Different spindle checkpoint proteins monitor microtubule attachment and tension at kinetochores in *Drosophila* cells

Elsa Logarinho[1,2,*], Hassan Bousbaa[1,2,*], José Miguel Dias[1], Carla Lopes[1], Isabel Amorim[1,3], Ana Antunes-Martins[1] and Claudio E. Sunkel[1,4,‡]

[1]Instituto de Biologia Molecular e Celular, Universidade do Porto, Rua do Campo Alegre 823, 4150-180 Porto, Portugal
[2]Instituto Superior de Ciências da Saúde-Norte, Grupo de Biologia Molecular e Celular, Rua Central de Gandra 1317, 4580 Gandra PRD, Portugal
[3]Departamento de Botânica da Faculdade de Ciências, Universidade do Porto, Rua do Campo Alegre 1119, 4150-181 Porto, Portugal
[4]Instituto de Ciências Biomédicas de Abel Salazar, Universidade do Porto, Largo do Prof. Abel Salazar 2, 4099-003 Porto, Portugal
*These authors contributed equally to this work
‡Author for correspondence (e-mail: cesunkel@ibmc.up.pt)

Accepted 3 December 2003
Journal of Cell Science 117, 1757-1771 Published by The Company of Biologists 2004
doi:10.1242/jcs.01033

Summary

The spindle assembly checkpoint detects errors in kinetochore attachment to the spindle including insufficient microtubule occupancy and absence of tension across bi-oriented kinetochore pairs. Here, we analyse how the kinetochore localization of the *Drosophila* spindle checkpoint proteins Bub1, Mad2, Bub3 and BubR1, behave in response to alterations in microtubule binding or tension. To analyse the behaviour in the absence of tension, we treated S2 cells with low doses of taxol to disrupt microtubule dynamics and tension, but not kinetochore-microtubule occupancy. Under these conditions, we found that Mad2 and Bub1 do not accumulate at metaphase kinetochores whereas BubR1 does. Consistently, in mono-oriented chromosomes, both kinetochores accumulate BubR1 whereas Bub1 and Mad2 only localize at the unattached kinetochore. To study the effect of tension we analysed the kinetochore localization of spindle checkpoint proteins in relation to tension-sensitive kinetochore phosphorylation recognised by the 3F3/2 antibody. Using detergent-extracted S2 cells as a system in which kinetochore phosphorylation can be easily manipulated, we observed that BubR1 and Bub3 accumulation at kinetochores is dependent on the presence of phosphorylated 3F3/2 epitopes. However, Bub1 and Mad2 localize at kinetochores regardless of the 3F3/2 phosphorylation state. Altogether, our results suggest that spindle checkpoint proteins sense distinct aspects of kinetochore interaction with the spindle, with Mad2 and Bub1 monitoring microtubule occupancy while BubR1 and Bub3 monitor tension across attached kinetochores.

Supplemental data available online

Key words: Mitosis, Checkpoint, Kinetochore, Microtubule, Tension, 3F3-2, Phosphoepitopes

Introduction

During mitosis, chromosome segregation is carefully monitored to prevent aneuploidy. For this, eukaryotic cells have evolved a surveillance mechanism known as the spindle assembly checkpoint (Murray, 1994), kinetochore attachment checkpoint (Rieder et al., 1994), chromosome distribution checkpoint (Nicklas, 1997) or the mitotic checkpoint. This checkpoint guarantees that anaphase onset is initiated only when all chromosomes are properly attached to microtubules and aligned at the metaphase plate.

Genetic screens in budding yeast identified many checkpoint components. The genes *MAD1-3* (Li and Murray, 1991), *BUB1 and BUB3* (Hoyt et al., 1991) were shown to be required for mitotic arrest in the presence of microtubule-depolymerising drugs. Homologues of many of these genes have been identified in *Saccharomyces pombe*, *Drosophila melanogaster*, *Xenopus laevis*, *Mus musculus* and *Homo sapiens* (reviewed by Musacchio and Hardwick, 2002). However, in human, mouse and *Xenopus*, instead of a Mad3 homologue, a protein with homology to both Mad3 and Bub1, called BubR1 kinase (Bub1-related kinase), was identified (Jablonski et al., 1998; Taylor et al., 1998; Chen, 2002). Analyses of mutants, antibody microinjection in tissue culture cells and expression of truncated proteins with a dominant negative phenotype, have shown that inhibition of a single protein inactivates the checkpoint (reviewed by Amon, 1999), allowing for sister chromatid separation in the absence of microtubules.

Accumulation of these proteins is high on unattached kinetochores and diminishes as microtubules attach and exert tension across kinetochore pairs. Kinetochore localization is probably necessary for these proteins to be able to relay the checkpoint signal. Accordingly, it has been shown that the presence of unattached or improperly attached kinetochores generates a ‘wait anaphase’ signal (Rieder et al., 1995) that ultimately acts to inhibit Cdc20, an activator of the anaphase-promoting complex/cyclosome (APC/C) and the downstream effector of the spindle checkpoint (reviewed by Skibbens and Hieter, 1998). APC/C is a multi-subunit E3 ubiquitin ligase that, upon its activation by Cdc20, targets anaphase inhibitors such as securin and cyclin B, for degradation by the 26S

proteasome, resulting in sister chromatid separation and exit from mitosis, respectively. Mad2 is able to bind Cdc20 and inhibit APC/C activation (Li et al., 1997; Fang et al., 1998; Hwang et al., 1998; Kim et al., 1998), and has a fast turnover at unattached kinetochores (Howell et al., 2000). The cycling of Mad2 at kinetochores is thought to be crucial for generating a soluble APC/C inhibitor, and Mad2 depletion from attached kinetochores may signal anaphase onset (Gorbsky et al., 1998; Waters et al., 1998; Hoffman et al., 2001). Similarly to Mad2, BubR1 has been recently shown to be able to bind Cdc20, either alone or in a complex, and inhibit APC/C activation (Sudakin et al., 2001; Tang et al., 2001; Fang, 2002). Sudakin et al. (Sudakin et al., 2001) purified a mitotic checkpoint complex that contains nearly stoichiometric amounts of BubR1, Bub3, Mad2 and Cdc20, and that is much more potent than purified Mad2 alone in inhibiting the APC/C. Other reports have indicated that in checkpoint-arrested cells, Cdc20 forms two separate complexes containing either BubR1 or Mad2, even though in vitro, addition of Mad2 was found to stimulate the inhibition of APC/C by BubR1 (Tang et al., 2001; Fang, 2002). Therefore, it is presently not clear whether BubR1 and Mad2 act synergistically or in a complex to transduce the checkpoint signal and inhibit APC/C activity.

Also, it is a matter of much debate whether the checkpoint sensors act in a branched or in a single pathway to monitor two different aspects of kinetochore attachment to the spindle, namely microtubule occupancy and tension across bi-oriented kinetochore pairs (Zhou et al., 2002b; Shannon et al., 2002). Li and Nicklas (Li and Nicklas, 1995) first observed that, in praying mantis spermatocytes, failure of the two X and the Y chromosome to be connected trivalently, yielded a mono-oriented X that could prevent anaphase onset. Nevertheless, anaphase could be triggered shortly after applying mechanical tension across the mono-oriented chromosome with a microneedle. Further evidence for the role of mechanical signals in regulating the checkpoint came from the analysis of the phosphoepitopes recognised by the 3F3/2 monoclonal antibody (Cyert et al., 1988). In mitotic culture cells, these phosphoepitopes localize at the kinetochores of misaligned chromosomes but are lost as chromosomes congress (Gorbsky and Ricketts, 1993). Microinjection of the 3F3/2 antibody into living cells protects dephosphorylation of the epitopes by phosphatases and simultaneously delays anaphase onset (Campbell and Gorbsky, 1995). In chromosome micromanipulation experiments with living insect spermatocytes, it was also found that tension applied artificially to kinetochores of misaligned chromosomes could promote dephosphorylation of the 3F3/2 epitopes and abrogate the spindle checkpoint (Nicklas et al., 1995; Li and Nicklas, 1997). Therefore, the tension-sensitive kinetochore phosphorylation detected by the 3F3/2 antibody reflects a chemical change at the kinetochores that is monitored by the spindle checkpoint.

Alternatively, checkpoint components might only be monitoring kinetochore-microtubule attachment. When the kinetochore is fully and stably saturated with microtubules the checkpoint signal is turned off, and tension might only serve to stabilise microtubule-kinetochore interactions. Indeed, in mitotic PtK1 cells, loss of Mad2 staining at kinetochores depends on microtubule attachment but not tension (Waters et al., 1998). Decreasing tension at kinetochores without detaching them from the spindle is not sufficient to induce relocalization of Mad2. Moreover, in mammalian cells, laser ablation of the unattached kinetochore of a mono-oriented chromosome is able to turn off the checkpoint signal allowing anaphase to occur (Rieder et al., 1995). The inability of the remaining, attached kinetochore to sustain checkpoint activity argues that microtubule occupancy, not tension, is regulating the checkpoint. However, polar ejection forces imposed by microtubule growth or by plus-end-directed microtubule motors on the chromosome arms, could counterbalance poleward forces at the non-ablated kinetochore generating sufficient tension to turn off the checkpoint signal (Waters et al., 1996).

More recently, it has been suggested that both attachment and tension are indeed monitored separately by the checkpoint. In maize, it was demonstrated that loss of Mad2 at kinetochores in mitosis follows microtubule attachment while in meiotic divisions loss of Mad2 correlates with tension (Yu et al., 1999). Furthermore, in lysed PtK1 cells it was shown that binding of Mad2 is governed by tension-sensitive kinetochore phosphorylation, whereas its loss occurs with accumulation of kinetochore microtubules (Waters et al., 1999). Also, studies in mammalian cells have suggested that the checkpoint proteins Mad2 and BubR1/Bub1 appear to sense distinct signals, attachment and tension, respectively (Skoufias et al., 2001; Zhou et al., 2002a). In the presence of low-doses of vinblastine, which arrest HeLa cells at mitosis with normal chromosome alignment yet without tension, Bub1 and BubR1 are recruited to kinetochores but Mad2 is not (Skoufias et al., 2001). Similar results were observed in noscapine-treated cells, which have bipolar spindles but do not complete chromosome alignment (Zhou et al., 2002a). However, current evidence for this model is controversial since other studies have produced contradictory results (Hoffman et al., 2001; Taylor et al., 2001).

To further explore this issue, we analysed how the *Drosophila* spindle checkpoint proteins Bub1, Mad2, BubR1 and Bub3 behave with respect to microtubule attachment and tension. Interestingly, we found that release of not only Mad2, but also Bub1 from kinetochores is governed by microtubule binding, whereas BubR1 and Bub3 kinetochore localization is exclusively dependent on tension-sensitive phosphorylation. The results support a model in which distinct spindle checkpoint proteins monitor different aspects of kinetochore interaction with the spindle.

Materials and Methods

Identification of *Drosophila* Bub1 and Mad2 homologues

The gene CG14030 encoding a 1100-amino acid protein with a predicted molecular mass of 125 kDa, closely related to Bub1 proteins, was identified from the *Drosophila* genome sequence project. The corresponding EST for *bub1* is LD22858. A genomic sequence (AE003565) with homology to human Mad2 was identified through a BLAST search. The full-length cDNA encoding a 207-amino acid protein with a predicted molecular mass of 23 kDa was amplified from a cDNA library.

Recombinant protein expression and antibody production

Full-length Mad2 and fragments of Bub3 and BubR1 were tagged with six histidines at the N terminus and expressed from the pQE30 vector (Qiagen) in *E. coli*. The recombinant proteins were purified

using the Ni-NTA Purification System (Qiagen). Bub1 recombinant protein was expressed from the pET-23b vector (Novagen) and purified from inclusion bodies. Polyclonal antibodies against Bub1, Mad2, BubR1 and Bub3 were generated in rabbits by immunisation with the purified fusion proteins corresponding to amino acids 1-273, 1-207, 103-697 and 7-255, respectively (Diagnostics Scotland, Edinburgh, UK). Anti-Bub1, anti-Mad2 and anti-Bub3 antibodies were affinity-purified against the recombinant proteins immobilised in a nitrocellulose membrane (Schleicher & Schuell) and used for all experiments described.

Cell culture, drug treatment and preparation of cell extracts

Drosophila S2 cells were cultured at 25°C in Schneider's medium (Gibco-BRL) containing 10% FBS. To arrest cells in mitosis, colchicine (Sigma) was added to culture medium to a final concentration of 25 μM and then incubated for 16 hours. For taxol treatment, 10 μM taxol in DMSO stock (Sigma) was diluted in medium to a final concentration of 10 nM and cells were incubated for 10 minutes. For preparation of mitotic extracts, cells incubated in colchicine were centrifuged at 4°C and resuspended in lysis buffer (150 mM NaCl, 1% NP-40, 50 mM Tris-HCl pH 8.0, 2 mM EDTA, 500 nM microcystin and protease inhibitors). After 30 minutes incubation at 4°C, cell lysate was homogenised, sonicated and centrifuged at 14,000 rpm for 20 minutes. Supernatant was saved and used in immunoblot analyses and immunodepletion experiments. For immunodepletion, magnetic beads coated with anti-rabbit IgGs (Dynal) were washed in TM buffer (50 mM Tris, 5 mM $MgSO_4$, pH 7.4) and incubated for 2 hours at 4°C with anti-BubR1 or anti-Bub3 serum diluted in TM buffer containing 0.1% bovine serum albumin (BSA; Sigma). Mitotic cell extract diluted 1:5 in TM buffer was then incubated twice with the magnetic beads coated with the primary antibody. The level of immunodepletion was monitored by western blotting.

Immunofluorescence

For immunofluorescence analysis, cells were centrifuged onto slides, fixed immediately with 1% formaldehyde/0.5% Triton X-100 in 1× PBS, for 5 minutes, and fixed additionally with 1% formaldehyde in 1× PBS, for 5 minutes. Cells were then washed in PBST (1× PBS + 0.1% Triton X-100) and blocked in PBST containing 10% fetal calf serum (PBSTB). Antibodies were diluted in PBSTB solution. Immunopurified anti-Bub1, anti-Mad2 and anti-Bub3 antibodies were used at 1:100. Anti-BubR1 serum was used at 1:1000. Anti-α-tubulin was purchased from Sigma. For chromosome labelling, cells were mounted in Vectashield medium containing DAPI (Vector). Immunofluorescence in detergent-extracted cells and isolated chromosomes was carried out as previously described (Logarinho and Sunkel, 1998; Bousbaa et al., 1997), respectively. Lambda phosphatase (New England Biolabs) treatments were performed according to the manufacturer's instructions and before chromosome fixation. To block dephosphorylation of 3F3/2 phosphoepitopes, isolated chromosomes were incubated with the 3F3/2 antibody, diluted 1:1000 in PHEM plus 5% BSA, before the lambda phosphatase treatment.

Rephosphorylation assay

After detergent extraction, additional incubation in TM was performed to ensure the complete dephosphorylation of the 3F3/2 epitopes. To rephosphorylate kinetochores, lysed and washed cells were incubated for 20 minutes in TM buffer containing 1.7 mM ATP, 1.7 mM DTT and 500 nM microcystin. Lysed cells, in which the kinetochores were to remain dephosphorylated, were incubated for 20 minutes in the same medium but without microcystin. For exogenous BubR1 and Bub3 binding experiments, cells were immediately incubated with S2 cell extract diluted 1:4 to 1:10 in TM buffer plus microcystin, for 20 minutes. Cells were then rinsed briefly in TM buffer plus microcystin, fixed and immunostained.

Fluorescence microscopy

Z-series optical sections of cells were collected using a BioRad MRC 600 confocal laser microscope (Figs 2, 3 and 4) or a Zeiss Axiovert microscope with an AxioCam camera (Zeiss, Germany) (Figs 5-9), and image projections were processed using Photoshop 6.0 (Adobe Microsystems, CA). In all figures requiring comparisons of antibody staining between panels, the exposure time on the digital camera was held constant, and all images were processed in the same fashion (Figs 2, 5, 6, 7 and 9). Measurement of interkinetochore distances was performed using calibrated Image J software (http://rsb.info.nih.gov). Distances were measured from the outer edge of each sister centromere as previously reported (Waters et al., 1996).

Results

Bub1-like protein kinases in *Drosophila*

Previous molecular and genetic analysis in *Drosophila* identified a gene (CG7838) that encodes a 165 kDa protein with significant homology to Bub1 proteins from other organisms and was reported as the *Drosophila* Bub1 homologue (Basu et al., 1999). However, completion of the *Drosophila* genome sequence revealed the existence of another gene (CG14030) that encodes a 125 kDa protein closely related to Bub1 proteins. Higher eukaryotes also have two genes that encode proteins closely related to Bub1, one of which has retained the name Bub1, while the other is called BubR1. The N-terminal domains of both Bub1 and BubR1 are highly homologous to yeast Mad3 (Fig. 1C) (Murray and Marks, 2001), but both Bub1 and BubR1 contain a C-terminal Ser/Thr kinase domain not found in Mad3 (Fig. 1B,C). Neither *Drosophila* nor vertebrate genomes appear to encode a Mad3-like protein without a kinase domain. Since Bub1 and BubR1 appear to respond to different checkpoint signals (Chan et al., 1999; Sudakin et al., 2001; Taylor et al., 2001), it became essential to determine which of the two *Drosophila* genes corresponds to Bub1 and BubR1. Phylogenetic analysis, including both Bub1-like *Drosophila* proteins, as well as Bub1 and BubR1 proteins from other species, did not provide a simple solution to this problem (Fig. 1D). The results show that the proteins encoded by the CG7838 and CG14030 genes are more closely related to each other and to the *Anopheles* homologues than to either Bub1 or BubR1 from other species, whereas the human, mouse and *Xenopus* proteins do fall into defined clusters. More recently, it has been suggested that a distinctive feature of BubR1 proteins is the presence of a conserved sequence, the KEN motif, in the N-terminal domain (Murray and Marks, 2001). The KEN motif is also present in yeast Mad3 and other Mad3 homologues, but not in Bub1. Sequence comparisons indicated that the previously described CG7838-encoded protein (165 kDa) contains a N-terminal KEN motif, while the new Bub1-like CG14030 protein (125 kDa) does not (Fig. 1A) (Murray and Marks, 2001). This suggests that CG7838 encodes the *Drosophila* homologue of BubR1 while CG14030 encodes the bona fide *Drosophila* homologue of Bub1. However, if the sequence alignment is restricted to the C-terminal kinase domain, CG7838 is found to encode a protein, which is significantly more similar to Bub1

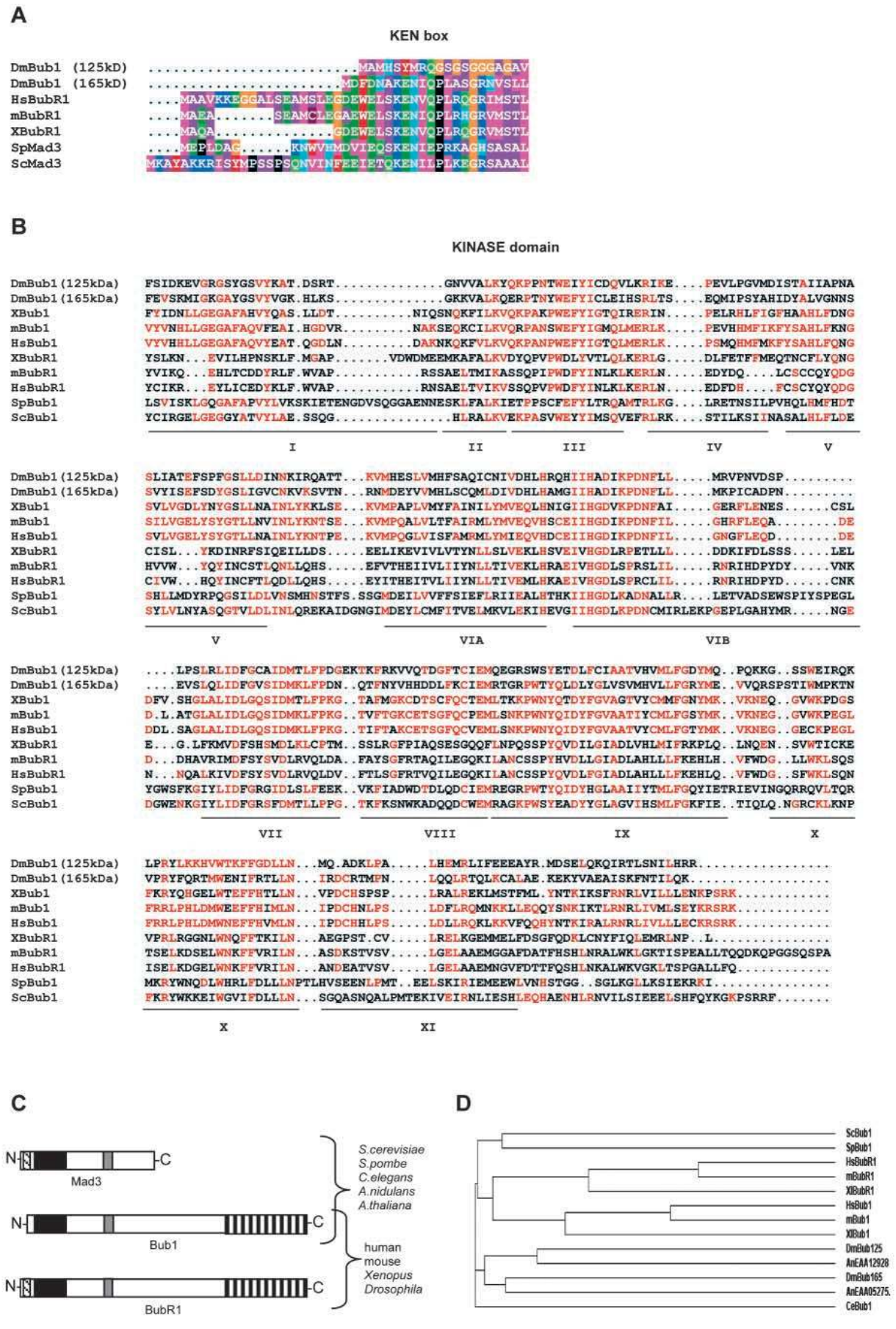
A
KEN box
DmBub1 (125kD)
DmBub1 (165kD)
HsBubR1
mBubR1
XBubR1
SpMad3
ScMad3
B
KINASE domain
DmBub1(125kDa)
DmBub1(165kDa)
XBub1
mBub1
HsBub1
XBubR1
mBubR1
HsBubR1
SpBub1
ScBub1
I
II
III
IV
V
V
VIA
VIB
VII
VIII
IX
X
X
XI
C
Mad3
Bub1
BubR1
S.cerevisiae
S.pombe
C.elegans
A.nidulans
A.thaliana
human
mouse
Xenopus
Drosophila
D
ScBub1
SpBub1
HsBubR1
mBubR1
XlBubR1
HsBub1
mBub1
XlBub1
DmBub125
AnEAA12928
DmBub165
AnEAA05275.
CeBub1

Fig. 1. Classification of Bub1-like proteins in *Drosophila*. Protein sequence analyses were done using Clustal X (http://www-igbmc.u-strasbg.fr/BioInfo/ClustalX/). Dm, *Drosophila melanogaster;* Hs, *Homo sapiens*; m, *Mus musculus*; X, *Xenopus laevis;* Sc, *Saccharomyces cerevisiae*; Sp, *Saccharomyces pombe*; An, *Anopheles gambiae*; Ce, *Caenorhabditis elegans*. (A) Sequence alignment of the KEN box motif uniquely present in the N-termini of BubR1 and Mad3 proteins. Note that DmBub1 (165 kDa) previously reported as Bub1 contains the KEN box motif similarly to other BubR1 proteins. (B) Sequence alignment of the C-terminal Ser/Thr kinase domains of Bub1 and BubR1 proteins. The 12 subdomains conserved among protein kinases (Hanks and Hunter, 1995) are underlined and the amino acids conserved within these subdomains are in red. In comparison with BubR1 kinases from other organisms, *Drosophila* BubR1 (DmBub1-165kDa) contains a much more conserved kinase domain. (C) Schematic representations of the Mad3, Bub1 and BubR1 proteins showing the relative positions of the KEN box motif present in Mad3 and BubR1 (crossed hatched box), the conserved Cdc20- and Bub3-binding domains present in all of them (black and grey boxes, respectively), and the conserved Ser/Thr kinase domain in Bub1 and BubR1 (vertical hatched box). Species that contain Mad3 and Bub1 or Bub1 and BubR1 are indicated on the right. (D) Phylogeny of the complete sequences of Bub1 and Mad3/BubR1 proteins.

than to BubR1 proteins (Fig. 1B). In vertebrates, BubR1 is clearly distinct from Bub1 because the kinase domain of the former is significantly less conserved (Taylor et al., 1998). Surprisingly, in *Drosophila,* the kinase domain of the KEN-box-containing protein (CG7838: 165 kDa) has almost as many conserved amino acids as the newly identified Bub1 protein (CG14030: 125 kDa) explaining why the protein encoded by the CG7838 gene was originally classified as Bub1. Even though from sequence analysis it seems difficult to determine unequivocally which *Drosophila* gene codes for Bub1 and BubR1, functional analysis of these proteins presented below clearly suggests that the newly identified CG14030 gene encodes the *Drosophila* homologue of *bub1*. Therefore, in contrast with our previous usage (Basu et al., 1998; Basu et al., 1999), CG7838 is more accurately described as the *Drosophila bubR1* gene, so the previously described mutant alleles *l(2)K06109* and *l(2)K03113* of CG7838 should be renamed $bubR1^1$ and $bubR1^2$. We suggest the use of this classification for future investigations and will adopt it in this work.

Mitotic localization of Bub1, BubR1, Bub3 and Mad2 in *Drosophila* culture cells

In addition to the *Drosophila* Bub1 homologue referred to above, we have also identified a genomic sequence (AE003565) encoding a protein with homology to human Mad2 (see Materials and Methods). To study the localization patterns of Bub1 and Mad2 during mitosis, we generated rabbit polyclonal antibodies, and new antibodies were also produced against Bub3 and BubR1. Western blot analyses of total protein extracts from S2 cultured cells showed that anti-Bub1 (Rb1112), anti-Mad2 (Rb1224), anti-Bub3 (Rb730) and anti-BubR1 (Rb666) sera are specific (Fig. S1, http://jcs.biologists.org/supplemental/). Firstly, their corresponding pre-immune sera do not react with the predicted antigens. Secondly, affinity-purified Rb1112, Rb1224 and Rb730 sera and crude serum Rb666 only recognise one band of the expected molecular mass in S2 cell extracts. Furthermore, anti-Bub1 antibodies do not cross-react with BubR1 since a single band of the predicted molecular mass is detected in extracts from $bubR1^1$ null mutant neuroblasts (data not shown).

Immunofluorescence analysis of S2 cells was performed with the specific antibodies against Mad2, Bub1, Bub3 and BubR1 to determine their distribution during mitosis (Fig. 2). The results show that Mad2 is mostly nuclear during prophase. At prometaphase, strong Mad2 labelling can be observed at kinetochores and spindle poles (see also Fig. 6A). At metaphase, Mad2 signal decreases dramatically and specifically in chromosomes that have congressed, but is still detected at the spindle poles and associated with microtubules in a punctuated pattern. In anaphase, Mad2 is no longer detected at kinetochores and staining of the spindle poles and microtubules decreases until telophase (Fig. 6A). The distribution of Mad2 in *Drosophila* cells is thus generally similar to that previously described for other organisms (Waters et al., 1998; Yu et al., 1999; Howell et al., 2000).

Bub1 immunolocalization shows that the polyclonal antibodies label the centrosomes strongly (Fig. 2), as well as some spindle microtubules, from prophase until late anaphase. Kinetochore staining is mostly weak and only detectable during prophase and prometaphase. This immunolocalization pattern observed for the Bub1 protein is specific since it is not observed with pre-immune serum and is completely abolished if antibodies are pre-incubated with the recombinant protein (data not shown).

Bub3 and BubR1 staining patterns (Fig. 2) are in agreement with our previous reports using chicken polyclonal antibodies (Basu et al., 1998; Basu et al., 1999). During prometaphase both proteins show strong kinetochore accumulation, which decreases significantly at metaphase and becomes undetectable at later mitotic stages. However, we consistently observed that whereas Bub3 and Bub1 localize to kinetochores during early prophase, as shown by anti-tubulin staining, BubR1 was never detected. This supports the classification adopted for the *Drosophila* Bub1-like proteins, as previous observations have shown that human Bub1 localizes to kinetochores at prophase before BubR1 (Jablonski et al., 1998; Taylor et al., 2001).

Drosophila S2 cells delay mitotic progression in response to microtubule poisons

In order to study the role of Bub1, BubR1, Bub3 and Mad2 in the spindle checkpoint, we first analysed the behaviour of *Drosophila* S2 cells when exposed to microtubule poisons (Fig. S2, http://jcs.biologists.org/supplemental/). Incubation of these cells with colchicine (25 μM) appears to inhibit cell proliferation without an effect upon cell viability. This is consistent with a significant increase in the mitotic index during the first 12 hours and a strong kinetochore localization of all spindle checkpoint proteins tested (data not shown). However, after this time the mitotic index starts to decrease and the culture shows an accumulation of polyploid cells (data not shown) suggesting that cells adapt and exit mitosis. The response of S2 cells to low doses of taxol (10 nM) is much less severe since the culture doubling time is only slightly delayed and the mitotic index shows a moderate increase. Overall these results indicate that S2 cells respond to microtubule poisons by delaying mitotic progression, and depending on the drug, the

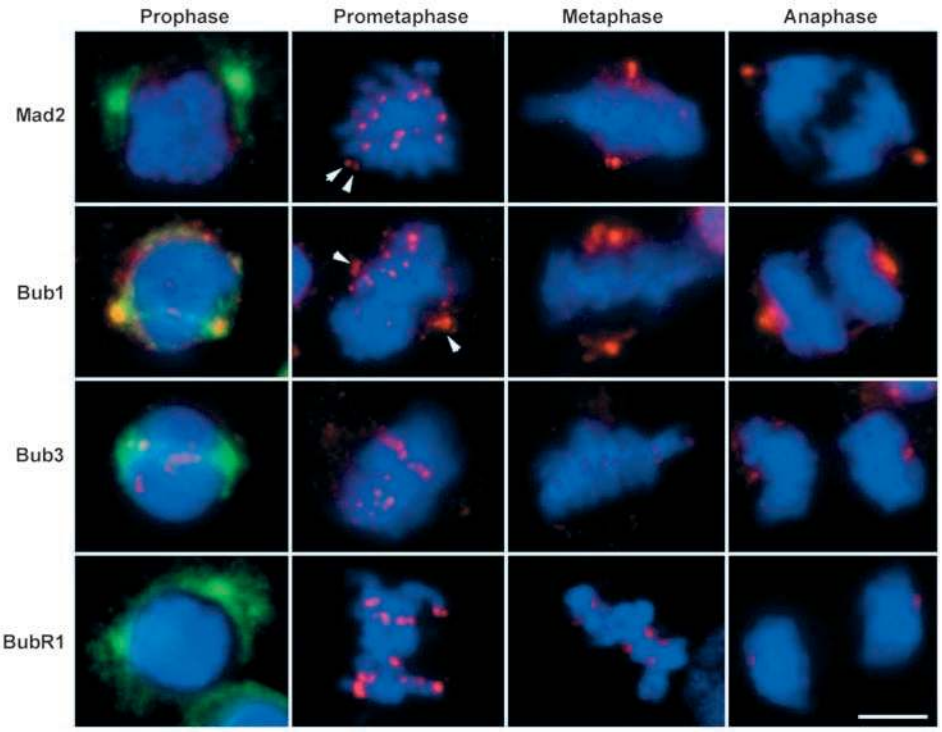

Fig. 2. Immunolocalization of Mad2, Bub1, Bub3 and BubR1 during progression through mitosis in *Drosophila* S2 cells. Cells were stained for DNA (blue) and antibodies against Mad2, Bub1, Bub3 or BubR1 (red). α-tubulin staining (green) was used to identify cells in prophase. Note that only Bub3 and Bub1 show kinetochore staining in prophase. All spindle checkpoint proteins show strong accumulation at kinetochores in prometaphase, weak or no staining in metaphase and are absent in anaphase. Staining of the spindle for Mad2 and Bub1 is observed during metaphase, and weak staining of centrosomes (arrowheads) is observed for all proteins at anaphase. Scale bar: 5 μm.

concentration used, and the incubation time, they display either a weak or a strong spindle checkpoint.

Taxol treatment reduces tension at metaphase kinetochores of *Drosophila* S2 cells

In mammalian cells, the distance between sister kinetochores at metaphase is considered to reflect the amount of tension exerted on them, since the microtubule poleward pulling forces cause the centromeric heterochromatin to stretch (Waters et al., 1998). Treatment of these cells with 10 μM taxol reduces centromeric stretch to nearly the 'rest length' (distance between sister kinetochores at prophase) when microtubules are unable to interact with kinetochores because of the nuclear envelope (Waters et al., 1996; Waters et al., 1998). To determine if S2 cells behave similarly, cells were fixed and co-immunostained for tubulin and the centromeric protein CID (Blower et al., 2001). We first collected Z-series optical sections of prophase and metaphase control cells and measured interkinetochore distances as previously described (Waters et al., 1996). The average interkinetochore distance in prophase cells was 0.73±0.12 μm (n=18). However, during metaphase the average interkinetochore distance increased significantly to 1.22±0.23 μm (n=23; Fig. 3A,B). To reduce the amount of tension across kinetochores, S2 cells were incubated for a brief period with low concentrations of taxol. Incubation with 10 nM taxol was sufficient to reduce tension without affecting microtubule attachment to kinetochores, as determined by measurement of interkinetochore distances in metaphase cells and tubulin immunostaining (Fig. 3A,B). The average interkinetochore distance in taxol-treated metaphase cells decreased to 0.82±0.13 μm (n=22), a value closer to the rest length. In order to confirm these results, control and taxol-treated cells were immunostained with the 3F3/2 monoclonal antibody (Fig. 3C). Tension is thought to induce a conformational change on the 3F3/2 kinetochore epitopes that promotes their dephosphorylation (Nicklas et al., 1995; Li and Nicklas, 1997). In *Drosophila*, 3F3/2 was shown to label kinetochores during early stages of mitosis becoming strongly reduced or absent at metaphase (Bousbaa et al., 1997). We found strong 3F3/2 kinetochore labelling in taxol-treated metaphase cells but not in control metaphase cells (Fig. 3C). These results suggest that, in S2 cells, tension can be reduced by incubation with nanomolar concentrations of taxol and monitored by staining with the 3F3/2 antibody.

Localization of BubR1, Mad2 and Bub1 in *Drosophila* cells under conditions of reduced kinetochore tension

Recent studies suggest that Mad2, Bub1 and BubR1 might be monitoring different aspects of kinetochore-microtubule interactions, namely attachment and tension (Skoufias et al., 2001; Zhou et al., 2002a). Therefore, we examined the kinetochore localization of *Drosophila* proteins under conditions of reduced tension induced by taxol treatment (Fig. 4A). We found that in taxol-treated cells, all metaphase kinetochores exhibit strong BubR1 staining in striking contrast to metaphase kinetochores from untreated cells, which only stain weakly (Fig. 2). However, Mad2 and Bub1 do not accumulate significantly at kinetochores after taxol treatment. Interestingly, the subcellular distribution of Mad2 appears to be affected by loss of microtubule dynamics since it was never found in centrosomes or spindle microtubules after incubation with taxol. We also characterised the kinetochore localization of these proteins in the mono-oriented chromosomes occasionally seen in the taxol-treated cells. While Mad2 and Bub1 stainings are mostly detected only at the unattached kinetochore, BubR1 labelling is usually detected at both

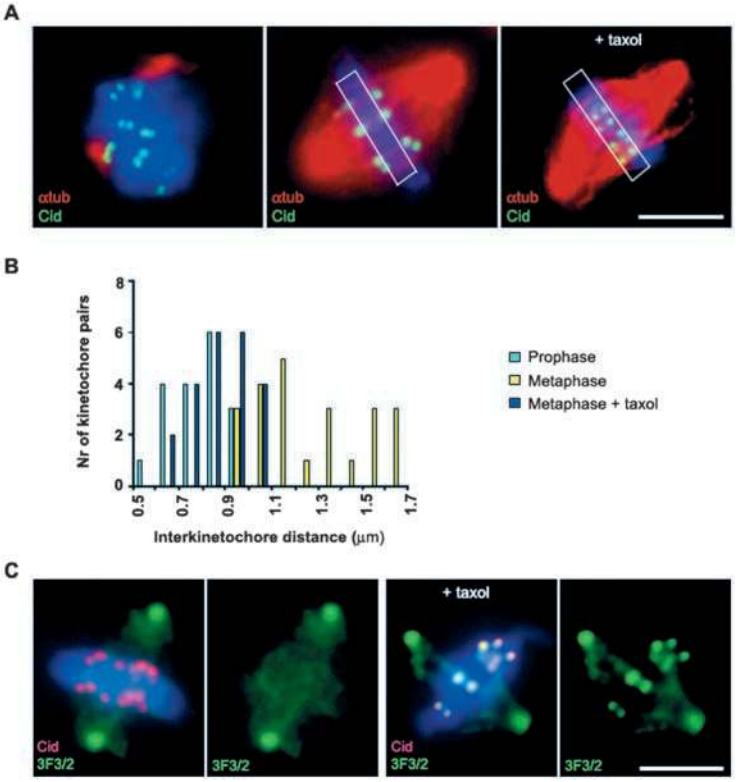

Fig. 3. Tension across bi-oriented kinetochore pairs of S2 metaphase cells is reduced after taxol treatment. (A) Cells in prophase or metaphase were stained for CID (green), α-tubulin (red) and DNA (blue). The distance between sister kinetochores in taxol-treated metaphase cells (right panel) is shorter than in control metaphase cells (middle panel) and closer to the distance in prophase cells (left panel). Boxes of identical size are used to compare interkinetochore distances between control and taxol treated cells. Tubulin staining shows that kinetochores still retain their microtubule fibers after taxol treatment. (B) Histogram showing the variation in distances between sister kinetochores in prophase, metaphase and taxol-treated metaphase cells. Interkinetochore distances in prophase cells (range: 0.48-0.92 mm). After sister kinetochores become bioriented poleward forces stretch centromeric heterochromatin and generate tension across kinetochore pairs (range: 0.88-1.59 mm). Taxol relieves tension by interfering with microtubule dynamics causing the distance between sister kinetochores to decrease (range: 0.59-1.00 mm). (C) S2 cells showing CID (red), 3F3/2 (green) and DNA (blue). After taxol treatment, all kinetochores in metaphase cells exhibit strong 3F3/2 labelling because of lack of tension (right panel). In a control metaphase cell, 3F3/2 labelling at the kinetochores is hardly detectable because of tension-sensitive dephosphorylation (left panel). Scale bars: 5 μm.

kinetochores. Staining at the attached kinetochore only was never observed for any of the antibodies (Fig. 4B). Quantification of the mono-oriented chromosome staining patterns showed that 75% and 65% exhibit Mad2 and Bub1 staining, respectively, only at the unattached kinetochore. However, most (78%) of the mono-oriented chromosomes were stained for BubR1 at both kinetochores. Taken together, these results show that BubR1 localizes to kinetochores whenever tension is absent and independently of their microtubule occupancy, whereas Mad2 and Bub1 localize preferentially to unattached kinetochores. These results are mostly consistent with recent data suggesting that localization of Bub1 is regulated by microtubule attachment, while that of BubR1 responds to tension, supporting the classification adopted for the two *Drosophila* Bub1-like proteins.

Tension-sensitive kinetochore phosphorylation can be controlled in *Drosophila* S2 lysed cells

The role of tension in spindle assembly checkpoint signalling is not easy to distinguish from that of attachment, as application of tension on kinetochores can enhance both the stability of individual microtubule attachments and the overall occupancy of microtubules (Nicklas and Koch, 1969; Nicklas

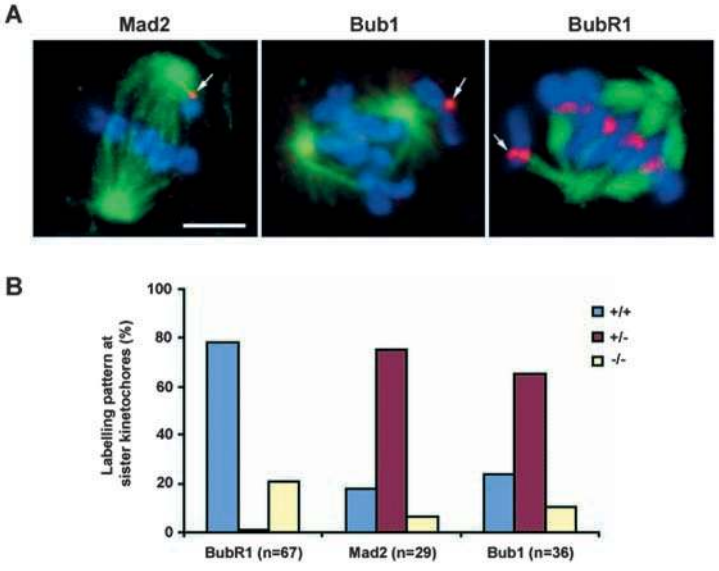

Fig. 4. Effect of reduced tension on the kinetochore localization of Mad2, Bub1 and BubR1. (A) Cells were incubated with 10 nM taxol for 10 minutes and then processed for immunofluorescence with antibodies against Mad2, Bub1 or BubR1 (red) and a-tubulin (green); DNA was stained with DAPI (blue). Mad2 and Bub1 are hardly detectable at kinetochores of aligned chromosomes, whereas BubR1 consistently localizes at all kinetochores of the metaphase plate. Mono-oriented chromosomes (arrows) exhibit BubR1 staining at both kinetochores while Mad2 and Bub1 labelling is only observed at unattached kinetochores. Scale bar: 5 μm. (B) Quantification of Mad2, Bub1 and BubR1 immunoreactivity patterns at sister kinetochores of mono-oriented chromosomes: labelling at both kinetochores (+/+), labelling only at unattached kinetochores (+/–) and no labelling at either kinetochore (–/–). Note that, whereas BubR1 labelling at mono-oriented chromosomes is mainly symmetric, Mad2 and Bub1 show staining only at unattached kinetochores.

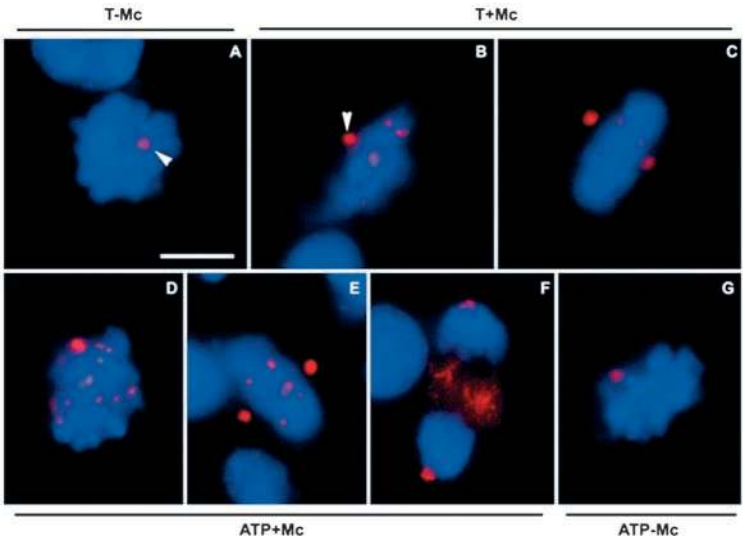

Fig. 5. Phosphorylation of 3F3/2 epitopes in S2 lysed cells. In all images DNA is shown in blue and 3F3/2 antibody staining in red. (A) S2 cell lysed in the absence of the phosphatase inhibitor microcystin (T-Mc). 3F3/2 epitopes are only detectable at the spindle poles (arrowhead). (B,C) S2 cells lysed in the presence of microcystin (T+Mc). Prometaphase cells (B) exhibit strong 3F3/2 staining at the kinetochores and spindle poles (arrowhead). At metaphase (C), 3F3/2 kinetochore labelling decreases significantly and is often undetectable. (D,E,F) S2 cells lysed in the absence of microcystin and then incubated with ATP plus microcystin (ATP+Mc). Kinetochores are strongly labelled with 3F3/2 antibody even at metaphase (E), but not at anaphase (F). (G) Dephosphorylated S2 cell (lysed with T-Mc) and incubated with ATP alone (ATP-Mc). 3F3/2 epitopes are not phosphorylated in the absence of microcystin. Scale bar: 5 μm.

and Ward, 1994; King and Nicklas, 2000). Therefore, we have developed a protocol using S2 lysed cells as an in vitro system to study 'tension in the absence of microtubules'. In this system, mechanical tension is analysed indirectly through the observation of 3F3/2 kinetochore phosphorylation, known to correlate with tension in vivo (Nicklas et al., 1998). Previously, it was shown that washed chromosomes from lysed cells (Campbell et al., 1995; Nicklas et al., 1998; Waters et al., 1999), as well as isolated chromosomes (Bousbaa et al., 1997), have dephosphorylated kinetochores that do not stain with 3F3/2 antibody. However, these kinetochores can be phosphorylated simply by incubation with ATP and a phosphatase inhibitor, showing that they contain a complete phosphorylation/dephosphorylation system, consisting of the kinase, the substrate and the phosphatase. In this system, kinetochore phosphorylation can be controlled experimentally rather than by tension, which is the situation in vivo. We found that S2 cells lysed with detergent in the absence of phosphatase inhibitors rapidly lose 3F3/2 phosphoepitopes at their kinetochores (Fig. 5A). However, if cells are lysed in the presence of the phosphatase inhibitor microcystin, 3F3/2 staining is clearly detectable, in particular before metaphase (Fig. 5B,C). When dephosphorylated S2 cells (lysed in the absence of microcystin) are incubated for 20 minutes with ATP and microcystin, all kinetochores label positively with the 3F3/2 antibody (Fig. 5D). Interestingly, even metaphase chromosomes exhibit strong labelling at their kinetochores (Fig. 5E), while anaphase kinetochores are no longer rephosphorylated (Fig. 5F). In control preparations incubated with ATP in the absence of microcystin, none of the kinetochores becomes labelled with the 3F3/2 antibody (Fig. 5G). Therefore, S2 lysed cells provide an in vitro phosphorylation system that can be manipulated in order to study the behaviour of checkpoint proteins with respect to tension-sensitive 3F3/2 kinetochore phosphorylation in the absence of microtubules.

Mad2, Bub1, BubR1 and Bub3 respond differently to tension-sensitive phosphorylation

In order to study the kinetochore localization of spindle checkpoint proteins in relation to tension-sensitive phosphorylation, S2 lysed cells were double immunostained to detect 3F3/2 and Mad2, Bub1, BubR1 or Bub3 (Figs 6, 7). S2 cells lysed in the presence of microcystin showed strong kinetochore labelling for 3F3/2 and all spindle checkpoint proteins prior to anaphase. In S2 cells lysed in the absence of microcystin, 3F3/2 labelling was, as expected, undetectable at all kinetochores, but spindle checkpoint proteins showed different staining patterns. Mad2 and Bub1 were easily detected in the absence of 3F3/2 kinetochore phosphoepitopes (Fig. 6). However, BubR1 and Bub3 kinetochore accumulation was not detected at any mitotic stage after detergent extraction in the absence of microcystin (Fig. 7). As a control, the centromeric protein CID was observed to behave independently of the kinetochore phosphorylation status, suggesting that BubR1 and Bub3 depletion from kinetochores is not caused by disruption of kinetochore structure resulting from the procedure. These results show that, unlike Mad2 or Bub1, BubR1 and Bub3 retention at kinetochores depends specifically on the presence of kinetochore phosphoepitopes.

BubR1 and Bub3 depletion from kinetochores is governed by 3F3/2 epitope dephosphorylation and is independent of microtubules

To unequivocally demonstrate that BubR1 and Bub3 depletion from dephosphorylated kinetochores is independent of microtubules, the phosphorylation assays were performed on isolated chromosomes (Fig. 8). These chromosomes were purified from S2 cells incubated with colchicine to depolymerise microtubules. Moreover, since microcystin was included during all purification steps, and since these chromosomes were isolated from cells with an activated spindle checkpoint, the kinetochores stained very brightly with the 3F3/2 and anti-BubR1 antibodies (Fig. 8A). Incubation of these chromosomes with lambda phosphatase removed completely the 3F3/2 phosphoepitopes from the kinetochores, as well as BubR1 (Fig. 8B). Control incubations with lambda phosphatase buffer or alternatively, with lambda phosphatase plus microcystin, showed that BubR1 loss is caused specifically by dephosphorylation (Fig. 8C; data not shown). The same

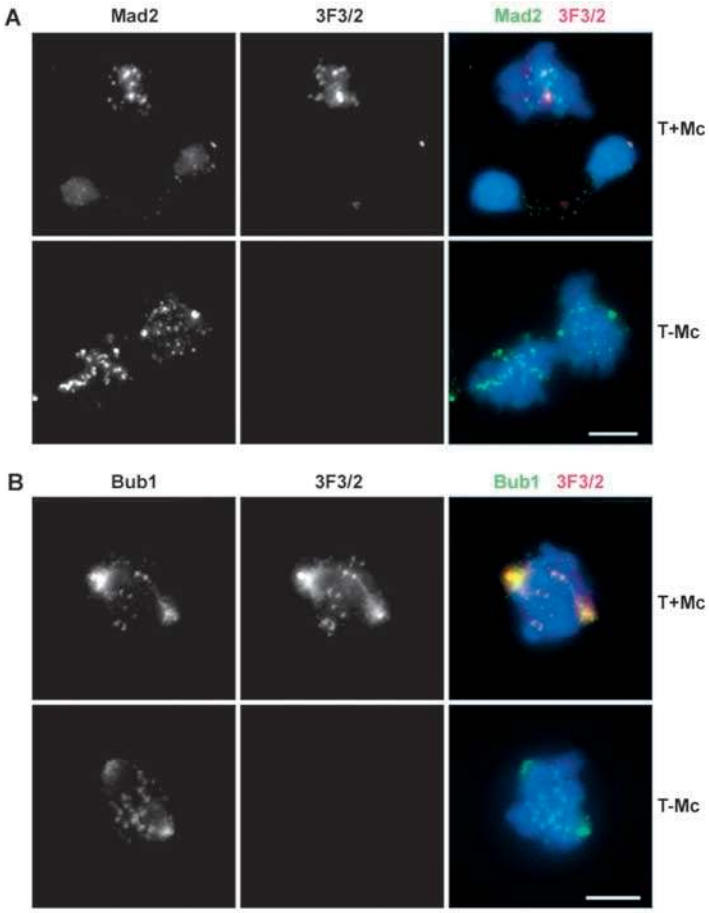

Fig. 6. Localization of Mad2 and Bub1 checkpoint proteins is not affected by kinetochore dephosphorylation. S2 cells were lysed with detergent either in the presence (T+Mc) or in the absence (T-Mc) of microcystin, fixed and immunostained. (A) Immunostaining with the anti-Mad2 (green) and 3F3/2 (red) antibodies shows that Mad2 labelling remains unchanged after dephosphorylation. (B) Immunostaining with the anti-Bub1 (green) and 3F3/2 (red) antibodies shows that Bub1 still localizes normally after dephosphorylation. Scale bar: 5 μm.

experiments were performed for Bub3 with similar results (Fig. 8E-G). To further determine whether the loss of BubR1 and Bub3 proteins from kinetochores is caused by dephosphorylation of 3F3/2 epitopes and not by dephosphorylation of some other epitopes, 3F3/2 phosphoepitopes were blocked with the antibody before lambda phosphatase treatment. Under these conditions, we found that BubR1 and Bub3 accumulation at the kinetochores is preserved (Fig. 8D,H). The results indicate that, even when other phosphoproteins are dephosphorylated, as long as 3F3/2 phosphoepitopes are present, BubR1/Bub3 proteins are retained at the kinetochores.

BubR1 and Bub3 relocalize to phosphorylated kinetochores

In the previous sections we showed that BubR1 and Bub3 retention or depletion from kinetochores correlates with the presence of 3F3/2 phosphoepitopes. To determine whether their accumulation at kinetochores also depends on 3F3/2 phosphorylated proteins we carried out relocalization studies.

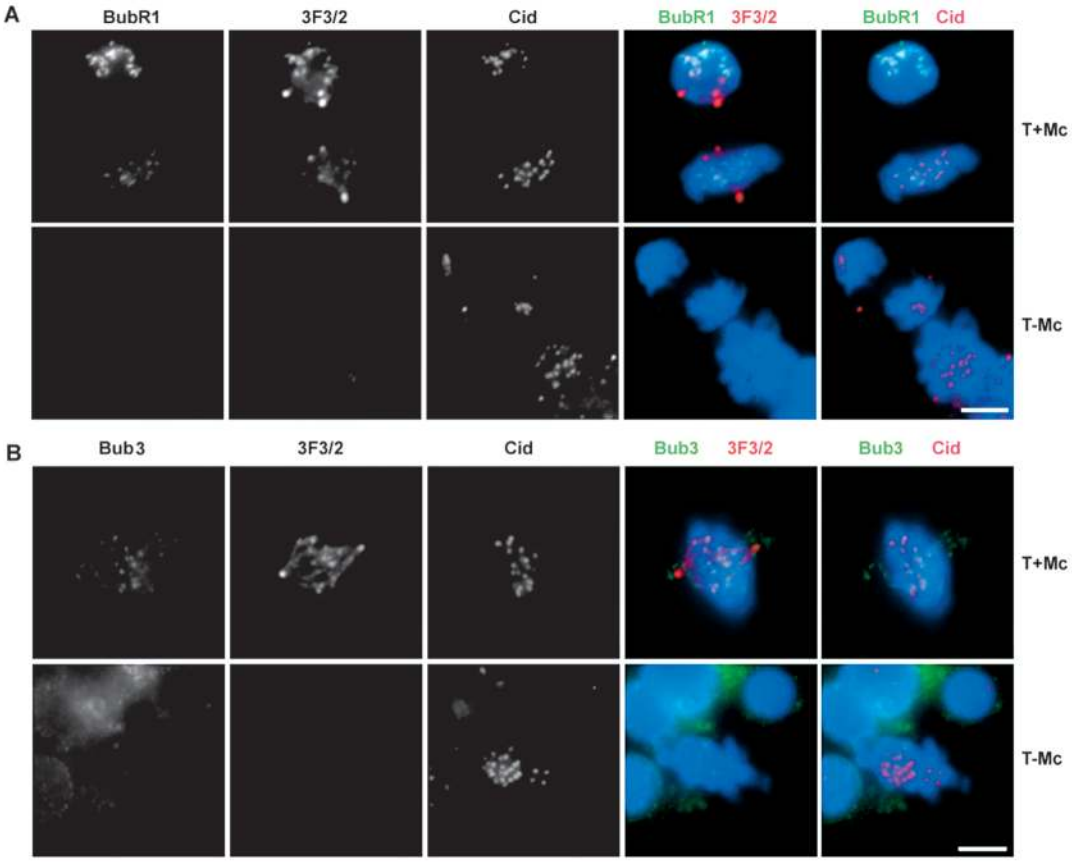

Fig. 7. Dephosphorylation of kinetochore phosphoepitopes releases BubR1 and Bub3. S2 cells were lysed with detergent either in the presence (T+Mc) or in the absence (T-Mc) of microcystin, fixed and immunostained. (A) Immunostaining with anti-BubR1 (green), 3F3/2 (red) and anti-CID (pink) antibodies shows that BubR1 staining is no longer detectable after dephosphorylation. CID localization is shown as a control and is not affected by dephosphorylation. (B) Immunostaining with anti-Bub3, 3F3/2 and anti-CID antibodies shows that Bub3 staining is lost after dephosphorylation, while CID labelling remains. Scale bar: 5 μm.

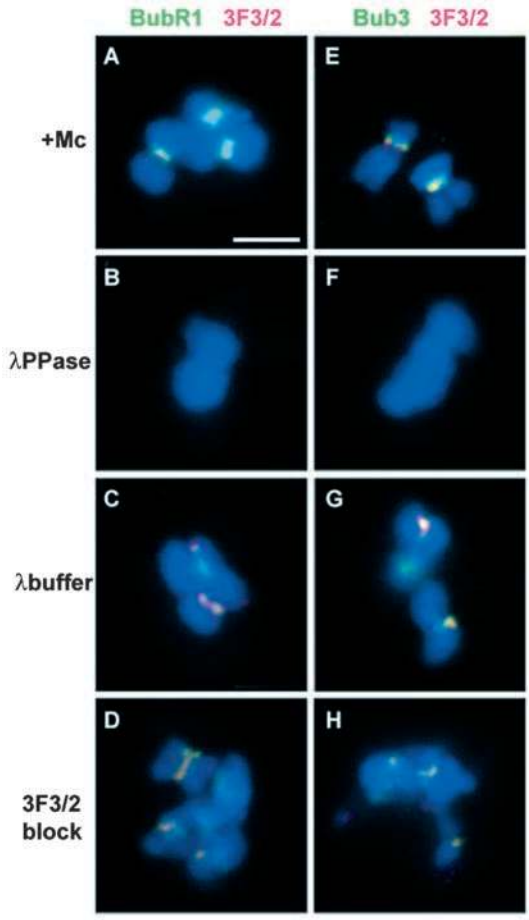

Fig. 8. BubR1 and Bub3 loss from kinetochores is specifically due to 3F3/2 epitope dephosphorylation. Condensed chromosomes were purified from S2 colchicine-arrested cells in the presence of microcystin to maintain kinetochore phosphoepitopes. In all preparations DNA was stained with DAPI (blue). (A,E) Isolated chromosomes were fixed and immunostained with 3F3/2, anti-BubR1 and anti-Bub3 antibodies. Note the strong kinetochore labelling for all antibodies. (B,F) Isolated chromosomes were incubated with lambda phosphatase (λPPase) leading to complete dephosphorylation of the 3F3/2 epitopes and to loss of BubR1 and Bub3 from kinetochores. (C,G) Chromosomes incubated with lambda phosphatase buffer alone (λ buffer) retain 3F3/2 phosphoepitopes, as well as BubR1 and Bub3 proteins. (D,H) Isolated chromosomes were first incubated with the 3F3/2 antibody and then treated with lambda phosphatase before immunostaining for BubR1 or Bub3. Preincubation with 3F3/2 antibodies blocks the accessibility of the phosphatase to the 3F3/2 phosphoepitopes causing BubR1 and Bub3 to remain at kinetochores. Scale bar: 5 μm.

Incubation of dephosphorylated lysed cells with ATP buffer without microcystin does not regenerate the 3F3/2 phosphoepitopes and BubR1 staining is also absent (Fig. 9A). However, if lysed cells are incubated with ATP plus microcystin, 3F3/2 phosphoepitopes are rephosphorylated and a very weak, mostly inconsistent, BubR1 signal can be detected (Fig. 9B). This result suggested that residual levels of BubR1 that had escaped detergent extraction could be recruited to kinetochores. Therefore, in order to test whether BubR1 reaccumulation at kinetochores, like its release, could be dependent on kinetochore phosphorylation, we examined the ability of exogenously added BubR1 to bind to dephosphorylated or phosphorylated kinetochores. We found that exogenously added BubR1 does not accumulate at dephosphorylated kinetochores (Fig. 9C), but it accumulates strongly at phosphorylated kinetochores (Fig. 9D). This recruitment is only observed before anaphase. Accordingly, rephosphorylated kinetochores stain brightly for 3F3/2 in metaphase but, at anaphase, 3F3/2 rephosphorylation does not occur. Incubation of rephosphorylated cells with a BubR1-depleted mitotic extract (data not shown) confirmed that BubR1 accumulation is specific (Fig. 9E). Similar experiments were performed for Bub3, with identical results (Fig. S3, http://jcs.biologists.org.supplemental/ and data not shown). These results indicate very clearly that BubR1 and Bub3 accumulate only at kinetochores containing 3F3/2 tension-sensitive phosphoepitopes.

Discussion

Classification of the *Drosophila* BubR1 and Bub1 proteins

We show that *Drosophila*, similarly to higher eukaryotes, contains two genes that encode Bub1-like proteins. Both proteins share homology at the N terminus with Mad3 and additionally have a putative kinase domain in the C terminus typical of other known Bub1 and BubR1 proteins (Taylor and McKeon, 1997; Taylor et al., 1998; Sharp-Baker and Chen, 2001). However, whereas phylogenetic analysis in vertebrates places Bub1 and BubR1 proteins into defined clusters, in *Drosophila* the two Bub1-like proteins are more closely related to each other than to either Bub1 or BubR1 from other species. Therefore, their classification on the basis of sequence analysis turned out to be rather difficult. This is mainly due to the fact that both proteins have highly conserved Ser/Thr kinase domains, while in vertebrates BubR1 proteins are easily distinguishable from Bub1 proteins because they are less conserved at their C termini. Nevertheless, our data suggests that the previously reported Bub1 protein (Basu et al., 1999) is BubR1 instead, and that the newly identified protein is Bub1, and we adopted this classification in this study. First, protein sequence analysis indicated that the previously described Bub1 protein contains a KEN-box motif at the N terminus while the new Bub1-like protein does not. The KEN box is an APC/C^{Cdh1} recognition signal and was identified on the N terminus of yeast Mad3 and Mad3/BubR1 homologues, but not in any Bub1 homologue. It is therefore thought to be the distinguishable feature between Bub1 and Mad3/BubR1 proteins (Murray and Marks, 2001). Since there are no species with both BubR1 and Mad3, the functions fulfilled by BubR1 in mammalian cells and by Mad3 in yeast cells may be partially analogous, even though Mad3 does not have a C-terminal kinase domain. Secondly, analysis of their intracellular pattern of localization showed that, during early prophase, only the newly identified Bub1 localizes to kinetochores, in agreement with previous observations showing that Bub1 localizes to kinetochores before BubR1 at prophase (Jablonski et al., 1998; Taylor et al., 2001). Finally, the *Drosophila* Bub1-like proteins were found to behave differently with respect to tension and microtubule attachment (see further discussion below). BubR1 proteins accumulate at kinetochores in the absence of tension (Skoufias et al., 2001; Zhou et al., 2002a; Shannon et al., 2002)

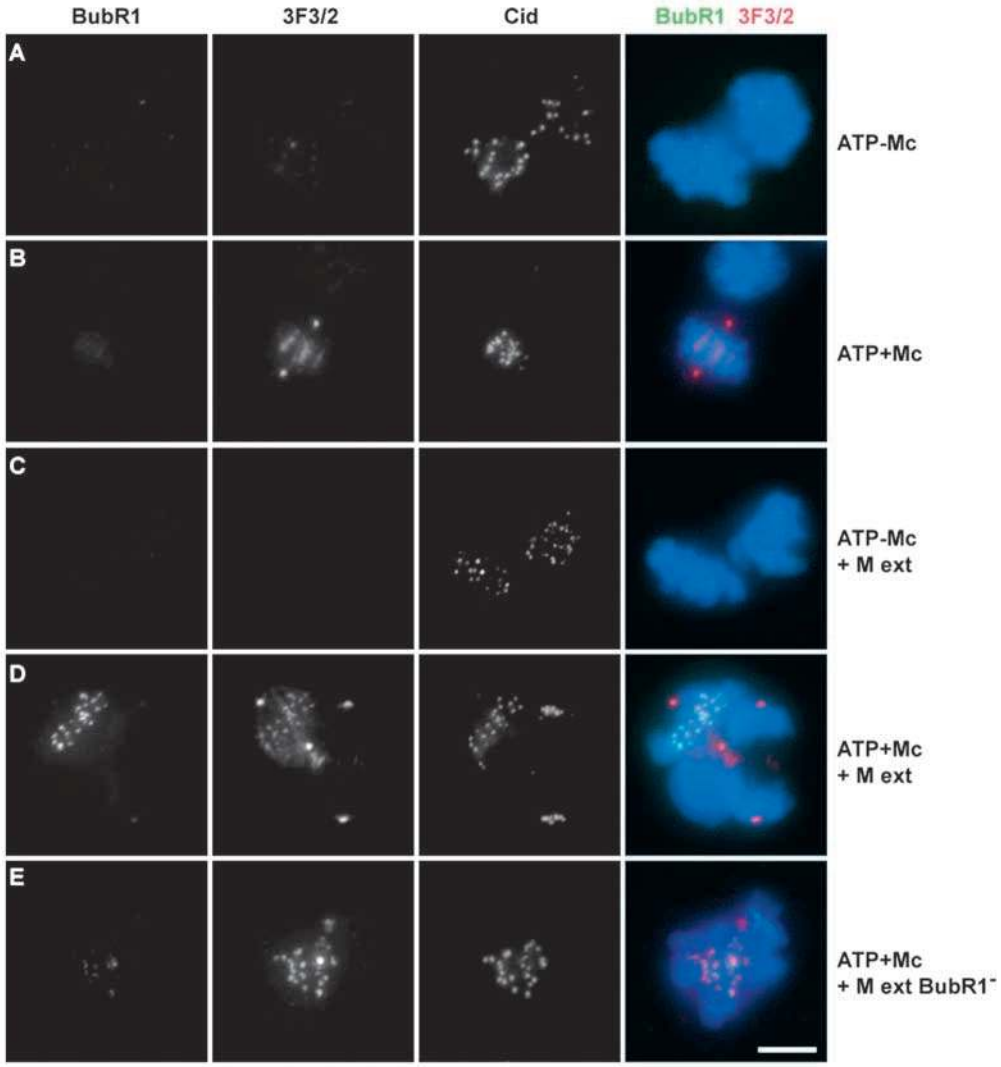

Fig. 9. BubR1 is recruited to phosphorylated but not to unphosphorylated kinetochores. All cells were initially lysed in the absence of microcystin to become dephosphorylated and, after different treatments, fixed and stained for 3F3/2, BubR1, CID and DNA (blue). (A) Cells treated with ATP in the absence of microcystin (ATP-Mc) retain normal CID staining but BubR1 and 3F3/2 labelling is abolished. (B) Cells treated with ATP plus microcystin (ATP+Mc) show CID and 3F3/2 staining but endogenous BubR1 is depleted during extraction. (C) Cells rephosphorylated with ATP but without microcystin and subsequently incubated with S2 mitotic extract (M ext). Note that 3F3/2 epitopes are not rephosphorylated and that exogenous BubR1 is not recruited to kinetochores. (D) Cells rephosphorylated in the presence of microcystin and subsequently incubated with S2 mitotic extract show rephosphorylated 3F3/2 epitopes and strong accumulation of BubR1 at kinetochores. (E) Cells rephosphorylated in the presence of microcystin and incubated with an S2 mitotic extract mostly depleted of BubR1, show strongly labelled 3F3/2 phosphoepitopes but only weak accumulation of BubR1. Scale bars: 5 μm.

and are not as sensitive to microtubule attachment as Bub1 (Taylor et al., 2001). In our study, the protein that contains the KEN-box is the one whose localization responds to changes in tension but not microtubule attachment, once again suggesting that is the BubR1 homologue.

Studying tension and attachment separately

It has remained unclear whether tension and attachment are separable events in terms of checkpoint function (King and Nicklas, 2000). Therefore, we used two different approaches to study tension and attachment separately to determine whether the spindle checkpoint monitors those events independently. To study attachment in the absence of tension, we have treated *Drosophila* S2 culture cells with nanomolar concentrations of taxol. At these low doses, microtubules can still attach to the kinetochores, but tension is severely reduced because of loss of microtubule dynamics (McEwen et al., 1997; Waters et al., 1998). Measurement of interkinetochore distances and tubulin staining confirmed that, in S2 cells treated with taxol, tension is lost without disturbing microtubule attachment. Furthermore, we showed that 3F3/2 kinetochore staining correlates with the presence or absence of tension as previously shown in other cell types (Gorbsky and Ricketts, 1993; Nicklas et al., 1995). In control cells, bi-oriented chromosomes showed dephosphorylated kinetochores as a result of tension while in taxol-treated cells, metaphase kinetochores were strongly phosphorylated.

To study ‘tension in the absence of attachment’, we have used detergent-extracted S2 cells. In these cells, microtubules are depolymerised and tension can be analysed indirectly through the observation of 3F3/2 kinetochore phosphoepitopes. Mechanically applied tension was shown to diminish kinetochore phosphorylation in lysed cells just as it does in living cells (Nicklas et al., 1998). Therefore, lysed cells can be used as an in vitro phosphorylation system to simulate the in vivo tension effect in the absence of microtubules. In vitro phosphorylated chromosomes mimic the in vivo improperly attached chromosomes, while in vitro dephosphorylated chromosomes mimic the in vivo bi-oriented chromosomes under tension.

Mad2 and Bub1 loss from kinetochores is regulated by microtubule attachment

We analysed how the kinetochore localization of spindle

checkpoint proteins is affected by disrupting tension but not microtubule attachment. Interestingly, we found different behaviours. When tension was reduced by low doses of taxol, BubR1 accumulated at kinetochores. This is in agreement with recent reports showing that reduced tension at kinetochores containing a full complement of microtubules induces a checkpoint-dependent metaphase delay associated with elevated levels of BubR1 at kinetochores (Skoufias et al., 2001; Zhou et al., 2002a; Shannon et al., 2002). However, Mad2 and Bub1 proteins did not localize at kinetochores of aligned bi-oriented chromosomes after taxol treatment. Nevertheless, in mono-oriented chromosomes, Mad2 and Bub1 staining was consistently detected at the unattached kinetochore. These results are fully consistent with previous observations showing that Mad2 depletion from kinetochores is governed by microtubule attachment (Waters et al., 1998). In the case of Bub1, there are contradictory results regarding its behaviour. Whereas some reports show kinetochore accumulation of Bub1 under conditions of reduced tension (Skoufias et al., 2001; Shannon et al., 2002), others show that its release from kinetochores is regulated by microtubule attachment (Taylor et al., 2001). Thus, either Bub1 responds to both attachment and tension, or the results reflect differences between cell types. Nevertheless, our results suggest that the behaviour of Bub1 is globally more similar to that of Mad2, suggesting that it is mainly sensitive to microtubule binding.

BubR1 and Bub3 loss from kinetochores is regulated by tension

We have also analysed how the spindle checkpoint proteins behave with respect to tension-sensitive kinetochore phosphorylation in the absence of microtubule attachment. These results were fully consistent with those obtained using taxol treatment. Whereas BubR1 and Bub3 were lost from kinetochores after dephosphorylation, Mad2 and Bub1 remained localized at kinetochores independently of their phosphorylation status, in agreement with the fact that these proteins are displaced by microtubule attachment. Previously, an inverse correlation between the amount of tubulin staining and the amount of Bub3 was shown for the two sister kinetochores of lagging chromosomes (Martinez-Exposito et al., 1999). However, this asymmetric labelling at lagging chromosomes might not necessarily reflect sensitivity to microtubule-attachment since 3F3/3 staining has been also reported to be asymmetric in lagging chromosomes. The phosphoepitopes are more strongly expressed on the leading kinetochore than in the trailing one (Gorbsky and Ricketts, 1993; Nicklas et al., 1995; Nicklas et al., 1998). Indeed, we showed that BubR1 and Bub3 depletion from kinetochores occurs specifically because of 3F3/2 epitope dephosphorylation. Dephosphorylation of 3F3/2 epitopes might induce a conformational change in kinetochore proteins rendering them unable to interact with BubR1 and Bub3. BubR1 and Bub3 are unlikely to bind directly to the phosphoepitopes since pre-blocking isolated chromosomes with the anti-BubR1 antibody, does not inhibit 3F3/2 dephosphorylation by the lambda phosphatase treatment. Finally, from the experiments with lysed S2 cells we found that, similar to their release, the binding of BubR1 and Bub3 to kinetochores is dependent on tension-sensitive kinetochore phosphorylation. Previous observations had demonstrated that Mad2 binding is also promoted by kinetochore phosphorylation (Waters et al., 1999). On the basis of all the results discussed above, we propose a model for the behaviour of spindle checkpoint proteins during microtubule-kinetochore interaction (Fig. 10).

Kinetochore phosphorylation in the spindle checkpoint

Phosphorylation establishes a biochemical difference between kinetochores that are under tension and those that are not. Since the disruption of normal microtubule dynamics is sufficient to cause rephosphorylation of the 3F3/2 epitopes at metaphase kinetochores, even though they still contain many microtubules, this biochemical signal must act independently of microtubule attachment (Campbell and Gorbsky, 1995; McEwen et al., 1997; Waters et al., 1998). Therefore, checkpoint proteins whose kinetochore localization is regulated exclusively by tension-sensitive phosphorylation are necessary to activate the checkpoint when microtubule dynamics is affected or when sister kinetochores are attached by microtubules from the same pole (syntelic attachment). Mad2 does not fit in this group of checkpoint proteins. Although Mad2 binding to kinetochores is initially governed by phosphorylation (Waters et al., 1999), it is inhibited later as kinetochores start being occupied by microtubules (Waters et al., 1998). However, our results show that BubR1 and Bub3 proteins behave differently from Mad2 because their removal from kinetochores is governed by dephosphorylation and is insensitive to microtubule attachment (Fig. 10). Compelling data suggests that BubR1 and Mad2 operate independently, with the first sensing tension and the second monitoring attachment. The strength of the checkpoint response induced by these two events also appears to be different since S2 cells show only a short mitotic delay when exposed to low taxol doses, while complete microtubule depolimerization by colchicine, strongly compromises mitotic progression.

However, even though BubR1 and Mad2 may sense different spindle assembly signals, these must be integrated at some point. Evidence for the convergence of the two sensing mechanisms comes from the observation that the metaphase delay induced by reduced tension and increased levels of BubR1 at the kinetochores, is Mad2 dependent (Shannon et al., 2002). This indicates that BubR1 and Mad2 cannot suppress the Cdc20-APC/C activity independently of each other. Indeed, Mad2 and BubR1 were found as components of the same APC/C-inhibiting complex (Sudakin et al., 2001).

The effect of tension on the spindle checkpoint might be direct. In the absence of tension, the 3F3/2 kinase(s) might directly activate spindle checkpoint proteins at kinetochores. Generation of tension, which pulls sister kinetochores apart, could then impair phosphorylation of 3F3/2 substrates by separating them from the kinase(s) or by changing their conformational structure. Only a few 3F3/2 epitopes have been identified so far (Daum et al., 1998; Daum et al., 2000). Interestingly, among those are the APC/C components Apc1 (Tsg24) and Cdc27, which concentrate at kinetochores during mitosis. Considering our results, which strongly suggest that BubR1 interacts closely with 3F3/2 phosphoproteins at kinetochores, and the recent evidence showing BubR1 interaction with the APC/C, it is possible that at unattached

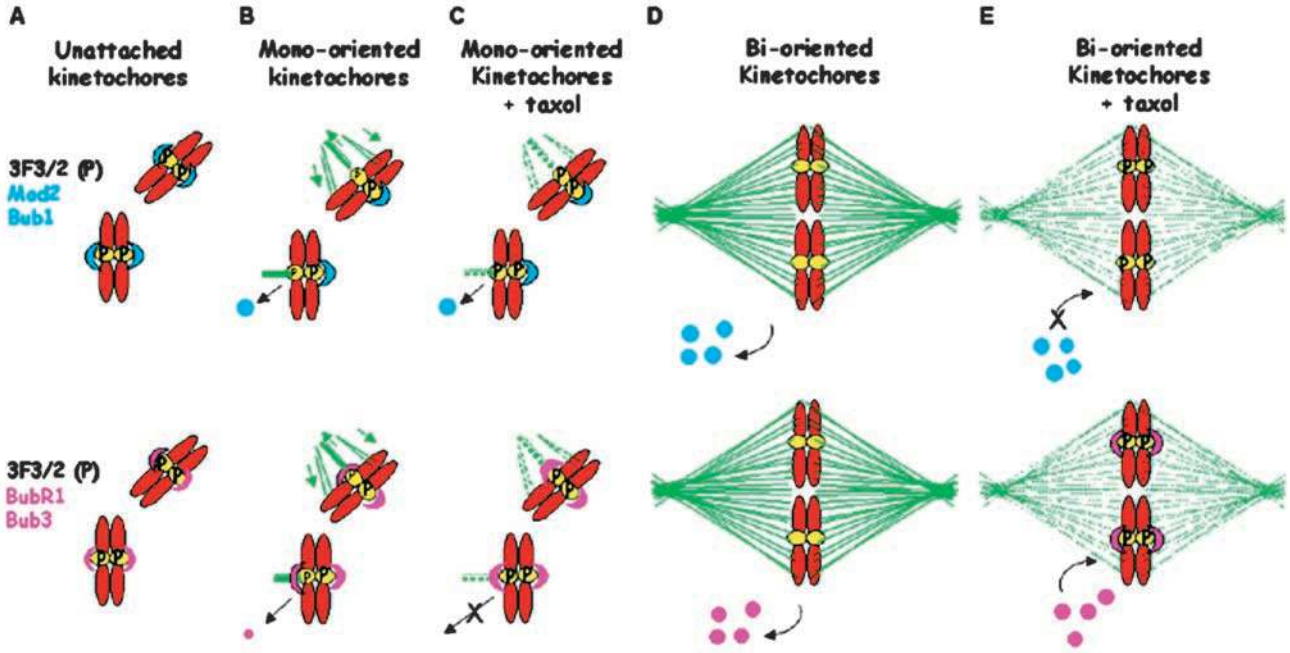

Fig. 10. Kinetochore localization of spindle checkpoint proteins is differentially regulated by spindle assembly events. (A) The conditions underlying binding to unattached kinetochores are similar for all proteins and require phosphorylated kinetochores. (B) Once chromosomes become mono-oriented, the attached kinetochore retains little or no Mad2 and Bub1, generating an asymmetrical localization pattern. BubR1 and Bub3 levels are only slightly asymmetrical with the attached kinetochore exhibiting reduced staining because of some tension generated from anti-poleward forces (arrows). (C) Under reduced tension from taxol treatment mono-oriented chromosomes show symmetrical BubR1/Bub3 staining while Mad2/Bub1 labelling remains asymmetrical. (D) When bipolar attachment is achieved, Mad2/Bub1 no longer localize to kinetochores, 3F3/2 epitopes are dephosphorylated because of tension and BubR1 and Bub3 are released. (E) Disruption of tension with taxol leads to recruitment of BubR1 and Bub3 to kinetochores because 3F3/2 epitopes become rephosphorylated. However, Mad2 and Bub1 cannot accumulate at kinetochores because of microtubule occupancy.

kinetochores, active kinases might catalyse phosphorylations that indirectly inhibit APC/C activity by enhancing the binding of BubR1 to the APC/C. Furthermore, we believe that BubR1 is not a 3F3/2 kinase, as *Drosophila bubR1* null mutants exhibit 3F3/2 staining (Basu et al., 1999). Curiously, *bubR1* mutant cells enter anaphase precociously and with strong 3F3/2 labelling at the kinetochores. This supports BubR1 as being a component of the spindle checkpoint pathway that monitors tension-sensitive kinetochore phosphorylation. When BubR1 is absent, cells can override an arrest that would be otherwise induced by the presence of phosphorylated kinetochores.

Alternatively, tension might inactivate the spindle checkpoint indirectly. Recent genetic work in budding yeast suggests that the Aurora kinase Ipl1 plays an important role in tension-dependent spindle assembly checkpoint signalling (Biggins and Murray, 2001). Aurora/Ipl1 is required for the spindle checkpoint activity induced by the absence of tension but not for the one induced by microtubule depolymerization. In addition, Aurora/Ipl1 was demonstrated to be critical for reorienting monopolar-attached sister chromatids whose kinetochores are not under tension so that they become attached to microtubules from opposite poles (Tanaka et al., 2002). The signal generated by lack of tension might therefore induce the release of microtubules from the syntelically attached sister kinetochores to allow the amphitelic reattachment. Accordingly, loss of tension indirectly maintains spindle checkpoint signalling by generating loss of microtubule occupancy, which is then sensed by Mad2. Recent results have shown that, in higher eukaryotes, Aurora B activity is also required to correct syntelic attachments and to activate the spindle checkpoint in the absence of tension (Ditchfield et al., 2003; Hauf et al., 2003). Furthermore, inhibition of Aurora B function in nocodazole-treated cells was shown to compromise kinetochore localization of the spindle checkpoint protein BubR1 but not Mad2. These results would be fully consistent with our observations if Aurora B/Ipl1 was the kinase that phosphorylates the 3F3/2 epitopes at kinetochores lacking tension.

We would like to thank G. Gorbsky for providing the 3F3/2 antibody and G. Karpen for providing the anti-CID antibody. We also thank M. Costa and N. Ribeiro for helpful support in cell culture procedures, and all the members of the lab for comments and suggestions. Work in the laboratory of C.E.S. is financed by grants from the Fundação para a Ciência e Tecnologia of Portugal and in part by the European Community's Human Potential Programme HPRNT-CT-2002-00260.

References

Amon, A. (1999). The spindle checkpoint. *Curr. Opin. Genet. Dev.* **9**, 69-75.

Basu, J., Bousbaa, H., Logarinho, E., Li, Z., Williams, B. C., Lopes, C., Sunkel, C. E. and Goldberg, M. L. (1999). Mutations in the essential spindle checkpoint gene *bub1* cause chromosome missegregation and fail to block apoptosis in *Drosophila*. *J. Cell Biol.* **146**, 13-28.

Basu, J., Logarinho, E., Herrmann, S., Bousbaa, H., Li, Z., Chan, G. K. T., Yen, T. J. and Sunkel, C. E. (1998). Localization of the *Drosophila*

checkpoint control protein Bub3 to the kinetochores requires Bub1 but not Zw10 or Rod. *Chromosoma* **107**, 376-385.

Biggins, S. and Murray, A. W. (2001). The budding yeast protein kinase Ipl1/Aurora allows the absence of tension to activate the spindle checkpoint. *Genes Dev*. **15**, 3118-3129.

Blower, M. D., Sullivan, B. A. and Karpen, G. H. (2001). Conserved organization of centromeric chromatin in flies and humans. *Dev*. *Cell* **2**, 319-330.

Bousbaa, H., Correia, L., Gorbsky, G. J. and Sunkel, C. E. (1997). Mitotic phosphoepitopes are expressed in Kc cells, neuroblasts and isolated chromosomes of *Drosophila melanogaster. J. Cell Sci.* **110**, 1979-1988.

Campbell, M. S. and Gorbsky, G. J. (1995). Microinjection of mitotic cells with the 3F3/2 anti-phosphoepitope antibody delays the onset of anaphase. *J. Cell Biol.* **129**, 1195-1204.

Campbell, M. S., Gersch, M. S., Daum J. and Gorbsky, G. J. (1995). Bound and soluble kinases can regenerate the 3F3/2 mitotic checkpoint phosphorylation at kinetochores in lysed cells. *Mol. Biol. Cell* **6 Supplement** 6a (abstr).

Chan, G. K. T., Jablonski, S. A., Sudakin, V., Hittle, J. C. and Yen, T. J. (1999). Human BUBR1 is a mitotic checkpoint kinase that monitors CENP-E functions at kinetochores and binds the cyclosome/APC. *J. Cell Biol.* **146**, 941-954.

Chen, R. H. (2002). BubR1 is essential for kinetochore localization of other spindle checkpoint proteins and its phosphorylation requires Mad1. *J. Cell Biol.* **158**, 487-496.

Cyert, M. S., Scherson, T. and Kirschner, M. W. (1988). Monoclonal antibodies specific for thiophosphorylated proteins recognize *Xenopus* MPF. *Dev. Biol.* **129**, 209-216.

Daum, J. R. and Gorbsky, G. J. (1998). Casein kinase II catalyzes a mitotic phosphorylation on threonine 1342 of human DNA topoisomerase IIalpha, which is recognized by the 3F3/2 phosphoepitope antibody. *J. Biol. Chem.* **273**, 30622-30629.

Daum, J. R., Tugendreich, S., Topper, L. M., Jorgens, P-M., Hoog, C., Hieter, P. and Gorbsky, G. J. (2000). The 3F3/2 anti-phosphoepitope antibody binds the mitotically phosphorylated anaphase-promoting complex/cyclosome. *Curr. Biol.* **10**, R850-R852.

Ditchfield, C., Johnson, V. L., Tighe, A., Ellston, R., Haworth, C., Mortlock, A., Keen, N. and Taylor, S. S. (2003). Aurora B couples chromosome alignment with anaphase by targeting BubR1, Mad2, and Cenp-E to kinetochores. *J. Cell Biol.* **161**, 267-280.

Fang, G. (2002). Checkpoint protein BubR1 acts synergistically with Mad2 to inhibit anaphase-promoting complex. *Mol. Biol. Cell* **13**, 755-766.

Fang, G., Yu, H. and Kirschner, M. W. (1998). The checkpoint protein MAD2 and the mitotic regulator CDC20 form a ternary complex with the anaphase-promoting complex to control anaphase initiation. *Genes Dev.* **12**, 1871-1883.

Gorbsky, G. and Ricketts, W. A. (1993). Differential expression of phosphoepitope at the kinetochores of moving chromosomes. *J. Cell Biol.* **122**, 1311-1321.

Gorbsky, G. J. (1995). Kinetochores, microtubules and the metaphase checkpoint. *Trends Cell Biol.* **5**, 143-148.

Gorbsky, G. J., Chen, R. H. and Murray, A. W. (1998). Microinjection of antibody to Mad2 protein into mammalian cells in mitosis induces premature anaphase. *J. Cell Biol.* **141**, 1193-1205.

Hanks, S. K. and Hunter, T. (1995). The eukaryotic protein kinase superfamily, kinase (catalytic) domain structure and classification. *FASEB J.* **9**, 576-596.

Hauf, S., Cole, R. W., LaTerra, S., Zimmer, C., Schnapp, G., Walter, R., Heckel, A., van Meel, J., Rieder, C. L. and Peters, J. M. (2003). The small molecule Hesperadin reveals a role for Aurora B in correcting kinetochore-microtubule attachment and in maintaining the spindle assembly checkpoint. *J. Cell Biol.* **161**, 281-294.

Hoffman, D. B., Pearson, C. G., Yen, T. J., Howell, B. J. and Salmon, E. D. (2001). Microtubule-dependent changes in assembly of microtubule motor proteins and mitotic spindle checkpoint proteins at PtK1 kinetochores. *Mol. Biol. Cell* **12**, 1995-2009.

Howell, B. J., Hoffman, D. B., Fang, G., Murray, A. W. and Salmon, E. D. (2000). Visualization of Mad2 dynamics at kinetochores, along spindle fibers, and at spindle poles in living cells. *J. Cell Biol.* **150**, 1233-1250.

Hoyt, M. A., Trotis, L. and Roberts, B. T. (1991). *S. cerevisiae* genes required for cell cycle arrest in response to loss of microtubule function. *Cell* **66**, 507-517.

Hwang, L. H., Lau, L. F., Smith, D. L., Mistrot, C. A., Hardwick, K. G., Hwang, E. S., Amon, A. and Murray, A. W. (1998). Budding yeast Cdc20: a target of the spindle checkpoint. *Science* **279**, 1041-1044.

Jablonski, S. A., Chan, G. K., Cooke, C. A., Earnshaw, W. C. and Yen, T. J. (1998). The hBUB1 and hBUBR1 kinases sequentially assemble onto kinetochores during prophase with hBUBR1 concentrating at the kinetochore plates in mitosis. *Chromosoma* **107**, 386-396.

Kim, S. H., Lin, D. P., Matsumoto, S., Kitazono, A. and Matsumoto, T. (1998). Fission yeast Slp1, an effector of the Mad2-dependent spindle checkpoint. *Science* **279**, 1045-1047.

King, J. M. and Nicklas, R. B. (2000). Tension on chromosomes increases the number of kinetochore microtubules but only within limits. *J. Cell Sci.* **113**, 3815-3823.

Li, R. and Murray, A. W. (1991). Feedback control of mitosis in budding yeast. *Cell* **66**, 519-531.

Li, X. and Nicklas, R. B. (1995). Mitotic forces control a cell-cycle checkpoint. *Nature* **373**, 630-632.

Li, X. and Nicklas, R. B. (1997). Tension-sensitive kinetochore phosphorylation and the chromosome distribution checkpoint in praying mantid spermatocytes. *J. Cell Sci.* **110**, 537-545.

Li, Y., Gorbea, C., Mahaffey, D., Rechsteiner, M. and Benezra, R. (1997). MAD2 associates with the cyclosome/anaphase-promoting complex and inhibits its activity. *Proc. Natl. Acad. Sci. USA* **94**, 12431-12436.

Logarinho, E. and Sunkel, C. E. (1998). The *Drosophila* kinase POLO localizes to multiple compartments of the mitotic apparatus and is required for the phosphorylation of MPM2 reactive epitopes. *J. Cell Sci.* **111**, 2897-2909.

Martinez-Exposito, M. J., Kaplan, K. B., Copeland, J. and Sorger, P. K. (1999). Retention of the Bub3 checkpoint protein on lagging chromosomes. *Proc. Natl. Acad. Sci. USA* **96**, 8493-8498.

McEwen, B. F., Heagle, A. B., Cassels, G. O., Buttle, K. F. and Rieder, C. L. (1997). Kinetochore fiber maturation in PtK1 cells and its implications for the mechanisms of chromosome congression and anaphase onset. *J. Cell Biol.* **137**, 1567-1580.

Musacchio, A. and Hardwick, K. G. (2002). The spindle checkpoint, structural insights into dynamic signalling. *Nat. Rev. Mol. Cell Biol.* **10**, 731-741.

Murray, A. W. (1994). Cell cycle checkpoints. *Curr. Opin. Cell Biol.* **6**, 872-876.

Murray, A. W. and Marks, D. (2001). Can sequencing shed light on cell cycling? *Nature* **409**, 844-846.

Nicklas, R. B. (1997). How cells get the right chromosomes. *Science* **275**, 632-637.

Nicklas, R. B. and Koch, C. A. (1969). Chromosome micromanipulation. 3. Spindle fiber tension and the reorientation of mal-oriented chromosomes. *J. Cell Biol.* **43**, 40-50.

Nicklas, R. B. and Ward, S. C. (1994). Elements of error correction in mitosis: microtubule capture, release and tension. *J. Cell Biol.* **126**, 1241-1253.

Nicklas, R. B., Campbell, M. S., Ward, S. C. and Gorbsky, G. J. (1998). Tension-sensitive kinetochore phosphorylation in vitro. *J. Cell Sci.* **111**, 3189-3196.

Nicklas, R. B., Ward, S. C. and Gorbsky, G. J. (1995). Kinetochore chemistry is sensitive to tension and may link mitotic forces to a cell cycle checkpoint. *J. Cell Biol.* **130**, 929-939.

Rieder, C. L., Cole, R. W., Khodjakov, A. and Sluder, G. (1995). The checkpoint delaying anaphase in response to chromosome monoorientation is mediated by an inhibitory signal produced by unattached kinetochores. *J. Cell Biol.* **130**, 941-948.

Rieder, C. L., Schultz, A., Cole, R. and Sluder, G. (1994). Anaphase onset in vertebrate somatic cells is controlled by a checkpoint that monitors sister kinetochore attachment to the spindle. *J. Cell Biol.* **127**, 1301-1310.

Shannon, K. B., Canman, J. C. and Salmon, E. D. (2002). Mad2 and BubR1 function in a single checkpoint pathway that responds to a loss of tension. *Mol. Biol. Cell* **10**, 3706-3719.

Sharp-Baker, H. and Chen, R. H. (2001). Spindle checkpoint protein Bub1 is required for kinetochore localization of Mad1, Mad2, Bub3, and CENP-E, independently of its kinase activity. *J. Cell Biol.* **153**, 1239-1250.

Skibbens, R. V. and Hieter, P. (1998). Kinetochores and the checkpoint mechanism that monitors for defects in the chromosome segregation machinery. *Annu. Rev. Genet.* **32**, 307-337.

Skoufias, D. A., Andreassen, P. R., Lacroix, F. B., Wilson, L. and Margolis, R. L. (2001). Mammalian Mad2 and Bub1/BubR1 recognize distinct spindle-attachment and kinetochore-tension checkpoints. *Proc. Natl. Acad. Sci. USA* **98**, 4492-4497.

Sudakin, V., Chan, G. K. and Yen, T. J. (2001). Checkpoint inhibition of the APC/C in HeLa cells is mediated by a complex of BUBR1, BUB3, CDC20, and MAD2. *J. Cell Biol.* **154**, 925-936.

Tanaka, T. U., Rachidi, N., Janke, C., Pereira, G., Galova, M., Schiebel, E., Stark, M. J. and Nasmyth, K. (2002). Evidence that the Ipl1-Sli15 (Aurora kinase-INCENP) complex promotes chromosome bi-orientation by altering kinetochore-spindle pole connections. *Cell* **108**, 317-329.

Tang, Z., Bharadwaj, R., Li, B. and Yu, H. (2001). Mad2-Independent inhibition of APCCdc20 by the mitotic checkpoint protein BubR1. *Dev. Cell* **1**, 227-237.

Taylor, S. S., Ha, E. and McKeon, F. (1998). The human homologue of Bub3 is required for kinetochore localization of Bub1 and Mad3/Bub1-related protein kinase. *J. Cell Biol.* **142**, 1-11.

Taylor, S. S. and McKeon, F. (1997). Kinetochore localization of murine Bub1 is required for normal mitotic timing and checkpoint response to spindle damage. *Cell* **89**, 727-735.

Taylor, S. S., Hussein, D., Wang, Y., Elderkin, S. and Morrow, C. J. (2001). Kinetochore localization and phosphorylation of the mitotic checkpoint components Bub1 and BubR1 are differentially regulated by spindle events in human cells. *J. Cell Sci.* **114**, 4385-4395.

Waters, J. C., Chen, R.-H., Murray, A. W. and Salmon, E. D. (1998). Localization of Mad2 to kinetochores depends on microtubule attachment, not tension. *J. Cell Biol.* **141**, 1181-1191.

Waters, J. C., Chen, R.-H., Murray, A. W., Gorbsky, G. J., Salmon, E. D. and Nicklas, R. B. (1999). Mad2 binding by phosphorylated kinetochores links error detection and checkpoint action in mitosis. *Curr. Biol.* **9**, 649-652.

Waters, J. C., Skibbens, R. V. and Salmon, E. D. (1996). Oscillating mitotic newt lung cell kinetochores are, on average, under tension and rarely push. *J. Cell Sci.* **109**, 2823-2831.

Yu, H. G., Muszynski, M. G. and Dawe, R. K. (1999). The maize homolog of the cell cycle checkpoint protein MAD2 reveals kinetochore substructure and contrasting mitotic and meiotic localization patterns. *J. Cell Biol.* **145**, 425-435.

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

Zhou, J., Yao, J. and Joshi, H. C. (2002b). Attachment and tension in the spindle assembly checkpoint. *J. Cell Sci.* **115**, 3547-3555.