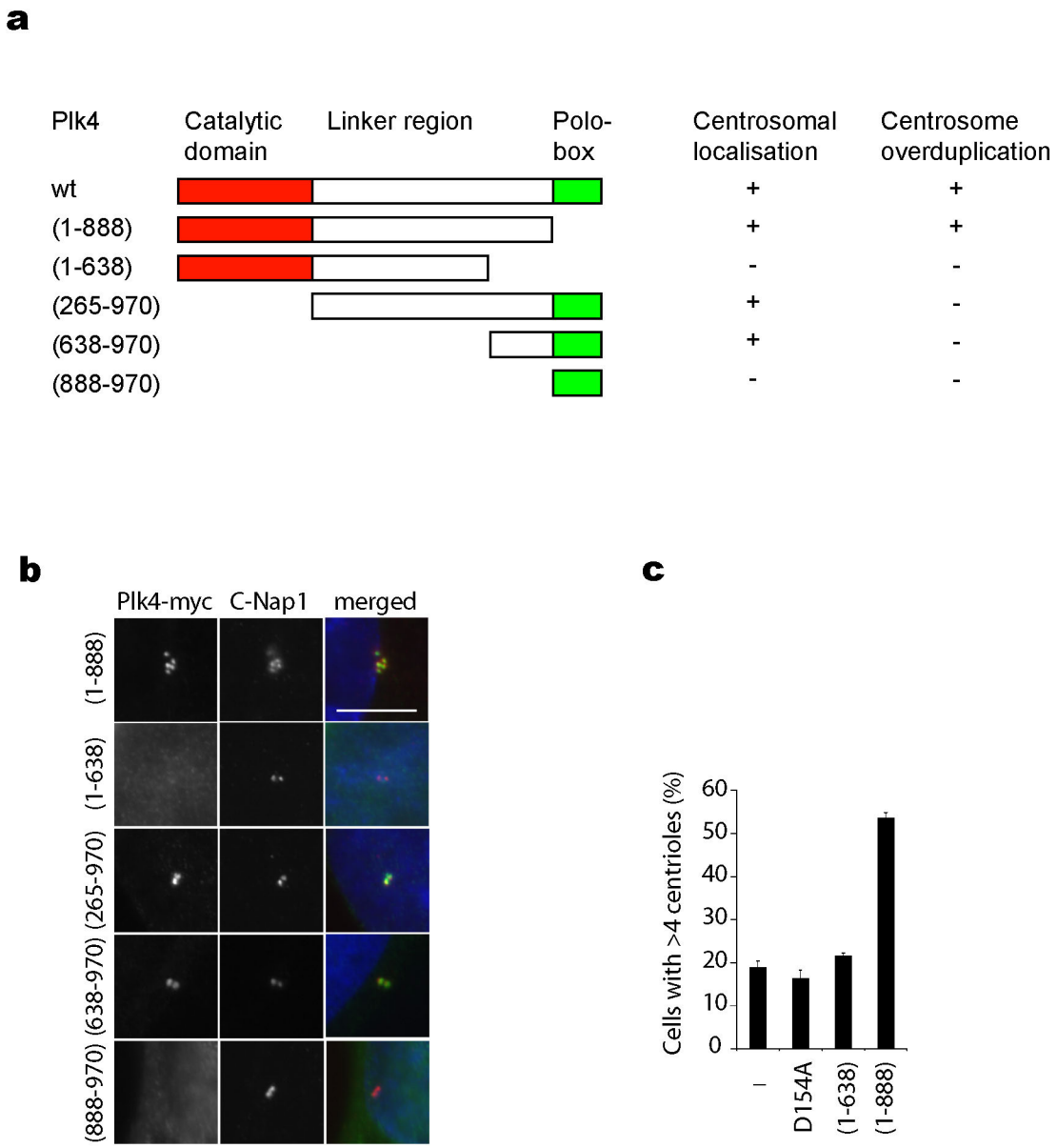

Figure 23. Centriole overduplication depends on centrosome localization of Plk4. (a) Schematic summarizing myc-tagged Plk4 deletion mutants that were assayed for localisation and ability to induce centrosome overduplication. **(b)** U2OS cells were transfected, incubated in medium containing aphidicolin for 48 h and processed for immunofluorescence microscopy, using anti-myc (9E10) antibody to visualize ectopic Plk4 (green) and anti-C-Nap1 antibody (red) to monitor centriole numbers. **(c)** Histogram shows percentage of cells with excess (>4) centrioles. Data are from three independent experiments, counting 120-150 cells each, with error bars indicating standard deviations.

Finally, we wanted to confirm the catalytic activity of these Plk4 deletion mutants. Using the approach shown in Figure 13, the various myc-tagged Plk4 deletion mutants were transfected into 293T cells for 24 hours and purified by immunoprecipitation. The purified proteins were then subjected to an *in vitro* kinase assay to investigate autophosphorylation. As seen in Figure 24, both the Plk4 (1-888) and the Plk4 (1-638) showed catalytic activity. The differences between these two mutants in the ability to induce centrosome overduplication therefore depended solely on the localisation of the overexpressed constructs in cells.

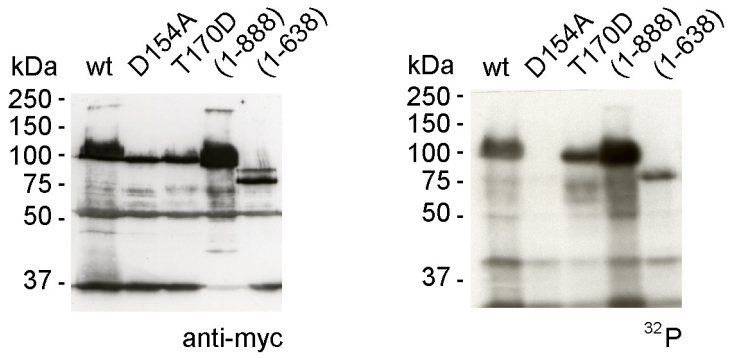

Figure 24. The C-terminal deletion mutants of Plk4 are catalytically active. N-terminal myc-tagged Plk4 constructs were immunoprecipitated with myc antibodies from total cell lysate prepared from overexpressing 293T cells, subjected to an *in vitro* phosphorylation assay, transferred to a nitrocellulose membrane and exposed to film. Left panel shows membrane probed with anti-myc antibody confirming immunoprecipitated deletion mutants, right panel shows autoradiogram revealing autophosphorylation.

Plk4 homodimerises

Previous work has revealed the dimerisation abilities of mouse Plk4 (Leung et al., 2002), this feature being dependent on C-terminal domains. Using directed yeast 2-hybrid analysis, full length Plk4 was found to interact with itself (Figure 25). In contrast, yeast cells transformed with full length Plk4 and empty bait or prey vectors did not grow on selective medium, showing that Plk4 was not autoactivatory. These results therefore confirm previous observations on the dimerisation of mouse Plk4.

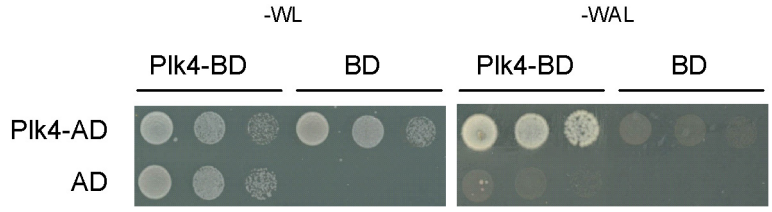

Figure 25. Plk4 interacts with itself in yeast 2-hybrid analysis. Yeast cells were cotransformed with plasmids encoding Plk4 fused to the GAL4 DNA binding domain (Plk4-BD) and the Plk4-activation domain fusion (Plk4-AD). After transformation, yeast cells were plated in three serial dilutions on non-selective (-WL) or selective medium (-WAL) for 5 days. Yeast cotransformed with bait Plk4-BD and empty activation domain vector (AD), or Plk4-AD and binding domain vector (BD) were used as controls. Note growth on selective medium of Plk4-AD/Plk4-BD transformed yeast only.

Domain mapping was used to determine which region was required for the dimerisation of Plk4. For this aim, myc-tagged Plk4 deletion mutants (see Figure 23) were assayed for their ability to bind Flag-tagged full length Plk4 in a co-immunoprecipitation assay. As shown in Figure 26, the Plk4(1-638) lacking C-terminal regions was incapable of interacting with the full length kinase. Conversely, the C-terminus of Plk4 (residues 638-970) was able to interact with wt Plk4, suggesting that this fragment functions as a dimerisation domain. When the polo-box domain was removed, the resulting mutant Plk4(1-888) still interacted with wt Plk4. This suggests that the polo-box is not sufficient for Plk4 dimerisation.

In summary, the data presented here confirms previous reports on the dimerisation of Plk4. Furthermore, this ability is dependent on C-terminal domains even though the polo-box domain alone is not essential. The role of dimerisation in the function of Plk4 is unclear and, moreover, the possibility of oligomerisation instead of dimerisation remains to be investigated.

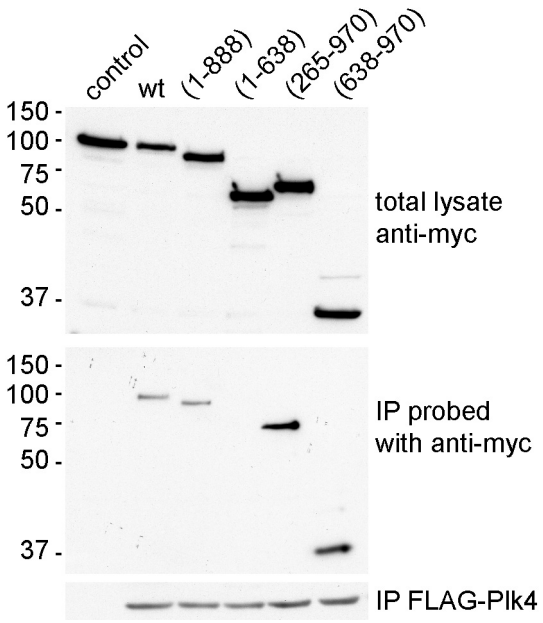

Figure 26. The C-terminus of Plk4 is required for dimerisation. Cos7 cells were co-transfected with Flag-tagged full length Plk4 and myc-tagged Plk4 constructs or myc vector alone as control. Success of overexpression was monitored by immunoblotting total lysate with myc-antibodies (top panel). Full length Flag-Plk4 was immunoprecipitated from total cell extracts using Flag-antibodies, eluted with Flag-peptide, and immunocomplexes were analysed for the presence of myc-tagged deletion constructs by probing with anti-myc antibodies (middle panel). Immunoprecipitation of Flag-Plk4 was confirmed by Western blot analysis using anti-Flag antibody (lower panel).

Overexpression of Plk4 leads to the formation of centriole precursors

Striking evidence for the direct action of Plk4 at the centrosome was found when centrosomes from cells induced to express wild type kinase for 16 hours were analysed by electron microscopy. Specifically, electron microscopy revealed conspicuous morphological changes in the form of multiple patches of electron dense material on the surface of centrioles, which often formed rosette-like structures in transverse cross-sections (Figure 27, top panels). Longitudinal cross-sections revealed that these unusual structures generally formed at the proximal ends of centrioles (Figure 27, lower panels). An intriguing interpretation of this data is that they could represent centriole precursor material recruited to centriole assembly sites at the proximal ends of centrioles.

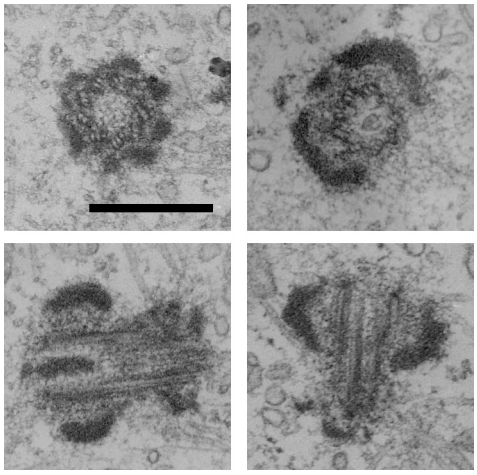

Figure 27. Overexpression of wt Plk4 induces morphological alterations of the centrosome. Centrosome alterations induced by excess Plk4 activity. Expression of Plk4-FLAG was induced in U2OS cells for 16 h before fixation in glutaraldehyde and analysis by electron microscopy. Electron micrographs of transverse (upper panels) and longitudinal sections (lower panels) of centrioles are shown emphasizing electron dense material at proximal ends. Bar = 500 nm. Electron microscopy kindly performed by Y-D Stierhof, ZMBP, University of Tübingen.

In support of this view, rosette-like structures induced by wild-type Plk4 could also be visualized by high-resolution immunofluorescence microscopy with antibodies against centrin (Figure 28, top row of panels), a centriolar marker implicated in centriole duplication (Salisbury et al., 2002). Plk4 did not exactly co-localize with rosettes (Figure 28), but was always located on the inside of these structures, possibly on the surface of centrioles.

To extend these findings and uncover additional proteins that localize to these rosettes, antibodies against other centrosomal proteins were used. Most notably, centrin positive rosettes were also stained with overexpressed GFP-CPAP or with antibodies directed against CPAP (Figure 28), the putative human homologue of Sas-4 required for centriole duplication in *C. elegans* (Leidel and Gonczy, 2003). In contrast, antibodies against C-Nap1, γ-tubulin or polyglutamylated tubulin did not stain rosettes (Figure 28), implying that these centriolar proteins are recruited after centrin and CPAP. These results therefore reveal a sequential recruitment of centriolar proteins to Plk4 induced precursor structures. An attractive conclusion from these data is that centrin and CPAP may form part of a putative complex required for the earliest stages of centriole assembly.

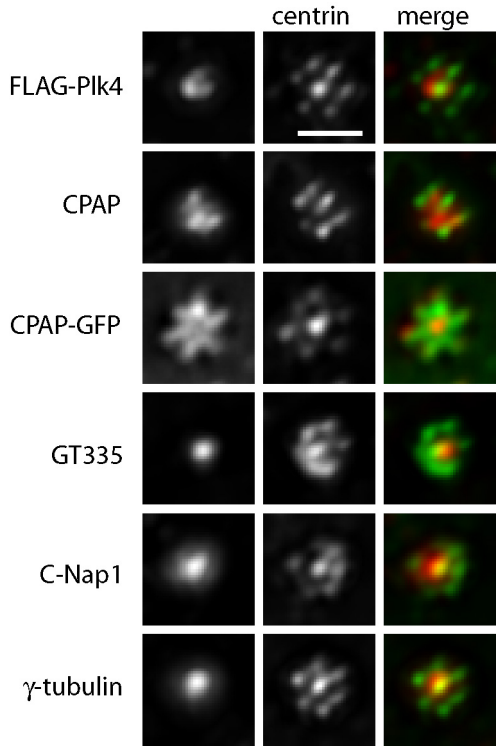

Figure 28. Plk4 induces the formation of centriole precursors. Expression of Plk4-FLAG was induced in U2OS cells for 16 h before cells were stained with antibodies to detect centrin (green) and Flag-Plk4 or several centrosomal proteins (red) in preparation for immunofluorescence microscopy. Bars = 0.5μm

To confirm whether the electron dense structures observed in Figure 28 were indeed centrin and CPAP positive rosettes observed by immunofluorescence, cells induced to overexpress wt Plk4 for 16h were used for pre-embedding immuno-gold electron microscopy. When cells were labelled with antibodies directed against the Flag epitope tag of over-expressed wt Plk4, gold particles were seen to decorate the base of prominent bud-like structures close to the parental centriole surface in both transverse and longitudinal cross-sections (Figure 29a). CPAP antibodies recognised similar bud-like structures, (Figure 29b) although transverse cross-sections of centrioles revealed that CPAP localized more peripherally, in close proximity to Flag antibodies (compare schematic diagrams in the top right-hand panels in Figure 29a and b). Furthermore, longitudinal views of centrioles revealed that electron dense structures at the proximal ends of centrioles were positive for both Flag and CPAP antibodies (compare lower left panels of Figure 29a, b and Figure 27). It is therefore likely that rosettes seen by electron and immunofluorescence microscopy are similar

and, moreover, the use of electron microscopy may permit the observation of earlier structures due to greater magnification.

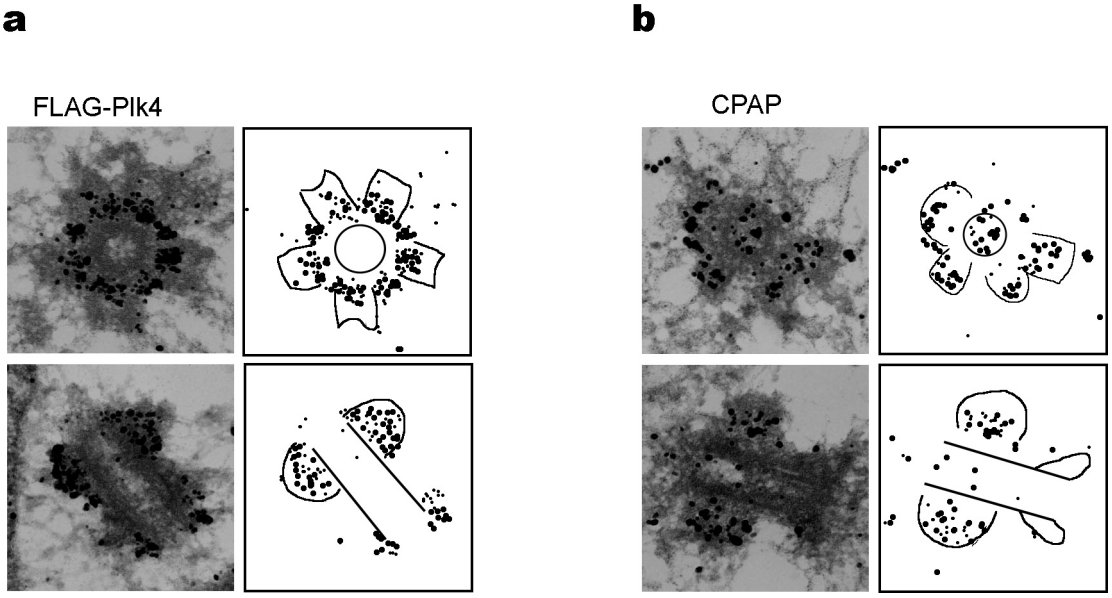

Figure 29. Plk4 and CPAP localise to centriole precursor material. Pre-embedding immuno-EM localisation of **(a)** Flag-Plk4 and **(b)** CPAP to centrosomes in Plk4 expressing cells. Panels show electron micrographs and schematic diagrams depicting nano-gold particles on centriole precursors for clarity, of transverse (upper panels) and longitudinal centrioles (lower panels). Electron microscopy kindly performed by Y-D Stierhof, ZMBP, University of Tübingen.

Taken together, the increased levels of wt Plk4 at the centrosome leads to distinct structural changes involving the recruitment of material containing centrin and CPAP. One tantalizing interpretation of these data is that the centriolar rosettes induced by Plk4 activity represent multiple initiation sites for centriole assembly. Further analysis of these putative precursor structures may thus lead to a better mechanistic understanding of centriole duplication. Another surprising finding is that the formation of nascent centrioles is not restricted to a single assembly point on the parent centriole. In these overexpression studies, excess Plk4 may therefore mark additional sites for the recruitment of centriolar material and permit the parallel assembly of multiple pro-centrioles on the circumference of the parent centriole.

Plk4 phosphorylates CPAP and its own C-terminus in vitro

Given the evidence so far in support of a direct activity of Plk4 at the centrosome, we asked whether this kinase could phosphorylate centrosomal proteins *in vitro*. Obvious substrate candidates for use in a kinase assay included proteins involved in centriole duplication, such as CPAP. To screen for *in vitro* substrates, the GST-tagged kinase domains of wt and catalytically inactive Plk4 consisting of residues 1-265 was used.

Since autophosphorylation of full length wt Plk4 had been observed in previous experiments (see Figure 13), the possibility of the C-terminus of Plk4 acting as an internal substrate was explored. A bacterially expressed and purified GST-tagged fusion of a previously used C-terminal fragment of Plk4 containing residues 638 to 970 (see Figure 13) was used as a possible substrate. The kinase domain of Plk4 phosphorylated this recombinant C-terminal domain (Figure 30) revealing additional phosphorylation sites besides those within the kinase domain. Whereas autophosphorylation of the Plk4 kinase domain most likely involves the conserved threonine residue 170 required for full catalytic activity, the role of phosphorylation sites in the C-terminus remains unclear and requires further analysis.

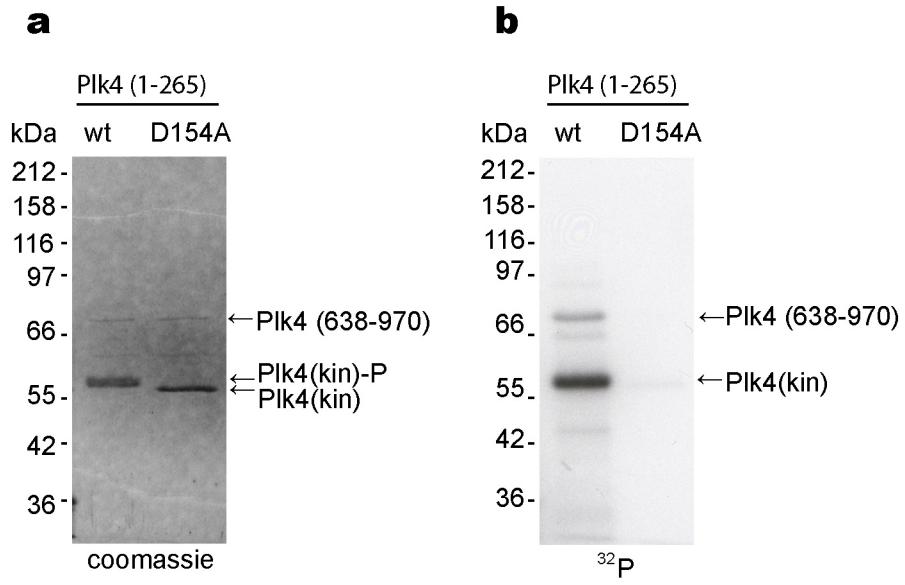

Figure 30. The C-terminus of Plk4 is a substrate. The bacterially expressed and purified GST-tagged C-terminus of Plk4 (residues 638-970) was used as substrate in an *in vitro* kinase assay together with recombinant Plk4 kinase domain as enzyme in the presence of [γ-^{32}P]-ATP . **(a)** The kinase assay was separated by SDS-PAGE and stained with Coomassie Blue. **(b)** Autoradiogram of kinase assay showing phosphorylation of the C-terminus.

Using a similar approach, full length and fragments of CPAP were purified from transfected COS7 cells by immunoprecipitation, and subjected to a kinase assay. The plasmid for full length GFP tagged CPAP was a kind gift of P. Gönczy (Swiss Institute for Experimental Cancer Research, Lausanne, Switzerland). Plk4 clearly phosphorylated N-terminal fragments of CPAP and the full length protein, although the latter only weakly (Figure 31). Unfortunately, no results for the C-terminus of CPAP could be obtained as this was not available.

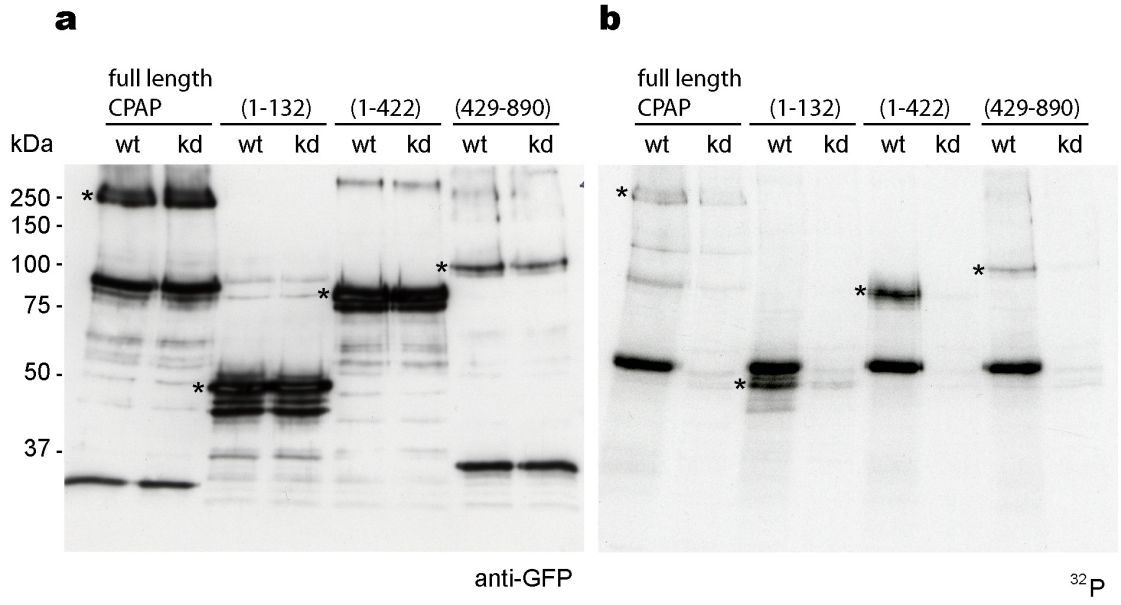

Figure 31. CPAP is an *in vitro* substrate of Plk4. GFP tagged full length and fragments of CPAP were expressed in Cos7 cells and immunoprecipitated from total cell extracts using anti-GFP antibodies. Immunocomplexes were subjected to an *in vitro* kinase assay with Plk4(1-265) in the presence of [γ-^{32}P]-ATP. **(a)** The kinase assays were separated by SDS-PAGE and immunoblotted with anti-GFP antibodies to quantify substrate. **(b)** Autoradiogram of the same immunoblot, showing phosphorylation of CPAP-GFP fragments marked by asterisks (*).

Since CPAP localised close to overexpressed Plk4 on putative centriole precursors observed by electron microscopy in Figure 29, this human homologue of Sas-4 could be a potential *in vivo* substrate and would thus be an attractive candidate protein for further study. Further characterization of *in vitro* phosphorylated CPAP would also provide data on currently unknown consensus motifs of Plk4 target sites.

Continued screening of centrosomal proteins is therefore a promising approach to identify additional substrates of Plk4.

Centrosome duplication is blocked by Plk4 inhibition

To corroborate a critical role for endogenous Plk4 in the regulation of centrosome duplication, loss-of-function experiments were performed. These experiments took advantage of the fact that certain mammalian cells such as U2OS reduplicate their centrosomes under a prolonged S-phase block. Inhibition or inactivation of proteins necessary for centrosome reproduction therefore prevent reduplication in such cells, as described in previous studies (Stucke et al., 2002; Meraldi et al., 1999a; Matsumoto et al., 1999b; Chen et al., 2002a).

As a first approach, purified anti-Plk4 antibodies, or non-immune immunoglobulins for control, were microinjected into the cytoplasm of asynchronously growing U2OS cells. Following treatment with aphidicolin for 64 hours to induce centrosome overduplication, cells were co-stained with secondary anti-rabbit antibodies to visualise injected cells, and with anti-γ-tubulin antibodies to monitor centrosome numbers (Figure 32a,b). Whereas approximately 50% of cells injected with control antibodies contained multiple centrosomes, most cells injected with anti-Plk4 antibodies displayed normal centrosome numbers, indicating that antibody-mediated inhibition of endogenous Plk4 prevented centrosome overduplication.

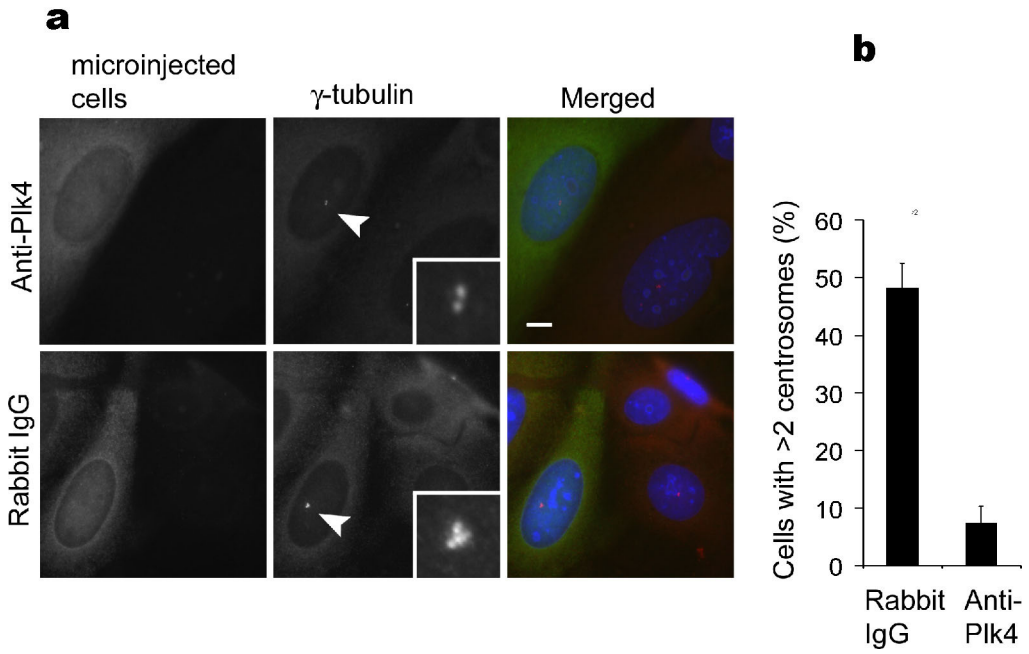

Figure 32. Microinjection of Plk4 antibody inhibits centrosome overduplication. (a) U2OS cells were microinjected with purified anti-Plk4 antibodies or control rabbit IgG, followed by incubation in medium containing aphidicolin for 64 h. After fixation, secondary anti-rabbit IgG antibodies were used to visualize injected cells (green) and centrosomes were detected with anti-γ-tubulin antibody (red). Insets emphasize inhibition of centrosome overduplication in anti-Plk4 antibody-injected cells as compared to controls. **(b)** Histogram shows the percentage of microinjected cells with increased numbers (>2) of centrosomes. Data are from three experiments, counting 150-200 injected cells each time, with error bars showing standard deviations.

As a second, complementary approach to abrogate Plk4 function, centrosome overduplication assays were performed in U2OS cells from which endogenous Plk4 had been depleted by siRNA (Figure 33). Cells were transfected with a Plk4-specific duplex RNA oligonucleotide for 24 hours, before aphidicolin was added for 64 hours. Staining with anti-γ-tubulin antibodies was then used to evaluate centrosome numbers in those cells that had been successfully depleted of endogenous Plk4, as judged by the complete absence of a centrosomal Plk4 immunofluorescence signal (Figure 33a). As compared to cells treated with a control siRNA duplex (GL2; (Elbashir et al., 2001), siRNA mediated depletion of Plk4 strongly suppressed the centrosome reduplication phenotype (Figure 33b) in these cells. Thus, antibody-microinjection and siRNA-mediated protein depletion concur to demonstrate that suppression of Plk4 function interferes with centrosome overduplication in U2OS cells.

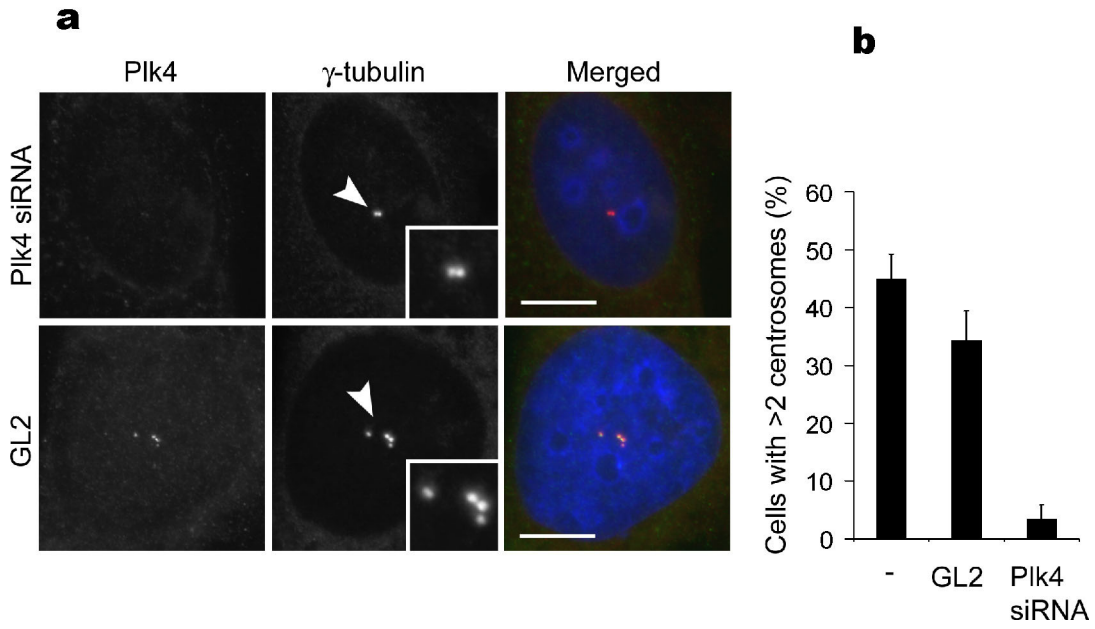

Figure 33. Depletion of Plk4 by siRNA blocks centrosome overduplication. (a) U2OS cells were transfected with either control (GL2) or Plk4 siRNA duplexes. 24 h after transfection, aphidicolin was added for an additional 64 h before cells were fixed and stained for Plk4 (green) and γ-tubulin (red). Bar = 10 μm. Insets highlight inhibition of centrosome overduplication in Plk4 depleted cells **(b)** Histogram shows the percentage of siRNA transfected or untransfected (-) cells with increased numbers (>2) of centrosomes. Results are from three independent experiments, counting 200 cells each, and error bars denote standard deviations.

Depletion of Plk4 causes progressive loss of centriole numbers

All results obtained so far indicated that Plk4 plays a critical role in triggering centriole duplication during S phase. However, we wanted to analyse asynchronously dividing cells and found that HeLa cells were better suited for these studies. More precisely, transfecting HeLa cells with siRNA duplexes targeting Plk4 for 48 hours resulted in a more efficient depletion of endogenous protein when observed by immunofluorescence microscopy (Figure 34). Furthermore, significant numbers of cells were observed to contain only one centriole when stained with anti-γ-tubulin antibodies. Since dividing cells were expected to give the most striking phenotype upon siRNA mediated Plk4 depletion, we focused our analysis on mitotic cells.

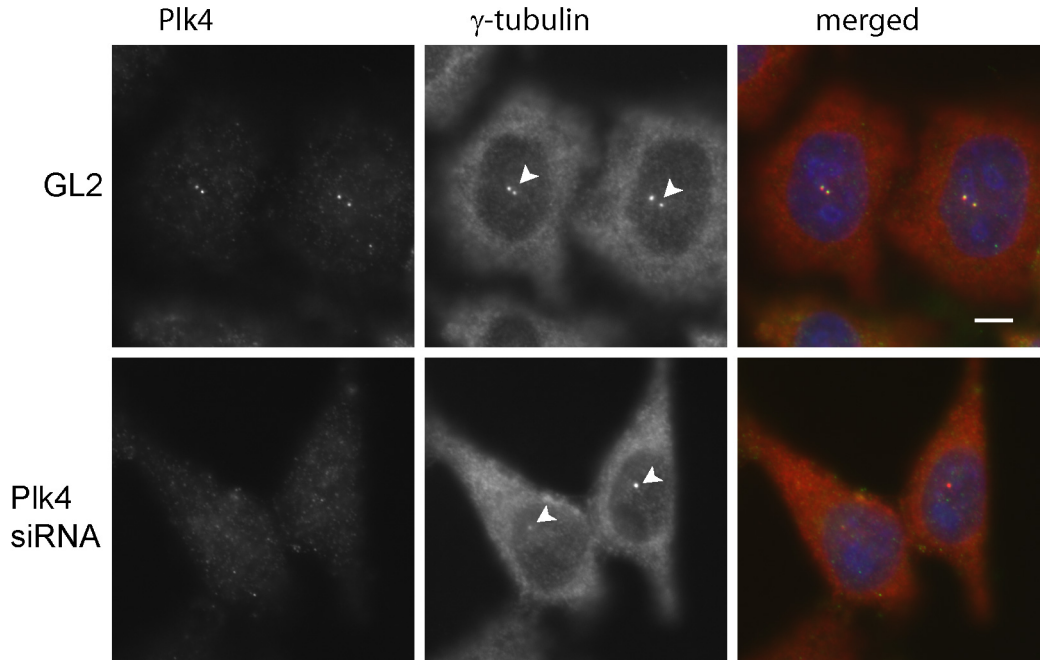

Figure 34. Depletion of Plk4 by siRNA leads to a reduction in centriole numbers in interphase cells. HeLa cells were transfected with Plk4 siRNA or GL2 duplex as control for 48 hours. Cells were fixed and prepared for immunofluorescence microscopy by staining with anti-Plk4 antibodies (green) and anti-γ-tubulin antibodies (red). Arrows point to centrioles. Bar = 10μm. Note efficient depletion of Plk4 signal and the presence of only one centrosome in Plk4 depleted cells.

In our approach to investigate mitotic figures, HeLa cells were transfected with siRNA duplexes targeting Plk4 and fixed after 24 to 48 hours. To simultaneously analyze both spindle structures and centriole numbers at spindle poles, fixed cells were co-stained with antibodies against α-tubulin and centrin-2, respectively. After depletion of Plk4 for 24 hours, the vast majority of all mitotic cells still appeared to form bipolar spindles, but after 48 hours, many monopolar spindles were observed (Figure 35b). Counting of centriole numbers revealed that virtually all cells treated with control siRNA-duplexes showed the expected four centrioles, two at each pole, regardless of the time of treatment (Figure 35a). In contrast, examination of the Plk4-depleted cells revealed progressive aberrations in spindle morphology and a stepwise reduction in centriole numbers (Figure 35a,b). At 24 hours after Plk4 depletion, most mitotic cells displayed bipolar spindles, but many had only single centrioles at each pole (Figure 35b, panel B). In addition, some monopolar spindles with 2 centrioles at the centre could be seen (Figure 35b, panel C).

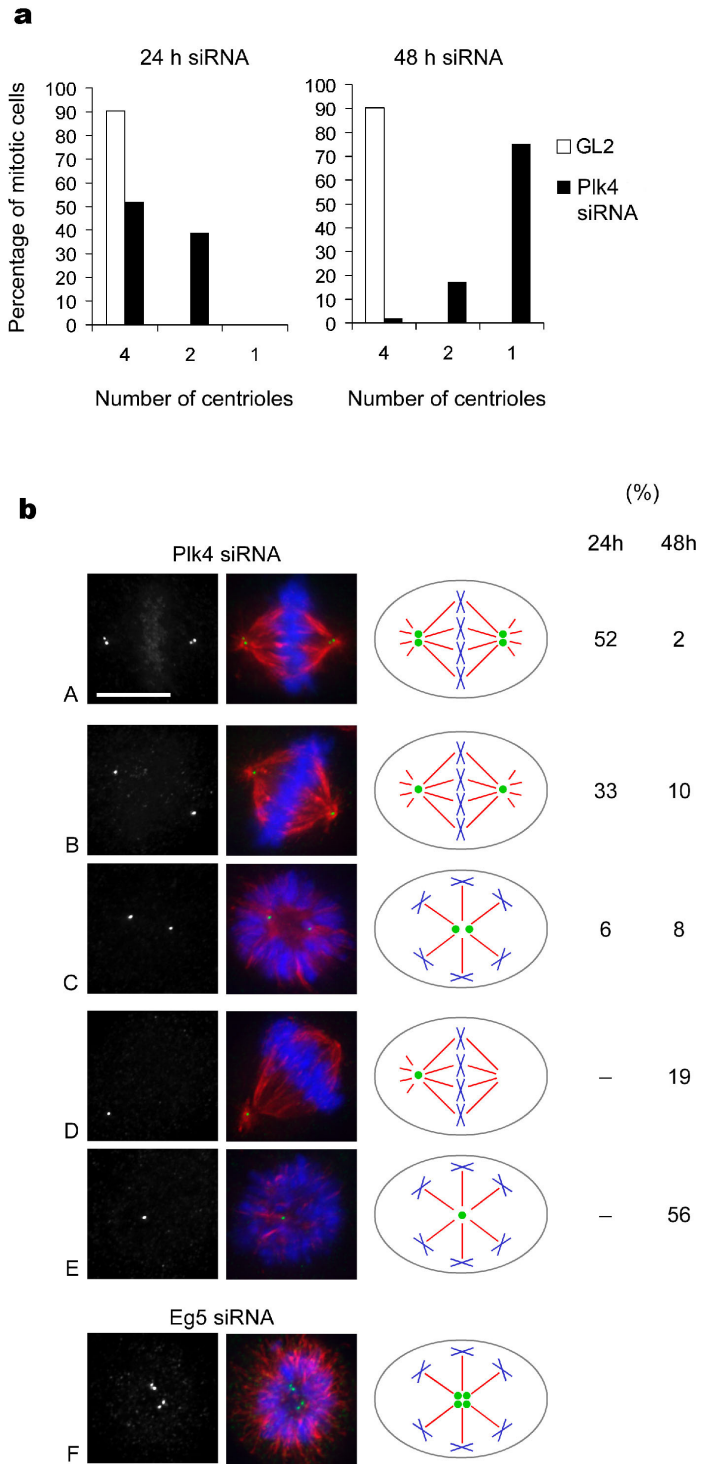

Figure 35. Depletion of Plk4 causes progressive reduction in centriole numbers. HeLa cells were transfected with control (GL2) or Plk4 siRNA duplexes for 24 or 48 h. After fixation, cells were stained with rabbit anti-centrin-2 (green), mouse anti-α-tubulin (red) antibodies and DAPI (blue). **(a)** Histogram shows the percentages of mitotic cells containing 1, 2 or 4 centrioles at 24 and 48 hours, counting 52 mitotic cells for each time point. **(b)** Left panels summarize the phenotypes of mitotic cells transfected with Plk4 siRNA duplexes, alongside with models depicting centriole numbers and spindle morphology for clarity. Left panels show centrioles (centrin) whereas colour panels show centrioles (centrin; green), α-tubulin (red) and DNA (DAPI, blue). Bar = 10μm. The numbers on the right indicate the frequency (in %; n=52) of each phenotype after 24 h and 48 h.

After 48 hours, bipolar spindles still formed occasionally, but most of these showed only a single centriole at one pole (Figure 35b, panel D). In these cells, the acentriolar pole was lacking astral microtubules (Figure 35, panel D), consistent with the notion that centrioles are required for astral microtubule formation (Khodjakov et al., 2000; Megraw et al., 2001). Most strikingly, the predominant 48 hour phenotype was constituted by monopolar spindles with a single centriole at the centre (Figure 35b, panel E). In control experiments, monopolar spindles produced by depletion of the class 5 kinesin motor Eg5 (Blangy et al., 1995; Harborth et al., 2001) were analyzed in parallel. Eg5 is required for centrosome separation at the onset of mitosis but its depletion would not be expected to affect centriole numbers. Indeed, four centrioles could readily be detected in the vast majority of monopolar spindles produced upon Eg5 depletion (Figure 35b, phenotype F). Taken together, the above results demonstrate that cells depleted of Plk4 progressively loose centrioles with continued passage through the cell cycle, indicating that Plk4 is indispensable for centriole duplication.

The above results suggested that the centrosome is not strictly required for the formation of a bipolar spindle, consistent with previously published data (Khodjakov et al., 2000). In support of this notion the proteins Aurora A and NuMa, two proteins required for the correct formation of bipolar spindles (reviewed in (Meraldi et al., 2004; Compton and Cleveland, 1994) were investigated in cells depleted of Plk4 (Figure 36). Cells were prepared for immunofluorescence microscopy by staining with anti-centrin antibody to visualise centrioles and antibodies directed against Aurora A or NuMa. As seen in Figure 36, both proteins still localised properly to both poles in cells exhibiting bipolar spindles with an acentriolar pole. This result suggested that the localisation of Aurora A and NuMa is not dependent on the presence of centrioles and therefore both proteins should be able to complete their role in spindle formation. In conclusion, the above data is consistent with reports showing that centrioles are not essential for spindle formation (Khodjakov et al., 2000), and suggest that these cells should be able to complete mitosis.

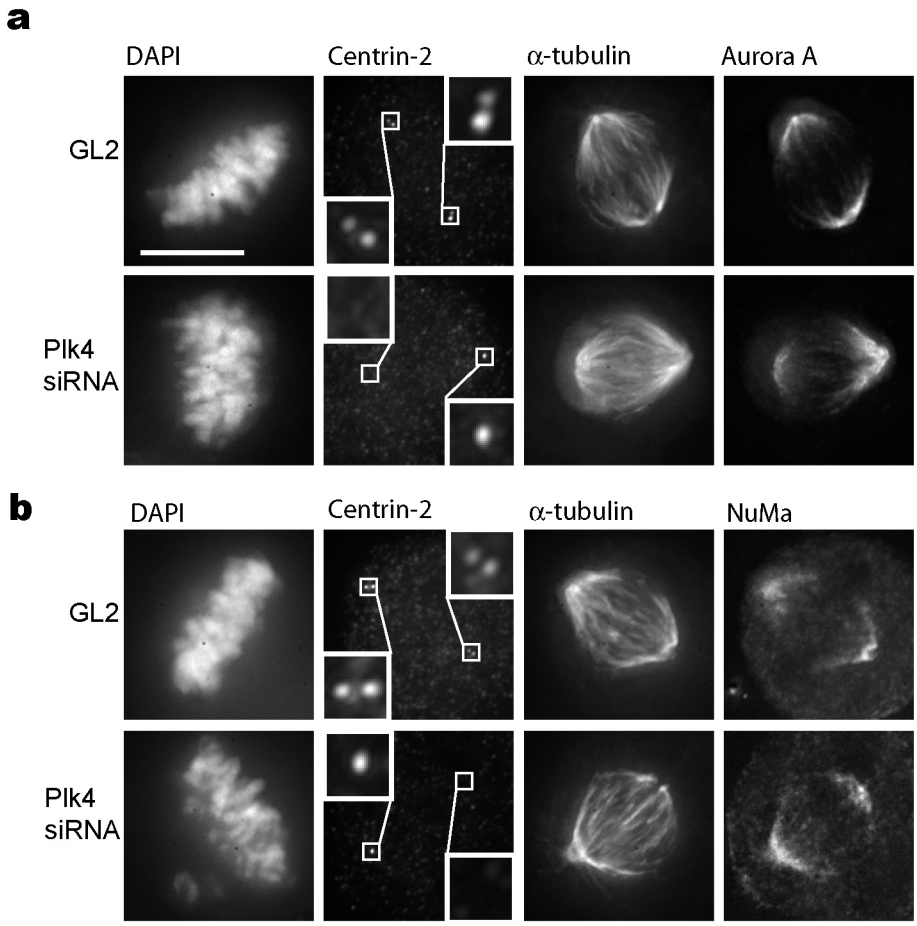

Figure 36. Normal localisation of NuMa and Aurora A to the acentriolar pole of spindles. HeLa cells transfected with control GL2 or Plk4 siRNA for 48 hours were prepared for immunofluorescence microscopy by labelling with antibodies against **(a)** Aurora A or **(b)** NuMa. Centrioles were visualised by staining with anti-centrin antibodies, spindles with anti-α tubulin. Bar = 10μm.

We wanted to confirm that cells depleted of Plk4 do not suffer a cell cycle arrest. HeLa cells transfected with Plk4 siRNA duplex for 48 hours were harvested and prepared for flow cytometric analysis, and depletion of kinase was confirmed by immunofluorescence microscopy (not shown). Cells transfected with GL2 duplex for the same length of time were used as control. As seen in Figure 37, the mitotic profile of Plk4 depleted cells appears normal, with G1-, S- and M-phase cells in similar proportions to the control transfected cells. A lack of this kinase therefore does not affect the normal mitotic progression of cells, at least not during the first 48 hours of depletion.

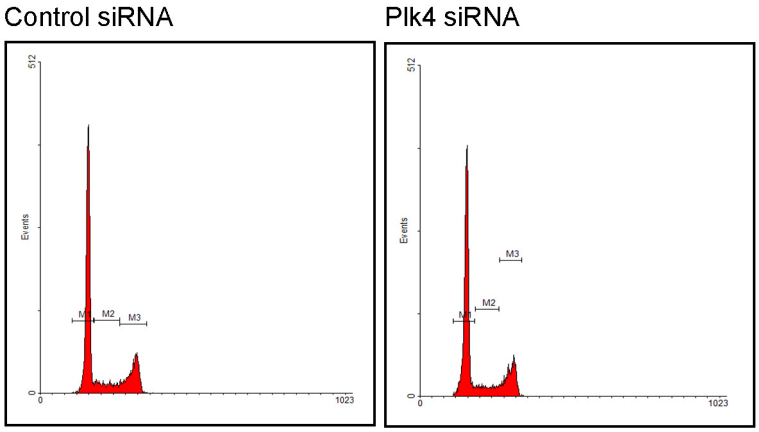

Figure 37. siRNA silencing of Plk4 does not arrest the cell cycle. HeLa cells were transfected with Plk4 or control GL2 siRNA duplex for 48 hours. After fixation, cells were stained with propidium idiodide and prepared for FACS analysis. Histograms show the mitotic profile of a representative 2000 cells from a total of 10000 cells transfected with control or Plk4 duplex.

The evidence so far suggested that cells depleted of Plk4 and exhibiting only one centriole but with bipolar spindles should be able to complete cytokinesis. We therefore searched for acentriolar progeny cells that should have inherited the acentriolar pole of the mitotic spindle. Since the centrosome is important for the organisation of the cytoskeleton, these cells were also predicted to have a disorganised microtubule network. HeLa cells were transfected with Plk4 siRNA duplex for 72 hours and stained with C-Nap1 antibodies to visualise the centrosome, and α-tubulin antibodies to stain the microtubule cytoskeleton. As shown in Figure 38, several cells depleted of Plk4 for 72 hours lacked centrioles indicating that acentriolar progeny cells were generated. The cytoskeleton also appeared diffuse and disorganised when compared to control siRNA duplex transfected cells but, surprisingly, this was irrespective of whether a cell still contained or centriole or not. Depletion of Plk4 therefore appears to affect the cytoskeletal organisation to some degree, and this may be unrelated to its centrosomal function.

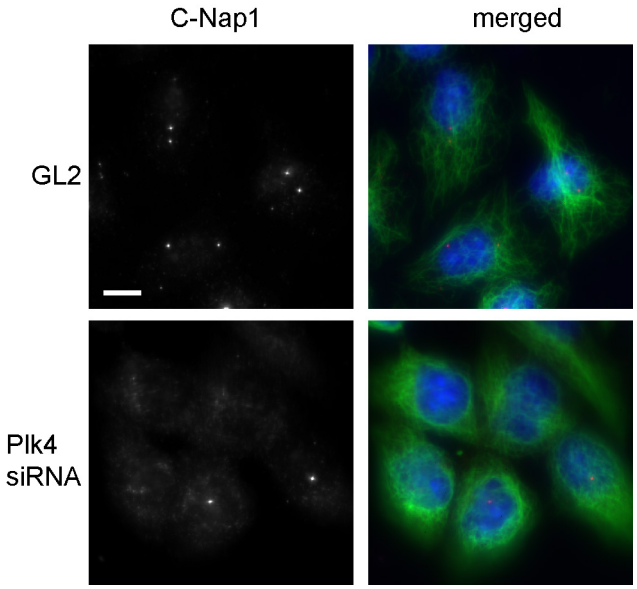

Figure 38. Depletion of Plk4 by siRNA leads to the formation of acentriolar cells. Centrosomes were monitored by labelling with anti-C-Nap1 antibodies (red), the cytoskeleton by staining with anti-α tubulin. Bar = 10μm.

In summary, depletion of Plk4 leads to the inhibition of centriole duplication. However, a lack of Plk4 was not consistent with a block in the cell cycle and therefore cells continue to divide until all centrioles are lost. Presently, it is unclear if acentriolar cells continue to cycle and enter the subsequent cell cycles.

Plk4 cooperates with Cdk2, CP110 and Hs-SAS6 to permit centriole duplication

As highlighted in the introduction, several proteins such as γ- and ε-tubulin, centrin, CP110 and Hs-Sas6 have previously been shown to be required for centrosome duplication (Ruiz et al., 1999; Shang et al., 2002; Chang et al., 2003; Salisbury et al., 2002; Chen et al., 2002b; Dammermann et al., 2004; Leidel et al., 2005; Sluder, 2004). Additionally, several protein kinases, including vertebrate Cyclin-dependent kinase 2 (Cdk2) (Meraldi et al., 1999b; Matsumoto et al., 1999a; Hinchcliffe et al., 1999a), Plk2 (Warnke et al., 2004), calcium-calmodulin kinase II (Matsumoto and Maller, 2002), *S. cerevisiae* Mps1 (Fisk and Winey, 2001) and the *C. elegans* kinase Zyg-1 (O'Connell et al., 2001), have been implicated in the regulation of this fundamental cellular process. However, our understanding of centrosome duplication and its integration with the chromosomal cell cycle remains scarce.

To elucidate a possible relationship between Plk4 and Cdk2 in controlling centrosome duplication, cells harbouring the inducible Plk4 kinase were transfected

with either the Cdk2 inhibitor p27 or a catalytically inactive Cdk2 that had previously been shown to act as a dominant negative mutant (Figure 39a, b) (Meraldi et al., 1999b). After induction of Plk4 expression, the transfected cells were fixed, stained with anti-C-Nap1 antibodies and examined for overduplicated centrosomes. Compared to cells transfected by empty vector, both dominant negative Cdk2 and p27 suppressed the Plk4 induced centrosome overduplication phenotype (Figure 39c). To confirm and extend these results, cells were also arrested at the G1/S boundary with aphidicolin for 16h before Plk4 expression was induced for 24 hours in the presence or absence of roscovitin, a small molecule inhibitor of Cdk2 (Meijer et al., 1997). In the presence of aphidicolin alone, about 38 % of the U2OS cells displayed centrosome overduplication (Figure 39d), consistent with previous results (Meraldi et al., 1999a). Additional induction of Plk4 increased the proportion of cells showing centrosome amplification to 62 %, but no increase over background was seen in the presence of roscovitin (Figure 39d). Together, these results strongly indicate that overexpression of Plk4 cannot cause centrosome amplification in the absence of Cdk2 activity. In a final series of experiments, we also explored the relationship between Plk4 and two structural centrosomal proteins, CP110 and Hs-SAS6. When either one of these two coiled-coil proteins was depleted by siRNA, the percentage of cells showing multiple centrosomes in response to Plk4 induction was strongly reduced (Figure 39e). This indicates that both CP110 and Hs-SAS6 are required for Plk4-induced centrosome amplification.

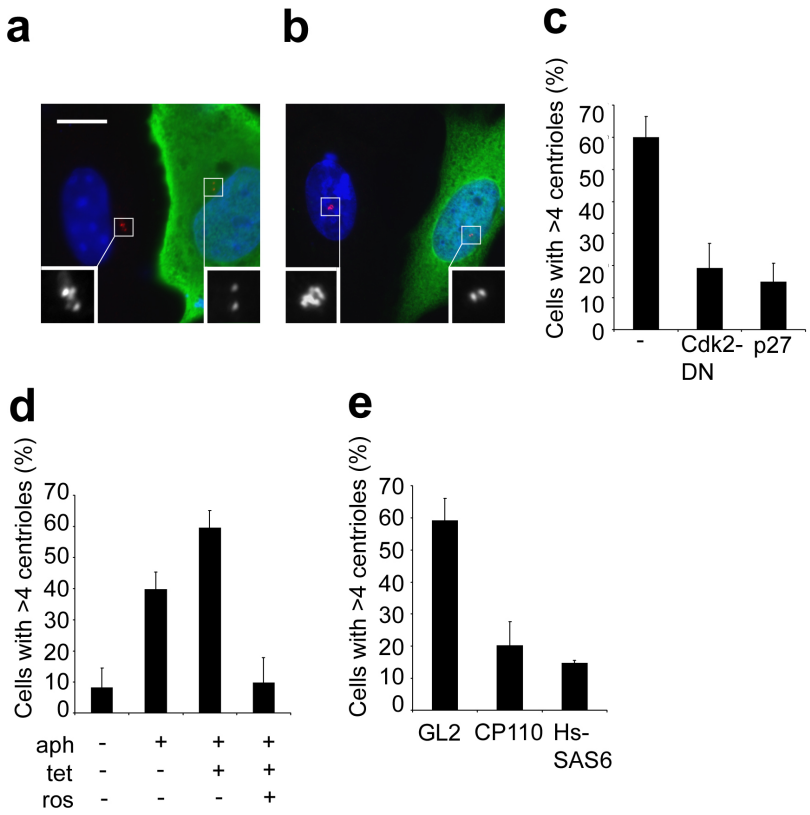

Figure 39. Cooperation of Plk4 with proteins implicated in centrosome duplication. A stable cell line carrying a tetracycline-inducible Plk4 cDNA was transiently transfected with epitope tagged dominant negative Cdk2 (Cdk2-DN) or p27 for 16h, after which Plk4 expression was induced for 24h. After fixation, centrioles were detected by staining with anti-C-Nap1 antibodies (red), whereas transfected cells expressing Cdk2-DN and p27 were identified using anti-HA and anti-myc antibodies, respectively (green). Cells were scored for overduplicated centrioles. Histograms summarize results from three independent experiments, counting 200 cells each, with error bars denoting standard deviations. (a,b) Immunofluorescence microscopy images showing cells transfected with Cdk2-DN (a) or p27 (b); bar denotes 10 μm and insets show enlargements of centrioles. Note the reduction in centriole numbers in cells expressing Cdk-DN or p27, compared to untransfected cells. (c) Histogram shows percentage of transiently transfected cells with overduplicated centrioles, using cells transfected with empty vector for control. (d) Cells were treated with aphidicolin (aph) for 16h before induction of Plk4 in the presence or absence of Roscovitin (ros) for 24h. Control cells were treated for 40h with aphidicolin only. All cells were scored for overduplicated centrioles. (e) Cells were transfected for 24h with siRNA duplexes specific for CP110 or Hs-SAS6, or GL2 for control, before Plk4 expression was induced. Histogram shows percentage of cells with overduplicated centrioles.

DISCUSSION

Plk4 is the unusual member of the polo-like kinase family due to its non-canonical structure by having a long linker region and by its conspicuous lack of a second polo-box motif (Swallow et al., 2005). Reports have shown that Plk4 localises to the centrosome and other intracellular structures (Leung et al., 2002) and have provided evidence for a role in cell proliferation for this kinase (Fode et al., 1994; Fode et al., 1996; Leung et al., 2002). Most importantly, mouse knock-out studies have shown that Plk4 is required for development and for proper cell progression. Specifically, mice deficient for Plk4 are embryonic lethal and Plk+/- heterozygotes show increased susceptibility to tumour formation in organs, showing that correct levels of Plk4 are essential for cell division (Ko et al., 2005). Here, we have identified the Plk family member Plk4 as a critical regulator of centriole duplication in human cells. Our conclusions are supported by both overexpression experiments and antibody-induced inactivation or siRNA-mediated depletion of Plk4 resulting in the formation of aberrant spindle morphologies. We are therefore able to provide an explanation based on the function of Plk4 for the tumorigenic properties of the deregulated enzyme.

Putative novel domains in Plk4

The available literature on Plk4 does not contain much data on the undefined linker region. A report has identified putative PEST domains which were revealed by a prediction program, and a further report suggested that deletion of these domains increases the half-life of the overexpressed kinase in mammalian cells (Fode et al., 1994, Yamashita et al., 2001). Furthermore, domain analysis has ascribed a dimerisation function to a region of this linker termed the cryptic polo box. This C-terminal region was also required for the centrosomal localisation of overexpressed Plk4 in mammalian cells (Leung et al., 2002). Here, secondary structure prediction programs suggest that the extensive linker region can be tentatively divided into two parts. Firstly, a genuine linker spans approximately half of the residues between the kinase and polo-box domains. Secondly, a region corresponding to residues 600-800 precedes the polo-box domain and consists of unidentified domains. Clearly, data generated by secondary structure prediction programs are only of limited value and may not reflect the actual structure of a protein in real life. However, the domain

analysis presented here shows that this C-terminal region is necessary for dimerisation and for the recruitment of Plk4 to the centrosome. This suggests that the C-terminus may be the domain interacting with other centrosomal proteins, and the evidence overall supports an *in vivo* function for the *in silico* predicted domains. Since these structures are not identified by domain identification programs, they represent novel and previously uncharacterised motifs. Indeed, since Plk4 lacks a second polo-box domain, these domains could function as a replacement, by acting as a targeting and interaction domain in conjunction with the single polo-box motif. Further structural analyses of the C-terminal domain of Plk4 by NMR or crystallography would be an attractive path for the further characterisation of this kinase.

Plk4 is required for centriole duplication

In order to investigate the role of Plk4 and its requirement for centriole reproduction, Plk4 microinjection and siRNA mediated depletion experiments were carried out. Both approaches demonstrated that Plk4 is essential for centrosome over-duplication in the particular assay used. In a further experiment, asynchronous cells were investigated. Depletion of Plk4 resulted in the sequential loss of centrioles in successive cell divisions, resulting in fewer and fewer centrioles at the spindle poles and a progressive increase in abnormal spindle formation. Occasional interphasic cells lacking centrioles were observed suggesting that cells with mitotic acentriolar bipolar spindles are able to complete cytokinesis. This is consistent with the notion that centrioles are not necessary for the completion of cytokinesis or for spindle formation, but for the correct orientation of the bipolar spindle (Khodjakov et al., 2000; Khodjakov and Rieder, 2004). Moreover, a report has shown a requirement of the centrosome for proper cytokinesis and cell abscission (Piel et al., 2001). In this report, mother centrioles were seen to travel to the cleavage furrow thus allowing putative signalling events to occur. Cells depleted of Plk4 and lacking centrioles demonstrate that this function of the centriole is not strictly necessary for cytokinesis. Reports indicate, however, that acentriolar progeny cells generated by laser surgery or microdissection arrest in the following G_1 phase (Maniotis and Schliwa, 1991; Hinchcliffe et al., 2001; Khodjakov and Rieder, 2001). Whether these acentriolar cells produced by Plk4 depletion continue to cycle and form offspring remains to be determined.

De novo centriole formation in certain differentiated tissues is necessary for the formation of multiple basal bodies during ciliogenesis (Beisson and Wright, 2003). In cell culture, *de novo* centriole formation can be observed after laser ablation of the centrosome (La Terra et al., 2005). In this particular experiment, new centrioles are formed that cluster to create a microtubule organising centre, albeit disorganised and lacking the orthogonal relationship between two centrioles. Since acentriolar cells were observed in the studies presented here, it is attractive to speculate that these cells are no longer able to reform a microtubule organising centre in the absence of Plk4. However, a direct role of Plk4 in *de novo* centriole formation is at present unknown.

It is interesting to compare Plk4 with Zyg-1, a kinase clearly shown to play an essential role in centriole duplication in *C. elegans* (O'Connell et al., 2001). Database searches do not reveal obvious homologues of Zyg-1 in vertebrate genomes and a more specific search for distant homologues in protein databases identified a putative chicken Plk4, amongst other kinases. However, any significant similarity in peptide sequences was restricted to the catalytic domain since no other domains were found in Zyg-1. Alignments of kinase domains used to assemble a phylogeny tree identify Polo-likes kinase and NimA related kinases as the closest homologues of Zyg-1. However, both kinase families were equally closely related to Zyg-1 such that no particular family member was classified as the closest human homologue of Zyg-1. Thus, although Plk4 and Zyg-1 do not show extensive sequence similarity, it remains possible that mammalian Plk4 may constitute the functional orthologue of the nematode kinase Zyg-1. The search for interactors and possible mechanisms should therefore focus on human homologues of *C. elegans* proteins that are involved in centriole duplication and that show a dependency on Zyg-1.

Overexpression of Plk4 induces the formation of 'centriole precursors'

Several proteins putatively involved in centriole duplication have been shown to cause centrosome amplification when overexpressed in cells (Mussman et al., 2000; Xu et al., 1999; Leidel et al., 2005). However, some proteins cause this phenotype upon overexpression by inducing a failure in cell division (Meraldi et al., 2002b; Sluder, 2004). In the work presented here, overexpression of active Plk4 in S-phase arrested cells resulted in genuine centriole overduplication in two different human cell lines,

suggesting that excess kinase activity is the cause for increased centriole over-production.

A seminal report has revealed the existence of a block to centriole reduplication that is intrinsic to the centriole itself (Wong and Stearns, 2003). Excess amounts of Plk4 are sufficient to overcome this intrinsic block to centriole reduplication in cultured cells, a feature shared with only one other protein, human Sas-6 (Leidel et al., 2004). It would be attractive to speculate that Plk4 and possibly Sas-6 are both involved in the mechanism that is responsible for preventing reduplication of centrioles.

Most interestingly, overexpression of Plk4 lead to the formation of early structures that could be interpreted as centriole precursors. These were seen as dark ring like structures by electron microscopy or as rosette-like staining patterns around the parental centriole in immunofluorescence studies. As yet, the exact nature of these structures is undetermined. However, two proteins that exhibited this particular staining pattern and localised to these structures were centrin and CPAP, whereas other centrosomal proteins did not. Since both proteins or their homologues have been implicated in centriole duplication (Leidel and Gonczy, 2003; Salisbury et al., 2002), a plausible involvement of both in the earliest stages of centriole assembly is an appealing interpretation. Further screening of all known centrosomal proteins in terms of localisation to these putative precursor structures in conjunction with a careful synchronisation of the centrosome duplication cycle would be required for an analysis of these structures.

Centrosomes are thought to duplicate according to the rule ‘once per cell cycle’ (Sluder, 2004; Delattre and Gonczy, 2004). The surprising conclusion derived from these early rosette-like structures is the concept that, in the presence of excess Plk4, parental centrioles are able to nucleate and assemble multiple centrioles at the same time. Given these results, an additional rule should therefore be added: ‘one new centriole per parent centriole’ (Figure 40) . Centriole overduplication is therefore a consequence of either a prolonged S-phase and thus multiple rounds of sequential duplication events (Figure 40a), or of a single round of multiple centriole reproduction (Figure 40b). It would be interesting to examine whether other proteins shown to induce centriole overduplication on overexpression proceed by this mechanism.

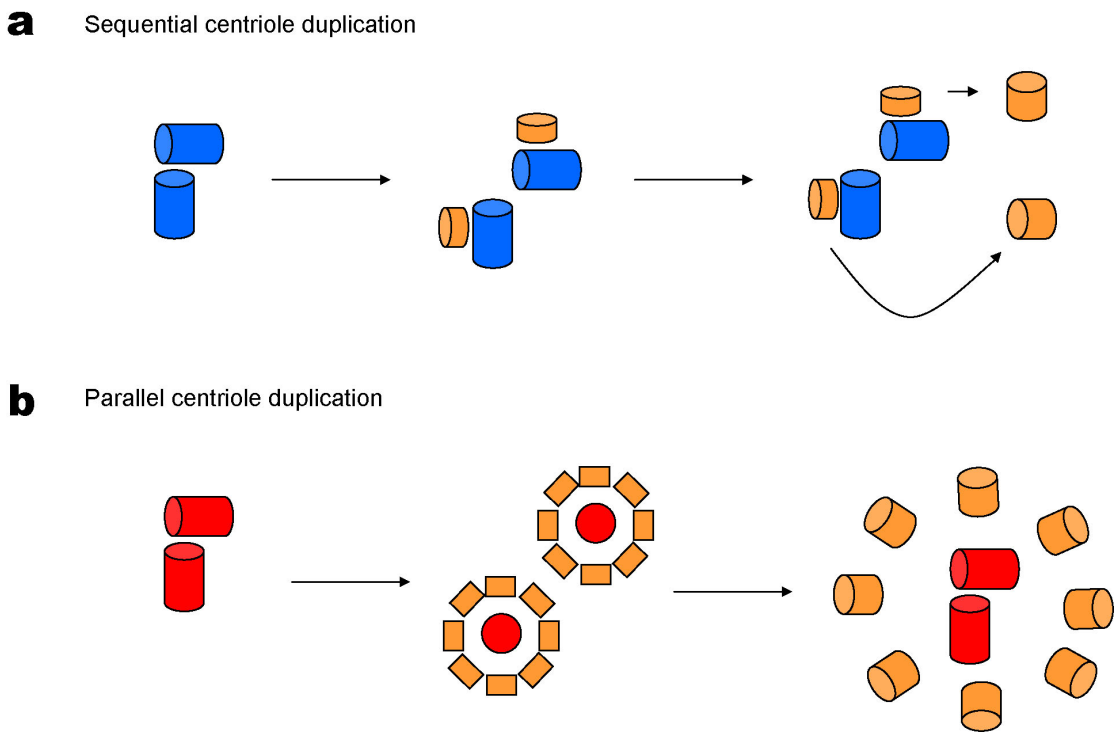

Figure 40. Two alternative modes of centriole overduplication in mammalian cells. (a) Model showing a sequential mode of centriole duplication in S-phase arrested U2OS or CHO cells, thus violating the 'only once per cell cycle' rule of centrosome duplication. **(b)** Alternatively, Plk4 overexpressing cells produce multiple centrioles at once, contrary to the rule 'only one daughter centriole per parent centriole'. In normal cells, both rules must apply to maintain correct centriole numbers.

Taking the above into consideration, the question arises as to the precise mechanistic function of Plk4. Since overexpression of this kinase leads to the formation of multiple centriole assembly points on the parental centriole surface, a simple explanation would involve Plk4 marking additional assembly sites already present, a so-called 'start gun' for pro-centriole formation. This would predict that the parental centriole has a limited and defined number of assembly sites located on the surface of its tubulin cylinder, and immediately poses the difficult question of how these sites are silenced during duplication in a normal wild type cell.

An alternative function for Plk4 would be the activation and subsequent recruitment of proteins that themselves form centriole assembly sites, thus defining how many are produced. If Plk4 is the functional homologue of Zyg-1, then the recruitment of proteins such as the human homologues of Sas-4 or Sas-6 to the centriole could be dependent on the activity of this kinase.

This latter model would predict that the level of endogenous Plk4 in normal cells would be strictly limited, such that sufficient kinase activity could permit the

formation of only one centriole assembly site. The limited Plk4 activity could initially be spread over the surface of the parent centriole, until a particular random location is more phosphorylated by chance than others. This hyperphosphorylated site would then recruit proteins involved in the earliest stages of centriole formation and thus become the kinetically favoured spot where the procentriole is formed. Interestingly, the phosphatase PP2C-β was identified in a mass-spectrometric analysis of proteins co-immunoprecipitated with epitope tagged and overexpressed Plk4. In a similar manner to the regulation of centrosome cohesion by Nek2 and the protein phosphatase 1 (Meraldi & Nigg, 2002), PP2C-β could act as an antagonistic phosphatase of Plk4. Like centrosome cohesion, the regulation of centriole duplication would be dependent on the balance of phosphorylation and dephosphorylation activity of these proteins. This would suggest that positive Plk4 phosphorylation activity would outstrip the negative phosphatase activity during the initial step in centriole duplication, resulting in the hyperphosphorylation of a centriole assembly site. Conversely, the effects of limited Plk4 activity would be dampened by the phosphatase during other stages of the cell cycle, thus preventing precocious centriole duplication.

Alternatively, Plk4 could be asymmetrically located to one side of the paternal centriole, therefore fixing the assembly site purely in a spatial manner. However, this would then beg the question of how such a polarised localisation would be achieved. In either case, a model requiring limited amounts of Plk4 would be consistent with the low level levels of this kinase seen by Western blot analysis of total cell extracts (Figure 17).

Secondly, this model would also provide explanations for the self-interaction of Plk4, not reported for other Plk family members. Similar to transcription factors or tubulin, dimerisation or oligomerisation would allow non-linear dose-dependent control over the formation of a complex involving Plk4. However, the immunofluorescence data presented in Figure 18 point to a constant protein level throughout the cell cycle for this kinase. Dimerisation may therefore be promoted by other mechanisms, such as phosphorylation by upstream kinases. Alternatively, dimerisation of Plk4 could mirror the mechanisms governing transmembrane receptor tyrosine kinase transactivation leading to the formation of signalling complexes localised to membranes. Dimerisation of this kinase could therefore collect sufficient

kinase activity at one point on the centriole surface to allow recruitment and activation of additional protein complexes and thus promote centriole formation. At present, all these points are speculative and await further analysis of this fascinating kinase.

Putative substrates of Plk4

The cellular substrates of Plk4 remain to be identified, but the present data demonstrate that overduplication of centrosomes requires the presence of active Plk4 at the centrosome. This suggests that the physiological substrates of this kinase will include centriolar and/or centrosomal proteins, and obvious candidates include the human homologues of Sas-4, -5 and -6. Although the *C. elegans* Zyg-1 kinase is required for the recruitment of Sas-4 and Sas-6 to the centriole (Leidel et al., 2005), these proteins were not shown to be substrates of Zyg-1.

Here, two *in vitro* kinase substrates were found, namely CPAP, the human homologue of Sas-4, and the C-terminus of Plk4 itself. The role of autophosphorylation of this kinase on the C-terminus is unclear. However, it is notable that CPAP localises in close proximity to Plk4 on newly forming centriole precursor 'buds' seen by electron microscopy, and is therefore at the right place for a phosphorylation event. CPAP could thus be an *in vivo* substrate of Plk4. The results presented here are only a starting point and whether the phoshorylation of these substrates is actually of any physiological relevance requires further analysis.

Identification of substrates would also allow the characterisation of a target consensus motif for this Polo-like kinase. Interestingly, the activities of Plk1 and 4 on the exogenous substrate casein are different. Plk1 and Plk4 were found to preferentially phosphorylate different subunits of casein, suggesting that these kinases act on different phosphorylation motifs. Although Plk1 colocalises with Plk4 during late S-phase and G2 at the centrosome, it is possible that these kinases phosphorylate entirely different targets.

In summary, it would be illuminating to reveal a complex regulating centriole duplication that is conserved from worms to mammals, containing evolutionarily related protein subunits. Such a discovery would also explain the absence of Plk4 homologues in higher plants which are deficient in centrioles. Indeed, no obvious plant Plk4 homologues were encountered in sequence homology searches presented here. Conversely, it would be interesting to speculate whether lower plants that are

able to form centriole-like structures (Gall, 2004) still have a functional homologue of Plk4. However, databases contain very few sequences for *Ginkgo* or Bryophyte proteins, and the only available sequenced genomes are from higher plants such as *Arabidopsis thaliana* and rice. Therefore, searches for plant Plk4 homologues will be difficult at present.

Plk4 and Cdk2 co-operate to regulate centriole duplication

Although information about the regulation of centriole duplication remains scarce, several studies point to an important role of Cdk2, acting in association with either cyclin E and/or A (Matsumoto et al., 1999b; Meraldi et al., 1999b; Hinchcliffe et al., 1999b). Using an established centrosome overduplication assay (Balczon et al., 1995; Meraldi et al., 1999b; Matsumoto et al., 1999b), we found that Cdk2 was not able to cause centrosome overduplication in the absence of Plk4. Conversely, overexpression of Plk4 did not result in centrosome amplification in the absence of Cdk2. With regard to structural proteins implicated in centrosome duplication, the data presented here show that both CP110 (Chen et al., 2002a) and human Sas-6 (Leidel et al., 2005) are required for Plk4-induced centrosome amplification. It is plausible that these coiled-coil proteins constitute important structural components required for centriole assembly. Taken together, these data indicate that Cdk2 and Plk4 cooperate in the regulation of centrosome duplication. However, whether Cdk2 directly phosphorylates Plk4 remains unknown and further analysis will be necessary to reveal the regulatory kinases directly upstream of Plk4.

Plk2 cannot substitute for Plk4 activity at the centrosome

Recently, Plk2 has also been localized to centrosomes and implicated in centriole duplication (Warnke et al., 2004). At present, it is not known whether Plk2 and Plk4 cooperate. However, it is to be emphasized that $Plk2^{(-/-)}$ mice are viable (Ma et al., 2003), indicating that Plk2 function at the centrosome is not essential or that redundant mechanisms can substitute for Plk2. Conversely, Plk4 is clearly required for murine embryonic development (Hudson et al., 2001) and, although $Plk4^{(-/-)}$ embryos develop to stage E7.5, this is likely to reflect maternal contribution. The embryonic lethality of the $Plk4^{(-/-)}$ animals thus points to an indispensable function of

Plk4 in a key cellular process. On the basis of the present data, we propose this key function to be the control of centrosome numbers.

***Plk4 is required for centriole duplication and flagella development in* Drosophila**

During the course of the work discussed here, Bettencourt-Dias, Glover and co-workers reported on the requirement of Plk4 for centriole duplication in flies (Bettencourt-Dias et al., 2005). *Drosophila* only has the two Plk family members Plk1 and Plk4, and lacks homologues of Plk2 or 3 (Glover et al., 1998). An siRNA screen of all *Drosophila* kinases by the same group had previously revealed centrosome aberrations in S2 cells depleted of Plk4 (Bettencourt-Dias et al., 2004) but not in cells lacking Plk1. Further analysis of Plk4 depleted S2 cells demonstrated a loss of centrioles and the formation of broad, disorganised spindle poles in dividing cells, lending support to the notion that centrioles may be required for mitotic fidelity but not for proper spindle formation (Khodjakov et al., 2000; Khodjakov and Rieder, 2001).

In a parallel approach, *Drosophila* mutant strains containing a hypomorphic allele consisting of a C-terminal deletion mutant of Plk4 were generated. Larval neurones examined for centrosomal markers exhibited reduced numbers of centrosomes, but otherwise appeared normal. Surviving adult mutant flies suffered from sterility and uncoordinated behaviour, and spermatocytes were found to lack flagella and basal bodies. Mathematical modelling of the centrosome cycles in the cell divisions leading to the formation of mature spermatocyte in the spermatocyte cyst elegantly demonstrated a defect in centriole duplication. In a final set of experiments, Glover and co-workers analysed the function of human Plk4 by siRNA depletion and showed its requirement for centriole duplication, thus providing an independent verification of the results presented here. Taken together, Plk4 has a conserved function in controlling centriole duplication in two evolutionarily distant organisms. It is therefore likely that the mechanisms involving Plk4 and its substrates in this fundamental process will be similar in both flies and humans.

Increased tumour formation in Plk4 +/- heterozygous mice may be caused by partially defective centrosome duplication.

At first glance, it may seem surprising that overexpression of both active and catalytically inactive Plk4 caused centrosome amplification, albeit to a different extent. However, this observation can readily be explained by considering that active Plk4 brings about genuine centrosome overduplication, whereas the catalytically inactive mutant causes an increase in centrosome numbers by the likely induction of division failure, through a probable dominant-negative mechanism. In line with earlier suggestions (Fode et al., 1996; Hudson et al., 2001; Swallow et al., 2005), this observation may hint at a second function of Plk4. Although endogenous Plk4 was not detected at the cleavage furrow, it is possible that Plk4 directly regulates cell division (Leung et al., 2002; Hudson et al., 2001). On the other hand, occasional division failure may also be a direct consequence of reduced centriole numbers caused by impaired centrosome duplication (Piel et al., 2001; Khodjakov and Rieder, 2001; Hinchcliffe et al., 2001; Gromley et al., 2003; Zou et al., 2005). In these cases, the centrosome is thought to act as a signalling platform for proteins required for the final stages of cytokinesis and abscission. Thus, the absence of the centrosome would be manifested in impaired signalling to the midbody and incomplete cytokinesis.

Mice heterozygous for Plk4 were recently shown to display an increased incidence in tumour formation, due to Plk4 haploinsufficiency (Ko et al., 2005; Swallow et al., 2005). Most interestingly, cells isolated from $Plk4^{+/-}$ animals showed multiple centrosomes, multipolar spindles and mitotic failures. This phenotype is remarkably similar to the one observed here upon overexpression of a dominant-negative Plk4 mutant in human cells. Conversely, reduced levels of Plk4 in mouse cells could also result in an aberrant spindle phenotype caused by impaired centriole duplication seen upon siRNA mediated Plk4 depletion. Therefore, the formation of aberrant spindles in $Plk4^{+/-}$ mouse cells upon mitogenic stimulation could be direct consequence of reduced numbers of centrosomes in dividing cells. Although most of these cells are expected to enter apoptosis, some may survive and generate progeny cells with aberrant ploidy. Since polyploid cells have been shown to promote tumorigenesis in mice (Fujiwara et al., 2005; Margolis, 2005), some Plk4 deficient cells are expected to become cancerous. It will be important to analyse how Plk4 haploinsufficiency causes the observed cellular defects and how these relate to the

increased incidence of tumours in animals. In particular, it will be interesting to test the hypothesis that a mechanism, centred on centrosome-related division failure and the generation of polyploid and aneuploid cells, may contribute to tumorigenesis in $Plk4^{+/-}$ animals.

The present data have implications for the development of anti-cancer therapeutics based on the inhibition of Polo kinases. On the one hand, the increased tumorigenesis observed in $Plk4^{+/-}$ animals (Ko et al., 2005; Swallow et al., 2005) may raise concerns as to the safety of such drugs. In particular, one could argue that partial inhibition of Plk4 by anti-Plk drugs may be oncogenic and should be avoided. On the other hand, we have shown that Plk4 is indispensable for centrosome duplication in human cells and, therefore, cell proliferation is expected to halt in the face of strong inhibition of this kinase. From this latter perspective, Plk4 might actually constitute an attractive target for anti-proliferative therapeutic approaches.

METHODS

Sequence analysis

Plk4 peptide sequences were submitted to the PsiPred server for secondary structure predictions (McGuffin et al., 2001). For identifying motifs and domains, sequences were analysed using ScanProsite (Gattiker et al., 2002) whereas coiled-coil domains were scored using the COILS program (Lupas et al., 1991). All programs were accessed from by web interface on www.expasy.ch. Protein-protein BLAST and pair-wise BLAST ((Altschul et al., 1997; Altschul et al., 1990; Tatusova and Madden, 1999) were performed using programs available on the National Centre for Biotechnology Information (NCBI) at www.ncbi.nlm.nih.gov. All other sequence analysis presented here was performed by H. Lutz and M. Vingron (Max-Planck Institute for Molecular Genetics, Berlin).

Plasmid constructions

All cloning procedures were performed according to standard techniques as described in Molecular Cloning, A Laboratory Manual, 2nd editition, Sambrook, J.,Fritsch, E.F., Maniatis, T., Cold Spring Harbor Laboratory Press 1989 and Current Protocols in Molecular Biology, Wiley, 1999. Restriction enzyme reactions were carried out as specified by the suppliers (NEB) and ligation reactions were done using T4 DNA Ligase (NEB). Extraction of DNA from agarose gels and preparation of plasmid DNA was performed using kits from QIAGEN according to the manufacturer's instructions. For PCR reactions, the Pfu DNA polymerase PCR System was used as recommended by the manufacturer (Promega) and reactions were carried out in a RoboCycler Gradient 96 (Stratagene). All PCR products were checked by sequencing at Medigenomix (Martinsried, Germany).

The EST clone AI561146 IMAGE:2210962 encoding human Plk4 was purchased from RZPD, Berlin. This clone was completely sequenced and found to contain the whole Plk4 coding sequence as well as portions of the 3′- and 5′-UTRs. PCR was used to amplify full-length Plk4 and to introduce BamHI and XhoI sites either side of the coding sequence, respectively (for primers and cloning strategy, see Table 5). The PCR product was phosphorylated with polynucleotide kinase (NEB, Frankfurt am Main, Germany) and blunt ligated into the SmaI site of the pBluescript II SK (-)

vector. This construct (HR4) was sequenced and found to match the Plk4 reference sequence NM_014264 on Medline/NCBI, with the exception of a silent T→C point mutation at position 210 relative to the start codon. To generate catalytically inactive Plk4, PCR used to introduce a point mutation in the conserved DFG motif of the kinase domain (Taylor et al., 1993b). Mutation of the aspartate of this motif to alanine has been shown to completely abolish catalytic activity (Taylor et al., 1993a). This was achieved by altering codon 154 GAT (aspartate) to GCT (alanine) creating the HR22 plasmid (see Table 6 for a list of plasmids used in the work presented here). Similarly, a T-loop mutant was generated by altering codon 170 (ACA to GAT) thereby replacing the threonine with an aspartate residue. All constructs were verified by sequencing.

Deletion mutants (see Figure 23) of Plk4 were produced by PCR using internal primers and blunt-end ligating PCR products into pBluescript II SK(-). Two deletion mutants (HR53 and HR54) were generated by virtue of a single EcoRV site present at the internal truncation point. The EcoRV-XhoI fragment was excised from HR9 and the remaining DNA fragment containing the vector backbone and the truncated Plk4 insert (residues 1-638) was blunt-ended and religated, thus generating the HR53 plasmid. Conversely, to generate HR54 containing the Plk4 C-terminus (residues 638-970), the BamHI-EcoRV fragment was removed from HR4 and the vector blunt-ended and religated. cDNAs were then subcloned into the BamHI-XhoI sites of mammalian expression vectors generating wt and mutant Plk4 constructs with N-terminal EGFP-, myc- or FLAG- tags.

Yeast two-hybrid analysis

The coding sequence of human K41R Plk4 was inserted into the two-hybrid prey vector pACT2 (Clontech Laboratories, Inc.) and the bait vector pFBT9 (a version of pGBT9 modified to encode kanamycin resistance; Clontech Laboratories, Inc.; a kind gift from F. Barr, Max-Planck-Institute for Biochemistry, Martinsried, Germany). These plasmids were cotransformed with into the yeast strain PJ69-4A to test for direct interactions. Alternatively, a lymphocyte cDNA library (Clontech) was screened with bait constructs consisting of full length K41R Plk4 or a N-terminal mutant K41R Plk4 (residues 1-380). Approximately 800,000 transformants were plated onto non-selective medium lacking either leucine and tryptophane (-WL) or on

selective medium lacking leucine, tryptophan and adenine with 2% (wt/vol) glucose as the carbon source (-WAL). All results were confirmed by streaking colonies onto both selective and nonselective plates and scoring growth after 3-5 days at 30°C.

Antibody Production

A Plk4 fragment spanning residues 314-817 was expressed as a poly-histidine-tagged fusion protein in *E. coli* and purified over a Ni^{2+}-column under denaturing conditions. 350-500 μg of purified antigen was injected subcutaneously together with Freund's adjuvant into New Zealand white rabbits every four weeks, to a total of six injections (animal house, Max-Planck Institute for Biochemistry, Martinsried, Germany). Antibodies were affinity purified by elution from immobilised antigen on nitrocellulose.

Cell culture, transfections and microinjections.

HeLa, U2OS, HEK293T or Cos-7 cells were cultured at 37°C in a 5% CO2 atmosphere in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% heat-inactivated fetal calf serum and penicillin-streptomycin (100 i.u./ml and 100μg/ml, respectively, Gibco-BRL, Karlsruhe, Germany). Cells adherent on acid treated glass coverslips were transiently transfected using FuGene (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol.

Tetracycline-inducible cell lines expressing FLAG-tagged wild type and D154A mutant Plk4 were generated by transfection of U2OS-TRex cells (Invitrogen) for 24 h prior to addition of selective reagent. Stable transformants were established by selection for two weeks with 1mg/ml G418 (Invitrogen, Carlsbad, CA) and 50μg/ml hygromycin (Merck, Darmstadt, Germany) after which colonies were picked and tested for Plk4 expression by immunofluorescence and by Western blot analysis. Cell lines were induced to express Plk4 by adding 1μg/ml tetracycline to growth medium.

Centrosome duplication assays

U2OS cells were seeded on acid treated, sterilised glass coverslips and transfected with either siRNA duplex oligonucleotides targeting Plk4 for 24 h or microinjected with anti-Plk4 antibodies. Cells were then incubated for 64h in the presence of 2 mM

hydroxyurea or 1,6 μg/ml aphidicolin before fixation. Centrosomes were stained using anti-γ-tubulin mouse monoclonal antibody (Sigma, Steinheim, Germany), and injected antibody was visualised by staining with Alexa green conjugated goat anti-rabbit IgG secondary antibodies (Jackson Immunoresearch, Soham, UK). Levels of Plk4 in cells transfected with siRNA duplex were monitored by staining with anti-Plk4 antibodies.

Antibody microinjections

Antibody injections into U2OS cells were performed as previously described. (Blangy et al., 1995). Briefly, U2OS cells were seeded onto acid treated coverslips and left to grow until 50-60% confluency for microinjection. Purified anti-Plk4 antibody or control rabbit IgG was concentrated to 2 mg/ml using Ultrafree-0.5 centrifugal filters (Amicon Bioseparation, Millipore, Schwalbach, Germany), followed by centrifugation at 55,000 rpm for 30 minutes at 4°C in a TLA-55 rotor and an Optima ultracentrifuge (Beckman, Krefeld, Germany) prior to microinjection. Antibodies were injected into the cell cytoplasm using 0.5μm Femtotips glass needles with a micromanipulator 5171 coupled to a FemtoJet/InjectMan microinjection apparatus (Eppendorf, Hamburg, Germany). After microinjection, cells were used for centrosome duplication assays (see above).

siRNA-mediated protein depletion.

Plk4 protein was depleted using three different siRNA duplex oligonucleotides (Dharmacon Research Inc, Lafayette, CO and Qiagen, Hilden, Germany) targeting the sequences 5'-ACTCCTTTCAGACATATAAG-3' (oligo 1; 2913-2938, relative to start codon, in the 3'UTR), 5'-AACTATCTTGGAGCTTTATAA-3' (oligo 2; 212-232, relative to start codon) and 5'-CTGGTAGTACTAGTTCACCTA-3' (oligo 3; 2420-2440, relative to start codon). Duplexes were used singly or in combination (oligo 1 plus 2), with similar results. For control, the luciferase duplex GL2 was used (Elbashir et al., 2001). Transfections were performed using Oligofectamine (Life Technologies, Karlsruhe, Germany) according to standard protocols.

Flow cytometric analysis

After transfection with siRNA duplexes, HeLa cells were detached by trypsinization and washed with ice-cold PBS. Cells were fixed in 70% ethanol, followed by an incubation for 30 minutes in PBS, 10µg/ml RNase A (Sigma-Aldrich) and 5µg/ml propidium iodide (Sigma-Aldrich). Analysis of the fixed cells was performed with a FACScan (Becton Dickinson) and the WinMDI software, according to the manufacturer's instructions.

Cell extracts, immunoprecipitations and immunoblotting

For immunoprecipitations of epitope-tagged overexpressed proteins, HEK293T or Cos-7 cells were plated to approx. 50% confluency in 10cm cell culture dishes. Cells were transfected as described above with 5µg of plasmid DNA using either FuGene (Roche Diagnostics, Mannheim, Germany) or by calcium phosphate precipitates as previously described elsewhere (Seelos, 1997). For co-immunoprecipitations, 1.5µg of each plasmid encoding either bait and interactor were co-transfected. 24-48 h after transfection, cells were washed three times with ice cold PBS and lysed on ice for 30 min in extraction buffer for centrosomal proteins (0.1% IGPAL, 100mM KCl, 20mM Tris-HCl pH 7.4, 1mM DTT, 1mM EDTA, 10% glycerol, 50mM NaF, 5mM PMSF, Complete Mini protease inhibitor cocktail, Roche Diagnostics, Mannheim, Germany). Lysates were cleared by centrifuging for 8 min at 16,000xg, 4°C and incubated with prot-G beads bearing epitope-tag targeted antibodies (mouse monoclonal 9E10 anti-myc; mouse M2 anti-FLAG affigel, Sigma, Steinheim, Germany; goat anti-GFP, a kind gift from F. A. Barr, Max-Planck Institute for Biochemistry, Martinsried, Germany) for 2h at 4°C. Immunocomplexes bound to beads were then washed thrice with co-IP wash buffer (0.1% IGPAL, 250mM KCl, 20 mM Tris-HCl pH7.4, 1mM DTT, 1mM EDTA, 10% glycerol, 50mM NaF, 5mM PMSF). Beads carrying bound proteins for use in kinase assays were treated as described below. Otherwise, immunoprecipitated proteins were eluted into Laemmli buffer or, in the case of Flag-tagged proteins, eluted with 0.5mg/ml of Flag peptide (Sigma, Steinheim, Germany) for 30 min at room temperature. Eluates were separated by SDS-PAGE and transferred to nitrocellulose membranes using a Hoefer semi-dry blotting apparatus (Amersham Biosciences, Little Chalfont, UK) and completed transfer was assayed by staining membrane bound proteins with Ponceau-red. For Western blot analysis,

membranes were incubated for one hour in blocking buffer (5% low-fat dry milk in PBS, 0.1% Tween-20). All antibody incubations were carried out in blocking buffer for 1 hour at room temperatur. Membranes were probed with purified anti-Plk4, anti-myc or anti-Flag antibodies in blocking buffer, followed by incubation with HRP conjugated goat anti-mouse or anti-rabbit antibodies (Jackson Immunoresearch). Signals were detected by enhanced chemiluminescence using ECL Supersignal reagents (Pierce Chemical Co.).

Purification of the recombinant Plk4 (1-265) kinase domain

The GST-tagged wt and catalytically inactive mutant of Plk4 (1-265) comprising the N-terminal catalytic domain was expressed in *E. coli* BRL RIL codon + (invitrogen). To express the protein, a starter culture of transformed bacteria was grown overnight under ampicillin selection and diluted to 1:5 with fresh medium in the morning. The culture was grown until and OD_{600} of 1.0 when the expression of the recombinant protein was induced with 1mM IPTG at 30°C for 4h. Cells were then pelleted by centrifugation, lysed in buffer (0.5% IGPAL, 100mM KCl, 20mM Tris-HCl pH7.4, 1mM DTT, 1mM EDTA, 10% glycerol, 5mM PMSF) and centrifuged to remove debris. Lysate was then incubated with glutathione coated beads (Amersham) for 1 hour to allow binding of expressed protein. After several washing steps with lysis buffer, GST-tagged Plk4(1-265) was then eluted by incubating beads in elution buffer (1mM glutathione, 20mM Tris-HCl pH7.4, 10 % glycerol, 1mM DTT). Purified Plk4(1-265) was then frozen as aliquots at -80°C.

In vitro *kinase assays*

Following immunoprecipitation, tagged Plk4 immune complexes were washed three times with lysis buffer (0.1% IGPAL, 100mM KCl, 20mM Tris-HCl pH 7.4, 1mM DTT, 1mM EDTA, 10% glycerol, 50mM NaF, 5mM PMSF, Complete Mini protease inhibitor cocktail, Roche Diagnostics, Mannheim, Germany) and once with Plk4 kinase buffer (50mM Hepes pH7.5, 10mM $MgCl_2$, 1mM DTT). Alternatively, 1 μg of recombinant GST-tagged Plk4(1.265) kinase domain was used as enzyme. Kinase reactions were carried out for 40min at 30°C in Plk4 kinase buffer supplemented with 10μM ATP and 2μCi of [γ32-P]-ATP (Amersham Corp.). Kinase reactions were

stopped by addition of sample buffer and heating at 95°C for 5 minutes. Proteins were then separated by SDS-PAGE and transferred to nitrocellulose in the case of immunoprecipitated kinase or substrate. Alternatively, when recombinant proteins were used proteins were stained by Coomassie Blue followed by drying on filter paper. ^{32}P incorporation was visualized by autoradiography.

Microscopic techniques.

Immunofluorescence microscopy was carried out as described (Meraldi et al., 1999a). Briefly, cells were fixed on coverslips in -20°C methanol for at least 5 minutes before incubation in blocking buffer (1% BSA in PBS). After rehydration in PBS, cells were incubated with primary antibodies in blocking buffer for 1 hour at room temperature, followed by staining with Alexa-Fluor conjugated goat secondary antibodies (Molecular probes). DNA was visualised by staining with DAPI (200 ng/ml). Coverslips were mounted onto glass slides using mounting medium (phenylenediamine in 90% glycerol). Slides were analyzed using a Zeiss Axioplan II microscope with Apochromat 63x and 100x/1.4 n.a. oil immersion objectives and images were taken using a CoolSNAP HQ CCD camera (Photometrics, Tuczon, AZ) and Metaview (Visitron Systems GmbH, Puchheim, Germany) software followed by processing with Adobe Photoshop (Adobe Systems, Mountain View, CA). Alternatively, slides were analyzed using a Deltavision microscope on a Nikon TE200 base (Applied Precision, Issaquah, WA) equipped with an APOPLAN 60x/1.4 n.a. oil immersion objective. Serial optical sections obtained 0.2 μm apart along the Z-axis were processed with a deconvolution algorithm and projected into one picture using Softworx (Applied Precision).

Electron microscopy and immunogold labelling

Electron microscopy was kindly performed by Y-D Stierhof (ZMBP, University of Tübingen, Germany). For electron microscopy, cells were induced for 48 h to express wt or D154A Plk4, fixed with 2.5% glutaraldehyde (Sigma-Aldrich, Steinheim, Germany) in PBS for 60 min, embedded in 2% low melting agarose (SeaPlaque Agarose; Marine Colloids, Rockland, USA) and post-fixed with 1% osmium tetroxide (Plano, Wetzlar, Germany) in PBS for another 60 min followed by staining with 1 % uranyl acetate (Science Services, München, Germany) in distilled water for 45 min.

After dehydration with ethanol, cells were embedded for ultrathin sectioning in epoxy resin (Epon; Roth, Karlsruhe, Germany). Sections were stained with 1% uranyl acetate and lead citrate (Science Services, München, Germany) and viewed in a LEO 906 transmission electron microscope.

Immunogold labelling

Mass-spectrometry

Proteins isolated by co-immunoprecipitation with FLAG-tagged wt Plk4 were kindly analysed by X. Li (Max-Planck Institute of Biochemistry, Martinsried, Germany) as previously described (Sauer et al., 2005). Briefly, Coomassie Blue stained protein bands were in-gel digested (Shevchenko et al., 1996) by trypsin (Promega, sequencing grade). Peptides were desalted and concentrated using C18 extraction tips, and analysed with a CAPLC nano HPLC system (Waters, Milford) coupled to a Q-TOF mass spectrometer (Q-ToF, Ultima, Micromass, London, UK). Data were searched against the Mass Spectrometry Protein Sequence Database (MSDB, csc-fserve.hh.med.ic.ac.uk/msdb.html) or the human International Protein Index database (www.ebi.ac.uk/IPI/IPIhelp.html) using in-house Mascot version 1.7 (www.matrixscience.com). Proteins identified by two or more peptides with a combined peptide score higher than 50 or by one single peptide with a score higher than 60 were considered significant, whereas all lower-scoring proteins were either included or discarded after inspection of individual spectra.

Miscellaneous techniques

Human centrosomes were purified from the T-lymphocyte KE-37 cell line according to the protocol reported in (Moudjou and Bornens, 1994). MT regrowth assays in asynchronously growing U2OS cells were performed by placing tissue culture plates on ice for 30 mins, before MT regrowth was induced by the addition of prewarmed growth medium for 30s. Cells were then immediately fixed in -20° methanol and prepared for immunofluorescence. Cdk2 was inhibited in aphidicolin treated cells by adding Roscovitin to growth medium at a final concentration of 25μM.

ABBREVIATIONS

All units are abbreviated according to the International Unit System.

AA: amino acid(s)

ATP: adenosine 5´-triphosphate

BSA: bovine serum albumin

DAPI: 4´,6-diamidino-2-phenylindole

DTT: dithiothreitol

ECL: enhanced chemiluminescence

EDTA: ethylenedinitrilotetraacetic acid

EGFP: enhanced green fluorescent protein

EM: electron microscopy

FCS: Fetal calf serum

GFP: green fluorescent protein

HCl: hydrochloric acid

HEPES: N-2-Hydroxyethylpiperazine-N`-2-ethane sulfonic acid

IgG: Immunoglobulin G

IP: Immunoprecipitation

IPTG: isopropyl-beta-D-thiogalactopyranoside

mAb: monoclonal antibody

MTOC: microtubule organising centre

PBS: Phosphate-buffered saline

PCR: Polymerase chain reaction

Plk4: Polo-like kinase 4

PMSF: phenylmethylsulfonyl fluoride

RNA: Ribonucleic Acid

RT: room temperature

SAK: Snk/Fnk akin kinase

SDS-PAGE: Sodium dodecylsulfate polyacrylamid gelelectrophoresis

siRNA: small interference Ribonucleic Acid

SPB: Spindle Pole Body

WT: wild-type

Table 5 List of primers used to introduce point mutations in Plk4

Primer name	Sequence (5´-3´)	mutation introduced
M1251 oHR033	GGAAGTTGCAATCCGGATGATAGATAA	K41R
M1252 oHR034	CTTATCTATCATCCGGATTGCAACTTCC	K41R
M1575 oHR039	CATCAAGATTGCTGCTTTTGGGCTGGCAAC	D154A
M1581 oHR042	GTTGCCAGCCCAAAAGCAGCAATCTTGATG	D154A
M1575 oHR041	CATGAAAAGCACTATGACTTATGTGGAACTCC	T170D
M1583 oHR044	GGAGTTCCACATAAGTCATAGTGCTTTTCATG	T170D

Table 6 List of plasmids. Epitope tags are N-terminal unless otherwise stated**.** Several plasmids used were generated by other people, in particular P. Meraldi (PM), L. Arnaud (LA) and J. Kleylein-Sohn (JK).

Name	Gene	Species	Insert	Vector	tag
HR9	Plk4	Human	full length; wt	pcDNA3.1-3xmycA	myc
HR57	Plk4	Human	full length; wt	pcDNA3.1-NFLAG	flag
HR38	Plk4	Human	full length; wt	pEGFP-C2	N-terminal EGFP
HR44	Plk4	Human	full length; wt	pEGFP-N3	C-terminal EGFP
HR26	Plk4	Human	full length; D154A mutation	pcDNA3.1-3xmycA	myc
HR63	Plk4	Human	full length; T170D mutation	pcDNA3.1-3xmycA	myc
HR46	Plk4	Human	full length; K41R	pFBT9	DNA binding domain
HR17	Plk4	Human	Full length; K41R	pACT2	activation domain
HR105	Plk4	Human	aa 1-380; K41R	pACT2	activation domain
HR57	Plk4	Human	aa 1-888; wt	pcDNA3.1-3xmycA	myc
HR53	Plk4	Human	aa 1-638; wt	pcDNA3.1-3xmycA	myc
HR55	Plk4	Human	aa 888-970	pcDNA3.1-3xmycA	myc
HR54	Plk4	Human	aa 638-970	pcDNA3.1-3xmycA	myc
HR56	Plk4	Human	aa 265-970	pcDNA3.1-3xmycA	myc
HR87	Plk4	Human	aa 1-265; wt	pGEX-5X-2	GST
HR88	Plk4	Human	aa 1-265; D154A	pGEX-5X-2	GST
JK	CPAP	Human	full length	pEGFP-C2	EGFP
JK	CPAP	Human	aa 1-132	pEGFP-C2	EGFP
JK	CPAP	Human	aa 1-422	pEGFP-C2	EGFP
P. Gönczy	CPAP	Human	aa 429-890	pEGFP-C1	EGFP
PM22	p27	Human	full length	pRcCMV	myc
LA178	Cdk2	Human	dominant negative	pRSC-EGFP	EGFP

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APPENDIX

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