Mlp1 Acts as a Mitotic Scaffold to Spatially Regulate Spindle Assembly Checkpoint Proteins in *Aspergillus nidulans*

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During open mitosis several nuclear pore complex (NPC) proteins have mitotic specific localizations and functions. We find that the *Aspergillus nidulans* Mlp1 NPC protein has previously unrealized mitotic roles involving spatial regulation of spindle assembly checkpoint (SAC) proteins. In interphase, An-Mlp1 tethers the An-Mad1 and An-Mad2 SAC proteins to NPCs. During a normal mitosis, An-Mlp1, An-Mad1, and An-Mad2 localize similarly on, and around, kinetochores until telophase when they transiently localize near the spindle but not at kinetochores. During SAC activation, An-Mlp1 remains associated with kinetochores in a manner similar to An-Mad1 and An-Mad2. Although An-Mlp1 is not required for An-Mad1 kinetochore localization during early mitosis, it is essential to maintain An-Mad1 in the extended region around kinetochores in early mitosis and near the spindle in telophase. Our data are consistent with An-Mlp1 being part of a mitotic spindle matrix similar to its *Drosophila* orthologue and demonstrate that this matrix localizes SAC proteins. By maintaining SAC proteins near the mitotic apparatus, An-Mlp1 may help monitor mitotic progression and coordinate efficient mitotic exit. Consistent with this possibility, An-Mad1 and An-Mlp1 redistribute from the telophase matrix and associate with segregated kinetochores when mitotic exit is prevented by expression of nondegradable cyclin B.

INTRODUCTION

The nuclear envelope (NE) functions as a physical barrier between the nucleoplasm and cytoplasm. The gateways through this barrier are the nuclear pore complexes (NPCs) which are embedded in the NE and regulate transport of proteins and nuclei acids in and out of the nucleus during interphase (Hetzer *et al.*, 2005; Tran and Went, 2006; Terry *et al.*, 2007). The basic NPC structure is conserved in all eukaryotes and is comprised of multiple copies of ~30 individual NPC proteins (nucleoporins or Nups; Hetzer *et al.*, 2005). Recently, a detailed overall NPC structure has been predicted in budding yeast (Alber *et al.*, 2007a,b). The largest structural component of the NPC is the core scaffold, which forms a ring-like structure that transits the NE and is thought to be anchored in the NE by the relatively small number of nucleoporins that contain transmembrane domains, although most surprisingly a triple mutant lacking the known fungal transmembrane Nups in *Aspergillus nidulans* is viable (Liu *et al.*, 2009). Occupying the central channel of the core scaffold are the unstructured FG-repeat nucleoporins that together act as a selective molecular sieve, re-

stricting diffusion of macromolecules through the central channel while also participating in active nucleocytoplasmic transport (Frey *et al.*, 2006; Lim *et al.*, 2006). In addition, other nucleoporins form cytoplasmic fibrils extending from the cytoplasmic side of NPCs, whereas nucleoporins such as the Mlp proteins form a nucleoplasmic basket structure (Hetzer *et al.*, 2005; Lim and Fahrenkrog, 2006). In organisms undergoing an open mitosis, NPCs are disassembled during mitosis and postmitotic NPC reassembly around daughter nuclei must be coordinated with other mitotic exit events. How this is regulated is not well understood. Interestingly, many nucleoporins and transport factors have mitotic functions at locations away from NPCs (Kerscher *et al.*, 2001; Harel *et al.*, 2003; Joseph *et al.*, 2004; Jeganathan *et al.*, 2005; Blower *et al.*, 2005; Zuccolo *et al.*, 2007), suggesting that they help ensure the fidelity of the mitotic process.

NPCs also help organize chromosomal positioning and nuclear architecture during interphase. For example NPCs play roles in regulating transcriptionally active or silent loci within the nucleus, likely by localizing such loci to different regions of the nuclear periphery (Galy *et al.*, 2000; Casolari *et al.*, 2004; Dilworth *et al.*, 2005; Brown and Silver, 2007; Akhtar and Gasser, 2007). In addition, enzymatic activities involved in SUMO (small ubiquitin-like modifier) modification, chromatin regulation, and DNA repair as well as nuclear transport are localized to the nuclear periphery by NPCs, providing a spatial aspect for these cellular functions within the nucleus (Saitoh *et al.*, 1998; Galy *et al.*, 2000; Zhao *et al.*, 2004; Mendjan *et al.*, 2006; Luthra *et al.*, 2007). Consistent with their localization, components of the nuclear basket of NPCs help organize nuclear architecture and in bud-

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Abbreviations used: NE, nuclear envelope; NORs, nucleolar organizing region; NPC, nuclear pore complex; SAC, spindle assembly checkpoint; SPB, spindle pole body.

ding yeast the myosin-like proteins Mlp1 and Mlp2 are particularly important for this (Galy *et al.*, 2000; Feuerbach *et al.*, 2002; Zhao *et al.*, 2004; Luthra *et al.*, 2007). Localization of Mlp orthologues, such as human Tpr (translocated promotor region), to the nuclear basket is conserved in evolution (Frosst *et al.*, 2002; Krull *et al.*, 2004; Xu *et al.*, 2007), suggesting that these large structural proteins may have a general function as a scaffold at the nucleoplasmic side of the NE. Interestingly, during mitosis the *Drosophila* Mlp orthologue Megator is part of a spindle matrix (Qi *et al.*, 2004; Johansen and Johansen, 2007), and a spindle-like localization has also been shown for the plant orthologue NUA (Xu *et al.*, 2007), suggesting that Mlp orthologues may also play structural roles in mitosis.

Eukaryotic cells utilize a mechanism called the spindle assembly checkpoint (SAC) to prevent sister chromatid segregation until after correct bipolar microtubule attachments have been made to all kinetochores (Musacchio and Salmon, 2007). In mammalian cells, SAC proteins such as Mad1 and Mad2 localize to kinetochores in prophase and generate a signal that inhibits the anaphase promoting complex (APC) until all kinetochores are properly attached to microtubules (Waters *et al.*, 1998; Howell *et al.*, 2001; Shah *et al.*, 2004; Musacchio and Salmon, 2007). When correct kinetochore microtubule attachments have been made, Mad1 and Mad2 are removed from kinetochores, the SAC is turned off, and sister chromatids segregate (Howell *et al.*, 2001; Shah *et al.*, 2004; Musacchio and Salmon, 2007). How cells respond to mitotic defects that occur after anaphase is not well understood although it has been shown in budding yeast that the SAC can be reactivated in anaphase (Palframan *et al.*, 2006).

Interestingly, during interphase the SAC proteins Mad1, Mad2, and MPS1 localize to NPCs (Campbell *et al.*, 2001; Liu *et al.*, 2003; Buffin *et al.*, 2005). The localization of Mad1 and Mad2 to NPCs also occurs in organisms undergoing closed mitosis, such as *Saccharomyces cerevisiae* (Iouk *et al.*, 2002; Gillett *et al.*, 2004; Scott *et al.*, 2005). In this organism, NPCs remain intact throughout mitosis and Mad1 and Mad2 remain at NPCs during a normal mitosis and only bind to kinetochores that lose their microtubule attachments (Iouk *et al.*, 2002; Gillett *et al.*, 2004; Scott *et al.*, 2005). Why these SAC proteins localize to NPCs during interphase is not understood. Intriguingly, several nucleoporins are found at mitotic kinetochores in mammalian cells. For example, Nup358 and all components of the vertebrate Nup107-Nup160 subcomplex localize to mitotic kinetochores and have been demonstrated to have functions at this locale (Belgareh *et al.*, 2001; Liodice *et al.*, 2004; Rasala *et al.*, 2006; Orjalo *et al.*, 2006; Zuccolo *et al.*, 2007; Franz *et al.*, 2007). In addition, the Rae1/Nup98 NPC subcomplex helps regulate spindle assembly (Blower *et al.*, 2005) and acts as a negative regulator of the APC (Jeganathan *et al.*, 2005). Therefore, the relationship between NPCs, kinetochores and mitotic regulation is likely complex.

We have previously shown that the filamentous fungus *A. nidulans* displays aspects of both open and closed mitosis in that its NPCs are partially disassembled within an otherwise intact NE (Osmani *et al.*, 1988, 1991, 2006a; De Souza *et al.*, 2004; De Souza and Osmani, 2007; Liu *et al.*, 2009). Peripheral and central channel FG-repeat nucleoporins disperse from NPCs during mitosis, whereas core NPC components remain associated with the NE. To better understand spatial regulation of the SAC and the role played by NPC components, we have examined how the *A. nidulans* orthologues of Mad1 and Mad2 are localized during the cell cycle. Both An-Mad1 and An-Mad2 localize to NPCs during interphase, and we show the An-Mlp1 NPC protein is required for this

localization. During early mitosis, An-Mlp1 as well as An-Mad1 and An-Mad2 concentrate around kinetochores, and the forming spindle and if the SAC is activated the kinetochore association of these three proteins is maintained. This is the first time localization of an Mlp orthologue to kinetochores has been demonstrated during an unperturbed mitosis. During late anaphase and telophase, An-Mad1, An-Mad2, and An-Mlp1 localize similarly in between segregating chromosomes. We present data consistent with An-Mlp1 being part of a mitotic spindle matrix, which acts as a scaffold to localize An-Mad1 and An-Mad2 near kinetochores and the telophase spindle. We propose that by correctly localizing these SAC proteins, An-Mlp1 helps the SAC monitor mitotic progression and also provides spatial and temporal regulation of these SAC components during mitotic exit.

MATERIALS AND METHODS

General Techniques

Media and general techniques for *A. nidulans* genetic manipulation were as previously described (Pontecorvo, 1953; Oakley and Osmani, 1993). Strains used in this study are listed in Supplemental Table S1. To generate genes endogenously tagged at their 3' end with green fluorescent protein (GFP) or mCherry, targeting constructs were generated using fusion PCR as described (Yang *et al.*, 2004; Szcwzyk *et al.*, 2006) and transformed into a *nkuA^{ku70}Δ* strain (strain SO451) to achieve a high frequency of homologous gene targeting (Nayak *et al.*, 2006). After confirmation of homologous integration, all transformants underwent at least one genetic cross. Confirmation of endogenous tagging was carried out by Western blotting using Living Colors anti-GFP or anti-DsRed antibodies (Clontech, Palo Alto, CA) to determine if protein chimeras of the predicted size were generated. Homologous integration of the tagging construct at the gene of interest was confirmed by diagnostic PCR using primers flanking the entire targeting construct. Growth of strains containing GFP or mCherry tagged proteins was compared with wild-type and SAC deficient mutants, with or without 0.4 μg/ml benomyl (Sigma, St. Louis, MO) at 32°C, to ensure no growth defects.

Heterokaryon Rescue

To follow protein localization in the absence of the essential *An-mlp1* gene, strains were first generated that contained the proteins of interest C-terminally labeled with either GFP or mCherry and also required uridine and uracil for germination because of the presence of *pyrG89* mutation. Heterokaryons were then generated by transformation of these *pyrG89* recipient strains with an *An-mlp1Δ::pyrG⁺* targeting cassette and heterokaryons identified as described (Osmani *et al.*, 2006b). Uninucleate conidiospores were germinated from heterokaryons in media lacking uridine and uracil. Conidiospores which were *An-mlp1Δ::pyrG⁺* were distinguished by their ability to germinate in the absence of uridine and uracil. In contrast, conidiospores that were *pyrG89* and wild type for *An-mlp1* did not form germ tubes in the absence of uridine and uracil. When germinated in the presence of 5 mM uridine and 10 mM uracil both types of conidiospores grew; however, those containing *An-mlp1Δ::pyrG⁺* could be distinguished due to the mis-localization of An-Mad1-GFP and growth defects.

Imaging and Analysis

For live cell imaging, conidiospores were germinated in minimal media containing 55 mM glucose as the carbon source and 10 mM urea as the nitrogen source in 35-mm glass-bottom microwell dishes (MatTek, Ashland, MA). Cells were imaged using an Orca-ER camera (Hamamatsu, Bridgewater, NJ) on a TE300 inverted microscope (Nikon, Melville, NY) configured with an Ultraview spinning disk confocal system (Perkin Elmer-Cetus, Norwalk, CT) controlled by Ultraview software (Perkin Elmer-Cetus). Cells grown for fixation were germinated in rich YG media. Fluorescence of GFP and mCherry was maintained following fixation in 1× PHEM buffer (45 mM PIPES, 45 mM HEPES, 10 mM EGTA, and 5 mM MgCl₂, pH 6.9) containing 6% paraformaldehyde (EM grade; Electron Microscopy Sciences, Hatfield, PA). For quantification of the nuclear/cytoplasmic ratios of An-Mad1-GFP and An-Mad2-GFP in G2 cells (see Figure 1A), the average gray-scale pixel intensity inside the circumference of the nucleus was measured along with an identical area of cytoplasm. Background levels in a region adjacent to each cell were subtracted from each and the ratio of levels in the nucleus to cytoplasm calculated for at least five nuclei per strain. Cells used for this analysis were in G2 just before mitotic entry as indicated by the subsequent NPC disassembly of An-Mad1 or An-Mad2. Similar analysis was performed to determine the nuclear/cytoplasmic ratios of An-Mad2-GFP levels in *An-mlp1Δ* cells and *An-mad1Δ* cells. All live-cell imaging was carried out at room temperature. Benomyl (Sigma) was used at a concentration of 2.4 μg/ml (Ovechkina *et al.*,

2003; Horio and Oakley, 2005). Image analysis, kymograph generation, and pixel intensity profiles were carried out using ImageJ freeware (NIH; <http://rsb.info.nih.gov/ij/>). Stacks of confocal slices were rotated in 3D space using the Volume Viewer plugin in ImageJ.

Induction of Nondegradable Cyclin B

Strains containing a nondegradable form of cyclin B under control of the *alcA* promoter (Waring *et al.*, 1989) were generated by crossing to strain MAT69 (*pabaA1; argB2::alcA::ΔnimE* cyclinB (nondegradable)::*argB*), a kind gift from Matthew O'Connell (Mount Sinai School of Medicine, New York, NY). Strains were germinated overnight in glucose-containing minimal media, which is repressing for the *alcA*. For induction of nondegradable cyclin B expression, exchange to media containing 1% ethanol, which is inducing for *alcA*, was carried out by washing cells twice in this media and allowing cells to grow for 1 h before fixation or time-lapse imaging.

RESULTS

A. nidulans An-Mad1 and An-Mad2 Associate with NPCs

We have previously demonstrated that *A. nidulans* undergoes partial NPC disassembly during mitosis such that central channel and peripheral nucleoporins disperse throughout the cell, but a core NPC structure remains associated with the NE (De Souza *et al.*, 2004; Osmani *et al.*, 2006a). However, these studies did not include analysis of the An-Mad1 and An-Mad2 SAC proteins, which are components of interphase NPCs in other systems (Campbell *et al.*, 2001; Iouk *et al.*, 2002; Gillett *et al.*, 2004; Buffin *et al.*, 2005; Scott *et al.*, 2005). To facilitate examination of the localization of An-Mad1 and An-Mad2 in *A. nidulans*, we endogenously tagged each with GFP or mCherry. These tagged versions of An-Mad1 and An-Mad2 were functional because they did not display the marked sensitivity to microtubule-depolymerizing drugs characteristic of loss of SAC function and as seen for the respective null alleles (Prigozhina *et al.*, 2004; Supplemental Figure S1).

During interphase, An-Mad1 and An-Mad2 both localized to the nuclear periphery, although the overall distribution of An-Mad2 differed from An-Mad1 in that significant levels of An-Mad2 were also present in the cytoplasm and nucleoplasm (Figure 1A), consistent with observations in other organisms (Chung and Chen, 2002; Iouk *et al.*, 2002). The localization of An-Mad1 and An-Mad2 at the nuclear periphery did not precisely resemble that of the central channel nucleoporin An-Nup49 (Figure 1B) but was almost identical to that of An-Mlp1, which is predicted to be a component of the nuclear basket of NPCs (Figure 1C). These data are consistent with An-Mad1 and An-Mad2 localizing to the nucleoplasmic side of NPCs during interphase in *A. nidulans*.

An-Mlp1 Is Essential for An-Mad1 and An-Mad2 Localization to NPCs

In *S. cerevisiae*, Nup53, Nup60, Mlp1, and Mlp2 are involved in localizing Mad1 and Mad2 to NPCs (Iouk *et al.*, 2002; Scott *et al.*, 2005). However, *A. nidulans* does not contain identifiable orthologues of Nup60 or Nup53 and has only a single Mlp orthologue (Mans *et al.*, 2004; Osmani *et al.*, 2006a). We therefore examined if An-Mlp1 was required for the association of An-Mad1 and An-Mad2 with NPCs. Although An-Mlp1 had previously been reported to be nonessential (Osmani *et al.*, 2006a), we found that strains previously thought to be haploid deletions were heterozygous diploids containing a wild-type and null allele of *An-mlp1* and that *An-mlp1* is in fact essential for viability (Supplemental Figure S2). Given that *An-mlp1* is essential, we utilized the heterokaryon rescue technique (Osmani *et al.*, 2006b) to study the phenotype of the null allele. We generated heterokaryons containing two types of haploid nuclei, one wild

Figure 1. An-Mad1 and An-Mad2 localize to NPCs. (A) Localization of An-Mad1-GFP and An-Mad2-GFP showing the characteristic ring-like pattern of NPCs (strains CDS487 and CDS578). The graph shows quantification of the relative levels of An-Mad1-GFP or An-Mad2-GFP in the cytoplasm and nucleus of late G2 cells. (B) A single confocal slice through a nucleus showing An-Nup49-mCherry and An-Mad2-GFP (strain CDS595) together with a pixel intensity profiles. (C) The An-Mad1-GFP and An-Mad2-GFP nuclear periphery localization overlaps with An-Mlp1-mCherry (strains CDS676 and CDS678). Bars, $\sim 5 \mu\text{m}$.

type for *An-mlp1* and one in which *An-mlp1* had been deleted by gene replacement with the *pyrG* nutritional marker (*An-mlp1Δ::pyrG*). Uninucleate conidiospores from the heterokaryons were inoculated in media in which germination only occurred if the conidiospores were *pyrG*⁺ and therefore *An-mlp1Δ*. We found that *An-mlp1* nulls germinated and underwent several nuclear divisions but could not continue growth to form viable colonies. Examination of *An-mlp1Δ* germlings indicated that although An-Nup49 located to NPCs normally, An-Mad1 failed to localize to the nuclear periphery and was found in the nucleoplasm (Figure 2A, *mlp1Δ*). In this figure, an *An-mlp1* wild-type cell that has not formed a germ tube is also present and acts as a control, displaying the characteristic ring-like NPC localization for both An-Nup49 and An-Mad1 (Figure 2A, *mlp1*⁺). Similarly, An-Mlp1 is required for the localization of An-Mad2 to NPCs (Figure 2B). Therefore, An-Mlp1 is essential for the correct localization of An-Mad1 and An-Mad2 to NPCs during interphase but is not required for their nuclear import.

We next examined whether either An-Mad1 or An-Mad2 were required for the localization of each other to NPCs. In the absence of An-Mad1, An-Mad2 localized throughout cells but was no longer enriched at NPCs, whereas the localization of An-Nup49 was unaffected (Figure 2C). In contrast, in the absence of An-Mad2, An-Mad1 as well as the An-Nup49 control localized to the nuclear periphery normally (Figure 2D).

Interestingly, although An-Mad2 failed to localize to NPCs in the absence of either An-Mad1 or An-Mlp1, An-Mad2 was enriched in the nuclei of *An-mlp1Δ* cells (nuclear/cytoplasmic ratio 1.8 ± 0.3) but not *An-mad1Δ* cells (nuclear/

Figure 2. An-Mlp1 is required for An-Mad1 and An-Mad2 localization to NPCs. (A) An *An-mlp1Δ* germling localizes An-Nup49-mCherry to NPCs normally but An-Mad1-GFP is mis-localized to the nucleoplasm. An *An-mlp1⁺* wild-type cell that cannot undergo polarized growth in this media acts as a control in which both An-Nup49-mCherry and An-Mad1-GFP localize to NPCs normally. Uninucleate spores of both genotypes originated from the heterokaryon hCDS662 and were inoculated in media selective for germination of only *An-mlp1Δ* conidiospores (see Materials and Methods). (B) As for A but showing the mis-localization of An-Mad2-GFP in an *An-mlp1Δ* germling (from strain hCDS659). (C) An-Mad2-GFP does not localize to NPCs in an *An-mad1* null (strain CDS687). (D) An-Mad1-GFP localizes to NPCs normally during interphase in an *An-mad2* null (strain CDS605). An-Nup49-mCherry is shown as a control in the same cell for C and D. Bars, ~5 μm.

cytoplasmic ratio 1.0 ± 0.1). This indicates that similar to budding and fission yeast (Ikui *et al.*, 2002; Iouk *et al.*, 2002; Scott *et al.*, 2005), An-Mad2 requires An-Mad1 for its nuclear localization.

Together, these data are consistent with An-Mlp1 acting as a scaffold to tether the An-Mad1/An-Mad2 complex to interphase NPCs via An-Mad1.

An-Mad1 and An-Mad2 Display Distinctive and Dynamic Localizations during Mitosis

To examine the localizations of An-Mad1 and An-Mad2 during mitosis, we used live cell time-lapse confocal imaging of the respective GFP-tagged proteins together with An-Nup49-mCherry as a marker for partial disassembly and reassembly of NPCs. When nuclei entered mitosis, as indicated by the onset of An-Nup49 dispersal, An-Mad1 also disassembled from NPCs but, unlike An-Nup49, concentrated in one area of the nucleus (Figure 3A, arrowheads; see also Supplemental Figure S3A video). Given that the kinetochores/centromeres of the eight duplicated chromosomes are in a single cluster adjacent to the spindle pole bodies (SPBs) during G2 in *A. nidulans* (see Figure 6H; Yang *et al.*, 2004) and that Mad1 localizes to kinetochores during mitotic entry in human cells (Waters *et al.*, 1998; Howell *et al.*, 2001; Shah *et al.*, 2004), we reasoned that the early mitotic focus of An-Mad1 corresponded to kinetochores. Further analysis indicated that An-Mad1 did concentrate around the An-Ndc80 kinetochore marker (Figure 3C, arrowheads) during prophase spindle formation (Figure 3B, arrowheads). As cells entered anaphase, the focus of An-Mad1 broadened and it remained in the vicinity of kinetochores and the expanding spindle (Figure 3, B and C). Most surprisingly, in telophase when kinetochores had segregated to the spindle poles, An-Mad1 displayed a distinctive localization near the spindle in between the segregating nuclei (Figure 3, A–C, arrows; see also Supplemental Figure S3C video). By this point of mitosis, An-Nup49 had begun to reassemble to NPCs (Figures 3A and 7C, arrows indicate An-Mad1 between nuclei that are reassembling An-Nup49). As nuclei

continued to form in early G1, An-Mad1 dispersed completely and did not appear in daughter nuclei until nuclear transport was reestablished in early G1 (Figure 3, vertical green line indicates period of An-Mad1 dispersal; Supplemental Figure S4A). Similar results were obtained for the localization of An-Mad2 during mitosis (Supplemental Figure S3). Therefore, An-Mad1 and An-Mad2 concentrate around the kinetochore/spindle region early in mitosis but in telophase localize near the spindle in a region distinct from the kinetochores.

An-Mlp1 Localizes Similarly to An-Mad1 and An-Mad2 during Mitosis

Given that An-Mlp1 is required to localize An-Mad1 and An-Mad2 to interphase NPCs, we determined if these proteins behaved similarly during mitosis. Interestingly, An-Mlp1 was mitotically dispersed from NPCs for close to 8 min, a period of time similar to An-Mad1 and An-Mad2 but that is almost twice as long as that of An-Nup49, An-Nup98, and An-Nup188 (Figure 3D). An-Mlp1 dispersal from NPCs began at the same time as An-Nup49, but, unlike An-Nup49, which dispersed throughout the cell, An-Mlp1 concentrated in one area of the nucleoplasm (Figure 4A, arrowheads; see also Supplemental Figure S4A video). This concentration of An-Mlp1 resembled that of An-Mad1 and An-Mad2 around the prophase kinetochore/spindle region (Figures 3C, 4C, and 7D, arrowheads) as the prophase spindle formed (Figure 4B, arrowhead). As the spindle elongated, the concentration of An-Mlp1 also elongated although the localization of An-Mlp1 was in part distinct from that of the spindle (Figure 4B). Interestingly, similar to An-Mad1 and An-Mad2, An-Mlp1 displayed a distinct localization between fully segregated kinetochores and reforming daughter nuclei before dispersing and reaccumulating in G1 nuclei (Figures 4, A–C, and 7D, arrows; Supplemental Figures S4B and S5). The kymographs in Figure 4A clearly demonstrate the association of An-Mlp1 with the mitotic apparatus throughout the period of mitosis when An-Nup49 is dispersed. Examination of An-Mad1-GFP and An-Mlp1-mCherry together by live cell imaging confirmed that their localization

Figure 3. An-Mad1 displays a dynamic mitotic pattern. Time-lapse imaging of An-Mad1-GFP or mCherry during mitosis in comparison to An-Nup49-mCherry (A; strain CDS604; see also Supplemental Figure S3A video), GFP-tubulin (B; strain CDS728) or An-Ndc80-mCherry (C; strain CDS578; see also Supplemental Figure S3C video). Pixel intensity profiles are shown for A and C. Arrowheads indicate localization of An-Mad1-GFP around the mitotic apparatus during mitotic entry, and arrows indicate An-Mad1-GFP localization between telophase nuclei. Time 0 indicates the onset of NPC disassembly at prophase. P, prophase; M, metaphase; A, anaphase; T, telophase. Vertical lines indicate the period of An-Nup49 dispersal (red in A), kinetochore segregation to the poles (blue in C) and An-Mad1 dispersal (green in A–C). In A the kymographs demonstrate that An-Mad1-GFP remains on the mitotic apparatus, whereas An-Nup49-mCherry is dispersed. The time course shown in the montage is indicated in the kymograph. Bars, $\sim 4 \mu\text{m}$. (D) Graph showing the time the indicated NPC proteins are dispersed from the nuclear periphery during mitosis.

during mitosis was nearly identical (Supplemental Figure S5). This pattern of An-Mlp1, An-Mad1, and An-Mad2 concentration around the mitotic apparatus in early mitosis and

between nuclei in telophase is not shared with other components of the *A. nidulans* NPC (De Souza *et al.*, 2004; Osmani *et al.*, 2006a).

Figure 4. Dynamic localization of An-Mlp1 during mitosis. Time-lapse imaging of An-Mlp1-GFP or mCherry during mitosis in comparison to An-Nup49-mCherry (A; strain CDS561; see also Supplemental Figure S4A video), GFP-tubulin (B; strain CDS726), or An-Ndc80-mCherry (C; strain CDS564; see also Supplemental Figure S4C video). Pixel intensity profiles are also shown for A and C. In prophase, An-Mlp1-GFP relocates from NPCs to the nucleoplasm, concentrating around kinetochores and the spindle (arrowheads). An-Mlp1-GFP remains near the spindle in between reforming daughter nuclei in telophase (arrows). Vertical lines indicate the period of An-Nup49 dispersal (red in A), kinetochore segregation to the poles (blue in C), and An-Mlp1 dispersal (green in A–C). In A kymographs demonstrate that An-Mlp1-GFP remains on the mitotic apparatus, whereas An-Nup49-mCherry is dispersed. The time course shown in the montage is indicated in the kymograph. Bars, $\sim 4 \mu\text{m}$.

An-Mlp1 Colocalizes with An-Mad1 and An-Mad2 at Kinetochore-associated Foci in SAC-activated Cells

As An-Mlp1, An-Mad1, and An-Mad2 display similar dynamic localizations at the mitotic apparatus, we wanted to assess if these proteins localized similarly during a SAC-arrested mitosis. To obtain SAC-arrested cells, we used the microtubule-depolymerizing drug benomyl at a concentration of $2.4 \mu\text{g}/\text{ml}$, which is sufficient to depolymerize all microtubules (Horio and Oakley, 2005). When microtubules were depolymerized during interphase, An-Mad1 and An-Mad2 remained at the nuclear periphery. In *A. nidulans*, the single cluster of interphase centromeres/kinetochores is

closely associated with the SPBs, and we found that this association was almost always maintained when cells entered mitosis without microtubule function (Figures 5 and 6, Ndc80 marker). When cells entered mitosis without microtubules, An-Mad1 and An-Mad2 concentrated on and around the An-Ndc80 kinetochore cluster and were maintained at this location during the SAC arrest (Figures 5A and 6, A, E, and F; see also Supplemental Figure S5A video). Importantly, the concentration of An-Mad1 was broader than that of the kinetochore cluster and An-Mad1 foci were often adjacent to the An-Ndc80 cluster (e.g., Figure 5A, arrowheads). The localization of An-Mad1 partially overlap-

Figure 5. Localizations of An-Mad1 and An-Mlp1 during SAC activation. Representative time-lapse series and pixel intensity profiles of cells entering mitosis in the presence of the microtubule poison benomyl at 2.4 $\mu\text{g/ml}$. (A) An-Mad1-GFP localization in comparison to the An-Ndc80-mCherry kinetochore marker in a wild-type cell (from strain hCDS831 germinated + uridine and uracil; see also Supplemental Figure S5A video). (B) As in A but during SAC activation in an *An-mlp1* Δ cell (from strain hCDS831 germinated + uridine and uracil in the same experiment as in A). (C) An-Mlp1-GFP localization in comparison to An-Ndc80-mCherry in a wild-type cell (strain CDS564; shown is every second frame of Supplemental Figure S5C video). Arrowheads indicate concentration of An-Mad1 or An-Mlp1 adjacent to the kinetochore cluster in A and C or An-Mad1 on kinetochores in B. Bar, $\sim 4 \mu\text{m}$.

ping with the An-Ndc80 kinetochore cluster was also observed when we examined fixed cells to eliminate the chance of movement during image capture and allow higher resolution imaging (Figure 6A). In these microtubule depolymerization experiments kinetochores that had dissociated from the kinetochore cluster invariably displayed high levels of An-Mad1 and An-Mad2 (Figure 6B, arrowheads; data not shown) as described previously in budding yeast (Gillett *et al.*, 2004).

We next examined the localization of An-Mlp1 as cells entered mitosis with depolymerized microtubules. Although no spindle was present, An-Mlp1 localized similarly to a normal prophase, concentrating in the region around the kinetochore cluster where the short prophase spindle would normally form (Figures 5C and 6C; see also Supplemental Figure S5C video). Similar to An-Mad1, An-Mlp1 was often concentrated adjacent to or partially overlapping with the kinetochore cluster (Figure 5C and 6C) and additionally was also present on kinetochores that had dissociated from the main kinetochore cluster (Figure 6D, arrowheads). Examination of An-Mlp1 together with An-Mad1 or An-Mad2 indicated that these proteins localized to the same foci during a SAC arrest (Figure 6, E and F). Given the proximity of the kinetochore cluster to the SPBs and that *S. cerevisiae* Mlp2 associates with SPB components (Niepel *et al.*, 2005), we compared the localization of the An-Mlp1 with SPBs during SAC activation. As shown in Figure 6G, the An-Mlp1 foci formed during SAC arrest are distinct from SPBs indicated by the γ -tubulin complex protein Gcp3.

We next examined if SAC activation was required for An-Mlp1 kinetochore association utilizing an *An-mad2* Δ

strain that does not have a functional SAC. When *An-mad2* Δ cells entered mitosis without microtubules, An-Mlp1 still concentrated in the expanded region around the kinetochore cluster in a normal manner (Supplemental Figure S6). Therefore, An-Mlp1 does not require either a spindle or SAC activation to concentrate around kinetochores during mitotic entry. However, An-Mlp1 may still be regulated by the SAC in some manner as SAC activation maintained the kinetochore association of An-Mlp1 (Figure 5C). This is similar to the case for An-Mad1 and An-Mad2 (Figure 5A, data not shown), whose localization to kinetochores is a hallmark of the SAC.

Therefore the dynamic mitotic localization of An-Mlp1 resembles, but is distinct from, the mitotic spindle. In the absence of a spindle, An-Mlp1 localizes normally around kinetochores where the short prophase spindle would form. These data are consistent with An-Mlp1 being part of a mitotic spindle matrix similar to its *Drosophila* orthologue Megator (Qi *et al.*, 2004).

An-Mlp1 Acts as a Mitotic Scaffold to Localize An-Mad1 during Mitosis

During interphase, An-Mlp1 acts as a scaffold to localize An-Mad1 and An-Mad2 to NPCs. During mitosis, An-Mlp1 is part of a potential spindle matrix that displays dynamic mitotic distribution similar to that of the An-Mad1 and An-Mad2 SAC proteins. Together this suggests that An-Mlp1 may be required for the correct mitotic localization of these SAC proteins. We therefore examined An-Mad1 localization in comparison to kinetochores during mitosis in *An-mlp1* null cells. During interphase, An-Mad1 mis-local-

Figure 6. An-Mad1, An-Mad2, and An-Mlp1 localize similarly in SAC-activated cells. Images are of SAC-arrested nuclei in fixed samples of cells treated with benomyl. Shown are maximum intensity projections (left) and the same Z-series rotated in 3D space. (A and B) An-Mad1-GFP with An-Ndc80-mCherry (strain CDS578). (C and D) An-Mlp1-GFP with An-Ndc80-mCherry (strain CDS564). (E) An-Mad1-GFP with An-Mlp1-mCherry (strain CDS678). (F) An-Mad2-GFP with An-Mlp1-mCherry (strain CDS676). (G) Mlp1-GFP with the SPB marker Gcp3-mCherry (strain CDS655). (H) Localization of Ndc80-mCherry kinetochores in comparison with SPBs indicated by Gcp3-GFP at the indicated cell cycle stages (strain CDS652). Arrowheads indicate kinetochores that have detached from the main kinetochore cluster. Bars, $\sim 2 \mu\text{m}$.

ized to the nucleoplasm as expected, but as *An-mlp1* null cells entered mitosis, An-Mad1 concentrated on the kinetochore cluster (Figure 7B, arrowheads). Although this indicates that An-Mlp1 is not required for An-Mad1 prophase kinetochore association, the prophase localization of An-Mad1 in wild-type and *An-mlp1* null cells was not identical. In wild-type cells the prophase kinetochore concentration of An-Mad1 is broad and extends well beyond the kinetochore cluster (Figure 7A, arrowheads). Contrasting this, in *An-mlp1* null cells An-Mad1 localizes almost exclusively at the kinetochore cluster during either normal mitotic entry (Figure 7B, arrowheads) or mitotic entry without microtubules (Figure 5B). The defects in An-Mad1 localization in *An-mlp1* null cells were also obvious as cells progressed through anaphase and telophase. In a normal anaphase An-Mad1 remains near segregating kinetochores (Figure 7A, the blue line indicates the period of kinetochore segregation). Contrasting this, in *An-mlp1* null cells An-Mad1 completely disperses after metaphase and does not localize near segregating anaphase kinetochores ($n > 20$) (Figures 7, B and C; see also Supplemental Figure S7B video). During telophase in *An-mlp1* null cells, An-Mad1 remained dispersed and was absent from the region in between segregating nuclei where it normally localizes at this stage of mitosis (Figure 7, A and B, arrows). These differences in the mitotic localization of An-Mad1 between wild-type and *An-mlp1*Δ mitoses are highlighted in the kymographs shown in Figure 7C. In wild-type cells An-Mad1 remains associated with the mitotic apparatus throughout the period of mitotic An-Nup49 dispersal, whereas in an *An-mlp1* null mitosis An-Mad1 is dispersed during the period of An-Nup49 dispersal (Figure 7C). After mitosis in both wild-type and *An-mlp1* null cells, An-Mad1 accumulated in G1 nuclei after return of An-Nup49 to NPCs, although in the absence of An-Mlp1, An-Mad1 did not reassemble to NPCs (Figure 7, B and C).

These data indicate that the SAC protein An-Mad1 requires An-Mlp1 for its proper mitotic localization. To deter-

mine if the mitotic localization of An-Mlp1 requires a functional SAC, we followed the mitotic localization of An-Mlp1 in an *An-mad2* null. We consistently found that *An-mad2*Δ SAC-deficient cells transit mitosis faster than wild-type cells (mitosis occurs 1.27 times faster in *An-mad2*Δ cells at 32°C; compare Figure 7, D and E). The results indicate that An-Mlp1 localization to the kinetochore/spindle region early in mitosis (Figure 7E, arrowhead) and near the spindle in telophase (Figure 7E, arrow) occurs independently of SAC function.

Therefore, similar to interphase, An-Mlp1 acts as a scaffold for An-Mad1 during mitosis. Although in interphase An-Mlp1 tethers An-Mad1 to NPCs, during mitosis An-Mlp1 is part of a potential spindle matrix, which acts as a scaffold to keep An-Mad1 near the spindle but is not required for An-Mad1 kinetochore association.

Redistribution of An-Mad1 and An-Mlp1 during a Telophase Arrest Caused by Nondegradable Cyclin B Expression

To further examine An-Mlp1 and An-Mad1 mitotic localization, we expressed a nondegradable form of cyclin B (also called NIME in *A. nidulans*; O'Connell *et al.*, 1992), which prevents mitotic exit in other organisms (Murray *et al.*, 1989; Wakefield *et al.*, 2000). This version of cyclin B lacks its destruction box sequences, and we utilized the *alcA* promoter to regulate its expression by exchange from repressing media to inducing media. Examination of cells before and after 1 h of induction indicated that the spindle mitotic index increased more than twofold from 4.5 to 10.7% and that cells arrested with segregated DNA on telophase spindles (Figure 8, D–F). Consistent with this, time-lapse imaging indicated that progression from G2 to metaphase was similar to a normal mitosis, but cells then arrested with segregated kinetochores and elongated telophase spindles (Figure 8, A–C). In these experiments, An-Mad1 relocated from its G2 location at NPCs and concentrated in the kinetochore/

Figure 7. An-Mlp1 is required for correct An-Mad1 mitotic localization. Time-lapse images of cells undergoing mitosis. (A) An-Mad1-GFP with An-Ndc80-mCherry in a wild-type cell (CDS578). (B) An-Mad1-GFP with An-Ndc80-mCherry in an *An-mlp1Δ* cell (from strain hCDS831; see also Supplemental Figure S7B video). (C) An-Mad1-GFP with An-Nup49-mCherry in a wild-type cell (strain CDS604) and an *An-mlp1Δ* cell (strain hCDS662; see also Supplemental Figure S7C video). The kymographs highlight that An-Mad1-GFP remains on the mitotic apparatus throughout a wild-type mitosis (WT, arrowhead) but disperses when cells undergo mitosis in the absence of An-Mlp1 (*mlp1Δ*, arrowhead). (D) An-Mlp1-GFP with An-Ndc80-mCherry in a wild-type mitosis and (E) in an *An-mad2Δ* mitosis. Vertical lines indicate the period of An-Nup49 dispersal (red in C), kinetochore segregation to the poles (blue in A, B, D, and E), or An-Mad1 dispersal (green in A-C). Arrowheads indicate the prophase location of kinetochores. Arrows indicate telophase localization between segregating nuclei or lack of this localization in the case of *An-mlp1* nulls. Bar, ~5 μm.

spindle region during mitotic entry as normal (Figure 8, A and B, single arrowhead). As cells entered telophase An-Mad1 was present on the spindle matrix between segregated kinetochores similar to a normal mitosis (Figure 8B, arrow), but then accumulated at the spindle poles where it was present in 96.7% of arrested cells (e.g., Figure 8, A and D,

paired arrowheads). Examination of An-Mad1 together with An-Ndc80 indicated that the An-Mad1 foci in these telophase-arrested cells corresponded with segregated kinetochores that had clustered at the spindle poles (Figure 8B). Interestingly, in these experiments, not all segregated kinetochores remained at the spindle poles during the arrest. For

Figure 8. Redistribution of An-Mad1 and An-Mlp1 during a telophase arrest caused by nondegradable cyclin B expression. Time-lapse images of nondegradable cyclin B-expressing cells entering mitosis and then arresting in telophase showing An-Mad1-mCherry together with GFP-tubulin (A; strain CDS833; see also Supplemental Figure S8A video), An-Mad1-GFP together with An-Ndc80-mCherry (B; strain CDS860; see also Supplemental Figure S8B video), or An-Mlp1-mCherry together with GFP-tubulin (C; strain CDS832; see also Supplemental Figure S8C video). Single arrowheads indicate An-Mad1 or An-Mlp1 accumulation around the mitotic apparatus during mitotic entry, and arrows indicate An-Mad1 localization between kinetochore clusters which are at the spindle poles. Paired arrowheads indicate localization of An-Mad1 or An-Mlp1 at the spindle poles of telophase cells. (D and E) Fixed samples of cells arrested in telophase with elongated spindles, segregated DAPI staining DNA, and either An-Mad1 or An-Mlp1 at the spindle poles. Bar, $\sim 5 \mu\text{m}$. (F) Quantification of the percentage of mitotic nuclei at the indicated stages of mitosis before and after 1-h induction of nondegradable cyclin B.

example in Figure 8B, all kinetochores are clustered at the spindle poles by 5 min, but later in the time course a kinetochore dissociates from this cluster (Figure 8B, *). Interestingly, in this experiment An-Mad1 relocated from the seg-

regated kinetochore cluster and concentrated near this isolated kinetochore (Figure 8B, *).

Similar to the case for An-Mad1, expression of nondegradable cyclin B had little effect on the localization of An-Mlp1

Figure 9. A pool of cyclin B persists in between reforming daughter nuclei in telophase. (A) Time-lapse imaging of cyclin B-GFP during mitosis in comparison to An-Ndc80-mCherry (strain CDS544). At prophase, the majority of nuclear cyclin B disperses but a pool remains near the SPBs and kinetochore cluster (0', single arrowhead). This focus segregates with the SPBs (paired arrowheads), while the kinetochores are segregated on the spindle. As kinetochores continue to segregate, cyclin B levels at SPBs decrease. After complete segregation of kinetochores, a pool of cyclin B persists in between the segregated kinetochores (arrow) before it too is degraded. Pixel intensity profiles of cross sections along the axis defined by the kinetochores are shown. (B) As for A but showing cyclin B-GFP in comparison to An-Nup49-mCherry (Strain CDS532). The telophase pool of cyclin B between segregated nuclei is still present as An-Nup49 begins to return to daughter nuclei. Bars, ~5 μ m.

from prophase to anaphase when it concentrated near the spindle/kinetochores as normal (Figure 8C, single arrowhead). As cells entered telophase, An-Mlp1 displayed a spindle-like localization similar to a normal mitosis, but then accumulated on the spindle poles in a manner similar to An-Mad1 (Figure 8C).

These data indicate that, unlike a normal telophase, during a telophase arrest induced by nondegradable cyclin B expression, An-Mad1 and An-Mlp1 localize to kinetochores in a manner similar to that occurring during SAC arrest.

Cyclin B Degradation is Regulated in a Temporal and Spatial Manner during Mitotic Exit

Cyclin B is an APC target whose degradation is inhibited by SAC activation (Musacchio and Salmon, 2007). Given this, and the spatial regulation of SAC protein localization, we examined where cyclin B-GFP localizes during mitosis to determine if its degradation is spatially regulated as occurs in *Drosophila* (Huang and Raff, 1999) and mammalian cells (Clute and Pines, 1999; Bentley *et al.*, 2007). Cyclin B-GFP accumulated in nuclei and at SPBs during G2, consistent with previous immunofluorescence studies (Wu *et al.*, 1998). On mitotic entry, cyclin B partially dispersed into the cytoplasm, although a nuclear pool remained, primarily concentrated at the SPBs (Figure 9, A and B, single arrowhead). During metaphase and early anaphase, cyclin B-GFP was present at the segregated SPBs (Figure 9, A and B, paired arrowheads) and also in the nucleoplasm in the vicinity of the segregating kinetochores. By telophase, the SPB-associated cyclin B had disappeared, presumably because of its degradation; however, a pool persisted in the same region between fully segregated kinetochores where An-Mlp1, An-Mad1, and An-Mad2 localize (Figure 9, A and B, arrows). By this stage of mitosis, An-Nup49 reassembly to NPCs around

daughter nuclei had begun (Figure 9B, 5 min). These data suggest that cyclin B degradation is regulated temporally and spatially during mitotic exit and that the pool of cyclin B localizing near the telophase spindle is degraded last.

DISCUSSION

Mlp proteins localize to the nucleoplasmic side of interphase NPCs where they have been implicated in numerous cellular processes (Saitoh *et al.*, 1998; Galy *et al.*, 2000, 2004; Luthra *et al.*, 2007). The basis for Mlp function in these processes appears to be its role as a scaffold to tether nuclear components to the interphase nuclear periphery. For example, An-Mlp1 localizes the SAC proteins An-Mad1 and An-Mad2 to interphase NPCs. When An-Mlp1, An-Mad1, and An-Mad2 disassemble from NPCs, they display a nearly identical pattern of localization throughout mitosis. Here we demonstrate that An-Mlp1 has a scaffolding role that spatially regulates these SAC proteins not only during interphase but during mitosis as well.

An-Mlp1 Is Part of a Spindle Matrix That Acts as a Mitotic Scaffold for An-Mad1

After its disassembly from NPCs in prophase, An-Mlp1 displays a dynamic localization pattern that resembles the spindle but is distinct from it. This suggests that An-Mlp1 may be part of a spindle matrix similar to its *Drosophila* orthologue Megator (Qi *et al.*, 2004; Johansen and Johansen, 2007). Supporting this, An-Mlp1 does not require spindle formation to concentrate around the kinetochore cluster where the spindle would normally form in prophase. Therefore the prophase spindle-like localization of An-Mlp1 is cell cycle-regulated in a manner that is independent of spindle formation. Providing further evidence that An-Mlp1 mitotic

localization is under cell cycle control, preventing mitotic exit stabilized the normally transient telophase spindle-like localization of An-Mlp1.

One potential role for a spindle matrix is to act as a scaffold to keep mitotic regulators near kinetochores and the spindle. Supporting such a role we find that the spindle matrix protein An-Mlp1 is required for the proper mitotic localization of An-Mad1. During prophase, An-Mlp1 concentrates in the nucleoplasm in an expanded area around the kinetochore/spindle region, perhaps acting as a spindle matrix. In a normal prophase, An-Mad1 is similarly present in this expanded region. Contrasting this, in cells without An-Mlp1, An-Mad1 is absent from the expanded area around the kinetochore/spindle region but is still present, in a more restricted location, at the kinetochore cluster. Later in mitosis An-Mad1 is normally maintained near anaphase kinetochores and the telophase spindle in a manner similar to An-Mlp1; however, in the absence of An-Mlp1, An-Mad1 is not present in these anaphase and telophase locations. Therefore, An-Mlp1 is required to keep a pool of An-Mad1 around the spindle throughout mitosis.

A role for Mlp proteins as a mitotic scaffold for Mad1 and Mad2 may not be unique to *A. nidulans*. For example, the telophase localization of An-Mlp1 resembles that of *Drosophila* Mlp, which is a component of the mitotic spindle matrix (Qi *et al.*, 2004; Johansen and Johansen, 2007), and a similar telophase localization has recently been described for *Drosophila* Mad2 (Katsani *et al.*, 2008). Interestingly, both *A. nidulans* germlings and early *Drosophila* embryos are multinucleate, suggesting that Mlp dependent scaffolding of SAC proteins in between reforming nuclei may be a general feature of syncytial mitoses. One possibility is that An-Mlp1 confines the SAC response to individual nuclei within a common cytoplasm. Such a mechanism may explain how the SAC inhibitory signal is restricted to one spindle in mammalian cells if they contain two independent spindles (Rieder *et al.*, 1997).

Also similar to *Drosophila* (Katsani *et al.*, 2008), after mitosis An-Mad1 and An-Mad2 first accumulated in the G1 nucleoplasm before reassembling to NPCs (Figure 3; Supplemental Figures S3 and S4). This suggests that this two-step mechanism for the reassembly of SAC proteins to NPCs is likely conserved.

Monitoring Mitotic Progression

Activation of the SAC maintained the kinetochore association of An-Mlp1 as well as An-Mad1 and An-Mad2. Together with the An-Mlp1-dependent localizations of An-Mad1 and An-Mad2, this suggests that these proteins may have a functional relationship. Supporting this, while this manuscript was under review, data were published indicating the human Mlp orthologue Tpr is important for Mad1 and Mad2 checkpoint function (Lee *et al.*, 2008). However, in *A. nidulans*, An-Mlp1 is not essential for SAC activation during mitotic entry without spindle function as cells still arrest in mitosis. This is likely because An-Mad1 can still associate with kinetochores when spindle formation is completely inhibited, even in the absence of An-Mlp1. Rather, our data indicate that the absence An-Mlp1 results in loss of An-Mad1 from the mitotic apparatus during mitotic progression, suggesting An-Mlp1 may be important if kinetochore microtubule attachments are initially made but subsequently lost. This situation could arise before completion of metaphase or potentially during anaphase or telophase, perhaps resulting in SAC reactivation. In this regard it is noteworthy that once the SAC is inactivated, it can subsequently be reactivated during metaphase in mammalian

cells (Clute and Pines, 1999) and during anaphase in budding yeast (Palframan *et al.*, 2006). Therefore, by maintaining the SAC machinery near the mitotic apparatus, An-Mlp1 may help facilitate the response to mitotic defects that occur after initial fulfillment of the SAC. Supporting this concept, our data indicate that if mitotic exit is prevented by expressing nondegradable cyclin B, An-Mad1 and An-Mlp1 redistribute from the telophase spindle matrix and accumulate at segregated kinetochores, an event that does not occur in a normal mitosis.

Coordinating Mitotic Exit

Mitotic fidelity requires daughter nuclei reformation to be coordinated with DNA segregation, which is likely achieved by the temporal and spatial control of mitotic regulators. Therefore, the regulated localization of Mlp, Mad1, Mad2, and cyclin B during mitotic exit likely helps coordinate mitotic exit. For example, by telophase *A. nidulans* chromosomes have migrated to the SPBs where cyclin B has been locally degraded, although a pool still remains between reforming daughter nuclei. At this stage, the NE undergoes very different fates with the NE around daughter nuclei reforming functional NPCs and transport competent nuclei, while the NE located between daughter nuclei does not reform functional NPCs (Ukil *et al.*, 2009). Therefore, the reduction of cyclin B/Cdk1 activity at the spindle poles may allow the observed onset of NPC reformation around daughter nuclei, whereas the pool of cyclin B/Cdk1 that remains between the daughter nuclei may locally inhibit NPC reformation in this region of the NE. In addition, we have recently demonstrated that daughter nucleoli do not segregate with the NORs but remain as a parental nucleolus which is expelled to the cytoplasm (Ukil *et al.*, 2009). This cytoplasmic nucleolus then undergoes sequential disassembly in the region that An-Mlp1, An-Mad1, An-Mad2, and cyclin B also transiently locate. Therefore, we speculate that the localization of these mitotic regulators may be important to coordinate the postmitotic disassembly of the nucleolus with other aspects of mitosis.

CONCLUSIONS

The localization of Mlp proteins to a mitotic spindle matrix supports the proposal that these proteins have structural roles in mitosis as well as interphase. During mitosis, An-Mlp1 acts as a scaffold that temporally and spatially localizes the SAC proteins An-Mad1 and An-Mad2. We propose that such temporal and spatial regulation of SAC proteins in turn regulates APC activation in a similar dynamic manner to help coordinate efficient mitotic exit. Finally, our data support the notion that the An-Mlp1-dependent scaffold can redistribute in response to mitotic defects after anaphase suggesting the SAC circuitry might have functions late in mitosis.

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