Bub1 and aurora B cooperate to maintain BubR1-mediated inhibition of APC/C^{Cdc20}

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Accepted 5 May 2005
Journal of Cell Science 118, 3639-3652 Published by The Company of Biologists 2005
doi:10.1242/jcs.02487

Summary

The spindle checkpoint maintains genome stability by inhibiting Cdc20-mediated activation of the anaphase promoting complex/cyclosome (APC/C) until all the chromosomes correctly align on the microtubule spindle apparatus via their kinetochores. BubR1, an essential component of this checkpoint, localises to kinetochores and its kinase activity is regulated by the kinesin-related motor protein Cenp-E. BubR1 also inhibits APC/C^{Cdc20} in vitro, thus providing a molecular link between kinetochore-microtubule interactions and the proteolytic machinery that regulates mitotic progression. Several other protein kinases, including Bub1 and members of the Ipl1/aurora family, also regulate anaphase onset. However, in human somatic cells Bub1 and aurora B kinase activity do not appear to be essential for spindle checkpoint function. Specifically, when Bub1 is inhibited by RNA interference, or aurora kinase activity is inhibited with the small molecule ZM447439, cells arrest transiently in mitosis following exposure to spindle toxins that prevent microtubule polymerisation. Here, we show that mitotic arrest of Bub1-deficient cells is dependent on aurora kinase activity, and vice versa. We suggest therefore that the checkpoint is composed of two arms, one dependent on Bub1, the other on aurora B. Analysis of BubR1 complexes suggests that both of these arms converge on the mitotic checkpoint complex (MCC), which includes BubR1, Bub3, Mad2 and Cdc20. Although it is known that MCC components can bind and inhibit the APC/C, we show here for the first time that the binding of the MCC to the APC/C is dependent on an active checkpoint signal. Furthermore, we show that both Bub1 and aurora kinase activity are required to promote binding of the MCC to the APC/C. These observations provide a simple explanation of why BubR1 and Mad2 are essential for checkpoint function following spindle destruction, yet Bub1 and aurora B kinase activity are not. Taken together with other observations, we suggest that these two arms respond to different spindle cues: whereas the Bub1 arm monitors kinetochore-microtubule attachment, the aurora B arm monitors biorientation. This bifurcation in the signalling mechanism may help explain why many tumour cells mount a robust checkpoint response following spindle damage, despite exhibiting chromosome instability.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/118/16/3639/DC1

Key words: Mitosis, Spindle checkpoint, Kinetochore, Aneuploidy

Introduction

Kinetochores are essential for successful chromosome segregation in eukaryotes (Biggins and Walczak, 2003; Cleveland et al., 2003; Hauf and Watanabe, 2004; Maiato et al., 2004). These large complex protein structures not only attach and move the chromosomes on the microtubule spindle apparatus, but they also generate the 'wait' signal, which prevents anaphase until all the chromosomes are correctly aligned on the spindle (Campbell and Gorbsky, 1995; Li and Nicklas, 1995; Rieder et al., 1995). This signal forms part of the spindle checkpoint mechanism, a highly conserved cell cycle checkpoint which ensures accurate chromosome segregation (Musacchio and Hardwick, 2002; Taylor et al., 2004). Studies in several model systems have identified a number of spindle checkpoint components including three protein kinases, Bub1, BubR1 and Mps1, as well as several non-enzymatic proteins such as Bub3 and Mad1-3 (Hoyt et al., 1991; Li and Murray, 1991; Weiss and Winey, 1996). Upon entry into mitosis, these proteins assemble at the kinetochore in a defined order that, at least in part, appears to reflect an underlying dependency of known protein-protein interactions (Jablonski et al., 1998; Johnson et al., 2004; Vigneron et al., 2004). The aurora B kinase, a component of the chromosome passenger complex (Vagnarelli and Earnshaw, 2004), has also been implicated in spindle checkpoint function (Kallio et al., 2002; Murata-Hori and Wang, 2002). However, whether this is a secondary consequence of its role in kinetochore assembly and/or regulating kinetochore-microtubule attachments is not clear.

Prevailing models propose that the assembly of the checkpoint signalling complex at kinetochores then catalyses the formation of a diffusible inhibitor (Shah and Cleveland, 2000; Musacchio and Hardwick, 2002; Cleveland et al., 2003; Taylor et al., 2004). This in turn then inhibits the anaphase promoting complex/cyclosome (APC/C), an E3 ubiquitin ligase that targets anaphase inhibitors and mitotic cyclins for

proteasome-mediated degradation (Peters, 2002; Yu, 2002; Murray, 2004). BubR1 and Mad2 can bind and inhibit the APC/C, explaining how an active checkpoint signal might prevent anaphase (Tang et al., 2001; Fang, 2002). Indeed, a mitotic checkpoint complex (MCC) consisting of BubR1, Bub3, Mad2 and Cdc20 has been purified from HeLa cells and shown to be a potent APC/C inhibitor (Sudakin et al., 2001). In yeast, however, the MCC exists in the absence of kinetochores (Fraschini et al., 2001). In addition, when purified from interphase cells and assayed in vitro, the MCC inhibits the APC/C (Sudakin et al., 2001). Thus, the relationship of the MCC with kinetochores is unclear. Indeed, it has recently been suggested that two MCC components, BubR1 and Mad2, are also part of a cytosolic timer mechanism that inhibits the APC/C during the early stages of mitosis (Meraldi et al., 2004). This timer may prevent premature APC/C activation while kinetochores are maturating, prior to them becoming competent to generate the 'anaphase wait' signal.

BubR1's role is unlikely to be restricted to MCC and/or timer function. Firstly, we recently discovered that BubR1 is required for chromosome alignment (Ditchfield et al., 2003), an observation subsequently confirmed by others (Lampson and Kapoor, 2004). BubR1 binds the kinesin-related motor protein Cenp-E (Chan et al., 1999; Yao et al., 2000) and is required to target Cenp-E to kinetochores (Johnson et al., 2004). Cenp-E binding stimulates the kinase activity of BubR1 and, importantly, when Cenp-E is bound by antibodies that mimic microtubule binding, BubR1's kinase activity is downregulated (Mao et al., 2003). BubR1's kinase activity is required for checkpoint signalling, consistent with the notion that Cenp-E-mediated regulation of BubR1 kinase activity controls the checkpoint. However, immunodepletion and add-back experiments in *Xenopus* egg extracts indicates that only ~20% of the BubR1 in the extract needs to be catalytically active (Mao et al., 2003). Thus, BubR1 may play three roles – one in mediating correct kinetochore-microtubule interactions, and two roles in the checkpoint: a catalytic role at the kinetochore that is Cenp-E dependent, and a kinetochore-independent role that relies on its stoichiometric presence, not its enzymatic activity.

An equally elaborate role is emerging for Bub1. Biochemical studies indicate that Bub1 phosphorylates Cdc20 and importantly, a non-phosphorylatable Cdc20 can activate the APC/C in vitro but is insensitive to Bub1-mediated inhibition (Chung and Chen, 2003; Tang et al., 2004a). However, the general significance of this is unclear: following immunodepletion of Bub1 from *Xenopus* egg extracts, checkpoint function can be restored by adding back a recombinant kinase mutant (Sharp-Baker and Chen, 2001). Furthermore, in budding yeast, the N-terminal 608 amino acids of Bub1 can perform all the checkpoint functions of the wild-type protein despite completely lacking the kinase domain (Warren et al., 2002). Is Bub1's kinase activity therefore required for something else? Interestingly, chromosome loss rates in Bub1-deficient yeast strains are 2-3 times higher than that in Mad1/2 mutants (Warren et al., 2002) suggesting that Bub1 may play another role in chromosome segregation. Indeed, we recently discovered that Bub1 is required for chromosome alignment in human cells (Johnson et al., 2004). Furthermore, in *S. pombe*, Bub1 targets cohesion factors to centromeres (Bernard et al., 2001; Yamaguchi et al., 2003; Kitajima et al., 2004), thus ensuring that sister chromatids segregate to the same pole in meiosis I. Strikingly, Bub1 also appears to protect centromeric cohesion in human somatic cells (Tang et al., 2004b).

Thus, although Bub1 has been implicated in checkpoint function and maintaining centromeric cohesion in several systems, many questions still remain. Indeed, rather surprisingly, Bub1-deficient human cells appear to have a robust checkpoint: following RNAi-mediated repression of Bub1, human tissue culture cells arrest transiently in mitosis when exposed to spindle toxins (Johnson et al., 2004). Here, we show that the ability of Bub1-deficient cells to maintain mitotic arrest following spindle damage is dependent on aurora kinase activity. This observation follows on from our previous report characterising a novel aurora kinase inhibitor, ZM447439 (Ditchfield et al., 2003). ZM447439 is a quinazoline derivative which, in in vitro kinase assays, inhibits aurora B with an IC_{50} ~100 nM. [Note that although ZM447439 inhibits both aurora A and B in vitro, the phenotypes it induces in cell based assays appear to be largely due to inhibition of aurora B (Ditchfield et al., 2003; Keen and Taylor, 2004). Therefore, we will refer to ZM447439 as an aurora B inhibitor. However, we will revisit this issue in the Discussion and consider the possible effects on aurora A]. Cells exposed to ZM447439 enter mitosis normally but then exit mitosis without aligning their chromosomes (Ditchfield et al., 2003). Analysis of another aurora kinase inhibitor, hesperadin, indicates that aurora B kinase activity is required to resolve syntelic orientations (Hauf et al., 2003). The fact that cells treated with ZM447439 or hesperadin exit mitosis prematurely indicates that the checkpoint is not fully functional. Indeed, kinetochore localisation of several checkpoint proteins, including BubR1 and Mad2, is reduced to ~10% in the presence of ZM447439 (Ditchfield et al., 2003). Furthermore, ZM447439 and hesperadin-treated cells fail to undergo mitotic arrest when microtubules are stabilised with taxol, or when centrosome separation is inhibited by the Eg5 inhibitor, monastrol (Ditchfield et al., 2003; Hauf et al., 2003). However, in the absence of aurora B kinase activity, cells do undergo mitotic arrest when microtubule polymerisation is inhibited with nocodazole. Although there are several explanations for this phenomenon (Ditchfield et al., 2003; Hauf et al., 2003), we show here that the ability of ZM447439-treated cells to maintain mitotic arrest following spindle destruction is dependent on Bub1. In other words, aurora kinase activity and Bub1 cooperate to maintain the spindle checkpoint. We also show that they do this by promoting the association of BubR1 with the APC/C.

Materials and Methods

Drug treatments

Small molecules were all used as described previously (Taylor et al., 2001; Ditchfield et al., 2003). Briefly, in all cases the aurora kinase inhibitor ZM447439 was used at a final concentration of 2 μM; the spindle toxins nocodazole and taxol were used at 0.2 μM and 10 μM, respectively; the proteasome inhibitor MG132 was used at 20 μM.

Molecular cell biology

Cell lines, culture conditions and drugs treatments were all as described (Taylor et al., 2001; Ditchfield et al., 2003). Transfection of

siRNA duplexes was basically as described (Johnson et al., 2004), except that DLD-1 cells were transfected in OptiMEM media (Invitrogen) lacking serum. Western blotting and immunofluorescence microscopy was also carried out essentially as described (Taylor et al., 2001; Johnson et al., 2004). A sheep polyclonal antibody against Bub3, a kind gift from K. Hardwick (ICMB, Edinburgh), was used at 1:750. Rabbit polyclonal antibodies against Cdc20 and APC7, kind gifts from Jan-Michael Peters (IMP, Vienna), were both used at 1:1000. A commercial anti-APC7 antibody (Abcam) was used at 1:500.

Time-lapse microscopy

An open reading frame encoding histone H2B was generated by RT-PCR amplification (Invitrogen) of mRNA prepared from HeLa cells and cloned as a GFP-tagged fusion into a pcDNA-5 based expression vector (Invitrogen). This plasmid was then integrated into the DLD-1 genome at a pre-integrated FRT recombination site as previously described (Tighe et al., 2004). DLD-1 cells expressing the GFP-histone fusion protein were then cultured and transfected with siRNA duplexes as described previously (Johnson et al., 2004). For time-lapse analysis, DLD-1 GFP-histone cells were seeded in 30 mm glass bottomed Petri dishes (MatTek Co), and then transferred to the microscope stage. Microscopy was performed on a manual Axiovert 200 (Zeiss) equipped with an environmental control chamber (Solent Scientific), which maintained the cells at 37°C and in a humidified stream of 5% CO_2, 95% air. Shutters and filter wheels were driven by Metamorph software (Universal Imaging) and images were taken every 30 seconds using a CoolSNAP HQ camera (Photometrics). When cultures were treated with nocodazole, images were taken every 2 minutes. Individual TIFF files were then imported into Photoshop (Adobe) for printing, or QuickTime (Apple) for movies. Nuclear envelope breakdown was judged as the point when the prophase chromatin lost a smooth, linear periphery, and the time of anaphase onset was judged to be first frame where coordinated pole wards movement was observed.

Analysis of BubR1 complexes.

HeLa cells were harvested, and then lysed in lysis buffer [0.1% Triton X-100, 100 mM NaCl, 10 mM Tris HCl (pH 7.4), 1 mM EDTA, 1 mM EGTA, 10 mM β-glycerophosphate, 1 mM DTT, 0.2 mM PMSF and a protease inhibitor cocktail] on ice for 30 minutes. Following centrifugation at 100,000 ***g*** for 30 minutes at 4°C, soluble BubR1 complexes were resolved by FPLC. For analytical separations, complexes were resolved using a SMART FPLC instrument (Amersham Biosciences) with either a Superose 6 PC 3.2/30 or a Mono Q PC 1.6/5 column. For preparative separations, proteins were resolved using an AKTA Basic instrument (Amersham Biosciences) and a Source 15 Q HR 16/10 column. Gel filtration columns were eluted with lysis buffer without the protease inhibitor cocktail. Ion exchange columns were eluted with a linear NaCl gradient (0.1-1.0 M) in lysis buffer without protease inhibitors. To affinity purify BubR1 complexes, sheep polyclonal anti-BubR1 antibodies, SBR1.1 (Taylor et al., 2001), were coupled to protein G Sepharose beads (Amersham Biosciences) using dimethyl pimelimidate according to standard procedures. Preimmune IgG antibodies were used as a negative control. Pooled peak fractions from preparative ion exchange separation were then incubated with the antibody beads for 1 hour at 4°C with end-over-end rotation. Following packing into a disposable column, the beads were washed with 10 volumes of lysis buffer. Bound proteins were then eluted with 100 mM glycine (pH 2.7) and analyzed by western blotting. Quantitative western blotting was done basically as described above using SBR1.1 but with an IRDye^{800}-conjugated donkey anti-sheep secondary antibody (Rockland). Bound secondary antibodies were then detected using a fluorescence infrared imager (Odyssey, LI-COR).

Results

RNAi reduces Bub1 levels to ~2% in ~70% of cells

To determine the role of Bub1 in spindle checkpoint function, we analyzed cells by time-lapse microscopy following RNAi-mediated repression of Bub1. Because we could not determine a priori the levels of Bub1 protein during the time-lapse analysis, we first evaluated the extent of Bub1 repression. Immunoblot analysis of HeLa cells indicates that, across a population, Bub1 was reduced to ~20% (Fig. 1A). Immunofluorescence analysis of DLD-1 cells revealed that the residual Bub1 protein was largely due to a relatively small number of non-transfected cells. Indeed, ~70% of the cells appeared almost completely depleted of Bub1 (Fig. 1B). Quantitation of individual kinetochores in these cells showed that Bub1 levels were reduced to ~2% of that in control cells (Table 1). In ~18% of the cells, Bub1 levels were similar to controls, suggesting that these cells were untransfected. In the remaining ~12%, Bub1 was readily detectable but reduced to about 20-50%, suggesting that these cells were transfected but only partially repressed. Thus, in population experiments, up to 70% of cells would be expected to behave like Bub1 nulls, while at least 30% of cells should behave as Bub1 wild-type or hypomorphs. (Note that although transfection/repression levels vary from experiment to experiment, HeLa and DLD-1 cells do, in general, behave similarly.)

Bub1-deficient cells enter anaphase with unaligned chromosomes

To determine the effect of repressing Bub1 by RNAi, we analyzed DLD-1 cells expressing a GFP-histone H2B fusion

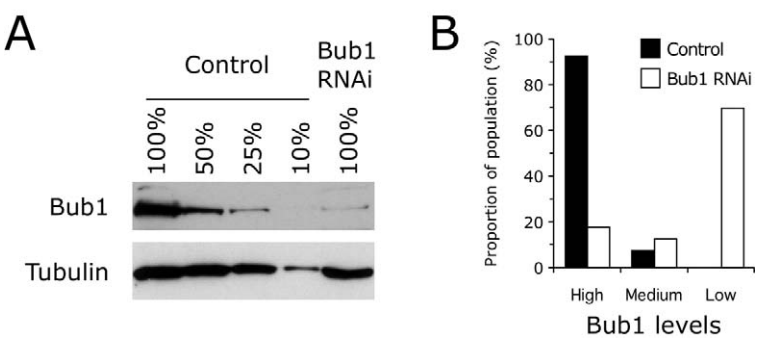

Fig. 1. RNAi reduces Bub1 levels to ~2% in ~70% of cells. HeLa and DLD-1 cells were transfected with either control or Bub1 siRNA duplexes then analyzed 48 hours later. (A) Blot of HeLa cell lysates showing that, across a population, RNAi reduces Bub1 levels to 10-25%. In order to quantitate the level of repression, the lysate from the Bub1 RNAi culture was analyzed side by side with a lysate from a control RNAi culture diluted in lysis buffer to 50, 25 and 10% of its original concentration. (B) Quantitation of immunofluorescence analysis in DLD-1 cells demonstrating that while ~70% of the mitotic cells in Bub1-RNAi cultures show low levels of Bub1 expression, ~18% appear untransfected and ~12% appear partially repressed.

Table 1. Normalised Bub1/ACA, BubR1/ACA and Mad2/ACA ratios in the presence of nocodazole following Bub1 RNAi and/or exposure to ZM447439

	Control +	RNAi	ZM	RNAi + ZM	Control –
Bub1	100.0±6.8 (70)	2.2±0.1 (68)	5.0±0.7 (62)	2.6±0.8 (67)	68.7±5.1 (64)
BubR1	100.0±5.5 (70)	13.9±1.0 (68)	8.5±0.8 (62)	9.5±2.7 (67)	2.5±0.3 (64)
Mad2	100.0±4.6 (64)	36.8±3.8 (62)	17.0±2.0 (61)	15.8±2.4 (61)	5.3±1.0 (64)

Values represent mean±s.e.m. and the number of kinetochore pairs measured. See Fig. 3 for associated graph and legend.

protein by time-lapse microscopy (Fig. 2). To provide appropriate negative and positive controls, we analyzed cells transfected with siRNA duplexes designed to target lamin B1 and BubR1, respectively. Following nuclear envelope breakdown (NEB), chromosomes in negative control cells typically formed prometaphase horseshoe shapes, and then aligned at the metaphase plate within 10-20 minutes. After a delay of ~10 minutes, anaphase initiated with the chromosomes separating into two clear masses (Fig. 2A; supplementary material Movie 1). Note that our previous analysis of fixed DLD-1 cells (Johnson et al., 2004) indicates that centrosomes are relatively close together in prometaphase horseshoes, suggesting that bipolar spindles do not form until late prometaphase (supplementary material Fig. S1).

Consistent with previous observations (Meraldi et al., 2004), when we repressed BubR1, progression through mitosis was accelerated, with anaphase often occurring prematurely. For example, Fig. 2B shows the appearance of a horseshoe configuration by ~10 minutes but then, after ~20 minutes, all the chromosomes move towards the centre of the horseshoe, indicating that anaphase initiated prior to bipolar spindle formation (Fig. 2B; supplementary material Movie 2). Note that all the chromatids move polewards, suggesting that both kinetochores on each chromosome were attached to the spindle poles at anaphase onset. A quantitative analysis (Fig. 3; Table 2) indicates that in BubR1-RNAi cultures the average time from NEB to anaphase was reduced to ~20 minutes, compared with ~32 minutes in controls. Furthermore, 27 out of 48 BubR1-RNAi cells entered anaphase with unaligned chromosomes (Table 2).

Time-lapse analysis of Bub1-RNAi cultures showed a very different phenotype. In particular, chromosome alignment and anaphase onset were frequently delayed, consistent with the chromosome alignment defect we described previously based on analyzing fixed cells (Johnson et al., 2004). For example, the cell shown in Fig. 2C took at least ~30 minutes to assemble a distinct metaphase plate. Even then, several chromosomes remain unaligned and, during the subsequent ~30 minutes, these chromosomes still failed to align, despite exhibiting oscillatory movements (supplementary material

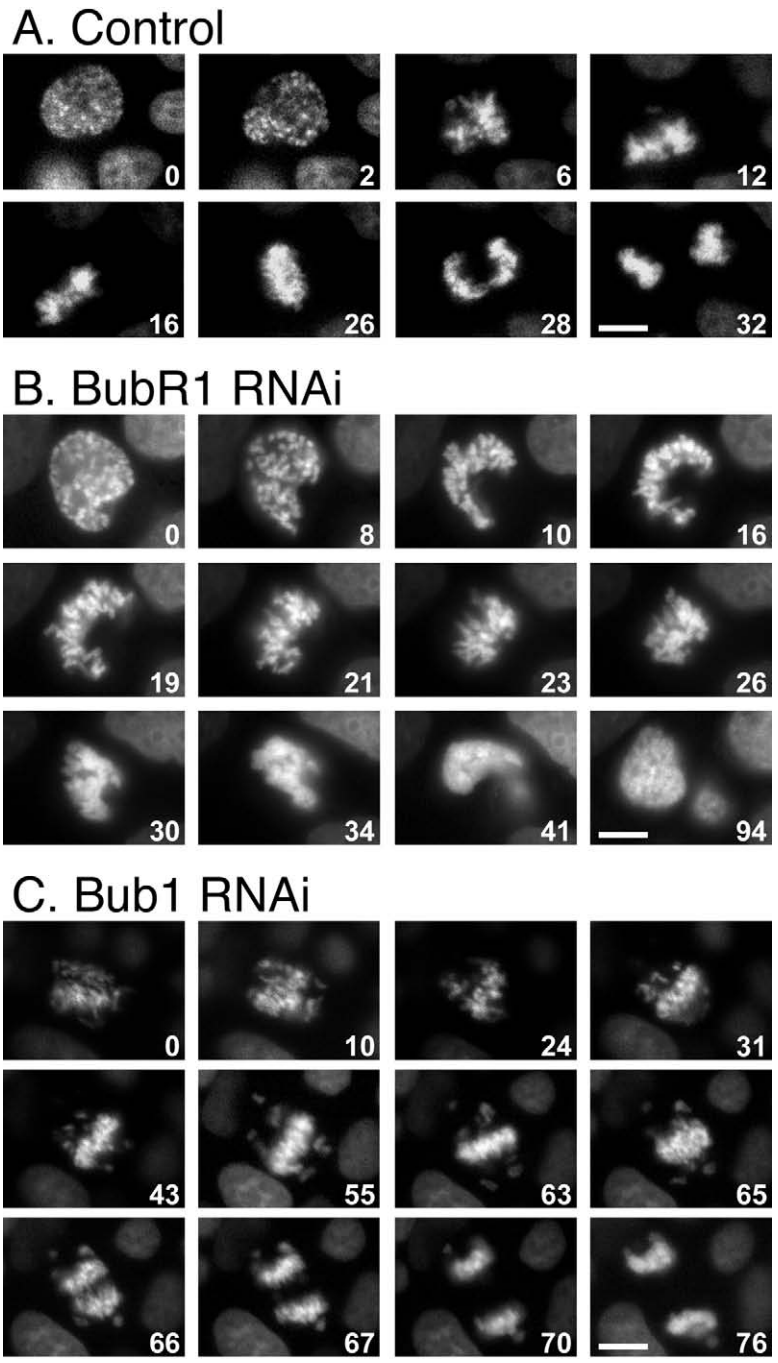

Fig. 2. Bub1-RNAi cells enter anaphase with unaligned chromosomes. DLD-1 cells expressing a GFP-histone fusion protein were transfected with siRNA duplexes designed to target (A) lamin B1, (B) BubR1 or (C) Bub1. The panels represent images taken from time-lapse sequences with the time shown in minutes. Bars, 5 μm. Note that the cell in panel C was already in prometaphase at the start of the time-lapse sequence, so the values underestimate the time spent in mitosis.

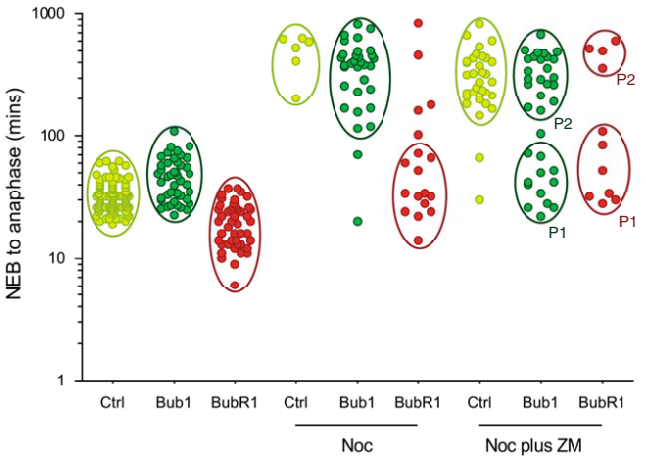

Fig. 3. Bub1-RNAi cells arrest in mitosis when the spindle is destroyed. DLD-1 cells expressing a GFP-histone fusion protein were transfected with siRNA duplexes against lamin B1 (yellow), Bub1 (green) and BubR1 (red), and then analyzed by time-lapse microscopy either in the absence of drugs, or in the presence of nocodazole or nocodazole plus ZM447439. The time from NEB to anaphase onset was then determined or, in the presence of nocodazole, the time from NEB to mitotic exit was measured. Note that each symbol on the scatter plot represents a single cell and the time is on a log scale. P1 and P2 are sub-populations; see Table 2 and text for more details. Importantly, in the presence of nocodazole, only 2 out of 32 Bub1-RNAi cells (6%) exited mitosis within 100 minutes. However, in the presence of nocodazole plus ZM447439, 12 out of 35 Bub1-RNAi cells (34%) exited mitosis within 100 minutes.

Movie 3). Significantly, this cell then entered anaphase without aligning these last few chromosomes (Fig. 2C). This suggests that repression of Bub1 is having two effects: firstly, chromosome alignment is inhibited, which delays anaphase; secondly, because anaphase then initiates in the presence of unaligned chromosomes, the spindle checkpoint is compromised. Indeed, the average time from NEB to anaphase increased from ~32 minutes to ~47 minutes following repression of Bub1 (Fig. 3; Table 2) but, whereas not a single control cell entered anaphase with unaligned chromosomes, 9 out of 43 Bub1-RNAi cells entered anaphase with one or more unaligned chromosomes (Table 2).

Bub1-deficient cells arrest in mitosis when spindle assembly is inhibited

The analysis described above indicates that repression of Bub1 by RNAi does indeed compromise the spindle checkpoint: cells enter anaphase with unaligned chromosomes. However, our previous analysis of fixed cells indicated that Bub1-RNAi cells arrest in mitosis when spindle assembly is inhibited (Johnson et al., 2004), indicating that under these conditions the checkpoint is intact. To confirm this, we analyzed RNAi cultures by time-lapse following exposure to nocodazole.

Whereas control cells remained in mitosis for several hours when cultured in nocodazole, many cells in the BubR1-RNAi culture exited mitosis rapidly (Fig. 3), with 52% exiting within 1 hour. Significantly, however, the vast majority of cells in the nocodazole-treated Bub1-RNAi culture remained in mitosis for prolonged periods. Indeed, the average time that Bub1-RNAi

Table 2. Time taken from NEB to anaphase in control, Bub1 or BubR1-RNAi cultures exposed to no drugs, nocodazole or nocodazole plus ZM447439

Drugs		RNAi	Median	Mean	s.e.m.	*n*	PA
None		Control	30	32	1	128	0
		Bub1	45	47	3	43	9
		BubR1	20	20	1	48	27
Noc		Control	558	497	68	6	–
		Bub1	388	377	34	32	–
		BubR1	34	119	46	19	–
Noc + ZM		Control	297	325	30	32	–
		Bub1	264	244	31	35	–
		BubR1	84	213	70	11	–
Noc + ZM	P1	Bub1	41	42	5	12	–
		BubR1	34	53	12	7	–
	P2	Bub1	350	360	28	22	–
		BubR1	508	495	50	4	–

The median and mean values represent the average time in minutes; s.e.m. represents the standard error of the mean; (*n*) is the number of cells analyzed; and PA is the number of cells that enter anaphase with unaligned chromosomes. P1 and P2 are the subpopulations shown in Fig. 3.

cells remained in mitosis was ~6 hours (Fig. 3; Table 2), confirming that, when the spindle is destroyed, Bub1-deficient cells can mount a robust checkpoint response. Clearly therefore, the effect of repressing Bub1 is different from that observed following inhibition of BubR1. Inhibition of BubR1 accelerates mitotic progression under all conditions (Chan et al., 1999; Shannon et al., 2002; Meraldi et al., 2004), indicating that BubR1 is essential for spindle checkpoint function. By contrast, repression of Bub1 only has a partial effect on the checkpoint: although Bub1-deficient cells delay anaphase onset when one or more chromosomes are aligned, they do enter anaphase with unaligned chromosomes. When the spindle is destroyed, however, Bub1-deficient cells sustain a mitotic arrest. This rather paradoxical phenotype is similar to that observed following inhibition of Cenp-E (Weaver et al., 2003). Although there are several ways to explain this observation, one possibility is that Bub1 is partially redundant in terms of checkpoint signalling.

Bub1 and aurora cooperate to maintain the spindle checkpoint

Aurora B has been implicated in spindle checkpoint function (Kallio et al., 2002; Murata-Hori and Wang, 2002). Consistently, when aurora kinase activity is inhibited with small molecules, the spindle checkpoint is compromised: cells enter anaphase prematurely and cannot sustain mitotic arrest in response to taxol or monastrol (Ditchfield et al., 2003; Hauf et al., 2003). However, aurora kinase-deficient cells do undergo mitotic arrest when exposed to nocodazole. Taken together with the results described above, we therefore speculated that Bub1 and aurora B may be partially redundant with respect to each other following nocodazole-mediated spindle destruction. To test this possibility, we used RNAi and ZM447439 to simultaneously inhibit Bub1 and aurora B activity respectively.

Consistent with the observations outlined above, Bub1 repressed cells accumulated in mitosis upon exposure to nocodazole (Fig. 4A). Likewise, when aurora kinase activity

was inhibited in control cells by exposure to ZM447439, they arrested in the presence of nocodazole. However, when aurora kinase activity was inhibited in Bub1-deficient cells, the accumulation of cells in mitosis following nocodazole-exposure was severely reduced, consistent with a defective checkpoint response (Fig. 4A).

To confirm that this effect was due to accelerated mitotic exit, control and Bub1-deficient cells were synchronized in mitosis, and then released into nocodazole plus and minus ZM447439. Bub1-deficient cells remained arrested in mitosis when released into nocodazole alone (Fig. 4B). Likewise, control cells released into ZM447439 plus nocodazole only exited mitosis slowly. Significantly, however, Bub1-RNAi cells released into ZM447439 plus nocodazole exited mitosis faster: whereas 56% of the control cells remained in mitosis 3 hours after release, only 30% of the Bub1-deficient cells were mitotic.

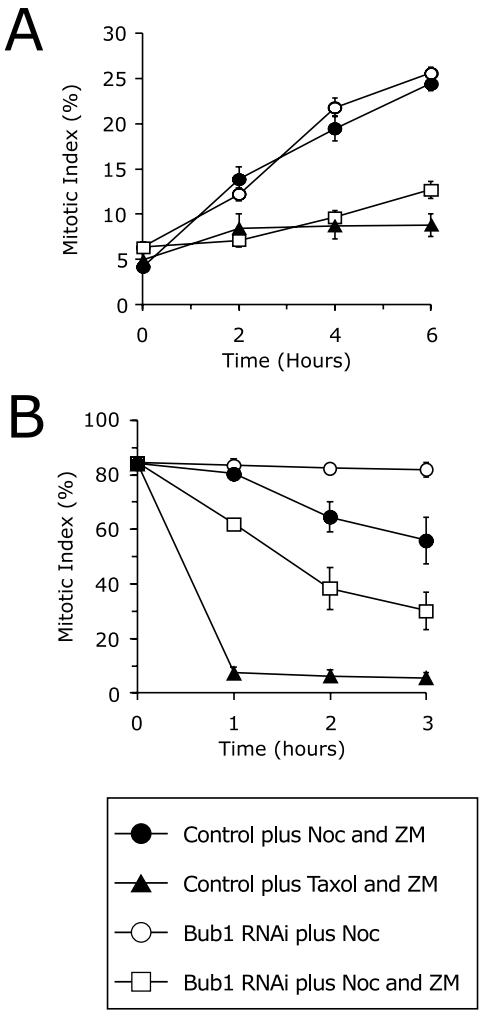

Fig. 4. Bub1 and aurora kinase activity cooperate to maintain the spindle checkpoint. (A) DLD-1 cells were transfected with control or Bub1 siRNA duplexes, and then exposed to spindle toxins, plus or minus ZM447439. Cells were then fixed, the DNA stained, and the mitotic index determined by microscopy analysis using chromosome condensation as a visual marker. Whereas control cells exposed to nocodazole plus ZM447439 (●) accumulate efficiently in mitosis, Bub1 repressed cells (□) do not. (B) HeLa cells were transfected with control or Bub1 siRNA duplexes, synchronised in mitosis by selective detachment following a 12 hour nocodazole block, and then released into spindle toxins plus or minus ZM447439. Cells were then reharvested, centrifuged onto glass slides, fixed, the DNA stained, and the mitotic index determined by microscopy analysis. Relative to the controls, Bub1-repressed cells exit mitosis faster in nocodazole plus ZM447439. Values represent means±s.e.m. from three independent experiments.

The effect of co-inhibition appears more dramatic when analysing asynchronous cells (Fig. 4A) compared with the analysis of synchronous cells (Fig. 4B). However, it is important to note that the synchronous experiment differs from the asynchronous experiment in two important ways. Firstly, the synchronised cells have been arrested in mitosis for up to 12 hours prior to the experiment and, as we have previously shown, ZM447439 does begin to induce mitotic exit following prolonged nocodazole arrest (Ditchfield et al., 2003). Secondly, in the synchronous experiment, the aurora inhibitor is added after mitotic entry, i.e. after the kinetochores have assembled and become checkpoint active.

Nevertheless, the synchronous experiment is consistent with the notion that the effect observed in Fig. 4A is due to accelerated mitotic exit. However, to test this more rigorously, we used time-lapse microscopy to analyse Bub1-RNAi cells following exposure to ZM447439 and nocodazole (Fig. 3; Table 2). Control cells exposed to ZM447439 and nocodazole remained arrested in mitosis for an average of ~5 hours. In addition, when BubR1-deficient cells were exposed to ZM447439 and nocodazole, many of them behaved in a similar manner to BubR1-RNAi cells exposed to nocodazole alone, indicating that ZM447439 had no additional effect.

By contrast, however, when Bub1-RNAi cells were exposed to ZM447439 and nocodazole, they behaved differently compared with Bub1-RNAi cells exposed to nocodazole alone. In particular, cells in the Bub1-RNAi culture now exhibited a bimodal distribution: whereas 63% remained in mitosis for >150 minutes, 30% exited within the hour. We suspect that the bimodal distribution reflects the fact that not all the cells in the population are efficiently repressed (Fig. 1). Specifically, a fraction of cells in the Bub1-RNAi culture are expected to be Bub1-proficient simply because the RNAi is not 100% efficient. (Note that the transfection efficiency in this experiment was lower than in Fig. 1 – data not shown.) Consequently, these cells act as internal controls, behaving like non-RNAi cells, arresting in mitosis when exposed to nocodaozle plus ZM447439. Nevertheless, taken together, the two population-based experiments (Fig. 4) and the single cell analysis (Fig. 3) indicate that the ability of Bub1-deficient cells to mount a robust checkpoint response following spindle destruction is dependent on aurora kinase activity.

Combined inhibition of Bub1 and aurora does not have an synergistic effect on the kinetochore localisation of BubR1 or Mad2

The above data demonstrates that simultaneous inhibition of Bub1 and aurora kinase activity has a ‘synthetic lethality’ effect on the ability of cells to maintain mitotic arrest in the presence of nocodazole: in the absence of either Bub1 or aurora kinase activity, the cells arrest; in the absence of both, they do

not. Because both Bub1-RNAi and exposure to ZM447439 significantly reduce, but do not abolish, the levels of kinetochore-bound BubR1 and Mad2 (Ditchfield et al., 2003; Johnson et al., 2004), a simple explanation for this observation is that simultaneous inhibition of Bub1 and aurora B has a 'double whammy' effect on kinetochore localisation of BubR1 and Mad2, further reducing their levels below the threshold required to activate the checkpoint (supplementary material Fig. S2). To determine if this was the case, we quantitated the amount of BubR1 and Mad2 at kinetochores following simultaneous repression of Bub1 and exposure to ZM447439. In all cases, cells were treated with nocodazole to inhibit kinetochore-microtubule interactions, thus creating conditions where all the checkpoint proteins should be maximally enriched at kinetochores.

In Bub1 RNAi cells, kinetochore-bound Bub1 was reduced to 2.2% (Fig. 5A; Table 1). In the presence of ZM447439, kinetochore-bound Bub1 was reduced to 5.0%. When both Bub1 and aurora kinase activity were inhibited, kinetochore bound Bub1 was reduced to 2.6%. Because this value is not significantly different from that obtained following repression of Bub1 alone, but is significantly different from that obtained following exposure to ZM447439 alone, it confirms that Bub1 was indeed repressed in these cells.

Upon repression of Bub1, kinetochore-bound BubR1 was reduced to 13.9% (Fig. 5B; Table 1). Upon exposure to ZM447439, the amount of BubR1 at kinetochores was reduced to 8.5%. When Bub1-repressed cells were exposed to ZM447439, the level of kinetochore-bound BubR1 was 9.5%. Because these latter two values are not significantly different ($P>0.05$), it indicates that simultaneous inhibition of Bub1 and aurora kinase activity does not further reduce the level of kinetochore-bound BubR1. Because the background value in this experiment (Ctrl- = 2.5%) is significantly different from the Bub1 RNAi plus ZM447439 value ($P<0.01$), it indicates that if a further reduction in BubR1 signal had occurred, it would have been detectable.

Similarly, the levels of Mad2 at kinetochores (Fig. 5C; Table 1) following exposure of Bub1 RNAi cells to ZM447439 (15.8%) did not differ significantly from the levels observed following exposure to ZM447439 alone (17.0%). The background value in this experiment (5.3%) was significantly different from the Bub1 RNAi plus ZM447439 value ($P<0.01$), again indicating that if a further reduction in Mad2 signal had occurred, it would have been detectable.

Thus, in the presence of nocodazole and ZM447439, similar amounts of BubR1 and Mad2 were present at kinetochores, regardless of whether or not Bub1 had been repressed, indicating therefore that simultaneous inhibition of Bub1 and aurora B activity does not further reduce the levels of kinetochore bound BubR1 and Mad2.

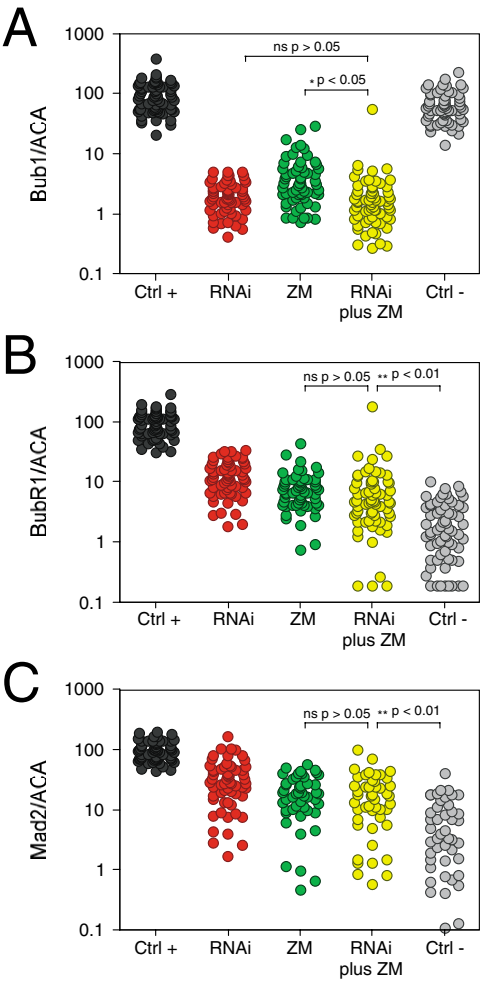

Fig. 5. Inhibition of Bub1 and aurora kinase activity does not have a synergistic effect on kinetochore localization of BubR1. DLD-1 cells were transfected with control or Bub1 siRNA duplexes. 48 hours after transfection, the cells were exposed to nocodazole plus or minus ZM447439 for 1 hour then fixed and stained to detect Bub1, BubR1 or Mad2, centromeres/kinetochores (ACA), and the DNA. In one sample, the anti-BubR1 or anti-Mad2 antibody was omitted to define the background signal. Image stacks of mitotic cells were acquired, deconvolved, then projected and the fluorescence pixel intensities at individual kinetochore pairs measured. At least 60 pairs in three or more cells were quantitated. The Bub1/ACA (A), BubR1/ACA (B) and Mad2/ACA (C) ratios were then calculated and the value for each kinetochore pair plotted on a log scale. Dark grey, control; red, Bub1-RNAi alone; green, ZM447439 alone; yellow, Bub1-RNAi plus ZM447439; light grey, control without BubR1 or Mad2 primary antibody. *P* values were determined using a nonparametric ANOVA (Kruskal-Wallis) test followed by a Dunn's post-test; ns, not significant ($P>0.05$); *significant ($P<0.05$); **very significant ($P<0.01$). See Table 1 for means and s.e.m.

BubR1 is part of a large complex in checkpoint activated cells

The above observations demonstrate that changes in kinetochore localization of BubR1 and Mad2 cannot necessarily be used as a marker to indicate whether or not the spindle checkpoint is active. When either Bub1 or aurora B kinase activity are inhibited, kinetochore localisation of BubR1 and Mad2 is severely diminished, yet the cells arrest in nocodazole (Ditchfield et al., 2003; Johnson et al., 2004). When Bub1 and aurora B kinase activity are simultaneously inhibited, BubR1 and Mad2 localisation is diminished to similar levels, but now the cells cannot maintain an active spindle checkpoint. Furthermore, changes in the phosphorylation status of BubR1 – as judged by mobility shift – does not provide an unambiguous marker for an active checkpoint signal: inhibition of aurora kinase activity prevents

BubR1 hyper-phosphorylation, yet ZM447439-treated cells arrest in nocodazole (Ditchfield et al., 2003) (supplementary material Fig. S3].

However, in addition to being phosphorylated and localizing to kinetochores, BubR1 also binds and inhibits the APC/C (Chan et al., 1999; Sudakin et al., 2001; Tang et al., 2001; Fang, 2002). We therefore set out to determine whether the effect observed following simultaneous Bub1-RNAi and exposure to ZM447439 could be explained by changes in the ability of BubR1 to bind the APC/C. To do this, we first analyzed BubR1 complexes in checkpoint-activated cells in order to develop a biochemical assay that would allow us to quantitate the amount of BubR1 bound to the APC/C. Consistent with previous observations (Sudakin et al., 2001; Tang et al., 2001), in asynchronous cells the majority of BubR1 is present in a single pool of about 500 kDa (Fig. 6A). In nocodazole-arrested mitotic cells, BubR1 is hyper-phosphorylated and a subpool is present in a larger complex of ~1.5 MDa. These two pools, referred to as BubR1-S and BubR1-L for small and large, respectively, can also be resolved by ion exchange (Fig. 6B).

BubR1-L corresponds to the MCC plus the APC/C

Following separation of BubR1-S and BubR1-L by ion exchange, these complexes were further purified using an anti-BubR1 affinity column, then analyzed by western blotting (Fig. 6C). Whereas both BubR1-S and BubR1-L contain BubR1, Bub3, Mad2 and Cdc20, only BubR1-L contains the APC/C component APC7. The simplest explanation, therefore, is that BubR1-S corresponds to the previously identified mitotic checkpoint complex (MCC) (Sudakin et al., 2001), and that BubR1-L corresponds to the MCC plus the APC/C. However, while it is clear that BubR1 and Mad2 can both inhibit APC/C in vitro, the composition and physiological relevance of the MCC is less clear (Sudakin et al., 2001; Tang et al., 2001). Indeed, our observations suggest that BubR1-S and BubR1-L are not simply the MCC, and the MCC plus the APC/C. When we analyzed purified BubR1 complexes by immunoblotting, per molecule of BubR1 or Bub3, there is more Mad2 and Cdc20 in BubR1-L than there is in BubR1-S (Fig. 6C). Furthermore, BubR1-S appears to contain at least two sub-complexes (supplementary material Fig. S4). Despite this, however, by using ion exchange to resolve BubR1-S and BubR1-L, and then measuring their relative abundance, we were able to determine the amount of BubR1 bound to the APC/C.

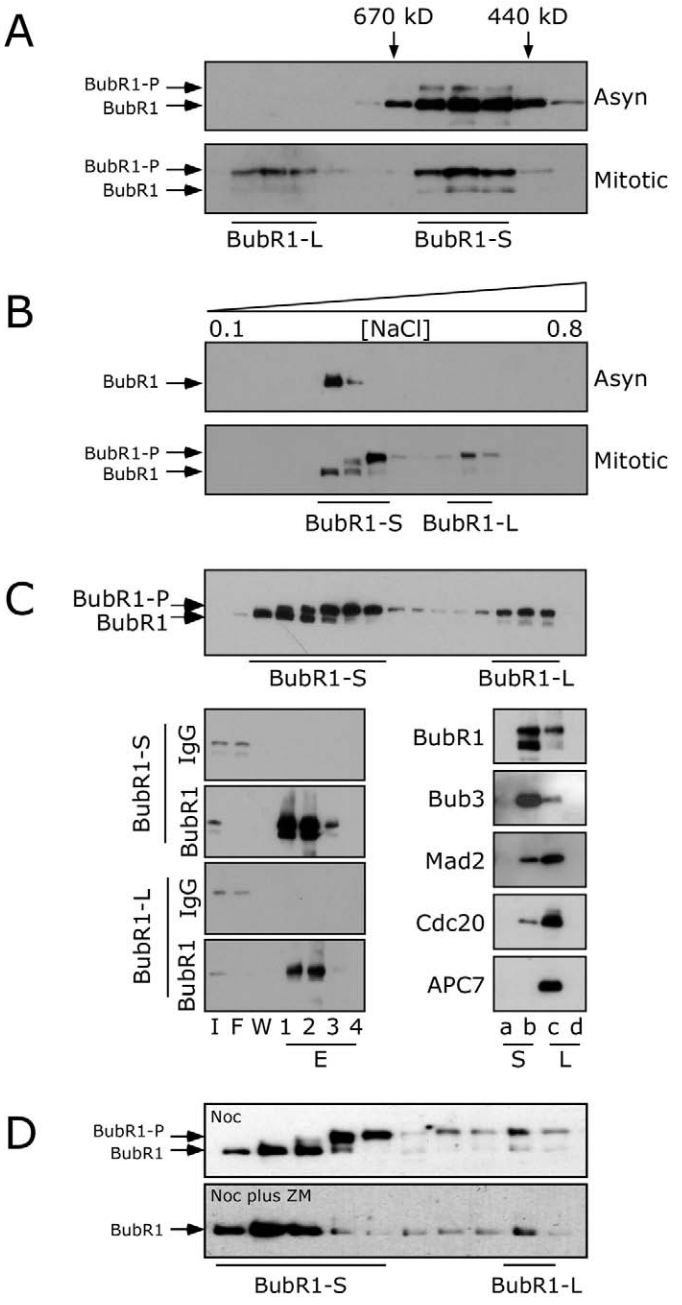

Fig. 6. BubR1 is part of a large complex in checkpoint-activated cells. (A,B) Soluble proteins were harvested from asynchronous or nocodazole-arrested HeLa cells, resolved by FPLC and the fractions blotted for BubR1. In mitotic-arrested cells, BubR1 is present in two pools, BubR1-S and BubR1-L, which can be resolved by gel filtration (A) and ion exchange (B). Horizontal arrows indicate the positions of the hypo- and hyper-phosphorylated forms of BubR1. The vertical arrows in panel A indicate the elution positions of thyroglobulin (670 kDa) and ferritin (440 kDa), while the triangle in panel B indicates the salt gradient. (C) Mitotic extracts were separated by preparative ion exchange to resolve BubR1-S and BubR1-L (top panel). Peak fractions were pooled and incubated with beads coupled to pre-immune IgGs or anti-BubR1 antibodies. Bound complexes were eluted and analyzed by western blotting to detect BubR1 (left panels). The first three fractions were pooled and analyzed by western blotting to detect BubR1, Bub3, Mad2, Cdc20 and APC7 (right panels). Labeled lanes correspond to: I, input; F, flow through; W, wash; E, eluted fractions 1-4; S and L refer to BubR1-S and BubR1-L, respectively, derived from the pre-immune fractions (a and d) or the anti-BubR1 fractions (b and c). Whereas the MCC components BubR1, Bub3, Mad2 and Cdc20 are present in both BubR1-S and BubR1-L, the APC/C component APC7 is only detectable in BubR1-L. (D) HeLa cells synchronized at G1/S were released into media and 8 hours later, prior to mitotic entry, nocodazole or nocodazole plus ZM447439 was added. 10.5 hours after release from G1/S, when the majority of cells were in mitosis, mitotic cells were harvested by selective detachment and then analyzed by ion exchange. BubR1-L is present in the cells exposed to ZM447439, indicating that aurora B kinase activity is not essential for the formation of BubR1-L.

Aurora kinase activity is not essential for BubR1 binding to the APC/C

To determine whether aurora B activity is required for the formation of BubR1-L, cells were synchronized at G1/S then released into nocodazole or nocodazole plus ZM447439. When the cells entered mitosis, they were then analyzed by analytical FPLC (Fig. 6D). BubR1-L clearly formed in the presence of ZM447439, indicating that aurora B activity is not essential to promote the binding of BubR1 to the APC/C. Indeed, the levels of BubR1-L appeared very similar both in the presence and absence of ZM447439. This is a striking observation: in the presence of nocodazole and ZM447439, kinetochore localisation of BubR1 is reduced to about 8.5% (Table 1). However, under similar conditions, the ability of BubR1 to associate with the APC/C is largely unaffected, raising the possibility that efficient kinetochore localization of BubR1 is not essential to promote its association with the APC/C. Note also that, as previously described by us (Ditchfield et al., 2003), and shown here (Fig. 6D; supplementary material Fig. S3), BubR1 is not hyper-phosphorylated when cells enter mitosis in the presence of ZM447439. Therefore, hyper-phosphorylation of BubR1 is also not required for its association with the APC/C.

BubR1-L decays prior to mitotic exit

In the presence of ZM447439 and nocodazole, kinetochore localisation and phosphorylation of BubR1 are dramatically reduced, yet the cells arrest in mitosis. By contrast, there is little difference in the amount of BubR1 bound to the APC/C. This raises the possibility that the binding of BubR1 to the APC/C may be dependent on an active spindle checkpoint signal, rather than a consequence of the cells simply being in mitosis. If this is the case, we predicted that BubR1-L levels should decay prior to mitotic exit. To test this, cells were synchronized in mitosis with nocodazole, and then released into media lacking nocodazole. Whereas the control cells were still in mitosis 60 minutes post release (Fig. 7A), the amount of BubR1-L had already fallen substantially (Fig. 7B). Furthermore, when we used ZM447439 to accelerate mitotic exit, BubR1-L levels were already down to ~25% within 30 minutes (Fig. 7B), yet the cells were still in mitosis (Fig. 7A). Thus, dissociation of BubR1 from APC/C does occur prior to mitotic exit, consistent with the notion that the binding of BubR1 to the APC/C is checkpoint dependent.

To test this more rigorously, we generated populations of cells with and without an active checkpoint signal by exploiting the fact that ZM447439 can override the checkpoint in the presence of taxol but not nocodazole (Ditchfield et al., 2003). However, in order to perform a biochemical analysis, we needed to maintain checkpoint-inactive cells in mitosis. Therefore, we used the proteasome inhibitor MG132 to prevent proteolysis of securin and mitotic cyclins, thereby maintaining the mitotic state. First, however, we had to determine whether MG132 would prevent ZM447439-induced mitotic exit. Importantly, following release from a nocodazole block, cells cultured in MG132, taxol and ZM447439 remained in mitosis for at least 3 hours (Fig. 7C), indicating that ZM447439's ability to induce mitotic exit is dependent on proteolysis.

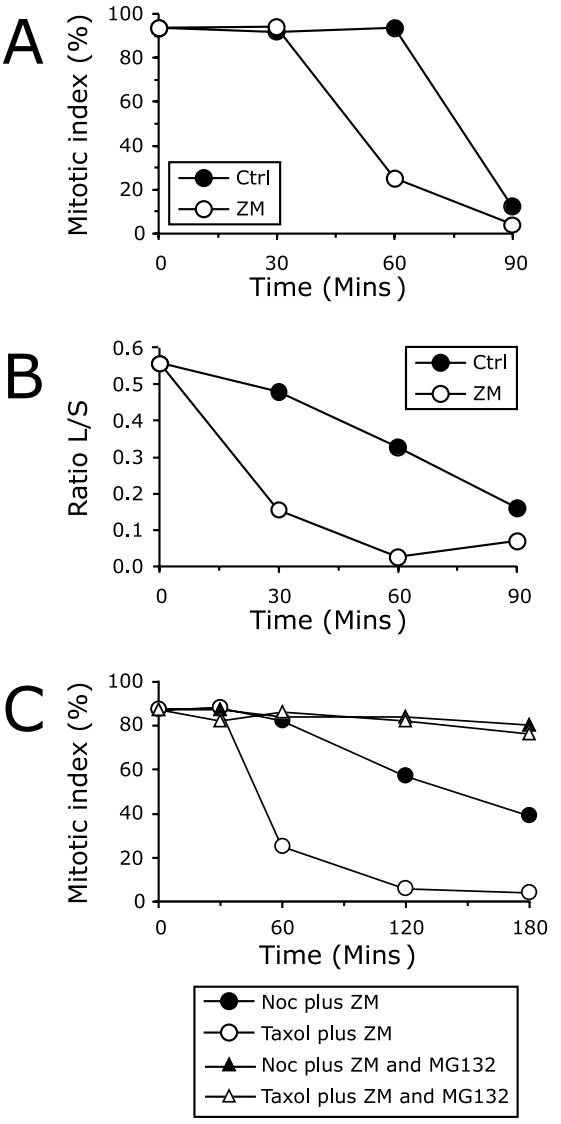

Fig. 7. BubR1-L decays prior to mitotic exit. HeLa cells were treated with nocodazole for 12 hours and the mitotic cells isolated by selective detachment. Following removal of the nocodazole, the cells were replated in various drug combinations. At the time points indicated, the cells were harvested, the mitotic index determined by microscopy, soluble proteins extracted and resolved by analytical ion exchange to determine the abundance of BubR1-L. (A) Plot of mitotic index, confirming that ZM447439-treated cells exit mitosis faster than controls. (B) Plot of the BubR1-L:BubR1-S ratio demonstrating that BubR1-L levels fall before mitotic exit, both in the presence and absence of ZM447439. (C) Plot of mitotic index following release, confirming that ZM447439 drives taxol-treated cells out of mitosis and that this can be inhibited by MG132.

BubR1 only binds the APC/C when the checkpoint is active

Having established that MG132 prevents mitotic exit when aurora B is inhibited, we generated populations of cells with and without an active checkpoint signal. HeLa cells were synchronized in mitosis with nocodazole, and the mitotic cells harvested by selective detachment. The nocodazole was then washed away and the cells replated in various drug

combinations. To generate mitotic cells with an active checkpoint, cells were released into nocodazole, ZM447439 and MG132. To generate cells without an active checkpoint signal, cells were released into taxol, ZM447439 and MG132. After 2 hours, the cells were harvested and analyzed by ion exchange.

When the checkpoint was activated in MG132-arrested cells, either by nocodazole or taxol, BubR1 was bound to APC/C, as judged by the presence of BubR1-L (Fig. 8A, panels i and ii). In addition, BubR1 was bound to the APC/C in cells released into nocodazole and ZM447439 (Fig. 8A, panel iii), confirming that aurora kinase activity is not essential for the BubR1-APC/C association. By contrast, however, BubR1 was not associated with the APC/C in cells released into taxol and ZM447439 (Fig. 8A, panel iv). Although these cells were still in mitosis due to the addition of MG132, the checkpoint was inactive because ZM447439 overrides taxol-mediated checkpoint activation (Fig. 7C). Therefore, the maintenance of BubR1-L is indeed dependent on an active spindle checkpoint. To confirm these observations, we directly immunoprecipitated BubR1 from cells released into the various drug combinations and probed for the presence of the APC/C. Consistent with the fractionation data in Fig. 8A, APC7 was present in the BubR1 immunoprecipitate from cells exposed to nocodazole plus ZM447439 (Fig. 8B). However, APC7 was not detectable in BubR1 immunoprecipitates from cells released into taxol and ZM447439.

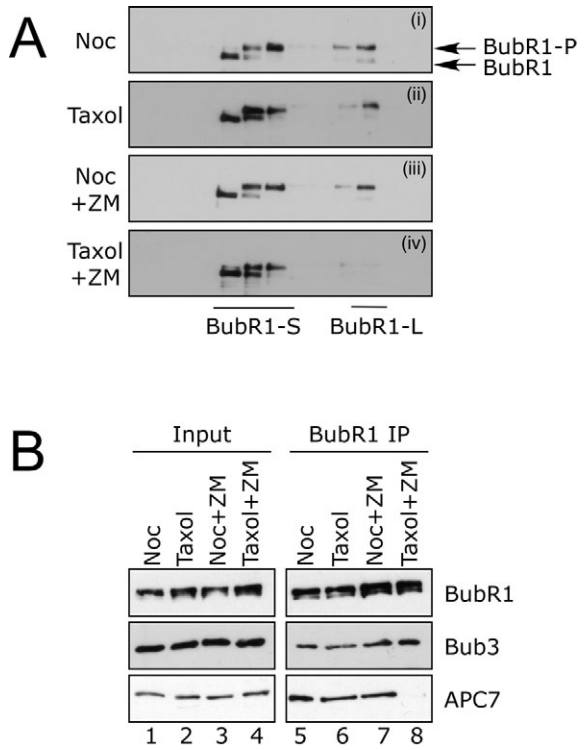

Fig. 8. BubR1 only binds the APC/C when the checkpoint is active. Nocodazole arrested HeLa cells were isolated by selective detachment, washed and then replated in either nocodazole or taxol, plus and minus ZM447439. MG132 was added to arrest the cells in mitosis downstream of the checkpoint. After 2 hours the cells were harvested, soluble proteins extracted and resolved by analytical ion exchange and immunoprecipitations to determine the amount of BubR1 bound to the APC/C. (A) In the absence of ZM447439, BubR1-L is abundant when the checkpoint is activated with either nocodazole or taxol (panels i and ii). However, in the presence of ZM447439, BubR1-L is abundant in nocodazole (panel iii) but not taxol-treated cells (panel iv). [Note that while ZM447439 inhibits BubR1 phosphorylation if added prior to mitotic entry (Ditchfield et al., 2003), BubR1 remains phosphorylated if ZM447439 is added to cells already arrested in mitosis, see supplementary material Fig. S4.] (B) APC7 is present in BubR1 immunoprecipitates from cells released into nocodazole plus ZM447439 (lane 7), but not taxol plus ZM447439 (lane 8).

Importantly, these observations indicate that BubR1-L provides a useful marker with which to monitor checkpoint status: under conditions where the checkpoint is on, BubR1-L is present at a level comparable with those in control cells, despite the fact that BubR1 is almost undetectable at kinetochores and it is not hyper-phosphorylated. However, under conditions where the checkpoint is off – but, importantly, when the cells are still in mitosis – BubR1-L is largely undetectable. Indeed, to our knowledge, the presence of BubR1-L is the only biochemical marker that unambiguously indicates that the checkpoint signalling pathway is active.

Bub1 and aurora cooperate to maintain BubR1 binding to the APC/C

Not only do the observations outlined above indicate that BubR1-L is a useful marker to determine checkpoint status, they also demonstrate that aurora kinase activity is required to maintain the BubR1-APC/C interaction in the presence of taxol, but not nocodazole. This latter observation therefore begs the following question: what maintains the BubR1-APC/C interaction in nocodazole-arrested cells that lack aurora B activity? Based on our observations described above (Figs 3, 4), we reasoned that Bub1 was a leading candidate. Therefore, we repeated the experiment shown above, but first repressed Bub1 by RNAi. Following transfection of siRNA duplexes, control and Bub1-RNAi cells were synchronized in mitosis with nocodazole, harvested, then replated in nocodazole plus and minus ZM447439. MG132 was also added to prevent mitotic exit. Two hours later, the cells were isolated and the ratio of BubR1-L:BubR1-S determined. Consistent with the data in Fig. 8, BubR1-L was abundant in control cells released into nocodazole and ZM447439 (Fig. 9A). Significantly, however, when Bub1 was repressed, BubR1-L was less abundant following release into nocodazole and ZM447439. Indeed, quantitative analysis revealed that repression of Bub1 reduced the BubR1-L:BubR1-S ratio from ~70% to ~45% (Fig. 9B). Note that this value underestimates the true effect of repressing Bub1, simply because up to 30% of the cells still contain substantial levels of Bub1 (Fig. 1C), and because ZM447439 does begin to induce mitotic exit following prolonged exposure to nocodazole (Ditchfield et al., 2003) (Fig. 4B, Fig. 7C). Regardless, this observation clearly demonstrates that, in the absence of aurora kinase activity, Bub1 is required to maintain the BubR1-APC/C association.

Discussion

Does BubR1 play a kinetochore-independent role?

Bub1 and BubR1 are two structurally related spindle checkpoint kinases that bind kinetochores. However, the localisation and

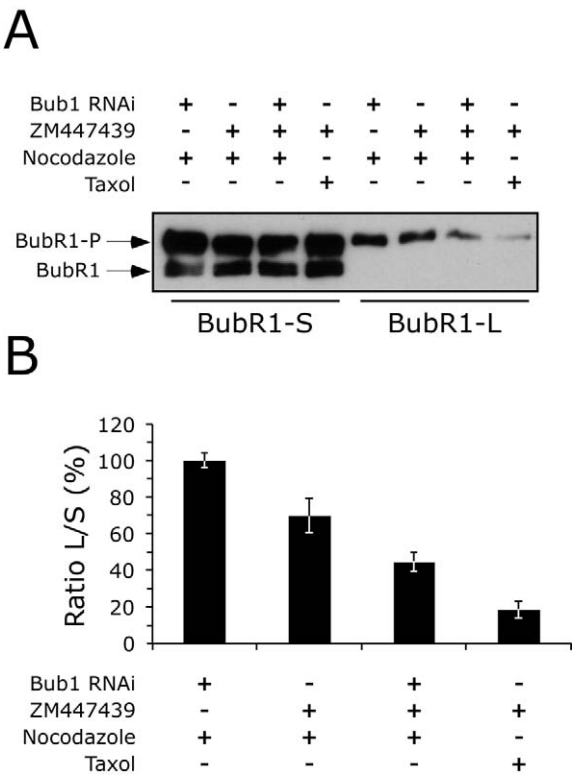

Fig. 9. Bub1 maintains the BubR1-APC/C interaction following loss of kinetochore–microtubule interactions. Cells were transfected with control or Bub1 siRNA duplexes and then synchronized in mitosis using nocodazole and selective detachment. Following removal of the nocodazole, the cells were then replated for two hours in nocodazole or taxol, plus or minus ZM447439 as indicated. MG132 was also added to maintain the mitotic state. The cells were then reharvested, and protein extracts prepared and analyzed by ion exchange to resolve BubR1-S and BubR1-L. (A) Western blot showing that when Bub1 is repressed, BubR1-L is less abundant in cells released into nocodazole and ZM447439. The blot shown is representative of three independent RNAi experiments. (B) Quantitation of the BubR1-L:BubR1-S ratio confirming that following repression of Bub1, BubR1-L is less abundant in cells exposed to nocodazole and ZM447439. Values represent the mean±s.e.m. derived from three western blots.

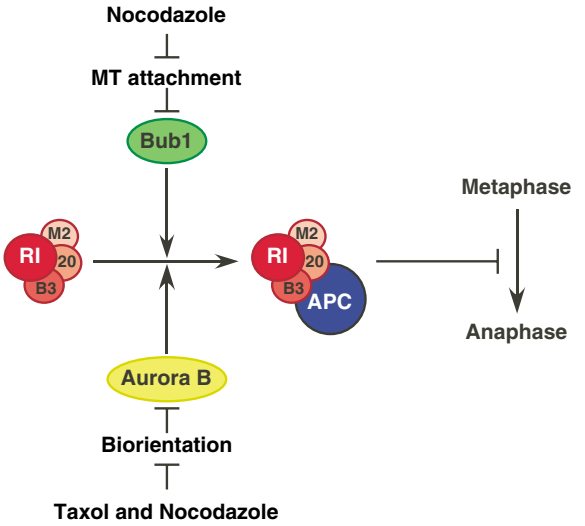

Fig. 10. The spindle checkpoint is composed of two arms, both of which promote and/or maintain BubR1 binding to the APC/C. The Bub1-dependent arm monitors microtubule attachment at kinetochores and is therefore activated by nocodazole. By contrast, the aurora-dependent arm monitors biorientation and is therefore activated by both nocodazole and taxol. Both arms converge on other checkpoint proteins including BubR1, Bub3, Mad2 and Cdc20, promoting their association with the APC/C and thereby preventing the metaphase to anaphase transition until all the chromosomes are correctly aligned on the spindle.

roles of these two proteins are rather different. Bub1 dissociates from kinetochores upon microtubule attachment and thus, like Mad2, is an 'attachment sensor' (Waters et al., 1998; Taylor et al., 2001). By contrast, BubR1 dissociates from kinetochores only after biorientation (Skoufias et al., 2001; Taylor et al., 2001). Furthermore, whereas BubR1 is clearly essential for checkpoint function, Bub1-deficient cells do mount a robust checkpoint response when the spindle is destroyed (Johnson et al., 2004) (Figs 3, 4). This latter observation presents a paradox because Bub1 appears to be a 'master regulator' of the kinetochore signalling domain (Johnson et al., 2004). Specifically, Bub1, which assembles at kinetochores very early in prophase, is required for kinetochore localisation of BubR1, Mad2 and Cenp-E. By contrast, BubR1, which assembles later, is required for kinetochore localisation of Cenp-E, but not Bub1 or Mad2. Therefore, if the role of BubR1 in terms of checkpoint activation was entirely at the kinetochore, one might expect that dislodging BubR1 from kinetochores by repression of Bub1 would yield a similar phenotype to that observed following repression of BubR1 itself. However, this is clearly not the case, raising the possibility that BubR1 plays a kinetochore-independent role. Indeed, it has recently been reported that BubR1 and Mad2 are part of a cytosolic, kinetochore-independent, mitotic timer mechanism, whereas the other checkpoint components are required only for kinetochore-dependent checkpoint control (Meraldi et al., 2004). Although our data is consistent with this recent report, our novel observations facilitate a simpler model (Fig. 10). Specifically, by inhibiting aurora kinase activity in Bub1-repressed cells, we show that the ability of Bub1-deficient cells to arrest in mitosis following spindle damage is dependent on aurora kinase activity. While this may be interpreted to suggest that that aurora B and Bub1 play redundant roles in the timer mechanism, another possibility is that the 'synthetic lethality' effect observed following inhibition of Bub1 and aurora kinase activity is because the checkpoint is composed of two arms, one dependent on Bub1, the other on aurora B. If this is the case, we suggest that BubR1 and Mad2 are common denominators to both pathways (Fig. 10), providing a simpler explanation as to why BubR1 and Mad2 are essential for checkpoint function, yet Bub1 and aurora B are partially redundant.

Why do aurora-deficient cells arrest in nocodazole but not taxol?

Following inhibition of aurora B kinase activity, or repression of the aurora B binding partner survivin, cells exit mitosis without biorienting their chromosomes, indicating a

compromised spindle checkpoint (Carvalho et al., 2003; Ditchfield et al., 2003; Hauf et al., 2003; Lens et al., 2003). In addition, upon inhibition of aurora B activity or survivin RNAi, cells cannot undergo mitotic arrest when microtubules are stabilised with taxol, or when spindle pole separation is prevented by the Eg5 inhibitor monastrol. Strikingly, however, the checkpoint is not completely defective in these cells: when microtubule polymerisation is inhibited with nocodazole, they do undergo mitotic arrest. Similarly, the budding yeast aurora kinase, Ipl1, is not required for checkpoint activation when microtubule polymerisation is inhibited (Biggins and Murray, 2001). However, it is required when kinetochores can attach microtubules but cannot come under tension.

Three possible explanations have been put forward to account for these observations. The first suggests that because aurora B/Ipl1 is required to destabilize inappropriate kinetochore-microtubule interactions, its apparent role in the checkpoint is simply a secondary consequence of its ability to generate unattached kinetochores (Tanaka et al., 2002; Hauf et al., 2003). If this explanation was universally true, one might expect that, in the absence of aurora B activity, spindle checkpoint proteins would localize to kinetochores that lacked bound microtubules. However, ZM447439 dramatically reduces the levels of kinetochore-bound BubR1 and Mad2 in nocodazole-treated cells (Ditchfield et al., 2003) (Fig. 5). Furthermore, in the absence of Bub1, aurora kinase activity becomes essential for nocodazole-induced mitotic arrest (Figs 3, 4). Together, these observations suggest that aurora B does indeed play a direct role in the spindle checkpoint.

A second explanation is that, although inhibition of aurora kinase activity reduces kinetochore-bound BubR1 and Mad2, perhaps the residual bound protein is sufficient to sustain mitotic arrest in the absence of kinetochore-microtubule interactions (Ditchfield et al., 2003) (supplementary material Fig. S2). If microtubule occupancy is sufficient to inactivate the remaining bound proteins, this may explain why aurora-deficient cells cannot arrest in the presence taxol. Although we cannot rule out this possibility, our data does not support it: when Bub1 and aurora are simultaneously inhibited, the ability to arrest in response to nocodazole is severely compromised (Figs 3, 4). Yet, kinetochore bound levels of BubR1 and Mad2 are not reduced below the levels observed when either Bub1 or aurora are inhibited alone (Fig. 5).

The third possible explanation suggests that the spindle checkpoint pathway is composed of two arms, one of which depends on aurora B, and one which does not (Ditchfield et al., 2003; Johnson et al., 2004). The data we present here is consistent with this model: whereas Bub1 does not appear to be essential for checkpoint activation in response to nocodazole (Johnson et al., 2004), it becomes essential in the absence of aurora kinase activity (Figs 3, 4). Thus, in light of the 'synthetic lethality'-like relationship observed following Bub1-RNAi and exposure to ZM447439, the simplest explanation for our observations is that the spindle checkpoint is composed of two arms, one dependent on Bub1 and one dependent on aurora B activity (Fig. 10).

What regulates the two arms?

Taken together with earlier observations (see below), the simplest explanation is that whereas the aurora B arm monitors biorientation, the Bub1 arm monitors kinetochore-microtubule attachment. Accordingly, in the absence of Bub1, the aurora B arm activates the checkpoint in response to either nocodazole or taxol, because both prevent biorientation. In the presence of nocodazole, the Bub1 arm is sufficient to trigger mitotic arrest, despite the absence of aurora B activity, because kinetochore-microtubule interactions are prevented (Fig. 10). However, in taxol- or monastrol-treated cells, although biorientation is prevented, kinetochores do bind microtubules. Consequently, if the aurora B arm is then inhibited, the lack of biorientation goes unnoticed and the checkpoint is silenced, triggering mitotic exit. Consistent with this, kinetochore localization of Bub1 is sensitive to microtubule attachment (Taylor et al., 2001). In addition, several reports argue that aurora B responds to changes in tension in order to promote biorientation (Tanaka et al., 2002; Hauf et al., 2003; Andrews et al., 2004; Dewar et al., 2004; Lan et al., 2004). While it is interesting to note that the APC/C is re-recruited to metaphase kinetochores following exposure to taxol, and that this is dependent on aurora kinase activity (Acquaviva et al., 2004), in the absence of any direct biochemical data supporting a link between aurora B and the MCC, it is premature to conclude that the 'tension'-sensing properties of aurora B feed directly into the checkpoint mechanism. Indeed, and especially because tension stabilizes attachment (Nicklas and Koch, 1969), it is still possible that the role of aurora B is mediated via regulating kinetochore structure and/or kinetochore-microtubule interactions. If this is the case, our data would suggest that in the complete absence of kinetochore-microtubule interactions, this mechanism operates in parallel with the Bub1-dependent mechanism.

Note that, in vitro, ZM447439 inhibits both aurora A and B (Ditchfield et al., 2003). On balance, the phenotypes induced by ZM447439 appear to be due to inhibition of aurora B, rather than aurora A (Keen and Taylor, 2004). However, we cannot rule out the possibility that the 'synthetic lethality' effect observed following Bub1-RNAi and ZM447439-exposure is due to inhibition of aurora A. In addition to regulating centrosome function (Blagden and Glover, 2003) and mitotic entry (Hirota et al., 2003), aurora A binds Cdc20, and its overexpression has been reported to compromise the spindle checkpoint (Farruggio et al., 1999; Anand et al., 2003; Jiang et al., 2003). Furthermore, aurora A localizes to spindle poles, where active APC/C resides, and where cyclin B degradation initiates (Clute and Pines, 1999; Kraft et al., 2003). Perhaps, therefore, the requirement for aurora kinase activity in the checkpoint reflects a role for aurora A in amplifying and/or localizing the checkpoint signal in order to prevent APC/C^{Cdc20} activation at the poles. Nevertheless, at present, we favor the simpler explanation that the phenotypes described here are due to inhibition of aurora B, not aurora A.

BubR1-L, the physiological anaphase inhibitor

While it remains to be determined exactly how spindle events regulate both arms, our data suggest that they both converge on BubR1, promoting and/or maintaining its association with the APC/C (Figs 8, 9). Consistent with previous observations, we show that BubR1 is part of a complex that also contains Bub3, Mad2 and Cdc20. Importantly, we show that this binds to the APC/C only when the checkpoint is active. That both arms converge on the MCC, promoting its binding to the

APC/C, explains why BubR1and Mad2 are essential for checkpoint function following spindle destruction, yet Bub1 and aurora B kinase activity are not. In addition, the recent observation that Bub1 phosphorylates Cdc20 provides a potential mechanism by which the Bub1-dependent arm could feed into the MCC-APC/C mechanism (Tang et al., 2004a). However, the notion that BubR1-S is the MCC and BubR1-L is the MCC plus the APC/C is clearly an oversimplification. Indeed, observations from yeast, *Xenopus* and HeLa cells indicate that multiple subcomplexes exist in checkpoint-activated cells (Musacchio and Hardwick, 2002) (supplementary material Fig. S4). Although the exact nature of these sub complexes, and their relative contribution in checkpoint signaling, remains to be solved, our data indicate that BubR1-L is the physiologically relevant anaphase inhibitor, and that both Bub1 and aurora kinase activity cooperate to promote and/or maintain the integrity of this complex.

The authors thank Kevin Hardwick and Jan-Michael Peters for antibodies. C.J.M. was supported by the Biotechnology and Biological Sciences Research Council (BBSRC) and GlaxoSmithKline; A.T. is supported by the A.I.C.R.; V.L.J. was supported by the BBSRC; M.I.F.S. is supported by the Wellcome Trust, C.D. was supported by AstraZeneca and S.S.T. is a Cancer Research UK Senior Fellow.

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