The role of the centriolar satellite protein PCM-1 in centrosome function

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Wellcome Trust Centre for Cell Biology
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Declaration

I hereby certify that I am the author of this work and the research presented is my own except where otherwise stated.
Acknowledgements

The work presented in this thesis was undertaken in the laboratory of Andreas Merdes. As his first Ph.D. student, it is to his credit that my time there went so smoothly. I would also like to thank him for giving me the opportunity to present my work at scientific conferences, an invaluable source of information and inspiration. I would also like to thank the members of the Merdes, Earnshaw, Heck, Rabouille and Sawin labs for their continued support throughout this project, both in kind and in advice, scientific or otherwise.

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This work was funded by the Wellcome Trust.
Dedication

I would like to dedicate this work to my family, in particular to my father, Dr. Rainer Dammermann, who encouraged me to pursue a career in Biology and who has been a role model for me in how to lead my life, and my sister Stephanie, who showed me it is possible to find happiness.

Abstract

In most animal cells centrosomes are responsible for the nucleation and organisation of the mitotic spindle microtubules and the interphase microtubule network. My work has concentrated on the role of one protein, PCM-1, that localises to small cytoplasmic granules known as ‘centriolar satellites’. These satellites have been observed moving between the centrosome and the surrounding cytoplasm. Although their presence had been correlated with the processes of centrosome duplication and ciliogenesis, the significance of this observation was unknown. The aim of my thesis was to determine the role of PCM-1 and centriolar satellites in centrosome function.

Several approaches were taken to inhibit PCM-1 activity: microinjection of antibodies into cultured cells, overexpression of a PCM-1 deletion mutant, and specific depletion of PCM-1 using siRNA. I found that inhibition or depletion of PCM-1 consistently led to mislocalisation of the centrosomal proteins pericentrin, centrin and ninein. Similar effects were observed upon disruption of the dynein/dynactin microtubule motor complex and prolonged exposure of cells to the microtubule-destabilising drug nocodazole. Furthermore, PCM-1 inhibition resulted in a loss of radial microtubule organisation. However, γ-tubulin localisation and microtubule nucleation were unaffected. These data are consistent with a model of PCM-1 as a shuttle protein required for the recruitment of centrosomal proteins involved in microtubule anchoring. Centriolar satellites may thus represent protein complexes in transit to and from the centrosome.

In addition, cells with impaired PCM-1 function frequently showed defects in cytokinesis and a failure to enter S phase. How PCM-1 is involved in these processes is unclear. I have laid the groundwork for studies to address these and other outstanding questions by creating a construct to express full-length PCM-1 tagged with green fluorescent protein allowing PCM-1 dynamics to be followed in vivo and by setting up a yeast two-hybrid screen to isolate interactors of PCM-1.
### Abbreviations used in this thesis

<table>
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<tr>
<td>BrdU</td>
<td>Bromo Deoxyuridine</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>DAPI</td>
<td>4′6-Diamidino-2-phenylindole</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetra acetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene Glycol-bis(beta-aminoethyl-ether)-N,N,N′,N′-TetraAcetate</td>
</tr>
<tr>
<td>gg</td>
<td>Chicken (Gallus gallus)</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione-s-transferase</td>
</tr>
<tr>
<td>hs</td>
<td>Human (Homo sapiens)</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Bertani medium</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-Buffered Saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenyl- methylsulfonyl fluoride</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>rpm</td>
<td>Rotations per minute</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse Transcriptase-PCR</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
</tr>
<tr>
<td>siRNA</td>
<td>Short interfering RNA</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-Buffered Saline</td>
</tr>
<tr>
<td>TE</td>
<td>Tris-EDTA</td>
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PCM-1 was originally used as an abbreviation for protein of the pericentriolar material-1. Since this description of PCM-1 as a pericentriolar material protein is incorrect and misleading, this protein will be henceforth referred to only by its abbreviation.
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Chapter 1. Introduction

1.1. Centrosomes, an overview

Centrosomes are full of contradictions. They were first described more than a century ago (Wilson, 1895), yet we still know very little about their molecular composition or function. For many years it was thought that their sole function was the nucleation and organisation of the microtubule network. Recent results however suggest that the centrosome as a whole may not be required for these processes to occur. At the same time a previously unsuspected role has been uncovered for the centrosome in cell cycle progression. The centrosome is a tiny organelle occupying a volume of only ~1-2μm³, yet it lacks a defined boundary and many centrosomal proteins are predominantly found elsewhere in the cytoplasm. Like DNA, the centrosome replicates once, and only once, per cell cycle, but apparently without the benefit of a nucleic acid template. As will be clear from this introduction there are many gaps in our knowledge about centrosomes, and what we do know is not always consistent. This may be expected in a field that is rapidly developing. In this section I will seek to summarise the available information about centrosome structure and function, in order to be better positioned to address the role of one particularly enigmatic structure, the centriolar satellite, and one of its constituent proteins, PCM-1.

1.1.1. Centrosomal ultrastructure

The centrosome in most animal cells consists of a pair of centrioles surrounded by an amorphous electron-dense mass called the pericentriolar material, or PCM, and is usually located in close proximity to the nucleus, to which it appears to be firmly anchored (Figure 1A).

The centrioles are cylindrical structures composed of nine triplet microtubules ~400 nm in length, and the two centrioles of a centriole pair generally
Figure 1. Centrosome structure.
A. Location of centrosome at the centre of the cell, adjacent to the nucleus. B. The centrosome consists of a pair of centrioles, each composed of 9 triplet microtubules, arranged at right angles to each other. Microtubules are nucleated in the surrounding pericentriolar material, their dynamic (plus) ends extending to the periphery of the cell. The older ('mother') centriole possesses two sets of appendages, distal (red) and subdistal (yellow); the latter may anchor microtubules. Centrioles appear linked at their proximal ends by interconnecting fibres (green). C. Immunoelectron micrograph showing centriolar satellites (labelled with PCM-1 antibody, arrows) and other granules (arrowheads) in the vicinity of the centrioles (asterisk). Bar 200nm. Reproduced with permission from Kubo et al., 1999 © The Rockefeller University Press.
are arranged at right angles to one another and in close proximity at one end (the proximal end, Figure 1B). The older ('mother') centriole in each centriole pair has additional appendages at the end farthest from the other centriole (the distal end). Centrioles show considerable structural similarity to basal bodies, which are located at the base of eukaryotic cilia and flagella, and the two structures may be considered interchangeable. In some organisms (Chlamydomonas, for example), the flagellar basal bodies become part of the mitotic spindle poles during each mitosis before returning to the base of the flagellum during the next interphase (Coss, 1974). And following fertilisation in most animals the basal body associated with the sperm flagellum is incorporated into the zygotic centrosome (Schatten, 1994). Conversely, in some cell types such as retinal photoreceptor cells the basal bodies are the only structures with microtubule organising centre activity and are also responsible for the nucleation of the cytoplasmic microtubule network (Troutt et al., 1990). In addition, many cells in culture and in vivo possess a single non-motile primary cilium, which is nucleated from the distal end of the oldest 'mother' centriole.

The centrioles are embedded in a cloud of pericentriolar material that is organised by an insoluble filamentous 'centromatrix' (Schnackenberg and Palazzo, 1999). While the pericentriolar material appears relatively unstructured, higher magnification electron microscopy revealed ring-like structures within the pericentriolar material that contained $\gamma$-tubulin and had diameters roughly similar to those of microtubules. These appear to be the site of microtubule nucleation (see below) (Moritz et al., 1995).

In addition to centrioles and pericentriolar material, electron microscopic observations identified electron-dense spherical granules ~70-100nm in diameter, localised around centrosomes in many vertebrate cell types (Figure 1C). These granules have been given a number of different names but will be referred to hereafter as 'centriolar satellites'. They were occasionally seen associated with microtubules radiating from the centrosome (de-Thé, 1964), and their number varies during the cell cycle (Rattner, 1992).

1.1.2. The relative contributions of the centrioles and the pericentriolar material to centrosome function
Although centrioles are the most distinctive feature of the centrosome complex, it is the pericentriolar material that is responsible for nucleating and anchoring microtubules. With the notable exception of axonemal microtubules, all microtubules including those that form the interphase microtubule array and the mitotic spindle terminate in the pericentriolar material, not at the centrioles themselves. Direct experimental proof that the pericentriolar material is the site of microtubule nucleation came from a number of experiments, including those of Gould and Borisy (Gould and Borisy, 1977). These experimenters separated an enriched centrosomal preparation into a pericentriolar material fraction and centrioles. Tubulin was added to each fraction, and was found to assemble into asters in the pericentriolar material preparation, but not on isolated centrioles. The functional components responsible for microtubule nucleation and anchoring are only loosely bound to the pericentriolar material, and can be removed by extraction with high salt concentrations leaving behind the aforementioned filamentous ‘centromatrix’ (Schnackenberg et al., 1998).

Although they do not nucleate cytoplasmic microtubules, the centrioles serve to localise the pericentriolar material into a focal body and thus determine the number of microtubule organising centres present in the cell. Thus, when centrioles are induced to disassemble by injecting cells with antibodies to polyglutamylated tubulin, the surrounding cloud of pericentriolar material is dispersed. When the antibodies are degraded, the pericentriolar material once again concentrates around the centrioles as they reform (Bobinnec et al., 1998). Centrosome duplication may therefore be considered to occur at the level of centriole replication, which occurs in higher animals only in association with a pre-existing centriole. If sea urchin cells are manipulated to contain a single centriole, centrosome replication is not compromised (Sluder and Rieder, 1985). However, if both centrioles are removed from sea urchin (Sluder et al., 1989) or vertebrate cells (Hinchcliffe et al., 2001; Khodjakov and Rieder, 2001; Maniotis and Schliwa, 1991), a new centriole and thus centrosome does not regenerate, even though a loosely focussed microtubule array eventually reforms.

The functional significance of the centriolar satellites has remained unknown until the present study. What information there is in the literature has been purely correlative. Thus, centriolar satellites have been observed in greater numbers in the vicinity of centrioles undergoing replication, both during centrosome duplication
(Rieder and Borisy, 1982; Robbins et al., 1968) and in the repeated rounds of replication prior to ciliogenesis (Anderson and Brenner, 1971; Sorokin, 1968), suggesting an involvement in these processes. Centriolar satellites continue to accumulate at the centrosome as cells approach prophase, but are then virtually absent during mitosis and only reappear in late telophase (Rattner, 1992; Rieder and Borisy, 1982).

1.2. Centrosome function in nucleation and organisation of microtubules

1.2.1. Microtubule nucleation, the role of $\gamma$-tubulin

Microtubules are hollow tubes 25 nm in diameter, consisting of several protofilaments arranged in a cylinder. Each protofilament is a head-to-tail polymer of $\alpha\beta$ tubulin heterodimers. The wall of the microtubule that assembles from a centrosome is composed of 13 protofilaments, while microtubules that assemble spontaneously \textit{in vitro} have a range of protofilament numbers (Evans et al., 1985). Due to its polar nature, the two ends of the microtubule polymer have different polymerisation rates, with a faster growing 'plus' end and a slower growing 'minus' end. A key feature of microtubule behaviour is dynamic instability, with stochastic and abrupt switches between growth and shortening phases. \textit{In vitro}, such behaviour is observed at both plus and minus ends. \textit{In vivo}, microtubule assembly is dominated by plus-end dynamic instability, while minus ends are often stabilized at centrosomes and spindle poles (Kirschner and Mitchison, 1986). Microtubule nucleation appears to occur primarily from centrosomes as cellular tubulin concentrations are below the concentration required for free assembly (Mitchison and Kirschner, 1984a; Mitchison and Kirschner, 1984b), although cases of non-centrosomal microtubule nucleation have also been reported (Bre et al., 1990). Centrosomes appear to contain only a finite number of microtubule nucleation sites. This has been demonstrated using both lysed cells and isolated centrosomes (Brinkley et al., 1981; Kuriyama, 1984). In each case, the number of microtubules
nucleated by centrosomes increased with increasing concentration of tubulin until reaching a plateau where further increases in tubulin levels had no effect.

What is the centrosomal template for microtubule nucleation? The key to answering this question came from the identification of γ-tubulin in a genetic screen for revertants of a conditionally lethal β-tubulin mutation in Aspergillus nidulans (Oakley and Oakley, 1989). This protein was subsequently found to be conserved from yeast to man. Though closely related to the α- and β-tubulins that make up the microtubule polymer, γ-tubulin localises specifically to the pericentriolar material of the centrosome (Horio et al., 1991; Stearns et al., 1991; Zheng et al., 1991), where it is required for microtubule nucleation (Joshi et al., 1992). Given its structural similarity to α- and β-tubulin, γ-tubulin is thought to act as a template for the addition of α- and β-tubulin subunits. The γ-tubulin nucleator model has been strongly supported by the discovery that γ-tubulin exists in the cytoplasm as part of a large complex (Stearns and Kirschner, 1994). This complex has the shape of an open ring of about the same diameter as a microtubule (Zheng et al., 1995) and is found at the minus ends of microtubules at the centrosome (Moritz et al., 1995). Originally purified from Xenopus egg cytoplasm, it is now clear that the γ-tubulin ring complex (γ-TuRC) is conserved in animals. Budding yeast have a much smaller, simpler γ-tubulin complex (Geissler et al., 1996; Knop et al., 1997), whereas other organisms have both large and small γ-tubulin complexes (Moritz et al., 1998; Oegema et al., 1999). The small γ-tubulin complex, and even γ-tubulin alone can nucleate microtubules, albeit with lower efficiencies (Leguy et al., 2000; Oegema et al., 1999). The three proteins that make up the small yeast complex – Spc97, Spc98 and Tub4 (γ-tubulin) – are conserved among other organisms (Murphy et al., 1998), suggesting that they form the core of γ-TuRCs. On the basis of these results and new biochemical and structural data (Keating and Borisy, 2000; Moritz et al., 2000; Wiese and Zheng, 2000), a model has been proposed for the structure of the γ-TuRC (Moritz et al., 2000), whereby six small γ-tubulin sub-complexes (each containing two molecules of γ-tubulin as well as one molecule each of the Spc97 and Spc98 homologues) are organised into a ring by other members of the γ-TuRC. The γ-tubulin molecules within the small complexes would act as templates for the nucleation of microtubule filaments perpendicular to the ring, while lateral
interactions between protofilaments would result in the formation of the tubular structure. The cytoplasmic γ-TuRCs (Moritz et al., 1995; Zheng et al., 1995) may be involved in the nucleation or capping of cytoplasmic microtubules. Alternatively, they may simply act as a source of nucleating complexes that are recruited to centrosomes when increased microtubule nucleation is required.

Little is known about how γ-TuRCs are recruited to and anchored at the centrosome. In budding yeast spindle pole bodies, Spc110 in the inner (nuclear) plaque and Spc72 in the outer (cytoplasmic) plaque anchor γ-tubulin complexes (Knop and Schiebel, 1997; Knop and Schiebel, 1998). In Drosophila, abnormal spindle protein (Asp), in conjunction with γ-TuRCs, can restore microtubule nucleation to salt-stripped centrosomes (do Carmo Avides and Glover, 1999), indicating that this protein may provide the centrosome anchor for γ-tubulin in mitosis. This anchorage is dependent on the phosphorylation of Asp by Polo kinase (do Carmo Avides et al., 2001). In C. elegans, another kinase, aurora A, is required for the recruitment of γ-tubulin prior to the onset of mitosis (Hannak et al., 2001). In vertebrates, pericentrin and CG-NAP/AKAP450, proteins which share a limited homology in their ‘PACT’ domain with the calmodulin binding region of Spc110 (Gillingham and Munro, 2000), have been implicated in the recruitment of γ-tubulin to and anchoring in the pericentriolar material (Dictenberg et al., 1998; Takahashi et al., 2002). The γ-TuRC cap structure appears to play a particular role in this process (Zhang et al., 2000). Whether microtubule-dependent transport by dynein or other motors is important in γ-tubulin recruitment is unclear. Whilst many labs have found that centrosomal targeting of γ-tubulin and other potential microtubule nucleation factors is independent of microtubules (Felix et al., 1994; Hannak et al., 2001; Khodjakov and Rieder, 1999; Li et al., 2001; Schnackenberg et al., 1998), others have reported a requirement for dynactin in γ-tubulin assembly (Quintyne et al., 1999; Young et al., 2000).

1.2.2. Microtubule release and anchoring

Once nucleated, microtubules need not remain anchored at the centrosome. For instance, in epithelial cells microtubules have been found to be released from
centrosomes and translocated to anchoring sites at the apex of the cell. These sites were found to lack the proteins γ-tubulin and pericentrin associated with microtubule nucleation, suggesting a distinction between microtubule nucleating and anchoring activities (Mogensen et al., 1997). Only one protein has so far been localised to these apical microtubule anchoring sites, ninein (Mogensen et al., 2000). This protein normally associates with the subdistal appendages of the maternal centriole, which act as microtubule anchoring sites in cell types such as fibroblasts that contain a microtubule network focused on the centrosome. Direct proof of its microtubule anchoring (or even binding) activity, however, has so far been missing. Other proteins which may play a similar role in anchoring microtubules have recently been identified (Ohta et al., 2002; Stein et al., 2002). Dynactin, a dynein-interacting complex, has also been implicated in centrosomal microtubule anchoring (Quintyne et al., 1999). This it may do by transporting microtubule anchoring proteins or by directly contributing to microtubule focusing in a similar manner to its action at mitotic spindle poles (see below).

In order for microtubules to be free to move to anchoring sites elsewhere in the cell, microtubules first have to be released from their centrosomal nucleation sites. One protein that has been suggested to perform this function is katanin, which has been shown to sever microtubules in vitro (Hartman et al., 1998) and in vivo (Ahmad et al., 1999; Buster et al., 2002). That microtubule release occurs in vivo has furthermore been demonstrated by video microscopy on living cells (Keating et al., 1997). The microtubule release and capture model, first proposed by De Brabander (De Brabander, 1982) has recently been revised by Bornens and colleagues in the light of their findings on the behaviour of mother and daughter centrioles of the centrosomal centriole pair (Piel et al., 2000). While both mother and daughter centrioles nucleate microtubules, only the mother centriole appears to be able to anchor them. Microtubules produced by the daughter centriole are free to be released into the cytoplasm once they have been severed from their nucleation sites – unless they too are anchored in the pericentriolar material surrounding the mother centriole. Microtubule release by the centrosome may therefore depend on the degree of separation between the two centrioles, which have been found to be far more dynamic than previously thought. Microtubule release and anchoring at sites nearby
in the pericentriolar material may also free nucleation sites for the production of further microtubules, enhancing the nucleation capacity of the centrosome.

1.3. Centrosome duplication

Centrosomes do not form de novo. In the few cases where this has been claimed, in the surf clam Spisula solidissima (Miki-Noumura, 1977), in parthenogenetically activated rabbit oocytes (Szollosi and Ozil, 1991) and in vertebrate cells where the centrosome has been destroyed by laser ablation (Khodjakov et al., 2002), the existence of centrosome precursors or in the latter case centrosome remnants cannot be ruled out. An exception to this is found in terminally differentiating ciliated epithelial cells, where several hundred centriole pairs destined to become basal bodies for an equivalent number of cilia are assembled. Some are formed in association with the centrosomal centriole pair (centriolar pathway). The majority of centrioles, however, appear to grow from spherical aggregates of fibres ~75-400nm in diameter, termed deuterosomes (acentriolar pathway) (Anderson and Brenner, 1971). Their origin and nature has remained unclear.

In normally dividing cells, the centriole pair splits and small procentrioles arise at the base of each parental centriole around the time of the G1/S phase transition, and elongate during S and G2 phases of the cell cycle, reaching full size as cells enter mitosis, whereupon the replicated centriole pairs and associated pericentriolar material separate to form the two poles of the mitotic spindle. Like DNA, centrioles are inherited in a semi-conservative manner. This was clearly demonstrated by Kochansky and Borisy (Kochanski and Borisy, 1990), by microinjecting cells with biotinylated tubulin. Only the replicated centriole incorporated tubulin, and following mitosis, each daughter cell received a centrosome containing an unlabelled and a labelled progeny centriole.

1.3.1. The molecular events underlying centrosome duplication
The first step in the centrosome duplication cycle is the splitting of the centriole pair which is generally thought to occur in late G1 (Kuriyama and Borisy, 1981a), although centrioles have been observed widely separated as early as telophase in some cells (Mack and Rattner, 1993; Piel et al., 2000). This key event may involve the degradation of a proteinaceous link between the centrioles. Evidence for such a model is the finding that components of the SCF ubiquitin-ligase complex, active during G1/S, localise to the centrosome and that proteasome inhibitors block centriole splitting \textit{in vitro} and centrosome duplication \textit{in vivo} (Freed et al., 1999). Furthermore, mutations in slimb, a *Drosophila* SCF complex component, result in aberrant centrosome duplication (Wojcik et al., 2000). The protein(s) that make up such a link remain to be identified.

In the next step, each parental centriole assembles a procentriole at a set distance from its proximal end, which subsequently elongates during the remainder of S and G2. This manner of reproduction, by putting out an 'offshoot', is strikingly similar to the situation in yeast, where a new spindle pole body arises from the so-called 'satellite' which is linked to the parent by the 'half bridge', a structure which acts as a spacer between old and new spindle pole body. Cdc31p, which forms part of this structure, has been found to be required for satellite formation and thus spindle pole body duplication. Interestingly, centrin-3, its vertebrate homologue has been found to be required for centrosome duplication, and disrupts spindle pole duplication if overexpressed in yeast (Middendorp et al., 2000). Similarly, depletion of centrin-2, a closely related protein, has recently been shown to lead to a failure of centriole duplication in HeLa cells, resulting in cells entering mitosis with one or, in subsequent mitoses, no centriole at each pole (Salisbury et al., 2002). Centrin-2, -3 and Cdc31p belong to a conserved family of proteins called centrins, part of the calmodulin superfamily of small Ca\textsuperscript{2+}-binding proteins. In green algae, centrins form contractile basal body associated fibres (Salisbury et al., 1984). Centrins may thus form the connecting structure between procentriole and centriole, which terminates at the procentriole in a cartwheel-like scaffold that appears to be the template for assembly of the singlet microtubules in the procentriole wall (Vorobjev and Chentsov Yu, 1982). Like other microtubules, the formation of centriolar microtubules appears to require \(\gamma\)-tubulin, at least in the unicellular ciliate *Paramecium* (Ruiz et al., 1999). The other rare tubulins, \(\delta\)-(Dutcher and Trabuco,
1998; Garreau De Loubresse et al., 2001), ε- (Dupuis-Williams et al., 2002), and η- (Ruiz et al., 2000) tubulin also appear to be involved in the formation of the triplet microtubules in Paramecium and Chlamydomonas, as their depletion or mutation results in the loss of one (usually the outer, C-tubule) or more of these structures. Vertebrate homologues of δ- and ε-tubulin have been described (Chang and Stearns, 2000), and may perform similar functions.

As the cell approaches mitosis, the two centriole pairs disjoin and separate. First, the physical link between the two parental centrioles is severed, perhaps by the action of the kinase NEK2 on its centrosomal substrate cNAP1 (Fry et al., 1998; Mayor et al., 2000). Another potentially relevant phosphorylation event at this junction is that of centrin, which may also form part of the link structure, although the functional consequences of this event are unknown (Lutz et al., 2001). The actual separation of the two centrosomes occurs at G2/M and involves aurora A kinase and its potential substrate, the kinesin related motor protein Eg5 (Giet et al., 1999). By prophase, the younger parental centriole has also finally acquired distal appendages and their associated proteins cenexin (Lange and Gull, 1995), ε-tubulin (Chang and Stearns, 2000), CEP110 and ninein (Ou et al., 2002), and outer dense fiber 2 (Nakagawa et al., 2001). As previously mentioned, some of these proteins have been implicated in the ability of centrosomes to anchor microtubules. Their recruitment, termed maturation, completes the centrosome duplication process and the two centrosomes that will form the two poles of the mitotic spindle are now fully equivalent.

The process of centrosome duplication requires the recruitment and incorporation of new centrosomal material. It is therefore perhaps not surprising that the inhibition of microtubule-dependent transport by depolymerisation of microtubules resulted in a block in centriole duplication in cells undergoing cell division or ciliogenesis (Boisvieux-Ulrich et al., 1989; Kuriyama, 1982). While embryonic cells contain stockpiles of centrosomal material and can therefore continue to duplicate their centrosomes in the absence of protein synthesis (Gard et al., 1990), somatic cells which do not possess such reserves fail to initiate procentriole formation if treated with protein synthesis inhibitors such as cycloheximide, but can elongate those procentrioles if they have already formed (Phillips and Rattner, 1976).
Centrosome duplication should occur once, and only once, per cell cycle. As was originally proposed by Boveri many years ago (Boveri, 1914), an irregular number of centrosomes may lead to the formation of mono- or multipolar spindles, resulting in aberrant chromosome segregation and aneuploidy, a frequent cause of tumorigenesis. Indeed, many, if not most, cancer cells contain supernumerary centrosomes and accumulations of centrosomal proteins (Lingle et al., 1998; Pihan et al., 1998). It is clear that cellular mechanisms must be in place to limit the occurrence of such events since cancer is still a rare event, arising only once in three lifetimes despite an average of $10^{14}$ cells in a human body (Evan and Vousden, 2001).

Centrosome duplication is tightly linked to the cell cycle. Centrosome duplication does not occur if Chinese hamster ovary cells are arrested in G1 with mimosine (Matsumoto et al., 1999), while a similar arrest in S phase with aphidicolin or hydroxyurea permits duplication, and indeed reduplication, of centrosomes (Balczon et al., 1995b). G2 phase does not support continued centrosome duplication (Balczon et al., 1995b). Conditions in mitosis similarly do not support centrosome replication, but do allow mother-daughter centriole splitting and separation. Thus the two centrioles at each spindle pole in sea urchin zygotes split but do not duplicate if mitosis is prolonged to 20 times its normal duration by treatment with mercaptoethanol (Mazia et al., 1960) or non-degradable cyclin B Δ90 (Hinchcliffe et al., 1998). Centrosome duplication will also not occur in mitotically arrested *Drosophila* embryos (Vidwans et al., 1999) or *in vitro* in *Xenopus* egg extracts (Lacey et al., 1999).

The latter system has been particularly useful in dissecting the control mechanisms regulating entry into the centrosome duplication cycle. Experiments with *Xenopus* eggs and embryos showed that inhibition of Cdk2 blocked centrosome duplication, just as it prevents entry into S phase and DNA replication (Hinchcliffe et al., 1999; Lacey et al., 1999). Similar experiments in cultured mammalian cells showed that Cdk2 is also required for centrosome duplication in somatic cells (Matsumoto et al., 1999; Meraldi et al., 1999). Cdk2 is known to function in
conjunction with two S phase cyclins, cyclin A and cyclin E, and either will serve to
drive centriole separation in *Xenopus* embryos and egg extracts. In somatic cells,
activated Cdk2-cyclin E, which peaks at the G1/S transition, phosphorylates the Rb
'pocket' protein, releasing the E2F transcription factor, which activates genes
required for S phase including cdk2 and cyclins E and A. While both cyclin E and
cyclin A were found to be able to drive centrosome duplication, only cyclin A could
do so in the presence of non-phosphorylatable mutant pRb, suggesting that it acts
downstream of cyclin E and pRb/ E2F in initiating centrosome duplication (Meraldi
et al., 1999). What does Cdk2 act on at the centrosome? One target is
nucleophosmin, a predominantly nucleolar protein that associates with the
centrosome from the end of mitosis until S phase, when phosphorylation by Cdk2-
cyclin E or A causes it to leave the centrosome (Okuda et al., 2000). Importantly,
overexpression of a mutant form of nucleophosmin lacking the Cdk2
phosphorylation site prevented centrosome duplication (Tokuyama et al., 2001),
suggesting that nucleophosmin may form part of the 'licensing' mechanism for
centrosome duplication. The actual trigger for centrosome duplication may be the
activation of calmodulin-dependent protein kinase II, which in *Xenopus* embryos
drives the initial and every subsequent round of centrosome duplication following
fertilisation of the egg in response to a sudden increase in intracellular Ca^{2+} levels
(Matsumoto and Maller, 2002). Its target(s) are presently unclear, although a number
of likely candidates are to be found at the centrosome, including calmodulin (Li et
al., 1999) and the centrins, two of which, as previously mentioned, appear to be
directly involved in the duplication process (Middendorp et al., 2000; Salisbury et
al., 2002). As mentioned above, centrosomes can be made to reduplicate in certain
systems (sea urchin zygotes (Sluder and Lewis, 1987) and *Drosophila* embryos (Raff
and Glover, 1988), as well as some cultured somatic cells (Balsezon et al., 1995b)),
by extending the duration of S phase with aphidicolin or hydroxyurea, drugs that
inhibit DNA synthesis. That this is not a general phenomenon, and that in vertebrate
somatic cells centrosome reduplication does not occur unless the cell cycle is
delayed for at least 40 hours, far longer than the duration of the entire cell cycle,
suggests that there is a powerful block to reinitiation of centrosome duplication. The
licensing model proposed above could account for this block. An interesting
observation in this regard is that overexpression of Mps1, another Cdk2 substrate,
drives the reduplication of centrosomes in S phase – arrested NIH3T3 cells, a cell line that does not reduplicate its centrosome during a prolonged arrest, in a Cdk2-dependent manner (Fisk and Winey, 2001). While Mps1 is likely to act downstream of Cdk2, the nature of its contribution to the centrosome duplication process is presently unknown. Like its yeast homologue, Mps1 in vertebrates may act at several steps during centrosome duplication (Schutz and Winey, 1998).

1.4. Centrosome function redefined

1.4.1. Centrosomes and mitotic spindle assembly

At the onset of mitosis, the interphase microtubule network is disassembled and mitotic spindle assembly begins. The centrosomal capacity to nucleate microtubules, constant throughout interphase, increases dramatically (Kuriyama and Borisy, 1981b), concomitant with an increase in centrosomal γ-tubulin levels (Khodjakov and Rieder, 1999). The nuclear envelope breaks down, weakened by the pulling forces exerted on it by interactions between centrosomal microtubules and nuclear membrane-associated cytoplasmic dynein (Salina et al., 2002). The microtubules nucleated by the centrosomes, made highly dynamic by the activities of microtubule stabilising and destabilising factors such as XMAP215 and XKCM1 (Tournebize et al., 2000), are captured and stabilised by kinetochores, thereby forming a bipolar spindle (Kirschner and Mitchison, 1986).

This neat model for centrosome involvement in spindle assembly has been called into question by a number of observations demonstrating the dispensability of centrosomes in several experimental systems. One longstanding observation in the centrosome field has been that bipolar spindles assemble in higher plant cells (Pickett-Heaps and Northcote, 1966) and in female meiosis of some animal species (Matthies et al., 1996; Szollosi et al., 1972) in the absence of recognisable centrosomes. In these systems, spindles appear to be organised around chromatin by the action of microtubule motors (Theurkauf and Hawley, 1992). This was clearly shown by experiments in *Xenopus* egg extracts (Heald et al., 1996), where bipolar
spindles were found to assemble around chromatin-coated beads in the absence of centrosomes through the movement and sorting of randomly nucleated microtubules. We now know some of the key participants in these processes, and there are indications that they may also play an important role in mitosis. The seeding of microtubules in the vicinity of chromosomes appears to involve the release by Ran-GTP, generated locally by the action of the chromatin-bound guanine nucleotide exchange factor RCC1, of factors that promote polymerisation and/or stabilisation of microtubules from their inhibitory embrace by importins (Gruss et al., 2001; Nachury et al., 2001; Wiese et al., 2001). A Ran-GTP gradient has recently been directly visualised around chromosomes in mitotic Xenopus egg extracts (Kalab et al., 2002), further supporting this model. Once nucleated, microtubules need to be sorted into a bipolar array. This may be achieved by the action of microtubule plus end-directed, multimeric motors such as the BimC family of plus end-directed kinesins that can crosslink and slide microtubules into an anti-parallel array (Sharp et al., 1999a). These microtubules are then bundled into convergent polar arrays by the action of microtubule minus end-directed motor cytoplasmic dynein (Gaglio et al., 1996), which acts in concert with dynactin (Gaglio et al., 1996), NuMA (Merdes et al., 1996) and possibly other spindle pole proteins. These motor activities appear to be required for bipolar spindle assembly irrespective of whether centrosomes are present or not (Heald et al., 1997; Sharp et al., 1999b).

What then is the role of the centrosomes in spindle assembly? Even in cells that normally do contain them, they could be removed experimentally without affecting spindle assembly or progress through mitosis (Dietz, 1966; Hinchcliffe et al., 2001; Khodjakov et al., 2000; Khodjakov and Rieder, 2001). One extreme view holds that centrosome association with the spindle poles is just a way of ensuring their correct segregation so that they can complete other, essential functions (Dietz, 1959; Friedländer and Wahrman, 1970). It ought to be remembered, though, that centrosomes, when present, exert a dominant effect on spindle assembly and thus determine spindle polarity (Heald et al., 1997). Furthermore, as previously noted, many centrosomal proteins have sizeable cytoplasmic pools, including γ-tubulin (Moudjou et al., 1996), centrin (Paoletti et al., 1996), NEK2 (Fry et al., 1998), and katanin (McNally and Thomas, 1998). In experiments where γ-tubulin was specifically depleted, microtubule nucleation and spindle assembly was severely
affected (Felix et al., 1994; Hannak et al., 2002; Sampaio et al., 2001; Stearns and Kirschner, 1994). Thus while centrioles may not be required for spindle assembly, the same cannot be said for the proteins of the pericentriolar material.

1.4.2. The role of centrosomes in cytokinesis

While the centrosome ablation experiments mentioned above failed to reveal any defects in mitosis, cells without centrosomes did show defects in other stages of the cell cycle. The first of these was a marked increase in the incidence of binucleate cells, indicative of a failure to complete cytokinesis (Hinchcliffe et al., 2001; Khodjakov and Rieder, 2001; Piel et al., 2001).

How do centrosomes contribute to the fidelity of cytokinesis? It has been known for many years that the radial astral microtubule arrays generated by mitotic centrosomes are involved in specifying the position of the cleavage furrow. They may do this directly, as suggested by the classic experiments of Rappaport on sand dollar eggs (Rappaport, 1961), or indirectly, by correctly positioning the mitotic spindle and thus the midzone microtubules, which appear to play an important role in signalling to the cortex to divide (Adams et al., 2001; Wheatley and Wang, 1996). Spindle positioning is known to require dynein-mediated interactions between astral microtubules and the cell cortex (Busson et al., 1998; Faulkner et al., 2000; O'Connell and Wang, 2000). One of the characteristic features of acentrosomal spindles is that they lack astral microtubules and thus the ability to position themselves correctly (Khodjakov and Rieder, 2001), although there is some evidence for non-centrosomal pathways of spindle positioning (Bonaccorsi et al., 2000).

Centrosomes may participate in cytokinesis by other means. γ-tubulin has been localised to the minus ends of the microtubule bundles that form the midbody, and inhibition of γ-tubulin function has been found to lead to abortive cytokinesis with poorly developed midbody microtubules (Julian et al., 1993; Shu et al., 1995). The origin of midbody microtubules is still a matter of debate, and γ-tubulin may be acting here as a microtubule minus end-capping protein, rather than as a microtubule nucleator as the authors suggest. Other centrosomal proteins may also localise to this region, although their function is unclear.
Finally, Bornens and colleagues (Piel et al., 2001) have recently drawn attention to an old observation of centrioles near the midbody in cells in late telophase, early interphase (Rattner and Phillips, 1973). Using centrioles marked with centrin-GFP, they showed a transient association of one centriole with the cytoplasmic end of the intercellular bridge, coinciding with the bridge narrowing and microtubule depolymerisation within the bridge. Movement of the centriole away from the bridge always preceded abscission. These observations are consistent with the centriole signalling to complete the cytokinesis process, perhaps acting within a checkpoint monitoring the completion of mitosis. Such a checkpoint operates in budding yeast, where migration of the spindle pole body into the bud activates the mitotic exit network by bringing the GDP-bound form of Tem1 at the spindle pole body into contact with the guanine nucleotide exchange factor Lte1p associated with the cortex of the bud, thereby linking nuclear migration with mitotic exit (Bardin et al., 2000; Pereira et al., 2000). It remains to be determined whether a similar checkpoint exists in vertebrates.

1.4.3. A requirement for centrosomes in S phase progression

Another defect displayed by acentrosomal cells is a failure to enter S phase following the first round of mitosis, irrespective of whether cells completed cytokinesis successfully or not (Hinchcliffe et al., 2001; Khodjakov and Rieder, 2001). Such cells showed no apparent defects in microtubule organisation or localisation of pericentriolar material proteins γ-tubulin and pericentrin. These results may be explained by the existence of a checkpoint monitoring centrosome integrity and/or duplication. Alternatively, core centrosomal components could bind cell cycle regulatory molecules in a way that activates their function. Such molecules may require dynein-mediated transport to the centrosome, as evidenced by the delay in cell cycle progression if dynein function is impaired (Quintyne and Schroer, 2002).

1.4.4. Centrosomes as anchors for regulatory molecules
Indeed, many regulatory molecules have been found to localise to the centrosome and/or the mitotic spindle poles. Several of these have been mentioned before as they play important roles in centrosome function.

Aurora A kinase localises to the pericentriolar material and later the spindle poles following centrosome duplication in S phase until the end of mitosis. In late G2/M it promotes centrosome separation (Glover et al., 1995), by phosphorylating and activating the kinesin related motor protein Eg5 (Giet et al., 1999). At the same time it promotes the recruitment of centrosomal proteins in preparation for mitosis. Thus, in *C. elegans*, aurora A is required for the recruitment of γ-tubulin and at least two other centrosomal proteins at the onset of mitosis and the assembly of the mitotic spindle (Hannak et al., 2001). In *Drosophila*, the XMAP215 homologue Msps and D-TACC fail to localise to the abnormal mitotic spindle poles formed in cells depleted of aurora A by RNA interference (Giet et al., 2002). The ‘core’ centrosomal proteins γ-tubulin and centrosomin may also require aurora A function, as these are delocalised in a kinase mutant (Berdnik and Knoblich, 2002). Aurora A function in spindle assembly appears to be conserved in vertebrates, since inhibition of aurora A function destabilises pre-existing bipolar spindles in *Xenopus* extracts (Giet and Prigent, 2000).

Another kinase with similar but apparently distinct roles is polo-like kinase, plk1. It localises to spindle poles from prophase until anaphase, when it transfers to the central spindle and later the midbody (Golsteyn et al., 1995). One of its functions early in mitosis appears to be as part of a positive feedback loop, phosphorylating and activating Cdc25 phosphatase in response to Cdc2 activity, thus further activating Cdc2-cyclin B and promoting mitotic entry (Kumagai and Dunphy, 1996). Antibody microinjection experiments further revealed a role for plk1 in centrosome maturation and separation, with injected cells failing to accumulate γ-tubulin and assembling monopolar spindles (Lane and Nigg, 1996). Later in mitosis, plk1 has been shown to promote mitotic exit by activating the APC (Descombes and Nigg, 1998). Finally, polo kinase in yeast (Ohkura et al., 1995) and *Drosophila* (Carmena et al., 1998) have been shown to be required for cytokinesis and this may also be the case in vertebrates (Liu and Erikson, 2002). In higher eukaryotes, this appears to involve an interaction with a kinesin-like protein (Adams et al., 1998; Lee et al., 1995). Both aurora A and polo-like kinase have been implicated in centrosome
duplication. However, while overexpression of pkl1 and aurora A, both wild type and kinase-dead, led to centrosome amplification, this occurred only through a failure in cytokinesis. Cells arrested in S phase failed to reduplicate their centrosomes in the presence of excess aurora A kinase (Meraldi et al., 2002). Thus there is little evidence for such a role at present.

Other key regulators of the cell cycle similarly localise to the centrosome. Thus the B-type cyclin, cyclin B1 localises to the centrosome throughout its accumulation in the cytoplasm and up to metaphase (Bailly et al., 1992). Degradation of cyclin B then initiates at the spindle poles before spreading to the remainder of the spindle and the cytoplasm (Clute and Pines, 1999; Huang and Raff, 1999). The anaphase promoting complex localises to the centrosome throughout the cell cycle (Tugendreich et al., 1995), as does the SCF ubiquitin ligase (Freed et al., 1999), as previously mentioned. Centrosomes thus appear to be involved in the localised degradation as well as activation (pkl1 activating Cdc2-cyclin B, passim) of global cell cycle regulators.

Another important class of signalling molecules are anchored at the centrosome through the action of A-kinase anchoring proteins (AKAPs). Their name derives from their targeting of cyclic AMP-dependent protein kinase A (PKA) to various cytoskeletal sites, including the microtubule and actin cytoskeleton. This enzyme is involved in numerous cellular processes, such as metabolism, gene transcription, actin cytoskeletal rearrangements and cell growth and division (Colledge and Scott, 1999). PKA has been known to localise to the centrosome for many years (Nigg et al., 1985), but although some putative centrosomal substrates have been identified (Inaba et al., 1998; Lutz et al., 2001) its role remains unclear. Targeting to the centrosome is mediated by two proteins, AKAP450/AKAP350/CG-NAP (Schmidt et al., 1999; Takahashi et al., 1999; Witczak et al., 1999) and pericentrin (Diviani et al., 2000). AKAPs are believed to integrate diverse signalling pathways by simultaneously interacting with multiple signalling molecules, such as kinases and phosphatases. Thus AKAP450 in addition to PKA interacts with PKN, protein phosphatase1 and 2a, and PKCe (Takahashi et al., 1999; Takahashi et al., 2002).

That centrosomes are involved in many of the complex signalling networks that control cellular function is not particularly surprising. An organelle involved in
many important cellular processes, including microtubule organisation, mitosis and cytokinesis needs to be tightly integrated into the other activities of the cell.

1.4.5. Centrosomes, why bother?

Despite its many and varied activities, the functional significance of the centrosome as a whole remains unclear. Indeed, a recent paper reported that a whole organism can develop in the absence of fully functional centrosomes. Megraw et al. (Megraw et al., 2001) show that Drosophila strain mutant for the centrosomal protein centrosomin, though sterile, develops to maturity practically without visible defects, despite mitotic spindle poles (but not interphase centrosomes) lacking γ-tubulin and possibly CP60 and CP190 and showing few if any astral microtubules. The same paper, however, notes that asymmetric divisions in mutant embryos appear randomised and are only subsequently corrected by developmental processes. Centrosomes, in particular the centrioles, are elaborate structures, and are structurally highly diverse even within higher eukaryotes. Like the tumour suppressor protein p53 they may not form part of the core machinery of cell division but act to improve the fidelity of this process, something that is crucial for cells to function effectively within highly complex multicellular organisms.

1.5. PCM-1 and centriolar satellites

We initially became interested in PCM-1 because of its reported cell cycle-dependent association with the centrosome (Balczon et al., 1994). While some of this early data on PCM-1 including its localisation to the pericentriolar material appears to be incorrect (see below and Results), PCM-1 retained our interest as within a few months of my beginning to work on this protein it was shown to localise to centriolar satellites, structures previously known only from electron microscopic studies (Kubo et al., 1999). These satellites turned out to be more dynamic than previously thought, continuously shuttling between the centrosomes and the surrounding cytoplasm.
Autoantibodies from patients with calcinosis, Raynaud’s phenomenon, esophageal dysmotility, sclerodactyly, and telangiectasia (CREST) have been used to identify many of the components of the centrosomes and spindle apparatus, including the kinetochore proteins CENP-A, B, and C (Earnshaw and Rothfield, 1985), the Nuclear and Mitotic Apparatus protein NuMA (Compton et al., 1992; Yang et al., 1992) and the centrosomal proteins pericentrin (Doxsey et al., 1994) and Cep250/ c-Nap1 (Mack et al., 1998). PCM-1 was similarly identified using a human autoimmune serum (SPJ serum) derived from a patient with systemic sclerosis and Raynaud’s phenomenon that specifically recognised centrosomes (Osborn et al., 1982). By immunoblotting of mammalian cell extracts this antibody reacted with several polypeptides, including three of molecular weights 39, 185, and 220kDa that appeared to be centrosomal using affinity-purified antibodies (Balczon and West, 1991). PCM-1 was eventually cloned using the autoimmune serum to screen a cDNA expression library (Balczon et al., 1994). Polyclonal antibodies raised against a fragment of the PCM-1 cDNA identified a band of ~220kDa by immunoblotting. By immunofluorescence on HeLa cells synchronised by mitotic shake-off, PCM-1 was found to be associated with the centrosome from G1 until late G2, when it appeared to dissociate from the centrosome and disperse into numerous foci throughout the cell, reassociating with the centrosome at the end of mitosis. This striking cell cycle-dependent redistribution of PCM-1 was not accompanied by a change in cellular protein levels. The sequence of PCM-1, a relatively acidic ($\pi_1$ of 4.82) protein of 2024 amino acids and a predicted molecular weight of 228kDa, did not provide any immediate clues as to its function, with database searches failing to identify clear homologues or functional motifs. Another study by the same group a year later (Balczon et al., 1995a) reported that synthesis and degradation of the mRNA encoding PCM-1 occurred in a cell cycle-dependent fashion in CHO cells, with peak levels in G1 and S phase cells, before becoming undetectable in G2 and mitosis. Notably, PCM-1 mRNA levels remained high in CHO cells arrested at the G1/S boundary with hydroxyurea, lending itself to an interpretation that PCM-1 may be
involved in the multiple rounds of centrosome duplication occurring under these conditions.

1.5.2. PCM-1, a centriolar satellite protein associated with the dynein microtubule motor

A somewhat different picture of PCM-1 emerged from work by the Tsukita laboratory, published within a few months of my beginning work on PCM-1 (Kubo et al., 1999). They cloned the *Xenopus* and mouse homologues of PCM-1, which were highly similar to the previously described human protein (57% identity at the amino acid level). By immunofluorescence microscopy on *Xenopus* A6 cells, PCM-1 was found to localise not to the pericentriolar material as previously reported (Balczon et al., 1994), but to punctate foci found throughout the cytoplasm but concentrated around the centrosome. By immunoelectron microscopy, PCM-1 antibody specifically labelled electron-dense spherical granules 80-90nm in diameter located around centrosomes (Figure 1C). These granules, which were not surrounded by membranes and were resistant to Triton X-100 treatment, were identified as the centriolar satellites known from previous electron microscopic studies and described earlier in this introduction. The authors went on to examine the dynamics of PCM-1 and centriolar satellites in living cells. They first showed that, like centriolar satellites (Boisvieux-Ulrich et al., 1989), PCM-1 localises to the centrosomal region in a microtubule-dependent manner, treatment with nocodazole (but not the actin-depolymerising drug cytochalasin B) resulting in PCM-1 foci being scattered throughout the cytoplasm. In cells stably expressing full-length or a middle portion of PCM-1 (amino acids 745-1271) fused to GFP, PCM-1 particles were shown to move to and from the centrosome at a maximal velocity of 0.7-0.8μm/s. Again, this movement was inhibited by nocodazole but not cytochalasin B. The authors then examined this movement *in vitro*, adding partially purified GFP-PCM-1-containing centriolar satellites (confirmed as such by immunoelectron microscopy) to asters formed with rhodamine-labelled tubulin. GFP-tagged centriolar satellites were observed accumulating at the centrosomes at rates comparable to those obtained *in vivo*. This accumulation was inhibited by agents specifically targeting the microtubule minus end-directed motor dynein (10μM vanadate, 2mM AMP-PNP,
anti-dynein intermediate chain antibody 70.1), but not those targeting kinesin (100µM AMP-PNP, a concentration that does not affect dynein motility (Schroer and Sheetz, 1989)). Their final set of observations concerns the expression pattern of PCM-1 in mouse epithelial cells undergoing ciliogenesis. As was described earlier in this introduction, the process of ciliogenesis involves the repeated replication of basal bodies and is accompanied by an accumulation of centriolar satellites as well as larger deuterosomes, from which new centrioles appear to grow. In cells induced to undergo ciliogenesis, PCM-1 expression was markedly upregulated in the apical cytoplasm, and immunoelectron microscopy showed that centriolar satellites but not deuterosomes labelled with PCM-1 antibody.

The concept of dynein-dependent microtubule transport of PCM-1 was further developed by Balczon and colleagues (Balczon et al., 1999), who demonstrated that PCM-1 co-pelleted with taxol-stabilised microtubules in the presence of GTP alone and three to fourfold more when AMP-PNP was added to induce motor binding to microtubules (Schroer and Sheetz, 1991). Cosedimentation was inhibited by immunodepletion of extracts with anti-dynein, but not kinesin antibodies, suggesting that PCM-1 binding to microtubules is dependent on the dynein motor.

It has not been determined how PCM-1 interacts with the dynein motor. Many of the diverse cargoes of this motor interact with one or more of the proteins that make up the multisubunit dynactin complex which is required for dynein motility (Gill et al., 1991). It is in this context interesting that PCM-1 and the p150Glued subunit of dynactin were isolated as interactors of huntingtin-associated protein 1 (HAP-1) by yeast two hybrid analysis (Engelender et al., 1997). The significance of this interaction is not clear, however, as HAP-1 expression is restricted to certain regions of the brain including the hypothalamus, where it may function in axonal transport of vesicles. A recently described targeted disruption of HAP-1 in mice revealed few defects in homozygous mutant animals other than postnatal lethality due to depressed feeding behaviour, a function associated with the hypothalamus (Chan et al., 2002).

1.5.3. The question to be addressed
As the above discussion makes clear, at the outset of my work on PCM-1 very little was known about the role of PCM-1 in centrosome function. Its localisation to centriolar satellites and dynein-dependent motility was clearly suggestive, as was its expression at times (G1/S phase) and places (apical cytoplasm in cells undergoing ciliogenesis) consistent with a role in centrosome duplication. However, no functional data had been reported at this time. The aim of my thesis, then, was to determine the function of PCM-1 and the centriolar satellites to which it localises, using all the cell biological tools at our disposal.

1.5.4. Recent reports on PCM-1

More recently, two papers have been published that go some way towards addressing the function of PCM-1, and as such their results show a degree of overlap with those I had previously obtained during the course of my work. These parallels will be pointed out in the Results section. In 2001, Li et al. (Li et al., 2001) showed that PCM-1 interacts with pericentrin, by coimmunoprecipitation and comigration on sucrose gradients. They further showed that neither protein was required for microtubule nucleation, since their immunodepletion from cellular extracts did not inhibit the ability of salt-stripped centrosomes to recover microtubule nucleation potential. Further, they showed that neither PCM-1 nor pericentrin could be recruited to salt-stripped centrosomes in the presence of nocodazole, while recruitment of \( \gamma \)-tubulin and restoration of microtubule nucleation potential took place normally. A second paper, published in July 2002 (Balczon et al., 2002) examines the effects of microinjecting PCM-1 antibodies into mouse oocytes or fertilised zygotes. Antibody microinjection did not affect spindle assembly in germinal vesicle stage oocytes or in fertilised embryos. However, embryos injected shortly after fertilisation showed a dispersal of centrosomal material and associated small cytoplasmic microtubule asters. These embryos frequently failed to incorporate BrdU into one or both pronuclei and rarely progressed through to the two cell stage, suggesting a disruption of centrosome integrity and cell cycle progression.
Chapter 2. Materials and Methods

2.1. Materials

2.1.1. Bacterial strain genotypes and growth conditions

_E. coli XL-1 Blue_ (Stratagene) - used for general cloning purposes:
Genotype: F'::Tn10 proA^B lac^B Δ(lacZ)M15/recA1 endA1 gyrA96 (Nal') thi hsdR17 (r_k^m_k^) glnV44 relA1 lac

_E. coli BL21(DE3) (Novagen)_ - used for expression of fusion proteins
Genotype: F' ompT gal [dcm] [lon] hsdS_B (r_B^m_B^-) (DE3)

_E. coli XL1-Blue MRF' (Stratagene)_ - host strain for λ cDNA library
Genotype:  Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac [F' proAB lac^B ΔM15 Tn10 (Tet')]

_E. coli SOLR (Stratagene)_ - used for phage excision
Genotype: e14 (McrA') Δ(mcrCB-hsdSMR-mrr)171 sbcC recB recJ uvrC umuC::Tn5 (Kan') lac gyrA96 relA1 thi-1 endA1 λ^R [F' proAB lac^B ΔM15] Su^-

Bacteria were grown in LB medium supplemented with the appropriate antibiotic (Ampicillin 1:1,000 dilution from 100 mg/ml stock, Kanamycin 1:1,000 dilution from 50mg/ml stock; stock solutions stored at -20°C) at 37°C. Liquid cultures were grown in well-aerated vessels (with a capacity at least 4 times the culture volume) in a shaker at 250rpm at 37°C. Strains were stored as frozen stocks in 15% glycerol at -80°C

2.1.2. Cell lines and culture conditions
Human HeLa, African green monkey COS-7, Chinese hamster ovary CHO, mouse NIH3T3 and rat kangaroo PtK2 cells were cultured in Dulbecco’s Modified Eagle’s Medium supplemented with 10% fetal bovine serum, 2mM L-glutamine and antibiotics (50 IU each of penicillin and streptomycin). Human osteosarcoma U-2 OS, Jurkat and chicken DU249 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2mM L-glutamine and antibiotics as above. These cell lines were grown in a humidified incubator at 37°C and 5% CO₂. Xenopus A6 cells were cultured at room temperature (22°C) in 65% Leibowitz L-15 medium, 35% dH₂O supplemented with 10% fetal bovine serum, 2mM L-glutamine and antibiotics as above. Cells were passaged every 2 to 3 days by washing plates with PBS and incubation with trypsin-EDTA until detached. Cells were stored at −80°C or in liquid Nitrogen in culture medium containing 20% fetal bovine serum and 10% DMSO. All media were obtained from Sigma.

2.1.3. Yeast strain genotypes and growth conditions


Y187 MATα, ura3- 52, his3- 200, ade2- 101, trp1- 901, leu2- 3, 112, gal4Δ, met–, gal80Δ, URA3 : : GAL1UAS-GAL1TATA-lacZ

Yeast were grown on agar plates prepared by single colony streak-out every 2 to 3 days. Liquid cultures were grown in a 30°C shaker at 200rpm unless otherwise indicated. Strains were stored on stock plates at 4°C for several weeks. For long-term storage, yeast were kept in 25% glycerol at -80°C.

Non-selective YPDA medium:

20g/l Difco peptone
10g/l Yeast extract
20g/l agar (for plates only)
15ml/l 0.2% adenine hemisulfate
pH adjusted to 6.5, then autoclaved
Let cool to ~55°C, then added sterile glucose to 2% final concentration

Selective Dropout medium:
1.7g/l Yeast Nitrogen Base without amino acids (QBiogene Bio 101)
5g/l ammonium sulfate
appropriate amount of Complete Synthetic Medium lacking one or more amino acids but supplemented with 40mg/ml adenine hemisulfate (QBiogene Bio 101)
2 pellets of sodium hydroxide
20g/l Bacto agar (for plates only, Difco) autoclaved
Let cool to ~55°C, then added sterile glucose to 2% final concentration

2.1.4. Primers

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2.1.5. **Vectors**

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<tr>
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<td>pGEM-T</td>
<td>PCR cloning vector (Promega)</td>
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<td>pGBK7-EV</td>
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<td>pGBT9</td>
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<tr>
<td>pRSET-C hs PCM-1 C-ter</td>
<td>above vector expressing amino acids 1665-2024 of human PCM-1 with N-terminal 6xHis tag (this study)</td>
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<td>chicken PCM-1 partial clone nucleotides 1492-3992 (Jean-Marie Buerstedde, University of Hamburg)</td>
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<td>pTD1-l</td>
<td>Yeast two-hybrid prey vector expressing amino acids 87-708 of SV40 T-antigen (+ve Control, Clontech)</td>
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2.1.6. Antibodies

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<th>Antibody</th>
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*Abbreviations:*

IF immunofluorescence, IB immunoblotting, IP immunoprecipitation
2.1.7. Commonly used buffers and media

LB medium (1l):
10g bacto-tryptone
5g bacto-yeast extract
10g NaCl
pH adjusted to 7.0 with NaOH
for plates added 15g agar/ l
autoclaved

1M Tris (1l):
121.1g Tris dissolved in 800ml dH₂O
adjusted pH to required value with concentrated HCl
added dH₂O to 1l; autoclaved

PBS (1l):
to 800ml dH₂O added
8g NaCl
0.2g KCl
1.44g Na₂HPO₄
0.24g KH₂PO₄
pH adjusted to 7.4 with HCl
added dH₂O to 1l; autoclaved

TBS (1l):
to 800ml dH₂O added
8g NaCl
0.2g KCl
3g Tris base
pH adjusted to 7.4 with HCl
added dH₂O to 1l; autoclaved
2.2. Nucleic acid methods

2.2.1. Plasmid minipreps by alkaline lysis

3 ml cultures were grown overnight (12-16 hours) at 37°C in LB medium supplemented with the appropriate antibiotic. 1.5 ml culture was transferred to a microcentrifuge tubes and pelleted by centrifugation at 6,000g for 3 min and resuspended in 200μl GTE buffer (50mM Glucose, 25mM Tris pH8.0, 10mM EDTA) by passing tubes over the surface of a rack. Cells were then lysed by the addition of 400μl 0.2M NaOH/1% SDS and samples mixed by inversion 6 times. Cell debris, protein and genomic DNA was precipitated by the addition of 300μl 3M potassium acetate pH5.2 and removed by centrifugation at 18,000g for 5 min. Supernatant containing plasmid DNA was decanted into fresh microcentrifuge tubes and DNA precipitated by the addition of 550μl isopropanol. Tubes were vortexed and centrifuged again at 18,000g for 5 min. DNA pellets were washed with 300μl 70% ethanol, dried briefly and resuspended in 30 to 50μl H2O containing 50μg/ml DNase-free RNaseA.

2.2.2. Plasmid midipreps by alkaline lysis

50ml cultures were set up by 1:500 dilution of a starter culture into selective LB medium and grown at 37°C for 12-16 hours. Cells were harvested and plasmid DNA isolated using the QIAfilter Plasmid Midi Kit (QIAGEN) following manufacturer's instructions. The yield and purity of DNA isolated was determined spectrophotometrically, given that 1 A260 unit = 50μg/ml of double-stranded DNA.
and pure DNA preparations have $A_{260}/A_{280}$ values of ~2.0. For multiple plasmid DNA preparations of the same construct, columns were regenerated after use by passing through another volume of elution buffer, followed by one volume of 0.5x TE, 50% ethanol. Columns were stored dry at room temperature.

2.2.3. Amplification of DNA using the Polymerase Chain Reaction

DNA templates were amplified by PCR using Taq DNA polymerase (Roche) or Pfu Turbo DNA polymerase (Stratagene) if 3' to 5' proofreading activity was required to increase fidelity. Reactions were performed on variable amounts of DNA template (<10µg/ml) in 1x reaction buffer with 250µM each primer (ordered from Sigma or MWG Biotech AG, Ebersberg, Germany) and 2.5 to 5U Taq/ Pfu DNA polymerase. The following cycles were carried out in a Biometra T3 Thermocycler: 1. 95°C 3min; 2. 95°C 1min; 3. (Tm of primer - 5)°C 1min; 4. 72°C (expected product size in kb) x1min; 5. return to 2. for 25 to 35 cycles; 6. 4°C hold

2.2.4. Agarose gel electrophoresis

DNA samples were separated according to size by standard agarose gel electrophoresis using the RunOne electrophoresis system (Embi Tec, San Diego CA). Samples in 1x DNA sample buffer were loaded on 1% agarose gels (Bioline, London) and run in 1x TAE buffer, with both gel and buffer containing 0.2µg/ml ethidium bromide. The bromophenol blue in the sample buffer runs at approximately the same rate as linear double-stranded DNA 300bp in length, whereas xylene cyanol FF runs at approximately the same rate as linear double-stranded DNA 4kb in length.

Stock solutions:

6x DNA sample buffer:
0.25% bromophenol blue
0.25% xylene cyanol FF
15% Ficoll Type400 (Pharmacia) in dH$_2$O
50x TAE (11):
242g Tris base
57.1ml glacial acetic acid
100ml 0.5M EDTA pH8.0 in dH2O

2.2.5. Extraction of DNA from agarose gels

After electrophoresis, relevant bands visualised by UV illumination were excised using a razor blade and DNA extracted using the QIAquick Gel Extraction Kit (QIAGEN) following manufacturer's instructions.

2.2.6. Restriction digestion of DNA

Restriction digests were performed using enzymes from New England Biolabs in the reaction buffer and at the temperature appropriate for the enzyme(s) used.

2.2.7. Blunting of 5' and 3' overhangs

After letting restriction digest proceed to completion, reactions were supplemented with 33μM each nucleotide and 2.5U DNA polymerase I Klenow Fragment (New England Biolabs) and incubated 15min at 25°C. Reactions were then stopped by addition of EDTA to 10mM and heat-inactivation at 75°C for 20min.

2.2.8. Clean-up of DNA from enzymatic reactions

DNA was purified from enzymatic reactions using the QIAquick PCR Purification Kit (QIAGEN) following manufacturer's instructions.
2.2.9. **Ligation**

Ligations were performed using T4 DNA ligase (New England Biolabs) with an excess of insert relative to vector. Control ligations were set up in parallel with buffer replacing the insert to estimate the background due to self-ligation of the vector. In blunt-end ligations, vector was pre-treated with Calf Intestinal Alkaline Phosphatase (New England Biolabs) to remove the 5' phosphoryl termini required by ligases and thus reduce vector-only background.

2.2.10. **Preparation of *E. coli* competent cells**

A single colony from a freshly streaked plate was inoculated into 2ml LB and grown overnight at 37°C. 1ml of this starter culture was then diluted into 500ml LB containing 10mM MgCl₂ and grown at 18°C with vigorous shaking until the OD₅₉⁵ reached 0.25 to 0.7 (36 to 72 hours). Flasks were then cooled on ice for 10min. Cells were harvested by centrifugation at 2500g for 10min at 4°C, resuspended in 80ml ice cold TB buffer and incubated in an ice bath for 10min before harvesting again as before. Cells were then gently resuspended in 20ml TB containing 7% DMSO and incubated on ice for another 10min before snap freezing in liquid nitrogen in single-use aliquots, which were stored at -80°C until use. Transformation efficiencies were typically in the range of 0.5-1x10⁸ colonies/μg plasmid DNA.

Stock solutions:

**TB buffer:**
10mM Pipes  
15mM CaCl₂  
250mM KCl  
pH adjusted to 6.7 with KOH, then added  
MnCl₂ to 55mM  
filter-sterilised with a 0.45μM filter and stored at 4°C
2.2.11. *E. coli* transformation

Competent *E. coli* prepared as described above were transformed by heat-shock. Aliquots of cells were thawed and incubated on ice with the plasmid DNA or ligation reaction for 30min. Cells were then transferred to a 42°C water bath for 90s, then transferred back onto ice for 2min. Cells were allowed to recover in 1ml SOC medium in a 37°C shaker for 40min before being spread on an LB agar plate containing the appropriate antibiotic.

Stock solutions:

**SOC medium (1):**
- 20g bacto-tryptone
- 5g bacto-yeast extract
- 0.5g NaCl
- 186mg KCl
- pH adjusted to 7.0 with NaOH, autoclaved, then added
  - glucose to 20mM
  - MgCl₂ to 10mM

2.2.12. DNA Sequencing

For 1 reaction, 3μl miniprep / 250ng midiprep DNA template was supplemented with 1.6pmol primer and 4μl BigDye Terminator v 3.0 Ready Reaction mix (Applied Biosystems) in 10μl total volume. PCR reactions were performed in a Biometra T3 Thermocycler using the following cycles: 1. 96°C 2min; 2. 96°C 30s; 3. 50°C 15s; 4. 60°C 4min; 5. return to 2. for 25 cycles; 6. 4°C hold.

Sequencing reactions were then precipitated by the addition of 1.5μl 3M sodium acetate pH 5.2 to each tube, followed by 31.25μl ethanol and 7.25μl H₂O. Tubes were vortexed to mix and placed into a -80°C freezer for 15min before centrifugation in a benchtop microfuge at 18,000g for 30min at 4°C. Pellets (invisible to the naked
eye) were washed with 300µl 70% ethanol, centrifuged again for 10min and dried for 1hr at room temperature. Samples were passed on to the ICAPB sequencing facility for loading onto an ABI Prism 377 DNA sequencer (Applied Biosystems).

2.2.13. Sequence analysis

ABI sequence files were imported into the Sequencher sequence analysis program (Gene Codes Corporation, Ann Arbor MI) for comparison with other sequences and editing. Newly cloned sequences were compared to the non-redundant and EST DNA databases using the BLAST programme on the NCBI server. Cloning strategies were developed with the help of hypothetical restriction digests performed using the Gene Jockey II programme (Biosoft, Cambridge). Multiple alignments of protein sequences were constructed using the ClustalW programme on the EMBL-EBI server.

2.2.14. Screening of chicken λ cDNA libraries

Hybridisation probes were labelled as follows: 60µM each of dATP, dGTP, dTTP and 50µCi α-32P-labelled dCTP were added to 100ng of a 500bp DNA template amplified by PCR in a radioactive isotope room. 10U DNA polymerase I Klenow Fragment (New England Biolabs) was added and reactions incubated at 37°C for 30min. Reactions were then stopped by the addition of EDTA to 50mM and incubation at 75°C for 20min. Labelled probes were found to be stable for several weeks if stored at 4°C. Probes were boiled for 10min and rapidly chilled on ice just before use.

Incorporation of label was confirmed by thin layer chromatography: 1µl of probe was spotted on a thin strip of PEI cellulose (Macherey-Nagel, Dueren, Germany). The strip was then inserted into a beaker containing a small volume of 0.75M NaH2PO4 pH3.45 such that the spotted probe remained above the liquid level. When liquid had risen to the top of the strip by capillary action, the strip was removed from the beaker and exposed to X-ray film in an autoradiography cassette.
While the labelled DNA probe is unable to move, any unincorporated label will rise with the liquid. Incorporation can thus be measured by comparison of the stationary and mobile phase on the exposed film.

A λZAP random-primed DU249 cDNA bacteriophage library (prepared by S. Kandels-Lewis, Earnshaw laboratory, University of Edinburgh using the Stratagene λZAP vector) was used to infect XL1-Blue MRF’ bacteria and plated out on large square dishes as per manufacturer's recommendations. Following induction of lysis, some of the phage on each plate was transferred onto Hybond-N nitrocellulose membranes (Amersham Pharmacia), taking care to mark the position on the plate using a pattern of holes punched through the membrane and underlying agar. Following denaturation, neutralisation, and washing steps (see manufacturer's protocol) DNA was fixed to the membranes by UV crosslinking in a UV Stratalinker (Stratagene). Membranes were pre-hybridised with 3x SSPE, 5x Denhardt's, 1% SDS, 25μg/ml salmon sperm DNA (boiled just before use and chilled on ice) for 1hr at 65°C, then hybridised by addition of probe (~0.5 to 2x10^6 counts/ml hybridisation buffer) overnight at 65°C. Membranes were then washed 2x10min with 2xSSPE, 0.1%SDS, followed by 2x15min with 1xSSPE, 0.1%SDS and finally 2x15min with 0.1xSSPE, 0.1%SDS, all steps being carried out at 65°C with pre-warmed solutions. Membranes were then blotted dry and exposed to X-ray film in an autoradiography cassette with intensifying screen at -80°C over several days. Spots of signal on the developed X-ray films were then traced back to regions on the agar plates that contained the phage of interest. Such regions were excised with the wide end of glass pipette and the phage recovered by briefly vortexing in 1ml SM buffer containing 20μl chloroform. This phage (which may be stored at 4°C) was then used to reinfect XL1-Blue MRF’ bacteria and plated on 10cm dishes, aiming for ~250 plaques per dish. Following this secondary and, where necessary, a tertiary screen, pure phage isolates were obtained. The pBluescript SK (-) phagemid was then excised from the λZAP phage as detailed in the manufacturer's protocol, and sequenced using the T3 and T7 primers.

Stock solutions:
20x SSPE (1l):
175.3g NaCl
27.6g NaH₂PO₄·H₂O
7.4g EDTA
pH adjusted to 7.4 with NaOH

50x Denhardt’s (50ml):
500mg Ficoll Type400 (Pharmacia)
500mg polyvinylpyrrolidone
500mg BSA
filter sterilised and stored at -20°C

2.2.15. RNA extraction and RT-PCR

HeLa RNA was extracted from 3x10⁷ cells as described by Comczinsky and Sacchi, 1987 (Chomczynski and Sacchi, 1987): Cells were washed twice in PBS and trypsinised. Trypsin was removed by washing cells with PBS. After centrifugation at 500g for 3min, the cell pellet was resuspended in 1 ml of lysis buffer (4M guanidine thiocyanate, 25mM sodium citrate, 0.5% sarcosyl, 1% β-mercaptoethanol). 0.1 volume of 2M sodium acetate pH4.0 and 1 volume of H₂O saturated phenol were added and the lysate chilled on ice for 10 min. 0.2 volume of chloroform was then added and the mixture centrifuged at 8,000g for 20 min. at 4°C. The RNAs present in the aqueous phase were precipitated with 1 volume of isopropanol for 1hr at -20°C and pelleted by centrifugation at 18,000g for 10min at 4°C. The pellet was then washed with 70% ethanol, and the RNA resuspended in water. The yield of RNA was determined spectrophotometrically at 260nm, given that 1 A₂₆₀ unit = 40μg/ml of single-stranded RNA. The reverse transcription reaction was then performed on 3μg of RNA using SuperScriptII RNaseH⁻ reverse transcriptase (Invitrogen) and random or specific primers as indicated, following manufacturer’s recommendations. PCR reactions were then performed using 1/10 of the RT product per reaction.
2.3. Protein methods

2.3.1. Expression of fusion protein in BL21 cells

BL21 bacteria were transformed with the prokaryotic expression construct of interest as described above. An overnight culture of cells grown from a single colony was diluted in LB medium containing the appropriate antibiotic and grown at 37°C until the OD₆₀₀ of the culture reached 0.6. Protein expression was then induced with 1mM of the galactose analogue IPTG, and cells were grown for at least two more hours. Bacteria were harvested by centrifugation at 3500g for 10min at 4°C, and pellets stored at -80°C until further use. The optimum conditions for protein expression, final concentration of IPTG, presence or absence of the protease inhibitor PMSF in the culture medium, and duration of induction, were determined experimentally for each construct with small 50ml cultures, taking 1ml aliquots at different timepoints after induction from induced and uninduced cultures. These were collected in microcentrifuge tubes, pelleted by centrifugation and resuspended in 200µl 1x SDS sample buffer by sonication for 20s and boiled for 5min. 15µl of each sample was run on an SDS-polyacrylamide gel and stained by Coomassie.

2.3.2. Purification of His-tagged fusion proteins under denaturing conditions

The bacterial pellet was thawed and resuspended in 20ml 8M urea 50mM NaH₂PO₄ pH7.6 containing 0.5mM PMSF, sonicated 3x 20s, and incubated 3 hours at room temperature with gentle agitation. Meanwhile, nickel sepharose was prepared as follows: 2.5ml chelating sepharose (Amersham Pharmacia) was washed several times by pelleting at 500g for 2min and resuspension in dH₂O, then charged by incubation.
with 2ml 0.1M NiSO₄ for 5min with gentle agitation, and unbound NiSO₄ was washed out with dH₂O. At the end of the 3 hour incubation, insoluble material in the lysate was removed by centrifugation at 76,000g for 20min at room temperature. The supernatant was then incubated with the nickel sepharose for 15min with gentle agitation. The nickel sepharose and bound protein were then recovered by centrifugation at 500g for 2min, and washed sequentially with 15ml 8M urea 50mM NaH₂PO₄ pH7.6 containing 0.5mM PMSF (lysis buffer) and 15ml 8M urea 50mM NaH₂PO₄ pH6.5 containing 0.5mM PMSF (wash buffer). The nickel sepharose resin was then transferred to a 'Poly-Prep' chromatography column (Bio-Rad), and bound protein eluted in 1ml fractions with 8M urea 50mM NaH₂PO₄ pH4.5 containing 0.5mM PMSF (elution buffer). Aliquots of original extract, insoluble pellet, washes and elution fractions were kept for analysis by SDS-polyacrylamide gel electrophoresis and Coomassie staining. Where necessary, fusion protein was further purified by passing over a hydroxyapatite column. For this, pooled fractions containing the fusion protein were first dialysed against 7M urea 10mM Na₂HPO₄ pH7.6 overnight at 4°C. Samples were then applied to 2.5ml washed hydroxyapatite (Bio-Rad), and incubated with the sample for 20min at 4°C with gentle agitation. Resin was recovered by centrifugation at 500g for 2min and washed 3x 20min with 10ml of 7M urea 10mM Na₂HPO₄ pH7.6 at 4°C. Resin was then transferred to a chromatography column as above and connected to a gradient mixer, exposing the column to a linear gradient of 7M urea 10mM Na₂HPO₄ pH7.6 increasing to 100mM Na₂HPO₄, and collecting the eluate in 30 fractions of 1ml. Elution fractions were stored at -20°C.

2.3.3. Purification of GST-tagged fusion proteins

Bacterial pellets were resuspended in 25ml PBS containing 1% Triton X-100 and 0.5mM PMSF, sonicated 3x 20s on ice and incubated for 30min at 4°C with gentle agitation. Insoluble material in the lysate was removed by centrifugation at 76,000g for 30min at 4°C. The supernatant was then incubated with 1ml washed glutathione
sepharose (Amersham Pharmacia) for 30min at 4°C with gentle agitation. Resin was recovered by centrifugation at 500g for 5min, and washed with 5x 10ml PBS before transfer into a chromatography column and elution with 10mM reduced glutathione in 50mM Tris pH8.0 in 15 fractions of 1ml.

2.3.4. Generation of antibodies

For immunisation of animals, protein samples were dialysed against 0.1x PBS and concentrated in a speed-vac. Animals were immunised with 100-150µg protein (rabbits) or 20-60µg protein (mice) in <250µl total volume of 1xPBS with adjuvant, with two-three boosters of the same amount. Rabbit antibodies were raised by Diagnostics Scotland (Law Hospital, Carluke, Lanarkshire). Mouse antibodies were raised in-house by Dr. Merdes in accordance with Home Office regulations. Importantly, pre-immune bleeds from all animals were screened by immunofluorescence staining on cultured cells to avoid immunising animals with serum reactivities against centrosomes or other cellular structures (Connolly and Kalnins, 1978).

2.3.5. Affinity purification of antibodies

Fractions containing 25mg fusion protein were dialysed against 0.25x coupling buffer (0.025M NaHCO₃, 0.125M NaCl, pH 8.3) containing 0.5mM PMSF overnight at 4°C. They were then concentrated four-fold in a vacuum centrifuge to 4ml of 5mg/ml protein. 0.5g CNBr-activated Sepharose 4B (Amersham Pharmacia) was washed 3x with 8ml 1mM HCl in a 15ml polypropylene tube and recovered by centrifugation at 200g for 90s. Washing steps were carried out rapidly (within 15min) to avoid loss of coupling efficiency due to hydrolysis. The washed gel was then incubated with the protein overnight at 4°C. At the end of the incubation, unbound protein solution ('flow-through') was recovered by centrifugation, and the resin washed 3x with alternating cycles of low pH (0.1M sodium acetate, 0.5M NaCl, pH 4.0) and high pH (0.1M Tris-HCl, 0.5M NaCl, pH 8.0). Resin was finally
transferred into a chromatography column in PBS containing 0.02% sodium azide, and stored at 4°C.

In each affinity purification, 2ml of serum was applied to the column, and the flowthrough reapplied 2x to maximise binding. 1ml fractions of PBS were then applied and the flowthrough collected until it turned clear. The column was then washed with another 20ml of PBS, before elution with 15x 1ml fractions of 200mM glycine, 0.5M NaCl, pH2.1-2.3. Eluted fractions were neutralised with 50μl 2M Tris, unbuffered, and the fractions containing affinity-purified antibody identified by Bradford analysis on 30μl eluate. Columns were regenerated by washing with PBS containing 0.02% sodium azide until pH was restored to ~7.0, and stored at 4°C.

2.3.6. Preparation of extracts from cultured cells

A near-confluent 10cm tissue culture dish (~0.5 to 1x10⁷ cells) was washed with PBS, and cells scraped off in ~1ml of PBS and transferred into a microcentrifuge tube. Tubes were then centrifuged briefly to pellet cells, and boiled in 100μl 1x SDS sample buffer for 5 min. 20 to 30μl of this extract were used for loading onto SDS polyacrylamide gels. Where accurate determination of protein concentration was required, cells were trypsinised and resuspended in 5 volumes of PBS containing 10μg/ml soybean trypsin inhibitor (Roche) and 1mM PMSF. Cells were then pelleted by centrifugation at 500g for 2min, resuspended in 1x SDS sample buffer lacking β-mercaptoethanol or bromophenol blue and boiled for 10min. This loading buffer is compatible with the BCA assay, which was carried out as described below.

Extracts for protein interaction experiments were prepared by washing plates with PBS and scraping off cells in a small volume of PBS. Cells were then pelleted by centrifugation at 500g for 2min and resuspended in 1ml of lysis buffer containing 1mM PMSF and 10μg/ml each of leupeptin, pepstatin and chymostatin. The choice of buffer depended on the type of experiment, 1x PBS in GST pulldown experiments, 1x TBS (10mM Tris pH8.0, 150mM NaCl, 0.1% NP-40) in immunoprecipitations with GFP antibody and 1x KHM buffer (78mM KCl, 50mM Hepes, pH7.0, 4mM MgCl₂, 2mM EGTA) in immunoprecipitations with PCM-1 antibody. Cells were disrupted by 50 strokes of the pestle of a tight-fitting Dounce homogeniser. Extracts were prepared on ice and used immediately.
2.3.7. Preparation of Xenopus egg extracts

Cytostatic factor-arrested Xenopus laevis extracts were prepared according to the protocol of Andrew Murray (Murray, 1991). Frog handling was performed by Dr. Merdes in accordance with Home Office regulations.

Frogs were primed for ovulation by injecting them with pregnant mare serum gonadotropin (PMSG). They were injected with 50U PMSG on day 1 and 25U of PMSG on day 3. Ovulation was induced on day 5 by injecting 150U of human chorionic gonadotropin (HCG). Eggs were collected on day 6 by gentle massage of the frogs and kept in 1X MMR buffer (100mM NaCl, 2mM KCl, 1mM MgCl₂, 2mM CaCl₂, 0.1mM EDTA, 5mM HEPES pH7.8. Eggs were dejellied using 2% cysteine pH7.8, in XB solution (100mM KCl, 1mM MgCl₂, 0.1mM CaCl₂) and washed in CSF-XB buffer (XB containing 10mM K-HEPES pH7.7, 50mM sucrose, 5mM EGTA pH 7.7). After addition of protease inhibitors (10μg/ml each of leupeptin, pepstatin and chymostatin) and 100 μg/ml cytochalasin B, the eggs were first compacted in a swinging bucket centrifuge at 300g for 1min at 4°C, then crushed by centrifugation at 11,000 g for 15min at 4°C. The cytoplasmic layer was collected using a needle and syringe via side puncture. The extract was either kept on ice to be used immediately or aliquotted and stored at -80°C.

2.3.8. Determination of protein concentration

Protein concentration was determined using the Bradford assay (Bradford, 1976). Samples were incubated in 1ml of Protein Assay Reagent (Bio-Rad) diluted 1/5 in dH₂O for ~5min. Absorbance at 595nm was then determined spectrophotometrically, and protein concentration determined by comparing readings with those obtained with known concentrations of BSA standards.
The protein concentration of cell extracts prepared in sample buffer containing SDS (but not yet β-mercaptoethanol or bromophenol blue) was determined by BCA assay (Smith et al., 1985) as follows:

1. ml of working reagent, made up from 100 parts of bicinechonic acid solution and 2 parts of 4% copper (II) sulfate pentahydrate (both Sigma), was added to 50μl of sample and incubated at 60°C for 15 min. Absorbance at 562 nm was then determined spectrophotometrically, and protein concentration determined by comparing readings with a standard curve of absorbance at 562 nm vs. protein concentration obtained with BSA standards.

2.3.9 Sucrose gradients

First, a 10-40% (w/w) sucrose gradient was prepared in a Beckman ultracentrifuge tube as follows: 1, 2, 3, or 4 g sucrose were mixed by vortexing with 9, 8, 7, or 6 g of CSF-XB (100 mM KCl, 0.1 mM CaCl₂, 2 mM MgCl₂, 10 mM Hepes pH 7.7, 50 mM sucrose, 5 mM EGTA). 250 μl of each layer was then carefully added to each ultracentrifuge tube starting with the highest percentage sucrose and left undisturbed overnight at 4°C for the layers to mix to give a continuous gradient. 500 μg of PtK₂ cell extract or 20 μl Xenopus egg extract in a total volume of 50 μl were then carefully added on top of the gradient and centrifuged at 50,000 rpm for 4 hrs at 4°C. In parallel, 50 μl of marker proteins, containing 6 μg each of thyroglobulin, catalase, BSA, and tubulin in CSF-XB, were run on a separate gradient. After centrifugation, 50 μl fractions were carefully taken from the very top of each gradient until all liquid had been removed. Any remaining pellet was then resuspended in CSF-XB. All fractions were then boiled in 1x SDS sample buffer and loaded on polyacrylamide gels for Coomassie staining (marker gradients) or immunoblotting (extract gradients). From the marker gradient, a linear graph could be plotted of fraction number of the peak of each marker protein against its known S value: thyroglobulin (173 kDa) 19S, catalase (60 kDa) 11.4S, BSA (70 kDa) 4.3S. Using this graph, the fraction numbers of each peak seen on the extract blots could then be converted into S values. Tubulin (50 kDa) in the marker gradient was used as
an internal control to compare with tubulin in the extract gradient as detected on a α-tubulin blot.

2.3.10. Immunoprecipitations

1 ml of extract (1.5 - 2 mg/ml) was pre-incubated with 25 μl washed protein A agarose beads (Invitrogen) for 1 hour at 4°C to reduce non-specific binding. Beads were then removed by centrifugation at 1,000g in a swinging bucket centrifuge, and the supernatant transferred to a fresh microcentrifuge tube. Antibody was then added to the extract at 2 (GFP antibody 3E6) or 8 μg (PCM-1 antibody) in the minimum volume of buffer so as not to dilute extracts, and incubated for 1 hour at 4°C. Rabbit non-immune IgG (Sigma) at equivalent concentration was used as a control in PCM-1 antibody immunoprecipitations. The antibody/extract mixture was then added to 25 μl washed protein A agarose beads and incubated for a further 3 hours at 4°C. Beads were then washed 5x with 1 ml of lysis buffer, and bound antibody eluted by boiling beads for 3 min in 1x SDS sample buffer.

2.3.11. GST-pulldown experiments

In each pulldown experiment, 200 μg GST-tagged protein or GST tag only (control) were added to 1 mg HeLa extract or 10 mg Xenopus egg extract, diluted to 1 ml in PBS and incubated for 1 hour at 4°C. GST fusion protein and associated interactors were then recovered by incubating the mixture with 100 μl glutathione sepharose beads for 30 min at 4°C. After extensive washes with PBS, bound protein was eluted with 10 mM reduced glutathione, pH 8.0, and precipitated with 1/10 volume of TCA for 1 hour on ice. Samples were then centrifuged at 18,000g for 30 min at 4°C and the protein pellet washed with acetone before resuspending in 1x SDS sample buffer and boiling samples for 5 min. Recovery of GST fusion protein was confirmed by SDS-PAGE and Coomassie staining, and the co-purification of other proteins tested by immunoblotting.
Two types of gel apparatus were used for electrophoresis of protein samples: Most samples were run on the EC120 Mini Vertical Gel System (E-C Apparatus Co, Holbrook NY). Samples for mass spectroscopy were run on a Protean II xi cell with 1.0mm spacers (Bio-Rad). The tables below give the recipes for separating and stacking gels. Minigels required ~5ml for the separating gel and ~2ml for the stacking gel. For larger gels, the volumes required were ~50ml and ~25ml, respectively. Samples were loaded in lx sample buffer and gels were run at ~15mA in lx electrode buffer until the dye front reached the bottom of the gel (~2 to 3 hours). Larger gels were run at ~30mA for ~7 hours.

<table>
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<th>10%</th>
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<td>2.5ml</td>
<td>2.08ml</td>
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<tr>
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<td>1.25ml</td>
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<tr>
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<tr>
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<td>30% acrylamide</td>
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<tr>
<td>Stacking gel buffer</td>
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<td>TEMED</td>
<td>2μl</td>
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Separating gel buffer:
1.5M Tris base
0.4% SDS
pH adjusted to 8.8 with HCl, and filter sterilised with 0.2μm filter

Stacking gel buffer:
0.5M Tris base
0.4% SDS
pH adjusted to 6.8 with HCl, and filter sterilised with 0.2μm filter

10x Electrode buffer:
0.25M Tris base
1.92M Glycine
1% SDS

4x Sample buffer:
250mM Tris pH 6.8
9.2% SDS
0.2% Bromophenol blue
40% glycerol
1% β-mercaptoethanol

2.3.13. Transfer of SDS-Polyacrylamide Gels

Gels were transferred onto nitrocellulose membranes (Millipore) using the MB140 Mini Blot Module in the gel tank of the EC120 Mini Vertical Gel System (E-C Apparatus Co, Holbrook NY) in 1x electrode buffer (see above for recipe) containing 20% methanol for 90min at 18V. Efficiency of transfer was monitored by staining of blots with 2% Ponceau S in 30% trichloroacetic acid.

For centrin immunoblots, Immobilon-P PVDF membranes (Sigma) were used instead of nitrocellulose. These were pre-soaked in dH₂O for 2min, then 100% methanol for 20s and finally transfer buffer for 15min before use. Transfer time was reduced to 45min, at the end of which transferred protein was fixed to the membrane with 0.2% glutaraldehyde in 10mM Tris pH7.4, 150mM NaCl for 45min. Membranes were then washed 3x 5min with d H₂O before blocking, etc.

2.3.14. Western blotting
After transfer, membranes were washed briefly in TBS containing 0.1% Tween, then blocked overnight in TBS Tween containing 5% (w/v) non-fat milk powder at 4°C. Membranes were then incubated in an appropriate dilution of primary antibody in milk (see table in Materials section) at 4°C for three hours. Where only small volumes of antibody were available, membranes were sealed in plastic pouches containing ~2ml of milk. These were the incubated on a rotating wheel to ensure complete coverage of the blot area. At the end of this incubation, membranes were washed extensively with milk for 1 hour at room temperature, then incubated with secondary antibody conjugated to horseradish peroxidase (Amersham Pharmacia) diluted 1/3,000 to 1/10,000 in milk for 1 hour. Membranes were then washed extensively in TBS Tween for 1 hour before detection by Enhanced Chemiluminescence.

For this, two solutions were prepared just before use, one containing 25μl 90mM coumaric acid/DMSO and 50μl 250mM luminol/ DMSO in 5ml 100mM Tris pH8.5, the other containing 3μl 30% H2O2 in 5ml 100mM Tris pH8.5. These were mixed together at the last minute and incubated with the membrane for 1 min before blotting it dry and exposing it to X-ray film in an autoradiography cassette.

Blots were stored in TBS at 4°C. Prior to re-blotting with a different antibody, blots were incubated with stripping buffer (2% SDS, 6.25mM Tris pH 6.7 containing 154μl/20ml β-mercaptoethanol) for 30min at 50°C. Blots were then washed 2x 15min with excess TBS Tween before blocking again as above.

2.3.15. Coomassie staining of SDS-Polyacrylamide Gels

Alternatively, gels were simultaneously fixed and stained for total protein using 0.25% Coomassie Brilliant Blue R250 in 50% methanol, 12% acetic acid for 1hr, then destained in 20% methanol, 10% acetic acid over several hours to overnight. A more rapid rate of destaining was achieved by the addition of a foam sponge to absorb the stain as it leaches from the gel. Once destained, gels were rinsed in dH2O and dried in a drying frame between two sheets of cellophane (Sigma).
2.3.16. Silver staining of SDS-Polyacrylamide Gels

Where improved sensitivity was required, gels were silver-stained as follows:

Gels were fixed for 2x 15min in 30% ethanol, 10% acetic acid, then incubated with 0.1% sodium thiosulfate in 30% ethanol, 100mM sodium acetate (pH adjusted to 6.0 with acetic acid)* for 30min. Gels were then rehydrated 3x 10min with dH₂O and incubated with 0.1% silver nitrate, 25μl 35% formaldehyde/100ml dH₂O* for 30min. After a brief rinse in dH₂O, gels were rinsed twice, then developed in 2.5% sodium carbonate, 50μl 35% formaldehyde/100ml dH₂O. To stop development, gels were then rinsed several times in 1% acetic acid over a period of at least 5min. Gels were then washed in dH₂O and stored as above.

*Note: These solutions were prepared fresh each time just before use.

2.3.17. Preparation of samples for mass spectroscopy

Gels were run and silver stained as described above, except for an extended rehydration step of 3x 20min. To have a permanent record of the banding pattern, the stained gel was scanned in while stored between two cleaned acetate sheets. Bands of interest were then excised using razor blades and stored at -80°C for processing for mass spectroscopy as follows:

Gel slices were first incubated in 300μl 200mM ammonium bicarbonate in 50% acetonitrile for 3x 30min at 30°C to remove SDS. Protein was then reduced in 300μl 20mM DTT, 200mM ammonium bicarbonate in 50% acetonitrile for 1 hour at 30°C. Samples were then washed 3x 5min in 300μl 200mM ammonium bicarbonate in 50% acetonitrile. Cysteines present in the protein were then alkylated in freshly prepared 100μl 50mM iodoacetamide in 200mM ammonium bicarbonate in 50% acetonitrile for 20min at room temperature in the dark. Gel slices were then washed 3x with 500μl 20mM ammonium bicarbonate in 50% acetonitrile before being cut into pieces of ~2 x 1mm with disposable scalpels. These were then incubated for ~10min in 100% acetonitrile until gel pieces had turned a greyish colour. Acetonitrile was then removed, and the gel pieces allowed to dry for ~15min. Finally, gel pieces were rehydrated in 59μl 50mM ammonium bicarbonate
containing 1μl sequencing-grade trypsin (Promega) for 30min at 30°C, then incubated for 16 to 24 hours at 32°C. All handling was performed wearing gloves, taking care to avoid contamination of samples with skin keratin. Samples were then given to the ICMB mass spectroscopy facility for further processing and analysis by MALDI-TOF.

2.4. Cell Biology methods

2.4.1. Transfections by the calcium phosphate method

Transfections of tissue culture cells with plasmid DNA were performed by precipitation of DNA with calcium phosphate as follows: 30μl of 2M CaCl₂ was added to 10μg DNA in 200μl TE, mixed and added to 240μl of 2x HEBS buffer (280mM NaCl, 50mM HEPES, 1.5mM Na₂HPO₄, pH7.15) with continuous vortexing, and the mixture incubated for 20 min. at room temperature. The DNA precipitate was then added to a subconfluent 6cm dish containing 5ml DMEM for 9 hours to overnight. The cells were then washed twice with PBS and fresh medium was added.

2.4.2. siRNA

21 nucleotide RNA oligomers corresponding to nucleotides 2190 to 2208 (GGGCUCUAAACGUGCCUCC, PCM-1.1) and 1465 to 1483 (UCAGCUUCGUGAUUCACUG, PCM-1.2) of human PCM-1, were synthesised in sense and antisense directions with dTdT overhangs at each 3’ terminus, deprotected and desalted (Xeragon AG, Huntsville, AL). Oligomers against centrin-3 (UGAAGUUGUGACAGACUGG), pericentrin (recognising both pericentrin A and B, GCAGCGAGCGUGAGAAGGAG), and ninein (UAUGAGCAUUGAGGCAGAG) were prepared in the same way. The
corresponding nucleotide sequences were identical in human and mouse mRNAs, except for PCM-1.1, which was human-specific. For annealing of siRNAs, 20µM single strands were incubated in annealing buffer (100mM potassium acetate, 2mM magnesium acetate, 30mM HEPES-KOH pH7.4) for 1min at 90°C, followed by 1 hour at 37°C (Elbashir et al., 2001). Transfections were carried out using Oligofectamine (Invitrogen) with 3µg siRNA on HeLa and U-2 OS cells grown overnight on 6-well dishes at 3x10⁴ cells/well. For timepoints beyond 60 hours, cells were split at 48 hours after the first transfection and then immediately subjected to a second treatment with siRNA. 3’Rhodamine-labelled and unlabelled control oligonucleotides (CGUACGCGGAAUACUUCGA plus 3’dTdT overhangs, control) were used to optimise transfection efficiency and to control for non-specific effects due to the presence of siRNAs in cells, respectively.

The level of protein depletion due to RNA silencing was determined by quantitative immunoblotting of cell extracts using ^125^I-labelled secondary antibody (Amersham Pharmacia Biotech). Equal amounts of protein extracts were separated by SDS-PAGE, and quantification was performed on immunoblots using a phosphoimager. Photometric quantification of immunofluorescence signals was performed from digital image files taken with a 40x/0.75NA lens that allowed a large depth of focus. Mean pixel values of 1-2 µm² areas were calculated using Adobe Photoshop. Control cells stained with non-immune serum and cells treated with control RNA, stained with the respective centrosomal antibodies, were used to calculate background levels and average control protein levels.

2.4.3. Microinjection experiments

Affinity-purified PCM-1 antibody was injected into Xenopus A6 cells cultured on glass coverslips at 2mg/ml in injection buffer (100mM KCl, 10mM potassium phosphate pH7.4). At 24 or 48 hours after injection, coverslips were fixed with methanol at -20°C and processed for immunofluorescence as above. Control injections were performed using rabbit IgG (Sigma) at the same concentration in injection buffer.
Purified dynamitin, prepared by Dr. Merdes, was injected into CHO cells at a concentration of 9mg/ml. Following two to four hours of incubation, cells were fixed and processed for immunofluorescence. Control cells were injected with fluorescently labelled secondary antibody.

Fertilised *Xenopus* embryos were injected with PCM-1 or control antibodies near the animal pole at the 2 to 4-cell stage and allowed to develop for three hours, during which time they were followed by video camera. Stills were captured and imported into Adobe Photoshop 6.0.

2.4.4. **Immunostaining and Immunofluorescence microscopy**

Cells grown on glass coverslips were fixed in one of three ways:

**Methanol fixation.**

Cells were fixed in -20°C methanol for 20 min, then washed and rehydrated in PBS 2x 5min. This method, which precipitates intracellular material, was used most often, as it is quickest and preserves antigens better than either formaldehyde or glutaraldehyde which act by covalently crosslinking structures (most antibodies against centrosome proteins other than PCM-1 did not work with these fixatives).

**Formaldehyde fixation.**

Cells were fixed in 3.7% formaldehyde in cytoskeletal buffer (100mM NaCl, 300mM sucrose, 10mM PIPES, 3mM MgCl₂, pH6.8 with KOH) for 10 min at room temperature. Unbound formaldehyde was quenched with 50mM ammonium chloride in PBS for 5min. Cells were permeabilised in 0.2% Triton X-100 in PBS for 5 min, then washed 2x 5min with PBS. This method was used for experiments involving GFP, as it preserved fluorescence better than methanol fixation. Microtubule staining was also found to be better with this fixative or with glutaraldehyde.

**Glutaraldehyde fixation.**

Cells were fixed in 0.5% glutaraldehyde in PHEM buffer (60mM PIPES, 25mM Hepes, 1mM EGTA, 2mM MgCl₂ pH6.9) for 10min at room temperature, then washed 3x 3min
with PHEM buffer. Cells were then permeabilised for 15min with 0.2% Triton X-100 in PHEM buffer, washed again 3x 3min with PHEM buffer and 3x 3min with PBS. Unbound glutaraldehyde was then quenched 2x 15min with 0.1% NaBH₄ in PBS, prepared just before addition onto the coverslips, while agitating coverslips to prevent the coverslips floating to the surface due to H₂ bubbles forming underneath. Finally, coverslips were washed 2x 5min with PBS.

Fixed cells were then permeabilised yet again for 5min with 0.1% Tween-20 in PBS, before blocking for 5min with 0.5% BSA in PBS-Tween. Cells were then incubated for 40min with primary antibody in blocking buffer at the dilution indicated in the table in the Materials section, washed 2x 5min with PBS and 1x 5min with PBS-Tween, and incubated with secondary antibody conjugated to FITC or Texas Red for 20min. At the end of this incubation, DNA was stained with DAPI at 2.5µg/ml for 2 1/2 min, before washing again 2x 4min with PBS and 1x 4min with PBS-Tween. Washing steps were carried out with coverslips in separate wells of a 6-well dish. Blocking and antibody incubation steps were carried out with coverslips placed on a stretch of parafilm surrounded with wet paper towels and covered with the lid of a 6-well dish to create a humid chamber. Coverslips were mounted on slides with Vectashield (Vector laboratories, Peterborough) to preserve fluorescence and sealed on the edges with nail varnish. For long-term storage, slide cases were kept at -20°C.

Slides were examined on a Zeiss Axioskop 2 fluorescence/ DIC microscope. Images were captured using a Zeiss Axiocam camera and AxioVision software and imported into Adobe Photoshop 6.0.

2.4.5. BrdU incorporation and modified immunostaining protocol

To identify cells undergoing S phase, the thymidine analogue BrdU (Sigma) was added to the growth medium at 20µM final concentration 20min before fixation. Cells were then processed for immunofluorescence as above for any marker in addition to BrdU, using a Texas Red - conjugated secondary antibody. Following
incubation with secondary antibody, coverslips were washed 3x 4min with PBS, then post-fixed with 3.7% formaldehyde in PBS for 10min. Unbound formaldehyde was quenched with 50mM ammonium chloride in PBS for 5min, and coverslips washed 1x 5min with PBS. DNA was rendered accessible to the anti-BrdU antibody by incubation with 2N HCl for 20min. Coverslips were then washed 2x 5min with PBS and 1x 5min with PBS-Tween before incubation with anti-BrdU antibody and anti-rat secondary antibody and DAPI as detailed above.

2.4.6. Treatment of cells with other reagents

Where applicable, the following reagents were added to the cell culture medium for the time indicated prior to fixation and staining: Brefeldin-A (Sigma) at 5μg/ml for 40 minutes; Nocodazole (Sigma) at 5μM for 40 minutes on ice; Taxol (Sigma) at 10μg/ml for 1 hour; Triton X-100 (Sigma) at 1% in PBS for 5min.

2.5. Yeast Two-Hybrid methods

2.5.1. Yeast transformations

This method is based on the protocol of Gietz and Woods (Gietz and Woods, 2002) and typically yields several hundred 100 transformants/μg of plasmid DNA. 25μl per transformation of yeast from a freshly streaked plate were resuspended in 1ml dH2O. Cells were pelleted at 18,000g for 15s, resuspended in 1ml of 100mM lithium acetate and incubated for 5min at 30°C. The volume representing a single transformation reaction was then pelleted and the following components added in the order listed: 240μl 50% (w/v) polyethylene glycol molecular weight 3350, 36μl 1M lithium acetate, 50μl 2mg/ml Salmon testes DNA DNA/TE (not sonicated, boiled for 5min and chilled on ice before use), 1μg plasmid DNA in 25μl dH2O. Samples were vortexed for 1min and incubated at 42°C for 20min. Cells were then pelleted by centrifugation at 18,000g for 25s and gently resuspended in 200μl dH2O. The cell
suspension was then spread on a dropout plate lacking the appropriate amino acid(s) and incubated at 30°C for 2-3 days until colonies appeared.

2.5.2. Preparation of yeast cell extracts for SDS Polyacrylamide Gel Electrophoresis

1.5ml overnight cultures were harvested by pelleting at 18,000g for 30s in a 2ml screw-cap microcentrifuge tube, and boiled for 5min. Pellets were then resuspended in 100μl PBS and ~200mg 0.5mm glass beads and the samples bead-beat 3x 45s in a Hybaid Ribolyser. Holes were then made in the top and bottom of the tubes and the tubes centrifuged in a swinging bucket centrifuge at 200g for 1min, collecting the flow-through in a 5ml polypropylene tube. Samples were then transferred into a microcentrifuge tube and boiled in 1x SDS sample buffer lacking β-mercaptoethanol or bromophenol blue for 5min. The protein concentration was then determined by BCA assay and 50μg sample loaded on an SDS polyacrylamide gel.

2.5.3. Mating (small-scale)

1 colony of each strain from a freshly streaked plate was grown overnight (20-24 hours) in 0.5ml YPDA in a single 10ml culture tube at 30°C with moderate shaking (200rpm).
100μl of the overnight culture was then spread on -Leu-Trp dropout plates to select for diploids and incubated at 30°C for 2-3 days until colonies appeared.

2.5.4. Mating (library-scale)

This method is based on the protocol of Bendixen et al. (Bendixen et al., 1994).
Several colonies of a yeast strain carrying the bait plasmid from a freshly streaked plate were grown overnight in 5ml of -Trp dropout medium in a sterile 50ml polypropylene tube at 30°C with moderate shaking (200rpm). Yeast were then diluted into 150ml of -Trp dropout medium such that the OD$_{600}$ of the culture the
next morning would be close to 1.0. This was achieved by using the following formulae:

Volume required = \( \text{Volume of culture} \times \text{final } \frac{OD_{600} \text{ of culture desired}}{\text{current } \frac{OD_{600} \text{ of culture}}{x} \times 2} \times \frac{\text{time of incubation} - \text{doubling time}}{\text{doubling time}} \)

Doubling time = \( \text{incubation time} \times \ln 2 \)

\[ \ln 2 + \ln \left( \frac{\text{final } OD_{600}}{\text{of culture}} \right) - \ln \left( \text{initial } OD_{600} \text{ of culture} \right) \]

One frozen vial of yeast strain carrying the library plasmid was then thawed and added to 20ml of YPDA containing 6μg/ml tetracycline in a sterile 100ml conical flask. Cells were then allowed to recover for 15min at 30°C with gentle agitation (120rpm). 80 OD 600 units of bait strain culture were then added and 1/12 of the total volume of the mixture pressed through a Swinnex 47 filter holder carrying a pre-wetted and air-dried 0.22μm MF-Millipore filter membrane (both Millipore). The filter membrane was then placed cells-up on a YPDA agar plate containing 6μg/ml tetracycline. This procedure was then repeated 11 times for the remainder of the mixture, and the plates incubated for 4 to 5 hours at 30°C. Cells were then washed off each membrane in an empty petri dish with 2x 1ml of -Leu-Trp-His dropout medium containing 6μg/ml tetracycline, and the ~25ml of cells collected in a sterile 50ml polypropylene tube. The cells suspension was then spread over 100 -Leu-Trp-His dropout plates containing 6μg/ml tetracycline and the plates incubated at 30°C for several days until colonies formed.

To determine the number of diploids screened and the mating efficiency, 50μl of a 1/1,000 dilution in -Leu-Trp-His dropout medium was spread on -Leu, -Trp, and -Leu-Trp plates. These numbers were then calculated as follows:

Number of diploids screened = number of colonies on -Leu-Trp plate x 25ml / 50μl

Mating efficiency = \( \frac{\text{number of colonies on -Leu-Trp plate}}{\text{number of colonies on -Leu plate} + \text{on -Trp plate}} \)

2.5.5. liquid β-galactosidase assay

The strength of a two-hybrid interaction was determined by liquid β-galactosidase assay as follows:
400μl of each overnight culture were diluted into 4ml of YPDA medium and incubated at 30°C on a rotating wheel until the OD_{600} of the culture reached 0.5-0.8. The OD_{600} was then recorded for all cultures and 1.5ml of each culture transferred to a microcentrifuge tube and pelleted by centrifugation at 18,000g for 30s. Pellets were then resuspended in 1.5ml Z buffer (10.7g/l Na_{2}HPO_{4}2H_{2}O, 6.22g/l NaH_{2}PO_{4}2H_{2}O, 0.75g/l KCl, 246mg/l MgSO_{4}, pH7.0) by vortexing, pelleted again and finally resuspended in 300μl Z buffer. 100μl cell suspension was then transferred into a fresh microcentrifuge tube and lysed by 3 cycles of freezing in liquid nitrogen and thawing at 37°C in a polystyrene rack. 700μl Z buffer containing 2.7μl/ml β-mercaptoethanol was then added to each tube as well as to 5 'blanks' containing 100μl Z buffer only, followed by 160μl 4mg/ml o-Nitrophenyl-β-D-Galactopyranoside (ONPG) in Z buffer, and samples incubated in a 30°C water bath in the dark until a yellow/straw colour developed (5min to overnight for weaker interactions). The reaction was then stopped by the addition of 400μl 1M sodium carbonate and the time elapsed since addition of ONPG, as well as the OD_{420} against a blank stopped at the same time recorded. The strength of interaction was determined according to the formula:

\[
\beta\text{-gal units} = 1,000 \times \frac{OD_{420}}{0.5 \times OD_{600} \times \text{time elapsed/min}}
\]

2.5.6. **Plasmid DNA isolation from yeast**

This method is based on the protocol of Alister Ward (Ward, 1990), and typically yields ~100 *E. coli* transformants per transformation experiment. 2ml overnight cultures in stationary phase were harvested by pelleting at 18,000g for 30s, and resuspended in 200μl 2.5M LiCl, 50mM Tris-HCl pH8.0, 4% Triton X-100, 62.5mM EDTA in a 2ml screw-cap microcentrifuge tube. 200μl 1:1 phenol chloroform and ~200mg of 0.5mm glass beads were then added and the samples bead-beat 3x 20s in a Hybaid Ribolyser. Holes were then made in the top and bottom of the tubes and the tubes centrifuged 3x 1min in a swinging bucket centrifuge at 200g, collecting the flow-through in a 5ml polypropylene tube. 160μl of the upper aqueous phase was transferred to a fresh microcentrifuge tube and DNA precipitated with 400μl ethanol and 16μl 3M sodium acetate pH5.2 at -80°C for at least 20min.
Samples were then centrifuged at 18,000g for 20min, and the pellet washed with 70% ethanol before resuspending in 20μl dH2O. Competent bacteria were then transformed with 5μl of the yeast miniprep DNA and plasmid DNA prepared as previously described.

2.6. Generation of plasmid constructs used in this study

2.6.1. pRSET-C hs PCM-1 C-ter

The C-terminus of human PCM-1 comprising nucleotides 4993-6095 after the start codon was amplified by PCR from a HeLa cDNA library (provided by S. Kandels-Lewis, University of Edinburgh) using primers AD01 and AD04 and cloned into the vector pGEM-T. The insert was then excised using PstI and NcoI and cloned into the bacterial expression vector pRSET-C.

2.6.2. pBS gg PCM-1 full-length

A partial chicken PCM-1 clone was obtained from a Bursal EST collection then managed by Dr. Jean-Marie Buerstedde, University of Hamburg, Germany (clone 4d19r1, Accession number AJ398048, now obtainable through RZPD, Berlin, Germany). This clone was fully sequenced and found to contain a 3.7kb insert with a stop codon and poly A sequence at the 3’ end, but incomplete at the 5’ end, lacking the first ~2,000 nucleotides of human PCM-1. I therefore screened a chicken λ cDNA library with a hybridisation probe derived from the 5’ end of this EST by PCR with primers AD06 and AD07. One clone (pBS 2.3A) was obtained that contained the first 2.6kb including an initiator ATG codon in the same place as in human PCM-1, and a ‘full-length’ clone assembled in pBluescript using BamHI and NotI to replace the 3’end of clone pBS 2.3A with that of pSPORT EST4d19r1. However, a
conceptual translation of the open reading frame of the chicken PCM-1 thus assembled yielded a protein with a predicted molecular weight of 185kDa, significantly less than that of human PCM-1. Immunoblots with PCM-1 failed to show such a difference in mobility. Furthermore, the 3’ end of chicken PCM-1 beyond nucleotide 5021 showed no sequence homology to PCM-1 in other vertebrate species (human, mouse, and frog full-length sequences had by then been submitted to databases, as well as short sequence reads from rat and zebrafish ESTs). I therefore screened the chicken λ cDNA library again with a hybridisation probe derived from the 3’ end of the confirmed sequence by PCR with primers AD23 and AD09 and, indeed, obtained several clones containing additional 3’ sequence including a stop codon in an equivalent position to that in human PCM-1 (nucleotide 5881/6202). A new, full-length PCM-1 construct was then assembled in several cloning steps: First, the new 3’ end was amplified by PCR with primers AD29 and AD30 from one 3’ clone, pBS 6.1, digested with BglIII and NotI and inserted into pBluescript. The 5’ end and middle of PCM-1 from pBS ‘full-length’ PCM-1 were then added using SalI and BglIII. Two BglIII sites 900 bases apart near the 3’ end of PCM-1 meant this section was lost in the previous two cloning steps. It was now reinserted from pBS 6.1 using BglIII, yielding the true full-length PCM-1 cDNA in pBluescript.

2.6.3. pRSET-A gg PCM-1 N-ter

Nucleotides 1-342 after the start codon were amplified by PCR from the chicken cDNA using primers Pcm1-D1-FG and Pcm1-R2-FG and cloned directly into the bacterial expression vector pRSET-A using BamHI and EcoRI.

2.6.4. pEGFP-N PCM-1 deletion constructs

pEGFP-N PCM-1 5’1 (amino acids 1-1468): A truncated 3’ end of PCM-1 (up to nucleotide 4570) was amplified by PCR with primers AD29 and AD40, digested with BglIII and SacII and inserted into pEGFP-N. The 5’ portion of PCM-1
(nucleotides 1-3971) was then reinserted from pEGFP-N PCM-1 full-length using BglII.

pEGFP-N PCM-1 5'2 (amino acids 1-1148): As with 5'1, a truncated 3' end (up to nucleotide 3610) was amplified by PCR with primers AD21 and AD41, digested with EcoRV and SacII and inserted into pEGFP-N. The 5' portion of PCM-1 of PCM-1 (nucleotides 1-2847) was then reinserted from pEGFP-N PCM-1 full-length using EcoRV.

pEGFP-N PCM-1 5'3 (amino acids 1-534): Nucleotides 1-1768 were amplified by PCR using primers AD39 and AD42 and inserted into pEGFP-N using XhoI and SacII.

pEGFP-N PCM-1 3'1 (amino acids 1463-1904): Here, a truncated 5' end (beginning nucleotide 4553) was amplified by PCR using primers AD46 and AD09 and inserted into pEGFP-N PCM-1 full-length using XhoI and AccI.

pEGFP-N PCM-1 3'2 (amino acids 1144-1904): Similarly, deletion construct 3'2 was generated by amplifying a truncated 5' end (beginning nucleotide 3596) was amplified by PCR with primers AD47 and AD14, digested with XhoI and EcoRI and inserted into pEGFP-N PCM-1 full-length.

pEGFP-N PCM-1 3'3 (amino acids 532-1904): Finally, deletion construct 3'3 was created by amplifying a truncated 5' end (beginning nucleotide 1760) with primers AD48 and AD22 and inserting it into pEGFP-N PCM-1 full-length with XhoI and EcoRV.

2.6.5. pEGFP-N gg PCM-1 5'1 GFP

This construct was generated by excising the GFP open reading frame from the pEGFP-N PCM-1 5'1 construct with SmaI and NotI, blunting and religating.

2.6.6. pGEX-4T-2 hs centrin-3

Human centrin-3 was obtained by PCR from a HeLa cDNA library (provided by S. Kandels-Lewis, University of Edinburgh, Scotland) using primers AD52 and AD54 and cloned into the bacterial expression vector pGEX4T2 (Amersham Pharmacia
Biotech) using BamHI and EcoRI. Sequencing confirmed it to be identical to the previously published human centrin-3 sequence (Middendorp et al., 1997).

2.6.7. pEGFP-C hs centrin-3

This construct was generated by excising the centrin-3 open reading frame from pGEX-4T2 centrin-3 with BamHI and EcoRI and cloning it into pEGFP-C linearised with BglII and EcoRI.

2.6.8. Yeast two-hybrid plasmid constructs

Human PCM-1 was cloned by PCR from a cDNA prepared by RT-PCR on RNA extracted from HeLa cells.

PCM-1 part A (corresponding to amino acids 1-720) was amplified by PCR in two fragments using the primer pairs AD57/AD60 and AD59/AD58 and each cloned into the PCR cloning vector pGEM-T. The latter was then cloned into pBluescript using PstI and SalI, followed by the former, which was amplified by PCR using the proofreading enzyme Pfu polymerase and primers AD57 and AD60, digested with PstI and inserted into the cloning intermediate opened up with SmaI and PstI.

PCM-1 part B (corresponding to amino acids 720-1380) was cloned using the same strategy, with the primer pairs AD61/AD64 and AD63/AD62.

For PCM-1 part C (corresponding to amino acids 1360-2063), two fragments were again amplified by PCR, using the primer pairs AD65/AD68 and AD67/AD66, and cloned into pGEM-T. The former was then amplified by PCR using the proofreading enzyme Pfu polymerase and primers AD65 and Sp6, digested with SalI and cloned into pBluescript opened up with SmaI and SalI. The second part was then inserted using EcoRV and SalI.

The three parts of PCM-1 thus assembled were then cloned into the two bait vectors pGBT9 (for low level expression) and pGBKT-7 (for high level expression). For pGBT9, both vector and insert were prepared by digestion with SmaI and SalI. For pGBKT-7, insert prepared by digestion with SmaI and SalI was inserted into the
positive control vector pGBKT7-53 cut with EcoRI (blunted with Klenow) and Sall. All baits were confirmed by sequencing the insert and junctions.

For control matings, empty vector bait pGBKT-7 was prepared by digestion of pGBKT7-53 with BamHI and EcoRI, blunting with Klenow and re-ligating. Prey vector pGADGH was made available to me by Dr. McNeill in the form of the yeast two hybrid positive pGADGH – cdc1. PCM-1 parts A, B, and C were inserted into this vector by digestion of pGBT9-hs PCM-1 A/B/C with SmaI and Sall and insertion into pGADGH-cdc1 cut with SmaI and Xhol. Centrin-3 was also inserted into this vector, by digestion of pGEX4T2-hs centrin-3 with BamHI (blunted with Klenow) and Xhol and insertion into pGADGH-cdc1 cut with Sphi (blunted with Klenow) and Xhol. Empty vector pGADGH was prepared by digestion of pGADGH-cdc1 with BamHI and Xhol, blunting with Klenow and re-ligating.
Chapter 3. Results

3.1. Generation of reagents and localisation of PCM-1

As a first step, antibodies were raised against PCM-1 to enable me to visualise the intracellular localisation of the protein and potentially to be able to perturb its function by microinjection of this antibody into cultured cells. At this time, only the human sequence of PCM-1 was known from the work of Balczon et al. (Balczon et al., 1994). Since the authors successfully raised antibodies to the C-terminus of the protein, a similar region was used to generate my antibody.

3.1.1. Generation of antibodies against the C-terminus of human PCM-1

The C-terminus of human PCM-1 comprising amino acids 1665-2024 was cloned by PCR from a HeLa cDNA library as described in the Materials and Methods section and expressed as a 6xHis-tagged fusion protein in bacteria. Fusion protein was then isolated using 8M Urea and purified over nickel sepharose and hydroxyapatite columns, concentrated and dialysed against PBS before injection into two rabbits, designated 816 and 817. Three booster immunisations were given to stimulate the immune response. A clear signal was observed by immunofluorescence in the second bleed and animals were sacrificed. Affinity-purified antibody was obtained by passing serum over a column of the same antigen coupled to a CNBr-activated Sepharose column. The same antigen was used to raise polyclonal antibodies in mice. One of the two mice immunised with PCM-1 antigen showed signs of distress and had to be sacrificed before completion of the experiment. By immunoblotting, a band of ~230 kDa was observed with extracts prepared from human cells and frog egg extracts using both rabbit antibodies, close to the expected size of 228kDa for human PCM-1 (Figure 2A).
Figure 2. Characterisation of PCM-1 antibody.
A. Immunoblot with PCM-1 antibody 817 on HeLa cell (H) and Xenopus egg (X) extract. Positions of molecular weight markers indicated on left. B. Immunofluorescence staining of interphase HeLa cell with anti-PCM-1 and anti-γ-tubulin antibodies. C. Interphase HeLa cells stained with anti-PCM-1 and anti-β-tubulin antibodies. DNA visualised with DAPI (blue). Bars in B and C 10 μm.
3.1.2. **Localisation of PCM-1 in cultured cells**

Immunofluorescence microscopy on HeLa cells gave a staining pattern for PCM-1 that was very different from the tight pericentriolar material staining observed by Balczon *et al.* (Balczon *et al.*, 1994). Instead, numerous punctae were seen scattered throughout the cell, but concentrated around the centrosome in most cells (Figure 2B,C). Upon entry into mitosis, PCM-1 did not disperse as reported, but first localised very tightly to the spindle poles in prophase and prometaphase and, indeed, also prior to recognisable mitotic events such as nuclear envelope breakdown and formation of the mitotic spindle (Figure 3). However, PCM-1 was found to be dispersed in most metaphase figures, and in all anaphase cells. By telophase, PCM-1 could be seen enriched around the centrosomal microtubule organising centre as well as proximal to the cleavage site, in an area where the minus ends of midbody microtubules terminate. This staining pattern was unaffected by the choice of fixative – methanol, formaldehyde or glutaraldehyde. It was subsequently confirmed by transfection of a full-length PCM-1 GFP construct, which localised indistinguishably from endogenous PCM-1 as detected by my antibody (see below).

In late 1999, Kubo *et al.* (Kubo *et al.*, 1999) showed that PCM-1 is not a pericentriolar material protein, but instead localises to centriolar satellites, electron-dense granules ~70-100nm in diameter found scattered around centrosomes. The staining pattern I obtained is in agreement with their results. The reason for the discrepancy between the localisation pattern of PCM-1 observed by Kubo *et al.* (Kubo *et al.*, 1999) and myself and the pericentriolar material localisation reported by Balczon *et al.* in 1994 (Balczon *et al.*, 1994) remains unclear, but may reflect a cross-reactivity of the original PCM-1 antibody.

3.1.3. **Association of PCM-1 satellites with Golgi membranes in late mitosis**

Since the cellular localisation of PCM-1 is strongly reminiscent of that observed for Golgi resident proteins, I co-stained HeLa cells for PCM-1 and GM130, a *cis*-Golgi matrix protein (Nakamura *et al.*, 1995), as well as p115, a protein involved in intra-Golgi transport (Waters *et al.*, 1992a). As can be seen in Figure 4A, PCM-1 staining
**Figure 3.** PCM-1 localisation during the cell cycle. HeLa cells in A. late G2. B. Prophase. C. Prometaphase. D. Metaphase. E. Anaphase. F. Cytokinesis. PCM-1 concentrates at the microtubule organising centre early in mitosis, before dispersing in metaphase. It relocates to the centrosomal area during cytokinesis, and to the minus ends of midbody microtubules. Bar in F. 10μm.
Figure 4. PCM-1 and the Golgi apparatus.
HeLa cells co-stained for PCM-1 (green) and GM130 (A, B) or p115 (C,D) (red). DNA visualised with DAPI (blue). Untreated cells in interphase (A) and cytokinesis (B). C. Cells treated with 1% triton for 5min prior to fixation. D. Cells treated with 5μg/ml BFA for 40min prior to fixation. Bars in B. and D. 10μm.
in interphase extended beyond the Golgi region and no significant association was observed between PCM-1 punctae and Golgi membranes. In mitosis, Golgi proteins initially concentrated near the microtubule organising centre in prophase and later dispersed throughout the cell as the Golgi apparatus fragmented into vesicles (Nelson, 2000). PCM-1 behaved in a similar manner, yet no colocalisation was observed at these stages (data not shown). However, interestingly, PCM-1 satellites and Golgi membranes were found in close proximity to each other at the intracellular bridge during late telophase/ cytokinesis, the significance of which is not clear (Figure 4B; see discussion below). It should be noted that, unlike Golgi residents, PCM-1 staining was unaffected by pre-extraction of cells with 1% Triton X-100/ PBS for 5min prior to fixation (Figure 4C). It was also unaffected by treatment of cells with 5µg/ml brefeldin A for 40min (Figure 4D), a drug that causes the redistribution of Golgi proteins to the endoplasmic reticulum by blocking a membrane-recycling pathway between the endoplasmic reticulum and the cis/medial Golgi (Lippincott-Schwartz et al., 1989). These results are in agreement with the detergent-resistance of PCM-1 granules observed by Kubo et al. by immunoelectron microscopy (Kubo et al., 1999), and suggest that while PCM-1 may be associated with membranous structures for example at the intracellular bridge in cytokinesis, PCM-1 particles themselves are not membrane-bound nor are they an integral part of the Golgi apparatus.

3.1.4. Taxol and Nocodazole experiments

Treatment of cells with taxol, a microtubule-stabilising drug is known to result in the formation of parallel bundles of microtubules in interphase cells and numerous non-centrosomal radial asters in mitosis (De Brabander et al., 1986; De Brabander et al., 1981). As can be seen in Figure 5, PCM-1 accumulated at the ends of these interphase microtubule bundles and at the centre of the mitotic asters. Interestingly, PCM-1 was also prominently seen at the cytoplasmic ends of midbody microtubules. While the polarity of the microtubule ends at which PCM-1 accumulated was not determined in my experiments, comparison to previously published studies employing the hook decoration method (Euteneuer and McIntosh, 1981) suggested that they are most likely minus ends. This result is consistent with the microtubule
Figure 5. Taxol experiment.
HeLa cells were treated with 10μg/ml taxol for 1 hour at 37°C prior to fixation and staining for PCM-1 (FITC) and β-tubulin (TexasRed). DNA visualised with DAPI (blue). PCM-1 can be seen accumulating at microtubule minus ends both in interphase and mitotic cells. Bar 10μm.

Figure 6. Nocodazole experiment.
HeLa cells were treated with 5μM nocodazole for 40min on ice prior to fixation and staining for PCM-1 (FITC) and β-tubulin (TexasRed). DNA visualised with DAPI (blue). PCM-1 localisation appears unchanged (compare with Figure 3). Bar 10μm.
minus end-directed movement of PCM-1 particles observed by Kubo et al. (Kubo et al., 1999).

In the converse experiment, microtubules were depolymerised by treatment of cells with the microtubule destabilising drug nocodazole on ice. As illustrated in Figure 6, PCM-1 localisation at all cell cycle stages was unaffected. This unexpected result may be explained by the large size of PCM-1 particles limiting their diffusion, especially at low temperatures. Indeed, as described later in this chapter, prolonged exposure of cells to nocodazole at 37°C resulted in the formation of cytoplasmic aggregates of PCM-1 (Figure 18).

3.1.5. Cloning and characterisation of chicken PCM-1

With a view towards the eventual generation of a targeted disruption of the PCM-1 gene in the chicken B-cell line DT40 (Buerstedde and Takeda, 1991; Wang et al., 1996), I cloned the chicken homologue of PCM-1 as described in the Materials and Methods section. The full-length PCM-1 cDNA sequence was submitted to GenBank (Accession number AJ508717) and is provided here in Figure 7A. A conceptual translation of chicken PCM-1 yields a protein of 1904 amino acids, 66% identical and 75% similar to human PCM-1. Two large gaps in the chicken sequence relative to the human one occur near the N-terminus. Using the GENSCAN gene prediction program on the human genomic sequence at the PCM-1 locus (chromosome 8p21.3-22) revealed that the missing sequence was the product of the first half of exon 3 and the whole of exon 4. RT-PCR with primers surrounding this sequence on RNA from chicken DU249 cells resulted in a number of minor bands that were larger in size than the predominant one which corresponded to the cloned chicken PCM-1 sequence. These bands were cloned in the PCR cloning vector pGEMT and sequenced. The larger size was found to be due to the presence of the missing exons 3.1 and 4, in all possible combinations (see Figure 7B,C). An even fainter band of larger size was found to contain exon 5 (not present in the published sequence of human or mouse PCM-1 but found in ESTs from these species as well as in the published sequence of frog and fugu PCM-1) as well as exons 3.1 and 4. However, this exon (isolated only once) contained an in-frame stop codon, which may be a PCR artefact (not shown). Nevertheless, these results indicate the existence
Figure 7. Sequence of chicken PCM-1.

A. Sequence of cDNA with conceptual translation, as submitted to GenBank (Accession number AJ508717). Arrows indicate positions of alternatively spliced exons 3.1 (B) and 4 (C).
of a large variety of alternative spliceforms both in chicken and other species, which may have functional significance. Sequence analysis reveals that PCM-1 is a 'pioneer protein', with no identifiable functional motifs (a predicted ATP/ GTP binding motif in human PCM-1 (Balczon et al., 1994) is not found in PCM-1 from other species), no similarities to other proteins or homologues in lower eukaryotes such as *Drosophila melanogaster* or *Caenorhabditis elegans*. What it does show is a preponderance of coiled coil regions in the N-terminal half of the protein, a characteristic of many centrosomal proteins. Sequence comparison between PCM-1 from different vertebrate species showed considerable sequence conservation spread throughout the protein and not limited to particular regions (Figure 8).

3.1.6. **Generation of a full-length chicken PCM-1 expression construct fused to GFP**

To confirm the immunofluorescence localisation of PCM-1, I generated a full-length PCM-1 expression construct fused to GFP in the vector pEGFP-N (C-terminal enhanced GFP tag) as described in the Materials and Methods section. Transfection of this construct into HeLa and other cultured cell lines resulted in a weak fluorescent signal localising indistinguishably from endogenous PCM-1 (Figure 9).

3.1.7. **Generation of a species-specific antibody against the N-terminus of chicken PCM-1**

Polyclonal antibodies were further generated in two mice against the N-terminus (amino acids 1-114) of the chicken PCM-1 protein, expressed as a 6xHis tagged fusion and purified as described above. Despite the N-terminus of PCM-1 being highly conserved between vertebrate species (see Figure 8), polyclonal antibodies from both animals were found to be species-specific, and reacted by immunofluorescence with PCM-1 in chicken cell lines such as DU249, but not in cultured cells from other species such as HeLa (Human), COS-7 (African Green Monkey), NIH3T3 (Mouse), CHO (Hamster), PtK2 (Rat Kangaroo) or A6 (*Xenopus*). They further reacted with chicken PCM-1 expressed in non-avian cells.
Figure 8. Multiple alignment for PCM-1 from various vertebrate species, prepared using the ClustalX alignment program. The colouring scheme used highlights conserved or similar amino acids. Also indicated are the alternatively spliced exons (underlined) located near the N-terminus. The numbering 1-38 refers to the intron-exon structure found in the human PCM-1 gene.
Figure 9. Cellular localisation of GFP-PCM-1 full-length. HeLa cells grown on glass coverslips were transiently transfected with pEGFP-N PCM-1 full-length. Coverslips were mounted on slides and examined ‘live’ by phase contrast (left, blue in merge) and GFP fluorescence microscopy (centre, green in merge). Panels show cells in interphase (A), prometaphase (B) and cytokinesis (C). Bar 10 μm.
3.1.8. Generation of deletion constructs and cellular localisation

In an attempt to dissect the function of PCM-1 and identify functional domains, serial deletion mutants were generated on the basis of the full-length PCM-1 GFP construct removing varying amounts of the N- and C-terminus of PCM-1 (see Figure 11) as described in the Materials and Methods section. These constructs were then transfected into HeLa and chicken DU249 cells to determine their intracellular localisation and possible interactions with the endogenous, full-length PCM-1. As illustrated in Figure 11, the rabbit polyclonal antibodies 816/817 raised against the C-terminus of human PCM-1 specifically detect the endogenous protein in cells transfected with the C-terminal deletion mutants 5’1 – 5’3, while the species-specific mouse polyclonal antibody raised against the N-terminus of chicken PCM-1 only recognises the endogenous protein in chicken cells transfected with the N-terminal deletion mutants 3’1 – 3’3. The results summarised in Figure 12 lead to a number of conclusions: First, deletions of the C-terminus of the protein up to amino acid 1148 (deletion mutants 5’1 and 2) resulted in a protein that is markedly overexpressed, compared to the full-length construct or N-terminal deletions. It is possible that a sequence motif regulating PCM-1 protein levels has been deleted in these mutants, and there is some evidence from the yeast two-hybrid screen performed with this region of the protein to support this (see below). Second, expression of all constructs containing the central portion of PCM-1 (amino acids 532-1148, deletion mutants 5’1 and 2, 3’3) affected the localisation of the endogenous protein, recruiting it to aggregates (5’1 and 2) or dispersing it throughout the cell (3’3). This suggests the existence of a self-interaction motif in this region. Again, there is yeast two-hybrid data to support this (see below). It is tempting to speculate that oligomerisation of PCM-1 is important in the formation of PCM-1-containing centriolar satellites, but more work is required to confirm this. Since PCM-1 is capable of interacting with other molecules of itself, it invalidates the use of deletion mutants in the presence of endogenous full-length PCM-1 to identify potential targeting motifs. In this respect it
Figure 10. Characterisation of mouse antibody raised against N-terminus of chicken PCM-1.
A. Two panels illustrating the staining pattern observed in chicken DU249 cells. Note that PCM-1 in these cells appears more clustered around the centrosome than in HeLa cells. The lower panel shows the characteristic midbody localisation pattern of PCM-1. B. HeLa cells transfected with chicken PCM-1 deletion mutant 5'1. The mouse anti-PCM-1 antibody fails to recognise the endogenous (human) PCM-1 but does react with the transfected chicken construct. DNA visualised with DAPI (blue). Bars 10µm.
Figure 11. Diagrammatic representation of chicken PCM-1 indicating amino acid positions of PCM-1 deletion constructs and immunogens used to raise PCM-1 antibodies.

Note that the N-terminal chicken-specific antibody only recognises the endogenous PCM-1 in chicken cells transfected with the N-terminal deletion mutants 3'1 to 3'3, while the C-terminal antibody likewise only recognises the endogenous PCM-1 in cells transfected with the C-terminal mutants 5'1 to 5'3.
Figure 12. Localisation of GFP PCM-1 deletion constructs and effects on endogenous PCM-1 protein. ~20x longer exposure required for constructs 5'3, 3'1, 3'2, and 3'3 due to weaker GFP signal. Bars 10μm.

<table>
<thead>
<tr>
<th>Construct</th>
<th>localises?</th>
<th>delocalises endogenous PCM-1?</th>
</tr>
</thead>
<tbody>
<tr>
<td>5'1 1-1468</td>
<td>(✓), aggregates</td>
<td>✓</td>
</tr>
<tr>
<td>5'2 1-1148</td>
<td>(✓), aggregates</td>
<td>✓</td>
</tr>
<tr>
<td>5'3 1-534</td>
<td>X, diffuse</td>
<td>X</td>
</tr>
<tr>
<td>3'1 1463-1904</td>
<td>X, diffuse</td>
<td>X</td>
</tr>
<tr>
<td>3'2 1144-1904</td>
<td>X, diffuse</td>
<td>X</td>
</tr>
<tr>
<td>3'3 532-1904</td>
<td>X, diffuse</td>
<td>✓</td>
</tr>
</tbody>
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is interesting to note that cells with lower levels of expression of deletion mutants 5'1 and 5'2 did show a normal localisation of the fusion protein.

### 3.2. Effect of PCM-1 inhibition on centrosomal proteins

Given that PCM-1 localises to centriolar satellites rather than the pericentriolar material that gave it its name, and that these only infrequently associate with the centrosome ('centriolar satellites' is also a misnomer), there was no a priori reason for PCM-1 involvement with centrosomes and centrosome function. As it turned out, PCM-1 plays an important part in centrosome function through its interaction with other centrosomal proteins. This was revealed using a number of approaches to inhibit PCM-1 function.

#### 3.2.1. Antibody microinjection experiments

Antibody microinjection experiments have been used successfully in the past to elucidate the function of other centrosomal proteins, for example the role of γ-tubulin in microtubule nucleation (Joshi et al., 1992). This method relies on the potential of injected antibody to delocalise the targeted protein and/ or prevent its interaction with other proteins through steric hindrance. *Xenopus* A6 cells were used in my experiments, since they grow at room temperature and environmental levels of CO₂.

Microinjection with affinity-purified PCM-1 antibodies 816 and 817 did not result in gross morphological defects, and positive cells could be identified 24-48 hours after injection. Injected cells were stained with all available antibodies against centrosomal proteins. The results are shown in Figure 13. PCM-1 was much reduced or no longer detectable with the mouse antibody against the C-terminus of PCM-1 in 89% of injected cells (N=88). Large cytoplasmic aggregates of centrin were observed, in addition to centrosome staining, in 67% of injected cells (N=284). In 10% of injected cells, these aggregates took on a filamentous or ribbon-like
Figure 13. Antibody microinjection experiments. *Xenopus* A6 cells were microinjected with PCM-1 or control antibody 24 hours prior to fixation, and stained for the injected antibody (red) and the centrosomal marker (green) indicated. PCM-1 antibody microinjection reduced centriolar satellite staining of PCM-1 and led to the formation of pericentriolar aggregates of centrin and pericentrin. γ-tubulin localisation was unaffected. DNA visualised with DAPI (blue). Bars 10μm.
character. Such aggregates were observed only once in control injected cells (<1%, N=180). In addition, a weak effect was seen on pericentrin, which was found in small pericentriolar aggregates in 17% of PCM-1 antibody injected cells (N=283). In control injected cells, such aggregates were rarely observed (3%, N=94). In contrast, γ-tubulin localisation was not significantly affected, with 1% of cells showing γ-tubulin abnormalities (N=174), a frequency comparable to that seen in untreated cells (<1%, N=424).

3.2.2. Experiments with the PCM-1 deletion mutant 5'I

How does a centriolar satellite protein like PCM-1 affect the localisation of centrosomal proteins? The answer to this question was provided by experiments using the C-terminal deletion mutant 5'I (Figure 14). As described above, this mutant delocalises endogenous PCM-1 to large cytoplasmic aggregates. Significant amounts of centrin-3 and pericentrin accumulated at the same protein aggregates in essentially all overexpressing cells (96% (N=200) and 93% (N=401) of transfected cells, respectively). In addition, the normally pericentriolar material-localised protein ninein was dispersed into numerous small foci (90% of overexpressing cells, N=249). However these only partly colocalised with the large PCM-1 aggregates. It should be noted that not all of the aggregating centrosomal protein is likely to originate from the centrosome. Many centrosomal proteins occur in large cytoplasmic pools (Moudjou et al., 1996; Paoletti et al., 1996) and it is likely that the majority of the protein was recruited from this pool. While it is difficult to be certain, double dots likely representing centrosomes were occasionally seen with centrin and pericentrin antibodies in overexpressing cells (not shown). Unlike centrin, pericentrin and ninein, the localisation of γ-tubulin was not significantly altered by mutant PCM-1 overexpression. Since PCM-1 aggregates recruit many of the proteins that characterise centrosomes, I wanted to test whether they in fact represent newly synthesised centrosomes. I therefore stained transfected cells with an antibody against acetylated tubulin, a marker for stable microtubules, including the 9 triplet microtubules of the centriolar cylinders (Piperno et al., 1987). As seen in Figure
Figure 14. Effect of overexpression of PCM-1 deletion mutant 5'1 on centrosomal proteins. HeLa cells transiently transfected with PCM-1 5'1 or β-galactosidase (control) were stained for transfected protein and centrosomal proteins indicated. Ninein experiments were carried out in CHO cells. DNA visualised with DAPI (blue). Bars 10μm.
14, acetylated tubulin was enriched at a double-dot representing one pair of centrioles, and at cytoplasmic fibres representing stable microtubules. The large protein aggregates of mutant PCM-1, however, did not show any enriched staining of acetylated tubulin, indicating that no new centrioles, and therefore no additional centrosomes had been formed in these cells. Overexpression of a control protein, β-galactosidase, had no effect on PCM-1 or other centrosomal proteins. These experiments were performed in a number of cell lines (HeLa, PtK₂, CHO, and NIH3T3), except those for ninein, where the available antibody limited me to rodent cells.

During my initial characterisation of this deletion mutant, I sometimes observed what appeared to be γ-tubulin staining as detected with the TexasRed secondary antibody colocalising with the strong GFP signal of PCM-1 5’1. This fluorescence pattern was not seen when the slides were first examined in the red channel. However, prolonged exposure of the sample to excitation by blue light in the green channel resulted in signal now appearing in the red channel, giving the false impression of γ-tubulin staining on PCM-1 aggregates (Figure 15). The explanation for this curious result is a little appreciated property of GFP, photoactivation (Elowitz et al., 1997; Sawin and Nurse, 1997). Prolonged excitation of GFP by blue light over several seconds results in a molecule that can now be excited by green light and emit in the red i.e. it behaves like a red fluorescent dye like rhodamine. This has serious implications for the use of GFP constructs in colocalisation studies, especially where, as in this case, the GFP signal is strong. Consequently, for the above experiments, an untagged version of PCM-1 5’1 was used, which was detected with the chicken-specific anti-PCM-1 antibody.

3.2.3. Depletion of PCM-1 by siRNA

The experiments above indicate that PCM-1 can, under certain circumstances, recruit large amounts of the centrosomal proteins centrin, pericentrin and ninein, suggesting that a similar interaction is occurring normally. Since the experiments of Kubo et al. (Kubo et al., 1999) showed that PCM-1-containing centriolar satellites are continually shuttling between the centrosome and the surrounding cytoplasm, one
Figure 15. Photoactivation of GFP
The panels above show γ-tubulin signal in the red channel before (left) and after (right) examining the slide in the green channel (middle panel) for 10 seconds. As explained in the text, photoactivation of GFP causes it to fluoresce over a wider spectrum, creating the false impression of γ-tubulin localising to the PCM-1 aggregates.
possibility is that this interaction is taking place in transit to the centrosome whereupon the centrosomal proteins are released and incorporated into the pericentriolar material, while PCM-1 recycles to the cytoplasm. This model predicts that in the absence of PCM-1, assembly of these proteins will be disrupted.

To test this hypothesis, I used the recently developed technique of short interfering RNAs (Elbashir et al., 2001) to specifically deplete PCM-1 mRNA. Two oligomer pairs directed against different regions of human PCM-1 were used, as well as labelled and unlabelled control oligomers (see Materials and Methods). Transfection efficiencies of 95% (N=522) were reached in HeLa cells using labelled control oligomers, and I was able to remove 34% (siRNA PCM-1.1) or 82% (siRNA PCM-1.2) of the original amount of PCM-1 in HeLa cell cultures, as judged by quantitative immunoblotting (Figure 16A). Photometric analysis on an individual cell level revealed depletion levels ranging from 69% to 99%, with an average depletion of 89% using siRNA oligomer PCM-1.2. Consistent with my hypothesis, centrosomal levels of centrin, pericentrin, and ninein were significantly reduced after PCM-1 depletion (Figure 16B). These effects were quantified by photometric analysis in U-2 OS cells, where centrosomal centrin was reduced to 39%, pericentrin to 36%, and ninein to 38% of control levels. By contrast, γ-tubulin levels remained unchanged (99%). Because previous work had indicated an interaction between PCM-1 and dynactin (Balczon et al., 1999), I also measured the centrosomal levels of the dynactin component p150Glued. These also remained largely unaffected after PCM-1 depletion (82% of control levels). Culturing of cells in the presence of PCM-1 siRNA for periods longer than 120 hours resulted in extensive cell death.

3.3. Mechanism of PCM-1 action at the centrosome

How does PCM-1 act in the recruitment of centrosomal proteins? The model outlined in the previous section proposes PCM-1-containing centriolar satellites functioning as shuttle complexes in the transport of proteins to the centrosome. It should therefore be possible to observe small amounts of centrin, pericentrin and ninein along with PCM-1 in transit to the centrosome. PCM-1 movement has
Figure 16. Depletion of PCM-1 by RNA interference leads to reduced centrosomal targeting of centrin, pericentrin, and ninein. A. Immunoblot on extracts prepared from HeLa cells that have been left untreated (untr.), or transfected with control oligomer or one of PCM-1 oligomers 1 and 2 (PCM-1.1, PCM-1.2) for the time indicated. Blots were probed with PCM-1 antibody as well as antibodies for NuMA and centrin-3 to control for loading. B. Immunofluorescence microscopy on U-2 OS cells treated with control or PCM-1.2 oligomer for 48 hours prior to fixation and staining for the antibody pair indicated. Bar 10μm.
previously been shown to occur along microtubules in a dynein-dependent manner (Kubo et al., 1999). Removing either the tracks (microtubules) or the motor (dynein) should therefore lead to similar defects in centrosomal protein assembly as inhibition of PCM-1 itself. Finally, if PCM-1 is engaged in the transport of centrosomal proteins, it should be possible to demonstrate an interaction, direct or indirect, between PCM-1 and these proteins.

3.3.1. Colocalisation of centrin, pericentrin and ninein with PCM-1 on centriolar satellites

In most cell types, including HeLa, COS-7 and NIH3T3, antibodies against centrin, pericentrin and ninein appeared to show an exclusively centrosomal localisation, with a variable cytoplasmic background. Occasionally, faint punctae were visible, especially when viewed over the dark background of a nucleus, but such low levels do not permit careful colocalisation studies. However, in other cell types, including mouse myoblasts, CHO and PtK₂ cells, satellite staining was clearly visible and coincided with PCM-1 (Figure 17). Centriolar satellite staining of γ-tubulin was never observed. While other interpretations are possible, the simplest is that the same holds true in all cell types, but that the relative amounts of centrosomal cargo vary. Interestingly, a fraction of dynein and dynactin was also found to colocalise with PCM-1 on some centriolar satellites, further evidence for an involvement of the dynein/dynactin microtubule motor complex in PCM-1 movement.

3.3.2. CHO nocodazole experiments

If PCM-1 transport occurs along microtubules, prolonged exposure of cells to microtubule-destabilising drugs should have a similar effect on centrosomal protein assembly as inhibition of PCM-1. To test this, CHO cells were exposed to 17μM nocodazole, sufficient to depolymerise all microtubules. Cells remained viable under these conditions for at least 40 hours, although there was an accumulation of multinucleate cells resulting from aberrant and aborted mitoses (data not shown). As early as 1 hour after addition of nocodazole, large cytoplasmic aggregates of
Figure 17. Centriolar satellites.
Certain cell lines such as CHO, PtK₂ and mouse myoblasts show centriolar satellite staining of centrin-3, pericentrin and ninein. As illustrated above for CHO cells, these satellites also contain PCM-1. The few pericentriolar punctae of dynein and dynactin also colocalise with PCM-1. γ-tubulin was never observed on centriolar satellites. Bar 10μm.
centrosomal proteins could be observed (Figure 18). These contained centrin, pericentrin and ninein as well as PCM-1, the motor protein dynein and dynactin. Aggregates in this case are thus likely to be motor-cargo complexes that are unable to move towards the centrosome in the absence of microtubules, instead increasing in size as more centrosomal proteins are recruited. γ-tubulin localisation by contrast remained unaffected, suggesting that this proteins accumulates at the centrosomes by other means.

3.3.3. **Dynamitin protein microinjections**

I next examined the involvement of the dynein microtubule motor in assembly of centrosomal proteins. An excess of p50/dynamitin, a subunit of the dynein-activating dynactin complex, has been reported to sequester other dynactin components and thereby inhibit dynein motor function (Echeverri et al., 1996; Quintyne et al., 1999). I therefore microinjected CHO cells with purified p50/dynamitin. As shown in Figure 19, within 2 to 4 hours after injection PCM-1 was found to be dispersed in 77% of cells (Control injections 2%). The centrosomal localisation of centrin was also disrupted, with only 40% showing a concentration of centrin at the centrosome compared to 98% of control injected cells. The same was true for pericentrin (33% vs. 95%) and ninein (55% vs. 97%). Again, γ-tubulin localisation remained unaffected (97% vs. 99% of control cells). 100-200 cells were counted per injection and staining condition. While nocodazole treatment and dynamitin overexpression are both rather blunt tools, affecting a multitude of cellular processes, the results obtained are at least consistent with the proposed transport of centrosomal cargo by PCM-1 in a dynein- and microtubule-dependent manner.

3.3.4. **Immunoprecipitation experiments on PCM-1**

If PCM-1 is engaged in transporting centrosomal proteins such as pericentrin, centrin and ninein, it should be possible to demonstrate an interaction between PCM-1 and these proteins. During the course of my studies it was reported that pericentrin and PCM-1 interact as determined by co-immunoprecipitation in CHO cell extracts (Li et
Figure 18. CHO nocodazole experiment. Prolonged exposure of cells to nocodazole leads to the formation of cytoplasmic aggregates containing centrin-3, pericentrin, ninein and PCM-1 as well as the motor proteins dynein and dynactin. Centrosomal γ-tubulin localisation is unaffected. CHO cells were treated for 2 hours with 17μM nocodazole at 37°C prior to fixation and staining. Bar 10μm.
Figure 19. Dynamitin microinjections.
Microinjection of p50/dynamitin protein into CHO led to dispersal of PCM-1, centrin, pericentrin, and ninein. γ-tubulin localisation was unaffected. Control cells injected with fluorescently labelled antibody were unaffected. Cells were fixed 4 hours after injection. DNA visualised with DAPI (blue). Bar 10μm.
al., 2001). It should however be noted that these experiment were performed on cells treated with hydroxyurea and nocodazole – conditions that lead to the aggregation of centrosomal proteins as demonstrated in Figure 18. The other centrosomal protein that I found to completely colocalise with PCM-1 in the aggregates formed by overexpression of the deletion mutant 5'1 is centrin-3. The following sets of experiments therefore sought to confirm an interaction between these proteins.

I first employed the affinity-purified PCM-1 antibody 816 to immunoprecipitate PCM-1 from HeLa and *Xenopus* egg extracts. As shown in Figure 20A and B, a small amount of centrin co-immunoprecipitated with PCM-1 in PCM-1 immunoprecipitates, but not with control antibody. To test whether this weak interaction could be reproduced in a different way, I transfected cells with GFP (control) or PCM-1 full-length or deletion 5'1 – GFP and immunoprecipitated the fusion protein with GFP antibody (Figure 20C). I then probed blots with PCM-1 (Figure 20D) and centrin (Figure 20E) antibodies. Again, a small amount of centrin co-immunoprecipitated with PCM-1 in GFP-PCM-1 immunoprecipitates, but not with GFP alone. Interestingly, endogenous PCM-1 also immunoprecipitated with PCM-1 deletion mutant 5'1, confirming the immunofluorescence results of Figures 12 and 14.

### 3.3.5. GST-Centrin-3 pulldown & PCM-1

The converse experiment of immunoprecipitating centrin was not possible due to a lack of sufficient reagents. However, it proved to be easy to clone the centrin-3 cDNA and express the protein with an N-terminal GST fusion tag. Bacterial fusion protein was purified on a glutathione sepharose column as described in the Materials and Methods section, and dialysed against PBS. GST centrin-3 fusion protein or GST alone was then added to HeLa or *Xenopus* egg extracts and recovered with glutathione sepharose beads. As shown in Figure 21, GST centrin-3 eluate, but not that from GST alone, contained PCM-1, which represented approximately 1-2% of the total PCM-1 in the original extract.

Why does so little centrin bind to PCM-1? One potential explanation is that the interaction between a shuttle protein and its cargo is necessarily unstable. Another is that centrin, like its close homologue calmodulin, is an EF-hand protein.
**Figure 20.** Immunoprecipitation experiments.
C, D and E. IP with GFP antibody on extract from HeLa cells transfected with pEGFP-N empty vector (EV), PCM-1 full-length (fl), and PCM-1 deletion mutant 5’1. C. GFP immunoblot. Arrows indicate position of GFP/GFP-PCM-1 bands. D. PCM-1 immunoblot. E. Centrin immunoblot. Arrows indicate position of PCM-1/centrin bands.
Figure 21. GST Pulldown experiment.
200μg GST-centrin-3 fusion protein or GST only were incubated with 1.5mg HeLa cell extract/ 10-40mg Xenopus egg extract for 1hr at 4°C. GST protein and interacting proteins were then recovered using glutathione beads. Following extensive washes with PBS, bound proteins were eluted, TCA precipitated and loaded on polyacrylamide gels. A. Coomassie-stained gel. Note quantitative recovery of GST fusion protein and absence of major extract bands in eluate lanes. B. PCM-1 Immunoblot. Bound PCM-1 in GST-Centrin-3 eluate represents 1-2% of total PCM-1 in the original extract.
and its localisation has been found to be extremely sensitive to changes in calcium levels (Baron et al., 1994). It may be difficult to maintain intracellular calcium levels in vitro, although precautions were taken (such as not adding calcium ions or EDTA/EGTA to buffers).

3.3.6. **GFP-Centrin-3 & PCM-1**

To further examine the relationship between centrin-3 and PCM-1, a GFP-tagged centrin-3 expression construct was generated as described in the Materials and Methods section. When expressed in HeLa cells, GFP-centrin-3 was predominantly cytoplasmically localised, but there was an enrichment on the centrosome (Figure 22A) and the spindle poles in mitosis (not shown). A minority of cells showed GFP-centrin-3 localising to what appear to be stress fibres. Interestingly, in these cells endogenous PCM-1 was delocalised from the centrosome and dispersed throughout the cell (Figure 22B). The significance of these results is not clear at present (though see below). Finally, cells were cotransfected with GFP-centrin-3 and the PCM-1 deletion mutant 5'1. In COS-7, the results were as expected, with GFP-centrin-3 being recruited to the PCM-1 aggregates (Figure 22C). In HeLa cells, however, no PCM-1 aggregates were seen in cells co-expressing GFP-centrin-3 and PCM-1 5'1. Instead, mutant PCM-1 localised like wild-type PCM-1 in an apparent 'rescue' of the mutant phenotype (Figure 22D). Clearly, modulating centrin-3 levels affects the behaviour of PCM-1, both wild type and mutant.

3.3.7. **Sucrose gradients**

Some attempt was also made to isolate PCM-1-containing centriolar satellites and determine their other protein constituents. Extracts from cultured cells as well as *Xenopus* egg extracts were run on sucrose gradients and fractions blotted for PCM-1 and other centrosomal proteins. As seen in Figure 23, both PCM-1 and centrin-3 were spread over much of the gradient, with PCM-1 in PtK₂ cells showing two 'peaks' of 12 and 20S. A similar result was obtained by Balczon and colleagues (Li et al., 2001). This apparent heterogeneity in the size of PCM-1 particles should not
Figure 22. Experiments with GFP-Centrin-3.
A. HeLa cells transiently transfected with GFP-Centrin-3. GFP-Centrin-3 localises to the centrosome in interphase and the spindle poles in mitosis (not shown). B. In a subset of cells, GFP-Centrin-3 localises to what appear to be stress fibres. In these cells PCM-1 satellites are dispersed. C. Cos-7 cells co-transfected with GFP-Centrin-3 and PCM-1 5’1. GFP-Centrin-3 colocalises with PCM-1 in aggregates. D. The same experiment performed in HeLa cells. No aggregates form, and the PCM-1 deletion mutant localises normally. DNA visualised with DAPI (blue). Bar in D 10μm.
Figure 23. Sucrose gradients.
A. Coomassie-stained gel of protein standards (catalase (cat) 60kDa 11.4S, BSA 70kDa 4.3S, thyroglobulin (thyr) 173kDa 19S) run on a parallel gradient. Tubulin (tub) was used as an internal control to compare with tubulin in the extract gradient as detected on an α-tubulin reblot (not shown).

B. PtK₂ cell extract was loaded on a 10 - 40% sucrose gradient, and centrifuged at 50000rpm for 4 hours. Samples of all fractions were then run on polyacrylamide gels and blots probed for PCM-1 and centrin. Peaks of PCM-1 signal (boxed) were assigned S values by reference to the standards gradient.
come as a surprise, as centriolar satellites in electron micrographs appear as irregular granules between 70 and 100nm in diameter (Kubo et al., 1999). It is clear from this data that there are significant obstacles to be overcome if one were to try to biochemically purify PCM-1-containing complexes.

3.4. Cellular effects of PCM-1 inhibition

As described above, inhibition of PCM-1 affects the localisation of many (but not all) centrosomal proteins. In this section I want to address the cellular consequences of this mislocalisation, by examining some of the cellular structures and events in which centrosomes have been shown to be involved.

3.4.1. Microtubule organisation

The primary role of centrosomes is in the nucleation and organisation of microtubules, both in interphase and in mitosis. Many cells in culture do not possess a radially organised interphase microtubule network, as microtubules once nucleated are released from centrosomes. There are some exceptions to this, including PtK2, COS-7 and U-2 OS cells, where most microtubules remain anchored at the centrosome.

Inhibition of PCM-1 by transfection of the deletion mutant PCM-1 5'1 resulted in a loss of radial microtubule organisation, both in COS-7 and PtK2 cells. Instead, microtubules were randomly distributed throughout the cell (Figure 24). There was no significant association of microtubules with the PCM-1 aggregates. This loss of microtubule organisation could be due to a defect in nucleation of microtubules by the centrosome or a failure to anchor microtubules once nucleated. To distinguish between these possibilities, transfected cells were treated with 25μM nocodazole on ice for 40 minutes to depolymerise microtubules. The drug was then washed out with fresh medium and cells allowed to recover at 37°C. As shown in Figure 25, initial microtubule nucleation from the centrosome occurred normally in
Radial microtubule organization in cells transfected with...

<table>
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<tr>
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<th>PCM-1 5'1-GFP</th>
<th>GFP</th>
<th>Untransfected</th>
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<tr>
<td><strong>Cos-7</strong></td>
<td></td>
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<tr>
<td>33% (N=636)</td>
<td>83% (N=724)</td>
<td>85% (N=958)</td>
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<tr>
<td><strong>PtK-2</strong></td>
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<td>25% (N=307)</td>
<td>77% (N=398)</td>
<td>81% (N=1028)</td>
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**Figure 24.** Effect of PCM-1 deletion mutant overexpression on interphase microtubule organisation. COS-7 and PtK2 cells were fixed and stained for β-tubulin 24 hours after transfection with PCM-1 5'1GFP/GFP. A. COS-7 cell expressing 5’1GFP, showing a disorganised microtubule network. B. Control cells, expressing GFP alone, showing a normal (radial) microtubule organisation. C. Summary of results obtained in COS-7 and PtK2 cells. Bar in B. 10μm.
Figure 25. Microtubule regrowth assay.
A. PtK₂ cells transiently transfected with PCM-1 deletion mutant 5'1-GFP were treated with 25μM nocodazole on ice for 40min. The drug was then washed out and cells incubated with fresh medium at 37°C for the time indicated. Transfected cells initially show small centrosomal asters, yet over time this radial organization was lost, and microtubules became randomly distributed in the cell. Bar 10μm. B. These results expressed in graphical form.
control cells and in cells overexpressing PCM-1 5'1. Note also that no microtubule nucleation occurred at the PCM-1 aggregates. Over time, however, in cells expressing mutant PCM-1 this radial organisation was lost, and microtubules were once again randomly distributed throughout the cell, indicating a defect in microtubule anchoring rather than microtubule nucleation. This result is consistent with the observed delocalisation of the proposed microtubule anchor ninein, while the microtubule nucleator γ-tubulin was unaffected (Figure 14).

To examine the requirement for PCM-1 transport in more detail, siRNA experiments were performed in the human cell line U-2 OS, which has a predominantly radial microtubule organisation. While control-depleted cells showed a focussed microtubule network in 74% of cells, PCM-1-depleted cells 48 hours after transfection exhibited a radial microtubule organisation in only 34% of cells (Figure 26, N= ~1,000 cells for each depletion). Similar effects were observed by Dr. Merdes and Charlie Payne in siRNA experiments on mouse myoblasts (not shown). Since PCM-1 granules do not localise to microtubule minus ends it is unlikely that PCM-1 itself is involved in microtubule anchoring. Rather, PCM-1 may be transporting one or more microtubule anchoring proteins. I therefore examined each of the proteins known to be transported to the centrosome in a PCM-1-dependent manner in turn, depleting the mRNA by siRNA. Transfection of cells with oligomers specific to centrin-3 resulted in a reduction of protein levels, to 23% of control extracts, as determined by quantitative immunoblotting. Similar levels of depletion were reached with oligomers targeting ninein (29%) and pericentrin (20%). Pericentrin levels were measured by photometric analysis, since the available antibodies were unable to recognise the denatured protein by immunoblotting. Pericentrin depletion did not affect the radial organisation of microtubules. There was however, a marked reduction in the microtubule density at the centrosome. This may reflect a loss in centrosomal microtubule nucleation potential, as predicted by its close association with the microtubule nucleator γ-tubulin (Dictenberg et al., 1998). Both centrin and ninein depletion, however, led to a loss of radial microtubule organisation, with only 18% and 31% of cells, respectively, exhibiting a focussed network. Since centrin-3 has no known affinity to microtubules, I examined the localisation of ninein in centrin-3-depleted cells. As demonstrated in Figure 27, centrin-3 depletion also resulted in a loss of ninein from the centrosome.
Figure 2. Effect of depletion of PCM-1, centrin-3, ninein and pericentrin on microtubule organisation.
A. U-2 OS cells were treated with siRNA oligomers targeting PCM-1, centrin-3, ninein, or pericentrin as indicated for 48 hours prior to fixation and staining for the targeted protein and β-tubulin. PCM-1, centrin-3, and ninein depletion resulted in a loss of radial microtubule organisation, while pericentrin depletion resulted in a decrease in microtubule density. Bar 10µm.
B. Immunoblot for centrin-3 (top) and ninein (bottom) performed on extracts prepared from control and siRNA-treated cells.
Figure 27. Effect of centrin-3 depletion on ninein localisation. U-2 OS cells were treated with oligomers targeting centrin-3 for 48 hours prior to fixation, and stained for centrin and ninein. As seen in the panels above, depletion of centrin-3 resulted in a loss of centrosomal localisation of ninein. Bar 10μm.
3.4.2. Effect on Golgi

The Golgi apparatus is an organelle composed of stacks of membranes. An association with microtubule minus end-directed motors helps it to maintain its position near the centrosome and its delicate and dynamic structure. Treatment of cells with reagents that disrupt the microtubule network or the function of the microtubule motor dynein has been reported to lead to a vesiculation of the organelle (Burkhardt et al., 1997; Corthesy-Theulaz et al., 1992). As shown in Figure 28, inhibition of PCM-1 function by overexpression of the deletion mutant 5'1 or depletion by siRNA had no such gross effect on Golgi morphology, although there may be a slight enlargement of the cis-Golgi stacks. This may be a consequence of the disruption of the microtubule network. Interestingly, Golgi localisation in cells undergoing cytokinesis, a stage where there was a degree of colocalisation of PCM-1 with Golgi stacks, also appeared unaffected (data not shown).

3.4.3. Effects on cell cycle progression - cytokinesis and entry into S phase

PCM-1 inhibition may have wider effects on cell cycle progression. To address this, I used the early developing *Xenopus* embryo, where several rapid rounds of division take place and cell division defects are easily visible (Middendorp et al., 2000). As detailed in the Materials and Methods chapter, embryos were microinjected with PCM-1 antibody at the 2 to 4 blastomere-stage and allowed to develop for several hours. As shown in Figure 29, injection of PCM-1 antibody, but not control antibody led to significant undercleavage in the injected region, indicative of a cell division defect. Similar results were reported by Balczon and co-workers in mouse embryos (Balczon et al., 2002).

The difficulty with this system is that the abundant yolk hinders further analysis by immunostaining although certain laboratories have done this successfully (Gard et al., 1990). To circumvent this problem, I turned to the *Xenopus* A6 tissue culture cell system. Here, injection of PCM-1 antibody resulted in a significant increase in the frequency of binucleate cells, indicative of a failure of cytokinesis.
Figure 28. Effect of PCM-1 inhibition on Golgi morphology.
A. HeLa cells transiently transfected with PCM-1 deletion mutant 5'1. B and C. siRNA treatment of HeLa cells with control (B) or PCM-1 oligomers (C). Cells co-stained for PCM-1 5'1 (A) or PCM-1 (B,C) (red) and GM130 (green). Bar 10μm.
Figure 29. Microinjection of PCM-1 antibody into *Xenopus* embryos leads to cleavage failure.
A. PCM-1 antibody or Control antibody was injected into one blastomere of a 2 cell-stage embryo which was then left to develop for ~3 hours. In PCM-1 antibody injected embryos see undercleavage in the injected region (marked with an asterisk *). B. Statistics for above experiment.
Similar results were obtained by overexpression of the PCM-1 deletion mutant 5'1 and depletion of PCM-1 by siRNA (see Table in Figure 30). No defects in mitotic spindle morphology or chromosome segregation were observed with any method of PCM-1 inhibition. It should be noted, however, that cells with PCM-1 aggregates formed after overexpression of deletion mutant 5'1 were never observed in mitosis. According to work presented by the Tsukita laboratory, this is because PCM-1 aggregates disassemble upon entry into mitosis (Kubo, A., Tsukita, S. 2002. ASCB meeting, San Francisco. Abstract L126).

Several laboratories have reported that removal of centrosomes, by laser ablation (Khodjakov et al., 2000; Khodjakov and Rieder, 2001) or microsurgery (Hinchcliffe et al., 2001; Piel et al., 2001) results in defects in cytokinesis but has no effect on the earlier stages of mitosis, suggesting that centrosomes somehow enhance the fidelity of this process. In addition, these experimenters reported an apparently unrelated block in entry into S phase. Since PCM-1 inhibition (potentially through a lack of recruitment of centrosomal proteins) affected the first of these processes, I examined whether it may also affect the second. Indeed, as detailed in Figure 31, all three means of inhibiting PCM-1 function resulted in a marked decrease in the number of cells undergoing S phase.

As will be discussed further in the Discussion, these results invite further analysis as to the way by which PCM-1 influences these processes.

3.5. Interactors of PCM-1 and Centrin-3

The work presented in the previous sections has provided a lot of information that neatly fits into the transport model of PCM-1 function. Yet certain aspects of PCM-1 function remain unclear. How does PCM-1 act in the transport of centrosomal proteins – does it provide a link between the dynein motor and cargo in a similar manner as dynactin has been proposed to do? Is there any link between PCM-1 and Golgi proteins? How does PCM-1 act in ensuring proper completion of cytokinesis and what is the requirement for PCM-1 in S phase entry? The answers to many of
Percentage of binucleate cells:

PCM-1 RNAi in HeLa cells (N=3000):
3.5% Control 0.7%

PCM-1 antibody microinjection in A6 cells (N=360):
14% Control 4%

Overexpression of PCM-1 deletion mutant5'1 in HeLa cells (N=300):
8% Control 2%

Figure 30. Effect of PCM-1 inhibition on cytokinesis.
A. A6 cell microinjected with PCM-1 antibody fixed 24 hours after injection and stained for injected antibody (green) and DNA (blue). Bar 10µm.
B. Statistics on percentage of binucleate cells following various means inhibiting PCM-1 function.
Percentage of cells incorporating BrdU:

**PCM-1 RNAi**
in U-2 OS cells (N=1000):
23%  Control 41%

**PCM-1 antibody microinjection in A6 cells** (N=200):
22%  Control 42%

**Overexpression of PCM-1 deletion mutant5'1** in HeLa cells (N=200):
4%  Control 42%

**Figure 31.** Effect of PCM-1 inhibition on S phase progression.
A. HeLa cells treated with control (top panels) or PCM-1 siRNA (lower panels) and co-stained for PCM-1 and BrdU incorporation. Bar 10μm.
B. Statistics on percentage of cells in S phase following various means inhibiting PCM-1 function.
these questions may be found by looking for interactors of PCM-1 and proteins like centrin-3 that are closely linked with PCM-1.

3.5.1. GST-pulldown experiments with centrin-3

The GST pulldown approach had already been used successfully to provide evidence for an interaction between PCM-1 and centrin-3 (see above). An obvious approach, then, was to repeat the experiment on a preparative scale and identify interactors of centrin by mass spectroscopy. Extracts were prepared from Jurkat cells, a human cell line that can be grown in suspension to high densities for the preparation of highly concentrated cell extracts. Use of a human cell line also facilitates the identification of interacting proteins, since the human genome is fully sequenced and most proteins may be found in the SwissProt database.

14 bands were found that were present in the eluate from GST-centrin-3 beads but not in the control eluate (Figure 32). Of these, only 4 could be unambiguously identified by mass spectroscopy, corresponding to three proteins. These were the Ras GTPase-activating-like proteins IQGAP1 (predicted molecular weight 203kDa, band 2 ~188kDa, MOWSE score 1.6 x 10^{25}, coverage 42%) and IQGAP2 (predicted molecular weight 180kDa, band 3 ~179kDa, MOWSE score 3.1 x 10^{10}, coverage 15%) as well as myosin Vc (predicted molecular weight 203kDa, band 1 ~211kDa, MOWSE score 6.8 x 10^5, coverage 9% and band 4 ~174kDa, MOWSE score 1.1 x 10^5, coverage 8%). All three have been reported to interact with calmodulin, a protein highly similar to centrin-3 and it is unclear which of the two (or both) is the true interactor, especially since the two have virtually indistinguishable localisation patterns. The potential significance of these results will be outlined in the Discussion.

3.5.2. Yeast two-hybrid experiments

Identifying interactors of PCM-1 itself in a similar manner proved to be difficult. Immunprecipitations of PCM-1 did not co-precipitate significant amounts of interacting proteins, and no sizeable portions of PCM-1 could be expressed in
Figure 32. Centrin-3 GST-pull down.
200μg GST/GST-Centrin-3 were incubated with 30mg Jurkat cell extract for 1hr at 4°C and recovered using 100μl glutathione sepharose beads; beads were washed with PBS, and bound protein eluted by boiling with 200 μl 1x sample buffer; loading on preparative gel = 1/10 total eluate; equivalent loading of GST Centrin-3 input. 14 bands found exclusively in GST Centrin-3 eluate were excised and analysed by mass spectroscopy.
bacteria. I therefore turned to the yeast two-hybrid system, first developed by Fields and Song (Fields and Song, 1989). This technique relies on the interaction between two proteins (or parts thereof) bringing together the DNA binding and transcription activation domains of a transcription factor to which they are fused, activating one or more reporter genes. By expressing your protein of interest as a fusion with the DNA binding domain (the 'bait') and the products of a cDNA library as a fusion with the transcription activation domain (the 'prey'), that library can be screened for proteins capable of directly interacting with your protein of interest.

In this case, I used the two-hybrid system developed by Clontech, which is based on the transcription factor GAL4. Bait and prey vectors are brought together by mating of two yeast strains, AH109 and Y187, carrying these plasmids. If bait and prey proteins are capable of interacting, transcriptional activation of the adenine and histidine biosynthesis genes permits growth on selective media lacking these amino acids. In addition, activation of the β-galactosidase reporter gene gives a quantitative read-out of the strength of the interaction. To capitalise on the large amount of sequence information available from the Human Genome Project a human cDNA library was screened, using baits derived from human PCM-1 to avoid inter-species incompatibilities. At ~230kDa, PCM-1 is a very large protein by yeast standards. To facilitate expression, I therefore decided to divide it into three approximately equal-sized portions at sites where a lack of sequence conservation suggested the end of one domain and the beginning of another. These parts, designated hs PCM-1 A (amino acids 1-720), B (amino acids 720-1380) and C (amino acids 1360-2063) (Figure 33A) were cloned into the two bait vectors pGBT9 (for low level expression) and pGBK7 (for high level expression) as detailed in the Materials and Methods section and used to transform the strain AH109. Expression of bait protein could be confirmed for yeast carrying baits in the vector pGBK7 by virtue of its myc tag (Figure 33B). The same parts of PCM-1 as well as centrin-3 were cloned into the prey vector pGADGH, and used to transform the strain Y187.

In control matings, the bait strain AH109 expressing PCM-1 parts A, B, C or bait vector only was mated with the prey strain Y187 expressing the same parts of PCM-1, centrin-3 or prey vector only and diploids plated on selective media. The results are summarised in Figure 34. A liquid β-galactosidase assay was also performed. As can be seen from the table, there was no clear interaction between
Figure 33. Yeast two-hybrid screen - overview.
A. Diagrammatic representation of human PCM-1, indicating positions of baits. These were cloned into vectors pGBT9 (low) & pGBKT7 (high expression level) and expressed in yeast strain AH109 for mating with library strain Y187. Bait and prey vectors confer growth on -Trp and -Leu medium, respectively; Bait-prey interaction permits growth on -His and -Ade, and results in expression of β-galactosidase
B. anti-myc immunoblot on extracts prepared from yeast transformed with baits pGBT9 empty vector, pGBKT7-53 (+ ve control), and pGBKT7 PCM-1A, -B, and -C
<table>
<thead>
<tr>
<th>Bait</th>
<th>vector</th>
<th>Prey vector pGADGH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>EV</td>
</tr>
<tr>
<td>EV</td>
<td>pGBT9</td>
<td>- / -</td>
</tr>
<tr>
<td></td>
<td>pGBK7</td>
<td>- / -</td>
</tr>
<tr>
<td>PCM-1A</td>
<td>pGBT9</td>
<td>- / -</td>
</tr>
<tr>
<td></td>
<td>pGBK7</td>
<td>- / ++</td>
</tr>
<tr>
<td>PCM-1B</td>
<td>pGBT9</td>
<td>- / ++</td>
</tr>
<tr>
<td></td>
<td>pGBK7</td>
<td>- / +</td>
</tr>
<tr>
<td>PCM-1C</td>
<td>pGBT9</td>
<td>- / -</td>
</tr>
<tr>
<td></td>
<td>pGBK7</td>
<td>- / -</td>
</tr>
</tbody>
</table>

**Figure 34.** Yeast two-hybrid - Results of control matings.

PCM-1 baits A, B, and C in vectors pGBT9 and pGBK7 were used to transform the strain AH109 which was then mated with the strain Y187 transformed with the same parts of PCM-1 as well as centrin-3 in the prey vector pGADGH. Diploid strains were then tested for growth on -Leu-Trp-His-Ade (1) and -Leu-Trp-His plates (2). ++ indicates strong growth, + weak growth, - no growth. As can be seen in the results table above, both PCM-1A and B bait strains show autoactivation activity, permitting growth on -Leu-Trp-His plates. Further, PCM-1A and B interact strongly with themselves and weakly with each other. PCM-1C also interacts weakly with itself. There may also be a weak interaction between centrin-3 and PCM-1A.
centrin-3 and any part of PCM-1. Further, PCM-1 parts A and B interacted strongly with themselves and weakly with each other. PCM-1 part C also weakly interacted with itself. These interactions were confirmed by liquid β-galactosidase assay (not shown). For a yeast two-hybrid bait to be used successfully in a library screen it should not inhibit growth of the yeast strain carrying it. As shown in Figure 35A, the PCM-1 baits met this criterion, since they did not dramatically reduce growth in liquid culture compared to the bait vector alone. Since bait strains expressing PCM-1 parts A and B (and to a limited extent C) displayed background growth on selective media lacking histidine, strains were plated on media containing increasing concentrations of 3-amino trizol to suppress bait autoactivation. As shown in Figure 35B, 2.5mM 3-amino trizol was sufficient to suppress this background growth.

Having thus completed the necessary preliminary tests, I proceeded to the library screen with bait pGBK7-hs PCM-1 C. The results are summarised in Figure 36. To verify interactions, promising candidates were used to transform the prey strain which was then mated with strains expressing one of the three parts of PCM-1 or bait vector only. As detailed in the figure, all but one of the candidate interactor proteins fulfilled the criterion of showing no growth in the absence of PCM-1 bait. The exception, RanBPM, is thus a classical ‘false-positive’. What, then, can be said about the remaining candidate interactors? The published literature and sequence analysis tells us little about the putative acid phosphatase or the hypothetical protein KIAA1574. Finding retinoblastoma binding protein 2 (RBP2), a binding partner and possible inhibitor of pRb (Fattaey et al., 1993; Kim et al., 1994), as an interactor of PCM-1 is certainly curious, given the results obtained above for S phase progression in cells with inhibited PCM-1 function. That this protein should interact with all three parts of PCM-1 however suggests a lack of binding specificity. If this interaction were to be pursued, one would need to show that RBP2 does not interact with other bulky baits such as perhaps the p53 positive control. No less interesting is the finding of the 26S proteasome subunit p58 and the SUMO-conjugating enzyme Ubc9 as interactors of PCM-1. Many proteins are degraded by the ubiquitin-proteasome pathway, and the simplest interpretation of finding the former protein is that PCM-1 is targeted for degradation via motifs in its C-terminus. This would also explain the marked overexpression of PCM-1 constructs lacking this region (Figure 12). However, no E3 ubiquitin ligase was identified in this screen.
Doubling time:

<table>
<thead>
<tr>
<th>Vector</th>
<th>Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGBKT7 EV</td>
<td>158 min</td>
</tr>
<tr>
<td>PCM-1A</td>
<td>165 min</td>
</tr>
<tr>
<td>PCM-1B</td>
<td>165 min</td>
</tr>
<tr>
<td>PCM-1C</td>
<td>163 min</td>
</tr>
<tr>
<td>pGBT9 EV</td>
<td>148 min</td>
</tr>
<tr>
<td>PCM-1A</td>
<td>152 min</td>
</tr>
<tr>
<td>PCM-1B</td>
<td>170 min</td>
</tr>
<tr>
<td>PCM-1C</td>
<td>149 min</td>
</tr>
</tbody>
</table>

Figure 35. Yeast two-hybrid - Further characterisation of baits.

A. Growth characteristics of AH109 transformed with baits PCM-1A, B and C in vectors pGBT9 and pGBKT7 in liquid culture starting from an OD₆₀₀ of 0.1. A slight increase in doubling time indicates some toxicity of bait vectors. B. The same strains were plated on -Leu-Trp-His plates containing increasing concentrations of 3-AT. As illustrated above, 2.5mM 3-AT was sufficient to suppress background growth.
### A

<table>
<thead>
<tr>
<th>Interactors (Prey)</th>
<th>Bait</th>
<th>EV</th>
<th>PCM-1A</th>
<th>PCM-1B</th>
<th>PCM-1C</th>
</tr>
</thead>
<tbody>
<tr>
<td>35x MAP1 light chain 3 related protein</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>7x similar to putative acid phosphatase</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6x Retinoblastoma binding protein 2</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6x ubiquitin-conjugating enzyme 9</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4x RanBPM</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3x KIAA1574 protein</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3x HIV-1 Tat interactive protein, 60kDa</td>
<td>N/D</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2x Ribosomal protein S20</td>
<td>N/D</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2x Transcription factor IIIIC102</td>
<td>N/D</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2x 26S proteasome subunit p58</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>1x KIAA0211 protein</td>
<td>N/D</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 36. Yeast two-hybrid - Results obtained with PCM-1C bait

Of 4.3x10⁷ diploids screened, ~600 grew on -Leu-Trp-His plates containing 7.5mM 3-AT. ~2/3 of these were positive in an X-gal overlay assay and grew on -Leu-Trp-His-Ade plates (strong interactors). For 64 of these the prey plasmid was isolated and the insert sequenced. Promising interactors were returned into the prey strain which was then mated with strains carrying the three PCM-1 baits or the empty vector (EV). The results are presented in (A). + indicates growth on -Leu-Trp-His-Ade (- no growth, N/D not determined). B. Sample results obtained with MAP1 light chain 3 related protein and RanBPM, an apparent false positive (growth in the absence of bait)
The importance of proteasome-mediated degradation in maintaining PCM-1 levels could be investigated by the use of proteasome inhibitors. SUMO-1 conjugation of PCM-1 may have even wider significance, since this modification usually results in a change in protein activity and/or localisation rather than degradation e.g. (Joseph et al., 2002). Finally, the interactor most frequently isolated in this screen was MAP1 light chain-3 related protein, also known as Golgi-associated ATPase Enhancer of 16kDa (GATE-16). This highly conserved protein belongs to a class of small adapter proteins involved in various cytoskeletal processes. In its GATE-16 incarnation, it has been shown to be essential for the NSF-driven reassembly of the Golgi following mitosis (Muller et al., 2002). The highly related proteins MAP1 LC3, a component of the neuronal microtubule-associated complex, and the GABA receptor-associated protein GABARAP, mediate interactions with the microtubule cytoskeleton by directly binding to tubulin (Mann and Hammarback, 1994; Wang et al., 1999). Whether this is also true for MAP1 light chain-3 related protein is not known, and no detailed immunolocalisation has been published for this protein. It is thus unclear if an interaction of PCM-1 with this protein may account for the observed association of PCM-1 with Golgi proteins at the end of mitosis and/or its interaction with microtubules.

While the interactors identified are certainly interesting, the screen has failed to identify known centrosomal proteins or components of the dynein/dynactin complex. These may interact with the remaining parts of PCM-1, and it is thus important to complete this screen with the other two baits, pGBKT7-hs PCM-1 A and B. Alternatively, these interactions are indirect or otherwise cannot be reproduced in this assay.
Chapter 4. Discussion

4.1. A transport model for PCM-1 function

4.1.1. The model

The data presented in this thesis are consistent with a model of PCM-1 acting as part of a transport complex, the centriolar satellite, that also includes the dynein microtubule motor and associated dynactin, as well as centrosomal protein cargo. According to this model, centriolar satellites are continually shuttling along microtubule tracks between centrosomes and the surrounding cytoplasm in a dynein-dependent manner, depositing their cargo at the centrosome. Proteins thus transported include the putative microtubule anchor ninein, the γ-tubulin associated protein pericentrin and centrin-3, a protein that may be involved in centrosome duplication. Other centrosomal proteins may take the same route but have not been investigated in this study. The only known exception so far is the microtubule nucleator itself, γ-tubulin, which appears to assemble at the centrosome independent of PCM-1, the dynein/dynactin motor or microtubules. This transport model for PCM-1 function is illustrated in Figure 37.

4.1.2. Evidence

Evidence for this model comes from a number of sources, primarily the present work but also previous reports on PCM-1. In particular, the interpretation of my data rests heavily on the dynamics of PCM-1-containing centriolar satellites reported by the Tsukita laboratory (Kubo et al., 1999). While the steady-state distribution of PCM-1 in interphase cells varies widely depending on cell type and other factors that are presently unclear, the strong concentration of PCM-1 in the vicinity of the
Figure 37. A Transport Model for PCM-1 function
PCM-1 acting as a shuttle protein, involved in the dynein/dynactin-dependent transport of centrosomal proteins, including centrin, pericentrin and ninein, along microtubules to the centrosome. These are required for centrosome-dependent processes including microtubule anchoring. The microtubule nucleator γ-tubulin, and perhaps other proteins, assemble at the centrosome independent of PCM-1, the dynein motor and microtubules.
centrosome prior to the onset of mitosis may reflect its role in recruiting centrosomal proteins during centrosome maturation.

My work has demonstrated that the assembly of three centrosomal proteins, centrin-3, pericentrin and ninein is dependent on PCM-1 function. When PCM-1 was depleted by siRNA, centrosomal levels of these proteins were much reduced (Figure 16). Centrosomal protein assembly also failed to occur properly if PCM-1 was mislocalised by antibody microinjection (Figure 13) or overexpression of a C-terminal deletion mutant (Figure 14). Assembly was further shown to be dependent on the presence of microtubules (Figure 18) and a functional dynein/dynactin motor complex (Figure 19). Small amounts of centrin-3, pericentrin and ninein were shown to colocalise with PCM-1 and subunits of the dynein/dynactin complex in a number of cell lines (Figure 17). While this centriolar satellite localisation could not be shown in other cell types, the colocalisation of these proteins with PCM-1 in cells overexpressing the C-terminal deletion mutant suggests it to be a general phenomenon. Not all centrosomal proteins assemble in a PCM-1-dependent way. γ-tubulin localisation to the centrosome was unaffected by inhibition or depletion of PCM-1 and, indeed, by depolymerisation of microtubules and disruption of the dynein/dynactin motor complex.

It is perhaps significant that mislocalised PCM-1 led to the aggregation of centrosomal proteins, while such aggregates did not form in the absence of PCM-1. ‘Aggregates’ may simply be centriolar satellites that are unable to move and instead steadily grow in size. This is what appears to occur if microtubules are depolymerised for extended periods of time (Figure 18). However, if PCM-1 was depleted or motor function disrupted by excess dynamitin (Figure 19), centriolar satellites did not form and centrosomal proteins remained dispersed in the cytoplasm. This suggests that PCM-1 and the dynein/dynactin complex are required for the formation of centriolar satellites, in which PCM-1 may play an important structural role.

Colocalisation aside, an interaction between centrin-3 and PCM-1 was demonstrated by immunoprecipitation with PCM-1 antibody (Figure 20) and GST pulldown with GST-Centrin-3 (Figure 21). The absence of positive data from yeast two-hybrid analysis suggests this interaction may not be direct. An interaction of PCM-1 with pericentrin had previously been demonstrated by Balcz on and...
colleagues (Li et al., 2001). It is still unclear whether PCM-1 interacts directly with the dynein/dynactin motor complex. However, as mentioned in the introduction, microtubule binding of PCM-1 has been shown to be dependent on the presence of dynein (Balczon et al., 1999), and movement along microtubules towards minus ends also depends on dynein activity (Kubo et al., 1999).

The model is also in broad agreement with the wider literature on centrosomal protein assembly, some of which has been already mentioned in my introduction. Dynein-independent microtubule binding and centrosomal assembly of pericentrin have previously been demonstrated (Young et al., 2000), and pericentrin has been shown to interact with the dynein light intermediate chain 1 by coimmunoprecipitation (Purohit et al., 1999; Tynan et al., 2000). Centrin localisation to the pericentriolar material has also been previously shown to be highly dynamic (Baron et al., 1994). Where the literature is divided is on the subject of γ-tubulin. In the same study that demonstrated a requirement for dynein in the binding of PCM-1 to microtubules (Balczon et al., 1999), γ-tubulin binding was not significantly reduced by depletion of the motor. In vitro, recruitment of γ-tubulin to Xenopus sperm heads or salt-stripped centrosomes occurs in extracts treated with nocodazole (Felix et al., 1994; Li et al., 2001; Schnackenberg et al., 1998). In vivo, recruitment of GFP-tagged γ-tubulin during centrosome maturation prior to the onset of mitosis (Hannak et al., 2001; Khodjakov and Rieder, 1999) as well as the slower turnover during interphase (Khodjakov and Rieder, 1999) have been found to occur with normal kinetics in the absence of microtubules. This would suggest that centrosomal assembly of γ-tubulin occurs by means other than the dynein and microtubule-dependent pathway that PCM-1 appears to be involved in. However, two conflicting reports disagree with this idea. The first (Young et al., 2000) showed that prolonged treatment of cells with nocodazole or dynein inhibitors (dynamitin or 70.1 anti-dynein antibody) from G1 phase until the onset of mitosis resulted in a failure of centrosomal assembly of γ-tubulin and pericentrin. The second (Quintyne et al., 1999), performed in the same cell type, showed a dispersal of γ-tubulin into several foci in cells transfected with dynamitin or other subunits of the dynactin complex, while the centrosomal localisation of pericentrin was unaffected. While it is unclear how these results can be reconciled with each other, the common observation of an effect on γ-tubulin localisation required treatment over a timeframe much longer than
the few hours used in my experiments with nocodazole and dynamitin. The most
careful experiments on γ-tubulin dynamics in somatic cells to date (Khodjakov and
Rieder, 1999) have revealed the existence of two different pools of γ-tubulin at the
centrosome with different exchange rates with the cytoplasm. It may be that only the
slowly exchanging pool of γ-tubulin requires dynein/dynactin function, not as a
transporter but as an anchoring activity at the centrosome.

Centrosomal proteins transported by PCM-1 need not be newly synthesised.
As previously mentioned in the introduction, many centrosomal proteins occur in
large cytoplasmic pools. Of the proteins apparently transported by PCM-1, this is
known to be the case for centrin (Paoletti et al., 1996). The above mentioned
dynamics of centrin observed in PtK2 cells (Baron et al., 1994) occur in response to
changes to temperature and intracellular calcium levels over a timeframe of seconds
more likely representing the redistribution of centrin already present in the cell than
the movement of newly synthesised protein. Such redeployment of centrosomal
proteins would enable the cell to respond quickly to changes in the extracellular
environment by remodelling its microtubule network and other centrosomal
activities.

4.2. Cellular roles for PCM-1 and PCM-1-dependent
transport

As outlined in the introduction, centrosomes are involved in numerous cellular
activities. It thus did not come as a surprise that a protein involved in the recruitment
of centrosomal proteins would affect many of these activities, including interphase
microtubule organisation, cytokinesis and S phase entry. For one of these,
microtubule organisation, I have tentatively identified one protein downstream of
PCM-1, ninein, that appears to mediate these effects. For the others, it remains
unclear whether PCM-1 is involved directly or indirectly.

4.2.1. Microtubule organisation
When PCM-1 was inhibited (Figure 24) or depleted (Figure 26), the normally radial organisation of interphase microtubules in cell lines that contain them was lost, and microtubules were instead randomly distributed throughout the cytoplasm. A microtubule regrowth experiment (Figure 25) revealed little change in the nucleation capacity of the centrosome, indicating instead a defect in the anchoring of microtubules once nucleated. This is in line with the observed mislocalisation of the putative microtubule anchor ninein, while the microtubule nucleator γ-tubulin remained unchanged at the centrosome (Figure 14, 16). To determine which of the three centrosomal proteins known to be transported in a PCM-1-dependent manner (if any) were required for microtubule organisation, these were depleted individually by siRNA (Figure 26). Depletion of pericentrin did not affect the radial organisation of microtubules, but resulted in an apparent decrease in the nucleation capacity of the centrosome, as predicted by its close association with γ-tubulin (Dictenberg et al., 1998). Depletion of both centrin-3 and ninein, like PCM-1, resulted in a loss of microtubule organisation. As mentioned in the introduction, ninein is amongst the proteins that localises to microtubule anchoring sites (Mogensen et al., 2000). The result reported here, however, is the first functional evidence that ninein may actually be required for microtubule anchoring. The unexpected effect of centrin depletion on microtubule organisation was accounted for by a concomitant loss of ninein from the centrosome (Figure 27), although how this occurred is unclear (failure of PCM-1-dependent transport, centrosomal assembly, or of structural integrity of the pericentriolar material/distal appendages to which ninein localises).

Disruption of the dynactin complex has previously been shown to result in a loss of microtubule anchoring at the centrosome, without affecting microtubule nucleation activity (Quintyne et al., 1999). The authors correlate this loss of anchoring activity with the failure of centrosomal targeting of the p150Glued subunit of dynactin. This subunit is known to interact with microtubules, and it may act at the centrosome to organise and anchor microtubules (Quintyne et al., 1999; Waterman-Storer et al., 1995). Further evidence for such a model is that p150Glued has been shown to interact with EB1, a protein that localises to both the centrosome and microtubule tips (Askham et al., 2002). Overexpression of deletion mutants of this protein had more severe effects on centrosome function, disrupting microtubule nucleation as well as anchoring, and inhibiting the centrosomal recruitment of γ-
The available data on dynactin does not distinguish between its function in dynein-dependent recruitment of centrosomal proteins involved in microtubule anchoring and a direct role in microtubule anchoring itself. These models are not mutually exclusive, and dynactin may well be engaged in both activities.

4.2.2. Cytokinesis

Unlike inhibition of the dynein/dynactin complex, PCM-1 inhibition or depletion did not affect mitotic spindle formation or chromosome segregation. It did, however, consistently result in an increase in the frequency of binucleate cells, indicative of a defect in cytokinesis (Figure 30). How PCM-1 may be involved in this process is unclear. It is notable, however, that PCM-1 localises to the cytoplasmic end of midbody microtubules, in close proximity to Golgi membranes as illustrated in Figure 4. Targeted membrane insertion has been shown to be important for cleavage furrow ingression, as inhibitors of syntaxin block cytokinesis in *C. elegans* and sea urchin embryos (Conner and Wessel, 1999; Jantsch-Plunger and Glotzer, 1999). Treatment of *C. elegans* embryos with brefeldin A similarly inhibited completion of cytokinesis (Skop et al., 2001). PCM-1 may thus act in positioning of the Golgi complex for its role in cytokinesis. This may involve PCM-1 interacting with the Golgi-associated MAP1 light chain-3 related protein/ GATE-16 as suggested by the yeast two-hybrid data. Whether PCM-1 is indeed involved in cytokinesis in this manner remains to be examined.

Of course, centrosomes as a whole are somehow required for cytokinesis, as shown by experiments in which the centrosome was removed by laser ablation (Khodjakov et al., 2000; Khodjakov and Rieder, 2001) or microsurgery (Hinchcliffe et al., 2001; Piel et al., 2001). Defective PCM-1 transport may prevent the centrosome from fulfilling its normal roles during cytokinesis. What these are, however, remains unclear (see introduction). In this context, the isolation of IQGAP and myosin V as potential interactors of centrin-3 may be significant. IQGAPs were originally identified as cytoskeletal targets of the small GTPases Cdc42 and Rac1 (Kuroda et al., 1996). IQGAP homologues in yeast (Eng et al., 1998; Epp and Chant, 1997; Osman and Cerione, 1998) and *Dictyostelium* (Adachi et al., 1997; Faix et al., 128
1998; Faix et al., 2001) have been found to be required for recruitment of actin and thus actomyosin ring formation in cytokinesis. They in turn appear to be recruited by calmodulin-like proteins (Boyne et al., 2000; Shannon and Li, 2000). Interestingly, the fission yeast IQGAP, Rng2p has been localised to the spindle pole body, although the significance of this observation is unclear (Eng et al., 1998). In vertebrates, IQGAPs have been primarily studied at the cell cortex, where they may be involved in cortical microtubule capture by interaction with CLIP-170 localised to microtubule plus ends (Fukata et al., 2002). Whether vertebrate IQGAP localises to centrosomes and/or cleavage furrows remains to be determined. Similarly, the unconventional myosin, myosin V, has been implicated in actin remodelling as well as in vesicle transport along microtubules (Waterman-Storer et al., 2000). While little work has been done on the ubiquitously expressed myosin Vc isolated here, the neuronal specific myosin Va has been shown to localise to centrosomes, spindle microtubules and the midbody (Espreafico et al., 1998; Wu et al., 1998). While the interactions between centrin-3 and IQGAP and myosin V remain to be confirmed, the involvement of these proteins in the actin dynamics that underlie cytokinesis may shed light on the currently mysterious involvement of the centrosome in cytokinesis.

4.2.3. S phase progression

Any discussion of the apparent requirement for PCM-1 in cell cycle progression into S phase (Figure 31) must similarly be highly speculative. As with proper completion of cytokinesis, entry into S phase has been shown to be dependent on the presence of centrosomes, except that here the requirement appears to be absolute (Hinchcliffe et al., 2001; Khodjakov and Rieder, 2001). As previously mentioned, inhibition of the dynein/ dynactin motor complex (Quintyne and Schroer, 2002) also affects S phase entry, albeit less severely. An appealing model, therefore, is that there is a ‘centrosome checkpoint’ that monitors the capacity of the centrosome to duplicate and will arrest the cell cycle if that is not possible. As illustrated in the introduction, a whole array of signalling molecules are concentrated at the centrosome, and these may transmit this signal to the cell cycle machinery. PCM-1 requirement for S phase progression most likely is due to it transporting centrosomal proteins that are directly or indirectly required for centrosome duplication and whose presence may be
monitored by the checkpoint. Of the currently known centrosomal cargo of PCM-1, centrin-3 most likely fits this description given its requirement for centrosome duplication. The observed two-hybrid interaction of PCM-1 with RBP2 (Figure 36), a binding partner and possible inhibitor of pRb (Fattaey et al., 1993; Kim et al., 1994), though curious given the key role played by pRb in S phase entry, requires confirmation by other experimental data before it is to be taken seriously.

4.3. Concluding remarks

I believe that in PCM-1 I have identified a key protein required for the assembly of centrosomal proteins, a process involving microtubule-dependent transport by the dynein/dynactin motor complex. Centriolar satellites, to which PCM-1 localises appear to be pre-assembled transport complexes en route to the centrosome. Not all centrosomal proteins are recruited in this manner. γ-tubulin, and perhaps others, assemble at the centrosome by other means not involving PCM-1, the dynein motor or microtubules. Through its involvement in centrosome assembly, it affects centrosome-dependent processes ranging from microtubule organisation to cytokinesis and cell cycle progression. Perhaps inevitably, questions remain.

While PCM-1 is clearly moving centrosomal cargo in a dynein and dynactin-dependent manner, how it interacts with motor or cargo remains unclear. The answer to this and perhaps other questions may come from the yeast two-hybrid screen with PCM-1 baits that I have initiated. While the potential interactors identified in the screen with the C-terminus of PCM-1 so far failed to provide clear answers, the success or failure of this approach will only become clear once the screen has been completed with the other two baits covering the remaining two-thirds of PCM-1.

Regarding the role of PCM-1 in S phase entry, several experimental strategies present themselves to further investigate this issue. First, given the availability of reagents to deplete centrin-3, pericentrin, and ninein by siRNA, the requirement for these proteins in S phase progression could easily be tested. Second, the issue of S phase progression is closely linked to the process of centrosome duplication. Centrosome reduplication has been shown to occur in U-2 OS cells, a human cell line, arrested in S phase with hydroxyurea (Liu and Erikson, 2002). The
requirement for PCM-1 and proteins downstream of PCM-1 in centrosome duplication could therefore be tested by depletion with siRNA.

Similarly, PCM-1 involvement in cytokinesis could be examined by depleting each of its centrosomal cargo proteins individually. The curious localisation of PCM-1 at the cytoplasmic end of midbody microtubules also deserves closer study. Given the availability of full-length PCM-1 tagged with GFP, the dynamics of this population could be investigated. Golgi morphology in cells undergoing cytokinesis in the absence of functional PCM-1 should also be examined.

In short, PCM-1 and centriolar satellites have revealed some of their secrets in this study. Many more remain to be discovered. Exciting times still lie ahead in this as in other areas of centrosome research.
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Publications

Assembly of centrosomal proteins and microtubule organization depends on PCM-1

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The protein PCM-1 localizes to cytoplasmic granules known as “centriolar satellites” that are partly enriched around the centrosome. We inhibited PCM-1 function using a variety of approaches: microinjection of antibodies into cultured cells, overexpression of a PCM-1 deletion mutant, and specific depletion of PCM-1 by siRNA. All approaches led to reduced targeting of centrin, pericentrin, and ninein to the centrosome. Similar effects were seen upon inhibition of dynactin by dynamin, and after prolonged treatment of cells with the microtubule inhibitor nocodazole. Inhibition or depletion of PCM-1 function further disrupted the radial organization of microtubules without affecting microtubule nucleation. Loss of microtubule organization was also observed after centrin or ninein depletion. Our data suggest that PCM-1-containing centriolar satellites are involved in the microtubule- and dynactin-dependent recruitment of proteins to the centrosome, of which centrin and ninein are required for interphase microtubule organization.

Introduction

Microtubule organization is essential for directional intracellular transport, for the modulation of cell morphology and locomotion, and for the formation of the spindle apparatus during cell division. With the exception of plants, most cells organize their microtubule network using specialized structures, such as the spindle pole body in yeast cells, the basal body of cilia and flagella in protozoan organisms, and the centrosome in animal cells. The centrosome consists of two centriolar cylinders surrounded by electron-dense pericentriolar material. The centriolar cylinders have diameters of ~0.2 μm and are each composed of nine triplets of short microtubules, arranged to form the wall of the cylinder. In addition to various tubulin isoforms (McKeen et al., 2001), centrin, a member of a larger calcium-binding protein superfamily, has been found associated with the centrioles (Paolletti et al., 1996). However, 95% of centrin in human cells is not bound to the centrioles, but fractionates with the cytoplasm or with nuclei in biochemical experiments (Baron et al., 1994; Paolletti et al., 1996). A large variety of proteins is attached to the periphery of the centrioles as part of the pericentriolar material (Kalt and Schliwa, 1993). Some of the proteins in this matrix have been characterized in recent years (for review see Doxsey, 2001). The primary function of the pericentriolar material is to nucleate microtubules, which are radically arranged from the centrosomal surface or subsequently released and anchored in other places of the cell (Mogensen, 1999). The initial step of microtubule nucleation is dependent on the function of 25S ring complexes of the protein γ-tubulin and associated proteins (Zheng et al., 1995). However, for stable anchoring of microtubules, another protein (ninein) is required (Bouckson-Castaing et al., 1996; Mogensen et al., 2000; Piel et al., 2000; Ou et al., 2002). Ninein is also found at noncentrosomal sites in specialized cell types such as polarized epithelial cells that undergo a change from a radial microtubule organization into an arrangement of fibers from the apical to the basal pole (Bacallao et al., 1989; Mogensen et al., 1989; Mogensen, 1999). To facilitate microtubule nucleation, it has been proposed that γ-tubulin complexes are embedded in the pericentriolar material, in a lattice formed by pericentrin (Dictenberg et al., 1998). Pericentrin is a large protein of which two isoforms have been described: a 220-kD form (pericentrin A; Doxsey et al., 1994), as well as a newly identified 350-kD form (pericentrin B; Li et al., 2001). It is transported to the centrosome by the microtubule-dependent motor dynein, a process apparently mediated through direct binding of pericentrin to the dynein light intermediate chain (Purohit et al., 1999; Tynan et al., 2000). Pericentrin has recently been found to bind to PCM-1, a 228-kD protein that localizes to small 70–100-nm granules in the cytoplasm of interphase cells (Balczac et al., 1994; Kubo et al., 1999; Li et al., 2001). These granules can move along microtubules in a dynein-dependent way and often concentrate near the microtubule organizing center (Balczac et al., 1999; Kubo et al., 1999). Detailed morphological analysis revealed that these PCM-1...
containing granules are identical to structures previously described as "centriolar satellites" (Kubo et al., 1999). Although centriolar satellites have been extensively studied by electron microscopy (Rattner, 1992), their function is so far unknown. In this paper, we test the potential role of their component protein PCM-1 in centrosome assembly and the organization of microtubule networks.

Results

Microinjection of antibodies against PCM-1 causes accumulation of centrin and pericentrin

To examine the cellular function of the protein PCM-1, we raised antibodies in rabbits against a hexahistidine-tagged fusion protein containing amino acids 1665-2024 of human PCM-1. As shown in Fig. 1 A, these antibodies specifically recognized a 230-kD band in HeLa cell extract, characteristic of full-length PCM-1. Because the region of the PCM-1 fusion protein used for immunization is highly conserved among different vertebrate species (Kubo et al., 1999), our antibodies also cross reacted with PCM-1 from other animals, including *Xenopus laevis*. PCM-1 staining in HeLa cells displays a pattern of cytoplasmic granules that are partly enriched near the centrosome, but clearly distinct from the strong centrosomal staining of γ-tubulin (Fig. 1, B and C). Although this is in agreement with data from Kubo et al. (1999), it contrasts with immunofluorescence data initially provided by Balczon et al. (1994), showing a concentrated staining of PCM-1 at the centrosome of CHO cells. Testing various cell lines, we found that the amount and localization of cytoplasmic granules varied between different cell types (unpublished data).

Expression of a highly conserved homologue of full-length PCM-1 from chicken (66% identity, 75% homology to human PCM-1 over the entire length of the protein) that was tagged with GFP at the carboxy-terminal end gave the same pattern of cytoplasmic granules as seen in our immunofluorescence, therefore excluding a staining artifact of our antibodies (Fig. 1 D). After centrosome duplication, we could see PCM-1 concentrating in two large foci, with the highest concentration as cells entered mitosis (compare Fig. 1 E with Fig. 1 F). During metaphase, a fraction of PCM-1 granules concentrated at the spindle poles, but the majority of the protein was found dispersed in the cytoplasm (Fig. 1 G). In telophase, PCM-1 could be seen enriched in two areas of each daughter cell: (1) distal from the cleavage site, in the area of the centrosomal microtubule organizing center, as well as (2) proximal to the cleavage site, in an area where the minus-ends of midbody microtubules terminate (Fig. 1 H).

We used affinity-purified rabbit antibodies for microinjection into the cytoplasm of cultured *Xenopus A6* cells. 24-48 h after microinjection, we found that PCM-1 granules were no longer detectable in 89% of the cells (n = 88), using a mouse antibody against PCM-1 for immunofluorescence (Fig. 2 B). Instead, only a weak staining in the centrosomal area remained. This could mean that the PCM-1 epitopes were masked by the microinjected antibody, and therefore, no longer detectable by immunofluorescence, or that the PCM-1 granules were dispersed upon microinjection. No apparent morphological defect was seen in injected cells, but when examining the distribution of other proteins, we observed large cytoplasmic aggregates of the centrosomal protein centrin, in addition to centrosome staining, in 67% of the cells (n = 284; Fig. 2, D-F). In 10% of the injected cells, these aggregates had acquired a filamentous or ribbon-like structure (Fig. 2, E and F). Further, there was a weak effect on pericentrin, with 17% of cells (n = 283) exhibiting small pericentriolar aggregates in addition to centrosome staining (Fig. 2 H). By contrast, the localization of γ-tubulin was not significantly affected by microinjection of PCM-1 antibodies (Fig. 2 J). Microinjection of control antibodies had no significant effect on the localization of centrosomal proteins or PCM-1 (Fig. 2, A, C, G, and I).

Overexpression of a PCM-1 deletion mutant causes aggregation of a subset of centrosomal proteins

Our microinjection data suggest that antibodies against PCM-1 can affect the intracellular distribution of centrosome components such as centrin and pericentrin. To ex-
amine the role of PCM-1 in centrosomal protein targeting using a different approach, we generated a set of PCM-1 deletion mutants lacking various parts of their carboxy-terminal end. Whereas full-length PCM-1 (aa 1–1904) localized to small cytoplasmic granules characteristic of endogenous PCM-1 (Fig. 1 D), mutants comprising amino acids 1–1468 or 1–1148 formed large cytoplasmic protein aggregates of various sizes, up to ten times the size of normal PCM-1 granules. When overexpressing these mutants, we found that all endogenous PCM-1 was segregated to these protein aggregates (Fig. 3, C and D). We were able to distinguish between the mutant and the endogenous form of PCM-1 using an antibody raised against the carboxy terminus, present only in the endogenous full-length PCM-1 (Fig. 3 D), and a species-specific antibody raised against amino acids 1–114 of the chicken homologue of PCM-1, from which the mutant expression construct was derived (Fig. 3 C). Overexpression of mutant PCM-1 also affected the correct localization of centrosomal proteins; the majority of centrin accumulated at the same large cytoplasmic aggregates that contained PCM-1.
and the deletion mutant (Fig. 3, E and F; detected in 96% of the transfected cells \( n = 200 \)).

These large protein aggregates also segregated significant amounts of pericentrin (Fig. 3, G and H; 93% of overexpressing cells \( n = 401 \)). In addition to centrin and pericentrin, the localization of the centrosomal protein ninein was also affected; whereas ninein localizes to the pericentriolar material in control cells, overexpression of mutant PCM-1 led to dispersion into multiple small foci of ninein in the cytoplasm in 90% of cells \( n = 249 \) (Fig. 3, K and L), which partly colocalized with the large PCM-1 protein aggregates. The localization of \( \gamma \)-tubulin, on the other hand, was not significantly altered by mutant PCM-1 (Fig. 3, I and J).

Next, we tested whether any of the protein aggregates induced by the PCM-1 deletion mutant 1–1468 represented newly replicated centrosomes. Therefore, we stained cells with an antibody against acetylated tubulin, a marker that labels stable microtubules, including those that form the 9 × 3 filaments of the centriolar cylinders. We found that cells expressing mutant PCM-1 stained for acetylated tubulin indistinguishably from controls; generally, acetylated tubulin was enriched at a double-dot representing one pair of centrioles, and at cytoplasmic fibers representing stable microtubules (Fig. 3, M and N). However, the large protein aggregates of mutant PCM-1 did not show any enriched staining of acetylated tubulin, indicating that no new centrioles, and therefore no additional centrosomes, had been formed in these cells. Overexpression of a control protein, \( \beta \)-galactosidase, had no effect on PCM-1 or the centrosome (Fig. 3, A and B).

Figure 4. **Depletion of PCM-1 by RNA silencing reduces centrosomal localization of centrin, pericentrin, and ninein, but not \( \gamma \)-tubulin or dynactin.**

A–J show U-2 OS cells treated with control or PCM-1 siRNA oligonucleotides, as indicated. Image pairs show cells double stained for (A and B) PCM-1 and centrin, (C and D) PCM-1 and pericentrin, (E and F) PCM-1 and ninein, (G and H) PCM-1 and \( \gamma \)-tubulin, and (I and J) PCM-1 and dynactin p150/glued. The amount of centrosomal protein localization after PCM-1 depletion was determined by photometric analysis to be 39% of centrin (± 17), 36% of pericentrin (± 21), 38% of ninein (± 20), 99% of \( \gamma \)-tubulin (± 58), and 82% of dynactin (± 37), as compared with control cells (\( n = 34 \) cells/each). (K) Immunoblots of extracts from untreated cells (untr.), and cells treated with control RNA oligomers siRNA PCM-1.1 or siRNA PCM-1.2, for different lengths of time as indicated. Blots were probed with antibodies against PCM-1, NuMA, and centrin-3. Bar (I), 10 \( \mu \)m.
RNA silencing of PCM-1 leads to reduced assembly of centrin, pericentrin, and ninein at the centrosome

Because antibody microinjections and overexpression of PCM-1 mutants could have dominant secondary effects on other proteins in the cell by steric hindrance or by segregation of interacting components, we tested the role of PCM-1 in an approach based on depletion rather than inhibition of this protein. A recently published technique using transfection of double-stranded RNA oligomers of 21 base pairs has demonstrated that depletion of specific mRNAs is possible (Elbashir et al., 2001). Using oligomer pairs from two different regions of human PCM-1, as well as control oligomers (see Materials and methods), we reached transfection levels of 95% (n = 522; as judged using labeled control oligomers) and were able to remove 34% (siRNA PCM-1.1) or 82% (siRNA PCM-1.2) of the original amounts of PCM-1 in cultures of HeLa cells, U-2 OS human osteosarcoma cells, and C2C4 mouse myoblasts.

We proceeded with the RNA oligomer pair PCM-1.2 that had the strongest effect on PCM-1 depletion, corresponding to nucleotides 1464–1484 in human PCM-1 cDNA. Efficient depletion of PCM-1 was observed at time points longer than 90 h (Fig. 4 K), which required prolonged culturing and retransfection with siRNA at 48 h. This may reflect a slow turnover rate of PCM-1 in the cells. When analyzing individual cells, we found that PCM-1 depletion removed centriolar satellite staining almost completely, with a few PCM-1 granules occasionally remaining near the centrosome or in the cytoplasm (Fig. 4, B, D, F, H, and J). Photometric analysis of PCM-1 fluorescence revealed that the depletion levels in individual cells ranged from 69 to 99%, with an average depletion of 89% of PCM-1 protein. As a consequence of PCM-1 depletion, we again saw an effect on the assembly of centrin, pericentrin, and ninein, but no significant effect on γ-tubulin (Fig. 4, A–H). In all cell types examined, we found that the amounts of centrin, pericentrin, and ninein at the centrosome were significantly reduced after PCM-1 depletion. We quantified the fluorescence intensity of these proteins in the centrosomal region of U-2 OS cells (see Materials and methods), and determined that only 39% of centrin, 36% of pericentrin, and 38% of ninein remained localized at the centrosome as compared with control cells. In contrast, the levels of γ-tubulin remained largely constant (99%). Because previous work has indicated an interaction between PCM-1 and dynactin (Balczon et al., 1999), we also measured the centrosomal levels of the dy-
Microtubules and dynactin are essential for the centrosomal accumulation of PCM-1, centrin, pericentrin, and ninein, but not γ-tubulin. (A–J) Image pairs of CHO cells after nocodazole treatment are shown, stained for (A and B) PCM-1 and centrin, (C and D) PCM-1 and pericentrin, (E and F) PCM-1 and ninein, (G and H) PCM-1 and γ-tubulin, and (I and J) PCM-1 and dynein intermediate chain. K and L show an untreated cell stained for PCM-1 and dynein intermediate chain. Arrowheads indicate pericentriolar dynein spots colocalizing with PCM-1 granules. (M–V) Image pairs of CHO cells microinjected with p50/dynamitin are shown. (M, O, Q, S, and U) Cells were stained for PCM-1, centrin-3, ninein, γ-tubulin, and pericentrin, respectively. (N, P, R, T, and V) Corresponding images showing dynamitin-injected cells (red) and DNA staining (blue). Dynamitin-dependent inhibition of centrosomal localization varied for different proteins; PCM-1 dispersed in 77% of injected cells (n = 106, controls 2%, n = 182), centrin was affected in 60% (n = 50, controls 2%, n = 191), ninein in 45% (n = 110, controls 3%, n = 169), pericentrin in 33% (n = 470, controls 5%, n = 73), and γ-tubulin in 3% (n = 76, controls 1%, n = 135). (W and X) Image pair of a control cell injected with labeled goat anti-rabbit antibody and stained for pericentrin. Bar (L), 10 μm.

Figure 6. Microtubules and dynactin are essential for the centrosomal accumulation of PCM-1, centrin, pericentrin, and ninein, but not γ-tubulin. (A–J) Image pairs of CHO cells after nocodazole treatment are shown, stained for (A and B) PCM-1 and centrin, (C and D) PCM-1 and pericentrin, (E and F) PCM-1 and ninein, (G and H) PCM-1 and γ-tubulin, and (I and J) PCM-1 and dynein intermediate chain. K and L show an untreated cell stained for PCM-1 and dynein intermediate chain. Arrowheads indicate pericentriolar dynein spots colocalizing with PCM-1 granules. (M–V) Image pairs of CHO cells microinjected with p50/dynamitin are shown. (M, O, Q, S, and U) Cells were stained for PCM-1, centrin-3, ninein, γ-tubulin, and pericentrin, respectively. (N, P, R, T, and V) Corresponding images showing dynamitin-injected cells (red) and DNA staining (blue). Dynamitin-dependent inhibition of centrosomal localization varied for different proteins; PCM-1 dispersed in 77% of injected cells (n = 106, controls 2%, n = 182), centrin was affected in 60% (n = 50, controls 2%, n = 191), ninein in 45% (n = 110, controls 3%, n = 169), pericentrin in 33% (n = 470, controls 5%, n = 73), and γ-tubulin in 3% (n = 76, controls 1%, n = 135). (W and X) Image pair of a control cell injected with labeled goat anti-rabbit antibody and stained for pericentrin. Bar (L), 10 μm.

nactin component p150/glued, which remained largely unaffected after PCM-1 depletion (82%; Fig. 4, I and J). Culturing of cells in the presence of PCM-1 siRNA for periods longer than 120 h led to extensive cell death.

PCM-1 and centrosomal proteins colocalize in a subset of centriolar satellites

Because antibody microinjection experiments, mutant overexpression experiments, and RNA silencing experiments consistently showed a dependence of centrin assembly on PCM-1, we wanted to test directly for an interaction between PCM-1 and centrin using biochemical methods. Therefore, we used glutathione-Sepharose beads on HeLa cell extract or Xenopus egg extract, preincubated with GST-tagged centrin isoform 3. As shown in Fig. 5 A, centrin-3 loaded beads, but not control beads, were able to copellet PCM-1 from both extracts, indicating that PCM-1 and centrin can bind to each other. Because previous reports have shown centrin localizing to electron-dense cytoplasmic granules during ciliogenesis and to dynamic pericentriolar spots in PtK2 cells (Baron et al., 1994), we examined whether PCM-1 colocalized with centrin in these cells. As shown in Fig. 5 B, ~79% of cytoplasmic granules of centrin-3 colocalized with PCM-1 (n = 518). We noticed that in specialized cell types, such as mouse myoblasts, the centrosomal proteins ninein and pericentrin could also be seen in small satellites surrounding the centrosome. As with centrin, these also partly colocalized with PCM-1 (Fig. 5 B).

Centrin, pericentrin, and ninein require PCM-1, dynactin, and microtubules for centrosomal localization

Together, our findings suggest that assembly of specific pericentriolar components depends on PCM-1. Because Kubo et al. (1999) have reported shuttling of PCM-1 granules in and out of the centrosome in a dynein-dependent manner, and because other reports provided evidence for pericentrin and PCM-1 transport dependent on microtubules and dynein–dynactin (Balczón et al., 1999; Purohit et al., 1999; Tynan et al., 2000), we wanted to examine whether prolonged treat-
ment of cells with microtubule-destabilizing drugs would have similar effects on the localization of centrosomal proteins as PCM-1 inhibition. When CHO cells were treated with 17 μM nocodazole for 2 h, leading to complete depolymerization of microtubules, we observed large protein aggregates in the cytoplasm that contained PCM-1 together with centrin, pericentrin, ninein, and also the motor protein dynein (Fig. 6, A—F, I, and J), but not γ-tubulin (Fig. 6, G and H). Interestingly, a fraction of dynein also colocalized with PCM-1 granules in untreated cells (Fig. 6, K and L, arrows). We then addressed the question whether assembly of these centrosomal proteins could be directly inhibited by destabilizing the dynein activating complex of dynactin. For this purpose, we microinjected CHO cells with purified p50/dynamitin, a dynactin subunit that sequesters other dynactin components and leads to dynactin disassembly when added in excess (Echeverri et al., 1996; Quintyne et al., 1999). We noticed that dynamitin led to the dispersion of most PCM-1 in injected cells (Fig. 6, M and N), and caused similar defects in centrosomal protein assembly as observed with PCM-1 inhibition or depletion; centrin, pericentrin, and ninein were dispersed or formed small cytoplasmic aggregates, whereas γ-tubulin localization was unaffected (Fig. 6, O—X).

Organization of a radial microtubule network depends on PCM-1

In a final set of experiments, we wanted to test whether the inhibition of PCM-1 had an effect on centrosome function, such as microtubule nucleation or organization of a radial microtubule network. In these experiments, we used Cos-7, U-2 OS, and PtK₂ cells, all of which contain a well focused microtubule network, radiating from a single microtubule-organizing center at the centrosome (Clark and Meyer, 1999; Quintyne et al., 1999). Transfection of PCM-1 deletion construct 1–1468 disrupted this microtubule organization, with most microtubules now randomly distributed throughout the cytoplasm (Fig. 7, A and B). To test whether this is due to a lack of microtubule nucleation at the centrosome, we performed a microtubule regrowth assay. Transfected cells were treated for 40 min with 25 μM nocodazole on ice, to depolymerize all microtubules (Fig. 7, E and F), after which time the drug was washed out to allow regrowth of microtubules at 37°C. As shown in Fig. 7 (G and H), both untreated control cells as well as cells overexpressing mutant PCM-1 showed initial growth of small centrosomal microtubule asters. Within 15 min, however, the radial microtubule organization in mutant expressing cells was lost, and the mi-
Figure 8. Depletion of PCM-1, centrin-3, or ninein results in loss of microtubule anchoring at the centrosome.

(A, C, E, and G) U-2 OS cells were transfected with control dsRNA oligomers and stained in green for (A) PCM-1, (C) centrin-3, (E) ninein, and (G) pericentrin. Microtubules were stained in red, DNA in blue. (B, D, F, and H) Corresponding image pairs showing cells after treatment with siRNA against (B) PCM-1, (D) centrin-3, (F) ninein, and (H) pericentrin. I, J, U-2 OS cells treated with (l) control oligomers or (J) centrin-3 siRNA. Red, centrin-3 immunofluorescence; green, ninein immunofluorescence. K, immunoblots of cells treated with control RNA or siRNA against (left) centrin-3, or (right) ninein, stained for centrin-3 or ninein, respectively. Bar in H, 10 μm.

crotubules became randomly distributed in the cytoplasm, as compared with control cells (Fig. 7, I–K). This indicates that although centrosomal microtubule nucleation was not affected by inhibition of PCM-1, the ability of the centrosomes to anchor microtubules was disturbed. No microtubules were seen nucleated or anchored at the protein aggregates formed by mutant PCM-1, suggesting that proteins segregated to these structures were not competent to nucleate or anchor microtubules by themselves.

Depletion of PCM-1, centrin, or ninein inhibits anchorage of microtubules to the centrosome

Then, we tested whether removal of PCM-1 had the same effect on radial microtubule organization as overexpression of mutant PCM-1. As shown in Fig. 8 (A and B), PCM-1 depletion from U-2 OS cells by siRNA induced loss of centrosomal microtubule anchorage, leaving only 34% of the cells with a radial microtubule network (n = 1,001, controls 74%, n = 1,001). Similar results were obtained in mouse C2C4 myoblasts (unpublished data). Because we described earlier in this paper that removal of PCM-1 affected the localization of centrin, ninein, and pericentrin to the centrosome, we examined whether loss of microtubule anchorage was mediated by one or more of these proteins. For this purpose, we depleted centrin, ninein, or pericentrin individually, using specific siRNA oligomers. Protein levels were reduced to 23% (centrin), 29% (ninein), or 20% (pericentrin) of control levels, as measured by quantitative immunoblotting (Fig. 8 K) or photometric analysis (available antibodies against pericentrin were unable to recognize the denatured protein by immunoblotting). Reduction of centrin-3 and ninein levels similarly affected microtubule organization (Fig. 8, C–F), with only 18% (n = 1,001) and 31% (n = 934) of cells, respectively, exhibiting a radial network. In contrast, pericentrin depletion had no significant effect on microtubule organization (Fig. 8, G and H). Radial microtubules were seen in 77% of the cells (n = 833), approximately at the same level as in control cells (74%). However, we noticed that removal of pericentrin resulted in a reduced density of microtubules emanating from the centrosome (Fig. 8 H).

Because centrin-3 is not known to bind directly to microtubules, we tested whether loss of microtubule anchorage at the centrosome after centrin depletion could be due to an indirect effect mediated by ninein. As shown in Fig. 8 (I and J), removal of centrin-3 from U-2 OS cells also resulted in loss of ninein localization to the centrosome.

Discussion

Microtubule nucleation and anchoring of the microtubule filament network are two functions associated with the centrosome. Our present work has directly shown that these two functions can be separated, and that microtubule anchoring is dependent on the protein PCM-1. Most interestingly, PCM-1 itself is not a classical component of the centrosome, but instead localizes to electron-dense protein granules in the cytoplasm. Because these were most easily recognized in electron micrographs near the centrosome, they were termed centriolar satellites (Rattner, 1992; Kubo et al., 1999). This raises the question of how a satellite component such as PCM-1 functions in anchoring microtubules to the centrosomal surface. Our data suggest that the role of PCM-1 in
microtubule anchoring is an indirect one, most likely mediated through other proteins that assemble at the centrosome in a PCM-1-dependent manner. As shown in this paper, the correct assembly of a subset of centrosomal proteins, including centrin, pericentrin, and ninein, depends on PCM-1 function. Beyond this, we have demonstrated that PCM-1 binds to an isoform of the protein centrin. Supporting evidence was also provided by Li et al. (2001), who reported that PCM-1 binds to pericentrin-B, and by Balczon et al. (2002), who noted changes in centrosome morphology after PCM-1 antibody injection into mouse oocytes.

To understand the role of PCM-1, it is important to note that the distribution of centrin, pericentrin and PCM-1 is very dynamic (Baron et al., 1994; Kubo et al., 1999; Young et al., 2000) and dependent on the action of dynine–dynactin motor complexes (Balczon et al., 1999; Kubo et al., 1999; Purohit et al., 1999). Intriguingly, small granules of GFP-tagged PCM-1 have been directly followed by video microscopy, shuttling along microtubules between the cytoplasm and the centrosome (Kubo et al., 1999). Therefore, a possible function of PCM-1 could be to mediate the transport of centrosome components from the cytoplasm to the centrosome, along microtubules. PCM-1 may serve as a carrier that associates with centrin, pericentrin, or ninein, and docks onto dynein–dynactin. Consistent with this idea is our observation that depolymerization of microtubules, as well as dynacin inhibition, led to dispersion of centrosomal proteins and cytoplasmic protein aggregates that contain centrin, pericentrin and ninein, as well as dynein and PCM-1. Transport complexes would contain only a small proportion of cellular centrin, pericentrin, or ninein, explaining why most cell types do not show significant centriolar satellite staining of these proteins. Specific cell types such as PtK2 or mouse myoblasts do exhibit recognizable cytoplasmic granules of these proteins, and we show that they colocalize with PCM-1. Centrin granules are very dynamic structures that can rapidly fuse with the pericentril material (Baron et al., 1994), consistent with our transport model. A role of PCM-1 in facilitating transport of centrosomal proteins could be important for the duplication of centrosomes during the cell cycle, when new pericentril material is recruited to the centrosomal surface, and to increase the potential of centrosomes to organize microtubules into mitotic spindles. This would explain why PCM-1 staining before mitosis is particularly concentrated at the centrosomes, with fewer cytoplasmic granules visible than in interphase, and why the signal becomes again more dispersed in metaphase, after spindle poles have fully formed.

Another explanation of our data could be that PCM-1 granules in the cytoplasm represent sites at which centrosomal proteins associate temporarily to undergo proper folding, or to assemble into complexes with other proteins. The two interpretations on PCM-1 function are not mutually exclusive. Several centrosomal proteins are not simply confined to the centrosome, but are also present in a large cytoplasmic pool (Moudjou et al., 1996; Paolletti et al., 1996). Cytoplasmic factors that support folding and assembly as well as factors that aid transport would contribute to a dynamic equilibrium between centrosome-bound and free protein (Baron et al., 1994).

As shown in this paper, not all centrosomal proteins follow a PCM-1-dependent assembly pathway. In particular, we show that recruitment of γ-tubulin to the centrosome is independent of PCM-1, and apparently of dynein–dynactin-dependent transport. Our data are further consistent with previous reports by Khodjakov and Rieder (1999), Hannak et al. (2001), as well as earlier biochemical studies by Klotz et al. (1990), Paoletti et al. (1994), Moreitz et al. (1995), and Schnackenberg et al. (1998), that centrosomal targeting of γ-tubulin and other potential microtubule nucleation factors is independent of microtubules. In contrast, work by Quintyne et al. (1999) and Young et al. (2000) clearly demonstrates a requirement for dynactin in γ-tubulin assembly. These seemingly contradictory findings may be reconciled by the existence of different pools of γ-tubulin at the centrosome with different rates of exchange with the cytoplasm, as shown by Khodjakov and Rieder (1999). If only the slowly exchanging pool of γ-tubulin required dynactin function, for example, as a microtubule anchor rather than as a transporter, then effects on γ-tubulin localization would only be observed after prolonged treatment of cells with dynein inhibitors, as in the experiments of Quintyne et al. (1999) and Young et al. (2000), and not over the shorter time frame of a few hours in our experiments. There may also be differences in the dynamics of centrosomal components between different cell types and cell cycle stages, and these may explain the observation of dynactin-independent pericentrin assembly by Quintyne et al. (1999), in contrast to data from this study and Young et al. (2000).

Consistent with our finding of γ-tubulin assembly independent of PCM-1, microtubule nucleation at the centrosome is not affected when PCM-1 is inhibited. Our data highlight the notion that microtubule nucleation and the organization of the microtubule network are distinct events. Earlier studies by Keating et al. (1997) provided direct evidence for microtubule release from the centrosome. In addition, Mogensen et al. (2000) have shown that in polarized cell types, microtubules can be transferred after nucleation from the centrosome to apical regions of the cell. It has been suggested that a protein involved in microtubule anchorage at these sites is ninein, previously identified as a component of the pericentril material (Bouckson-Castaing et al., 1996). It has further been shown in a paper by Piel et al. (2000) that immature daughter centrioles, lacking ninein localization, are able to nucleate microtubules, but fail to anchor them. Here, we provide direct evidence for a role of ninein in microtubule anchorage to the centrosome by demonstrating that depletion of ninein causes loss of centrosomal microtubule organization. Our data further suggest that the effects of PCM-1 or centrin depletion on the microtubule network organization are mediated through ninein, because ninein levels at the centrosome decrease when PCM-1 or centrin are depleted.

Therefore, the potential of the centrosome to anchor microtubules may depend on the correct assembly of a subset of proteins; PCM-1 may be involved in targeting centrin to the centrosome, where it would be necessary for the assembly of ninein, and thereby regulate microtubule anchorage. As discussed above, this targeting of microtubule-anchoring factors appears to be mediated by dynein–dynactin-depen-
dent transport, consistent with observations by Quintyne et al. (1999) and Clark and Mayer (1999), who showed that dynactin inhibition interferes with microtubule organization at the centrosome. Pericentrin was also found to assemble at the centrosome in the cell decreased after pericentrin depletion, indicating that pericentrin either stabilizes microtubules or aids microtubule nucleation, as suggested by Dictenberg et al. (1998), due to its close association with γ-tubulin. Additional factors may be involved in the regulation of microtubule anchoring, such as the microtubule-severing protein katanin (Hartman et al., 2002), which was grown at RT under atmospheric conditions. Transient transfections were performed by calcium phosphate precipitation as described in Sambrook et al. (1989).

Materials and methods

Cell culture

HeLa, U-2 OS human osteosarcoma cells, C2C4 mouse myoblasts, Ptk2, and COS-7 cells were cultured in DME, CHO cells in McCoy's 5A medium, and Xenopus A6 cells in 0.6x L15 medium, all supplemented with 10% FBS. Cells were grown at 37°C and at 5% carbon dioxide, except A6 cells, which were grown at RT under atmospheric conditions. Transient transfections were performed by calcium phosphate precipitation as described in Sambrook et al. (1989).

Cloning of chicken PCM-1 cDNA and construction of expression vectors

An EST clone containing the middle 3.5-3.9 kb fragment of the chicken homologue of PCM-1 was obtained from a Bursal EST collection managed by S. Kandels-Lewis. University of Edinburgh, UK) using primers against centrin-3 (anti-HsCen3p; Laoukili et al., 2000) and against ninein (Mogensen et al., University of California, Berkeley, CA; Heald et al., 1997). The full-length cDNA (GenBank/EMBL/DDBJ accession no. AJ508717) assembled in the cloning vector pBluescript (Stratagene) in a series of cloning steps. A full-length PCM-1-GFP expression construct was then generated by modifying the cDNA insert at its 3' end by PCR to remove the stop codon, and cloning it in frame into the multiple cloning site of pEGFP-N (CLON-S. Kandels-Lewis, University of Edinburgh, Edinburgh UK) using hybridization probes derived from this EST, and the full-length cDNA (GenBank/EMBL/DDBJ accession no. AJ508717) assembled in the cloning vector pBluescript in a series of cloning steps.

The COOH terminus of human PCM-1 comprising nucleotides 4993-6095 after the start codon was amplified by PCR from a HeLa cDNA library provided by S. Kandels-Lewis, University of Edinburgh, Edinburgh UK) using primers CAGATCACCCAGCAACAGGAAGGCG and AGACATCACGGAGGCTCACCC, annealed at 57°C after the start codon was amplified by PCR, and the GFP tag removed by cutting the vector with Smal and NotI, blunting, and religating. A control vector for the expression of β-galactosidase was obtained from Dr. Adrian Bird (University of Edinburgh, Edinburgh UK).

Antibodies, immunofluorescence, and immunoblotting

The COOH terminus of human PCM-1 comprising nucleotides 4993-6095 after the start codon was amplified by PCR from a HeLa cDNA library (provided by S. Kandels-Lewis, University of Edinburgh, Edinburgh UK) using primers CGAAAAGGTGCGACGCGCAGCATC and CATGTCCTGAGCGCCTAC, annealed at 54°C after the start codon was amplified by PCR, and the GFP tag removed by cutting the vector with SmaI and NotI, blunting, and religating. A control vector for the expression of β-galactosidase was obtained from Dr. Adrian Bird (University of Edinburgh, Edinburgh UK).

Microinjection experiments

Affinity-purified PCM-1 antibody was injected into Xenopus A6 cells cultured on glass coverslips at 2 mg/ml in injection buffer (100 mM KCl, 10 mM potassium phosphate, pH 7.4). Cells were fixed with ethanol at −20°C and processed for immunofluorescence as above. Control injections were performed using rabbit IgG (Sigma-Aldrich) at the same concentration in injection buffer. Purified dy
naminatin (Wittmann and Hyman, 1999) at a concentration of 9 mg/ml was injected into CHO cells. After 2-4 h of incubation, cells were fixed and processed for immunofluorescence. Control cells were injected with fluorescently labeled secondary antibody.

**Centrin copurification experiments**

Centrin-3 was obtained by PCR from a HeLa cDNA library (provided by S. Kandels-Lewis, University of Edinburgh, UK) using primers ATGGATCATGTTAAGATGAGATGACCC and TAAATCTT-TAAATGTCACCCATATAAGGCA and cloned into the bacterial expression vector pGEX4T2 (Amersham Biosciences) using BamHI and EcoRI. Sequencing confirmed it to be identical to the previously published human centrin-3 sequence (Middendorp et al., 1997). Bacterial fusion protein in PBS was loaded on a gluthathione Sepharose 4B column and purified using reduced gluthathione according to the manufacturer’s instructions (Amersham Biosciences), and dialyzed against PBS.

HeLa cell extracts were prepared by resuspending cell pellets from 6 ml confluent 10-cm plates (~6 x 10⁹ cells) in 1 ml PBS using a Dounce homogenizer. Xenopus egg extracts were prepared as described by Murray (1991). Protein concentrations of the extracts prepared varied between 2-5 mg/ml for HeLa cell extracts and 40-100 mg/ml for Xenopus egg extracts. In each copurification experiment, 200 μg GST-centrin-3 or GST alone was added to 1 ml of HeLa extract or 10 mg Xenopus egg extract, diluted to 1 ml total volume in PBS, and incubated for 1 h at 4°C. GST fusion protein and associated interactors were then recovered by incubating the mixture with 100 μl glutathione Sepharose beads for 30 min at 4°C. After extensive washes with PBS, bound protein was eluted with 10 mM reduced glutathione in 8 M urea containing SDS and mercaptoethanol. Recovery of the GST fusion protein was confirmed by SDS-PAGE and Coomassie staining, and the copurification of PCM-1 tested by immunoblotting.

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