A role for glycogen synthase kinase-3 in mitotic spindle dynamics and chromosome alignment

James G. Wakefield*,‡, David J. Stephens and Jeremy M. Tavaré

Department of Biochemistry, School of Medical Sciences, University of Bristol, Bristol BS8 1TD, UK

*Author for correspondence (e-mail: james.wakefield@zoo.ox.ac.uk)

‡Present address: Department of Zoology, University of Oxford, South Parks Road, Oxford OX1 3PS, UK

Accepted 14 November 2002

Journal of Cell Science 116, 637-646 © 2003 The Company of Biologists Ltd

doi:10.1242/jcs.00273

Summary

Glycogen synthase kinase-3 (GSK-3) is a conserved, multifunctional kinase that is constitutively active in resting cells, and inactivated through phosphorylation by protein kinase B (PKB). We have investigated the temporal and spatial control of GSK-3 phosphorylation during the cell cycle in mammalian cells. We show that GSK-3 is present along the length of spindle microtubules and that a fraction of GSK-3 is phosphorylated during mitosis. Phospho-GSK-3 is abundant at the centrosomes and spindle poles but absent from other areas of the spindle. GSK-3 phosphorylation occurs concomitantly with the appearance of phosphorylated and active PKB at the centrosome, which suggests that PKB is the kinase responsible for phosphorylating and inactivating GSK-3 at the centrosome during mitosis.

We demonstrate that lithium and two structurally distinct inhibitors of GSK-3 promote defects in microtubule length and chromosomal alignment during prometaphase. Treated cells contain mono-oriented chromosomes concentrated at the plus ends of astral microtubules, which are longer than in untreated cells. Live microscopy of cells expressing Histone-2B-GFP confirms that the inhibition of GSK-3 suppresses mitotic chromosome movement and leads to a prometaphase-like arrest. We propose that GSK-3 is regulated in a temporal and spatial manner during mitosis and, through controlling microtubule dynamics, plays an important role in chromosomal alignment on the metaphase plate.

Movies available online

Key words: GSK-3, Mitotic spindle, Microtubule, PKB

Introduction

GSK-3 was identified over 20 years ago as a kinase that phosphorylates glycogen synthase (for a review, see Frame and Cohen, 2001; Embi et al., 1980). The kinase consists of two closely related isoforms, GSK-3α and GSK-3β, which are ubiquitously expressed. GSK-3 is constitutively active in resting cells and can be inactivated by phosphorylation on an N-terminal serine residue (Ser21 of GSK-3α and Ser9 of GSK-3β). Insulin causes the inactivation of GSK-3 through phosphorylation by the upstream kinase protein kinase B (PKB; also known as Akt) (Cross et al., 1995). Thus, insulin leads to dephosphorylation of glycogen synthase and a concomitant increase in glycogen synthesis. Studies in *Drosophila* also identified GSK-3 as a crucial component of the conserved developmental wingless signalling pathway (Seigfield et al., 1992). In the absence of a signal, GSK-3 is part of a complex containing Axin, the adenomatous polyposis coli protein (APC), and β-catenin, all of which are substrates for GSK-3. β-catenin is a transcriptional activator which, when phosphorylated by GSK-3, is targeted for degradation through ubiquitin-mediated proteolysis. Inactivation of GSK-3 causes disruption of the complex and leads to elevated levels of free β-catenin, which translocates into the nucleus and activates transcription of wingless-specific genes (for a review, see Ferkey and Kimelman, 2001; Rubinfeld et al., 1996).

Several studies have also pointed towards a role for GSK-3 in the regulation of microtubule dynamics during interphase. In neuronal cells, GSK-3 is able to phosphorylate a number of microtubule-associated proteins, such as MAP2C, MAP1B and Tau (Lovestone et al., 1996; Goold et al., 1999; Sanchez et al., 2000). Phosphorylation of these proteins by GSK-3 decreases their ability to stabilise microtubules (Lovestone et al., 1996; Wagner et al., 1996; Utton et al., 1997).

Microtubule dynamics need to be exquisitely controlled during mitosis in order to produce a spindle apparatus capable of successfully segregating chromosomes. As cells enter mitosis, microtubule nucleation from the centrosomes dramatically increases and microtubules become more 10-100 times more dynamic (for a review, see Inoue and Salmon, 1995; Compton, 2000). The rapidly growing and shrinking microtubules are captured and stabilised by chromosomes, allowing formation of a bi-polar mitotic spindle. These changes in microtubule dynamics occur concomitantly with the phosphorylation of many proteins, through the activation of a number of mitotic kinases, such as cdc2 kinase, polo kinase and aurora kinase (for a review, see Cassimeris, 1999; Nigg, 2001).

We sought to examine a possible role for GSK-3 in regulating microtubule stability during mitosis in cultured mammalian cells. Here, we report that GSK-3 is phosphorylated at the minus ends of the mitotic spindle, where active PKB is also localised. Treatment of HeLa cells with inhibitors of GSK-3 leads to an increase in the length of mitotic microtubules and defects in chromosome congression on the

metaphase plate, suggesting that GSK-3 is involved in regulating the balance of microtubule dynamics during mitosis.

Materials and Methods

Cell culture

HeLa and HEK293 cells were grown in DMEM (GIBCO/BRL) supplemented with 10% FBS (HyClone) and maintained in a humid incubator at 37°C in a 5% CO_2 environment. Cells were synchronised using a double thymidine block (Stein et al., 1994). In some experiments mitotic cells were obtained by treatment with 1 μM nocodazole for 15 hours. Identical results were obtained using either procedure. Stimulation of HeLa cells was achieved by serum starving cells overnight, followed by incubation in DMEM with 10% FBS for 10 minutes prior to extraction. The GSK-3 inhibitors were a kind gift of GlaxoSmithKline (Harlow, UK) and were used at 10 μM (SB-216763) or 30 μM (SB-415286); concentrations previously shown to elicit an effect on GSK-3 whilst retaining high specificity (Coghlan et al., 2000). To depolymerise microtubules, cells were treated with 1 μM nocodazole for 2 hours before fixing and staining.

Microtubule sedimentation experiments

Mitotic cells were briefly washed in Buffer A (50 mM Hepes pH 7.4, 50 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM $MgCl_2$), shaken off and centrifuged at 1000 ***g***. The cell pellet was resuspended in Buffer B [Buffer A + 0.5% Triton X-100, 1 mM NaF, 1 mM PMSF and 1 μM protease inhibitors (pepstatin, leupeptin and antipain)] before centrifugation at 14,000 ***g*** for 5 minutes. The clarified supernatant was then centrifuged for 10 minutes at 100,000 ***g***, followed by a further 40 minutes at 100,000 ***g***. Dithiothreitol (DTT) and GTP were added to the high speed supernatant (1 mM final concentration), which was then divided into two equal aliquots. The supernatants were warmed to 37°C for 5 minutes to allow polymerisation to initiate, and then taxol was added to a final concentration of 10 μM. In control supernatants, buffer without taxol was added. Extracts were left at 37°C for a further 10 minutes, and then transferred to ice for 10 minutes. The supernatants were then layered onto an equal volume cushion of Buffer A with 40% sucrose, before centrifugation at 100,000 ***g*** for 40 minutes. Equal fractions of supernatant and pellet were resuspended in protein sample buffer and subjected to SDS-PAGE.

Immunofluorescence microscopy and live cell imaging

Cells were fixed either with 4% paraformaldehyde for 20 minutes or in methanol (–20°C) for 5 minutes (for γ-tubulin and phospho-PKB) before being permeabilised and blocked in PBS plus 0.1% Triton with 3% BSA for 45 minutes. When visualising GSK-3, coverslips were briefly washed in MTSB (100 mM PIPES pH 6.8, 1 mM EGTA, 5 mM $MgCl_2$) before incubation at 37°C for 1 minute with MTSB + 0.5% Triton. Cells were then fixed as described above. Primary antibody incubations were carried out for 1 hour at room temperature. The following primary antibodies were used: GSK-3 (Upstate Biotechnology, USA), Phospho-GSK-3α/β (Ser21/9) (Cell Signalling Technologies, NEB, USA), PKB (Oncogene, Germany), phospho-PKB (T308) (Biosource, USA), α-tubulin, DM1A and γ-tubulin, GTU-88 (Sigma). Staining was visualised using the appropriate secondary antibodies (Alexa 488 and Alexa 568; Amersham Bioscience, UK). Coverslips were mounted on slides with Vectashield mounting medium containing DAPI (Vector Labs, USA). Images were collected with a Leica SP2 Laser Scanning Confocal Microscope and the images processed with Adobe Photoshop 5. All movies of Histone2B-GFP dynamics were acquired using an Olympus/TILL Photonics imaging system. Images were acquired with a PLAPO 100×, NA 1.40, oil immersion objective; illumination was provided by a 150W Xenon lamp controlled by a Polychrome IV monochromator coupled by a Quartz light guide to an Olympus IX-70 microscope. Emission filters for GFP/Cy3 were from Chroma (Brattleboro, VT). Images were captured with a TILL IMAGO SVGA camera controlled by TILL visION v.4.0 software and processed using Image J (http://rsb.info.nih.gov/ij), QuickTime Pro v. 5.0 (Apple, Cupertino, CA) and Adobe Photoshop v6.0 (Adobe Systems, San Jose, CA). Live cells were cultured in 35 mm glass-bottomed dishes (Mat-Tek Co, USA), mounted in MEM (Gibco-BRL) supplemented with 5% serum and imaged at 37°C with the microscope enclosed in a heated Perspex box (Solent Scientific, Portsmouth, UK). Images were taken every 3 or 4 seconds using a 50 millisecond exposure. A total of approximately 20 untreated and GSK-3 inhibitor treated mitotic cells were imaged. All untreated cells were followed until chromosome decondensation to ensure they were able to exit mitosis. To measure chromosome movements, distances were calculated from the kinetochore region of the mitotic chromosome (seen as a constricted, less fluorescent area in the images) to the centre of the metaphase plate. Maximal chromosome velocities and trajectories were measured using a manual particle tracking macro plug-in to Scion Image kindly provided by Jens Rietdorf of the Advanced Light Microscopy Facility at EMBL Heidelberg.

SDS-PAGE and western blotting

Antibody detection was performed using either enhanced chemiluminescence (Amersham Bioscience, UK), or the Supersignal kit (Pierce, USA) according to the manufacturer's instructions. Primary antibodies were used at 1:1000 dilution. Horseradish-peroxidase-conjugated anti-rabbit IgG secondary antibodies were obtained from Sigma and used at 1:10,000 dilution for the GSK3β (Transduction Laboratories, USA) and tubulin antibody, and at 1:2000 for the phospho-specific antibodies. Blots were stripped using Pierce Restore Western reagent for 30 minutes before re-probing with subsequent antibodies.

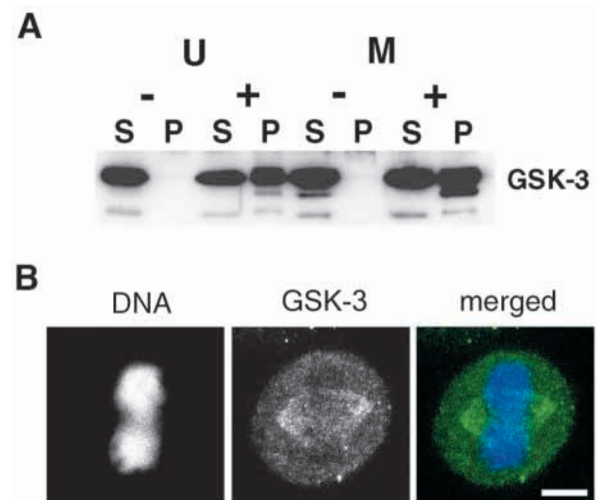

Fig. 1. GSK-3 interacts with spindle microtubules during mitosis. (A) A microtubule co-sedimentation assay using cycling or mitotic HeLa cell extracts. S, high speed supernatant; P, microtubule pellet; –/+ indicates the absence or presence of taxol in the assay; U, unsynchronised cells; M, Mitotic cells. Western blots were probed with antibodies against GSK-3β. (B) HeLa cells permeabilised with 0.5% Triton before fixation with 4% paraformaldehyde and stained with affinity purified antibodies raised against GSK-3. Bar, 10 μm.

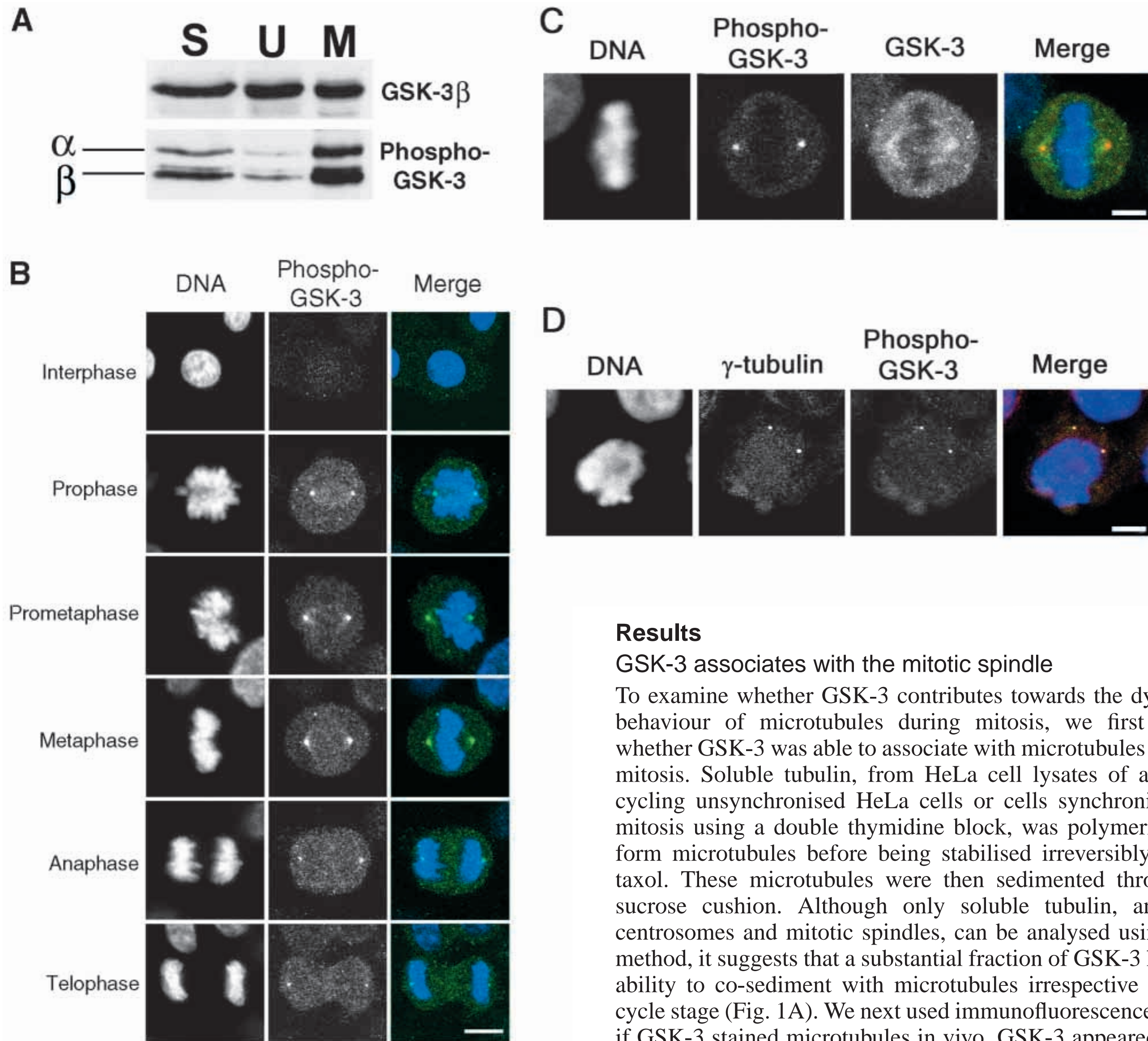

Fig. 2. GSK-3 is phosphorylated during mitosis and accumulates at centrosomes and spindle poles. (A) GSK-3 phosphorylation increases during mitosis. Total HeLa cell lysates were probed using antibodies against GSK-3β and Phospho-GSK-3α/β (Ser21/9). S, stimulated HeLa cells (see Materials and Methods); U, unsynchronised, unstimulated HeLa cells; M, mitotic HeLa cells. (B) HeLa cells stained for Phospho-GSK-3α/β (Ser21/9) and DNA. (C) HeLa cell stained for Phospho-GSK-3α/β (Ser21/9), total GSK-3 and DNA. (D) Localisation of phospho-GSK-3 and γ-tubulin in HeLa cells treated with 1 μM nocodazole for 2 hours. Bar, 10 μm.

Online supplemental material

The movies show chromosomal movements in prometaphase HeLa cells stably expressing Histone-2B-GFP. 600 images were acquired at 3 second intervals (total imaging time of 30 minutes) and converted into movies using QuickTime Pro v.5.0. QuickTime movies were assembled to play back at 10 frames per second. Movie 1, an untreated cell; Movie 2, a cell treated with 30 μM SB-415286 for 60 minutes prior to imaging.

Results

GSK-3 associates with the mitotic spindle

To examine whether GSK-3 contributes towards the dynamic behaviour of microtubules during mitosis, we first asked whether GSK-3 was able to associate with microtubules during mitosis. Soluble tubulin, from HeLa cell lysates of actively cycling unsynchronised HeLa cells or cells synchronised in mitosis using a double thymidine block, was polymerised to form microtubules before being stabilised irreversibly using taxol. These microtubules were then sedimented through a sucrose cushion. Although only soluble tubulin, and not centrosomes and mitotic spindles, can be analysed using this method, it suggests that a substantial fraction of GSK-3 has the ability to co-sediment with microtubules irrespective of cell cycle stage (Fig. 1A). We next used immunofluorescence to see if GSK-3 stained microtubules in vivo. GSK-3 appeared to be evenly distributed throughout the cell (J.G.W., unpublished). However, when we removed the soluble fraction of GSK-3 from cells by permeabilising cells using 0.5% Triton prior to fixation, we found that GSK-3 was present along the length of the mitotic spindle (Fig. 1B). Taken together, this suggests that GSK-3 can associate with spindle microtubules in vivo.

GSK-3 is phosphorylated during mitosis and is present at centrosomes and spindle poles

To investigate the phosphorylation status of GSK-3 in mitotic cells, we western blotted cell lysates with a phosphospecific antibody that recognises both phosphoserine-21 of GSK-3α and phosphoserine-9 of GSK-3β. In lysates from unsynchronised cells, GSK-3 is constitutively active and possessed a very low basal level of phosphorylation (Fig. 2A). However, there was a marked increase in GSK-3 phosphorylation in extracts derived from cells synchronised in mitosis using either nocodazole (Fig. 2A) or a double thymidine block (data not shown). We next examined whether the phosphorylation of GSK-3 was spatially regulated during

the HeLa cell cycle. Confocal microscopy revealed that during interphase, phospho-GSK-3 was diffusely distributed throughout the cell (Fig. 2B). In contrast, we observed a striking accumulation of phospho-GSK-3 at the centrosomes upon entry into mitosis. As the cells progressed from prophase to metaphase, the intensity of phospho-GSK-3 immunoreactivity increased and extended to the surrounding minus ends of the mitotic spindle (Fig. 2B). As cells exited mitosis the level of phospho-GSK-3 on the spindles returned to basal levels, although weak centrosome staining was seen until late telophase (Fig. 2B). Similar results were obtained using HEK293 cells (data not shown). Co-staining cells for GSK-3 and phospho-GSK-3 confirmed that GSK-3 was present along the length of the spindle, while phospho-GSK-3 staining was seen only at the centrosomes and spindle poles (Fig. 2C). The staining was specific to this antibody; we did not observe centrosomal and/or spindle pole staining with a number of other phosphospecific antisera (e.g. phospho-Histone H3; data not shown). To see if the accumulation of phospho-GSK-3 was dependent on microtubules, we treated cells with 1 μM nocodazole before fixing and staining (Fig. 2D). We found that although phospho-GSK-3 could still be detected at centrosomes in cells lacking microtubules, the intensity of staining was greatly reduced compared with that in untreated cells, suggesting that GSK-3 can associate both with centrosomes and with spindle microtubules.

PKB is phosphorylated during mitosis and is present at the centrosome

As GSK-3 is phosphorylated on the inhibitory serine by the upstream kinase PKB (Cross et al., 1995), we next asked whether PKB is also regulated during mitosis. PKB is phosphorylated on two specific sites, Thr308 and Ser473 (Alessi and Cohen, 1998). Thr308 is phosphorylated by an upstream kinase, PDK1 (which is itself activated by phosphoinositide-lipids), leading to activation of PKB (Alessi et al., 1997; Alessi and Cohen, 1998). Activity is further increased by phosphorylation on Ser473 although the kinase(s) involved are not yet fully defined. We used antibodies specific for PKB phosphorylated on Thr308 to determine its activity either in interphase or during mitosis. By western blotting HeLa cell lysates we found that phosphorylation of PKB on Thr308 was greatly increased in cells synchronised in mitosis when compared with that in non-synchronised cells, which strongly suggested that PKB is activated during mitosis (Fig. 3A).

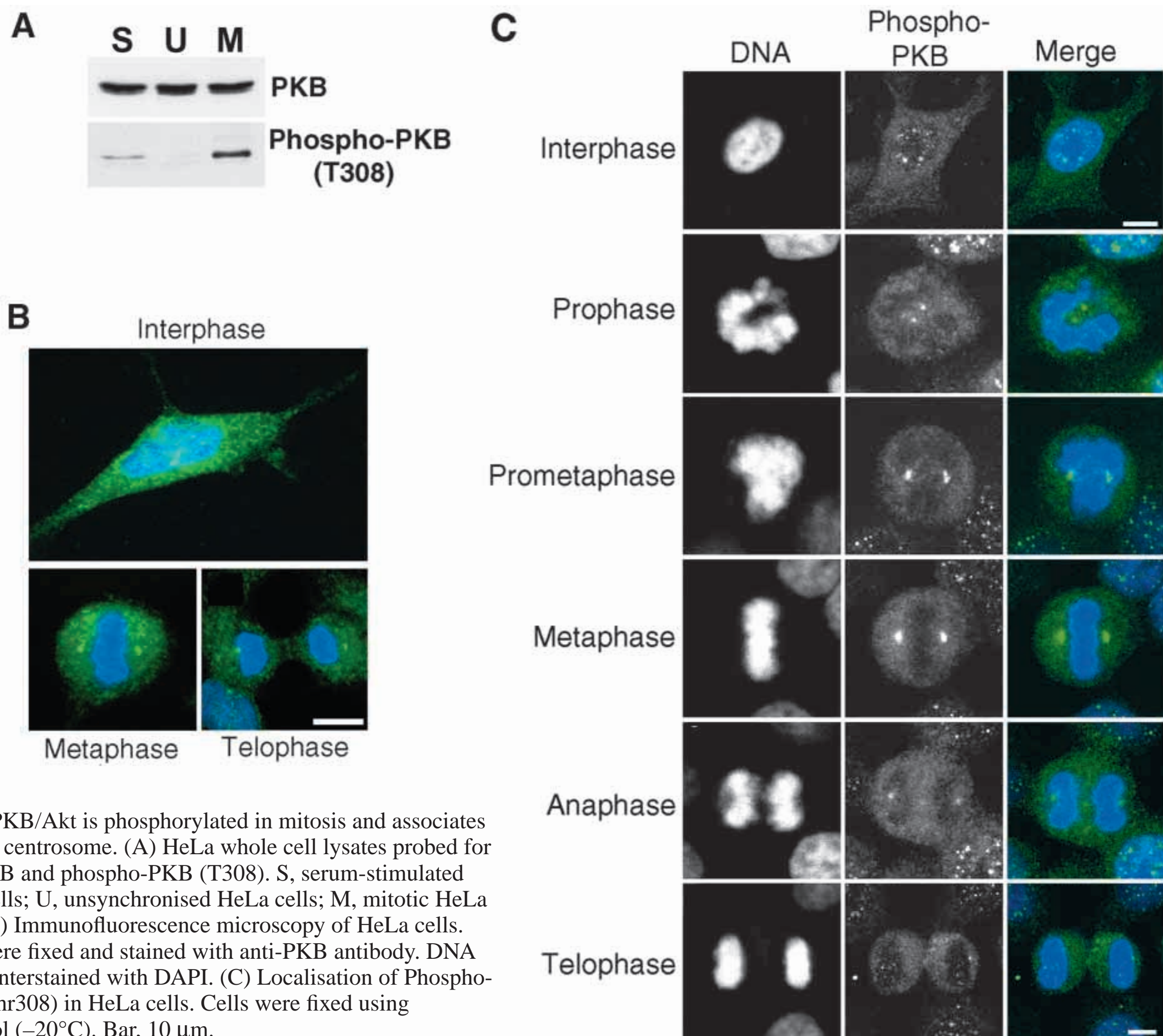

Fig. 3. PKB/Akt is phosphorylated in mitosis and associates with the centrosome. (A) HeLa whole cell lysates probed for total PKB and phospho-PKB (T308). S, serum-stimulated HeLa cells; U, unsynchronised HeLa cells; M, mitotic HeLa cells. (B) Immunofluorescence microscopy of HeLa cells. Cells were fixed and stained with anti-PKB antibody. DNA was counterstained with DAPI. (C) Localisation of Phospho-PKB (Thr308) in HeLa cells. Cells were fixed using methanol (–20°C). Bar, 10 μm.

We next examined the regulation of PKB phosphorylation in HeLa cells during the cell cycle by confocal microscopy. PKB was weakly localised to the centrosomes throughout mitosis (Fig. 3B). However, using anti-phospho-PKB (Thr308) antibodies we found that the phosphorylation of the centrosomal PKB rapidly increased as the cells entered mitosis (Fig. 3C). Phosphorylation on Thr308 was initiated during prophase, steadily increased until metaphase, and then returned to basal levels following sister chromosome separation. Immunostaining of HEK293 cells produced similar results (data not shown). Thus PKB, like GSK-3, appears to be regulated in a spatio-temporal manner during mitosis.

Inhibition of GSK-3 causes defects in astral microtubule length and chromosome alignment

To determine whether the accumulation of phosphorylated GSK-3 at the minus ends of the mitotic spindle was indicative of a role for GSK-3 in the regulation of microtubules in cell division, we sought to inhibit GSK-3 with either of two structurally distinct and selective inhibitors of GSK-3, SB-216763 and SB-415286 (Coghlan et al., 2000). We incubated cycling HeLa cells with inhibitors for 90 minutes and then fixed and stained with anti-tubulin antibodies and DAPI, to visualise microtubules and DNA, respectively.

Clear and specific defects in chromosomal alignment were observed as a result of these treatments (Fig. 4). Bi-polar mitotic spindle assembly did not appear to be greatly affected; however, many spindles contained extended astral microtubules emanating from the centrosomes (Fig. 4B-D). In some cases, additional long microtubules were formed from one pole, which appeared to force the centre of the spindles away from the centre of the cell (Fig. 4C). Careful examination of the position of chromosomes on these metaphase spindles revealed that, in approximately half of the metaphase-like cells, many chromosomes were not correctly aligned (Table 1; Fig. 4B-D). These chromosomes were able to attach to microtubules but remained mono-oriented and displaced from the main spindle. In some examples many of the chromosomes were mono-oriented, and seemed to cluster around one spindle pole (Fig. 4B,C). In addition, we saw many

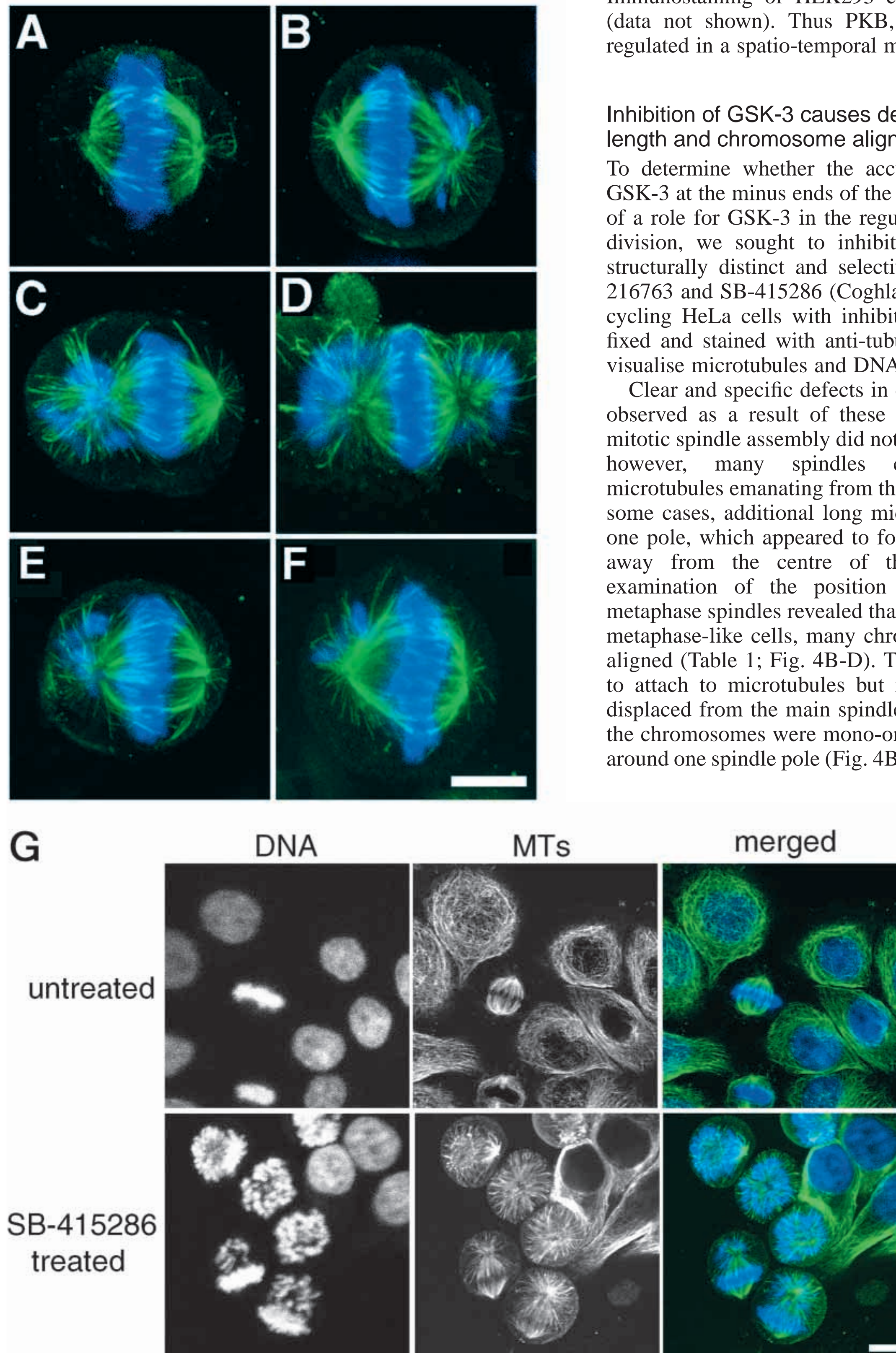

Fig. 4. Inhibition of GSK-3 during mitosis causes spindle defects and chromosome mis-alignment. HeLa cells were incubated with 10 μM or 30 μM of the GSK-3 inhibitors SB-216763 and SB-415286, respectively for 90 minutes. DNA, blue; tubulin, green. (A) Untreated metaphase cell; (B) SB-216763-treated metaphase cell; (C,D) SB-415286-treated metaphase cell; (E) Mitotic cell treated with 40 mM LiCl for 90 minutes; (F) Mitotic cell treated for 90 minutes with 1 nM taxol. Bar, 10 μm. (G) Effect of treating HeLa cells with SB-415286 for 16 hours. Bar, 10 μm.

Table 1. Treatment of HeLa cells with GSK-3 inhibitors causes chromosome mis-alignment

	Aligned		Non-aligned		
	no.	%	no.	%	Total
Untreated	132	95	7	5	139
SB-216763	85	59	59	41	144
SB-415286	84	58	62	42	146
Lithium	98	68	47	32	145

Cells were treated with SB-216763 (10 μM), SB-415286 (30 μM) or lithium chloride (40 mM) for 90 minutes before fixing and staining for microtubules and DNA. Only mitotic cells containing a bi-polar spindle upon which the majority of chromosomes were aligned were scored. Cells were scored as non-aligned if at least one chromosome was not present in the centre of the spindle (e.g. Fig. 4A and B-D for comparison).

examples where chromosomes were present close to both poles (Fig. 4D).

To see if the perturbation in spindle dynamics was transient, we incubated cells with SB-415286 for 16 hours before fixation. Overnight treatment with the GSK-3 inhibitor increased the proportion of cells in mitosis and the defects in chromosome alignment and spindle structure described above were more prominent (Fig. 4G). Cells exhibited bipolar spindles containing astral arrays of microtubules that were more dense and much longer than in untreated cells. Furthermore, these poles contained many mono-oriented chromosomes concentrated at the ends of the extended astral microtubules. Similar results were obtained using HEK293 cells (data not shown).

Lithium is a well established inhibitor of GSK-3 (Klein and Melton, 1996; Stambolic et al., 1996). Importantly, incubation of HeLa cells with concentrations of LiCl known to inhibit GSK-3 (20-40 mM) brought about the same phenotypic effects as either SB-216763 or SB-415286 (Fig. 4E; Table 1).

The presence of long astral microtubules in cells in which GSK-3 has been inhibited is suggestive of a disruption of mitotic microtubule dynamics. Indeed, the above observations are similar to those reported for cells treated with low doses of the microtubule-stabilising drug taxol (Ault et al., 1991; Jordan et al., 1996). We confirmed these results by treating HeLa cells with 1 nM taxol for 90 minutes. We found that a proportion of cells showed chromosomal displacement phenotypes similar to those observed with the GSK-3 inhibitors (Fig. 4F). The above results strongly argue that GSK-3 activity is required in mitosis to correctly align chromosomes on the metaphase plate. As GSK-3 is normally inactive at the spindle poles through phosphorylation, the inhibitors must be affecting the non-phosphorylated and active GSK-3 that is both present along the main body of the spindle as well as the GSK-3 present in the cytosol.

Inhibition of GSK-3 during mitosis does not affect centrosome separation but does disrupt mitotic chromosome movement

As the abnormally long microtubule asters seen in cells treated with GSK-3 inhibitors seemed to emanate predominantly from one pole, we examined whether this was due to a perturbation in microtubule dynamics or as a result of a failure to separate duplicated centrosomes (Khodjakov et al., 2000). To distinguish between these possibilities we treated HeLa cells (Fig. 5) or HEK293 cells (not shown) with the GSK-3 inhibitor SB-415286 for 90 minutes and stained them with antibodies to gamma-tubulin. In cells containing mono-oriented chromosomes two dots were seen on opposite sides of the metaphase plate (Fig. 5). Identical results were obtained using SB-216763 (data not shown). Thus we conclude centrosome duplication and separation are not affected by inhibiting GSK-3.

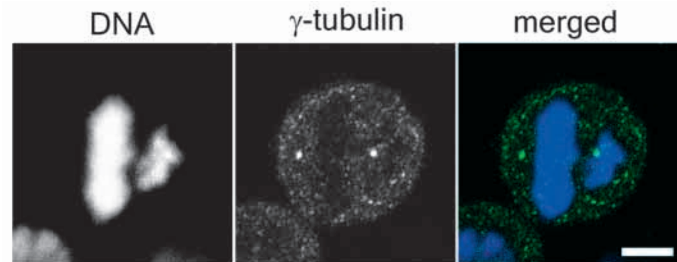

Fig. 5. Inhibition of GSK-3 does not affect centrosome separation. Actively cycling HeLa cells were treated with the GSK-3 inhibitor SB-415286 for 90 minutes before fixation with methanol (–20°C). Coverslips were stained with antibodies to γ-tubulin and with DAPI to visualise DNA. In cells possessing mono-oriented chromosomes γ-tubulin staining is seen as two discrete dots, one at either side of the metaphase plate. Identical results were obtained using SB-216763.

If the inhibitors affect microtubule dynamics during mitosis, they would be expected to disrupt the oscillations of the chromosomes as they congress on the metaphase plate. To address this, we captured images of chromosomes throughout mitosis using a HeLa cell line stably expressing Histone 2B-GFP (Kand et al., 1998) and an Olympus/TILL Photonics imaging system (see Materials and Methods). By imaging of untreated cells, we were able to observe fast reversible oscillations associated with mono-oriented chromosomes, as well as general chromosome movements (Fig. 6A; see also Movie 1, http://jcs.biologists.org/supplemental). The cell shown in the time sequence in Fig. 6A shows chromosome congression on the metaphase plate (0-600 seconds), with subsequent separation (1200-1500 seconds); both daughter cells subsequently underwent chromosome decondensation (not shown). The arrow in panel '300 sec' shows the position of a chromosome undergoing significant movement. This is shown in more detail in the zoomed images (279-458s, the arrow marks the same starting position in all frames) and is clearly seen in the associated time-lapse sequence (Movie 1). The maximum chromosome velocity observed in untreated cells was 5.8 $\mu m\ min^{-1}$. Imaging of cells treated with either of the GSK-3 inhibitors for 60 minutes revealed that chromosome movements were highly suppressed; the maximal chromosome velocity was reduced to 1.0 $\mu m.min^{-1}$ [Fig. 6B (using SB-415286); see Movie 2, http://jcs.biologists.org/supplemental]. Notably, cells treated with GSK-3 inhibitors for 90 minutes were not seen to enter anaphase up to 60 minutes after the start of imaging (120 minutes in the continued presence of the inhibitor, not shown). Similar results were obtained from time-lapse imaging of more than 20 cells in the presence or absence of the inhibitors.

Particle tracking of these data clearly shows the absence of movement in SB-415286 treated cells (Fig. 7A,C) compared

with that in control cells (Fig. 7B,D). Tracks of two dynamic chromosomes were superimposed onto the first image of each time sequence (Fig. 7A,B). Fig. 7C and D show the total distance moved (including movements away from as well as towards the metaphase plate) by these highlighted chromosome over time. The severe reduction in chromosome movement seen in cells treated with GSK-3 inhibitors was further analysed by measuring the distance between the oscillating chromosomes and the centre of the metaphase plate against time (Fig. 7E,F). Whereas chromosomes from untreated prometaphase cells showed several rapid oscillations followed by alignment on the metaphase plate (Fig. 7E), mono-oriented chromosomes in treated cells failed to show similar movement (Fig. 7F). As microtubules are the force behind chromosome congression, these results suggest that inhibiting GSK-3 affects chromosome oscillations and alignment on the metaphase plate through a suppression of normal microtubule dynamics.

Discussion

GSK-3 and PKB are phosphorylated in a spatio-temporal manner during mitosis

Our data shows that GSK-3 is regulated during mitosis in a temporal and spatial manner. While total GSK-3 is present on microtubules throughout the spindle, phospho-GSK-3 is confined to an area including the centrosomes and spindle poles. As phosphorylation of GSK-3 leads to its inactivation, it seems likely that, during mitosis, the GSK-3 that resides close to the centrosomes is inactive, whereas the GSK-3 associated with the main body of the spindle is active and able to phosphorylate and regulate downstream targets. Our data also suggests that PKB is activated during mitosis. This is consistent with recent reports that PKB is required for the G2/M transition in both *Xenopus* and starfish oocytes (Andersen et al., 1998; Okumura et al., 2002). It is also in agreement with a recent report that found a dramatic increase in PKB activity at the G2/M boundary in HeLa cells, as measured by phosphorylated PKB and phosphorylation of a

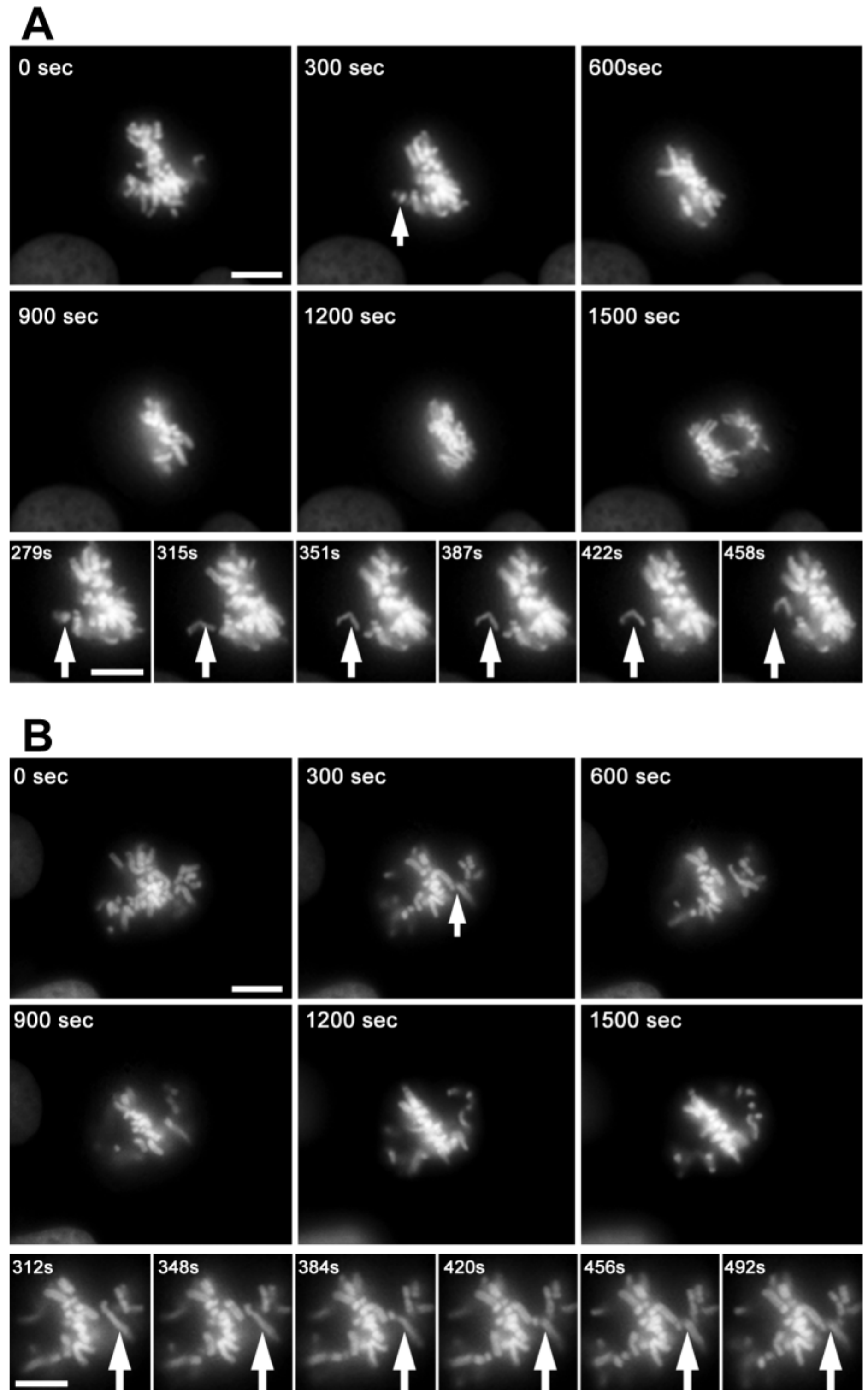

Fig. 6. Inhibition of GSK-3 dramatically reduces chromosome dynamics in mitosis. HeLa cells stably expressing Histone 2B-GFP were imaged at 37°C at 3 second intervals for 30 minutes in the absence (A) or presence (B) of the GSK-3 inhibitor, SB-415286. (A) The arrow in the image at 300 seconds marks the position of a chromosome that undergoes significant movement. This movement is shown enlarged in panels 279-458s. (B) The arrow in the image at 300 seconds marks the position of a chromosome that does not undergo significant movement. This is shown enlarged in panels 312-492s. Bars, 10 µm. QuickTime™ movies of time-lapse sequence shown in A and B are available online as Movies 1 and 2, respectively (http://jcs.biologists.org/supplemental).

substrate peptide (Shtivelman et al., 2002). It is well established that the phosphorylation of PKB on Thr308 leads to its activation and its ability to phosphorylate and inhibit GSK-3 (Cross et al., 1995; Frame and Cohen, 2001). We find that phospho-PKB is present only at centrosomes during mitosis. Consistent with this, we have also localised the PKB Thr308 kinase, PDK1, to centrosomes during mitosis (data not shown). As phosphoinositide lipids are able to activate PDK1 and PKB, they might be expected to be present around the area of PKB phosphorylation. Indeed, remnants of the Golgi apparatus are known to cluster around the spindle poles during mitosis (Corthesy-Theulaz et al., 1992). However, as inhibitors of PI 3-kinase (such as LY294002 and wortmannin) do not appear to affect the mitosis-specific phosphorylation of PKB or GSK-3 (data not shown), it may be that PKB is activated in a phosphoinositide-independent manner during mitosis.

We suggest that the cell-cycle-dependent accumulation of phospho-PKB at the centrosomes and of phospho-GSK-3 at the centrosomes and spindle poles is indicative of a role for these proteins at these sites during mitosis. Thus the active PKB associated with the centrosomes is likely to be capable of phosphorylating the GSK-3 present on the centrosome and nearby spindle microtubules. This would result in the generation of phospho-GSK-3 at centrosomes and spindle poles, while leaving GSK-3 active elsewhere in the cell.

Inhibition of GSK-3 activity during mitosis affects astral microtubule length and chromosome dynamics

To investigate the role of active GSK-3 during mitosis, we sought to inhibit GSK-3 throughout the cell using specific inhibitors. One advantage of probing protein function using inhibitors is that behaviour of cells in which GSK-3 has been inactivated can be assessed within minutes. Other methods such as direct gene knockouts and siRNA are subject to the plasticity of biological systems. The phenotypes analysed are usually the result of much greater lengths of time without normal levels of the protein of interest and, as such, the primary defects in cellular function can be extremely difficult to interpret. In addition, as some proteins, such as GSK-3, fulfil many different cellular roles, reducing the amount of protein

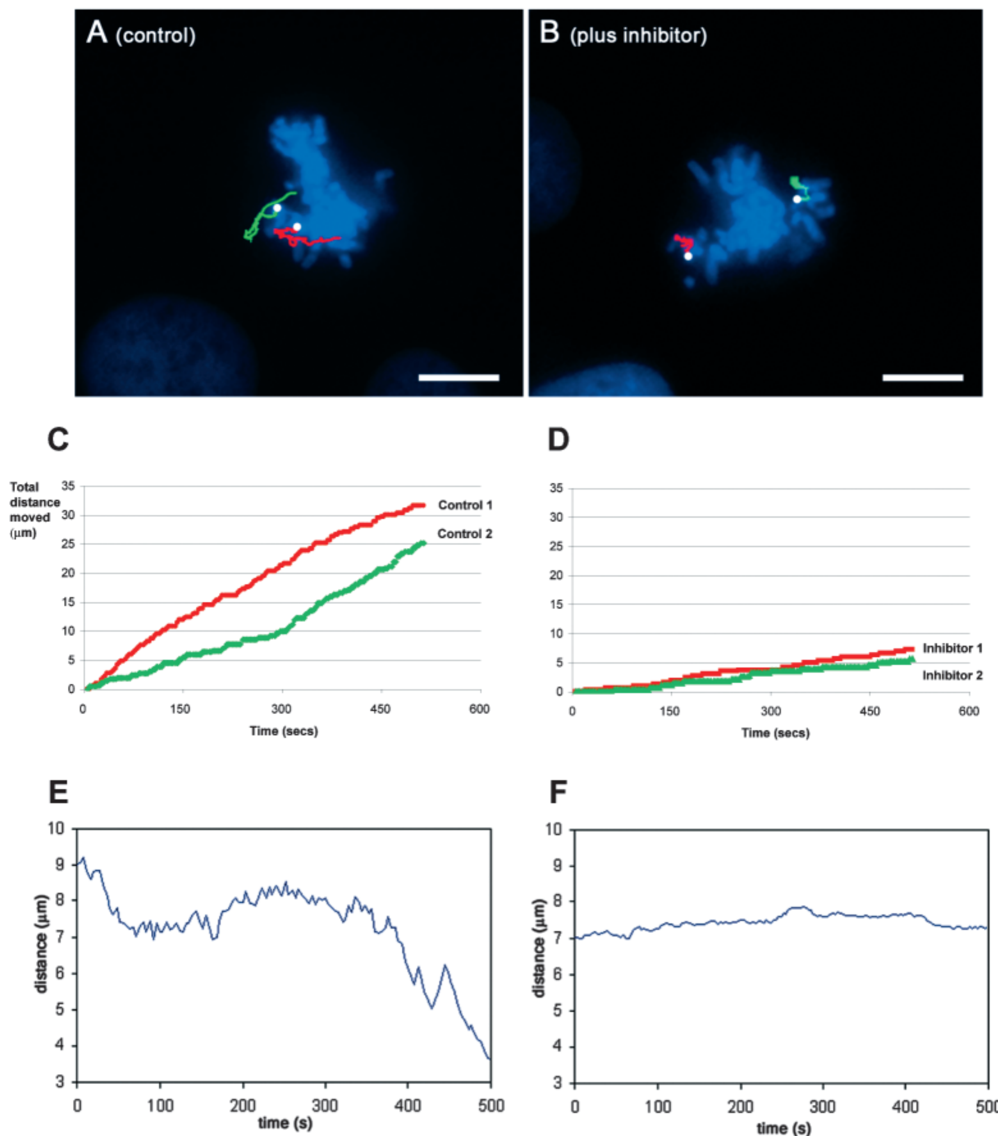

Fig. 7. Inhibition of GSK-3 results in inhibition of chromosome oscillation and congression. Manual particle tracking was used to quantitate the time-lapse data from Fig. 6. Tracks shown in green in Fig. 7 represent movements of the chromosomes highlighted in the zoomed images in Fig. 6C. Starting points of two dynamic chromosomes for control (A) or inhibitor treated cells (B) are shown as white spots with their subsequent trajectories during the time-lapse sequence shown as red or green overlays. (C,D) The total distance moved of each of these chromosomes is shown as a function of time. Bars, 10 µm. (E-F) Tracking record of mono-oriented chromosomes. (E) In an untreated cell, the chromosome performs several rapid, reversible movements before moving into the metaphase plate. (F) A cell treated with SB-415286. The unaligned chromosome fails to show similar oscillations. Even after a further 30 minutes this chromosome does not approach the metaphase plate (D.J.S., unpublished) (for the movies of these chromosomes, see http://jcs.biologists.org/supplemental).

using these methods can result in the accumulation of numerous phenotypes within a cell. One caveat with inhibitor studies, however, is the specificity of the compounds used. Recently, Leclerc and co-workers have investigated the effect on the cell cycle of a compound that inhibits CDK1 (Leclerc et al., 2001). Interestingly, this compound was also noted to inhibit GSK-3 (Leclerc et al., 2001). However, incubation of unsynchronised cells with this inhibitor resulted in a G2 arrest and the absence of metaphase figures (Damiens et al., 2001). Furthermore, treatment of mitotic cells led to premature exit from mitosis without cytokinesis, resulting in aneuploidy and endoreplication (Damiens et al., 2001). Therefore, it is extremely unlikely that the effects on spindle microtubules and chromosome alignment we observe in cells treated with three distinct GSK-3 inhibitors (SB-216763, SB-415286 and lithium) are a result of inhibiting CDK1.

By accurately inhibiting GSK-3 in cells about to enter mitosis we can assess the function of this kinase at this time of the cell cycle. As GSK-3 is normally inactive at the spindle poles through phosphorylation, the inhibitors must be affecting both the non-phosphorylated and active GSK-3 that is present along the main body of the spindle as well as the GSK-3 that is free in the cytosol. Treatment of mitotic cells with the GSK-3 inhibitors causes an increase in the length of the astral microtubules, such that some contain bi-polar spindles that are pushed away from the centre of the cell. It also promotes the accumulation of mono-oriented chromosomes on bi-polar spindles. One possible interpretation of these results is that inhibition of GSK-3 activity during mitosis causes a perturbation of normal microtubule dynamics that leads to defective congression of chromosomes on the metaphase plate. This hypothesis is further strengthened by our observations of live HeLa cells expressing Histone-2B-GFP. The frequent transitions between microtubule growth and microtubule shrinkage that occur during mitosis are responsible for causing the chromosome oscillations normally found in prometaphase cells. Importantly, we find that these rapid, reversible chromosome oscillations are inhibited in cells in which GSK-3 is inhibited. Chromosome movements still occur, but do so more slowly, while many of the mono-oriented chromosomes fail to align on the metaphase plate, even after many hours. These cells contain unattached kinetochores and, as such, remain in mitosis presumably through the activation of the spindle checkpoint.

This phenotype is reminiscent of cells treated with low doses of drugs that interfere with microtubule dynamics and we have confirmed that similar chromosome displacements occur when HeLa cells are treated with nanomolar concentrations of taxol. In addition, a recently identified microtubule-interacting drug, noscapine, leads to a mitotic arrest phenotype that is similar to the one seen when GSK-3 is inhibited (Zhou et al., 2002). However, while noscapine inhibits the rate of microtubule catastrophe without affecting astral microtubules and microtubule polymerisation, the GSK-3 inhibitors (like taxol) cause an increase in astral microtubule length and number. These results suggest that inhibiting GSK-3 promotes stabilisation of microtubules. This is wholly consistent with several studies which show that GSK-3 destabilises microtubules in interphase cells (Lovestone et al., 1996; Goold et al., 1999; Krylova et al., 2000; Zumbrunn et al., 2001). It is therefore likely that GSK-3 is acting in a similar manner during mitosis, and that by globally inhibiting the kinase, microtubules become stabilised.

The role of spatially regulating GSK-3 during mitosis

We have shown that GSK-3 is present on the mitotic spindle where it presumably acts to phosphorylate target proteins. However, in normal cells there appears to be a phosphorylated and inactive fraction of GSK-3 at the centrosome and spindle poles. Why should GSK-3 be inactivated here during mitosis? We propose that this spatial regulation of GSK-3 along the spindle normally contributes towards differences in microtubule dynamics between those microtubules near to the centrosomes and those in other areas of the cell. During mitosis, active PKB would inactivate GSK-3 in the vicinity of the centrosomes. This would contribute towards the stabilisation of microtubules in this area of the cell, allowing centrosomes to become the dominant site of microtubule growth. Conversely, GSK-3 would remain active along the main body of the spindle, de-stabilising microtubules further away from the poles, and contributing towards the highly dynamic search for chromosomes by microtubules. The use of GSK-3 inhibitors during mitosis would mimic the effect of phosphorylation of GSK-3 but would act throughout the cell, not just at the spindle poles. As a result, this global inhibition of GSK-3 would lead to stabilisation of all microtubules, not just those close to the centrosome.

The substrates for phosphorylation by GSK-3 that lead to destabilisation of spindle microtubules are not known at present. In interphase cells, GSK-3 has been reported to phosphorylate Tau and other MAPs (Lovestone et al., 1996; Goold et al., 1999). However, these MAPs are predominantly expressed in neurons and are unlikely to be important in regulating microtubule stability in other cell types. GSK-3 is also able to phosphorylate APC. Recently APC has been found to bind to microtubules both in vitro and in vivo, increasing their stability (Zumbrunn et al., 2001). Phosphorylation of APC by GSK-3 decreases the interaction between APC and microtubules, making microtubules less stable (Zumbrunn et al., 2001). Furthermore, mutations in APC cause defects in chromosome segregation (Kaplan et al., 2001; Fodde et al., 2001). Another possible target for GSK-3 is the microtubule-associated protein CLASP2 (Akhmanova et al., 2001). CLASP2 associates with CLIP-115 and CLIP-170, cytoplasmic linker proteins that specifically associate with the ends of microtubules. Inhibition of GSK-3 increases the accumulation of CLASP2 at the plus end of microtubules, again leading to their stabilisation (Akhmanova et al., 2001). Whether CLASP2 has a role in mitosis has not yet been tested. However, by uncovering a previously unknown role for GSK-3 in chromosomal alignment and mitosis, identifying the substrates for GSK-3 involved in spindle microtubule assembly is now an important goal.

This work was supported by grants from the Medical Research Council and Diabetes UK (J.G.W. and J.M.T.). D.J.S. is supported by a non-clinical Research Career Development Fellowship (G120/617) from the Medical Research Council. We thank GlaxoSmithKline for the gift of the GSK-3 inhibitors, Jon Pines for the Histone-2B-GFP stable cell line (constructed originally in the lab of Geoff Wahl) and Jens Rietdorf for the manual particle tracking macro. We also thank Gavin Welsh, Harry Mellor and Kate Heesom for helpful discussions, and Eric Friar for technical assistance.

References

Ahkmanova, A., Hoogenraad, C. C., Drabek, K., Stepanova, T., Dortland, B., Verkerk, T., Vermeulen, W., Burgering, B. M., de Zeeuw, C. I., Grosveld, F. and Galjart, N. (2001). Clasps are CLIP-115 and -170 associating proteins involved in the regional regulation of microtubule dynamics in motile fibroblasts. *Cell* **104**, 923-935.

Alessi, D. R. and Cohen, P. (1998). Mechanism of activation and function of protein kinase B. *Curr. Opin. Genet. Dev*. **8**, 55-62.

Alessi. D. R., James, S. R., Downes, C. P., Holmes, A. B., Gaffney, P. R., Reese, C. B. and Cohen, P. (1997). Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balpha. *Curr. Biol*. **7**, 261-269.

Andersen, C. B., Roth, R. A. and Conti, M. (1998). Protein kinase B/Akt induces resumption of meiosis in *Xenopus* oocytes. *J. Biol. Chem.* **273**, 18705-18718.

Ault, J. G., DeMarco, A. J., Salmon, E. D. and Rieder, C. L. (1991). Studies on the ejection properties of asters: astral microtubule turnover influences the oscillatory behavior and positioning of mono-oriented chromosomes. *J. Cell Sci*. **99**, 701-710.

Cassimeris, L. (1999). Accessory protein regulation of microtubule dynamics throughout the cell cycle. *Curr. Opin. Cell Biol*. **11**, 134-141.

Coghlan, M. P., Culbert, A. A., Cross, D. A., Corcoran, S. L., Yates, J. W., Pearce, N. J., Rausch, O. L., Murphy, G. J., Carter, P. S., Roxbee Cox, L. et al. (2000). Selective small molecule inhibitors of glycogen synthase kinase-3 modulate glycogen and gene transcription. *Chem. Biol.* **7**, 793-803.

Compton, D. A. (2000). Spindle assembly in animal cells. *Annu. Rev. Biochem.* **69**, 95-114.

Corthesy-Theulaz, I., Pauloin, A. and Rfeffer, S. R. (1992). Cytoplasmic dynein participates in the centrosomal localization of the Golgi complex. *J. Cell Biol.* **118**, 1333-1345.

Cross, D. A., Alessi, D. R., Cohen, P., Andjelkovich, M. and Hemmings, B. A. (1995). Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature* **378**, 785-789.

Damiens, E., Baratte, B., Marie, D., Eisenbrand, G. and Meijer, L. (2001). Anti-mitotic properties of indirubin-3′-monoxime, a CDK/GSK-3 inhibitor: induction of endoreplication following prophase arrest *Oncogene* **20**, 3786-3797.

Embi, N., Rylatt, D. and Cohen, P. (1980). Glycogen synthase kinase-3 from rabbit skeletal muscle. Separation from cyclic-AMP-dependent protein kinase and phosphorylase kinase *Eur. J. Biochem*. **107**, 519-526.

Ferkey, D. M. and Kimelman, D. (2000). GSK-3: new thoughts on an old enzyme. *Dev. Biol*. **225**, 471-479.

Fodde, R., Kuipers, J., Rosenberg, C., Smits, R., Kielman, M., Gaspar, C., van Es, J. H., Breukel, C., Wiegant, J., Giles, R. H. and Clevers, H. (2001). Mutations in the APC tumour suppressor gene cause chromosomal instability. *Nat. Cell Biol.* **3**, 433-438.

Frame, S. and Cohen, P. (2001). GSK3 takes centre stage more than 20 years after its discovery. *Biochem. J*. **359**, 1-16.

Goold, R. G., Owen, R. and Gordon-Weeks, P. R. (1999). Glycogen synthase kinase 3beta phosphorylation of microtubule-associated protein 1B regulates the stability of microtubules in growth cones. *J. Cell Sci.* **112**, 3373-3384.

Inoue, S. and Salmon, E. D. (1995). Force generation by microtubule assembly/ disassembly in mitosis and related movements. *Mol. Biol. Cell*. **6**, 1619-1640.

Jordan, M. A., Wendell, K., Gardiner, S., Derry, W. B., Copp, H. and Wilson, L. (1996). Mitotic block induced in HeLa cells by low concentrations of paclitaxel (Taxol) results in abnormal mitotic exit and apoptotic cell death. *Cancer Res*. **56**, 816-825.

Kand, T., Sullivan, K. F. and Wahl, G. M. (1998). Histone-GFP fusion protein enables sensitive analysis of chromosome dynamics in living mammalian cells. *Curr. Biol.* **8**, 377-385.

Kaplan, K. B., Burds, A. A., Swedlow, J. R., Bekir, S. S., Sorger, P. K. and Nathke, I. S. (2001). A role for the Adenomatous Polyposis Coli protein in chromosome segregation. *Nat. Cell Biol.* **3**, 429-432.

Khodjakov, A., Cole, R. W., Oakley, B. R. and Rieder, C. L. (2000). Centrosome-independent mitotic spindle formation in vertebrates. *Curr. Biol.* **10**, 59-67.

Klein, P. S. and Melton, D. A. (1996). A molecular mechanism for the effect of lithium on development. *Proc. Natl. Acad. Sci. USA* **93**, 8455-8459.

Krylova, O., Messenger, M. J. and Salinas, P. C. (2000). Dishevelled-1 regulates microtubule stability: a new function mediated by glycogen synthasekinase-3beta. *J. Cell Biol*. **151**, 83-94.

Leclerc, S., Garnier, M., Hoessel, R., Marko, D., Bibb, J. A., Snyder, G. L., Greengard, P., Biernat, J., Mandelkow, E. M., Eisenbrand, G. and Meijer, L. (2001). Indirubins inhibit glycogen synthase kinase-3 beta and CDK5/p25, two protein kinases involved in abnormal tau phosphorylation in Alzheimer's disease. A property common to most cyclin-dependent kinase inhibitors? *J. Biol. Chem.* **276**, 251-260.

Lovestone, S., Hartley, C. L., Pearce, J. and Anderton, B. H. (1996). Phosphorylation of tau by glycogen synthase kinase-3 beta in intact mammalian cells: the effects on the organization and stability of microtubules. *Neuroscience* **73**, 1145-1157.

Nigg, E. A. (2001). Mitotic kinases as regulators of cell division and its checkpoints. *Nat. Rev. Mol. Cell Biol*. **2**, 21-32.

Okumura, E., Fukuhara, T., Yoshida, H., Hanada, Si. S., Kozutsumi, R., Mori, M., Tachibana, K. and Kishimoto, M. (2002). Akt inhibits Myt1 in the signalling pathway that leads to meiotic G2/M-phase transition. *Nat. Cell Biol.* **4**, 111-116.

Rubinfeld, B., Albert, I., Porfiri, E., Fiol, C., Munemitsu, S. and Polakis, P. (1996). Binding of GSK3beta to the APC-beta-catenin complex and regulation of complex assembly. *Science* **272**, 1023-1026.

Sanchez, C., Perez, M. and Avila, J. (2000). GSK3beta-mediated phosphorylation of the microtubule-associated protein 2C (MAP2C) prevents microtubule bundling. *Eur. J. Cell Biol*. **79**, 252-260.

Shtivelman, E., Sussman, J. and Stokoe, D. (2002). A Role for PI 3-Kinase and PKB Activity in the G2/M Phase of the Cell Cycle. *Curr. Biol.* **12**, 919-924.

Siegfried, E., Chou, T. B. and Perrimon, N. (1992). Wingless signaling acts through zeste-white 3, the *Drosophila* homolog of glycogen synthase kinase-3, to regulate engrailed and establish cell fate. *Cell* **71**, 1167-1179.

Stambolic, V., Ruel, L. and Woodgett, J. R. (1996). Lithium inhibits glycogen synthase kinase-3 activity and mimics wingless signalling in intact cells. *Curr Biol.* **6**, 1664-1668.

Stein, G. S., Stein, J. L., Lian, J. B., Last, T. J., Owen, T. and McCabe, L. (1994). Synchronisation of normal diploid and transformed Mammalian cells. *In Cell Biology: A Laboratory Handbook (*ed. J. Celis), pp. 282-287. San Diego: Academic Press.

Utton, M. A., Vandecandelaere, A., Wagner, U., Reynolds, C. H., Gibb, G. M., Miller, C. C., Bayley, P. M. and Anderton, B. H. (1997). Phosphorylation of tau by glycogen synthase kinase 3beta affects the ability of tau to promote microtubule self-assembly. *Biochem. J*. **323**, 741-747.

Wagner, U., Utton, M., Gallo, J. M. and Miller, C. C. (1996). Cellular phosphorylation of tau by GSK-3 beta influences tau binding to microtubules and microtubule organisation. *J. Cell Sci.* **109**, 1537-1543.

Zhou, J., Panda, D., Landen, J. W., Wilson, L. and Joshi, H. C. (2002). Minor alteration of microtubule dynamics causes loss of tension across kinetochore pairs and activates the spindle checkpoint. *J. Biol. Chem.* **277**, 17200-17208.

Zumbrunn, J., Kinoshita, K., Hyman, A. A. and Nathke, I. S. (2001). Binding of the adenomatous polyposis coli protein to microtubules increases microtubule stability and is regulated by GSK3 beta phosphorylation. *Curr. Biol.* **11**, 44-49.