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# Kinetochores Localization of Spindle Checkpoint Proteins: Who Controls Whom?<sup>□</sup>

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The spindle checkpoint prevents anaphase onset until all the chromosomes have successfully attached to the spindle microtubules. The mechanisms by which unattached kinetochores trigger and transmit a primary signal are poorly understood, although it seems to be dependent at least in part, on the kinetochores localization of the different checkpoint components. By using protein immunodepletion and mRNA translation in *Xenopus* egg extracts, we have studied the hierarchic sequence and the interdependent network that governs protein recruitment at the kinetochores in the spindle checkpoint pathway. Our results show that the first regulatory step of this cascade is defined by Aurora B/INCENP complex. Aurora B/INCENP controls the activation of a second regulatory level by inducing at the kinetochores the localization of Mps1, Bub1, Bub3, and CENP-E. This localization, in turn, promotes the recruitment to the kinetochores of Mad1/Mad2, Cdc20, and the anaphase promoting complex (APC). Unlike Aurora B/INCENP, Mps1, Bub1, and CENP-E, the downstream checkpoint protein Mad1 does not regulate the kinetochores localization of either Cdc20 or APC. Similarly, Cdc20 and APC do not require each other to be localized at these chromosome structures. Thus, at the last step of the spindle checkpoint cascade, Mad1/Mad2, Cdc20, and APC are recruited at the kinetochores independently from each other.

## INTRODUCTION

During cell division, accurate transmission of the genome is essential for survival. The entry into mitosis is controlled by checkpoints that monitor DNA damage and replication, whereas the exit of mitosis is controlled by checkpoints that monitor assembly and position of the mitotic spindle. The spindle assembly checkpoint restrains cells from entering anaphase until all replicated chromatids are correctly attached to the bipolar spindle. The major components of this surveillance mechanism, originally identified in budding yeast, include Mad1-3 (Li and Murray, 1991), Bub1-3 (Hoyt *et al.*, 1991; Roberts *et al.*, 1994), and Mps1 (Weiss and Winey, 1996). These proteins are conserved in all eukaryotic genomes sequenced to date except for Bub2 and Mad3. The functional ortholog of yeast Mad3 is the protein named BubR1, which seems to be a hybrid of yeast Mad3 and Bub1 proteins (Chen *et al.*, 1996; Li and Benezra, 1996; Taylor and McKeon, 1997; Jablonski *et al.*, 1998; Taylor *et al.*, 1998; Chan *et al.*, 1999; Martinez-Exposito *et al.*, 1999). In addition to these basic checkpoint components, other proteins such as CENP-E (Abrieu *et al.*, 2000; Yao *et al.*, 2000), Rod, ZW10 (Basto *et al.*, 2000; Chan *et al.*, 2000), Aurora B (Biggins and Murray, 2001; Kallio *et al.*, 2002b; Ditchfield *et al.*, 2003), and

mitogen-activated protein kinase play a role in the spindle checkpoint. Subcellular localization studies have placed all these checkpoint proteins at the kinetochores. Some of them (Mad1, Mad2, Bub1, Bub3, and BubR1) are present only in unattached but not in fully microtubule-attached kinetochores (Chen *et al.*, 1998; Waters *et al.*, 1998; Abrieu *et al.*, 2001; Skoufias *et al.*, 2001; Taylor *et al.*, 2001; Campbell and Hardwick, 2003). In this regard, real-time visualization in living cells of one of these proteins, Mad2, demonstrates that it is dynamically and exclusively associated with unattached kinetochores (Howell *et al.*, 2000). From these results, a model for the role of Mad2 in the generation of a delaying anaphase signal has been proposed. In this model, unattached kinetochores would generate an active diffusible signal (probably through Mad2 activation) that would prevent anaphase onset.

How this checkpoint signal is transmitted to induce metaphase arrest is not clear; however, it probably blocks entry into anaphase by inhibiting the proteolytic destruction of Securin, an anaphase inhibitor whose degradation is required for sister chromatid separation. Securin and other mitotic proteins are targeted for destruction by the covalent addition of ubiquitin (Alexandru *et al.*, 1999). This ubiquitination is mediated by the ubiquitin ligase anaphase promoting complex (APC) (Alexandru *et al.*, 1999). Activation of the APC at the metaphase-to-anaphase transition requires its association with the WD-motif containing protein Cdc20 (Sigrist and Lehner, 1997; Fang *et al.*, 1998; Kramer *et al.*, 1998; Lorca *et al.*, 1998). A first series of studies showed that Mad2 binds to Cdc20 and thereby inhibits APC/Cdc20 ubiquitination activity (Li *et al.*, 1997; Fang *et al.*, 1998; Hwang *et al.*, 1998; Kim *et al.*, 1998). These findings suggest that the different checkpoint proteins act upstream, at the unattached

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kinetochores, to convert Mad2 to its diffusible APC inhibitory form. However, two recent studies have demonstrated the presence of a complex capable of inhibiting APC/Cdc20 activity formed by different checkpoint proteins. This complex was found to inhibit APC/Cdc20 ubiquitination in vitro 3000-fold more efficiently than purified recombinant Mad2 alone (Sudakin *et al.*, 2001; Tang *et al.*, 2001). A major discrepancy between these two studies concerns the composition of this complex. Sudakin *et al.* (2001) reported the presence of a complex in HeLa cells that the authors named mitotic checkpoint complex (MCC) that contains an equivalent proportion of the proteins BubR1, Bub3, Cdc20, and Mad2. In contrast, the complex purified from the same type of cells by Tang *et al.* (2001) showed equal amounts of BubR1 and Bub3, a lower amount of Cdc20 and a completely absence of Mad2. Thus, it is not clear whether there is a single checkpoint pathway that induces a MCC signal to block APC/Cdc20 activity or whether this checkpoint pathway bifurcates into separate Mad2- and BubR1-dependent branches. Whatever the signal used by the checkpoint, it seems clear that kinetochore localization of these checkpoint proteins is essential, first for sensing chromosome-microtubule attachment and subsequently to inhibit the APC/Cdc20 complex.

The nature of the direct molecular interactions between checkpoint proteins and kinetochores is poorly understood. Similarly, the network of interactions and interdependencies of the different checkpoint proteins for kinetochore localization are only partly defined. Mad2 protein requires Mad1 to be correctly localized at the kinetochore (Chen *et al.*, 1998). Bub1 and BubR1 require Bub3 (Taylor *et al.*, 1998). *Xenopus* Bub1 is required for Bub3, Mad1, Mad2, and CENP-E localization (Sharp-Baker and Chen, 2001); *Xenopus* Mps1 is required for CENP-E, Mad1, and Mad2 localization (Abrieu *et al.*, 2001). Finally, immunodepletion of *Xenopus* BubR1 reduces the levels of Bub1, Bub3, Mad1, Mad2, and CENP-E (Chen, 2002). These results show that the localization of the different checkpoint proteins is interdependent on each other.

To better understand the sensor and the signaling pathways of the spindle checkpoint, in this study we have characterized the whole interdependent network that governs the timing and dependence of kinetochore localization of the different checkpoint components.

## MATERIALS AND METHODS

### Isolation of Survivin cDNA, Immunization Procedures, and In Vitro Production of mRNAs

*Xenopus* Survivin cDNA was amplified from total mRNA of *Xenopus* metaphase II-arrested eggs by using the primers 5'ATGTATTCTGCCAAGAA-CAGG3' and 5'GTGGTCGAGATCTATGGAGCAC3'.

The *Xenopus* affinity-purified anti-Aurora B, anti-Cdc20, anti-CDC27, and anti-Mad2 antibodies have been described previously (Lorca *et al.*, 1998; Castro *et al.*, 2002). Anti-CENP-A and anti-INCENP antibodies were kindly provided by Dr. P.T. Stukenberg (University of Virginia, Medical School, Charlottesville, VA) (McClelland *et al.*, 2003), and Dr. S. Dimitrov (Institut Allbert Bonniot, La Tronche, France), respectively. Anti-NTD Mps1 antibodies, against the N-terminal domain of *Xenopus* Mps1 (residues 1–626) and anti-NP Mps1 antibodies produced against the peptide MDDEDISERKLIKIA (residues from 1–14 of *Xenopus* Mps1) were obtained as described previously (Abrieu *et al.*, 2001). CENP-E was detected and immunodepleted by using anti-tail CENP-E polyclonal antibodies (Wood *et al.*, 1997). Anti *Xenopus* Bub1 antibodies were generated against a glutathione S-transferase fusion protein corresponding to the N-terminal domain of this protein (residues 1–850). Purified fusion protein was used to immunize rabbits and serum was affinity purified on immobilized Bub1 fusion protein. In some preliminary experiments, an anti-Bub1 antibody kindly provided by Dr. J. Maller (University of Colorado School of Medicine, Denver, CO) was used. Finally, rabbit polyclonal antibodies anti-*Xenopus* Bub3 and Mad1 were generated against two peptides (MNTQDMDTGSNE and MDDSEDNTTVIS, respectively) corre-

sponding to the N-terminal sequence of these proteins. Peptides were coupled to thyroglobulin by using *m*-maleimidobenzoyl-*N*-hydroxysulfosuccinimide ester for immunization and to immobilized bovine serum albumin for affinity purification.

mRNAs encoding full-length wild-type forms of *Xenopus* Bub1 and Mps1 were transcribed in vitro with T7 or SP6 RNA polymerase.

### Preparation of *Xenopus* Egg Extracts, Immunofluorescence, and Immunoprecipitation

CSF and extracts were prepared from unfertilized *Xenopus* eggs that were arrested at metaphase of the second meiotic division by CSF. The CSF extract and demembrated sperm nuclei were prepared as described previously (Murray, 1991).

For immunofluorescence staining of unreplicated chromosomes, 20  $\mu$ l of CSF extract was incubated with nocodazole (final concentration 10  $\mu$ g/ml) and sperm nuclei (2000/ $\mu$ l extract) for 75 min at room temperature. The chromosomes were isolated and processed for immunofluorescent staining as described previously (Abrieu *et al.*, 2001). When the immunofluorescence was performed under spindle checkpoint conditions, 9000 sperm nuclei/ $\mu$ l instead of 2000/ $\mu$ l were used.

For immunodepletion, 50  $\mu$ g of affinity-purified antibodies (anti-Aurora B, anti-INCENP, anti-NP Mps1, anti-Bub1, anti-CENP-E, anti-Mad1, anti-Cdc20, and anti-Cdc27) or nonimmune rabbit IgG was bound to 250  $\mu$ l of Dynal beads protein A for 30 min at 4°C and then added to 250  $\mu$ l of CSF extract for 1 h at 4°C. When anti-Bub1, anti-CENP-E, anti-Cdc27, and anti-INCENP antibodies were used, three successive immunodepletions of 30 min each were performed to completely remove these two endogenous proteins.

For the rescue experiments, Mps1, Bub1, or Aurora B immunodepleted CSF egg extracts were supplemented with dithiothreitol (1 mM), RNAGuard (0.4 U/ $\mu$ l extract; Amersham Biosciences, Piscataway, NJ), tRNA (0.1  $\mu$ g/ $\mu$ l), and the corresponding mRNA (0.05  $\mu$ g/ $\mu$ l extract) and incubated for 2 h at 20°C. Expression of the corresponding wild-type proteins was verified by immunoblotting. When Survivin mRNA was used, [<sup>35</sup>S]methionine (AGQ0080; Amersham Biosciences) was added to the extract (1.5  $\mu$ l of [<sup>35</sup>S]methionine/20  $\mu$ l extract) to allow translated Survivin detection.

### Light Microscopy

A DMR A Leica microscope with a 100 $\times$  immersion oil objective (HCX PL APO), tube factor 1 was used for epifluorescence imaging (A4, GFP, N2.1 cube filters, excitation HBO light bulb). Images were captured with a Roper MicroMax 1300 Y/HS camera, and the whole set was driven by MetaMorph (Universal Imaging, Downingtown, PA).

When confocal microscopy was developed, a DMR B Leica microscope with a 100 $\times$  immersion oil objective (HCX PL APO), tube factor 1.6 was used. Confocal fluorescence imaging was obtained with a PerkinElmer Ultraview system fitted with Sutter filter wheels, excitation was obtained with a double band Ar-Kr Melles Griot laser (488–568 nm). Images were captured with a Photometrics CoolSnap HQ camera, and the whole setup was driven by MetaMorph.

### H1 Kinase Assay

Extract (1  $\mu$ l) was frozen in liquid nitrogen at the indicated times. Extract samples were then thawed by the addition of 9  $\mu$ l of H1 buffer including [ $\gamma$ -<sup>32</sup>P]ATP (Chen and Murray, 1997) and incubated for 10 min at room temperature. Reactions were stopped by adding Laemmli gel sample buffer and analyzed by SDS-PAGE.

## RESULTS

### Aurora B/INCENP Is Required for the Maintenance of the Spindle Checkpoint by Inducing the Recruitment of Mps1, Bub1, CENP-E, Bub3, Mad1, and Mad2 to the Kinetochores

Aurora B, in association with the proteins INCENP and Survivin is localized in late prophase and metaphase at the inner centromere where it has been implicated in the regulation of the spindle checkpoint. The exact role of Aurora B in this pathway is confusing. In budding yeast, the Aurora B ortholog Ipl1 seems to participate in the spindle checkpoint activation by loss of tension at centromeres (Biggins and Murray, 2001; Tanaka *et al.*, 2002). However, this protein seems to monitor both, microtubule attachment and tension in higher eukaryotes (Kallio *et al.*, 2002b; Ditchfield *et al.*, 2003; Hauf *et al.*, 2003). Moreover, despite the fact that kinetochore localization of BubR1 clearly seems to be regulated by Aurora B, there are contradictory results concerning

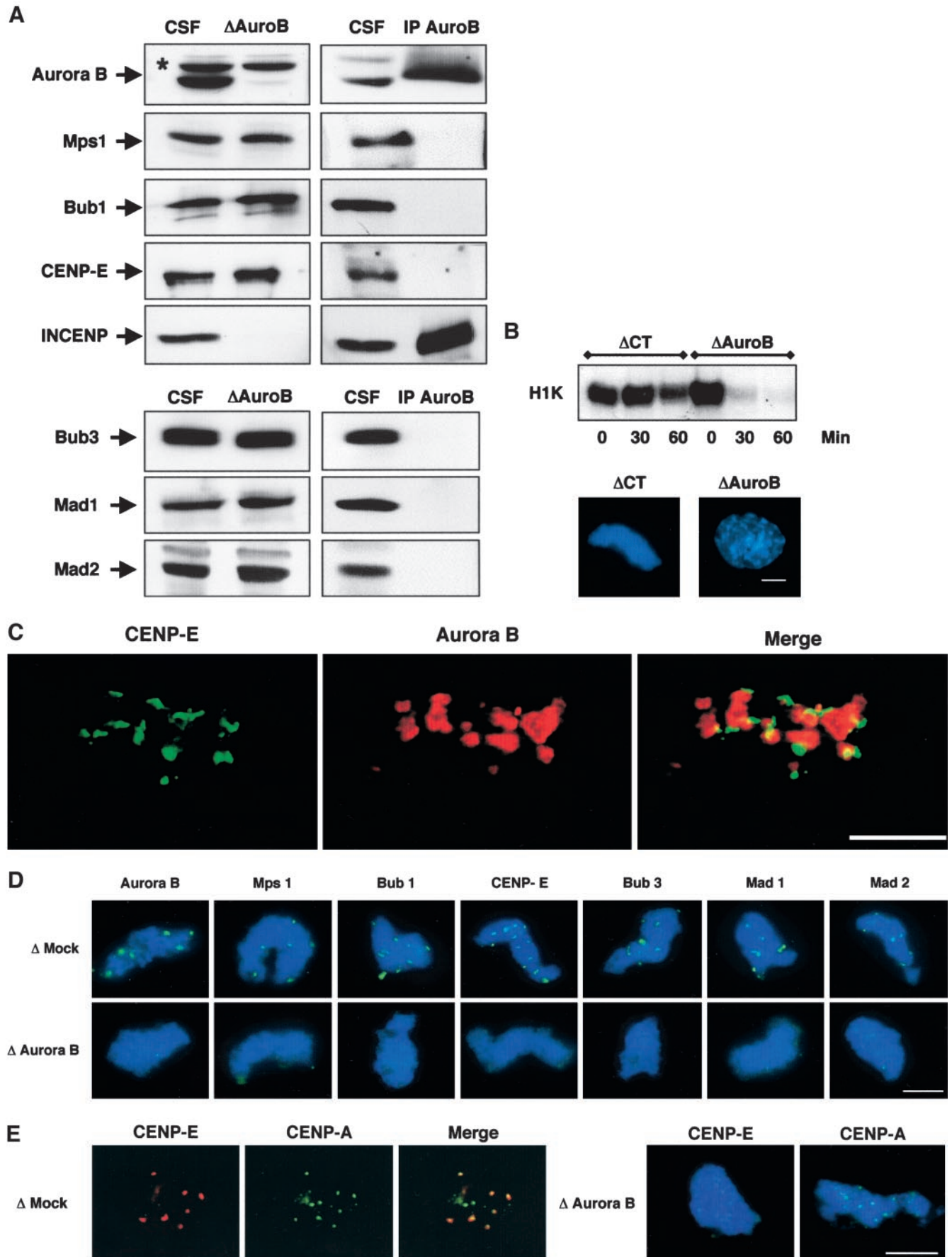
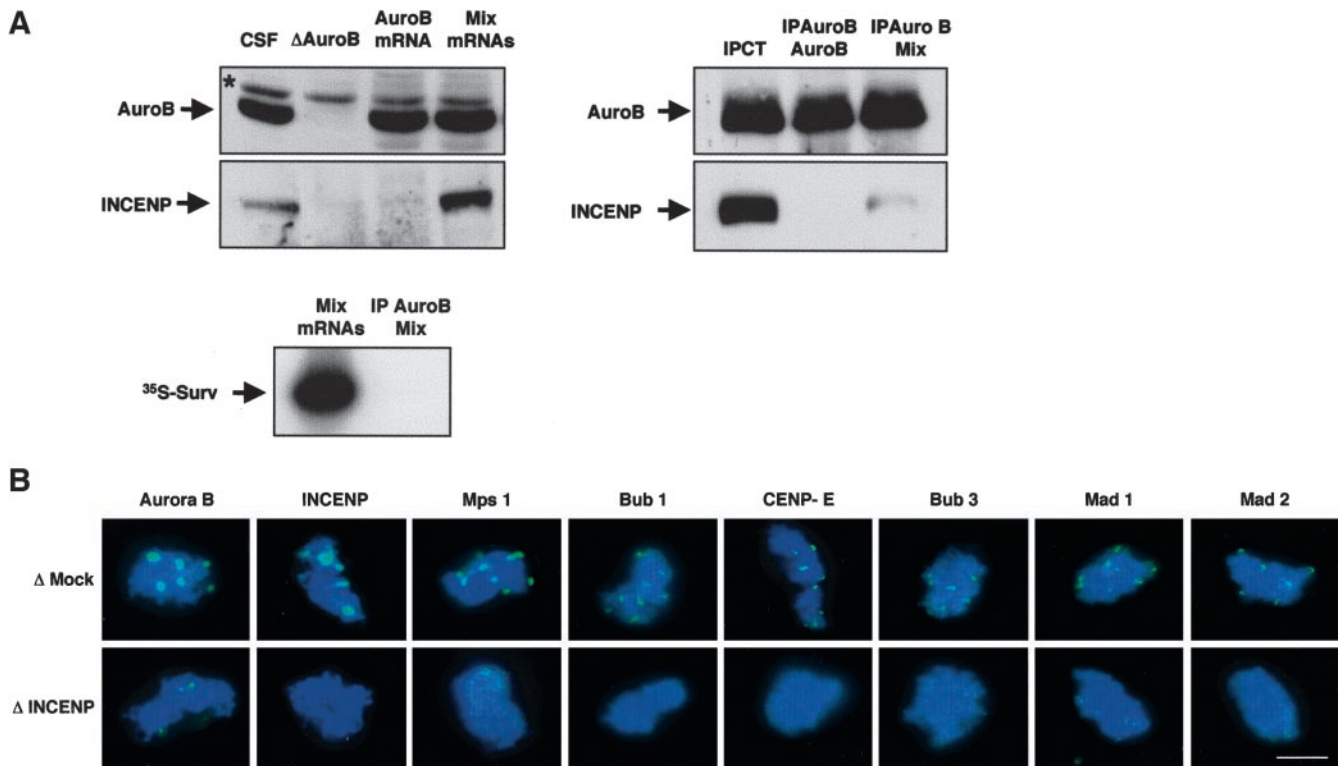


Figure 1.





**Figure 2.** The complex Aurora B/INCENP regulates the kinetochore localization of all the other checkpoint proteins under spindle checkpoint conditions. (A) Aurora B-immunodepleted CSF extracts ( $\Delta$ AuroB) were supplemented with either Aurora B, INCENP, or Survivin mRNAs. A sample of 1  $\mu$ l of the Aurora B mRNA-supplemented extract was taken to analyze the levels of Aurora B and INCENP by Western blot (Aurora B mRNA). Another sample of 5  $\mu$ l of Aurora B-translated extract was immunoprecipitated with anti-Aurora B antibodies, and the immunoprecipitate was used to evaluate the levels of Aurora B and INCENP (IP AuroB AuroB). Finally, a third sample of the Aurora B-translated CSF extract was mixed with an equal volume of INCENP and Survivin-translated CSF extracts (Mix mRNAs). One microliter of the mix was used to analyze the levels of Aurora B, INCENP (Western blot), and Survivin (autoradiography), and 5  $\mu$ l of the same mix was immunoprecipitated with anti-Aurora B antibodies to analyze the association of Aurora B, INCENP, and Survivin (IP AuroB Mix). (B) CSF extracts supplemented with nocodazole and sperm nuclei (9000/ $\mu$ l) were immunodepleted of INCENP, incubated for 75 min, and used for immunofluorescence staining to analyze the kinetochore localization of the Aurora B, INCENP, Mps1, Bub1, CENP-E, Bub3, Mad1, and Mad2 proteins.

the regulation by this kinase of the localization of CENP-E and Mad2 at the centromeres (Kallio *et al.*, 2002b; Ditchfield *et al.*, 2003; Hauf *et al.*, 2003). To determine the interdependence network of Aurora B with the other checkpoint components, we took advantage of the techniques of immunodepletion and rescue of different spindle checkpoint proteins developed in *Xenopus* extracts obtained from metaphase II-arrested eggs (CSF extracts) (Chen and Murray,

1997), and we studied the implication of Aurora B in the regulation of the kinetochore localization of Mps1, Bub1, CENP-E, Bub3, Mad1, and Mad2 under spindle checkpoint conditions. To that, we supplemented Aurora B-depleted CSF extracts with nocodazole and 9000 sperm nuclei per microliter of extract. A sample of this mix was used to examine the consequence of Aurora B depletion on the establishment of the spindle checkpoint by measuring H1

**Figure 1 (facing page).** Aurora B is required for the maintenance of the spindle checkpoint by the recruitment of Mps1, Bub1, CENP-E, Bub3, Mad1, and Mad2 to kinetochores. (A) Cytostatic factor (CSF) egg extracts were immunodepleted with anti-Aurora B antibodies. A sample of 1  $\mu$ l of CSF extract (CSF) or of immunodepleted supernatant ( $\Delta$ AuroB), as well as the Aurora B immunoprecipitate corresponding to 5  $\mu$ l of CSF extract (IP AuroB) were then taken to analyze by Western blotting the endogenous levels of Aurora B, Mps1, Bub1, CENP-E, INCENP, Bub3, Mad1, and Mad2 proteins. \* represents an unspecific band recognized by the anti-Aurora B antibodies (B) CT ( $\Delta$ CT) and Aurora B-immunodepleted ( $\Delta$ AuroB) CSF extracts were supplemented with 9000 sperm per microliter of extract and nocodazole. After a 75-min incubation, a sample was taken to measure H1 kinase activity (H1K) at 0, 30, and 60 min after calcium addition (0.4 mM) and chromatin condensation at 60 min after calcium addition. (C) Isolated chromosomes from nocodazole-treated CSF extracts used in A were double stained with anti-CENP-E and anti-Aurora B antibodies and analyzed by confocal microscopy. Anti-CENP-E antibody labeled with biotin-(long arm)-NHS was used and detected with streptavidin-AlexaFluor. Anti-Aurora B immunostaining was visualized by using anti-rabbit rhodamine secondary antibody (D) CT ( $\Delta$ Mock) and Aurora B ( $\Delta$ AuroB) immunodepleted CSF extracts supplemented with nocodazole, and sperm nuclei used in A were incubated during 75 min and subsequently used to analyze kinetochore localization of Aurora B, Mps1, Bub1, CENP-E, Bub3, Mad1, and Mad2 by immunostaining. Locations of the different proteins were visualized with anti-rabbit fluorescein isothiocyanate (FITC) secondary antibody (dilution 1/200; Vector Laboratories, Burlingame, CA) and image merged with chromatin (0.5  $\mu$ g/ $\mu$ l 4,6-diamidino-2-phenylindole). (E) CENP-E and CENP-A were coimmunostained in chromosomes isolated from control ( $\Delta$ Mock, CENP-E, CENP-A, and Merge) and anti-Aurora B ( $\Delta$  Aurora B, CENP-E, and CENP-A)-immunodepleted CSF extracts by using anti-rabbit rhodamine and anti-chicken FITC (dilution 1:100; Zymed Laboratories, South San Francisco, CA) secondary antibodies, respectively. Location of these two proteins in Aurora B-immunodepleted CSF extracts was imaged merged with chromatin. Bars, 5  $\mu$ m.

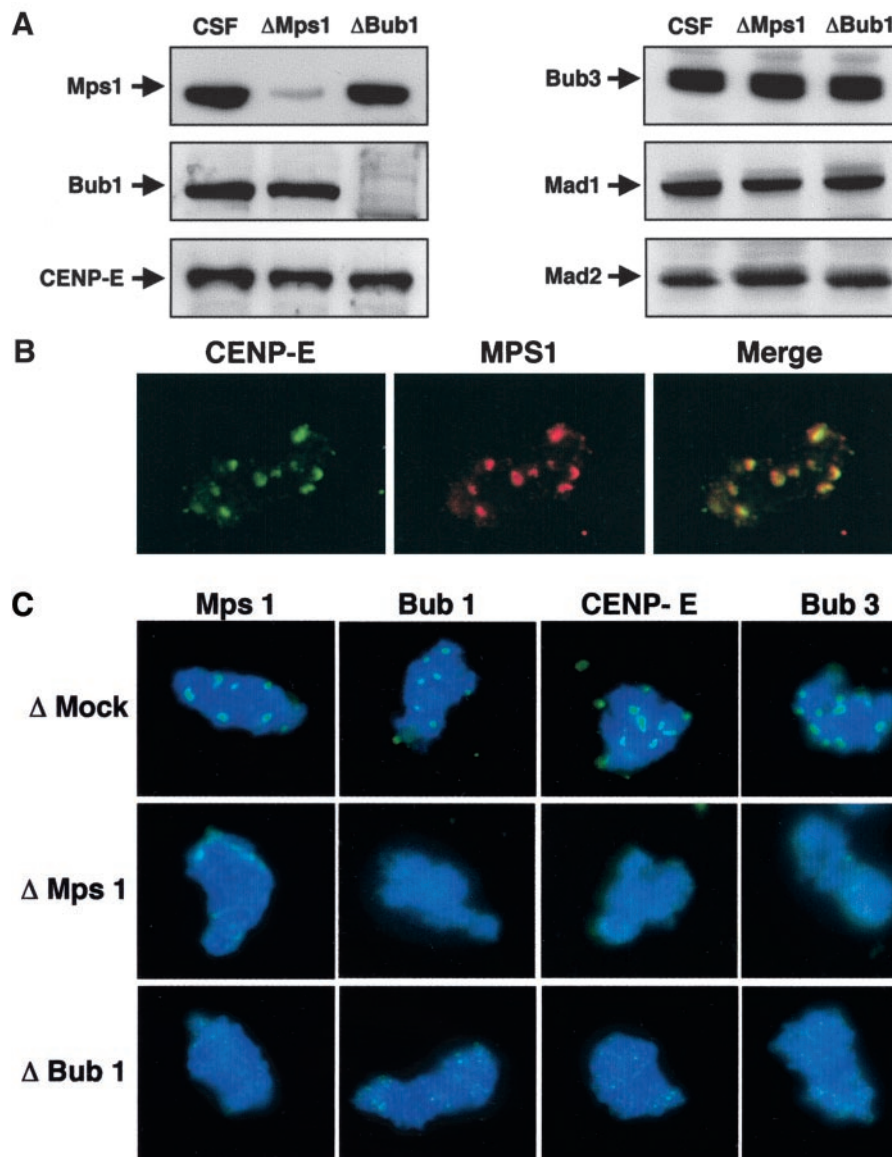
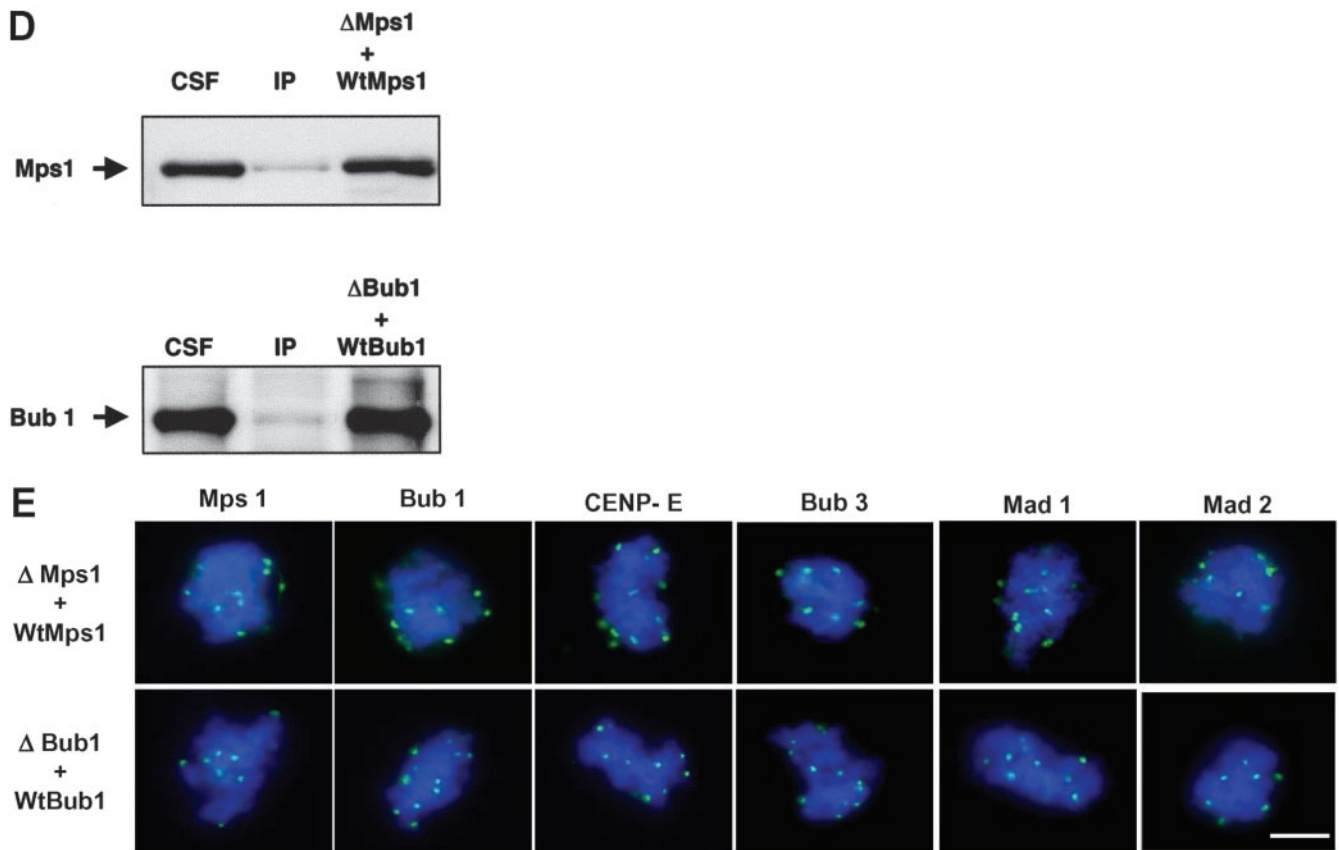


Figure 3.

kinase activity and by assessing chromatin condensation after calcium addition. Another sample was used to isolate chromosomes and to study the kinetochore localization of Mps1, Bub1, CENP-E, Bub3, Mad1, and Mad2. Immunodepletion of Aurora B removed >95% of this kinase and of the Aurora B-associated protein INCENP but did not immunoprecipitate either of the other checkpoint proteins (Figure 1A, IP AuroB). Accordingly, the endogenous levels of these checkpoint proteins were not affected (Figure 1A,  $\Delta$ AuroB). As shown in Figure 1B, control-depleted CSF extracts presented an active spindle checkpoint because the H1 kinase activity remained constant and the chromatin condensed despite the addition of calcium (Figure 1B,  $\Delta$ CT). In contrast, depletion of Aurora B induced a decrease of the H1 kinase activity and a clear decondensation of chromatin indicative of a loss of the mitotic checkpoint (Figure 1B,  $\Delta$ AuroB). Thus, according to the results of Kallio *et al.* (2002b), our data show that Aurora B is required to maintain the spindle checkpoint in *Xenopus* egg extracts. We next ana-

lyzed whether the failure of the spindle checkpoint in Aurora B-immunodepleted CSF extracts was associated with a modification of the localization pattern of the different checkpoint proteins. We first tested the specificity of our antibodies by performing a colocalization analysis of CENP-E and Aurora B. In agreement with published data, the double immunostaining revealed a localization of Aurora B at the inner centromeres (Adams *et al.*, 2001; Cleveland *et al.*, 2003), confirming the specificity of our antibodies (Figure 1C). Moreover, the analysis of the localization of Mps1, Bub1, CENP-E, Bub3, Mad1, and Mad2 in Aurora B-immunodepleted CSF extracts revealed a loss of the kinetochore binding of all these proteins (Figure 1D,  $\Delta$ Aurora B). The absence of this association was not the result of a destruction of the basic centromere organization because the dot-staining pattern of the centromere marker CENP-A was not disrupted in Aurora B-depleted extracts (Figure 1E,  $\Delta$ Mock and  $\Delta$ Aurora B). Altogether, these results indicate that Aurora B association to the kinetochores is required to

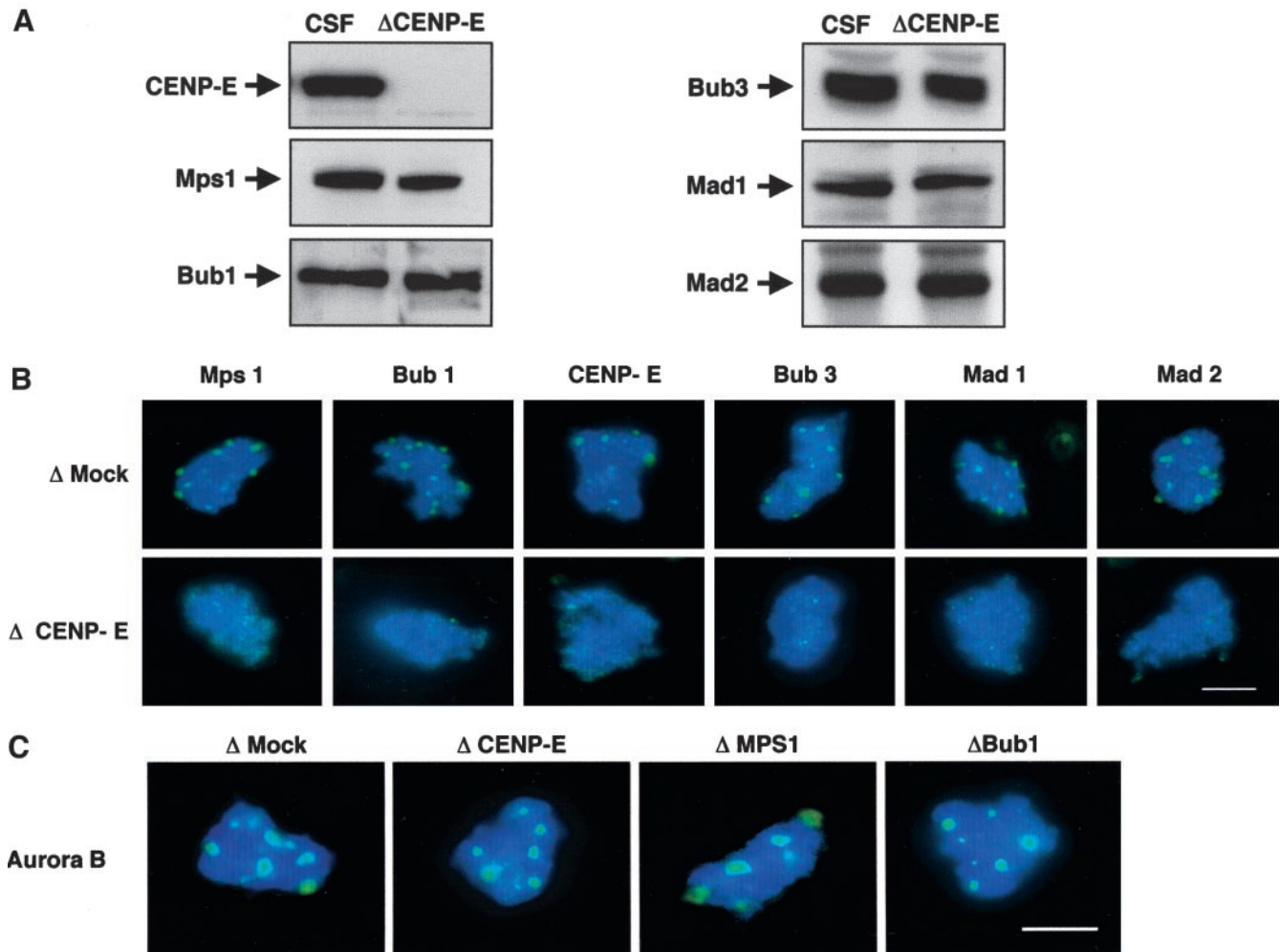


**Figure 3 (cont).** Bub1 and Mps1 are dependent on each other for their kinetochore localization and the localization of the other checkpoint proteins. (A) CSF extracts (250  $\mu$ l) were depleted either with control (CSF), anti-Mps1 ( $\Delta$ Mps1), or anti-Bub1 ( $\Delta$ Bub1) antibodies (50  $\mu$ g). A sample of 1  $\mu$ l was then taken and endogenous Mps1, Bub1, CENP-E, Bub3, Mad1, and Mad2 levels were analyzed by Western blotting. (B) Anti-CENP-E (CENP-E), anti-Mps1 (Mps1), and costaining of both antibodies (Merge) (top row) and anti-CENP-E (CENP-E), anti-Bub1 (Bub1), and costaining of both antibodies (Merge) (bottom row) in chromosomes purified from CSF egg extracts. Anti-CENP-E antibody was labeled with biotin-(long arm)-NHS and detected using streptavidin-AlexaFluor (dilution 1/1000; Molecular Probes, Eugene, OR). Anti-Mps1 and anti-Bub1 immunostaining were visualized by using anti-rabbit rhodamine secondary antibody (dilution 1/200; Cappel Laboratories, Durham, NC) (C) The same immunodepleted CSF extracts with control ( $\Delta$ Mock), anti-Mps1 ( $\Delta$ Mps1), and anti-Bub1 ( $\Delta$ Bub1) antibodies used in A were supplemented with nocodazole and sperm nuclei (2000/ $\mu$ l) and incubated for 75 min at room temperature. Chromosomes were then isolated and processed for immunofluorescent staining with anti-Mps1 (Mps1), anti-Bub1 (Bub1), anti-CENP-E (CENP-E), anti-Bub3 (Bub3), anti-Mad1 (Mad1), or anti-Mad2 (Mad2) antibodies. Locations of the different proteins were visualized with anti-rabbit fluorescein isothiocyanate secondary antibody (dilution 1/200; Vector Laboratories) and image merged with chromatin (0.5  $\mu$ g/ $\mu$ l 4,6-diamidino-2-phenylindole). (D) CSF extracts were first immunodepleted with anti-Mps1 or anti-Bub1 antibodies as in A and subsequently supplemented with the mRNAs coding for the wild-type form of these proteins. A sample of 1  $\mu$ l was taken before immunodepletion (CSF), after immunodepletion (IP), and after the translation of the wild-type form of Mps1 and Bub1 ( $\Delta$ Mps1+WtMps1 and  $\Delta$ Bub1+WtBub1, respectively), and the levels of these two proteins were analyzed by immunoblotting. (E) Egg extracts immunodepleted and mRNA translated in D were supplemented with nocodazole and sperm nuclei (2000/ $\mu$ l). Chromosomes were then isolated and used to analyze Mps1, Bub1, CENP-E, Bub3, Mad1, and Mad2 by immunofluorescent staining as described in C. Bars, 5  $\mu$ m.

maintain checkpoint activation by allowing the localization of the other checkpoint proteins to these chromosome structures, placing Aurora B at the most upstream protein in the spindle checkpoint pathway. However, even if Aurora B immunodepletion did not affect the endogenous levels of the other checkpoint proteins, we wanted to test whether the effect of this immunodepletion was specific to the sole removal of Aurora B. To test that, we analyzed the localization of the different checkpoint proteins in Aurora B-depleted egg extracts where the levels of this kinase were restored by the addition of its mRNA. Surprisingly, despite the fact that the ectopic translation of this mRNA induced protein levels comparable with the endogenous kinase (Figure 2A, AuroB mRNA), they did not restore its kinetochore localization (unpublished data).

To explain these results, we hypothesized that because Aurora B is associated to the kinetochores as a complex with INCENP and Survivin, and because the depletion of Aurora B in CSF extracts induces the removal of INCENP and vice versa (Figure 1A and supplementary data Figure S1), the effect of Aurora B immunodepletion is likely not to be due to the removal of Aurora B but to the removal of the complex Aurora B/INCENP/Survivin. If this is the case, the immunodepletion of INCENP in CSF extracts also should perturb the localization of the different checkpoint proteins to the kinetochores. Actually, this was the case, because this immunodepletion also prevented the kinetochore loading of all the other checkpoint proteins, including Aurora B (Figure 2B,  $\Delta$ INCENP).





**Figure 4.** Mps1, Bub1, and CENP-E act together at the same upstream level of the spindle checkpoint pathway. (A) CSF egg extracts were immunodepleted with control (CSF) or anti-CENP-E ( $\Delta$ CENP-E) antibodies. A sample of 1  $\mu$ l was then taken to analyze by Western blotting the endogenous levels of CENP-E, Mps1, Bub1, Bub3, Mad1, and Mad2 proteins. (B) The immunodepleted extracts obtained in A were supplemented with nocodazole and sperm nuclei (2000/ $\mu$ l) and used for immunofluorescence staining to analyze the kinetochore localization of the Mps1, Bub1, CENP-E, Bub3, Mad1, and Mad2 proteins. (C) Kinetochore localization of Aurora B was analyzed by immunostaining in chromosomes isolated from control ( $\Delta$ Mock), anti-CENP-E ( $\Delta$ CENP-E), anti-Mps1 ( $\Delta$ MPS1), and anti-Bub1 ( $\Delta$ Bub1) immunodepleted CSF extracts. Bars, 5  $\mu$ m.

We next tried to rescue the phenotype observed by either Aurora B or INCENP-immunodepletion by supplementing Aurora B-depleted egg extracts simultaneously with the mRNAs coding for Aurora B, INCENP, and Survivin. The three proteins were cotranslated at a similar amount to the endogenous levels (Figure 2A, Mix mRNAs), but, again, they did not restore Aurora B kinetochore localization. We then tested whether the Aurora B/INCENP/Survivin complex formation took place in these conditions by analyzing the amount of INCENP and Survivin present in Aurora B immunoprecipitates. Neither INCENP nor Survivin were detected in these immunoprecipitates (Figure 2A, IPAuroB Mix), indicating that one or more unknown components are probably required to induce the formation of this complex and thus to allow its kinetochore localization.

Thus, the complex containing Aurora B/INCENP and probably also Survivin and other unknown proteins acts as the most upstream regulator of the spindle checkpoint pathway, allowing the loading of all the other checkpoint proteins to the kinetochores.

#### *Mps1, Bub1, and CENP-E Are Dependent upon Each Other to Induce Their Kinetochore Localization and the Localization of the Other Spindle Checkpoint Components*

The results mentioned above show that the checkpoint proteins Mps1 and Bub1 act downstream the Aurora B/INCENP complex in the spindle checkpoint pathway. Moreover, the results obtained in other laboratories indicate that Mps1 and Bub1 act upstream in the spindle checkpoint pathway because they control the kinetochore localization of CENP-E, Bub3, Mad1, and Mad2 (Abrieu *et al.*, 2001; Sharp-Baker and Chen, 2001). However, despite the fact that both kinases act at the same level of the spindle checkpoint pathway, no data exist about the interdependence on each other.

To study this interdependence, we removed either Mps1 or Bub1 from CSF extracts by immunodepletion before the addition of nocodazole and sperm nuclei. Subsequently, chromosomes were isolated and the kinetochore localization of the different spindle checkpoint proteins analyzed. Anti-Mps1 and anti-Bub1 immunodepletion removed >95% of these two proteins without affecting the bulk of the endog-



enous Bub1/Mps1, CENP-E, Bub3, Mad1, or Mad2, respectively (Figure 3A,  $\Delta$ Mps1 and  $\Delta$ Bub1).

Both anti-Mps1 and anti-Bub1 staining colocalize with the kinetochore staining of anti-CENP-E antibodies, indicating that they specifically recognize these two proteins (Figure 3B). As expected, immunodepletion of CSF extracts with control antibodies did not affect the kinetochore localization of the different checkpoint proteins (Figure 3C,  $\Delta$ Mock). According to our previous results (Abrieu *et al.*, 2001), in Mps1-depleted egg extracts, kinetochore staining of CENP-E, Mad1, and Mad2 completely disappeared. However, in contradiction to Liu *et al.* (2003), we also observed a disappearance of Bub1 from these chromosomal structures, results that were confirmed by the concomitant loss in these extracts of the kinetochore localization of Bub3 (Figure 3C,  $\Delta$ Mps1).

As previously described by Sharp-Baker and Chen (2001), depletion of Bub1 from egg extracts prevented CENP-E, Bub3, Mad1, and Mad2 from localizing to the kinetochores (Figure 3C,  $\Delta$ Bub1). However, in addition, we also observed a removal of Mps1 from these chromosome structures. These results indicate that both Mps1 and Bub1 are dependent upon each other to induce their robust kinetochore localization and the localization of the other downstream spindle checkpoint proteins.

To determine whether the loss of kinetochore staining of the different analyzed checkpoint proteins was the consequence of the removal of Mps1 or Bub1, we translated the mRNAs for the wild-type forms of these two proteins in the Mps1- and Bub1-depleted extracts, respectively. The levels of translated wild-type Mps1 and Bub1 were equivalent to those observed for the endogenous proteins in nondepleted egg extracts (Figure 3D, CSF vs. IP vs.  $\Delta$ Mps1+Wt and  $\Delta$ Bub1 + Wt, respectively). As shown in Figure 3E ( $\Delta$ Mps1+Wt and  $\Delta$ Bub1+Wt), the restoration of Mps1 and Bub1 levels induced kinetochore relocalization of all the analyzed checkpoint proteins.

Thus, these results demonstrate that Bub1 and Mps1 are dependent on each other for their kinetochore localization.

Besides Mps1 and Bub1, the kinesin-like protein CENP-E also is required in *Xenopus* egg extracts for the checkpoint activation and maintenance (Abrieu *et al.*, 2000). It has been demonstrated that Mps1 and Bub1 regulate CENP-E kinetochore localization. Moreover, up to now only a CENP-E-dependent regulation of two late components of the spindle checkpoint, Mad1 and Mad2, has been described. Thus, despite the fact that the regulation of the kinetochore association of Mps1 and Bub1 by CENP-E has not been analyzed, it has been assumed that CENP-E is a downstream factor of the spindle checkpoint pathway. To investigate whether there is a regulation of these two upstream checkpoint proteins by CENP-E, we examined kinetochore localization of Bub1 and Mps1 in CSF extracts where CENP-E was removed before the addition of nocodazole and sperm nuclei. Immunodepletion of CENP-E removed nearly 100% of the endogenous protein without affecting the levels of Mps1, Bub1, Bub3, Mad1, or Mad2 (Figure 4A). Analysis of kinetochore staining showed a complete disappearance of the immunofluorescence signal of Mps1, Bub1, CENP-E, Bub3, Mad1, and Mad2 in anti-CENP-E depleted extracts (Figure 4B,  $\Delta$ CENP-E), whereas all these proteins localized at the kinetochores when the extracts were depleted with mock antibodies (Figure 4B,  $\Delta$ Mock). The removal of these proteins from these chromosomal structures is not the result of a failure of kinetochore assembly, because as we have previously demonstrated, XKCM1, another known centromere component, is normally localized in these depleted extracts

(Abrieu *et al.*, 2001). These results suggest that the kinetochore localization of Mps1, Bub1 and CENP-E depend on each other and that they act together at the same upstream level of the spindle checkpoint pathway.

At least five proteins are required upstream in the spindle checkpoint pathway to induce correct kinetochore localization of the other checkpoint components: Aurora B, INCENP, Mps1, Bub1, and CENP-E. The results presented above demonstrate that Mps1, Bub1, and CENP-E act at the same step in the checkpoint pathway. However, we do not know whether Aurora B/INCENP complex acts together with or upstream of these three checkpoint proteins. To investigate this question, we analyzed the centromere association of Aurora B in CSF extracts depleted of CENP-E, Mps1, or Bub1. As shown in Figure 4C, immunodepletion either of CENP-E, Mps1, or Bub1 did not induce removal of Aurora B from the kinetochores, indicating that this protein acts upstream of the Mps1/Bub1/CENP-E-dependent step of the spindle checkpoint pathway.

#### **Regulation of the Kinetochore Localization of Cdc20 and the APC Proteins Cdc27 and Cdc23 by the Spindle Checkpoint**

The mechanism by which the spindle checkpoint may induce APC/Cdc20 inhibition before alignment of the chromosomes at the metaphase plate is not known. Accumulating data lead to a model in which unattached kinetochores foster the assembly of inhibitory proteins that will subsequently control APC/Cdc20 activity. The exact cellular localization at which these inhibitory proteins will block APC/Cdc20 is not known, although it seems likely that both unattached chromosome detection and APC inhibition could be performed at the kinetochores. In agreement with this hypothesis, it has been shown that the APC regulator Cdc20 is localized at the kinetochores from prometaphase to telophase (Kallio *et al.*, 1998, 2002a; Raff *et al.*, 2002). Moreover, a concentration of Cdc27 (APC3), APC1, and APC10, three components of the APC complex, at these chromosomal structures also has been reported (Jorgensen *et al.*, 1998; Kurasawa and Todokoro, 1999; Topper *et al.*, 2002). The fact that APC/Cdc20 could be inhibited at the centromeres, raises the intriguing possibility that the spindle checkpoint may modulate the metaphase-to-anaphase transition not only by regulating APC/Cdc20 activity but also by controlling APC/Cdc20 localization. We examined the latter point by analyzing kinetochore localization of Cdc20, Cdc27 (APC3), and Cdc23 (APC8) in CSF extracts that have been depleted of Aurora B, Mps1, Bub1, CENP-E, and Mad1 checkpoint proteins before the addition of nocodazole and sperm nuclei (Figures 1A, 3B, 4A, and supplementary data Figure S2). The results of the double immunostaining analysis with either anti-Cdc20, anti-Cdc27, or anti-Cdc23 and anti-CENP-E antibodies demonstrate a kinetochore localization of these proteins in purified chromosomes from egg extracts (Figure 5A). Moreover, as shown in Figure 5B, the depletion of CSF extracts with control antibodies ( $\Delta$ Mock) did not affect the kinetochore localization of Cdc27, Cdc20, and Cdc23, whereas removal of Aurora B, Mps1, Bub1, and CENP-E ( $\Delta$ Aurora B,  $\Delta$ Mps1,  $\Delta$ Bub1, and  $\Delta$ CENP-E, respectively) completely inhibited centromere association of these three proteins. Unlike Aurora B, Mps1, Bub1, and CENP-E depletion, Cdc27, Cdc20, and Cdc23 remained at the kinetochores when Mad1 was removed from the CSF extracts. These results indicate that the upstream spindle checkpoint proteins Aurora B, Mps1, Bub1, and CENP-E are required to induce the correct kinetochore localization of Cdc20, Cdc27 and Cdc23 during prometaphase and that Mad1 does not participate in this regulation.

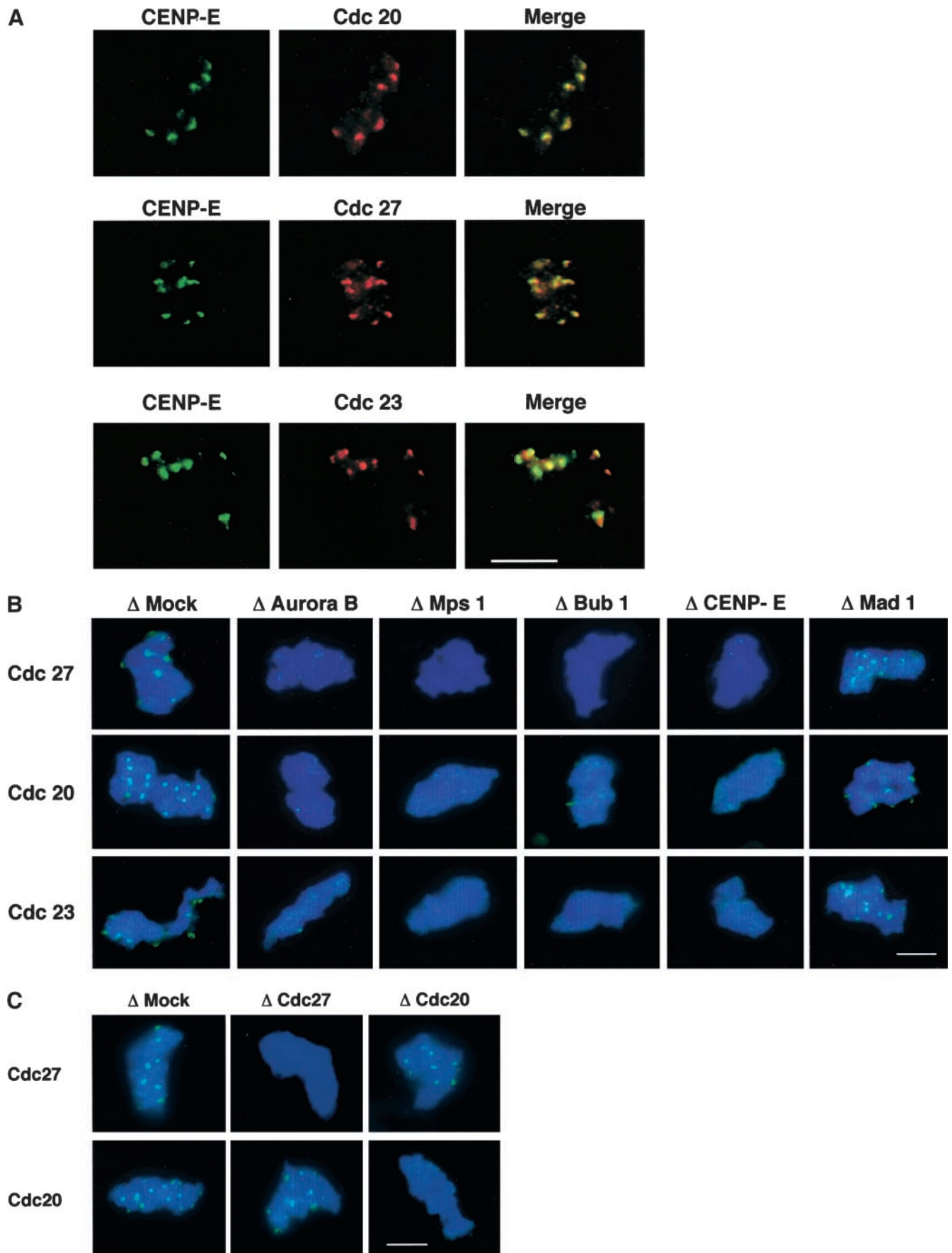


Figure 5.

We next investigated whether the APC modulator Cdc20 and the APC complex itself could regulate each other their association with the kinetochores. To answer this question, we immunodepleted either Cdc20 or Cdc27 from CSF extracts (supplementary data Figure S3) and subsequently we analyzed the presence of the different checkpoint proteins at the kinetochores. As shown in Figure 5C, removal of either Cdc27 or Cdc20 had no effect on the centromere association of Cdc20 and Cdc27, respectively; moreover, they did not affect the localization of any other of the proteins of this pathway (unpublished data). Thus, Cdc20 and Cdc27 localize independently of each other to the kinetochores. Moreover, Aurora B/INCENP, Mps1, Bub1, and CENP-E, the upstream components of the spindle checkpoint and not Mad1, a late component of this pathway, regulate both localizations.

## DISCUSSION

The spindle checkpoint blocks the metaphase-to-anaphase transition when kinetochores fail to bind spindle microtubules or are not under tension. The signal induced by this checkpoint stimulates recruitment at the kinetochores of several spindle checkpoint proteins, including Mps1, Bub1, Bub3, BubR1, CENP-E, Mad1, and Mad2 (for review, see Musacchio and Hardwick, 2002). Apart from these checkpoint components, Aurora B and its associated proteins INCENP and Survivin have emerged as new factors important for the spindle checkpoint. In budding yeast, the Aurora B ortholog, Ip11 is required for the delay triggered by the spindle checkpoint when the kinetochores are not under tension but is dispensable for the arrest induced by spindle depolymerization (Biggins and Murray, 2001). In mammalian cultured cells, Aurora B seems to modulate the spindle checkpoint in both cases (Kallio *et al.*, 2002b; Ditchfield *et al.*, 2003; Hauf *et al.*, 2003). How this protein may participate in the checkpoint signaling is not known, although the inhibition of its kinase activity induces a loss of the kinetochore localization of Bub1, BubR1, CENP-E, and Mad2 proteins (Ditchfield *et al.*, 2003; Hauf *et al.*, 2003). In this work, we investigated the regulation of the kinetochore localization of Mps1, Bub1, Bub3, CENP-E, Mad1, and Mad2 by Aurora B. Our results demonstrate that, in *Xenopus*, Aurora B controls the kinetochore localization of all the analyzed checkpoint proteins, whereas Aurora B centromere localization is not affected by the absence of any of the other checkpoint components. As far as we know, this is the first work that demonstrates a regulation of all the checkpoint proteins by Aurora B,

placing this protein as the more upstream regulator in the checkpoint cascade. Thus, because it may act as the first activated factor of this pathway, it is likely that it detects both, loss of tension and microtubule attachment at kinetochores.

Similarly to the immunodepletion of Aurora B, the removal of INCENP induces a loss of the kinetochore presence of the downstream checkpoint components, thus it makes sense to hypothesize that, despite Aurora B, INCENP and Survivin also could participate directly (by modulating spindle checkpoint signaling) or indirectly (by inducing kinetochore localization of Aurora B) in the modulation of the spindle checkpoint (Carvalho *et al.*, 2003; Lens *et al.*, 2003). Accordingly, we have been unable to restore the kinetochore localization of Aurora B and the other checkpoint proteins in Aurora B-depleted extracts by the sole translation of Aurora B. Moreover, surprisingly, the cotranslation of Aurora B, INCENP, and Survivin was neither sufficient to induce the localization of Aurora B to the kinetochore. This was due to the fact that there was not Aurora B/INCENP/Survivin-complex formation, indicating that in addition to these three proteins, more unknown components of this complex are required to restore its endogenous function and to restore spindle checkpoint.

Two other spindle checkpoint components, Mps1 and Bub1, have been suggested to act upstream in the spindle checkpoint cascade. The overexpression of both kinases leads to the activation of the spindle checkpoint in the apparent absence of spindle damage. This activation is dependent on the other spindle checkpoint proteins (Bub1/Mps1, Bub3, Mad1, and Mad2) (Farr and Hoyt, 1998; Hardwick *et al.*, 1996). Moreover, they regulate the kinetochore localization of CENP-E, Mad1, and Mad2 (Abrieu *et al.*, 2001; Sharp-Baker and Chen, 2001; Liu *et al.*, 2003). In this work, we have analyzed the mutual dependent kinetochore localization of these two kinases. Our results demonstrate that they are dependent on each other to induce their robust kinetochore localization, indicating that, despite the fact that they act downstream Aurora B/INCENP, they may both act together at an early step in the checkpoint signaling pathway.

In addition to Aurora B/INCENP, Mps1, and Bub1, the kinesin-like protein CENP-E is required for the kinetochore localization of the checkpoint proteins Mad1, Mad2, and to a lesser extent, BubR1 (Abrieu *et al.*, 2000; Weaver *et al.*, 2003). It has been assumed that this protein acts downstream of Mps1 and Bub1 because these two kinases regulate its kinetochore localization (Abrieu *et al.*, 2001; Sharp-Baker and Chen, 2001). Surprisingly, our results demonstrate that CENP-E acts at the same Mps1/Bub1 step of the spindle checkpoint pathway.

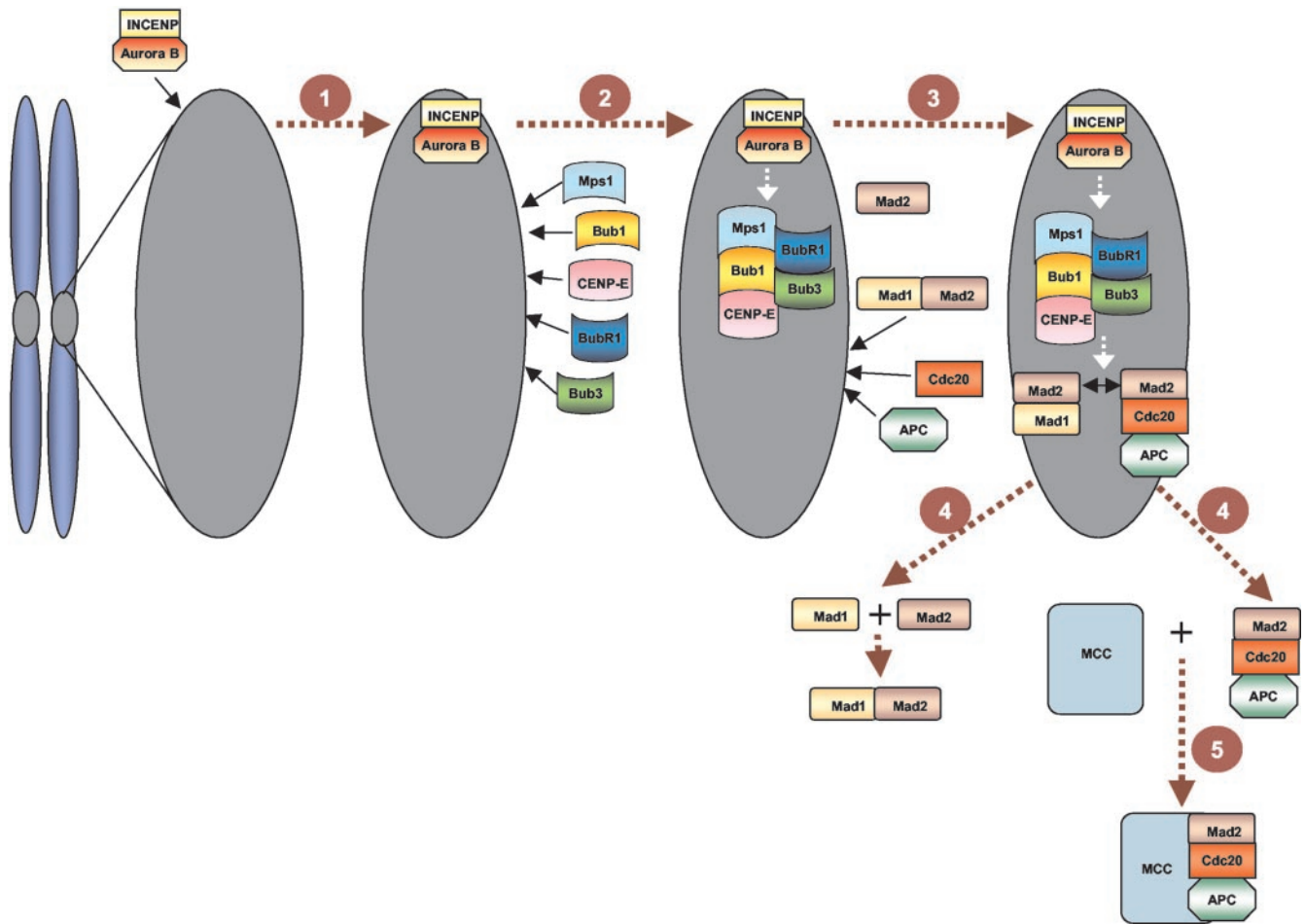
To continue with the view of the checkpoint cascade, two other proteins, Bub3 and BubR1, also could work at this stage of the checkpoint pathway. Unfortunately, we do not dispose of immunoprecipitating anti-Bub3 and anti-BubR1 antibodies. However, because it has been described that kinetochore recruitment of Bub1 is dependent on Bub3 (Taylor *et al.*, 1998) and that loading of Bub1, CENP-E, Mad1, and Mad2 to these chromosome structures is dependent on BubR1 (Taylor *et al.*, 1998; Chen, 2002; Mao *et al.*, 2003), it is likely that these proteins also participate at the Mps1/Bub1/CENP-E-dependent level of the spindle checkpoint pathway.

Finally, Mad1 and Mad2 are not capable of regulating in any case the kinetochore localization of the other upstream checkpoint components, indicating that they represent the downstream proteins of the checkpoint signaling pathway (Sharp-Baker and Chen, 2001; unpublished data).

The picture that emerges from our work and from those of other laboratories is that there is a network of interactions and interdependencies among the different checkpoint pro-

**Figure 5 (facing page).** The kinetochore localization of the APC regulator Cdc20 and the APC structural components Cdc23 and Cdc27 is regulated by the upstream checkpoint proteins Aurora B, Mps1, Bub1, and CENP-E. (A) Colocalization analysis of CENP-E with the APC proteins Cdc20, Cdc27, and Cdc23 in chromosomes isolated from nocodazole and sperm nuclei (2000/ $\mu$ m)-supplemented CSF extracts. Anti-CENP-E antibody labeled with biotin-(long arm)-NHS was used and detected with streptavidin-AlexaFluor. Anti-Cdc20, anti-Cdc27, and anti-Cdc23 immunostaining was visualized by using anti-rabbit rhodamine secondary antibody. Merged images of every costaining are shown. (B) The kinetochore localization of Cdc27, Cdc20, and Cdc23 were examined in the CSF extracts used in (A), which were immunodepleted with control ( $\Delta$ Mock), anti-Aurora B ( $\Delta$ Aurora B), anti-Mps1 ( $\Delta$ Mps1), anti-Bub1 ( $\Delta$ Bub1), anti-CENP-E ( $\Delta$ CENP-E), or anti-Mad1 ( $\Delta$ Mad1) antibodies. (C) Locations of Cdc20 and Cdc27 were analyzed in the chromosomes isolated from CSF extracts used in A, which were depleted with control ( $\Delta$ Mock), anti-Cdc27 ( $\Delta$ Cdc27), and anti-Cdc20 ( $\Delta$ Cdc20) antibodies. Images were visualized merged with chromatin. Bars, 5  $\mu$ m.





**Figure 6.** Proposed schematic diagram of the spindle checkpoint cascade in *Xenopus* egg extracts. Kinetochore localization of Aurora B/INCENP stimulates the formation of a kinetochore complex, including at least Mps1, Bub1, and CENP-E and probably also Bub3 and BubR1. This complex will independently modulate the kinetochore localization of Mad1/Mad2, Cdc20, and the APC, allowing the inhibition of the APC first by the association of APC/Cdc20 to Mad2 at the kinetochore and subsequently by the binding of APC/Cdc20/Mad2 complex to the MCC at the cytosol. The dissociation of APC/Cdc20/Mad2 from the kinetochore will allow the generation at this chromosomal location of a new formed complex by recruitment from the cytosol of free APC and Cdc20 and of a new formatted Mad1/Mad2 complex. Generation of the latter takes place by the association of free cytosolic Mad2 and the Mad1 protein liberated from the kinetochore during the APC/Cdc20/Mad2 complex formation.

teins that probably induces the formation of a stable structure at the kinetochore, conferring like this, their robust association to this chromosomal structure. One possibility is that some checkpoint proteins could act as scaffolds for the kinetochore recruitment of the others. Alternatively, the association of each partner of the checkpoint complex to the kinetochore may induce a conformational change of this protein, allowing its subsequent interaction with the others. Only when all the proteins of the complex would be associated the checkpoint-dependent kinetochore structure would be stabilized and the checkpoint pathway would be activated. This hypothesis implies the existence of a direct or indirect Mps1, Bub1, CENP-E interaction, and probably of other unidentified proteins. In this regard, we have not detected an Mps1-Bub1-CENP-E complex in *Xenopus* egg extracts, although this may not be surprising because these interactions are probably only present as a stable complex at the kinetochores.

The final target of the spindle checkpoint signaling is the ubiquitin-ligase APC/Cdc20. Unattached kinetochores induce the formation of a checkpoint complex that will inhibit

this ubiquitin-ligase and as a consequence will block Securin degradation and the metaphase-to-anaphase transition. The nature of this inhibitor checkpoint complex is not clear. First, results indicate that Mad2 could directly bind and inhibit APC, indicating that this protein could act as the "wait for anaphase signal" generated by the kinetochores (Li *et al.*, 1997; Fang *et al.*, 1998). However, two studies from different laboratories have recently demonstrated the presence of a new complex named MCC whose APC-inhibitory activity is 3000-fold greater than that of recombinant Mad2 (Sudakin *et al.*, 2001; Tang *et al.*, 2001). It is as yet unclear whether Mad 2 acts as a component of the MCC; thus, it is possible that two different wait for anaphase signals could independently block the APC/Cdc20, the first one by the MCC and the second one mediated by Mad2. Interestingly, Sudakin *et al.* (2001) have demonstrated that MCC generation is uncoupled from kinetochores, but these chromosome structures could enhance or prolong the inhibition of the APC by the MCC. They also have demonstrated that the MCC exists at all stages of the cell cycle, but this complex is only capable to inhibit the APC purified from mitotic cells, indicating that



the APC must be modified during mitosis to be recognized by the MCC. Whatever the mechanism used by the kinetochores to induce APC inhibition, it is clear that these chromosome structures orchestrate unattached chromosomes detection, activation of the spindle checkpoint, and inhibition of APC/Cdc20.

In this work, we demonstrate a colocalization of the CENP-E checkpoint protein with Cdc20, and with the APC (Cdc27/Cdc23) at the kinetochore in *Xenopus* egg extracts. Moreover, we demonstrate that the localization of both Cdc20 and APC are regulated by the spindle checkpoint. Thus, depletion from the extracts of the upstream components of the checkpoint pathway, Aurora B, Mps1, Bub1, and CENP-E induces a loss of the kinetochore localization of Cdc20 and of Cdc27 and Cdc23, two structural subunits of the APC. Unlike Aurora B, Mps1, Bub1, and CENP-E, the downstream checkpoint protein Mad1 does not regulate this kinetochore localization. Finally we demonstrate that the APC activator Cdc20 and the APC complex itself localize independently from each other to the kinetochores.

On the basis of these results, we propose a model in which the localization of Aurora B/INCENP in unattached kinetochores stimulates the formation of a kinetochore complex, including at least Mps1, Bub1, and CENP-E and probably also Bub3 and BubR1 (Figure 6). Recently, Mao *et al.* (2003) have proposed that a direct association of BubR1 with CENP-E in these kinetochores induces BubR1 activation, leading to a signaling cascade that finally inhibits APC activity. They also hypothesize that the capture of a microtubule by kinetochore-associated CENP-E alters CENP-E-BubR1 interaction and induces, as a consequence, the inactivation of BubR1 kinase and the silencing of the spindle checkpoint (Mao *et al.*, 2003). Our results show that the formation of a stable complex in the kinetochore is only possible if all the proteins of this complex are present. Thus, it is likely that the formation of Mps1-Bub1-CENP-E-Bub3-BubR1 complex is required to allow the correct interaction between CENP-E and BubR1, and, like this, the transmission of the spindle checkpoint signal along the checkpoint pathway. This signal would induce on the one hand the generation of a wait for anaphase signal, probably mediated by Mad2, and on the other hand the kinetochore localization of both Cdc20 and the APC. The presence of Mad2, Cdc20, and the APC at the kinetochores could then promote a first APC modification (such as subunit phosphorylation) (Topper *et al.*, 2002) and a subsequent APC-Cdc20-Mad2 association that would render a source of APC sensitive to the inhibition by the MCC. Once formed, this APC/Cdc20/Mad2 complex would be dissociated from the kinetochore and liberated to the cytosol where it would bind to the MCC, allowing the generation of a newly formed complex at this chromosomal location.

**Note added in proof.** While this manuscript was in press, a paper was accepted for publication that shows a kinetochore localization of the APC in HeLa cells (Acquaviva, C., Herzog, F., Kraft, C., and Pines, J. (2004). *Nat. Cell Biol.* 6, 892–898.

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