Dnt1 acts as a mitotic inhibitor of the spindle checkpoint protein dma1 in fission yeast

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ABSTRACT The Schizosaccharomyces pombe checkpoint protein Dma1 couples mitotic progression with cytokinesis and is important in delaying mitotic exit and cytokinesis when kinetochores are not properly attached to the mitotic spindle. Dma1 is a ubiquitin ligase and potential functional relative of the human tumor suppressor Chfr. Dma1 delays mitotic exit and cytokinesis by ubiquitinating a scaffold protein (Sid4) of the septation initiation network, which, in turn, antagonizes the ability of the Polo-like kinase Plo1 to promote cell division. Here we identify Dnt1 as a Dma1-binding protein. Several lines of evidence indicate that Dnt1 inhibits Dma1 function during metaphase. First, Dnt1 interacts preferentially with Dma1 during metaphase. Second, Dma1 ubiquitin ligase activity and Sid4 ubiquitination are elevated in dnt1Δ cells. Third, the enhanced mitotic defects in dnt1Δ plo1Δ double mutants are partially rescued by deletion of dma1+, suggesting that the defects in dnt1Δ plo1Δ double mutants are attributable to excess Dma1 activity. Taken together, these data show that Dnt1 acts to restrain Dma1 activity in early mitosis to allow normal mitotic progression.

INTRODUCTION

In the fission yeast, Schizosaccharomyces pombe, the septation initiation network (SIN) triggers actomyosin contractile ring constriction once chromosomes have segregated to opposite sides of the cell (for reviews see McCollum and Gould, 2001; Krapp et al., 2004). However, when cells are arrested in metaphase by the spindle checkpoint, SIN activity must be restrained to prevent premature mitotic exit and cytokinesis. Indeed, if the SIN is precociously activated in cells arrested in metaphase, cells exit mitosis prematurely (Fankhauser et al., 1993; Guertin et al., 2002b). To prevent precocious mitotic exit and cytokinesis, the checkpoint protein Dma1 inhibits SIN activity when cells are arrested by the spindle checkpoint (Murone and Simanis, 1996; Guertin et al., 2002b).

Fission yeast Dma1 belongs to a small FHA-RING ubiquitin ligase family, comprising two human proteins—Chfr (comprising with FHA and RING) and Rnf8 (RING finger 8)—as well as two budding yeast proteins—Dma1 and Dma2 (Scolnick and Halazonetis, 2000; Fraschini et al., 2004; Tuttle et al., 2007; Brooks et al., 2008; Loring et al., 2008). These FHA-RING ligases negatively regulate the cell division cycle, apparently by coupling protein phosphorylation events to specific ubiquitination of target proteins (reviewed in Brooks et al., 2008; Chin and Yeong, 2010). Recently a cytosolic and centrosomal protein Stil was identified as the first negative regulator of the mammalian Chfr. Stil reduces Chfr’s stability and thus protein levels by increasing its autoubiquitination (Castiel et al., 2011).

Dma1 contains both an FHA domain, which is essential for its localization at spindle pole bodies (SPBs) and the division site, and a RING finger (RF) domain, which confers E3 ubiquitin ligase activity.
to the protein (Murone and Simanis, 1996; Guertin et al., 2002b; Johnson and Gould, 2011). Dma1 localizes at the SPB through interaction with the SIN scaffold protein Sid4 and ubiquitinates Sid4 to prevent recruitment of the Polo-like kinase Plo1 during a mitotic checkpoint arrest (Chang and Gould, 2000, Guertin et al., 2002a,b; Johnson and Gould, 2011). Antagonizing Plo1 recruitment to SPBs prevents SIN activation and mitotic exit. Dma1 does not seem to inhibit Plo1 function in early mitotic events such as spindle assembly, suggesting that Dma1 itself may be regulated to limit its ability to antagonize Plo1.

Fission yeast Dnt1 was first identified in a genetic screen for suppressors of the cytokinesis checkpoint defect in a weakened SIN mutant, cdc14-118 myo2-E1 (Jin et al., 2007). The majority of Dnt1 accumulates in the nucleolus throughout the entire cell cycle. The amino acid sequence of Dnt1 shows weak similarity to those of budding yeast nucleolar proteins Net1/Cfi1 and Tof2, but no clear homologues have been found in higher eukaryotes. However, unlike Net1/Cfi1, which regulates the mitotic exit network through the Cdc14 phosphatase, Dnt1 inhibits the SIN independently of Clp1, the fission yeast homologue of Cdc14 (Jin et al., 2007). The detailed mechanism of Dnt1’s negative regulation of the SIN has remained unclear.

In this study, we identified the protein Dnt1 as a negative regulator of Dma1 in early mitosis. Dnt1 binds to Dma1 specifically in mitosis, and loss of Dnt1 results in increased levels of Dma1 at SPBs, higher Dma1 enzymatic activity, elevated Sid4 ubiquitination, and interference with early mitotic events such as spindle assembly. dnt1Δ cells also display negative genetic interactions with plo1 mutants, which are reversed by dma1 deletion, further supporting Dnt1 as a negative regulator of Dma1. Overall, we present evidence that Dnt1 restricts Dma1 function in early mitosis in order to prevent Dma1 from interfering with normal mitotic progression.

RESULTS
Identification of Dnt1 as a Dma1-binding protein
To better understand Dma1 function, we used a tandem affinity purification (TAP) strategy (Rigaut et al., 1999; Gould et al., 2004) to identify potential Dma1-associated proteins from S. pombe cells. Dma1 protein complexes were subjected to two dimensional (2D) liquid chromatography (LC) mass spectrometric analysis. One major Dma1-associated protein, Dnt1, was identified reproducibly (Figure 1A; see Supplemental Tables S2 and S3 for complete lists of copurifying proteins). Reciprocally, Dma1 was identified by 2D LC mass spectrometric analysis as a major component of purified Dnt1-TAP complexes (Figure 1A; see Supplemental Table S4 for complete list of copurifying proteins). To validate these results, we constructed a yeast strain in which Dma1 and Dnt1 were C-terminally tagged at their endogenous loci with green fluorescent protein (GFP) and 13Myc, respectively, and performed coimmunoprecipitation experiments. Our results established that Dma1-GFP stably bound Dnt1-13Myc (Figure 1B). Furthermore, by using the yeast two-hybrid system, we found that Dma1 and Dnt1 interacted with each other, suggesting that the two proteins might interact directly (Figure 1C).

**Dma1 and Dnt1 interaction is cell cycle dependent**
Dma1 levels do not vary in abundance during the cell cycle (Guertin et al., 2002b). Similarly, immunoblot analyses of cells growing asynchronously or arrested in G1, S, or metaphase indicated that Dnt1 protein levels did not change appreciably as cells progress through the cell cycle (Figure 2A). Nevertheless, we wondered whether Dma1 and Dnt1 interact at particular phases in the cell cycle. To test this, we arrested cells producing both Dma1-GFP and Dnt1-13Myc at different stages in the cell cycle using a drug (hydroxyurea [HU]) or various cell cycle mutants and subjected them to anti-GFP immunoprecipitation. Of interest, Dnt1 copurified with Dma1 the most in cells arrested in metaphase using either the proteasome mutant mts3-1 or the β-tubulin mutant nda3-KM311 (Figure 2A). We also explored whether the strong interaction between Dma1 and Dnt1 established at metaphase could remain into anaphase. We enriched anaphase cells by performing a block-and-release experiment using the nda3-KM311 mutation and found that Dnt1 bound Dma1 throughout anaphase, but the interaction decreased significantly when cells underwent cytokinesis (Figure 2B). The fact that the overall levels of Dma1 and Dnt1 do not change throughout the cell cycle (Guertin et al., 2002b) indicates that the ability of the two proteins to interact is cell cycle regulated.

**Dma1 binds a phosphorylated form of Dnt1 in metaphase**
Given that Dma1 contains a phospho-binding FHA domain, we reasoned that the interaction between the two proteins might be regulated by the phosphorylation status of Dnt1. We first tested whether

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**FIGURE 1:** Identification of Dnt1 as a Dma1-binding protein. (A) Results of tandem mass spectrometry analysis of protein mixtures from two independent Dma1-TAP purifications (1st and 2nd) and from one Dnt1-TAP purification. (B) Confirmation of the physical association between Dma1 and Dnt1 in vivo. Lysates were prepared from unsynchronized yeast cells expressing no tags, either Dma1-GFP or Dnt1-13myc, or both Dma1-GFP and Dnt1-13myc. Dma1-GFP was immunoprecipitated, and samples were analyzed by immunoblotting using anti-GFP and anti-Myc antibodies as indicated. (C) Dma1 interacts with Dnt1 by yeast two-hybrid assay. Dma1 was fused with the DNA-binding domain of GAL4 (BD) and Dnt1 with the transcriptional activation domain of GAL4 (AD). S. cerevisiae host strain PJP6-4A was cotransformed with plasmids as indicated, and growth on synthetic defined medium/-Leu, -Trp and synthetic defined medium/-Leu, -Trp, -His, +2 mM 3-aminotriazole is shown (left). As controls, coexpressions of Dma1 and empty AD vector and of empty BD vector with Dnt1 (negative control) or an AD fusion with S. pombe Sid4 (positive control) are shown. The two-hybrid interaction between Dma1 and Sid4 has been shown previously (Guertin et al., 2002b). Mean β-galactosidase activity units from liquid β-galactosidase assay are also shown (right). Error bars, SD from three independent experiments.
Dma1 binds to a phosphorylated form of Dnt1. Bacterially expressed Dma1 was used to pull down Dnt1 from metaphase-arrested yeast cell lysates, and the complexes were treated with phosphatase to determine whether the fraction of Dnt1 that was pulled down by Dma1 was phosphorylated. Indeed, phosphatase treatment significantly enhanced the gel mobility of Dnt1 protein compared with the untreated sample, indicating that Dma1 can pull down a phosphorylated form of Dnt1 (Figure 2C). Next we examined whether the Dma1 FHA domain was required for the Dnt1–Dma1 interaction. Immunoprecipitation experiments using various mutant Dma1 proteins expressed in yeast cells showed that either the complete absence of the FHA domain or mutation of critical residues in the FHA domain abolished binding between Dma1 and Dnt1. In contrast, the RF domain was dispensable for their interaction (Figure 2, D and E). We also found that pretreating yeast cell lysates with phosphatase before the pull down reduced binding of Dnt1-13myc to bacterially expressed MBP-Dma1 (Figure 2F). Taken together, these results show that Dnt1 phosphorylation is important for its ability to bind to Dma1’s FHA domain.

**Dnt1 antagonizes Dma1 function by inhibiting its SPB localization and E3 ligase activity**

As the first step to delineate the significance of the interaction between Dma1 and Dnt1, we analyzed whether Dma1 and Dnt1 affect each other’s protein level or intracellular localization. Our biochemical analyses demonstrated that the abundance of both Dma1 and Dnt1 proteins was not influenced by the presence or absence of the other (Figure 3A and unpublished data), and we did not observe any changes in Dnt1-GFP localization in the nucleolus or on anaphase spindles when Dma1 was absent or overexpressed (Supplemental Figure S1). However, we noticed that Dma1-GFP seemed brighter at SPBs when Dma1 was absent or overexpressed (Figure 3A and Dnt1-13myc cells). Compared with wild-type ∆dnt1-13myc cells (Figure 3B), the fraction of Dnt1 that was pulled down by Dma1 was phosphorylated. In contrast, the RF domain was dispensable for their interaction (Figure 2, D and E). We also found that pretreating yeast cell lysates with phosphatase before the pull down reduced binding of Dnt1-13myc to bacterially expressed MBP-Dma1 (Figure 2F). Taken together, these results show that Dnt1 phosphorylation is important for its ability to bind to Dma1’s FHA domain.
intensities at SPBs in dnt1Δ cells compared with wild-type cells (Supplemental Figure S2A). These data suggest that the effect of Dnt1 on Dma1 localization is specific to metaphase. We also investigated Dma1 localization at SPBs when Dnt1 was overexpressed from the strong nmt1 promoter. Although overproducing Dnt1 does not cause any obvious phenotypes in chromosome segregation and cytokinesis (unpublished data), it significantly reduced Dma1 localization at SPBs in anaphase and telophase cells (Supplemental Figure S2B).

We previously observed that Dma1 can autoubiquitinate (Johnson and Gould, 2011). If Dnt1 inhibits Dma1 ubiquitin ligase activity during early mitosis, we would anticipate that Dma1's autoubiquitination activity would be enhanced in the absence of Dnt1. To test this, we assayed the ubiquitin ligase activity of Dma1 after its purification from cells arrested in early mitosis using the proteasome mutant mts3-1. Consistent with Dnt1 acting as a Dma1 inhibitor, Dma1 had elevated ubiquitin ligase activity in metaphase-arrested dnt1Δ cells compared with metaphase-arrested wild-type cells (Figure 3D). Collectively these data suggest that Dnt1 inhibits Dma1 function by antagonizing its localization to SPBs and inhibiting its E3 ligase activity.

Absence of dnt1⁺ compromises Plo1 function, which is rescued by deletion of dma1⁺

Our previous study demonstrated that ubiquitination of Sid4 by Dma1 in early mitosis inhibits the polo kinase Plo1 by interfering with its localization to the SPB (Johnson and Gould, 2011). Thus we examined whether dnt1Δ displays negative genetic interactions with mutations in the plo1 kinase (plo1-24C, plo1-25, plo1-ts4), which have been shown to be defective in spindle formation and cytokinesis (Bahler et al., 1998a; Tanaka et al., 2001). Double mutants between dnt1Δ and each plo1 mutant had more severe growth defects compared with the single mutants, which could be partially rescued by deletion of dma1⁺ (Figure 4A and Supplemental Figure S4). For dnt1Δ plo1-24C, the synthetic growth defects were also similarly rescued by removal of FHA or ring finger domains of Dma1 (Supplemental Figure S3). The dnt1Δ plo1-24C double mutants also displayed a delay in early mitosis as judged by an increase in the percentage of cells with unsegregated condensed chromosomes, which was also rescued by deletion of dma1⁺ or absence of FHA or ring finger domains (Figure 4B and Supplemental Figure S3B). All these growth defects and the mitotic delay observed in dnt1Δ plo1-24C double mutants were
Cells that arrested with condensed chromosomes exhibited spindle formation defects: either a dot-like, tubulin-containing structure or a short, monopolar spindle (Figure 4C). These spindle defects were partially suppressed by deletion of dma1+ (Figure 4C). These data show that when Plo1 function is reduced, the absence of dnt1+ disrupts spindle formation and leads to a metaphase arrest. Taken together, these results support the notion that Dnt1 inhibits Dma1 activity in early mitosis to keep it from interfering with Plo1 function and potentially other regulators of early mitosis.

Overexpression of Dma1 in dnt1Δ cells causes metaphase arrest

Previous studies showed that overexpression of Dma1 caused inhibition of the SIN signaling pathway, which resulted in failed cytokinesis (Murone and Simanis, 1996; Guertin et al., 2002b). Of interest, we found that deletion of dnt1+ rendered early mitotic cells very sensitive to overexpression of Dma1, with up to 50% of cells accumulating hypercondensed chromosomes, which is typical for metaphase-arrested cells (Figure 5, A and B). This phenotype was abolished if the ring finger or FHA domain in Dma1 was deleted or key residues in these domains were mutated (Figure 5B), suggesting that the ubiquitin ligase activity of Dma1 and proper localization of Dma1 at SPB are critical for the overexpression phenotype. The presence of Cut2-GFP (securin) and Cdc13-GFP (cyclin B) signals in dnt1Δ cells overproducing Dma1 confirmed that these cells arrested in a preanaphase state, because these proteins are normally degraded in anaphase (Figure 5C). To test whether the mitotic arrest caused by Dma1 overexpression in dnt1Δ cells depends on the spindle checkpoint, we examined the effects of deleting the spindle checkpoint regulators Mad2 and Bub1. Deletion of either mad2+ or bub1+ suppressed the mitotic arrest caused by Dma1 overproduction (Figure 5D), demonstrating that in the absence of dnt1+ this arrest depends on the spindle assembly checkpoint. Therefore we examined spindle organization in dnt1Δ cells overproducing Dma1. Consistent with our previous study (Guertin et al., 2002b), overexpression of Dma1 in the presence of dnt1+ did not impair spindle assembly (Figure 5E). However, upon Dma1 overexpression in the absence of dnt1+, all cells that arrested with condensed chromosomes exhibited spindle formation defects. The spindle defects fell into three major classes: 1) complete failure to form a bipolar spindle, 2) a dot-like or monopolar spindle, and 3) a long spindle without segregated DNA (Figure 5F). We never observed any of these spindle formation defects in dnt1Δ cells without
FIGURE 5: Overexpression of Dma1 in dnt1Δ cells leads to arrest in early mitosis. (A) Examples of nmt1-dma1* and nmt1-dma1* dnt1Δ cells after overexpression of Dma1 in EMM liquid media without thiamine for 20 h at 30°C. Cells were fixed and stained with DAPI. In the dnt1Δ strain, open arrows and triangles indicate cells with two clustered nuclei in a postmitotic configuration and multinucleate cells, respectively. Asterisks indicate cells with condensed chromosomes in the dnt1Δ strain. Note that the major phenotype in Dma1-overexpressing dnt1Δ cells is defective cytokinesis, whereas cells with condensed chromosomes mainly accumulate upon overexpression of Dma1 in dnt1Δ. (B) The functional ring finger (RF) and FHA domains in Dma1 are required for inducing metaphase arrest when Dma1 is overproduced in dnt1Δ cells. The dma1* open reading frame with deleted RF domain (Dma1ΔRF), two point mutations in RF domain (Dma1C210H212A), deleted FHA domain (Dma1ΔFHA), or two point mutations in FHA domain (Dma1R64A or Dma1H88A) was integrated into the yeast genome under the full-strength nmt1 promoter and overexpressed in wild-type or dnt1Δ cells. The indicated strains were fixed and DAPI stained after Dma1 overexpression was induced as in A, and the frequencies of cells with cytokinetic defects (i.e., binucleate with the nuclei in a postmitotic configuration and multinucleate) and condensed chromosomes were quantified in wild-type cells or in dnt1Δ cells, respectively. We counted n > 200 cells for each strain. Error bars, SD from three independent experiments. (C) Securin Cut2 and mitotic cyclin Cdc13 persist in dnt1Δ cells upon overexpression of Dma1. nmt1-dma1* dnt1Δ cells carrying Cut2-GFP or Cdc13-GFP were induced for Dma1 overexpression as in A, fixed, and examined for localization of Cut2-GFP or Cdc13-GFP. Asterisks indicate cells with condensed chromosomes. (D) The phenotype of hypercondensed chromosomes in nmt1-dma1* dnt1Δ cells is dependent on the spindle assembly checkpoint. Dma1 was overexpressed in the indicated strains as in A, and cells with hypercondensed chromosomes determined by DAPI staining were counted (n > 200). Error bars, SD from three independent experiments. (E) Normal spindle formation upon overexpression of Dma1 in wild-type cells. Microtubules were labeled by indirect immunofluorescence with TAT1 antibody in wild-type cells induced for 20 h at 30°C for dma1* overexpression. Nuclei were stained with DAPI. Left, the nucleus shows condensed chromosomes at metaphase with a fully assembled spindle; right, an early-anaphase nucleus shows an intact spindle. (F) Microtubules were stained in dnt1Δ cells overproducing Dma1. These cells showed no spindle, partial spindles, or monopolar spindles, and the percentages of each type in cells with hypercondensed chromosomes (n > 200) are indicated.

DISCUSSION

Dma1 mediates SIN inhibition by preventing the Polo-like kinase Plo1, an upstream activator of the SIN, from localizing to the SPB (Guertin et al., 2002b). Sid4 ubiquitination by Dma1 prevents SIN activation and cytokinesis during a mitotic checkpoint arrest by delaying recruitment of Plo1 to SPBs while at the same time prolonging the residence of the SIN inhibitor Byr4 (Johnson and Gould, 2011). However, Dma1 does not seem to inhibit Plo1 function in early mitotic events such as spindle assembly, suggesting that Dma1 itself may be regulated to limit its ability to antagonize Plo1.

In this study, we identified Dnt1 as a factor that negatively regulates the extent of Dma1 recruitment to SPBs and Dma1’s E3 ubiquitin ligase activity in early mitosis, possibly in response to a mitotic checkpoint. Given that we detected a strong interaction between Dma1 and Dnt1 in both metaphase and anaphase, Dnt1 might modulate Dma1 and SIN activity throughout mitosis, with the interaction in early mitosis being important for Plo1 regulation and spindle assembly. Previous studies showed that both Dma1 and Dnt1 are negative regulators of the SIN pathway (Murose and Simanis, 1996; Guertin et al., 2002b; Jin et al., 2007). These seemingly contradictory results could be reconciled if Dnt1 acts to inhibit Dma1 in early mitosis, and then the two proteins function together or separately to inhibit the SIN in anaphase.

Similar to mammalian polo-like kinase 1 (Plk1), Plo1 in fission yeast is critical for proper mitotic progression, and its association with the SPB is important for microtubule nucleation and function (Ohkura et al., 1995; Bahler et al., 1998a). Previous studies identified Sid4, Cut12, and Pcp1 as major factors that recruit Plo1 to SPBs in fission yeast (Mulvihill et al., 1999; Morrell et al., 2004; Fong et al., 2010). On the basis of the results from the present study, we propose a model in which Dnt1 regulates Plo1 kinase through inhibition of Dma1 (Figure 6). Dnt1 overexpression of Dma1 (unpublished data). Taken together, our results suggest that the spindle formation defects and metaphase arrest phenotype in dnt1Δ cells overexpressing Dma1 are most likely due to a loss of Dma1 inhibition when dnt1Δ is deleted.
might function to fine tune Dma1 activity at the SPB to avoid problematic inhibitory effects on Plo1. However, because the rescue of the growth defects in \textit{dnt1Δ plo1} double mutants by removal of Dma1 was not complete (Figure 4A and Supplemental Figure S3), there might be additional functions for Dnt1 in the regulation of the SPB and mitosis.

The mammalian protein Chfr functions in an early mitotic checkpoint that delays the cell cycle in response to microtubule-targeting drugs by inhibiting polo kinase (Plk1) and the Aurora A kinase (Scollnick and Halazonetis, 2000; Kang et al., 2002; Yu et al., 2005). Like Chfr, Dma1 also inhibits the Polo kinase Plo1 to maintain a cell cycle arrest after microtubule depolymerization (Murone and Simansky, 1996; Guertin et al., 2002b), although, unlike Chfr, which blocks progression into prophase (Scollnick and Halazonetis, 2000; Summers et al., 2005), Dma1 blocks initiation of cytokinesis. Of interest, a recent study identified a cytosolic and centrosomal protein, Stil, as the first negative regulator of the mammalian Chfr in mouse embryonic fibroblasts (Castiel et al., 2011). Stil limits Chfr's inhibition of Plk1 in early mitosis to allow normal mitotic progression and proper centrosome assembly by affecting Chfr's stability and protein level (Castiel et al., 2011). Therefore the activities of both Chfr and Dma1 seem to be carefully modulated to keep them from interfering with normal mitotic progression (Castiel et al., 2011, and this study). Although fission yeast Dnt1 and mammalian Stil do not show any amino acid sequence similarity, they do share similar functions in antagonizing their respective E3 ubiquitin ligase.

It is not known how Dnt1–Dma1 interaction is regulated. However, our results show that the interaction depends on Dnt1 phosphorylation. Because the interaction between the two proteins is strongest in early mitosis, when multiple kinases are active, phosphorylation of Dnt1 would provide a mechanism for the cell cycle–specific interaction between the two proteins. Clearly, finding the kinase(s) responsible for Dnt1 phosphorylation is an important goal for future studies.

In summary, we showed that in the absence of Dnt1, Dma1 can inhibit SPB function and spindle formation much like Chfr in human cells. Thus it is possible that Dma1 acts like Chfr to inhibit mitotic progression in response to certain stimuli. Because the ability of Dma1 to block mitotic progression requires loss of Dnt1, it will be interesting in future studies to determine the conditions that modulate the interaction between Dnt1 and Dma1 to regulate passage through early mitosis.

**MATERIALS AND METHODS**

**Yeast media, strains, and genetic manipulations**

The fission yeast strains used in this study are listed in Supplemental Table S1. Genetic crosses and general yeast techniques were performed as previously described (Moreno et al., 1991). \textit{S. pombe} strains were grown in rich medium (yeast extract [YE]) or Edinburgh minimal medium (EMM) with appropriate supplements (Moreno et al., 1991). EMM with 5 \(\mu\)g/ml thiamine was used to repress expression from the \textit{nmt1} promoter. YE containing 100 mg/ml G418 (Sigma-Aldrich, St. Louis, MO) was used for selecting Kan\(^R\) cells. For serial dilution drop tests for growth, three serial 10-fold dilutions were made, and 5 \(\mu\)l of each was spotted on plates with the starting cell number of \(10^4\). Cells were pregrown in liquid YE or EMM at 25°C and then spotted onto YE or EMM plates at the indicated temperatures and incubated for 3–5 d before photography. Saccharomyces cerevisiae strain PJ69-4A was used as the host strain in two-hybrid analyses (James et al., 1996) and was transformed using the LiAc/PEG procedure (Gietz et al., 1995). Leu\(^+\) and Trp\(^+\) transformants were selected and scored for positive interactions by spotting onto synthetic dextrose plates lacking histidine or with 2 mM 3-aminotriazole (Sigma-Aldrich) added. Liquid \(\beta\)-galactosidase assays were performed as described (Pryciak and Hartwell, 1996).

**Molecular biology methods**

Carboxy-terminal-GFP and 13Myc epitope tagging of Dnt1 was done by PCR-based gene targeting (Bahler et al., 1998b). To construct the \textit{dnt1} deletion strains, the entire \textit{dnt1}-coding region was replaced with the \textit{ura4} gene or \textit{kanR} cassette by homologous recombination. Fission yeast cells were transformed using a lithium acetate–based procedure (Keeney and Boeke, 1994). All plasmids were generated by standard molecular biology techniques. cDNAs for two-hybrid analysis were cloned into the bait plasmid pGBT1 or the prey plasmid pGAD-XP (Clontech Laboratories, Mountain View, CA). \textit{dnt1} constructs in the pREP41-GFP expression vector were created by PCR amplification from \textit{S. pombe} genomic DNA or subcloned from pREP42-GFP plasmids (Guertin et al., 2002b) using \textit{NdeI}/\textit{BamHI} sites. To generate the vector for recombinant fusion protein production of MBP-dma1, a gene fragment was amplified by PCR using \textit{S. pombe} genomic DNA, \textit{cDNA} library (Clontech Laboratories), or plasmid carrying the genes as templates and then inserted into pMAL-2c (New England BioLabs, Ipswich, MA). To generate a plasmid overexpressing \textit{dnt1}\(^+\), we amplified the full-length 1852 nucleotide \textit{dnt1}\(^+\) open reading frame by PCR using \textit{S. pombe} genomic DNA as template and then inserted it into the \textit{NdeI}/\textit{Xmal} sites of the pREP1 vector (Maundrell, 1993).

Strains carrying chromosomal \textit{dma1}\(^+\), \textit{dma1}\(\Delta{\text{H212A}}\), \textit{dma1}\(\Delta{\text{H212A}}\), \textit{dma1}\(\Delta{\text{R244A}}\), or \textit{dma1}\(\Delta{\text{R244A}}\) expressed from the \textit{nmt1} promoter were constructed by first cloning the Plst–BamHI fragments harboring the \textit{nmt1} promoter and \textit{dma1} from pREP1-dma1 plasmids (Guertin et al., 2002b) and then subcloning them into vector pJK148 (Keeney and Boeke, 1994). The resulting plasmids were linearized and integrated at the \textit{leu1}\(^+\) locus by homologous
recombination as previously described for construction of nmt1-dma1-fs-long (Guertin et al., 2002b).

**Protein methods**

To prepare TAP complexes for mass spectrometry analyses, we purified Dma1-TAP and Dnt1-TAP from 8- to 10-l cultures using TAP as previously described (Gould et al., 2004) and analyzed the protein composition of the complexes by 2D liquid chromatography tandem mass spectrometry as previously described (MacCoss et al., 2002). For coimmunoprecipitation and Western blot experiments, whole-cell lysates were prepared in NP-40 buffer (6 mM Na2HPO4, 4 mM NaH2PO4, 1% NP-40, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 0.1 mM Na2VO4), and lysates were subjected to immunoprecipitation with anti-GFP (3E6; Molecular Probes, Eugene, OR) antibodies and Western blot analyses with anti-GFP or anti-Myc (9E10; Santa Cruz Biotechnology, Santa Cruz, CA) antibodies as previously described (Guertin et al., 2002b). The in vivo Sis4 ubiquitination assay was performed as previously described (Johnson and Gould, 2011). Sis4 ubiquitination was detected by immunoblotting using ubiquitin antiserum (Sigma-Aldrich) and fluorescently labeled streptavidin (LI-COR Biosciences, Lincoln, NE).

**In vitro binding assays**

All recombinant bacterially produced MBP-Dma1 proteins were expressed in Escherichia coli BL21(DE3) cells and purified on amylose beads (MBP; New England BioLabs) according to the manufacturer’s instructions and as previously described (Carnahan and Gould, 2003). Purified proteins were incubated with clarified whole-yeast-cell lysates made from dnt1-13Myc cells for 1–2 h at 4°C to examine the association between Dma1 and Dnt1. Proteins were resolved by SDS–PAGE, followed by Coomassie blue staining or Western blot analysis with anti-Myc (9E10, Santa Cruz Biotechnology) to detect proteins. Phosphatase treatment was performed either on the protein extracts pulled down by MBP-Dma1 or on yeast cell lysates before pull downs. For the phosphatase treatment after pull downs, beads were washed three times in AP buffer (50 mM Tris-HCl, pH 8.5, 1 mM EDTA) containing protease inhibitors. Either 15 U of calf-intestinal alkaline phosphatase (Fermentas, Glen Burnie, MD) or 120 U of lambda phosphatase (λ-PPase) (New England BioLabs) was added and incubated without phosphatase for 75 min at 30°C. Phosphatase treatment before pull downs, yeast cell lysates from dnt1-13Myc cells were incubated with 480 U of PPase (New England BioLabs) and without phosphatase inhibitors for 75 min at 30°C, then mixed with bacterially produced MBP-dma1 for in vitro affinity binding using amylose beads as described.

**In vitro ubiquitination assay**

Dma1-HA-TAP complexes were purified as described (Tasto et al., 2001) except that the final 1-ml elution contained 40 mM EGTA. A 5.8-μl amount of each eluate was incubated with 100 nM E1 enzyme (Boston Biochemical, Cambridge, MA), 5 μM human Ubc4 (E2) produced in bacteria as a hexahistidine fusion as described (Leverson et al., 2000), 10 μM bovine ubiquitin (Sigma-Aldrich), and 2 mM ATP in a 10-μl final volume of 50 mM Tris-HCl, pH 7.5, 2.5 mM MgCl2, and 0.5 mM EDTA for 90 min at room temperature (∼23°C). Reactions were stopped with 2× SDS–PAGE sample buffer, resolved on 3–8% Tris-acetate gels (Invitrogen, Carlsbad, CA), and analyzed by immunoblotting with anti-ubiquitin antibody (Sigma-Aldrich) or anti-hemagglutinin (12CA5).

**Immunofluorescence techniques and microscopy**

GFP or red fluorescent protein (RFP)–fusion proteins were observed in cells after fixation with cold methanol or in live cells. DNA was visualized with 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) at 2 μg/ml. Indirect immunofluorescence microscopy was done as described previously (Balasubramanian et al., 1997). Primary antibodies used were TAT1 monoclonal antibody (1:200; a gift from K. Gull, University of Oxford, Oxford, United Kingdom) for tubulin detection and mouse anti-GFP antibody (1:100; Roche, Indianapolis, IN) for detection of GFP-tagged proteins. Secondary Cy3-conjugated anti-mouse antibody (1:2000; Chemicon, Temecula, CA) and BODIPY FL-conjugated anti-mouse antibody (1:100; Molecular Probes) were used. Photomicrographs were obtained using an Eclipse E600 fluorescence microscope (Nikon, Melville, NY) coupled to a cooled charge-coupled device (CCD) camera (ORCA-ER; Hamamatsu Photonics, Hamamatsu, Japan), and image processing and analysis was carried out using IPLab Spectrum software (Signal Analytics, Vienna, VA).

For quantitative microscopy, GFP, RFP, and mCherry fusion proteins were imaged live on a spinning disk confocal microscope (UltraView LCI; PerkinElmer, Waltham, MA) with a 100×/numerical aperture 1.40 Plan Apochromat oil immersion objective. A 488-nm argon ion laser was used for GFP, and a 594-nm helium neon laser was used for RFP and mCherry fusion proteins. Images were collected using a CCD camera (ORCA-ER) and processed using MetaMorph 7.1 software (MDS Analytical Technologies, Sunnyvale, CA). Average fluorescence intensities were measured using ImageJ software (National Institutes of Health, Bethesda, MD), and final values are expressed as green/red ratios.

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