The *S. pombe* aurora-related kinase Ark1 associates with mitotic structures in a stage dependent manner and is required for chromosome segregation

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SUMMARY

Metazoans contain three aurora-related kinases. Aurora A is required for spindle formation while aurora B is required for chromosome condensation and cytokinesis. Less is known about the function of aurora C. *S. pombe* contains a single aurora-related kinase, Ark1. Although Ark1 protein levels remained constant as cells progressed through the mitotic cell cycle, its distribution altered during mitosis and meiosis. Throughout G2 Ark1 was concentrated in one to three nuclear foci that were not associated with the spindle pole body/centromere complex. Following commitment to mitosis Ark1 associated with chromatin and was particularly concentrated at several sites including kinetochores/centromeres. Kinetochore/centromere association diminished during anaphase A, after which it was distributed along the spindle. The protein became restricted to a small central zone that transiently enlarged as the spindle extended. As in many other systems mitotic fission yeast cells exhibit a much greater degree of phosphorylation of serine 10 of histone H3 than interphase cells. A number of studies have linked this modification with chromosome condensation. Ark1 immuno-precipitates phosphorylated serine 10 of histone H3 in vitro. This activity was highest in mitotic extracts. The absence of the histone H3 phospho-serine 10 epitope from mitotic cells in which the *ark1*$^+$ gene had been deleted (*ark1.Δ1);* the inability of these cells to resolve their chromosomes during anaphase and the co-localisation of this phospho-epitope with Ark1 early in mitosis, all suggest that Ark1 phosphorylates serine 10 of histone H3 in vivo. *ark1.Δ1* cells also exhibited a reduction in kinetochore activity and a minor defect in spindle formation. Thus the enzyme activity, localisation and phenotype arising from our manipulations of this single fission yeast aurora kinase family member suggest that this single kinase is executing functions that are separately implemented by distinct aurora A and aurora B kinases in higher systems.

Key words: Aurora, *S. pombe*, INCENP, Mitosis, Histone H3

INTRODUCTION

Upon commitment of a typical eukaryotic cell to mitosis the microtubule organising centre (MTOC) splits into two and each MTOC then generates an elaborate microtubule array. These arrays interdigitate to form the bipolar mitotic spindle (Wittmann et al., 2001). The chromosomes condense as the spindle forms and associate with the spindle microtubules via their kinetochores. Association of all of the kinetochores with the spindle triggers the degradation of key molecules such as securin and cyclin B and the chromatids split and move to either pole (Nasmyth et al., 2000). Genome separation is followed by cytokinesis as an acto-myosin ring constricts to cleave the cell into two (Glotzer, 1997). The timing and execution of these mitotic events involves the concerted action of several evolutionarily conserved protein kinases, including the nimA, polo and aurora-related kinases (Nigg, 2001).

Members of the aurora kinase family regulate a range of mitotic processes. The link between altered expression of human aurora kinases and cancer suggest that these proteins play a vital role in maintaining genome integrity (Adams et al., 2001a; Giet and Prigent, 1999). Metazoans appear to contain three distinct aurora-related kinases, each one of which regulates a different set of mitotic events. In each system, aurora A kinase controls spindle formation while aurora B kinase regulates chromosome disjunction and cytokinesis. Less is known about the function of the aurora C kinase.

Consistent with its role in spindle formation, aurora A kinase associates with the centrosome and microtubules. Mutation of the gene encoding *Drosophila* aurora A, results in the formation of monopolar rather than bipolar mitotic spindles (Glover et al., 1995). A similar defect in spindle formation occurs when the *Xenopus* aurora A, Eg2, is depleted from *Xenopus* egg extracts (Roghi et al., 1998). The *Xenopus* protein Eg5 is a member of the BimC family of kinesin-related proteins that are required for bipolar spindle formation in a range of systems (Walczak and Mitchison, 1996). Eg5 inhibition, like loss of aurora A function, results in the formation of monopolar rather than bipolar spindles (Kapoor et al., 2000). The ability of Eg2 to bind and phosphorylate Eg5 suggests that one aspect of Eg2 function may be to control the anti-parallel sliding of microtubules during mitosis (Giet et al.,

1999). Aurora A may control additional events during spindle formation because the *Xenopus* and mammalian molecules associate with centrosomes in the absence of microtubules (Gopalan et al., 1997; Roghi et al., 1998).

Aurora B kinases associate with chromatin and the spindle mid-zone (Adams et al., 2001a). This association requires the function of two different conserved molecules, INCENP and a member of a distinct sub-class of the proteins that share a common motif called the BIR domain, which we shall refer to as the BIRMs (for BIR domain proteins of mitosis) (Silke and Vaux, 2001). Mammalian INCENP was first identified as a chromosome-associated antigen that accumulated with the inner centromere regions of mammalian chromosomes (Cooke et al., 1987). Like aurora B, INCENPs and BIRM proteins move from the centromere to the overlap zone during anaphase B and are required for cytokinesis.

Recent data have highlighted a correlation between disruption of aurora B, INCENP or BIRM function and a decrease in chromosome condensation in a number of different systems. The loss of chromosome condensation correlates with the reduction in the level of phosphorylation of serine 10 of the N-terminal tail of histone H3 (Adams et al., 2001b; Giet and Glover, 2001; Hsu et al., 2000). Phosphorylation of histone H3 at this site was first shown to be associated with mitotic chromosome condensation in *Tetrahymena* (Wei et al., 1998). Mutation of serine 10 to alanine in this system resulted in perturbation of mitotic chromosome transmission and reduced chromosome condensation (Wei et al., 1999). Immunofluorescence revealed a correlation between the location of regions where histone H3 was phosphorylated on serine 10 and chromosome condensation (Schmiesing et al., 2001). This suggested that this phosphorylation event may drive chromosome condensation by recruiting condensin. Consistently depletion of aurora B from a *Drosophila* tissue culture cell line reduced serine 10 histone H3 phosphorylation and resulted in a concomitant reduction in chromatin association of a condensin sub-unit, *Barren* (Giet and Glover, 2001).

We describe the characterisation of the *S. pombe* aurora-related kinase Ark1. We describe the association of Ark1 with mitotic structures and show that Ark1 is required for spindle formation, kinetochore microtubule interactions and chromosome resolution during anaphase. We show that the chromosome resolution defects correlate with a reduction in the level of phosphorylation of histone H3 on serine 10 and that immunoprecipitates containing Ark1 can phosphorylate histone H3 on serine 10 in vitro. Ark1 protein levels do not change as cells progress through the cell cycle.

MATERIALS AND METHODS

Strains and culture

Strains used are listed in Table 1. Standard fission yeast techniques and media were employed (Moreno et al., 1991). With the exception of h^{90} mating, cells were either grown in EMM2 minimal or YES complete medium. Cells were grown in EMM2 with 4 μM thiamine to repress the transcription of Eg2 from the *nmt1*$^+$ promoter in pRep3×Eg2 (Maundrell, 1993). For studying meiosis, IH2124 was cultured as previously described (Petersen et al., 1998).

Molecular manipulations

The XlEg2 cDNA (Roghi et al., 1998) was cloned into the *Sma1* site of pRep3X creating pRep3xEg2. The assignation of all nucleotide positions for *ark1*$^+$ is according to the *S. pombe* genome sequencing project at the Sanger Centre (SPCC320). pINTArk1PKC was generated as follows. pArk1BS contains the genomic sequences stretching from 2287-5219. The *ark1*$^+$ stop codon (position 4214) was converted to an *Nde1* site using the Quickchange kit (Stratagene) to generate pArk1Nde1STOP. Sequencing determined that the desired change was the only alteration to this sequence. The region extending from the *Nde1* site to the *Pst1* site (position 2287) of pArk1Nde1STOP was cloned between the *Pst*I and *Nde*I sites of pRep42PKC (Craven et al., 1998) to generate pArk1PKC. A *Pst*I linker was inserted into the *Hinc*II site of pINT6 (Drummond and Hagan, 1998) to make pINTA. The *Pst*I *Sac*I fragment from pArk1PkC was inserted between the *Pst*I and *Sac*I sites of pINTA. This generated pINTArk1PkC in which the *leu1*$^+$ gene is disrupted with a cassette that contains the *ura4*$^+$ gene and a version of the *ark1*$^+$ gene that is regulated by its endogenous promoter, fused to three copies of the PK tag and the termination sequences of the *nmt1*$^+$ gene at its C-terminus. The following strategy was used to delete the *ark1*$^+$ coding sequences. An *Nde1* site was created at position 3062 by the Quickchange strategy to make pArk1D1. pArk1D1 was digested with *Nde*I and *Eco*47III, treated with Klenow and a *Not*I linker was inserted to substitute for residues 3062-4291 in pArk1.D2. The *LEU2*$^+$ gene was excised as a *Not*I fragment from pLeu2Not1[2] (F. H. MacIver, D. M. Glover and I.M.H., unpublished) and two copies of this fragment were inserted into the *Not*I site of pArk1.D2 to generate pArk1.D3. The 2287-5219 fragment from pArk1.D3 was transformed into IH634 strain to generate strain IH1877. The correct insertion of the fragment was confirmed by PCR (not shown). Sequence alignments were done with Lasergene software package.

Table 1. Strains used in this study

Strain number	Genotype	Source
IH365	h$^-$ ura4.*D18 leu1.32*	Lab collection
IH376	*h$^-$ cdc10.v50 leu1.32 ura4.d18 ade6.M210*	Reymond et al., 1992
IH534	*h$^-$/h$^+$ ura4.d18/ura4.d18 leu1.32 /leu1.32 ade6.M210/ade6.M216 his2$^-$/his2$^+$*	Lab collection
IH738	*h$^-$ cdc7.A20 ura4.D18*	Lab collection
IH1308	*h$^-$ ura4.d18*	This study
IH1877	*h$^-$/h$^+$ ura4.d18/ura4.d18 leu1.32 /leu1.32 ade6.M210/ade6.M216 ark1.Δ1::LEU2$^+$ LEU2$^+$/ark1$^+$ his2$^-$/his2$^+$*	This study
IH2034	*h$^-$ leu1.32 ura4.d18* pRep3XEg2	This study
IH2035	*h$^+$ Nuf2GFP:kanMX pintark1.pkC::leu1:ura4$^+$ ark1.Δ1::LEU2$^+$LEU2$^+$ ura4.d18 his2$^-$*	This study
IH2036	*h$^-$ pintark1. pkC::leu1:ura4$^+$ ark1.Δ1::LEU2$^+$ LEU2$^+$ ura4.d18*	This study
IH2116	*h^{90} cdc10.v50 pintark1.pkC::leu1:ura4$^+$ ark1.Δ1::LEU2$^+$LEU2*	This study
IH2117	*h$^-$ cdc25.22 pintark1.pkC::leu1:ura4$^+$ ark1.Δ1::LEU2$^+$LEU2$^+$ ura4.d18*	This study
IH2124	*h^{90} Nuf2GFP:kanMX pintark1.pkC::leu1:ura4$^+$ ark1.Δ1::LEU2$^+$LEU2$^+$ ura4.d18*	This study

Western blotting to monitor Ark1.PkC levels

Total cell extracts were prepared by TCA precipitation (Caspari et al., 2000), run on 10% SDS-PAGE and blotted as described previously (Mulvihill et al., 1999) using alkaline phosphatase coupled secondary antibodies.

Kinase assays

A cell pellet containing 1.2×10^8 cells was washed once in stop buffer and snap frozen. The pellet was resuspended in 100 μl HEN buffer (50

mM Hepes (pH 8.0), 150 mM NaCl, 5 mM EGTA, 5 mM EDTA, 1% NP-40, 50 mM β-glycerophosphate, 50 mM NaF, 1 mM Na_3VO_4, 1 mM leupeptin, 1 μg/ml aprotinin, 1 mM PMSF) (Cueille et al., 2001). 1 ml ice-cold glass beads was added to the suspension and the tube was shaken in a Ribolyser at 4°C for 4 seconds at full speed. The cell lysate was cleared by spinning at 16,000 ***g*** for 10 minutes at 0°C. 336 anti-PK epitope monoclonal antibody (Serotec MCA 1360) (Southern et al., 1991) was covalently coupled to sepharose beads (Harlow and Lane, 1988) and the beads were then equilibrated with HEN buffer. 95 μl of soluble extract was added to 10 μl of packed beads and the mixture was incubated at 4°C for 30 minutes. The beads, with the associated immunoprecipitate, were washed three times in 0.5 ml HEN buffer and three times in 0.5 ml KAB buffer (50 mM Hepes (pH 7.5), 150 mM NaCl, 1 mM DTT, 10 mM $MgCl_2$) before final resuspension in 20 μl KAB buffer. After incubation at 32°C for 5 minutes, 5 μl of substrate mix containing 20 μM ATP and 5 μg purified Histone H3 (Roche Molecular Biochemicals) were added to the IP complex. After a further 20 minutes at 32°C the reaction was stopped by the addition of 10 μl of 3× SDS-page loading buffer and boiling for 5 minutes. A nitrocellulose blot of a 15% SDS-PAGE gel was cut in half and the upper part (30-90 kDa) was probed with α-PK antibodies to detect Ark1.PkC. The lower part (10-30 kDa) of the same membrane was probed with rabbit poly-clonal anti-phospho-serine 10-histone H3 (H3SP) (cat. no. 06-570 lot no:19633; Upstate Biotechnology, NY). Both primary antibodies were detected with SuperSignal ULTRA (Pierce).

Fluorescence microscopy

Immunofluorescence was as described (Hagan and Asycough, 2000). For anti-tubulin immunofluorescence with TAT1 antibodies (Woods et al., 1989) standard combined aldehyde fixation was used. For other staining cells were fixed with 3% formaldehyde alone. For localisation of Pk-tagged Ark1 with the monoclonal antibody 336, which recognises the PK epitope (Southern et al., 1991), and Cut7 with cAP5LB antibodies (Hagan and Yanagida, 1992) fixation was carried out for 2 minutes. With the 2 minutes fixation it was vital to incubate in antibodies at 4°C. Cells were fixed for 5 minutes in preparation for H3SP (Upstate biotechnology, NY). Images were acquired with a Quantix (Photometrix) slow scan CCD camera and processed with Metamorph (Universal Imaging).

RESULTS

Ectopic expression of *Xenopus* Eg2 kinase in *S. pombe* results in defective mitotic chromatin architecture and spindle formation

As an initial approach to the study of aurora function in *S. pombe* the *Xenopus* aurora A kinase, Eg2, was expressed from the strong inducible *nmt1*$^+$ promoter (Maundrell, 1990). Gene induction was associated with mitotic defects (Fig. 1). At early time points in the induction the chromatin was stretched along elongating spindle in 30% of mitotic cells (Fig. 1A,C, open circles). At later time points spindle formation defects became apparent as multiple microtubule bundles extended from a single focus of the spindle pole marker *Sad*I (Fig. 1B,C, open squares). Diploid cells with large amorphous nuclei accumulated in the culture as the incubation period extended (Fig. 1C, filled squares).

ark1$^+$ encodes an aurora-related kinase that is required for chromosome segregation and spindle formation

In order to identify molecules with aurora-related functions we isolated multi-copy plasmids that could suppress the lethal consequences of *Xenopus* aurora A overproduction. Two plasmids were retrieved that contained the *S. pombe* aurora-related kinase, which we will refer to as *ark1*$^+$ (aurora-related kinase; SPCXC320.13C at Sanger centre; CAA18315 at NCBI). Increasing *ark1*$^+$ gene dosage suppressed both the spindle formation and chromosome segregation defects arising from *Xenopus* aurora A expression (data not shown). We conclude that *Xenopus* aurora A generated mitotic defects because it perturbed Ark1 function. Searching the *S. pombe* genome database has failed to identify a second aurora-related kinase suggesting that *S. pombe*, like *S. cerevisiae*, contains a single aurora kinase. Using the Jotun Heim alignment algorithm to compare *ark1*$^+$ with the two *Drosophila* aurora kinases gave virtually identical scores suggesting that *ark1*$^+$ is no more closely related to the aurora A or aurora B isoforms (data not shown; for a full alignment see Giet and Prigent) (Giet and Prigent, 1999).

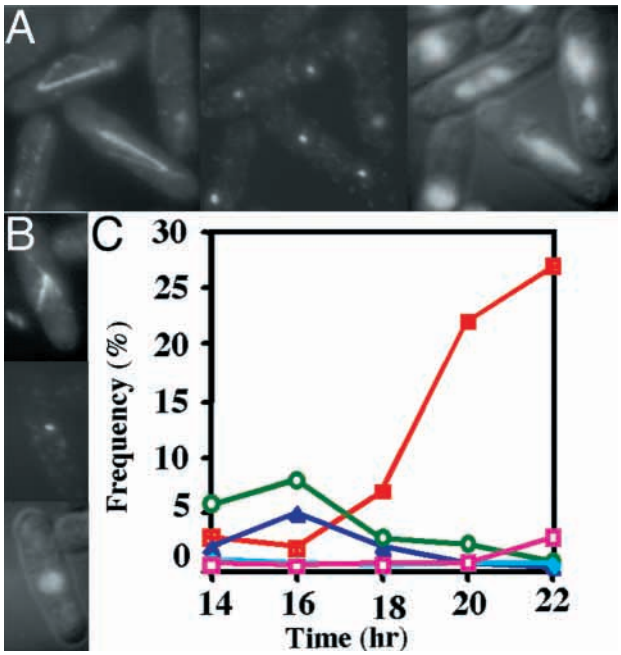

Fig. 1. Spindle formation and chromosome segregation defects upon overproduction of *Xenopus* Aurora A in *S. pombe*. Panels A and B show immunofluorescence/DAPI staining. The first panel in each series bears the label and shows microtubules, the next spindle poles, while the last one shows a combined DAPI and DIC image. Expression of Eg2 induces defects in chromosome segregation (A) and spindle formation (B). Wild-type cells bearing a pREP3XEg2 plasmid were grown at 30°C so that 20 hours after induction they would be in mid-log phase and samples were fixed every 2 hours from 14-22 hours later. The three mitotic cells in A are clearly unable to segregate their chromosomes. The microtubules in B emanating from a single focus of Sad1 staining indicates that spindle formation is defective. (C) Quantitation of the phenotypes arising from expression of *Xenopus* aurora A. The graph shows the frequency with which each particular mitotic defect is seen in an entire population of cells. Open squares, monopolar spindles; open circles, chromatin stretched along an anaphase spindle; triangles, 'cut' phenotype; green diamonds cells with bi-polar spindles and condensed chromatin; filled squares, diploid cells with large amorphous nuclei.

Cells lacking ark1⁺ exhibit defects in spindle formation and chromosome segregation

The region of the genome encoding one of the two *ark1*$^+$ genes present in the diploid strain IH1877 was replaced with *LEU2*$^+$ encoding sequences. The deletion allele this generated was called *ark1.Δ1*. Tetrad dissection of IH1877 gave two viable leu$^-$ spores indicating that the *ark1*$^+$ gene is essential for viability. Expression of *ark1*$^+$, but not *Eg2*, from a heterologous locus supported growth of *ark1.Δ1* cells (see below). In order to determine the consequences of loss of Ark1 function a preparation of spores from IH1877 was grown in the absence of leucine to specifically favour for growth of only *ark1.Δ1* spores and not *ark1*$^+$ spores. The distribution of microtubules and chromatin revealed four phenotypes. The most prevalent phenotype at early time points following germination at 25°C was a stretching of chromatin along an elongating anaphase B spindle (Fig. 2A). This phenotype suggested a defect in chromosome condensation in prophase or resolution during the metaphase/anaphase transition. In the second phenotypic class, condensed chromosomes were seen alongside robust short spindles. The chromosomes in these cells often failed to associate with the spindle or were found at one end of the spindle (Fig. 2B, arrow). The latter phenotype suggested that kinetochore function is perturbed by loss of Ark1 function while the former is reminiscent of the '*dis*' phenotype (Ohkura et al., 1988). (*dis* mutants form a mitotic spindle that elongates but does not completely disappear at the end of anaphase B. As sister chromatids do not separate in a '*dis*' mitosis, the unsplit chromosomes randomly associate with either of the two poles.) The third phenotype that was very rare, but consistently seen at 30°C, was a spindle formation defect as microtubules emanated from a single focus of SPB staining (Fig. 2C). The final phenotype that became apparent 36 hours after germination at 30°C was the appearance of exceptionally elongated cells (Fig. 2D). The chromatin of these cells was either compact (Fig. 2D) or a diffuse amorphous staining.

The proportion of histone H3 that is phosphorylated on serine 10 is lowest in G1 and highest in mitosis

Given the correlation between histone H3 phosphorylation and aurora B activity (Adams et al., 2001a; Adams et al., 2001b) we used characterised antibodies that specifically recognise phosphorylated histone H3 (Wei et al., 1998) to monitor the extent of this modification in *S. pombe*. We will refer to these antibodies as H3SP.

Logarithmically growing wild-type *S. pombe* cells have fulfilled the size requirement for passage through START when

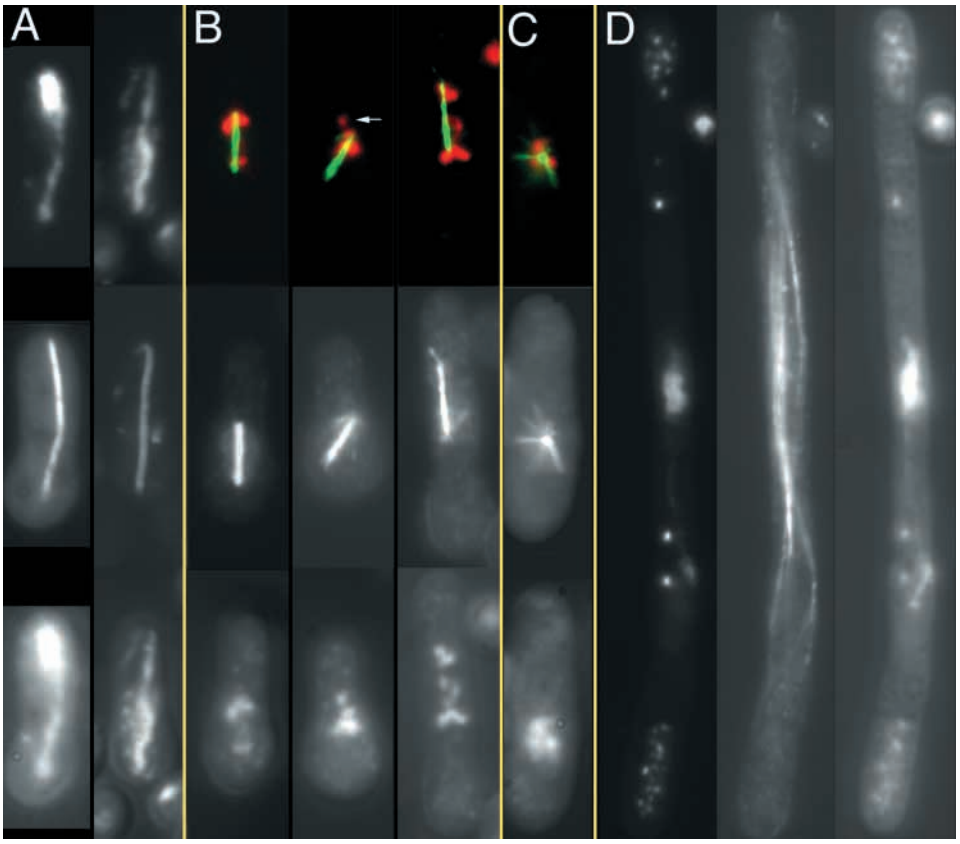

Fig. 2. Cells lacking Ark1 function have defects in spindle formation, chromosome segregation, chromosome resolution during anaphase and cell cycle progression. Spores from diploid strain IH1877 were germinated in minimal medium supplemented with all amino acids except leucine in order to select for those spores containing the *ark1.Δ*1 allele. 24 hours after germination cells were stained to reveal microtubules and chromatin. (A-C) The first panel in each series bears the label and shows chromatin, the next microtubules, the third a combined chromatin and DIC image.
(A) Chromosome resolution is defective in *ark1.Δ1* cells. Chromatin is stretched out along the spindle. (B) *ark1.Δ1* cells have abnormal mitotic figures in which condensed chromosomes associate with a normal spindle. In each case the chromosomes have not split into two sister chromatids. In the left and middle cells the chromosomes are clustered at one end of the spindle. In the cell on the right, one chromosome is at either end and one is in the middle of the spindle. As the spindle on the right is elongating the chromosomes should already have split into two sister chromatids and moved to the spindle poles. The fact that a chromosome is lagging on this spindle and that one is apparently unattached to the spindle (arrow) indicates that kinetochore function is compromised when Ark1 function is compromised. (C) Ark1 is required for spindle formation. Instead of forming a bipolar spindle, microtubules extend from a single focus around which the chromosomes cluster. These figures became prominent at later time points when the germination was at 30°C, suggesting that spindle formation does not require as much Ark1 activity as chromosome resolution in anaphase B, the first defect to be seen. (D) *ark1.Δ 1* cells exhibit a novel *cdc*$^-$ phenotype. From left to right; DAPI, microtubules, DAPI/DIC. An extensive microtubule network extends between the cell tips while a small compact nucleus is present in the middle of the cell. Note that this panel is printed to 80% of the scale of the others in the figure.

mitosis is complete. Cells therefore commit to the next cell cycle before cytokinesis has produced two daughter cells (Baum et al., 1997). G1 phase is therefore very short in logarithmically growing cultures. Binucleate, post-mitotic cells are therefore in either an extremely short G1 or S phase of the cell cycle, whereas all uni-nucleate interphase cells in a culture are in G2. Thus cell 1 in Fig. 3A is in G2, cell 2 and 3 are in mitosis while cell 4 is in G1 or S phase. Comparison of the H3SP staining patterns (Fig. 3A, top) in these cells shows little difference in the intensity of H3 staining between cells 1-3 but a considerable decrease in cell 4. In order to determine whether the reduction in staining in cell 4 corresponded to a reduction of staining in G1 or S phase we stained for H3SP following arrest of cell cycle progression in G1 or S phase of the cell cycle. The nuclei of cells that had been arrested in early S phase by incubation with hydroxyurea stained strongly with H3SP (Fig. 3B). By contrast, staining in *cdc10.v50* cells that had been arrested in G1 before START (Fig. 3C, uninucleate cells) was much lower than that of the control *cdc7.A20* mutant cells that had been mixed into the culture to provide an internal reference for the basal level of H3SP staining (Fig. 3C, multinucleate cells). We conclude that H3SP reactivity was lowest in G1 phase of the cell cycle.

Switching from the very sensitive detection system (CY3 conjugated secondary antibodies at a concentration of 1 in 2000) used in Fig. 3A-C to a less sensitive regime (FITC-conjugated secondary antibodies at a concentration of 1 in 100) revealed a further level of complexity to the pattern of H3SP staining. The less sensitive detection system was unable to detect H3SP staining in interphase cells but entry into mitosis was accompanied by the appearance of discrete H3SP foci over a fainter general H3SP chromatin staining (Fig. 3D; Fig. 4A). Because the behaviour of the spots of strong H3SP staining was consistent with the behaviour of kinetochores, we localised H3SP in a strain in which GFP was fused to the kinetochore marker Nuf2 (Wigge and Kilmartin, 2001). The strongest spots of H3SP staining did indeed show a very strong association with Nuf2-GFP staining (Fig. 3F). As cells entered anaphase the fainter general chromatin H3SP staining diminished and the number of zones of H3SP staining decreased (Fig. 3F). By mid-late anaphase B each nucleus contained one to three spots of H3SP staining (Fig. 3D-F). One spot was always tightly associated with the Nuf2-GFP spot at the nuclear periphery while the other had a more random location (Fig. 3F).

Mitotic phosphorylation of histone H3 on serine 10 is absent from *ark1.Δ1* cells

We next asked whether H3SP reactivity was affected in *ark1.Δ1* cells. Because *ark1*$^+$ is essential it was necessary to stain germinating *ark1.Δ1* spores with H3SP. We therefore

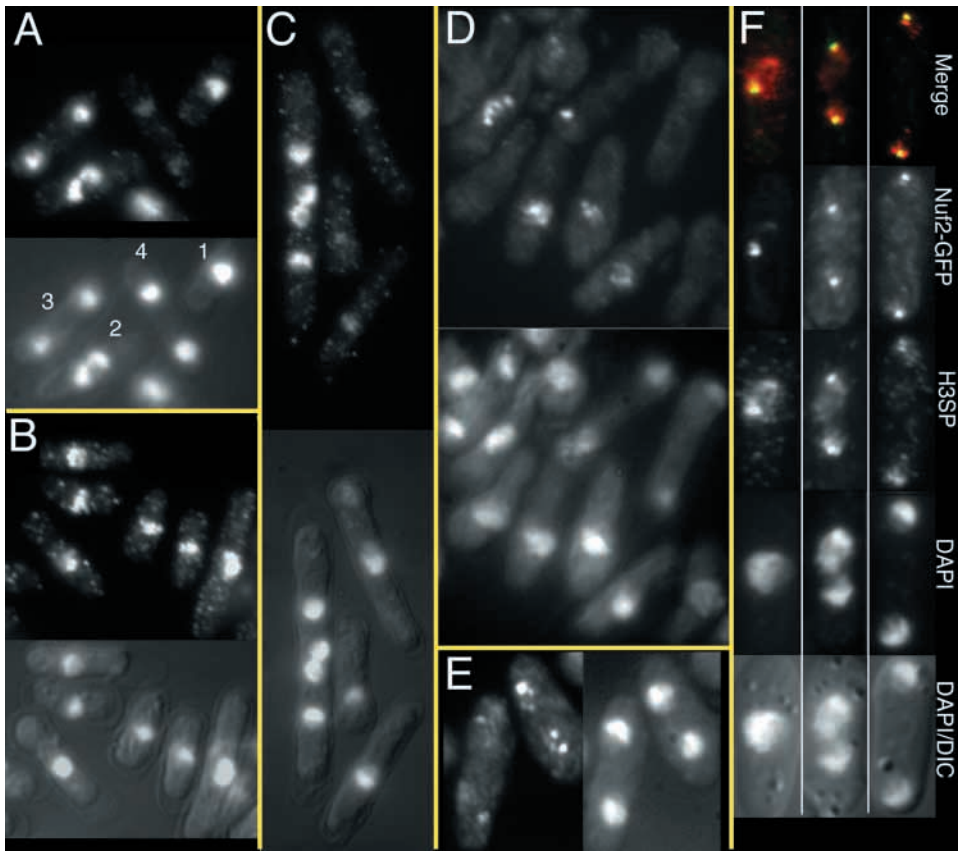

Fig. 3. Antibodies against phospho-serine 10 of histone H3 stain *S. pombe* nuclei through the cell cycle, but staining is radically reduced in G1 cells and highest on mitotic centromeres. In each case, the top panel shows H3SP staining and the bottom DAPI/DIC. All panels use the same concentration of primary antibodies. For (A-C) Cy3 anti-rabbit antibodies were used at a dilution of 1:2000, for (D-H) FITC anti-rabbit antibodies were used at a dilution of 1:100. (A) Asynchronous wild-type cells. Cell 1 is in G2, cells 2 and 3 are in M, and cell 4 is in G1/S phase. Note the strong H3SP chromatin fluorescence in all cells except for 4. (B) H3SP staining is strong in cells arrested in early S phase. Cells were grown to early log phase in rich medium at 30°C before the addition of hydroxyurea. The chromatin of these early S phase cells had bright H3SP staining. (C) A mixed culture of *cdc10.v50* and cdc7.A20 cells were grown to early log phase in rich medium at 25°C before the temperature was changed to 36°C for a further 6 hours. The H3SP staining of the single nuclei in the three *cdc10.v50* arrested cells is much lower than that of the multi-nucleate, actively cycling *cdc7.A20* mutant cell on the left. (D-E) Asynchronous wild-type cells stained by a less sensitive procedure to visualise H3SP than that used in A-C. Nuclear staining is only seen in the mitotic cells. (F) H3SP and Nuf2.GFP localisation. In each case the first (top) image is a merge of the Nuf2.GFP fluorescence (second panel) and H3SP staining (third panel). The fourth panel shows DAPI staining, while the fifth is stained with DAPI/DIC. In each case the pole proximal spot of H3SP associates with Nuf2.GFP kinetochore fluorescence. The two signals are not entirely overlapping as revealed by the presence of red, yellow and green sectors in many such pole associated spots - for example, in the middle cell.

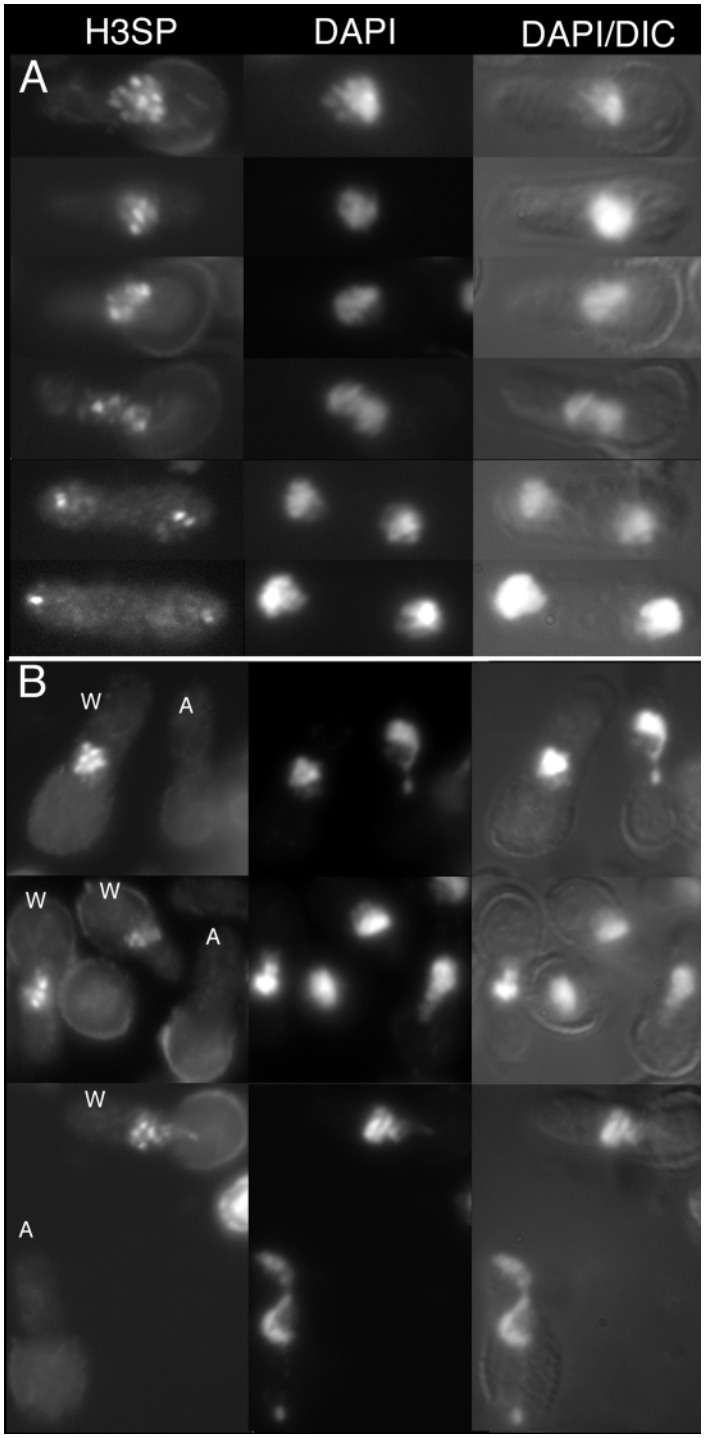

Fig. 4. H3SP staining of germinating spores reveals a deficiency of H3SP reactivity in *ark1.Δ* cells. (A) Spores arising from sporulation of wild-type strain IH534 were inoculated into full supplemented minimal medium and processed for immunofluorescence using the procedures for Fig. 3D-F 24 hours later. Left panels, H3SP staining; middle, DAPI; right; DAPI/DIC. No staining was seen in interphase spores. Upon mitotic commitment a lumpy pattern of general chromatin staining was seen alongside some much brighter spots of H3SP staining. The number of spots of strong H3SP staining decreased with mitotic progression. During anaphase B a spot of strong staining was always seen at the extreme ends of the nuclei indicative of close proximity to the spindle pole. Towards the end of anaphase B staining generally diminished to two chromatin-associated spots. (B) Spores arising from sporulation of IH1877 were inoculated into full supplemented minimal medium. Left, H3SP; middle, DAPI; right, DAPI/DIC images of the same fields of cells. Strong H3SP staining is clearly visible in the mitotic wild-type nuclei (W) but undetectable in the stretched *ark1.Δ1* mitotic nuclei (A).

started this analysis by staining germinating wild-type spores. It was important to examine these wild-type germinating spores because the first mitosis after germination in *S. pombe* is unique in forming a metaphase plate before committing to anaphase A. This means that extensive chromosome condensation is seen. As it is unclear why this mitosis should differ from those in actively cycling it remained possible that the staining of an antigen associated with chromosome condensation at this stage of the life cycle may have some special features. However, as Fig. 4A shows, the staining pattern was virtually identical to that pattern seen in logarithmically growing cells. By contrast, when spores were prepared from IH1877 to permit growth of both *ark1*$^+$ and *ark1.Δ1* spores a novel staining pattern was seen. While discrete regions of strong H3SP staining were seen in wild-type mitotic cells (Fig. 4B, cells labelled W) H3SP staining was completely absent from *ark1.Δ1* cells (Fig. 4B, cells labelled A). The fact that chromatin condenses and a metaphase plate is formed during the first division after germination in *S. pombe* accentuated the difference between the *ark1*$^+$ and *ark1.Δ1* spores. The lack of staining in the *ark1.Δ1* spores may either be due to a reduction in H3SP levels or a secondary consequence of epitope masking arising from defects in chromosome resolution during mitosis. We therefore stained H3SP in cells in which the function of topoisomerase II or the essential condensin subunit Cut3 was abolished by temperature-sensitive mutations. H3SP staining remained strong throughout mitosis despite the lack of chromosome resolution in these strains (data not shown) indicating that the absence of staining in *ark1.Δ1* cells was a direct consequence of the loss of Ark1 function.

The levels of epitope tagged Ark1 remain constant as cells progress through the cell cycle

Having established the phenotypic consequences of deleting the *ark1*$^+$ gene we tagged the gene at its C-terminus with three copies of the Pk epitope (Southern et al., 1991) in order to initiate a biochemical and cytological characterisation of Ark1 protein. This C-terminally tagged protein will be referred to as Ark1.PkC. Because we experienced repeated difficulties in directing integration to the *ark1*$^+$ locus throughout the course of this study the tagged gene was integrated at the *leu1* locus. The expression of this *ark1.PkC* allele at the *leu1* locus (*leu1::ark1.PkC ura4*$^+$) was regulated by the endogenous *ark1*$^+$ promoter. The *ark1.PkC* allele was combined with the *ark1.Δ1* allele in strain IH2036 so that the tagged Ark1.PkC was the only Ark1 protein in the cell. These cells undergo a normal mitosis (see below) (Fig. 8) indicating that the protein is fully functional. Small G2 IH2036 cells were isolated from an asynchronous culture by centrifugal elutriation and Ark1.PkC levels were monitored as the cells progressed through two successive cell cycles. No appreciable changes in

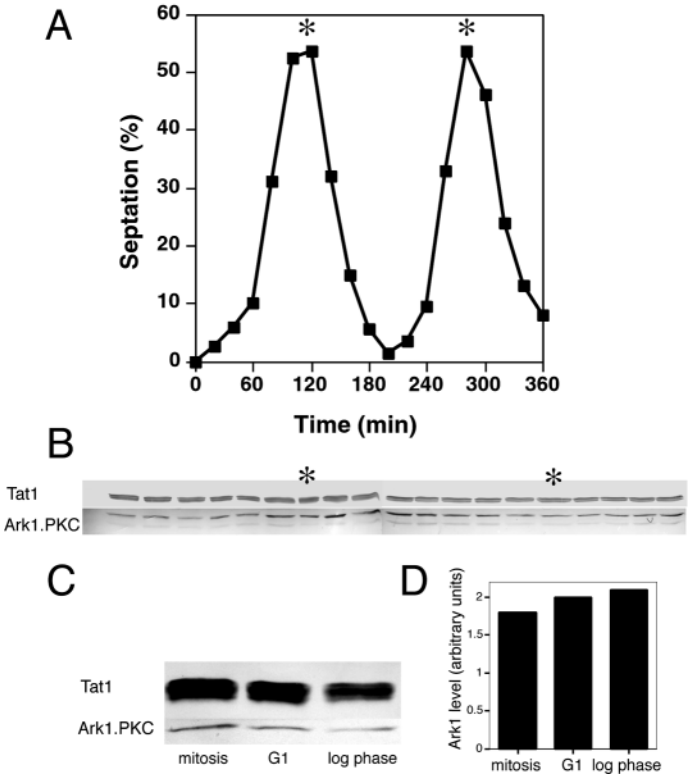

Fig. 5. Ark1.PkC levels do not fluctuate as cells progress through the cell cycle. A cell-cycle synchronised population of strain IH2036 was generated by elutrient centrifugation at 25°C in rich YES medium. The small G2 cells were harvested at the beginning of the experiment and samples were taken every 20 minutes to score the septation index (A) and for western blot analysis of whole cell extracts (B). The blot was cut in two and the top portion was probed with antibodies to a tubulin (TAT1) to act as a loading control for the Ark1.PkC blot (below). The position of the peaks seen in the septation index are indicated by asterisks above the septation profile and the western blot. Quantitation of the ratio of the TAT1 to Ark1.PkC signals for two independent blots led to the conclusion that there is no great fluctuation in Ark1.PkC levels as cells progress through the cell cycle (data not shown). (C) Ark1 protein levels remain constant after arrest in the G1 phase of the cell cycle. Extracts were prepared from cells which were manipulated to be in distinct cell cycle stages. IH2117 was blocked at 37°C for 4 hours 15 minutes and released to 25°C for 45 minutes (mitotic sample). In this mitotic sample 60% of the cells had either condensed chromosomes or were binuclear without septa. A culture of IH2116 was split in two and one half was incubated at 37°C for 5 hours 30 minutes to arrest cell cycle progression at the *cdc10* execution point (G1-phase sample). (D) Quantification of the bands shown in C. In each case the intensity of the Tat1 signal in the upper panel was divided by the intensity of the Ark1.PkC signal in the lower panel.

protein level were detected (Fig. 5A,B). As discussed above, when cells are growing in logarithmic phase, G1 events are initiated upon commitment to mitosis (Baum et al., 1997). Thus G1 phase is effectively absent from the synchronous culture shown in Fig. 5. The constant level of Ark1.PkC throughout the time course may be seen because the phase of the cell cycle in which the protein is unstable, G1 is effectively missing. We therefore used a different approach to ask whether Ark1.PkC levels in G1 phase cells were lower than those in a log phase or an M phase population. Protein extracts were prepared from a culture of *cdc10.v50* cells grown at the permissive temperature of 25°C or from a portion of the same culture that had been incubated at the restrictive temperature of 37°C for 5.5 hours to arrest cell cycle progression in G1 phase. To prepare a mitotic extract, strain IH2117 was incubated for 4.25 hours at 36°C to arrest cells in G2 and then the temperature was returned to 25°C to induce a synchronous mitosis. The M phase population was harvested 45 minutes after the return to 25°C. There was no appreciable difference in Ark1.PkC levels between these samples or those in mitotic cells (Fig. 5C,D). These data suggest that, unlike other aurora-related kinases, Ark1.PkC is not subject to bulk degradation after mitosis.

Phosphorylation of serine 10 of histone H3 in Ark1 immunoprecipitates is highest in mitosis

The dependency of H3SP staining upon the presence of Ark1 suggested that the Ark1 may directly phosphorylate serine 10 of histone H3. In turn, the constant level of Ark1.PkC as cells transit the cell cycle suggested that if Ark1 possesses such activity, it must be subject to cell cycle stage specific regulation. We precipitated Ark1.PkC from cell extracts in order to conduct kinase assays to address this question. The monoclonal antibody which recognises the Pk epitope, 336, was coupled to sepharose beads. These beads were incubated with G1, S, G2 and M cell extracts that were prepared according to the procedures described in the previous section. The level of H3SP reactivity following incubation of histone H3 with Ark1.PkC immunoprecipitates from G1, S or G2 cells was slightly higher than the background levels seen in control reactions (Fig. 6). There was a very strong signal in the lane in which Ark1.Pk had been precipitated from a mitotic population. These data show that Ark1.PkC associated kinase activity can phosphorylate serine 10 of histone H3 and that this activity is enhanced during mitosis.

Ark1 shows stage-specific association with the mitotic spindle and centromeres

Staining IH2036 cells to reveal the distribution of Ark1.PkC revealed punctate nuclear patterns which were reminiscent of the behaviour of centromeres and spindle pole bodies (SPBs) (Funabiki et al., 1993; Wigge and Kilmartin, 2001). We therefore started this analysis by charting kinetochore movements as the spindle formed by staining a strain containing Nuf2.GFP (IH2035) with the SPB marker Sad1 (Hagan and Yanagida, 1995). As FISH analysis of centromere behaviour has previously shown (Funabiki et al., 1993) the single interphase Nuf2.GFP spot was tightly associated with the SPB. During the early stages of mitotic commitment kinetochore distribution was highly variable. While kinetochores remained associated with the very short spindle in some cases (Fig. 7B), in other spindles of exactly the same length, they had wandered a long way from the SPB (Fig. 7C). After the spindle had elongated to span roughly one third of the diameter of the nucleus all the kinetochores were found at some point along the spindle axis, which is defined as the shortest distance between the two SPBs at this stage of mitosis (Fig.7D) (Hagan and Yanagida, 1995).

Having established the behaviour of kinetochores during spindle formation we determined the distribution of Ark1.PkC

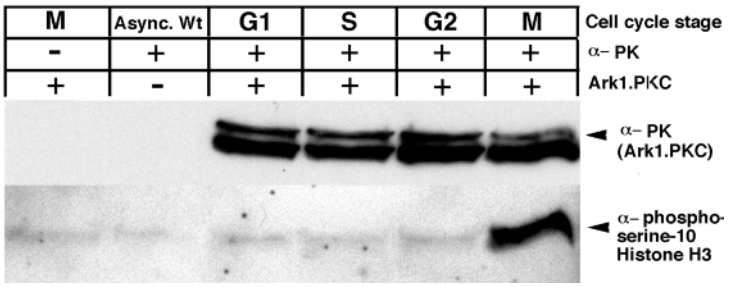

Fig. 6. Ark1-associated phosphorylation of histone H3 at serine 10 is highest in mitosis. This figure shows a cold kinase assay that uses anti-phospho-serine 10-histone H3 antibodies to detect phosphorylation of serine 10 of histone H3 in immunoprecipitates. Extracts were prepared from cells that were manipulated to be the equivalent of mid-log phase growth state but be enriched in one of four distinct cell cycle stages. IH2117 was blocked at 37°C for 4 hours 15 minutes (G2-phase sample) and released to 25°C for 45 minutes (mitotic sample). In this mitotic sample, 60% of the cells had either condensed chromosomes or were binuclear without septa. IH2116 was blocked at 37°C for 5 hours 30minutes (G1-phase sample). 100 mM hydroxyurea was added to IH2036 and the culture was incubated for 5 hours at 36°C (S-phase sample). The presence of α-Pk antibodies on the beads or Ark1.PKC protein in the strain used to make the extract is indicated above each lane. The band corresponding to the full length protein is weaker than in Fig. 5B and a number of degradation products appear below Ark1.PkC in the Ark1.PkC panel. Despite this uniform degradation there is dramatic increase in H3SP reactivity in mitotic extracts over the interphase extracts (right-hand panel).

relative to the kinetochore maker Nuf2.GFP in strain IH2035. The results are presented in Fig. 8. The merged images have been processed to reveal the relative locations of the individual signals rather than portray all of the information seen with each individual channel. The merged images in panels 1-14 are twice the size of the individual panels. Panels 15-17 are marked with an asterisk to show that they are presented at the same scale as the adjacent single channel figures of the same cells.

Ark1.PkC was present in discrete nuclear foci throughout interphase (Fig. 8, cells 1-3). These interphase foci did not associate with the single spot of Nuf2.GFP staining. When cells reached the critical length for commitment to mitosis the entire nucleus became more reactive to anti-Pk antibodies and a bright spot of Ark1.PkC staining was seen in tight association with a single Nuf2-GFP dot (Fig. 8, cells 4-7). Spots were still seen in other regions of the nucleus, but they were fainter than this Nuf2.GFP-associated signal. From this point on until anaphase the strongest regions of Ark1.PkC staining were associated with a Nuf2.GFP signal. The two signals often overlapped, but on occasion it was possible to see two spots of Nuf2.GFP staining either side of an Ark1 spot. A good example of such a pattern is seen in Fig. 8, cell 8 and the enlarged version of this image in Fig. 9A. The kinetochores in this cell are neither maximally separated nor aligned in the linear fashion that would indicate spindle association. Given the striking resemblance between this pattern and the Nuf2.GFP staining in Fig. 7C, we conclude that these cells have very short spindles and that the kinetochore in question is not yet associated with it. This relationship between the Ark1.PkC and Nuf2.GFP signals shown by the arrows in Fig. 9A is consistent with Ark1.PkC localisation to the inner centromere region. As the kinetochores moved towards the SPBs during anaphase A, Ark1.PkC association with the kinetochores became less pronounced (Fig. 8, cells 9,10; Fig. 9B,C). Several kinetochores did not have any associated Ark1 signals. At this level of resolution it is not possible to determine whether this was due to the disassociation of Ark1.PkC from the kinetochores or because of deformation of the kinetochore/centromere complex prior to sister chromatid separation. After anaphase A was complete and the chromosomes had reached the SPBs, Ark1.PkC was seen along the short spindle (Fig. 8, cell 11). The protein accumulated in the mid-zone during spindle elongation (Fig. 8, cell 12). The region of Ark1.PkC staining in the spindle extended as the spindle extended (Fig. 8, cells 13,14) until late anaphase B when it was once more a restricted region of punctate staining (Fig. 8, cell 16). Once the spindle had broken down and cytokinesis had started, punctate Ark1.PkC staining, which was not associated with the SPB/centromere complex, was seen once more (Fig. 8, cell 17).

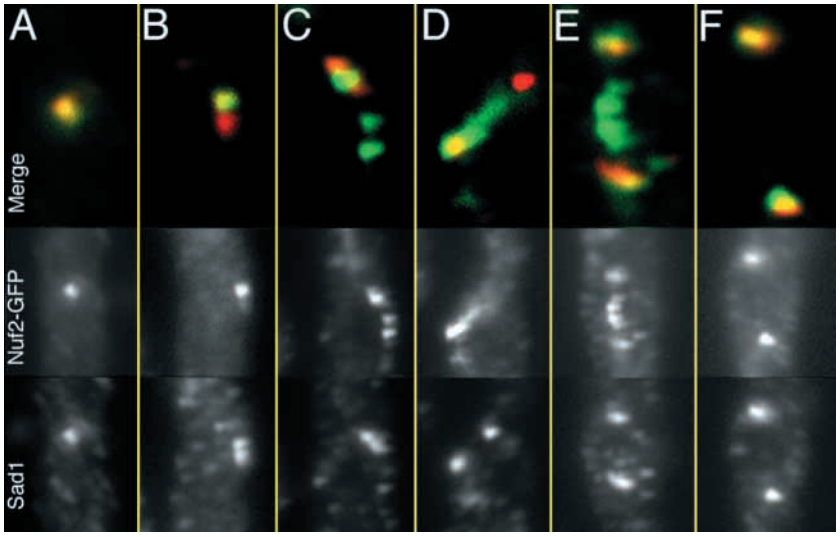

Fig. 7. The kinetochores can wander some distance from the spindle as it forms. IH2035 cells were grown to mid-log phase in rich medium at 30°C and processed for anti-Sad1 immunofluorescence. In each case the top panel is a merge of the middle Nuf2.GFP signal and the lower Sad1 staining. It is shown at twice the magnification of the images of the individual channels to show the juxtaposition of the signals most clearly. During interphase a single spot of Nuf2.GFP always associated with the Sad1 SPB stain. Upon spindle formation the association of Nuf2.GFP with Sad1 staining became much more varied. In some cases (B) association was maintained, in others (C) it was not. The two lower kinetochores in B have wandered a long way from the spindle that is forming in the upper right portion of the nucleus. At later stages of spindle formation the kinetochore marker was always seen on a line that marked the shortest route between the two Sad1-reactive SPB spots (D-E) until the kinetochores reached the poles at the end of anaphase B (F).

Ark1.PkCand Cut7 association with the central spindle during anaphase B

The association of Ark1.PkC with the central spindle during anaphase B was highly reminiscent of the distribution of the kinesin related protein Cut7 at this time (Hagan and Yanagida, 1992). It has been reported that the *Xenopus* Cut7 homologue, Eg5, associates with the *Xenopus* aurora A, Eg2 (Giet et al., 1999). This raised the possibility that Cut7 may be taking Ark1.PkC from the polar regions to the mid zone. We therefore stained cells to simultaneously visualise Ark1.PkC and Cut7 (Fig. 10). The two staining patterns showed very little co-registration, suggesting that any co-localisation in the central regions is more likely to be due to the independent accumulation of both proteins in the same part of the spindle rather than a direct physical association.

Because the association of aurora B kinases with the mid-zone of the spindle has been associated with a role in cytokinesis once the spindle disassembles it is possible that the accumulation of Ark1 in the central spindle is anticipating a function in cytokinesis in fission yeast. If this were its sole role it would not be required in the middle of the meiotic spindles as dissolution of these spindles is not followed by cytokinesis. We therefore monitored Ark1.PkC distribution in meiosis in an h^{90} strain. The distribution was essentially the same as that seen in mitotic cells up until spore formation (Fig. 11). The protein was found in the anaphase B overlap zone of both the first (Fig. 11B) and second meiotic spindle (Fig. 11D).

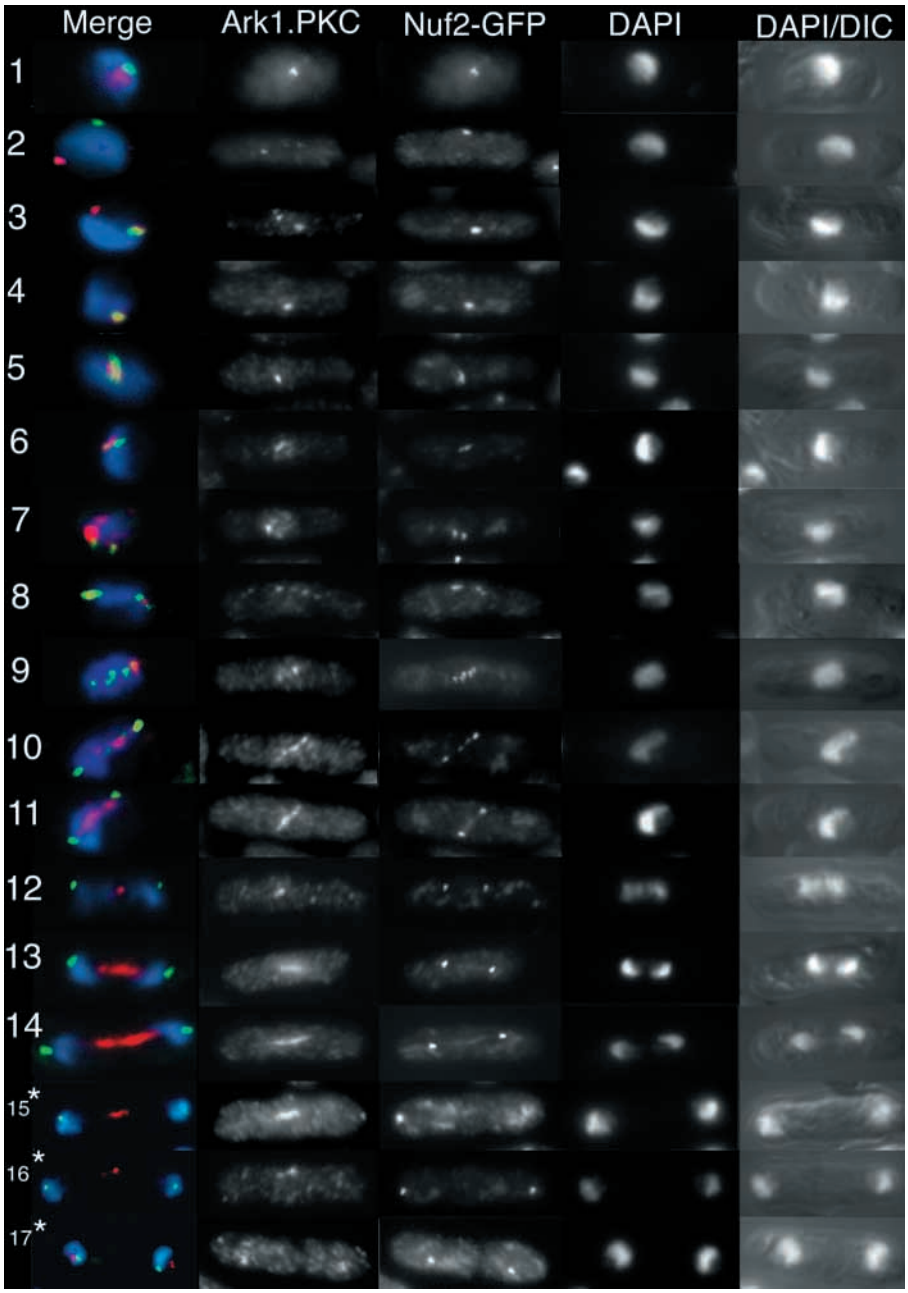

Fig. 8. Ark1.PkC and Nuf2.GFP staining as cells transit through the cell cycle. IH2035 was grown to mid-log phase before preparation for anti-Pk immunofluorescence microscopy. In each case the panels show (from right to left): DAPI/DIC, DAPI, Nuf2.GFP fluorescence, Ark1.PkC staining and a merge of the two middle panels on the left. For cells 1-14 the merged image is double the size of the other images of these cells, whereas the merged image in 15-17* is the same scale as all the other panels. For full details see the text. The figure shows images of cells following 2 minutes fixation. The same patterns were seen if fixation time was extended to 30 minutes with the exception that the intensity diminished and the interphase nuclear spots and mitotic nuclear accumulation were no longer detected.

DISCUSSION

We have characterised the role played by the aurora-related kinase Ark1 in regulating mitotic progression in fission yeast. In contrast to metazoans, where aurora A is required for spindle formation and aurora B required for both chromosome condensation and cytokinesis, a single aurora kinase, Ark1, is required for mitosis in *S. pombe*. The phenotypes arising in cells that lack Ark1 suggest that this single kinase is performing functions that are related to the functions of each of the two isoforms in higher systems. The spindle formation defects of *ark1.Δ1* cells resemble the consequences of perturbing aurora A function in *Drosophila* and *Xenopus* (Glover et al., 1995; Roghi et al., 1998), while the lack of chromosome resolution during anaphase resembles aurora B deficiencies in *C. elegans* and *Drosophila* (Adams et al., 2001b; Giet and Glover, 2001; Kaitna et al., 2000; Severson et al., 2000b). Although we failed to detect a requirement for Ark1 during cytokinesis, it remains a possibility that, like

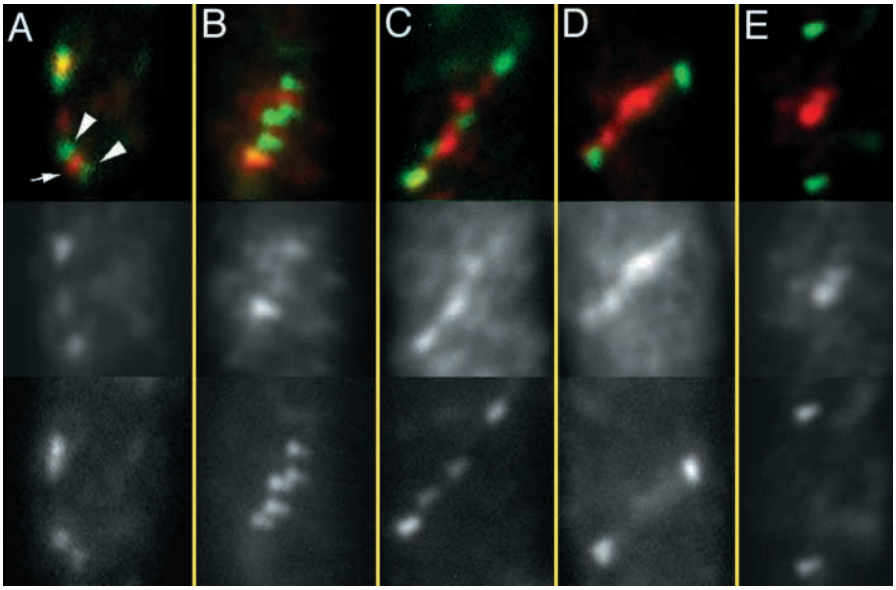

Fig. 9. Ark1.PkC and Nuf2.GFP staining from pro-metaphase to early anaphase B. Images from panels 8-12 of Fig. 8 processed to superimpose (top) the distribution of Nuf2.GFP fluorescence (bottom) upon the Ark1.PkC staining pattern (middle) rather than give the best representation of the signal from each individual channel. The merged images are twice the size of the single signals. For full details see text. Note the two green Nuf2.GFP signals (arrowheads) either side of the red Ark1 spot (arrow) at the bottom of the image in A.

aurora B, Ark1 is required for cytokinesis but that this requirement is much less sensitive to the reduction of Ark1 levels in germinating *ark1.Δ1* cells than chromosome condensation or spindle formation. The fact that *S. pombe* Ark1 appeared to conduct an amalgam of the functions executed by aurora A and B may explain why *Xenopus* aurora A is unable to substitute for Ark1. It would also explain why enhanced expression of *ark1*$^{+}$ can suppress the deleterious consequences of *Xenopus* aurora A expression in *S. pombe*. Although it is unable to perform the same functions as Ark1, the *Xenopus* protein may be interacting with one or more Ark1 binding partners. Increasing Ark1 levels would successfully compete for *Xenopus* aurora A and reintroduce functional Ark1 to these complexes, thus restoring sufficient activity for a normal mitosis.

The mitotic distribution of Ark1 is reminiscent of the distribution of metazoan aurora B (Adams et al., 2001a). Before mitosis it was localised to between one to three nuclear foci. The nature of these foci and whether they are reflecting an important role for Ark1 in interphase remains unclear at present. It is clear that they do not represent the centromere/SPB complex (Funabiki et al., 1993). It is unclear whether they are stable structures that persist at particular sites throughout interphase, or whether Ark1 is accumulating in different regions of the nucleus at different times. What could Ark1 be doing at these sites? A strong link between aurora B function and phosphorylation of histone H3 on serine 10 during mitosis has been established (Adams et al., 2001a). Phosphorylation at this site also plays a critical role in interphase when it is important to modulate transcriptional activity by controlling chromatin architecture (Cheung et al., 2000). We show that Ark1 can phosphorylate serine 10 of histone H3 in vitro and that this modification is virtually absent from mitotic cells from which *ark1*$^{+}$ has been deleted. It is therefore possible that during interphase Ark1 is conducting functions that are analogous to those carried out by other histone H3 kinases such as JIL1 (Wang et al., 2001), which control transcription by regulating chromatin architecture. This would be entirely consistent with the similarity between the

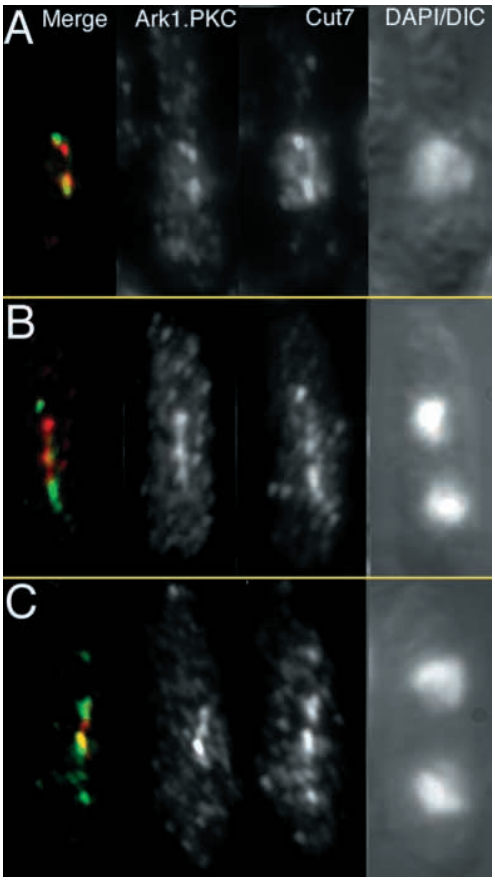

Fig. 10. Cut7 and Ark1.PkC do not show appreciable co-localisation as the spindle elongates during anaphase B. IH2035 cells were grown to mid-log phase in rich medium at 30°C and processed for Cut7 and Ark1.PkC staining. Cut7 staining of the termini of the metaphase spindle does not show a strong overlap with Ark1.PkC staining in the same cells (A). Similarly the distribution of the two molecules along the anaphase B spindle is very different (B,C). The restricted nature of the co-localisation of the two signals is graphically shown by the predominance of green and red signals over the co-localisation yellow signals in the merged image.

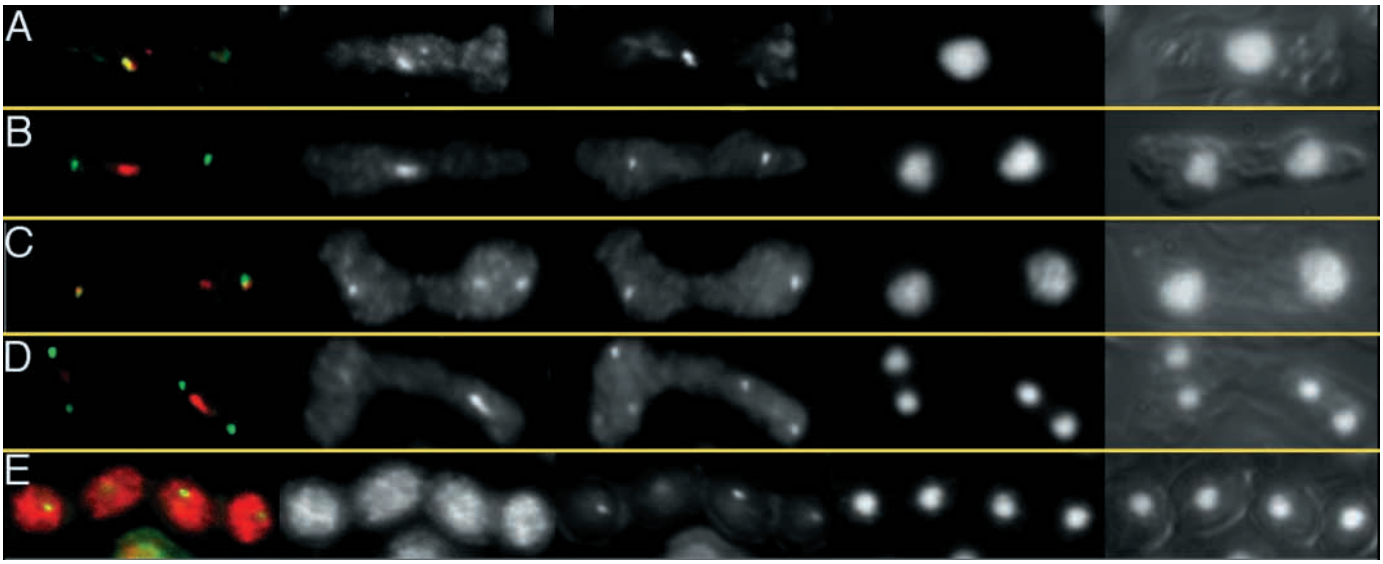

Fig. 11. Ark1 distribution in meiosis. A mid log culture of IH2124 was spread on MSA plates and incubated overnight to induce mating. Cells were washed off and processed for Pk staining. In each case the panels show (from right to left): DAPI/DIC, DAPI, Nuf2.GFP fluorescence, Ark1.PkC immunofluorescence and a merge of the two middle panels on the left. Ark1.PkC distribution is highly reminiscent of that in mitosis. As the spores form Ark1.PkC reactivity accumulates.

distribution of interphase Ark1 spots and the foci of sites at which histone H3 is phosphorylated on serine 10 during interphase. The slight enhancement of H3SP reactivity in interphase extracts over the background level seen in control samples may represent an interphase Ark1 kinase activity. However, although the current assay does demonstrate a very strong mitotic activation of Ark1 activity, it is not sensitive enough to draw firm conclusions about a possible interphase activity at present.

The progressive acquisition of phosphorylation of histone H3 on serine 10 shows a strong correlation with progressive chromosome condensation during mitotic commitment in mammalian cells (Hendzel et al., 1997). Moreover, the reduction in this phosphorylation event that occurs after RNAi-mediated ablation of *Drosophila* aurora B is accompanied by an inability to recruit the condensin sub-unit *Barren* (Giet and Glover, 2001). Ark1 also appears to play a crucial role in both chromosome condensation and phosphorylation of histone H3 (H3SP) on serine 10 as chromosome segregation fails and antibodies that recognise H3SP stain wild-type, but not *ark1.Δ1* cells and Ark1 immunoprecipitates can phosphorylate this serine residue. Punctate interphase staining for both Ark1 and H3SP was succeeded by staining throughout the early mitotic nucleus and strong association of both epitopes with the centromeres. Pronounced H3SP reactivity of centromeres is reminiscent of the accumulation of H3SP staining in pericentromeric regions in late G2 of mammalian cells (Hendzel et al., 1997). H3SP staining of centromere/kinetochore persisted throughout mitosis, but Ark1 lost its affinity for these regions during anaphase A and associated with the spindle microtubules after anaphase A was complete. Ark1 maintained its association with the central spindle to varying degrees for the duration of anaphase B. After spindle dissolution Ark1 was once more localised to discrete nuclear spots.

Both aurora A and B have been reported to associate with the microtubules of the mitotic spindle (Adams et al., 2001a; Giet and Prigent, 1999). *Xenopus* aurora A has been shown to associate with and phosphorylate the motor protein Eg5 (Giet et al., 1999). This raised the possibility that in addition to a direct association with microtubules (Roghi et al., 1998) aurora A associates with microtubules via an interaction with Eg5-related molecules. However, we failed to find a strong correlation between the distribution of the *S. pombe* Eg5 homologue Cut7 and the distribution of Ark1 suggesting that Ark1 is not associating with the spindle as a consequence of an interaction with Cut7. However, Ark1 does resemble aurora A in being required for spindle formation. Aurora A associates with the spindle pole in a microtubule independent manner (Gopalan et al., 1997; Roghi et al., 1998). Despite a concerted effort we were unable to detect Ark1 on the SPB from prometaphase to telophase. However, we cannot rule out a transient association of Ark1 with the SPB in the very early stages of mitosis because the proximity of the centromere associated Ark1 to the SPB at this time would obscure such a signal.

Aurora B behaves like a classical chromosomal passenger protein in that it leaves the centromeres and associates with the spindle microtubules in anaphase B (Earnshaw and Bernat, 1991). Abolition of the function of aurora B or its binding partners INCENP or BIRM blocks cytokinesis (Giet and Glover, 2001; Kaitna et al., 2000; Severson et al., 2000a; Uren et al., 2000). It has been proposed that this effect is due to the weakening of the integrity of the spindle mid-zone that occurs when this complex is not operative. These abnormalities in spindle structure correspond to the loss of a conserved kinesin-related protein that is required to locate the mitotic kinase Polo to the mid-zone where it is thought to control cytokinesis (Adams et al., 1998). Alternatively, because INCENP binds to the cleavage furrow (Eckley et al., 1997) the INCENP/aurora B complex may play a direct role in regulating cytokinesis. What are the parallels with fission yeast? It seems unlikely that

Ark1 is required to maintain the integrity of the central spindle as the morphology of anaphase B spindles was normal when the chromosomes were clearly not being resolved. As in other systems, *S. pombe* polo kinase Plo1 is required to define the architecture of the acto-myosin ring; however, Plo1 executes this role much earlier than in higher systems and it is complete by metaphase (Bähler et al., 1998). Although *S. pombe* does then use Plo1 to regulate the contraction of the actin ring during cell division this appears to be mediated through the control of the SPB-located septum initiation network rather than an effect in the cleavage furrow (Tanaka et al., 2001). Despite these differences does it remain possible that Ark1 is associating with the spindle to directly regulate aspects of cytokinesis? Because *S. pombe* has a closed mitosis, spindle associated Ark1 is shielded from directly influencing the pre-assembled cleavage furrow by the nuclear membrane. Also, Ark1 still accumulates in the central overlap zone of both meiotic spindles, neither of which is followed by cytokinesis. Although this may rule out an influence on the acto-myosin ring, it is worth noting that, when aurora B is depleted from *C. elegans* or *Drosophila* by RNAi, it is the late, rather than early, stages of cytokinesis that fail (Adams et al., 2001b; Giet and Glover, 2001; Kaitna et al., 2000; Schumacher et al., 1998; Severson et al., 2000a). Fusion of the plasma membrane to generate two separate membranes is an integral part of the later stages of cytokinesis. A similar problem confronts systems with a closed mitosis, but it is more complex as they must resolve both the nuclear and plasma membranes. It is therefore possible that Ark1 associates with the spindle to get to the regions where it can regulate membrane fusion events in telophase.

In line with our ability to detect Ark1 in interphase cells by immunofluorescence, microscopy western blot analysis of synchronous cultures and cells arrested in G1, S and G2, as well as an M phase population, indicated that Ark1 levels did not change appreciably as cells progressed through the mitotic cell cycle. This contrasts with mammalian aurora A whose abundance is highest during mitosis (Gopalan et al., 1997). While the protein levels of aurora B have not been characterised, transcription of the mammalian aurora B AIM-1 peaks in mitotic cells (Kawasaki et al., 2001). The levels of the *S. cerevisiae* protein Ipl1p are lower in G1 arrested cells than in actively cycling cells or cells arrested in mitosis (Biggins et al., 1999). It has been proposed that the presence of a ‘KEN’ box in aurora-related molecules may target them for cell cycle stage-specific destruction and so account for fluctuations in protein level (Pfleger and Kirschner, 2000). However, if there is a relation between the KEN box and cyclical degradation of aurora-related kinases it is likely to be more complex than currently appreciated as Ark1 contains a KEN box but does not appear to be degraded in a cell cycle-dependent fashion in logarithmically dividing cells.

In many respects other than protein stability, Ark1 resembles the single *S. cerevisiae* aurora-related kinase Ipl1p. This kinase associates with the anaphase B spindle and is required for chromosome segregation and, like Ark1, has a minor defect in spindle architecture (Chan and Botstein, 1993; Kim et al., 1999). Errors in chromosome disjunction are common in *ipl1.2* mutant cells and probably arise from a reduction in the affinity of kinetochores for microtubules (Biggins et al., 1999). This effect is likely to be mediated by a direct regulation of kinetochore function as both Ipl1p and its partner Bir1p bind to the kinetochore protein Ndc10 and a GST-Ipl1p fusion protein can phosphorylate an GST-Ndc10p fusion protein in vitro (Biggins et al., 1999; Yoon and Carbon, 1999). A role for aurora kinases in regulating kinetochore activity may well be conserved as depletion of aurora B from *Drosophila* cell lines leads to lagging chromosomes in anaphase B (Adams et al., 2001b; Giet and Glover, 2001). It is therefore significant that a subset of *ark1.Δ1* cells have defects in chromosome association with an apparently normal spindle. In addition we observed several cells with the ‘dis’ phenotype in which chromosomes with unseparated chromatids are seen at the spindle poles (Ohkura et al., 1988). One of the dis mutants *dis2.11* encodes a protein phosphatase 1 catalytic subunit (Ohkura et al., 1989). Significantly, mutations in the budding yeast PP1 *GLC7* or overproduction of a Glc7p inhibitor can suppress the chromosome segregation defects of Ipl1 mutants (Francisco et al., 1994; Sassoon et al., 1999). Along with the hyperphosphorylation of Ndc10p in *glc7.10* mutants these data indicate that Glc7p antagonises the activity of Ipl1p. If the defect in *ark1.Δ1* cells is just a defect in kinetochore activity one is forced to ask why the sister chromatids are not separating in anaphase *ark1.Δ1* cells that have the ‘dis’ phenotype (Fig. 2B). One explanation may lie in a recently postulated role for aurora-related kinases in the regulation of sister chromatid cohesion (Adams et al., 2001a).

The localisation and functional interactions between Ipl1p, the INCENP-related protein Sli15p, Bir1p and kinetochore proteins suggests that Ipl1p is more akin to an aurora B than an aurora A. A similar image is likely to emerge from *S. pombe* as cells lacking the BIRM protein Pbh1 have the same chromosome segregation defects as *ark1.Δ1* cells (Rajagopalan and Balasubramanian, 1999). However, mutation of both Ark1 and Ipl1p results in the formation of monopolar rather than bipolar spindles, a feature most commonly attributed to aurora A molecules. Intriguingly, in experiments in which the kinase domain was swapped with that from aurora A and aurora B it was only the catalytic domain of aurora A that was able to support growth of Ipl1 mutants (Bischoff et al., 1998). Ark1 and Ipl1p may therefore constitute a distinct class of aurora-related kinase. The divergence of function may reflect differences in the biology of these two systems from higher eukaryotes. Because the actin ring forms before anaphase in each yeast (in budding yeast when the bud emerges) the cell does not need to use auroras to control ring formation. Similarly, the inner face of the SPB is only used for nucleating spindle microtubules. It does not therefore require an aurora to drive the upregulation of microtubule nucleating activity that is integral to converting the interphase centrosome to an active mitotic pole in higher systems. Further study of these two proteins and their partner molecules will therefore clearly complement the extensive body of data that is rapidly accumulating from the analysis of the two main higher eukaryotic isoforms aurora A and B.

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