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Bub3p Facilitates Spindle Checkpoint Silencing in Fission Yeast

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Although critical for spindle checkpoint signaling, the role kinetochores play in anaphase promoting complex (APC) inhibition remains unclear. Here we show that spindle checkpoint proteins are severely depleted from unattached kinetochores in fission yeast cells lacking Bub3p. Surprisingly, a robust mitotic arrest is maintained in the majority of *bub3*Δ cells, yet they die, suggesting that Bub3p is essential for successful checkpoint recovery. During recovery, two defects are observed: (1) cells mis-segregate chromosomes and (2) anaphase onset is significantly delayed. We show that Bub3p is required to activate the APC upon inhibition of Aurora kinase activity in checkpoint-arrested cells, suggesting that Bub3p is required for efficient checkpoint silencing downstream of Aurora kinase. Together, these results suggest that spindle checkpoint signals can be amplified in the nucleoplasm, yet kinetochore localization of spindle checkpoint components is required for proper recovery from a spindle checkpoint-dependent arrest.

INTRODUCTION

Microtubule drugs are extensively used as anti-cancer agents. Yet it remains unclear how these drugs actually kill cancer cells or why they kill some cancer cells better than others (for review, see Gascoigne and Taylor, 2009). More work is needed to understand why cells survive or die after exposure to microtubule stress. Here we use fission yeast to dissect the pathways required for cell survival upon microtubule de-polymerization.

Two major mechanisms play critical roles in ensuring faithful chromosome segregation following microtubule stress in mitosis: (1) the spindle checkpoint and (2) the Aurora kinase–dependent error correction mechanism that destabilises kinetochore-microtubule connections failing to produce tension across the centromeres. We and others have shown previously that, in fission yeast, this Aurora-dependent correction mechanism requires Shugoshin2 (Sgo2)-dependent localization of Aurora on centromeres (Hauf *et al*., 2007; Kawashima *et al*., 2007; Vanoosthuyse *et al*., 2007).

On exposure to microtubule drugs, the spindle checkpoint delays anaphase onset until all sister chromatids are attached in a bipolar manner and are under tension. Only then is cohesion between sister chromatids destroyed allowing their segregation to the two daughter cells. Many components of the spindle checkpoint have been identified and their modes of action are becoming clear (Musacchio and Salmon, 2007; Burke and Stukenberg, 2008). Far less is understood about how this checkpoint is turned off upon chromosome biorientation.

In most organisms, all checkpoint components and their targets (Cdc20-APC) (Acquaviva *et al*., 2004) have been localized to unattached kinetochores. It is thought that Bub1 and Mad1 act there as scaffolds to efficiently recruit and activate downstream signaling molecules, such as Mad3/BubR1 and Mad2, which then become "anaphase inhibitors." These APC inhibitors act locally at unattached kinetochores, but the checkpoint must also transmit a signal throughout the mitotic machinery, so that cohesion is protected on all sister chromatids and cyclin B levels are maintained on centrosomes and the spindle. Similarly, cohesion must be destroyed in a concerted manner at anaphase onset. The nature of these long-range signals remains unclear but it is predicted that the checkpoint kinases Bub1, Mps1, and Aurora play an important role in signal amplification. When these kinases phosphorylate a substrate to sustain a checkpoint arrest, it is likely that the substrate will then need to be de-phosphorylated upon chromosome biorientation. Consistent with this, we have recently shown that a kinetochore-localized pool of PP1 phosphatase is crucial to turn-off checkpoint signals and activate the APC (Vanoosthuyse and Hardwick, 2009).

Here we show that Bub3p, the binding partner of the spindle checkpoint kinase Bub1p, has two main functions upon microtubule depolymerization: (1) Bub3p is necessary for efficient spindle checkpoint silencing and (2) Bub3p is necessary for proper chromosome biorientation. These two functions are independent of each other and contribute to maintain cell viability upon microtubule stress.

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MATERIALS AND METHODS

Yeast Strains

A table listing all the strains used in this study is shown in Supplemental Table S1.

Microscopy

Most microscopy was performed as described previously (Vanoosthuyse *et al*., 2007).

Chromatin Immunoprecipitation (ChIP) Analysis

ChIP was performed as described previously (Vanoosthuyse *et al*., 2004) with the following modification: the DNA was purified using a Promega kit (Wizard SV Gel and PCR clean-up system, Promega, Madison, WI) according to the manufacturer's instructions.

Checkpoint Silencing Assay

Midlog *ark1-as3 cdc13-GFP nda3-KM311* cells were first arrested in early mitosis in liquid cultures by shifting the temperature to 18°C for 6 h. 5 μM 1NMPP1 was then added to the culture. 800 μL of cells were fixed every 10–15 min by resuspending them with 1 ml of 100% methanol and mounted immediately in media containing DAPI (to label the DNA) and calcofluor (to label cell walls and septa). 100 cells for each condition (DMSO or 1NMPP1) were counted immediately under the microscope in the interval before the next time-point.

Mitotic Checkpoint Complex (MCC)–APC Interaction

Cells expressing from their endogenous loci, TAP-tagged Lid1 (Apc4) and Mad2p and Mad3p both tagged with GFP, were presynchronized in G2 using the *cdc25-22* mutation. Proteins were extracted in lysis buffer (50 mM Hepes pH 7.6, 75 m MKCl, 1 mM MgCl2, 1 mM EGTA, 0.1% Triton X-100, 1 mM Na Vanadate, Microcystin-LR, Leupeptin, Pepstatin, Chymostatin, and Pefabloc) from ≈2.10^8 cells as described previously (Hardwick and Murray, 1995). Extracts were then incubated for 30 min with IgG-coupled Dynabeads, which bind to Lid1-TAP. The immunoprecipitated complexes were washed three times with lysis buffer and once with PBS containing 0.02% Tween 20. The immunoprecipitated complexes were then analyzed by immunoblot using a sheep anti-GFP antibody.

nda3-KM311 Release Assay

Midlog *nda3-KM311* cells were first arrested in early mitosis in liquid cultures by shifting the temperature to 18°C for 6 h. Cells were then filtered on a Durapore Filter 0.45 μM HV (Millipore, Bedford, MA) and released from the filter into prewarmed media (32°C) by shaking. At each time-point after the release at 32°C, 2 ml of cells were fixed by mixing with 20 ml of 100% Methanol precooled at −80°C. Cells were then processed for immunofluorescence with an anti-tubulin antibody (TAT1, a kind gift of Prof Keith Gull; University of Oxford, Oxford, United Kingdom).

mto1 and nda3 Kinetochore Retrieval Assay

The *nda3* retrieval assay was performed as described (Grishchuk and McIntosh, 2006) but with release to 30°C from a 10-h block at 18°C. Analysis of kinetochore retrieval in *mto1*Δ was performed on lactose gradient synchronized live cells in an imaging chamber (CoverWell PCI-2.5, Grace Bio-labs, Bend, OR) filled with 1 ml of 1% agarose in minimal medium and sealed with a 22 × 22 mm glass coverslip. Fluorescence microscopy was performed at 30°C using a Photometrics system containing a Photometrics CoolSnapHQ2 CCD camera (Photometrics, Tucson, AZ) and a Nikon TE2000E inverted microscope with a 100 × 1.49 NA objective equipped with MetaMorph for data collection and analysis. Stacks of 6 z-sections (0.6 μm apart) were taken at 1-minute intervals with exposure times of 1 s for GFP and CFP. Projected images were made for each time point followed by intensity adjustments and transfer to Photoshop for figure preparation.

RESULTS

On Spindle Disassembly, Fission Yeast Bub3 Is Largely Dispensable for Spindle Checkpoint Arrest but Is Essential for Recovery

We have previously shown that *bub3*Δ cells die rapidly if their microtubules are depolymerised using the cold-sensitive tubulin mutant *nda3-KM311* (Hiraoka *et al*., 1984; Vanoosthuyse *et al*., 2004). Although we could detect some cells showing the characteristic "cut" phenotype indicative of a spindle checkpoint defect (Vanoosthuyse *et al*., 2004), further examination showed that *bub3*Δ *nda3* cells were arresting in mitosis much more effectively than other checkpoint mutants (see also Tange and Niwa, 2008; Windecker *et al*., 2009). To confirm this we used *nda3* cells expressing cyclin B-GFP (cdc13-GFP) or securin-GFP (cut2-GFP), whose accumulation on spindle pole bodies (SPBs) indicates early mitosis. Mutants were shifted to the restrictive temperature (18°C) and analyzed for accumulation of these mitotic markers. Unlike *mad2*Δ or *bub1*Δ mutants, the majority (≈70%) of *bub3*Δ*nda3* cells were able to arrest in mitosis in a Mad2p-dependent manner (Figure 1A). In addition, their chromosomes condensed (Figure 1B) and only 15% of cells went through cytokinesis and septated (Figure 1C). Thus, *bub3*Δ*nda3* cells are able to establish and maintain a robust spindle checkpoint arrest, although not quite as effectively as wild-type cells.

To monitor loss of viability, we first subjected *bub3*Δ*nda3* cells to spindle stress by growing them at 18°C for 6 h, and then isolated single cells on solid rich medium at the permissive temperature (32°C). Only 30% of *bub3*Δ*nda3* cells were able to form colonies (Figure 1D) and those that did were typically slow growing, most likely due to aneuploidy (see below). Analysis by microscopy of single cells on these plates showed that the majority of *bub3*Δ *nda3* cells died during the first division (Figure 1E).

Bub3p Is Required for the Enrichment of Checkpoint Components to Unattached Kinetochores but Is Dispensable for Mad2p/Mad3p-APC Binding

The Mad and Bub proteins are recruited to the central domain of fission yeast centromeres in mitosis (Vanoosthuyse *et al*., 2004). However, there is no detectable centromeric enrichment of Mad3p or Bub1p in *bub3*Δ cells (Vanoosthuyse *et al*., 2004). To analyze Mad1p and Mad2p localization, we carried out imaging and ChIP on arrested *nda3bub3*Δ cells expressing Mad1-, Mad2-, or Mad3-GFP. As reported recently (Windecker *et al*., 2009), there was, by imaging, no detectable enrichment of any of these checkpoint components at unattached kinetochores in *nda3bub3*Δ (Figure 2A). ChIP quantification indicated that the levels of all three Mad proteins were reduced by 90% or more in arrested *bub3*Δ cells (Figure 2B). Note that the kinetochore recruitment of another outer kinetochore protein, Nuf2p, was not affected in *bub3*Δ cells (Figure 2B). Thus the absence of Bub3p has a profound but specific effect on the kinetochore recruitment of all the Mad and Bub proteins. Nonetheless, as the mitotic arrest is robust in the majority of these *bub3*Δ cells, we conclude that these levels are sufficient for spindle checkpoint arrest.

The fact that Bub3 is necessary for kinetochore enrichment of checkpoint components yet is largely dispensable for a checkpoint arrest is surprising. In most systems Bub3p is an integral component of the mitotic checkpoint complex (MCC; typically Mad3p/BubR1-Mad2p-Bub3p-Cdc20p), which is an important effector of spindle checkpoint arrest (Hardwick *et al*., 2000; Sudakin *et al*., 2001; Fang, 2002; Millband and Hardwick, 2002). Fission yeast Mad3p and Mad2p, but not Bub3p, stably bind to the APC in mitotic cells, and none of the upstream checkpoint proteins (Mph1p, Bub1p, Mad1p) or Bub3p were required for this interaction when assayed in prolonged mitotic arrests (Sczaniecka *et al*., 2008). To test these dependencies more carefully, we analyzed the kinetics of MCC–APC association in synchronized cells. In particular we tested whether Mad1p or Bub3p were required for efficient formation of the MCC–APC complex. Cultures containing TAP-tagged APC (Lid1p is fission yeast Apc4) were synchronized by *cdc25* block (G2) and release. Cells were released from G2 and after 20 min anti-microtubule drugs were added (carbendazim, CBZ) to activate the spindle checkpoint. This experiment demonstrates that although lack of Mad1p dramatically reduces the efficiency of MCC–APC interaction, the absence of Bub3p does not (Figure 2C). We conclude that fission yeast Bub3p is not directly

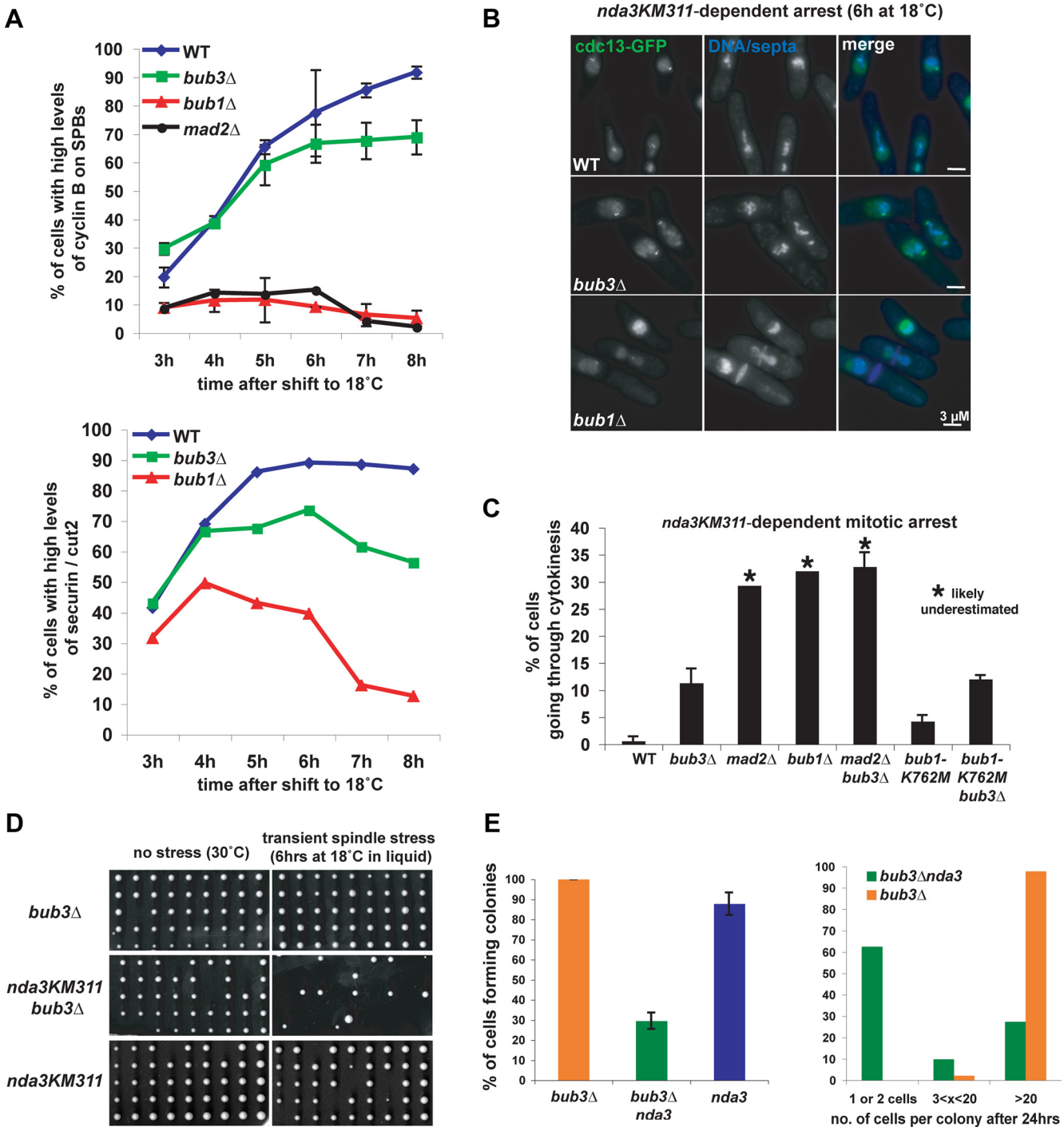

Figure 1. Bub3p is largely dispensable for spindle checkpoint arrest in response to unattached kinetochores, but is required for recovery from the arrest. (A) When shifted to their restrictive temperature, of 18°C, *nda3-KM311* cells depolymerise microtubules and thereby activate the spindle checkpoint in response to unattached kinetochores. Over time (here up to 8 h after the shift to 18°C), *nda3-KM311* cells accumulate in early mitosis with high levels of cyclin B (cdc13-GFP) and securin (cut2-GFP) in the nucleus and on spindle poles (SPBs). Checkpoint mutants such as *bub1*Δ and *mad2*Δ failed to accumulate in mitosis, but *bub3*Δ did, although not as efficiently as *bub3+* cells. This data are derived from a minimum of three independent experiments (error bars are SD). (B) Images of representative cells after 6 h at 18°C. *Bub3+* and *bub3*Δ cells accumulate cyclin B on SPBs, condense their chromosomes, and inhibit septation. Checkpoint defective *bub1*Δ cells fail to arrest in mitosis and instead go through cytokinesis (septate) without segregating their chromosomes, leading to the characteristic "cut" phenotype. (C) Percentage of cells going through cytokinesis (septation index) 6 h after the shift to 18°C. This number was negligible in *nda3KM311* cells, whereas more than 40% of *bub1*Δ and *mad2*Δ cells had septa. Note this underestimates the number of cells failing to arrest in response to unattached kinetochores in the absence of Bub1p or Mad2p, as it does not include the cells that have already completed cytokinesis after checkpoint failure. The number of cells failing to accumulate cyclin B (>90% of *bub1*Δ or *mad2*Δ cells 6 h after the shift to 18°C) is a more accurate estimate of checkpoint failure in these cells. Reproducibly, 10% to 15% of *bub3*Δ cells failed to prevent cytokinesis and septated, suggesting that Bub3p is required for a fully efficient mitotic arrest. Mitotic arrest in *bub3*Δ cells was dependent on Mad2p, as the phenotype of *mad2*Δ*bub3*Δ was indistinguishable from *mad2*Δ, but was not affected by lack of Bub1 kinase activity (*bub1-K762M*).

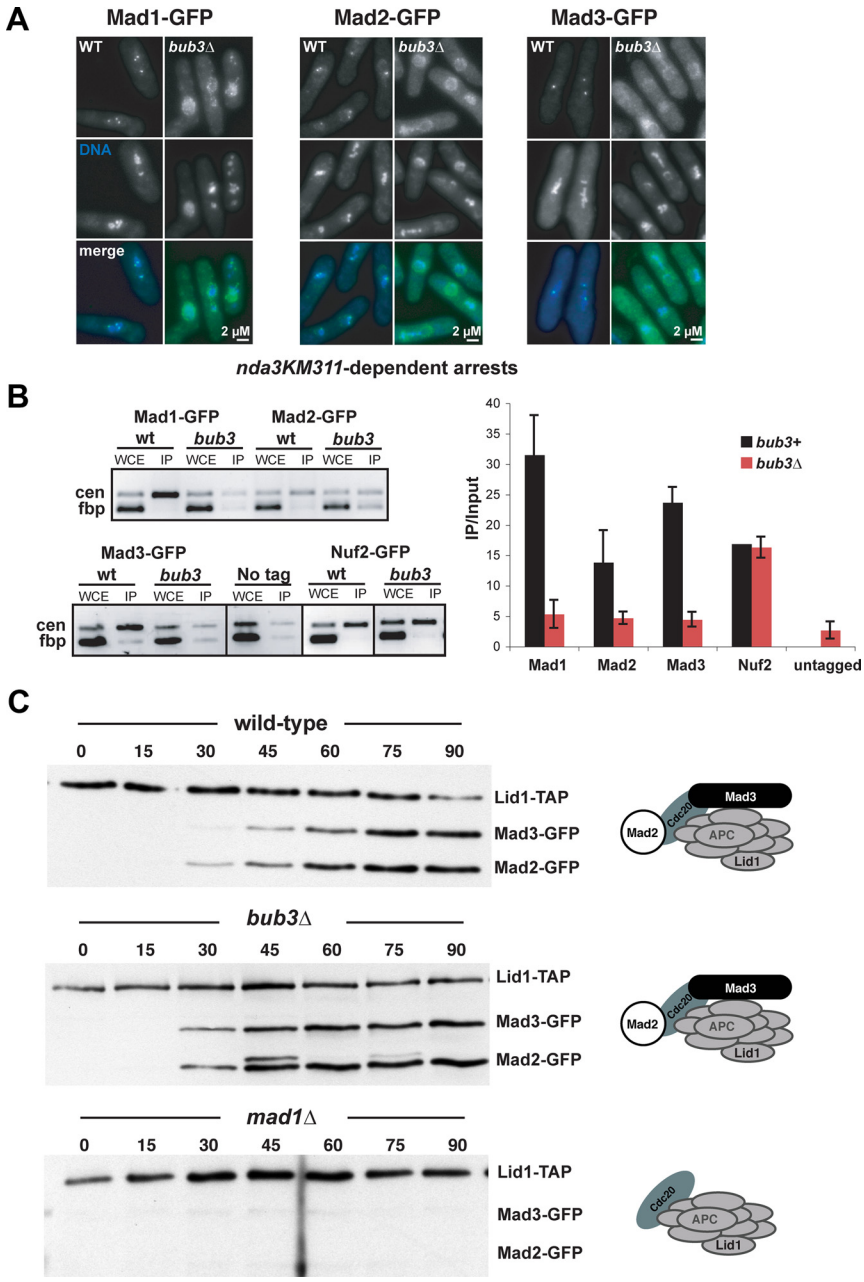

Figure 2. Bub3p is required to enrich checkpoint proteins at centromeres, but not for efficient MCC–APC complex assembly. Cells expressing Mad1-GFP, Mad2-GFP, or Mad3-GFP under the control of their normal promoters at their endogenous loci were arrested in mitosis (*nda3KM311*-dependent mitotic arrest) in the presence (wild-type [WT]) or absence of Bub3p (*bub3*Δ). Six hours after the shift to 18°C, the localization of each of these proteins was analyzed by microscopy and by chromatin immunoprecipitation (ChIP). (A) As reported previously (Vanoosthuyse *et al*., 2004), Mad1p, Mad2p, and Mad3p are targeted to unattached kinetochores in checkpoint-arrested cells, and form up to three pairs of bright GFP dots in the nucleus. In the absence of Bub3p, all three Mad proteins failed to localize to kinetochores and were released into the nucleus. (B) These observations were confirmed and quantitated by ChIP. The immunoprecipitated DNA was amplified by PCR using primers specific for the central core (cc), which is the site of kinetochore assembly, and control primers that amplify a noncentromeric, euchromatic negative control (fbp). Left, Representative PCRs are shown for each tagged protein, as well as an untagged negative control. Right, Quantification of the ChIP data. The data presented are derived from three independent ChIP experiments. (C) To analyze MCC–APC binding in mitosis, a strain expressing a TAP-tagged Apc4 subunit of the APC (Lid1p), and Mad2p and Mad3p both tagged with GFP (*cdc25-22* Lid1-TAP Mad2-GFP Mad3-GFP) was pre-synchronized in late G2 using the *cdc25-22* mutation, and then released into mitosis in the presence of the microtubule-depolymerising drug carbendazim (CBZ) to activate the spindle checkpoint (CBZ was added 20 min after the release from the G2 block). Lid1p was immunoprecipitated from samples collected every 15 min. In the wild-type, a complex between Lid1p, Mad2p, and Mad3p was detectable 30 min after release from the G2 block and persisted for more than 120 min. In the absence of Mad1p, no significant complex formation between Lid1p, Mad2p, and Mad3p was detectable, showing that MCC–APC formation requires Mad1p for efficient formation. In the absence of Bub3p, MCC–APC complex formation was unaffected.

involved in APC inhibition, nor in the production of MCC–APC, and this probably explains why it is not required for robust spindle checkpoint arrest. However, Bub3p is required for Mad and Bub protein enrichment at kinetochores (Figure 2, A and B, and Vanoosthuyse *et al*., 2004; Kadura *et al*., 2005), which strongly argues that the low levels observed in *bub3*Δ cells must be sufficient for spindle checkpoint arrest (see Discussion).

Figure 1 (cont). (D) *nda3-KM311bub3*Δ cells die. After 6 h at 18°C in liquid cultures, individual *nda3-KM311bub3+*, *nda3-KM311bub3*Δ, or *nda3+bub3*Δ cells were isolated and placed on solid media at the permissive temperature of 32°C. *nda3-KM311bub3*Δ cells formed far fewer colonies than *nda3-KM311bub3+* cells, showing that Bub3p is required to maintain cell viability upon spindle stress although it is largely dispensable for spindle checkpoint activation. The viability of *nda3+bub3*Δ cells was not affected by the transient cold-shock, showing that Bub3p is required to maintain cell viability specifically upon spindle checkpoint activation. (E) Left, Quantitation of strains ability to form colonies in D. This data are derived from three independent experiments (error bars are SD). Right, The number of cells per colony was counted 24 h after isolation on solid media at 32°C. More than 60% of *bub3*Δ*nda3-KM311* cells had not divided at all or had divided only once, whereas nearly all *nda3+* cells had already divided more than four times.

Loss of Bub3p Accentuates the Cell Cycle Delay in Response to Other Kinetochore-Microtubule Attachment Defects

In the above experiments *nda3* cells completely lack microtubules at 18°C, and all kinetochores are unattached. To test whether Bub3p is also necessary to respond to more subtle mitotic defects, we tested whether lack of Bub3p affected the viability and mitotic index of various mutants known to activate the spindle checkpoint: the temperature-sensitive kinetochore mutant *nuf2-3* (Nabetani *et al*., 2001) at its semi-

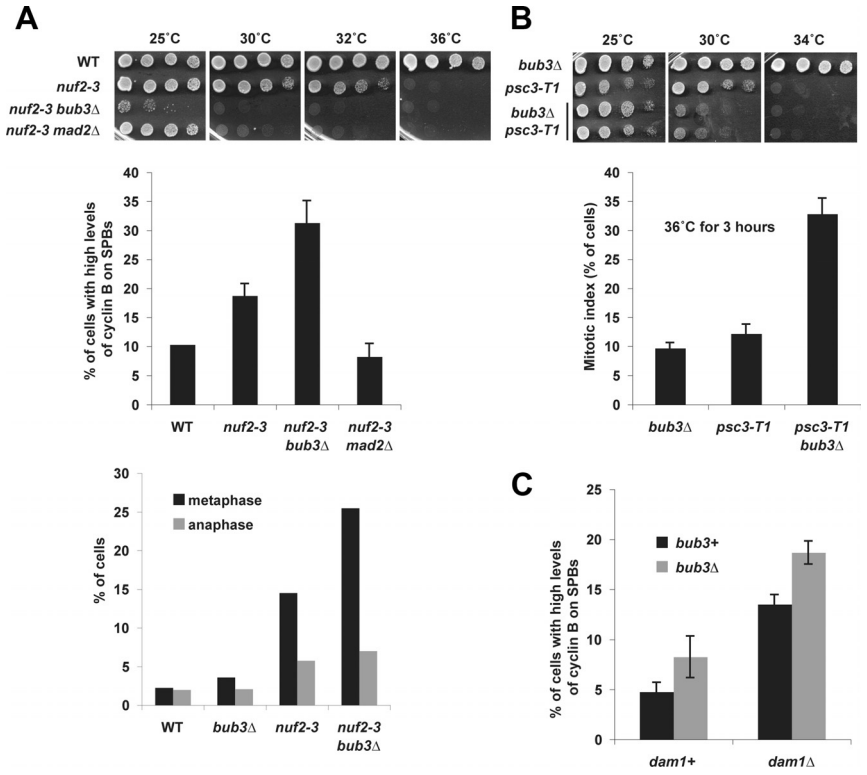

Figure 3. Lack of Bub3p accentuates checkpoint arrest in response to a wide range of mitotic defects. (A) The temperature-sensitive kinetochore mutant *nuf2-3* activates the spindle checkpoint (Nabetani *et al*., 2001). Even at the permissive temperature of 25°C, the mitotic index of *nuf2-3* cells is increased compared with wild-type cells, as judged by the number of cells with high levels of cyclin B on SPBs (left) or with a metaphase spindle (right). This mitotic delay was abrogated in the absence of Mad2p but was enhanced by lack of Bub3p. *mad2* and *bub3* mutations significantly decrease the restrictive temperature of the *nuf2-3* mutation, showing that both proteins are essential to maintain the viability of *nuf2-3* cells. This data are derived from three independent experiments, where 300 cells were counted for each experiment. (B) Bub3p is also necessary to maintain the viability of the temperature-sensitive cohesin mutant *psc3-T1* but is not required for the spindle checkpoint-dependent delay (Kawashima *et al*., 2007) triggered by the mutation at the restrictive temperature of 36°C. The mitotic index of *psc3-T1* cells is increased in the absence of Bub3p, as judged by spindle staining. This data are derived from two independent experiments, where 300 cells were counted for each experiment. (C) Lack of the Dam1p (*dam1*Δ) subunit of the DASH complex (which is required for kinetochore-microtubule attachment) triggers a mitotic delay as judged by the increased number of cells with high levels of cyclin B on SPBs. Here again, the lack of Bub3p enhanced the mitotic index of the *dam1*Δ mutant.

permissive temperature (Figure 3A); the cohesin mutant *pcs3-T1* (Nonaka *et al*., 2002; Kawashima *et al*., 2007; Figure 3B); and the *dam1*Δ mutant (Figure 3C), which impairs the function of the DASH complex in kinetochore-microtubule attachment (Sanchez-Perez *et al*., 2005). In all three mutants, lack of Bub3p caused no impairment in spindle checkpoint-dependent delay/arrest, consistent with other recent studies (Meadows and Millar, 2008; Tange and Niwa, 2008; Windecker *et al*., 2009), but resulted in a dramatic loss of viability. Moreover, our data suggest that lack of Bub3 accentuates the cell cycle delay in response to these spindle attachment defects(see below). Taken together these experiments (Figures 1–3) show that Bub3p is largely dispensable for spindle checkpoint arrest, but is required for recovery from these mitotic arrests.

Why do *bub3*Δ *nda3* cells die if they checkpoint arrest this efficiently? Recovery from *nda3* arrest requires: (1) DASH- and Klp2p-dependent retrieval of unclustered kinetochores (Franco *et al*., 2007; Grishchuk *et al*., 2007), (2) Sgo2p- and Aurora-dependent chromosome biorientation (Kawashima *et al*., 2007; Vanoosthuyse *et al*., 2007), and (3) spindle checkpoint silencing. In the following, we tested which of these steps are defective in *bub3*Δ cells.

Bub3p Is Not Required for Kinetochore Capture or Retrieval

Vertebrate cells lacking Bub1 or Bub3 appear to display kinetochore attachment defects (Meraldi and Sorger, 2005; Logarinho *et al*., 2008; Klebig *et al*., 2009). To test directly whether Bub3p is required for kinetochore attachment after spindle stress in fission yeast, we first monitored the retrieval of unclustered kinetochores after spindle reassembly in live cells. At the restrictive temperature *nda3* cells lack microtubules and unattached kinetochores can drift some distance from spindle poles. After release to the permissive temperature, microtubules are nucleated allowing capture and retrieval of these unclustered kinetochores and their subsequent biorientation (Grishchuk and McIntosh, 2006; Vanoosthuyse *et al*., 2007). Cells expressing markers for both kinetochores (Ndc80-GFP) and spindle poles (Cdc11-CFP) were released at the permissive temperature after an *nda3* block and fixed every five minutes. At each time-point, we measured the percentage of cells with unclustered kinetochores (Figure 4A). As a control, we used *dam1*Δ, a mutant of the Dam1/DASH complex required for kinetochore retrieval (Franco *et al*., 2007). No significant retrieval defect was apparent in *nda3 bub3*Δ cells during spindle reassembly after the *nda3* block and release (Figure 4B). In a second assay, we monitored retrieval of kinetochores that were unclustered from the spindle poles due to the lack of the Mto1p microtubule organizer (≈10% of *mto1*Δ cells display at least one pair of unclusteredkinetochores [Franco *et al*., 2007]). Once again we found that there was no defect in kinetochore retrieval in *mto1 bub3*Δ cells (Figure 4C). We conclude that kinetochore capture, attachment, and retrieval are all carried out efficiently in cells lacking Bub3p.

*bub3*Δ *Mutants Mis-Segregate Chromosomes after Spindle Disassembly/Reassembly*

Next we analyzed the ability of *bub3*Δ cells to accurately segregate their chromosomes during the first anaphase after release from *nda3* arrest. Segregation of chromosome 2 was scored using strains expressing lacI-GFP with a tandem array of lac operators integrated 5 kb from cen2 (Ding *et al*., 2004). Around 25% mis-segregation was observed, where the two centromeres of chromosome 2 ended up in the same daughter cell (Figure 5A). This is reminiscent of *bub1*kinase and *sgo2* mutants, which checkpoint arrest but then die during recovery, probably due to their inability to correct chromosome biorientation defects (Fernius and Hardwick, 2007; Indjeian and Murray, 2007; Kawashima *et al*., 2007;

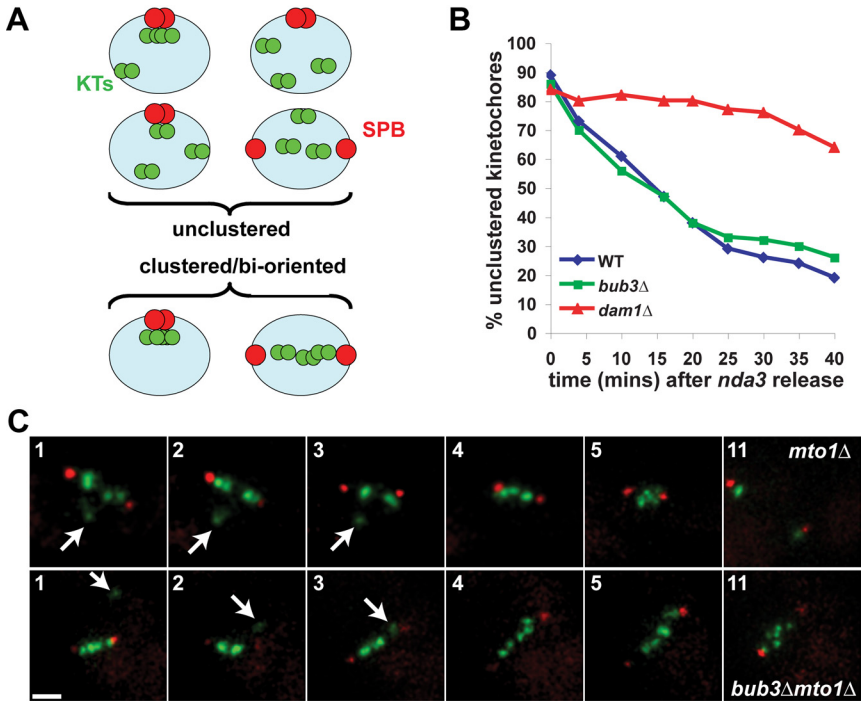

Figure 4. Bub3 is not required for the capture or retrieval of unclustered kinetochores. (A) Schematic of SPBs (red: cdc11-CFP) and kinetochores (green: Ndc80-GFP) imaged in B and C. (B) Kinetochores are captured and retrieved efficiently in *bub3*Δ *nda3* cells during spindle reassembly. 100 cells were analyzed per strain for each time point. (C) Kinetochores are captured and retrieved efficiently in *bub3*Δ *mto1*Δ cells. White arrows indicate unclustered kinetochores. Time frames are labeled in minutes. Note that the *mto1*Δ has undergone anaphase A by 11 mins, whereas the *bub3*Δ *mto1*Δ strain is still in metaphase even though all six sisters appear to be bioriented. The incidence of successful retrieval was 9/10 *mto1*Δ movies and 7/7 *bub3*Δ *mto1*Δ movies. Scale bar is 1 micron.

Vanoosthuyse *et al*., 2007; Windecker *et al*., 2009). Consistent with this, lack of Bub3p did perturb the centromeric localization of Sgo2p and other key components of the Aurora-dependent error correction mechanism in *nda3*-arrested cells (Bir1p and Ark1p, Figure 5, C and D and data not shown). Lack of Bub3p did not affect their protein levels (Figure 5B and data not shown). Importantly, the double mutant *bub3*Δ*sgo2*Δ shows a very similar rate of chromosome mis-segregation as either single mutant in this assay (Figure 5A). Taken together, these observations suggest that the chromosome mis-segregation phenotype of *bub3*Δ cells upon spindle reassembly is due to a weakened Aurora-dependent error correction mechanism. Note however that these results do not rule out that Bub3p has also Sgo2p-independent functions, as has been suggested recently (Windecker *et al*., 2009 and see below).

Bub3p is required to target Bub1p to kinetochores (Vanoosthuyse *et al*., 2004). Furthermore, Bub1 kinase activity is required for Sgo2p localization on chromatin (Supplemental Figure S1). The simplest interpretation of our data are that Bub3p is required to target Bub1 kinase activity to kinetochores, which in turn acts to enrich Sgo2p at centromeres. This is particularly important after checkpoint arrest, as many errors are encountered when sister-chromatid pairs attach to microtubules of the reforming mitotic spindle, and these need to be corrected before anaphase onset (Fernius and Hardwick, 2007; Indjeian and Murray, 2007; Vanoosthuyse *et al*., 2007; Windecker *et al*., 2009). The failure to correct inappropriate attachments probably explains the loss in viability in *nda3*-arrested *bub3*Δ cells. However, chromosome segregation is not significantly affected in *bub3*Δ cells in a normal cell cycle (Vanoosthuyse *et al*., 2004; Tange and Niwa, 2008).

Bub3p, but not Sgo2p, Is Required for Spindle Checkpoint Silencing

We observed a very reproducible ≈20-min delay in anaphase entry, judged by spindle elongation, in *bub3*Δ cells upon recovery from an *nda3* arrest (Figure 6A). Consistent with a delay in anaphase onset, we also observed prolonged hyper-phosphorylation of the checkpoint kinases Bub1p and Mph1p in *bub3*Δ cells upon recovery from an *nda3* arrest (data not shown). This mitotic delay is unlikely to be due to the fact that the Aurora-dependent error correction mechanism is weakened in the absence of Bub3p (Figure 5). Indeed, abrogating the Aurora-dependent error correction mechanism by deleting Sgo2p (as opposed to simply mislocalizing Sgo2p as in *bub3*Δ cells) does not alter the metaphase delay observed in *bub3*Δ cells (Figure 6A). Moreover, such a delay in anaphase entry upon recovery from an *nda3* arrest is not observed in a *sgo2*Δ mutant (Vanoosthuyse *et al*., 2007). We conclude that it is unlikely that the metaphase delay observed in *bub3*Δ cells is due to their failure to localize Sgo2p properly or correct inappropriately attached kinetochores. As we also failed to detect significant kinetochore capture and retrieval defects in *bub3*Δ cells (Figure 4), an alternative explanation for this delay is that lack of Bub3p during spindle reassembly reduces the efficiency of checkpoint silencing, because it directly perturbs checkpoint inactivation mechanisms.

To test whether Bub3p is required for checkpoint silencing we used the novel spindle checkpoint silencing assay that we recently developed (Vanoosthuyse and Hardwick, 2009). Cells expressing a conditional ATP analogue–sensitive allele of Ark1 kinase (*ark1-as3*) were checkpoint arrested using the cold-sensitive tubulin mutant *nda3KM311*. On inhibition of Ark1 kinase activity, by addition of the ATP analogue 1NMPP1, the spindle checkpoint is silenced and APC reactivation leads to cyclin B destruction and mitotic exit (Vanoosthuyse and Hardwick, 2009). We have previously demonstrated that kinetochore-localized Protein Phosphatase 1 ($PP1^{Dis2}$) is required for spindle checkpoint silencing (Vanoosthuyse and Hardwick, 2009), and we used this assay to test directly whether Bub3p also has a role. Importantly, in the *bub3*Δ mutant cyclin B degradation was delayed by 40–60 min (Figure 6B). These data provide direct evidence of a role for Bub3p in spindle checkpoint silencing,

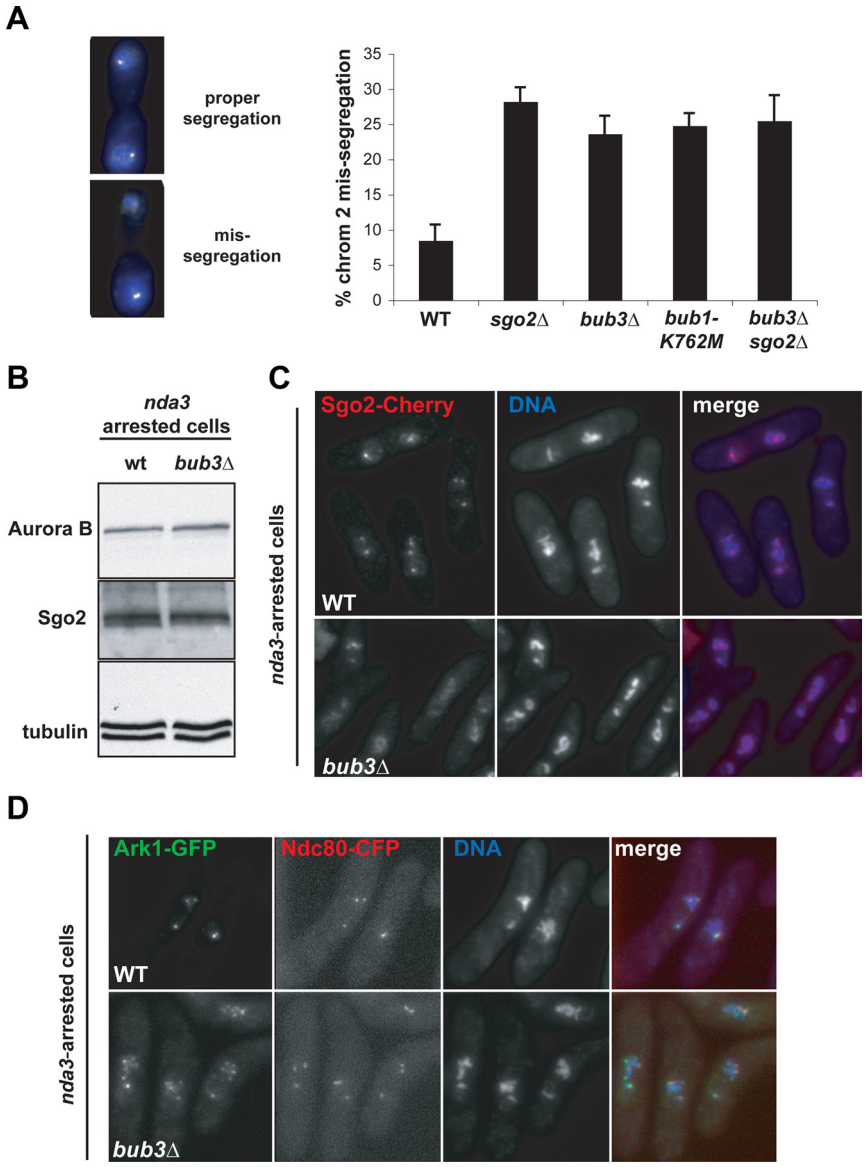

Figure 5. *bub3*Δ cells mis-segregate chromosomes in the first anaphase after spindle reformation. (A) *nda3-KM311* cells were grown at 18°C for 6 h to activate the spindle checkpoint and subsequently shifted to their permissive temperature of 32°C. At 32°C, cells reformed a spindle and committed to anaphase within 10 min (see Figure 6). Chromosome segregation was monitored in the first anaphase after checkpoint release using a GFP-marked chromosome (GFP array inserted 5 kb away from centromere 2). A mis-segregation event was scored when both copies of the GFP-marked chromosome segregated to the same daughter cell (left). As described previously, *sgo2*Δ mutants, which mis-localize the Aurora B–containing chromosomal passenger complex (CPC; Kawashima *et al.*, 2007; Vanoosthuyse *et al.*, 2007), mis-segregate chromosome 2 in approximately one-third of cells. So did cells lacking Bub1 kinase activity (*bub1-K762M*), in which Sgo2p is mis-localized (Supplemental Figure S1). Cells lacking Bub3p lost chromosome 2 at a comparable rate to both *sgo2*Δ and *bub1-K762M*. Deleting Sgo2p in a *bub3*Δ background (*bub3*Δ*sgo2*Δ) did not increase the chromosome loss, which is consistent with the idea that Bub3p and Sgo2p work in the same pathway to prevent chromosome loss. Note, however, that *bub3*Δ cells show no significant chromosome loss in a normal mitosis (Vanoosthuyse *et al.*, 2004; Windecker *et al.*, 2009). (B) Lack of Bub3p does not affect the protein levels of Sgo2p or Aurora B. Protein extracts were prepared from *nda3KM311*-arrested cells and immunoblotted with tubulin as a loading control. (C) In the absence of Bub3p, Sgo2p failed to concentrate on centromeres in *nda3KM311*-arrested cells and instead decorated the whole chromatin. Consequently, localization of the Aurora kinase Ark1 was also affected (D): instead of forming bright centromeric foci, Ark1 was found in a punctate signal all over the chromatin. Some of these foci represent telomeres (not shown).

and this explains why lack of Bub3p accentuates the cell cycle delay in response to kinetochore-microtubule attachment defects (Figure 3). Note that lack of Bub3p does not affect anaphase entry (and thus APC activation) to the same extent in the *nda3* release experiment (Figure 6A, 20-min delay) and in the silencing assay (Figure 6B, 40- to 60-min delay). We see two potential explanations for this discrepancy. The *nda3* release experiment is performed at 32°C, whereas the silencing assay is performed at 18°C (a temperature at which the rate of APC activation might be slower). Alternatively, as the *nda3* release experiment but not the silencing assay is performed in the presence of microtubules, it is possible that the presence of microtubules stimulates APC activation.

We predicted that Bub3p acts through its binding-partner Bub1p. To test this prediction, a deletion was made in Bub1p, removing residues 264–299 and thereby the conserved Bub3p binding site (Larsen *et al.*, 2007), sometimes referred to as the "GLEBS" domain (Wang *et al.*, 2001; Windecker *et al.*, 2009). Coimmunoprecipitation studies confirmed that this mutant Bub1 protein was stable but unable to bind Bub3-Myc (Supplemental Figure S2A). Analysis of *nda3bub1*Δ*GLEBS* cells showed that they display a phenotype remarkably similar to *bub3*Δ: they formed the MCC–APC complex and checkpoint arrested rather effectively (data not shown and Supplemental Figure S2B), but when Ark1 kinase was inactivated they could only silence the checkpoint and degrade cyclin B inefficiently (Figure 6C). We conclude that the silencing defect observed in *bub3*Δ cells is most likely due to an inability to target Bub1p, Mad3p, and perhaps other checkpoint proteins to kinetochores. Note that the kinetochore localization of $PP1^{Dis2}$ whose activity is necessary for APC activation upon Ark1 inhibition (Vanoosthuyse and Hardwick, 2009) is not affected by lack of Bub3p (Supplemental Figure S3). Note also that Sgo2p, whose proper enrichment on centromeres requires Bub3p (Figure 5), is not required for spindle checkpoint silencing. Indeed, *sgo2*Δ cells efficiently activate the APC upon Ark1 inhibition in our assay (Supplemental Figure S4), and they do not delay anaphase onset upon *nda3KM311* block and release (Vanoosthuyse *et al.*, 2007). Together these data indicate that Bub3p has at least two

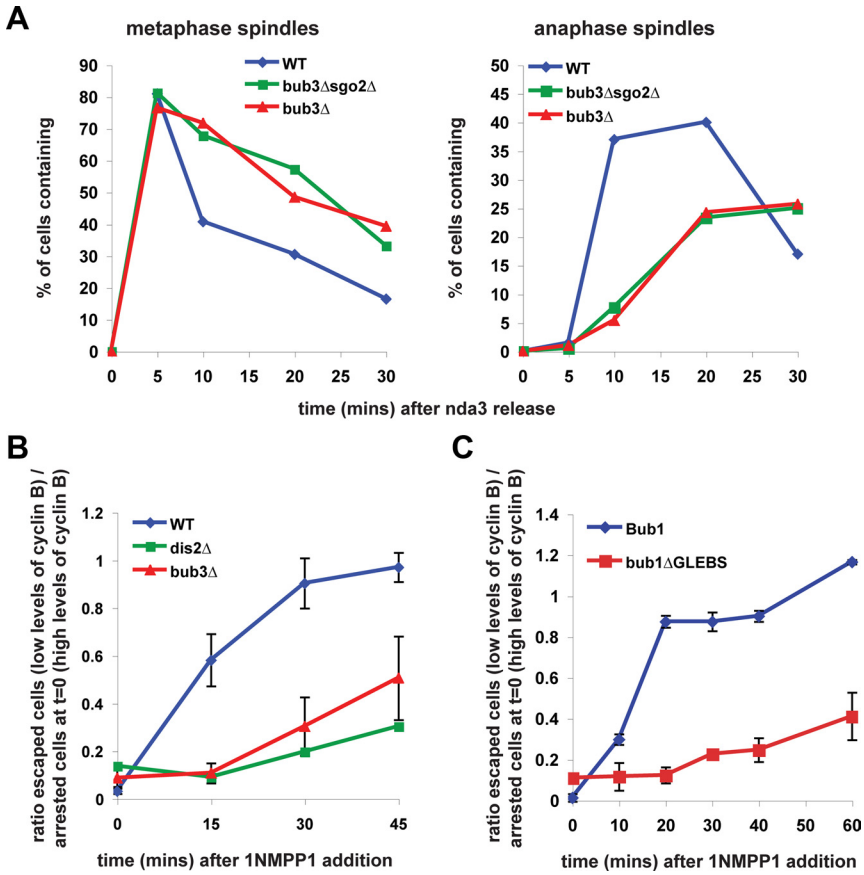

Figure 6. Bub3 facilitates spindle checkpoint silencing. (A) *nda3KM311*-arrested cells (6 h at 18°C) were allowed to reform their spindle at the permissive temperature of 32°C (see Methods). Progression through mitosis was then followed at regular intervals using spindle length as a marker. On recovery from *nda3KM311* arrest, lack of Bub3p induced a ≈20 min metaphase delay. This delay was Sgo2-independent, suggesting that it is not due to Sgo2 mis-localization in *bub3*Δ cells. (B) When the ATP analogue 1NMPP1was added to *nda3*-arrested cultures to inhibit Ark1 kinase, the APC was activated and cyclin B was degraded. In the absence of $PP1^{Dis2}$ (Vanoosthuyse and Hardwick, 2009) or Bub3p, cyclin B degradation was significantly delayed. For each time-point, the number of cells having degraded cyclin B (escaped cells) was divided by the number of arrested cells at t = 0 (initial number of arrested cells). These data are derived from a minimum of 4 experiments, and error bars represent SD. (C) The Bub3p-binding domain of Bub1 was deleted (*bub1ΔGLEBS*). Similarly to *bub3*Δ, *bub1ΔGLEBS* cells fail to degrade cyclin B efficiently after Ark1 inhibition.

separable roles in recovery from spindle checkpoint arrest (1) Sgo2p-independent checkpoint silencing and (2) Sgo2p-dependent chromosome biorientation.

DISCUSSION

Our data highlight roles played by fission yeast Bub3p in two key aspects of recovery from spindle stress, chromosome biorientation, and spindle checkpoint silencing. Importantly, these two functions can be separated genetically, suggesting that they are independent of each other. Our major findings can be summarized as follows:

(1) Lack of Bub3p reduces by >90% the levels of checkpoint components on kinetochores, yet *bub3*Δ cells can establish and maintain a robust spindle checkpoint arrest (Figures 1–3). It was recently argued that fission yeast Bub3p is totally dispensable for checkpoint arrest (Tange and Niwa, 2008; Windecker *et al*., 2009). However, we believe that Bub3p-dependent kinetochore enrichment of the checkpoint machinery is required for full checkpoint activity as we found that ≈15% of *bub3*Δcells failed to maintain mitotic arrest upon complete microtubule de-polymerization (Figure 1A). Nonetheless, these observations demonstrate that, at least in fission yeast, checkpoint signals can be amplified efficiently at sites other than kinetochores.

(2) The Bub1p–Bub3p complex facilitates spindle checkpoint silencing, suggesting that kinetochore enrichment of the checkpoint machinery is required for checkpoint silencing. This is consistent with the idea that, even if checkpoint signals can be amplified away from kinetochores, these checkpoint signals are silenced on kinetochores. One possibility is that activated checkpoint components are more efficiently silenced on kinetochores by the $PP1^{Dis2}$ phosphase-dependent spindle checkpoint silencing mechanism we have recently described (Vanoosthuyse and Hardwick, 2009). To fully demonstrate this model, one would need to identify the substrates $PP1^{Dis2}$ required for checkpoint silencing and see whether their phosphorylation/de-phosphorylation is affected by lack of Bub3p.

(3) Kinetochore localization of Bub1p-Bub3p is required to maintain Sgo2p and the chromosomal passenger complex (CPC) on centromeres upon microtubule depolymerization (Figure 5 and data not shown), and thereby contributes to proper chromosome biorientation, particularly after prolonged spindle stress (Vanoosthuyse *et al*., 2007).

Together, our observations explain why fission yeast cells lacking Bub3p are extremely sensitive to microtubule drugs and kinetochore-microtubule attachments defects. Our data also clarify the roles played by kinetochores in spindle checkpoint signaling.

Roles of Bub3p in the Spindle Checkpoint in Fission Yeast?

In most systems Bub3p is a core component of the spindle checkpoint, but this is not the case in fission yeast (Figures 1 and 3 and Tange and Niwa, 2008; Windecker *et al*., 2009). Bub3p is not part of the fission yeast MCC–APC complex (Sczaniecka *et al*., 2008), nor is it required for efficient formation of that complex (Figure 2). Note, the latter is not true for the Mad1p and Bub1p scaffold proteins, whose absence has a dramatic effect on the efficiency of MCC–APC formation (Figure 2C and not shown).

Although Bub1p and Mad1p normally act as scaffolds at kinetochores, our findings suggest that, in the absence of

Bub3p, they can also act as scaffolds in the nucleoplasm. We believe this is not a fission yeast exception. Recent studies in metazoan have reported that the checkpoint signaling function of Mad3p/BubR1 is independent of its kinetochore localization (Essex *et al*., 2008; Kulukian *et al*., 2009; Malureanu *et al*., 2009). Moreover, recent studies in vertebrate cells have reported situations where cells exhibited prolonged checkpoint-dependent metaphase delays, even if Mad2 was not enriched on kinetochores (for example, see Chan *et al*., 2009; Daum *et al*., 2009).

We would argue that fission yeast Bub3p ensures efficient checkpoint activation and silencing through its ability to target and enrich checkpoint components at centromeres. This increases the local concentration of checkpoint components. One possibility is that it does this by increasing their residence time at kinetochores. Their increased concentration facilitates enzymatic reactions (both phosphorylation and de-phosphorylation) necessary for APC inhibition and reactivation. The low levels of Mad1p-Mad2p remaining at kinetochores in *bub3*Δ cells may well have an essential role to play in checkpoint signaling. We propose that this pool is activated on unattached kinetochores and that the signal it produces is then amplified via soluble nonkinetochore-based pathways so that the "wait-anaphase" signal is transmitted throughout the mitotic machinery and cohesion is protected on all sister chromatids. This amplification is powerful enough to compensate for the reduced amount of Mad1p and Bub1p scaffolds activated on kinetochores in *bub3*Δ cells. It is possible that this amplification mechanism is particularly powerful in fission yeast, for example to compensate for the small number of kinetochores (six on three chromosomes).

Bub3p appears to work primarily by targeting Bub1p to kinetochores. We have previously demonstrated that ectopic targeting of Bub1p is sufficient to recruit both Bub3p and Mad3p to ectopic sites on chromosomes, such as telomeres (Rischitor *et al*., 2007). Here we have shown that the absence of Bub3p dramatically reduces levels of Bub1p, Mad3p, Mad1p, Mad2p, and the CPC at kinetochores of mitotically-arrested cells. We suspect that most of these effects are due to the loss of the Bub1p scaffold from kinetochores. To test this hypothesis directly, we have tried by several ways to target Bub1p to kinetochores independently of Bub3p. However Bub1p tethered to kinetochores throughout the cell-cycle appears to make fission yeast cells very sick, making this hypothesis particularly difficult to test (data not shown).

Biorientation Functions of Bub3p-Bub1p

Our data suggest that the targeting of the Bub1p-Bub3p complex to kinetochores is critical for efficient kinetochore biorientation after a cycle of microtubule de-polymerization/repolymerization. This is consistent with a recent study (Windecker *et al*., 2009). After microtubule de-polymerization/repolymerization, scattered chromosomes need first to be captured by microtubules (step 1) and then biorientated on the spindle (step 2). In a *bub3* mutant, there do not appear to be major kinetochore attachment defects (step 1), as we have shown in two independent assays that the retrieval of unclustered kinetochores takes place efficiently (Figure 4). However, we have shown that chromosome biorientation (step 2) is defective (Figure 5) and we believe that this function of the Bub1p-Bub3p complex is, at least in part, due to the malfunction of the Aurora-dependent error correction mechanism. Indeed (1) the localization on centromeres of Sgo2p and the CPC is significantly altered in the absence of Bub3p upon microtubule depolymerization (this study and Windecker *et al*., 2009), and (2) the mis-segregation phenotype is not enhanced by the concomitant loss of both Bub3p and Sgo2p (*bub3*Δ*sgo2*Δ mutant), suggesting that these proteins work in the same pathway.

Recently, it was reported that Bub1p and Bub3p have Sgo2-independent functions in kinetochore biorientation (Windecker *et al*., 2009). Our data do not exclude this possibility, and we believe that this is probably the case in an unperturbed mitosis. The rate of chromosome mis-segregation is greater in a *bub1* mutant than in a *sgo2* mutant, and *bub1* mutants exhibit lagging chromosomes in anaphase while *sgo2* mutants do not. Finally, *bub3*, but not *sgo2* mutants, have spindle checkpoint–silencing defects. Together, these observations show that Bub1p and Bub3p do indeed have Sgo2p-independent functions. Further experiments will be required to uncover the additional roles of Bub1p and Bub3p in chromosome biorientation.

CONCLUSION

Here we have explained how Bub3p contributes to the survival of fission yeast cells exposed to microtubule de-polymerization. Furthermore, we put forward the idea that, although potent APC inhibition can occur even if there is only very little Mad1p or Mad2p stably associated with kinetochores, their enrichment does enhance checkpoint activation and silencing reactions. It remains to be understood how these activation and silencing reactions are coordinated with chromosome biorientation.

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