Chemical Genetics Reveals a Role for Mps1 Kinase in Kinetochore Attachment during Mitosis

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Summary

Accurate chromosome segregation depends on proper assembly and function of the kinetochore and the mitotic spindle. In the budding yeast, *Saccharomyces cerevisiae*, the highly conserved protein kinase Mps1 has well-characterized roles in spindle pole body (SPB, yeast centrosome equivalent) duplication and the mitotic checkpoint [1]. However, an additional role for Mps1 is suggested by phenotypes of *MPS1* mutations that include genetic interactions with kinetochore mutations and meiotic chromosome segregation defects [1] and also by the localization of Mps1 at the kinetochore, the latter being independent of checkpoint activation [2]. We have developed a new *MPS1* allele, *mps1-as1*, that renders the kinase specifically sensitive to a cell-permeable ATP analog inhibitor, allowing us to perform high-resolution execution point experiments that identify a novel role for Mps1 subsequent to SPB duplication. We demonstrate, by using both fixed- and live-cell fluoresence techniques, that cells lacking Mps1 function show severe defects in mitotic spindle formation, sister kinetochore positioning at metaphase, and chromosome segregation during anaphase. Taken together, our experiments are consistent with an important role for Mps1 at the kinetochore in mitotic spindle assembly and function.

Results and Discussion

A Selectively Inhibitable Kinase, Mps1-as1

We generated an Mps1 ATP binding pocket mutation that confers specific sensitivity to a class of bulky ATP analog inhibitors as a tool for investigating Mps1 kinetochore function (reviewed in [3]). Only the genetically modified kinase is inhibited because the ATP binding pockets of unmodified kinases are too small for the inhibitor. By changing a single residue within the ATP binding site of the kinase domain from a methionine to a

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glycine (Figure 1A, subdomain V), we created an analog-sensitive allele of the Mps1 kinase and have termed this allele *mps1-as1*.

Inhibition of In Vitro Mps1-as1 Kinase Activity

We tested in vitro protein kinase activity of both glutathione-S-transferase (GST)-tagged Mps1 and GST-Mps1-as1 and assayed for inhibition of activity by addition of the ATP analog 4-amino-1-(*tert*-butyl)-3-(1′-naphthylmethyl) pyrazolo [3,4-*d*] pyrimidine (1NM-PP1) (Figure 1B). In the absence of inhibitor, Mps1-as1 shows slightly lower autophosphorylation activity than wild-type Mps1 (most likely the cause of the faster mobility of the altered protein in the gel [4]) and substantially lower phosphorylation of the exogenous substrate Myelin basic protein (MBP). A lowered basal activity also has been seen with analog-sensitive alleles of other kinases [5]. Importantly, however, addition of inhibitor leads to a dosage-dependent decrease in kinase activity of Mps1-as1, indicating that *mps1-as1* encodes an analog-sensitive kinase. This is in contrast to wild-type Mps1 kinase activity, which is not affected by the addition of even the highest level of inhibitor.

mps1-as1 Cells Treated with 1NM-PP1 Show Defects in Spindle Pole Body Duplication and the Spindle Checkpoint

We replaced the wild-type gene at the *MPS1* locus with the *mps1-as1* allele. The *mps1-as1* strain is viable at all temperatures tested (17°C–38°C; data not shown) and shows no detectable defect in growth rate in the absence of inhibitor when it is compared to the wild-type strain (Figure 1C). In contrast, *mps1-as1* cells, but not wild-type cells, grown in the presence of inhibitor show a severe growth defect (Figure 1C), with fewer than 1% of cells being viable after three cell doublings in the presence of inhibitor (data not shown).

To confirm the specificity of the inhibitor 1NM-PP1 in *mps1-as1* cells, we assayed its effect on the two cellular processes in which Mps1 is known to play a role: spindle pole body (SPB) duplication and the spindle checkpoint. We demonstrated that, similar to cells containing other *mps1⁻* conditional alleles [4], *mps1-as1* cells treated with 1NM1-PP1 exhibit defects in SPB duplication (Figure S1B, available in the Supplemental Data with this article online). We observed that Mps1 checkpoint function also is impaired because *mps1-as1* cells treated with 1NM-PP1 do not arrest in the presence of microtubule-depolymerizing drugs (data not shown). Furthermore, *mps1-as1* cells that are arrested with microtubule-depolymerizing drugs in the absence of inhibitor cannot maintain the arrest upon subsequent treatment with 1NM-PP1 (Figure S2A), showing that continuous Mps1 signaling is needed to maintain the arrest. We have shown previously that overexpression of wild-type Mps1 leads to mitotic arrest [6] (Figure S2B). We observe the same result with Mps1-as1 in the absence of inhibitor, but not with either a kinase-dead (kd) version of Mps1

A

B

C

Figure 1. Cells Containing *mps1-as1* Are Viable but Die in the Presence of 1NM-PP1

(A) Alignment of a portion of the nucleotide binding domain of the kinases Mps1, Cdc28p, and Src to show positions of mutations. *mps1-as1* contains a single amino acid change, M to G, at residue 516. *mps1-kinase dead* (*mps1-kd*) contains a D-to-A change at residue 580 [25]. Other mutations that render the kinase inactive are M516G with I579A, M516A with I579A, and I579G.

(B) Autoradiograph showing kinase activity inhibition of Mps1-as1 by 1NM-PP1, whereas wild-type Mps1 is not affected. Kinase assays were performed as described [2]. Relative protein levels were determined from a Western blot probed with anti-GST antibody and secondary antibody with an Odyssey imaging system and software from LI-COR Biosciences.

(C) Growth curves showing inhibition of *mps1-as1* cells but not wild-type cells in the presence of 10 μM 1NM-PP1 (3013 and 1522 strains, Table S1). One representative experiment of three trials is shown.

(Mps1-kd sequence, Figure 1A) or Mps1-as1 in the presence of inhibitor (Figure S2B). In summary, we have shown that *mps1-as1* is an effective allele for analyzing Mps1 function because inhibition with 1NM-PP1 mimics known *mps1* mutant phenotypes.

mps1-as1 Cells Treated with 1NM-PP1 Show Defects in Spindle Formation

Next, we introduced the *mps1-as1* allele into a strain designed to allow one to examine events specifically between SPB duplication and metaphase, when the mitotic spindle is formed. The strain contains *cell division cycle 34-2* (*cdc34-2*), which is a temperature-sensitive mutation in a gene encoding the SKP1-Cullin-F-box E2 enzyme [7] and leads to arrest of cells after SPB duplication [8]. The strain also contains a transcriptionally controlled allele of *CDC20*, a gene required for anaphase [9]. *CDC20* is expressed when the cells are grown in galactose-containing medium but not in glucose-containing medium, and metaphase arrest results. Cells were arrested with *cdc34-2*, then released into glucose for 1.5 hr to deplete Cdc20 (doubling time under these conditions is 4.5 hr) in the absence or presence of 1NM-PP1, and spindles were analyzed by immunofluorescence. In the absence of 1NM-PP1, normal metaphase spindles were observed at the Cdc20 depletion arrest

in *mps1-as1* cells (Figure 2A, "−1NM-PP1") and in wild-type *MPS1* cells in the presence or absence of inhibitor (data not shown). However, in *mps1-as1* cells in the presence of 1NM-PP1, we observed a predominance of discontinuous/sparse spindles (Figure 2A, "+1NM-PP1"). We confirmed these results by using electron microscopy (Figure 2B). The defect we observe is clearly after SPB duplication because we verified that duplication was complete at the *cdc34-2* arrest (Figure 2B). The phenotype is not due to premature exit from Cdc20 depletion arrest because we observe stabilized levels of Pds1 (Securin) in both the absence and presence of inhibitor (Figure S3). Furthermore, cells that are first arrested by Cdc20 depletion in the absence of inhibitor are able to maintain a metaphase arrest when inhibitor is added, and they are able to do so for at least 2 hr, as indicated by normal spindle arrest morphology and G2 DNA content (Figure S4). This shows again that the observed phenotype is not due to a defect in the Cdc20 metaphase arrest and also argues against a defect in spindle maintenance. Overall, our results indicate that Mps1 has a role in proper metaphase spindle assembly. Mps1 function is not required after metaphase because *mps1-as1* cells brought to metaphase arrest in the absence of inhibitor and released in the presence of inhibitor continue through mitosis and into G1 normally (data not shown). This result is consistent with previous data
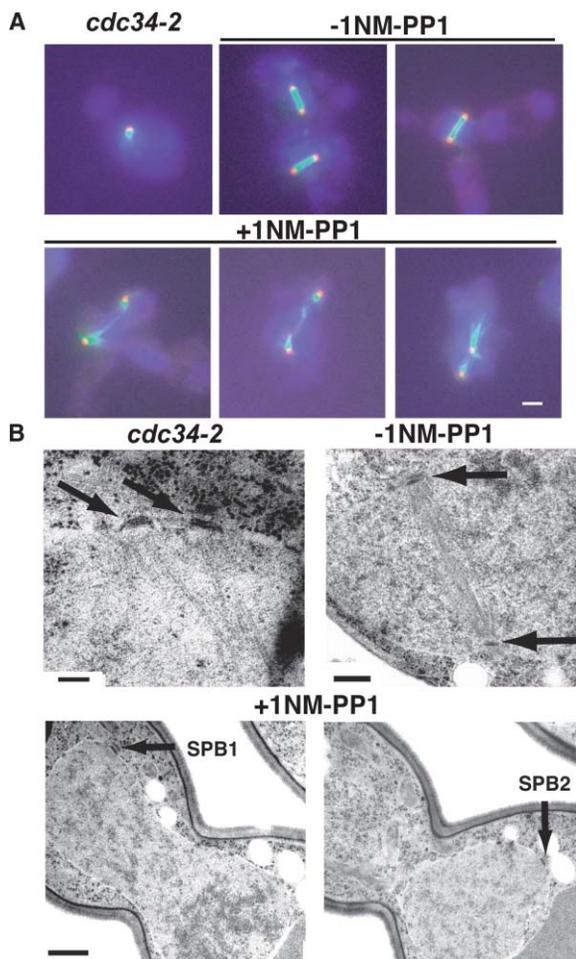
Figure 2. *mps1-as1* Cells Treated with 1NM-PP1 after SPB Duplication Are Unable to Form a Normal Mitotic Spindle

(A) Immunofluorescence images of *mps1-as1* cells showing SPBs (red, γ tubulin, antibody, gift of T. Stearns) and microtubules (green, α tubulin); immunofluorescence was performed as described [23]. Two side-by-side SPBs are detected as one focus in cells at the *cdc34-2* arrest. −1NM-PP1 = untreated *mps1-as1* cells; viability, approximately 100%. +1NM-PP1 = *mps1-as1* cells treated with 10 μM 1NM-PP1, 2% normal spindles, 98% defective spindles, n = 200; viability, approximately 40%. Spindles were considered "normal" if continuous and unbroken and "defective" if discontinuous. The scale bar represents 1 μm.

(B) EM images of *mps1-as1* cells. The top left panel shows a *cdc34-2* arrest with two duplicated side-by-side SPBs (arrows, 100%, n = 9; scale bar represents 0.1 μm). The right panel shows released, untreated cells (−1NM-PP1) with a normal spindle and two SPBs (arrows, 91%, n = 11; scale bar represents 0.3 μm). The bottom images (+1NM-PP1; scale bar represents 0.5 μm) are two serial sections of a single *mps1-as1* cell released into inhibitor; two SPBs (arrows) are separated but misoriented with respect to each other and lack spindle integrity (36% of cells examined showed a complete severing of the spindle, n = 14). Cells were prepared and viewed as described [26].

obtained with conditional *MPS1* alleles [10]. The spindle integrity defects observed when Mps1-as1 is inhibited are quite similar to those we have observed previously in cells containing a kinetochore mutation, *dam1-1* [11]; interestingly, *dam1-1* has been shown to be synthetically lethal with certain *MPS1* mutations [11].

Mps1 Inhibition Leads to Defects in Kinetochore Position in Metaphase

Because of the similarity of *mps1-as1* and *dam1-1* mutant phenotypes and their genetic interaction, we tested whether localization of a Dam1-GFP (green fluorescent protein) fusion was perturbed under the same conditions as those used in the above spindle assembly experiment. In *mps1-as1* cells at metaphase arrest, in the absence of 1NM-PP1, Dam1-GFP localizes as two closely spaced dots (Figure 3A, left panel). This reflects normal sister kinetochore separation at metaphase, as we and others have shown previously in wild-type cells for localization of Dam1 and other kinetochore proteins and for centromeric DNA [11–13]. Interestingly, in metaphase-arrested *mps1-as1* cells containing 1NM-PP1, Dam1-GFP still colocalizes with the spindle between the SPBs (data not shown), but as a single dot instead of two (Figure 3A, right panel). The collapsed configuration is reminiscent of the effect of microtubule-depolymerizing drugs and kinetochore mutations on the bi-lobed conformation of centromeric DNA [12, 14], suggesting that inactivation of Mps1 may cause a decrease in kinetochore tension.

The abnormal Dam1-GFP localization suggests that when Mps1 is inactivated, kinetochores may not be properly attached or positioned along the metaphase spindle. To address this further, we determined kinetochore/centromere position relative to spindle pole bodies in asynchronous live cells as we have done previously [15]. As before, we used the histone-like centromere binding protein Cse4, fused to GFP to allow identification of centromere position (which is observed as two clusters, as described above), and a spindle pole protein Spc29, fused to CFP (cyan fluorescent protein) to allow identification of spindle pole bodies. We placed asynchronous *mps1-as1* cells containing Cse4-GFP and Spc29-CFP on gelatin slabs in the presence of 1NM-PP1 (or with DMSO as a control) for 1 hr. Cells that had recently duplicated and separated their spindle poles (determined by a separation of Spc29-CFP of >1 μm and <2.5 μm) were analyzed. Similar to reported results for wild-type cells (and to wild-type cells treated with 1NM-PP1, data not shown), the untreated *mps1-as1* cells achieved a normal bi-lobed metaphase position of Cse4-GFP between the two SPBs in the majority of the cells (67%; Figure 3B; no inhibitor). In contrast, we found that only 37% of 1NM-PP1-treated cells achieved a distinct metaphase positioning, as shown by Cse4-GFP localization. In the remainder, the Cse4-GFP signal was not resolvable as two clusters but was present either in only one cluster or was diffuse between the two SPBs (Figure 3B, plus inhibitor). The collapsed configuration of centromeric protein localization observed in live cells is similar to that observed for Dam1-GFP in fixed cells. The defect was observed less frequently in the live-cell experiment, most likely because the assay surveyed any cell with duplicated and separated spindle poles, and such cells include those in which kinetochore attachment and spindle assembly have occurred before Mps1 is inactivated. Nonetheless, we observe that inactivation of Mps1 in live cells disrupts kinetochore positioning during metaphase, similar to what has been observed for several mutations in genes encoding kinetochore proteins [16–18].

A

-1NM-PP1   +1NM-PP1

B

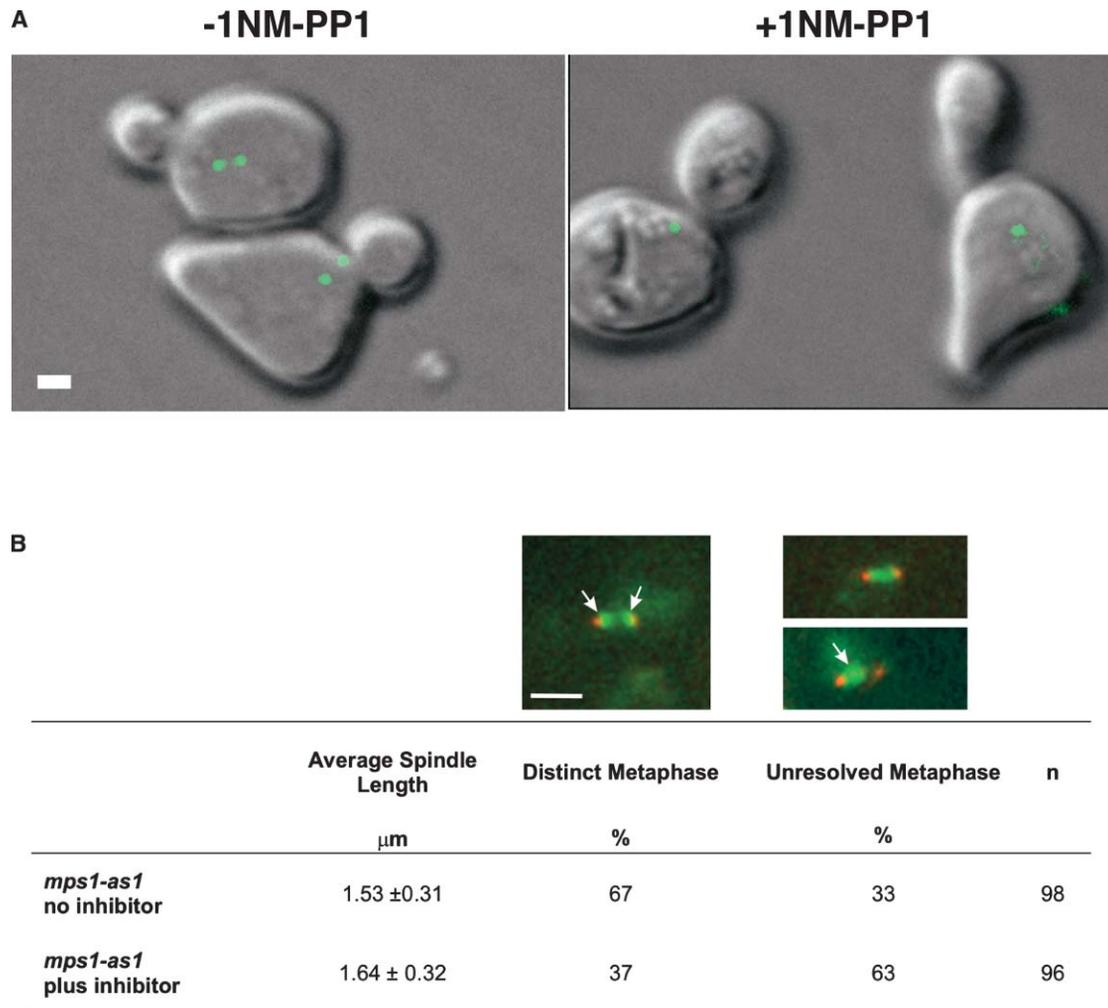|	Average Spindle Length	Distinct Metaphase	Unresolved Metaphase	n
	μm	%	%	
mps1-as1 no inhibitor	1.53 ±0.31	67	33	98
mps1-as1 plus inhibitor	1.64 ± 0.32	37	63	96

Figure 3. Mps1 Is Required for Normal Dam1 Localization and Centromere Positioning at Metaphase

(A) GFP autofluorescent and DIC (*differential interference contrast*) images of *mps1-as1 cells* (strain 3379, Table S1) synchronized at 37°C and then released into glucose for 1.5 hr, in the absence ("−1NM-PP1," 94%, n = 50) or presence ("+1NM-PP1," 97%, n = 50) of inhibitor. Samples were then processed for microscopy. The scale bar represents 1.5 μm.

(B) Centromere position was measured in *mps1-as1* cells by fluorescence imaging (Cse4-GFP = green, Spc29-CFP = red) 30 min after treatment with DMSO (no inhibitor control) or 1NM-PP1 (plus inhibitor). Wild-type cells with separated spindle pole bodies show the metaphase position of distinct separation of sister centromere clusters equally distant from the spindle equator (white arrows). In cells in which Mps1p is inhibited, centromeres did not resolve into a distinct metaphase as frequently as the wild-type. Inhibitor-treated cells exhibited unresolved fluorescence along the spindle length (top panel) or single clusters of centromeres (lower panel). Panels show representative images, and the percentages below indicate the observed frequency. Spindle length is the distance between the centers of the Spc29-CFP signal. A normal metaphase position of centromeres has been reported at 78% in wild-type cells [16]. The slightly lower frequency (67%) observed may be the result of reduced kinase activity in the *mps1-as1* strain background. Cell and media preparation as well as imaging techniques were previously described [15]. The scale bar represents 2 μm.

Mps1 Inhibition Causes Severe Defects in Chromosome Segregation

The defect in kinetochore positioning at metaphase suggests a decrease in kinetochore attachment. As an independent assay of kinetochore function, we tested whether sister chromatids segregate properly to opposite poles during mitosis in *mps1-as1* cells treated with 1NM-PP1. We visualized the location of an individual chromosome by introducing TetR binding sites on chromosome V and a gene encoding a GFP-TetR fusion protein [19]. We again used the *cdc34-2* allele to synchronize cells after SPB duplication. In this experiment, we also used a mutant allele of the cyclin Clb2 to arrest the cells with long spindles in late anaphase [20], when segregation of sister chromatids is easily monitored. Cells containing *mps1-as1* that are released from the *cdc34-2* arrest in the absence of 1NM-PP1 show normal chromosome segregation with a tagged chromosome segregating to each pole of the anaphase spindle (Figure 4, "−1NM-PP1"). In contrast, when the *mps1-as1* cells are released from *cdc34* arrest in the presence of 1NM-PP1, the majority of cells (98%, n = 200) contain both chromosomes at one pole (Figure 4, "+1NM-PP1"). Chromosome missegregation is consistent with a significant defect in kinetochore function and has been observed previously with mutations in genes such as *SPC34* [21] and *DAM1*
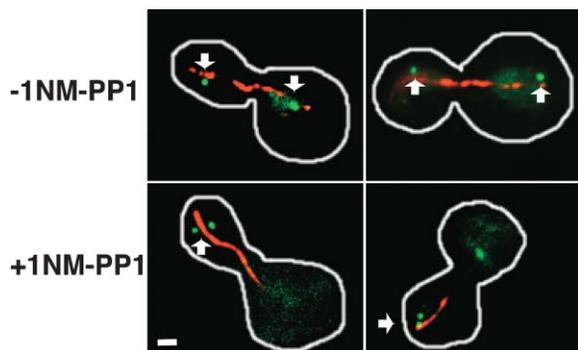
Figure 4. Mps1 Is Required for Proper Chromosome Segregation

mps1-as1 cells were released from *cdc34-2* arrest and induced as described [6] for nondestructible Clb2 in the absence (−1NM-PP1, 98% were as shown, n = 200) or presence (+1NM-PP1, 88% were as shown, n = 200) of inhibitor and processed for immunofluorescence. Only cells with intact spindles were monitored. Autofluorescence of GFP-TetR bound to chromosome V at 35 kb from the centromere, or "arm" location. (green, white arrows) and microtubules (red) stained with anti-tubulin primary antibody and Cy3 anti-rat secondary antibody are shown. Cell outlines are shown with white lines. The scale bar represents 1.5 μm.

[22, 23], which encode kinetochore components. We also tested for bias of the marked chromosomes at the old SPB (in the daughter cell), a phenotype observed previously for mutations in the yeast Aurora B kinase *IPL1* [24] and proposed to reflect defects in turnover of the connection between SPB and kinetochores. However, we observed no bias in that the two GFP-marked chromosomes were at the old SPB in only 48% of the cells (n = 100).

Conclusions

We have developed a new ATP analog-sensitive allele of *MPS1* and confirmed its effectiveness by showing that known phenotypes of *MPS1* mutants are reproduced in *mps1-as1* strains in the presence of the inhibitor 1NM-PP1. Furthermore, we demonstrated that cells containing the Mps1-as1 kinase in the presence of inhibitor show defects in the following: spindle morphology, centromere/kinetochore position at metaphase, and chromosome segregation at anaphase. We favor a role for Mps1 at the kinetochore because the combination of phenotypes we observe has been shown for other kinetochore mutations, and because previous genetic and localization data places Mps1 at the kinetochore. However, given that Mps1 has been shown to affect several steps of SPB duplication, it would not be surprising if Mps1 had roles during multiple steps of spindle assembly and function. Clearly, the next step will involve identifying kinetochore proteins with which Mps1 interacts and/or that are substrates for Mps1 kinase.

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