# Dbf4-dependent kinase and the Rtt107 scaffold promote Mus81-Mms4 resolvase activation during mitosis

Lissa N Princz[1], Philipp Wild[2], Julia Bittmann[1], F Javier Aguado[3], Miguel G Blanco[3] (ID), Joao Matos[2] & Boris Pfander[1,*] (ID)

## Abstract

DNA repair by homologous recombination is under stringent cell cycle control. This includes the last step of the reaction, disentanglement of DNA joint molecules (JMs). Previous work has established that JM resolving nucleases are activated specifically at the onset of mitosis. In case of budding yeast Mus81-Mms4, this cell cycle stage-specific activation is known to depend on phosphorylation by CDK and Cdc5 kinases. Here, we show that a third cell cycle kinase, Cdc7-Dbf4 (DDK), targets Mus81-Mms4 in conjunction with Cdc5—both kinases bind to as well as phosphorylate Mus81-Mms4 in an interdependent manner. Moreover, DDK-mediated phosphorylation of Mms4 is strictly required for Mus81 activation in mitosis, establishing DDK as a novel regulator of homologous recombination. The scaffold protein Rtt107, which binds the Mus81-Mms4 complex, interacts with Cdc7 and thereby targets DDK and Cdc5 to the complex enabling full Mus81 activation. Therefore, Mus81 activation in mitosis involves at least three cell cycle kinases, CDK, Cdc5 and DDK. Furthermore, tethering of the kinases in a stable complex with Mus81 is critical for efficient JM resolution.

## Introduction

Many DNA transactions are under cell cycle control to adjust them to cell cycle phase-specific features of chromosomes (Branzei & Foiani, 2008). Homologous recombination (HR) is cell cycle-regulated at several steps including the first, DNA end resection, and the last, JM removal (Heyer et al, 2010; Ferretti et al, 2013; Mathiasen & Lisby, 2014; Matos & West, 2014). Given that JMs provide stable linkages between sister chromatids, they will interfere with chromosome segregation and therefore need to be disentangled before sister chromatid separation during mitosis. Accordingly, JM resolvases, such as budding yeast Mus81-Mms4 (Interthal & Heyer, 2000; Schwartz et al, 2012) or Yen1 (Ip et al, 2008), become activated during mitosis (Matos et al, 2011, 2013; Gallo-Fernández et al, 2012; Szakal & Branzei, 2013; Blanco et al, 2014; Eissler et al, 2014). In contrast, the alternative JM removal pathway, JM dissolution by the Sgs1-Top3-Rmi1 complex, is thought to be constantly active throughout the cell cycle (Mankouri et al, 2013; Bizard & Hickson, 2014). The activation of JM resolvases in mitosis therefore leads to a shift in the balance between JM removal pathways, with dissolution being preferred outside of mitosis, but JM resolution becoming increasingly important in mitosis (Matos et al, 2011, 2013; Gallo-Fernández et al, 2012; Dehé et al, 2013; Saugar et al, 2013; Szakal & Branzei, 2013; Wyatt et al, 2013). It has been hypothesized that JM resolvases are downregulated at cell cycle stages other than mitosis in order to counteract crossover-induced loss of heterozygosity or to prevent over-active resolvases from interfering with S phase by, for example, cleaving stalled replication forks (Gallo-Fernández et al, 2012; Szakal & Branzei, 2013; Blanco et al, 2014).

Budding yeast Mus81-Mms4 has previously been shown to be targeted by two cell cycle kinases, cyclin-dependent kinase Cdc28 (CDK) and the yeast polo-kinase Cdc5 (Matos et al, 2011, 2013; Gallo-Fernández et al, 2012; Szakal & Branzei, 2013). The corresponding Mms4 phosphorylation events were shown to correlate with and to be required for activation of Mus81-Mms4 in mitosis. In 2014, we showed that in mitosis Mus81-Mms4 also forms a complex with Slx4-Slx1 and the scaffold proteins Dpb11 and Rtt107 (Gritenaite et al, 2014). Interestingly, mass spectrometric analysis of this complex (Gritenaite et al, 2014) revealed that Cdc5 and a third cell cycle kinase Dbf4-Cdc7 (Dbf4-dependent kinase, DDK) are also a stable part of this protein assembly (see Appendix Fig S1A). Here,

1   Max Planck Institute of Biochemistry, DNA Replication and Genome Integrity, Martinsried, Germany
2   Institute of Biochemistry, Eidgenössische Technische Hochschule, Zürich, Switzerland
3   Department of Biochemistry and Molecular Biology, Center for Research in Molecular Medicine and Chronic Diseases, Universidade de Santiago de Compostela, Santiago de Compostela, Spain
    *Corresponding author. Tel: +49 89 85783050; Fax: +49 89 85783022; E-mail: bpfander@biochem.mpg.de

we investigate the role of DDK in Mus81-Mms4 regulation and find that DDK can phosphorylate Mms4 and that DDK and Cdc5 target Mus81-Mms4 in an interdependent manner. Moreover, we show that Rtt107 promotes the association of both kinases with the Mus81-Mms4 complex. The DDK-dependent regulation of Mus81-Mms4 is critical for Mus81 activity thus revealing DDK as a novel regulator of homologous recombination.

# Results

### Mus81-Mms4 is a DDK phosphorylation target

The cell cycle regulation of JM resolution by Mus81-Mms4 is intricate and involves phosphorylation by the cell cycle kinases CDK and Cdc5 (Matos *et al*, 2011, 2013; Gallo-Fernández *et al*, 2012; Szakal & Branzei, 2013) as well as complex formation with the scaffold proteins Dpb11, Slx4 and Rtt107 (Gritenaite *et al*, 2014). To study this protein complex, we performed an analysis of Mms4[3FLAG] interactors in mitosis by SILAC-based quantitative mass spectrometry (Gritenaite *et al*, 2014) and found in addition to Dpb11, Slx4, Rtt107 and Cdc5, also Cdc7 and Dbf4 as specific interactors of Mms4 (Appendix Fig S1A). We verified that Cdc7 binds to Mus81-Mms4 in an Mms4[3FLAG] pull down from mitotic cells analysed by Western blots (Fig 1A). The fact that Mus81-Mms4 binds to DDK suggested that it might be involved in the phosphorylation cascade that occurs on Mms4 and controls Mus81 activity in mitosis. Accordingly, we found that purified DDK was able to phosphorylate both subunits of purified Mus81-Mms4 *in vitro* (Fig 1B, lane 3). When we furthermore compared the DDK-dependent
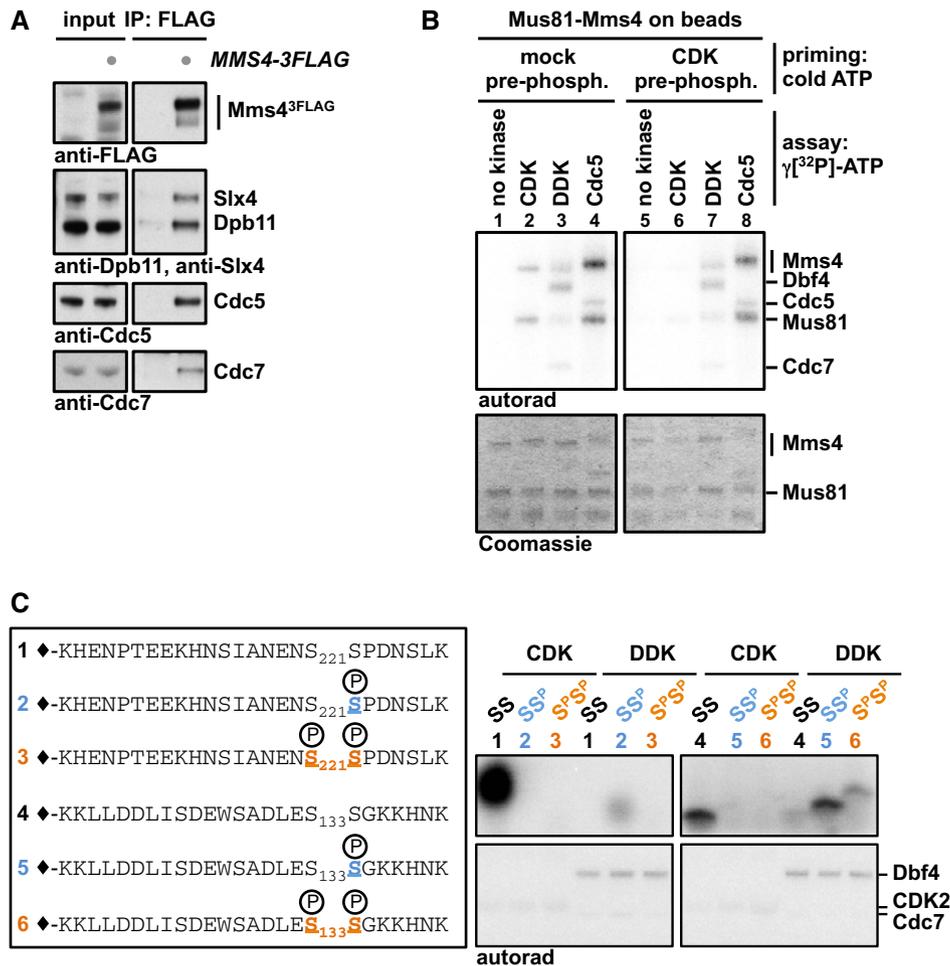


**Figure 1. Dbf4-dependent kinase (DDK) binds to the Mus81-Mms4 complex in mitosis and can phosphorylate Mms4 at (S/T)(S/T) motifs.**

A  Cdc7 and Cdc5 are specifically enriched in Mms4[3FLAG] co-IPs from cells arrested in mitosis (with nocodazole). Under the same conditions, Mus81-Mms4 associates with scaffold proteins such as Dpb11 and Slx4 (Appendix Fig S1A and Gritenaite *et al*, 2014).

B  DDK can phosphorylate Mus81-Mms4 *in vitro*. Purified, immobilized Mus81-Mms4 is incubated in an *in vitro* kinase assay with purified CDK2/cycA[N170] (a model CDK), DDK or Cdc5 (lanes 1–4). Additionally, Mus81-Mms4 is incubated with respective kinases after a non-radioactive priming step with CDK (lanes 5–8).

C  DDK phosphorylates Mms4 peptides at (S/T)(S/T) motifs and is enhanced by priming phosphorylation. Mms4 peptides including (S/T)(S/T) motifs (221/222; 133/134) were synthesized in different phosphorylation states (depicted in left panel) and incubated in an *in vitro* kinase assay with either CDK or DDK. CDK targets unphosphorylated Mms4 peptides 1 and (to a weaker extent) 4 consistent with its substrate specificity (Mok *et al*, 2010), while DDK primarily targets Mms4 peptides 2 and 5, which harbour a priming phosphorylation at the C-terminal (S/T) site (see Appendix Fig S1B for in-gel running behaviour of peptides).

phosphorylation signal to Mms4 phosphorylation by CDK and Cdc5 (Fig 1B, lanes 2–4), we observed different degrees of phosphorylation shifts indicating that the three kinases phosphorylate Mms4 at distinct sites and/or to different degrees. DDK target sites on other proteins have been studied in detail, and in several cases, DDK was found to target (S/T)(S/T) motifs, where phosphorylation was stimulated by a priming phosphorylation usually on the second (S/T) (Masai *et al*, 2006; Montagnoli *et al*, 2006; Randell *et al*, 2010; Lyons *et al*, 2013). Intriguingly, Mms4 contains 15 of these motifs and we therefore tested whether these could be targeted by DDK and would depend on priming phosphorylation. We therefore turned to a peptide-based assay where Mms4 phosphorylation states are precisely defined. To this end, we synthesized peptides corresponding to two (S/T)(S/T) motifs of Mms4. We chose two representative motifs: S222, as it harbours a minimal CDK consensus motif (S/T)P, and S134, as it contains a non-(S/T)P consensus for CDK [(S/T)X(K/R)(K/R) (Suzuki *et al*, 2015)]. For each of these motifs, we generated peptides in three different phosphorylation states: non-phosphorylated, phosphorylated at the second serine and doubly phosphorylated (Fig 1C and Appendix Fig S1B). When using such peptides as substrates in *in vitro* kinase reactions, we saw that CDK targeted specifically only the second serine in each peptide, although much stronger for S222 than for S134, consistent with these residues matching CDK consensus motifs (Fig 1C). In contrast, DDK showed only little activity towards the non-phosphorylated peptides, but was strongly stimulated when the second residue in the (S/T)(S/T) motif was in a phosphorylated state (Fig 1C). DDK may thus be stimulated by priming phosphorylation in order to efficiently phosphorylate Mms4 on (S/T)(S/T) sites. However, using the full-length protein as a phosphorylation substrate, we did not obtain evidence for a stimulatory effect on DDK by prior CDK phosphorylation (Fig 1B and Appendix Fig S1C), perhaps because over the whole 15 (S/T)(S/T) motifs CDK phosphorylation plays a minor role. We also did not reveal any priming activity of either CDK or DDK for Mms4 phosphorylation by Cdc5 (Fig 1B and Appendix Fig S1D). Overall, the data in Fig 1 thus identify Mus81-Mms4 as an interaction partner and potential substrate of DDK.

## Mus81-Mms4 is phosphorylated by a mitotic Cdc5-DDK complex

DDK is present and active throughout S phase and mitosis until anaphase when the Dbf4 subunit is degraded by APC/C[Cdc20] (Cheng *et al*, 1999; Weinreich & Stillman, 1999; Ferreira *et al*, 2000). We therefore tested at which cell cycle stage DDK would associate with Mus81-Mms4 using cells synchronously progressing through the cell cycle. Figure 2A shows that DDK did not associate with Mus81-Mms4 in S phase, but only once cells had reached mitosis. Strikingly, DDK binding therefore coincided with binding of Cdc5, Slx4 and Dpb11 and most notably the appearance of the hyperphosphorylated form of Mms4[3FLAG] (Fig 2A).

Given this late timing of the association, we tested in co-immunoprecipitation (co-IP) experiments whether DDK binding to Mus81-Mms4 would depend on CDK or Cdc5 activity. Using analog-sensitive mutant yeast strains for CDK [*cdc28-as1* (Bishop *et al*, 2000)] and for Cdc5 [*cdc5-as1* (Snead *et al*, 2007)], we observed that inhibition of these kinases in mitotically arrested cells strongly reduced the hyperphosphorylation shift of Mms4 (see also Matos

*et al*, 2013) and compromised the association with DDK (Fig 2B and C, and Appendix Fig S2A–C). Notably, both conditions also interfered with Cdc5 binding (Fig 2B and C, and Appendix Fig S2A), suggesting that the association of DDK may follow a similar regulation as Cdc5.

Next, we tested whether conversely DDK is involved in Mms4 phosphorylation. To bypass the essential function of DDK in DNA replication, we used the *mcm5[bob1-1]* allele (Hardy *et al*, 1997), which allowed us to test a *cdc7Δ* mutant. Using Western blot and SILAC-based mass spectrometry as a read-out of Mms4[3FLAG] co-IPs from cells arrested in mitosis, we found that Cdc5 association with Mus81-Mms4 was strongly reduced in the *cdc7Δ* mutant strain (Fig 2D and E). Moreover, we observed that Mms4[3FLAG] phosphorylation as indicated by mobility shift was decreased in the absence of DDK, although not to the same extent as upon CDK or Cdc5 inhibition (Fig 2D and Appendix Fig S2C). Additionally, as an alternative way to deregulate DDK, we used the *cdc7-1* temperature-sensitive mutant. Even with WT cells, we observed that elevated temperature (38°C) leads to a slight reduction in Cdc5 binding to Mus81-Mms4. However, in *cdc7-1* mutant cells, incubation at 38°C leads to the complete disappearance of Cdc5 binding to Mus81-Mms4 (Appendix Fig S2D). Therefore, we conclude from these data that DDK and Cdc5 bind to Mus81-Mms4 in an interdependent fashion.

Interestingly, Cdc5 was previously shown to interact with DDK via a non-consensus polo-box binding site within Dbf4 (Miller *et al*, 2009; Chen & Weinreich, 2010). The proposed model based on genetic experiments suggested that DDK binding antagonizes mitotic functions of Cdc5. However, the catalytic activity of Cdc5 was not inhibited in this complex (Miller *et al*, 2009) and we reason that DDK may simply target Cdc5 to a specific set of substrates. Since the Cdc5 binding site was mapped to the N-terminal portion of Dbf4 (Miller *et al*, 2009), we tested whether N-terminal truncations of Dbf4 would affect DDK or Cdc5 association with Mus81-Mms4. While the *dbf4-ΔN66* truncation lacking the first 66 amino acids (including a D-box motif) did not influence DDK or Cdc5 binding to Mms4[3FLAG], the *dbf4-ΔN109* truncation, which additionally lacks the Cdc5 binding motif (Miller *et al*, 2009), showed strongly decreased DDK and Cdc5 binding to Mus81-Mms4 (Fig 2F). Additionally, also mitotic hyperphosphorylation of Mms4 was diminished when DDK and Cdc5 could not interact with each other (Fig 2F). Overall, these data strongly suggest that Cdc5 and DDK interact with and target Mus81-Mms4 in an interdependent manner. Furthermore, it is currently unclear whether collaboration of DDK and Cdc5 is a widespread phenomenon that may affect other Cdc5 substrates as well, given that mitotic phosphorylation of two candidate Cdc5 substrates, Ulp2 and Scc1 (Alexandru *et al*, 2001), was affected to varying degree by the *cdc7Δ* mutation (Appendix Fig S2E).

Given the known cell cycle regulation of Cdc5 and DDK (Shirayama *et al*, 1998; Cheng *et al*, 1999; Weinreich & Stillman, 1999; Ferreira *et al*, 2000; Mortensen *et al*, 2005), the limiting factor for the temporal regulation of this complex and its restriction to mitosis is expected to be Cdc5 and not DDK, which is present already throughout S phase. Consistently, we observed that forced expression of Cdc5 (using the galactose-inducible *GAL* promoter) in cells that were arrested in S phase by hydroxyurea (HU) led to the premature occurrence of Mms4
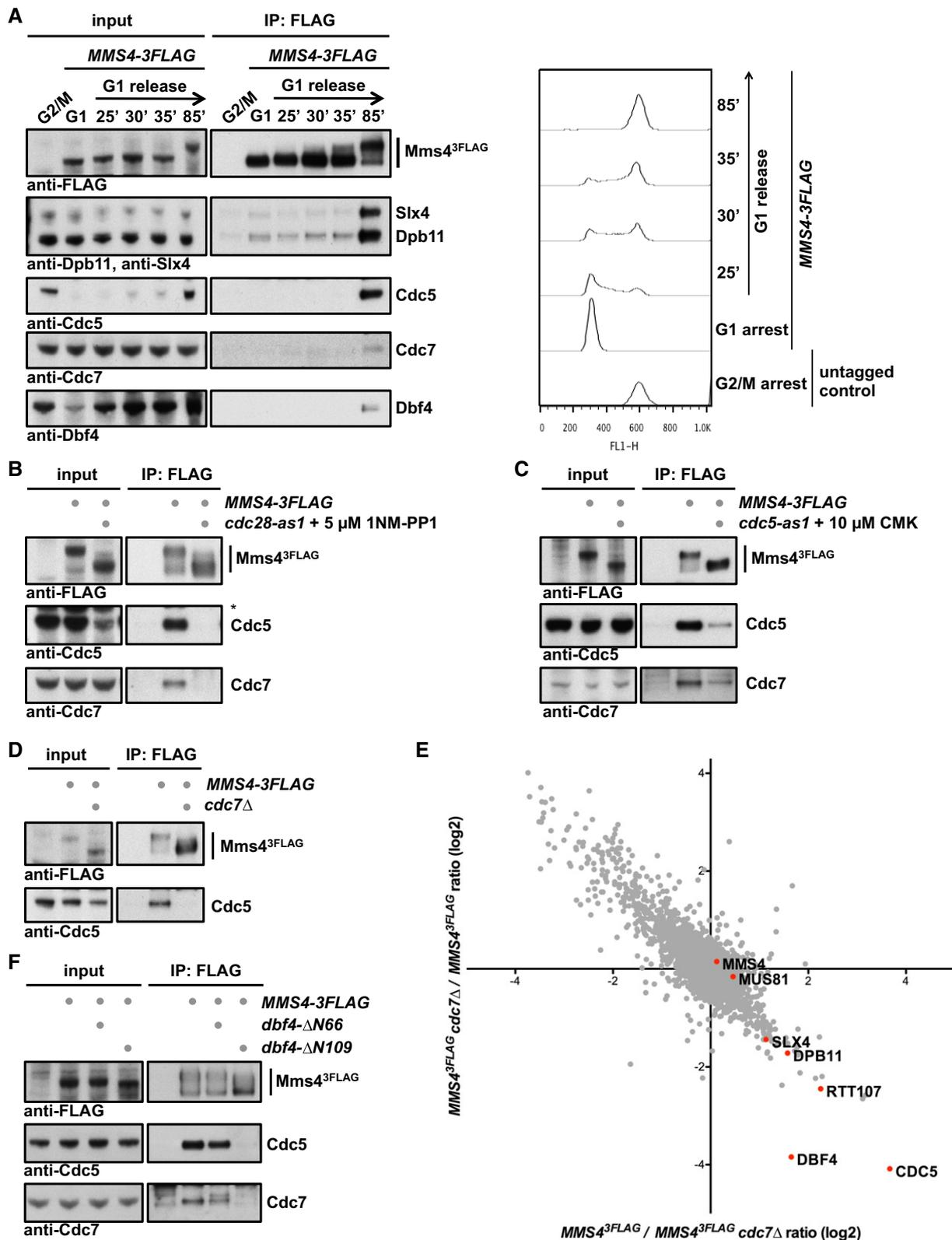
**Figure 2.**

hyperphosphorylation (Fig EV1A; Matos *et al*, 2013), suggesting that S-phase DDK is in principle competent for Cdc5 binding and joint substrate phosphorylation.

Furthermore, we performed additional experiments that addressed the regulation of Mus81-Mms4 by the DNA damage response. In M-phase-arrested cells, association of DDK and

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**Figure 2.  DDK and Cdc5 target Mus81-Mms4 in an interdependent manner.**

A    DDK stably associates with Mus81-Mms4 in mitosis, but not in S phase or G1. Mms4$^{3FLAG}$ pull down experiment (left panel, as in Fig 1A) from cells arrested in G1 (with alpha-factor) or in cells progressing synchronously through S phase until mitosis (arrest with nocodazole) reveals that DDK binds specifically in mitosis concomitant with the raise in Cdc5 levels and Cdc5 binding to Mus81-Mms4. A nocodazole-arrested untagged strain was used as a control. Right panel shows measurements of DNA content by FACS from the respective samples.

B    CDK activity is required for DDK and Cdc5 association with Mus81-Mms4. Mms4$^{3FLAG}$ pull down as in (A), but in mitotic WT or *cdc28-as1* mutant cells treated with 5 μM 1NM-PP1 for 1 h. Additional Western blots of this experiment are shown in Appendix Fig S5B, including as a control the identical anti-FLAG Western blot.

C    Cdc5 activity is required for DDK association with Mus81-Mms4. Mms4$^{3FLAG}$ pull down as in (A), but with mitotically arrested WT or *cdc5-as1* mutant cells treated with 10 μM CMK for 1 h.

D, E    DDK is required for Cdc5 binding to Mus81-Mms4 in mitosis and the mitotic Mms4 phospho-shift. (D) Mms4$^{3FLAG}$ pull down using mitotically arrested cells as in (A), but using a *bob1-1* background (all samples), where the DDK subunit Cdc7 could be deleted. (E) SILAC-based quantification of Mms4$^{3FLAG}$ pull downs in mitotically arrested *bob1-1* vs. *bob1-1 cdc7Δ* cells. Plotted are the H/L ratios of two independent experiments including label switch.

F    The Cdc5 binding region on Dbf4 is required for interaction of DDK and Cdc5 with Mus81-Mms4 and for efficient Mms4 phosphorylation. Mms4$^{3FLAG}$ pull down as in (A), but using mitotically arrested cells expressing N-terminal truncation mutants of Dbf4 lacking aa2–66 (including a D-box motif) or 2–109 [additionally including the Cdc5 binding site (Miller *et al*, 2009)].

Cdc5 with Mus81-Mms4 was reduced after induction of DNA damage with phleomycin (Appendix Fig S2F), but this treatment was not sufficient to induce a significant reduction in the Mms4 phosphorylation shift. Interestingly, when we forced Cdc5 expression in S-phase cells and compared normal S-phase cells to cells treated with hydroxyurea (HU), we observed that the Mms4 phosphorylation shift was less pronounced in the presence of hydroxyurea (HU) (Fig EV1B). These data are therefore consistent with the current view that DNA damage, specifically the DNA damage checkpoint, negatively influences Mus81 resolution activity (Szakal & Branzei, 2013; Gritenaite *et al*, 2014). Since DDK is known to be targeted and inhibited by the DNA damage checkpoint (Weinreich & Stillman, 1999; Lopez-Mosqueda *et al*, 2010; Zegerman & Diffley, 2010), it could become particularly critical to regulate Mms4 phosphorylation after DNA damage.

Even though DDK and Cdc5 seem to target Mus81-Mms4 in unison, we tested whether it was possible to resolve differences on the level of individual phosphorylation sites. Therefore, we analysed Mms4 phosphorylation sites in M-phase cells after Cdc5 inhibition (Fig 3A and C) or *CDC7* deletion (Fig 3B and D) by SILAC-based mass spectrometry. We also applied two different experimental set-ups that used either endogenously expressed Mus81-Mms4 (Fig 3A and B) or overexpressed Mus81-Mms4 (Fig 3C and D), as the latter set-up allowed much better coverage of Mms4 phosphopeptides in higher order phosphorylation states (peptides harbouring > 1 phosphorylated site). Cdc5 inhibition or lack of DDK led to overlapping, but distinct changes in Mms4 phosphorylation sites, suggesting that each kinase phosphorylates specific sites on Mms4. After Cdc5 inhibition, phosphorylation of many sites was reduced and among those were sites that match to a putative Cdc5 consensus [(D/E/N)X(S/T), blue, Fig 3A and C; Mok *et al*, 2010]. Overall, *CDC7* affected Mms4 phosphorylation less than Cdc5 inhibition, but nonetheless, we found widespread changes in the phosphorylation of (S/T)(S/T) motifs (Fig 3B and D). (S/T)(S/T) motifs were found less abundantly in the doubly phosphorylated state (Fig 3D, red), while conversely these motifs were found more abundantly in the state where only the second (S/T) was singly phosphorylated (Fig 3B and D, yellow), as expected for a substrate–product relation. These data are thus consistent with phosphorylation of the second (S/T) priming for phosphorylation at the preceding (S/T) (Appendix Table S1 and Appendix Fig S3).

## DDK phosphorylation is required for activation of Mus81-Mms4 during mitosis

Phosphorylation of Mms4 by CDK and Cdc5 has previously been shown to be required for the upregulation of Mus81-Mms4 activity during mitosis (Matos *et al*, 2011, 2013; Gallo-Fernández *et al*, 2012; Szakal & Branzei, 2013). Based on our finding that hyperphosphorylation of Mms4 was impaired in the absence of DDK (Fig 2D and Appendix Fig S2C), we predicted that also Mus81-Mms4 activity would be influenced. Therefore, we tested the activity of endogenous Mus81$^{9myc}$-Mms4$^{3FLAG}$ immunopurified from G2/M arrested cells (approx. 5 fmol) on a nicked Holliday junction (nHJ) substrate (500 fmol) using an assay related to those in (Matos *et al*, 2011, 2013; Gritenaite *et al*, 2014). Notably, the activity of the endogenous purified Mus81-Mms4 from G2/M cells exceeded the activity of recombinant Mus81-Mms4 (subjected to a dephosphorylation step during the purification), indicating that it is the mitotically activated form (Appendix Fig S4A). Moreover, the activity of endogenous purified Mus81-Mms4 was not influenced by 350 mM NaCl salt washes. This indicates that the presence of accessory, salt-labile factors such as Rtt107 or Cdc5 in the reaction is unlikely to contribute to Mus81 activity (Appendix Fig S4B and C).

Importantly, when we used this assay to test immunopurified Mus81$^{9myc}$-Mms4$^{3FLAG}$ from mitotic cells lacking DDK (*cdc7Δ* or *dbf4Δ*), we observed a reduced activity compared to Mus81$^{9myc}$-Mms4$^{3FLAG}$ from WT cells (Fig 4A and Appendix Fig S4D; also observed with an RF substrate, Appendix Fig S4E). In order to exclude that indirect effects of the *CDC7* deletion may cause the reduction in Mus81 activity, we furthermore created an Mms4 mutant that specifically lacks candidate DDK phosphorylation sites. We chose to mutate (S/T)(S/T) motifs (SS motifs in particular) and created an *mms4-8A* mutant that harboured eight S to A exchanges at the N-terminal (S/T) of the motifs (see Appendix Fig S3A). This mutant appeared less phosphorylated in mitosis as judged by a less pronounced phosphorylation shift (Fig 4B). Furthermore, we observed a reduction in the association of DDK and Cdc5 with the Mus81-Mms4-8A complex in pull-down experiments (Fig 4B), suggesting that phosphorylation of Mms4 also plays a role in tethering these kinases. Notably, Mus81$^{9myc}$-Mms4$^{3FLAG}$-8A from mitotic cells showed a moderate but reproducible reduction in resolution activity on nHJ substrates compared to WT Mus81$^{9myc}$-Mms4$^{3FLAG}$ (Fig 4C and Appendix Fig
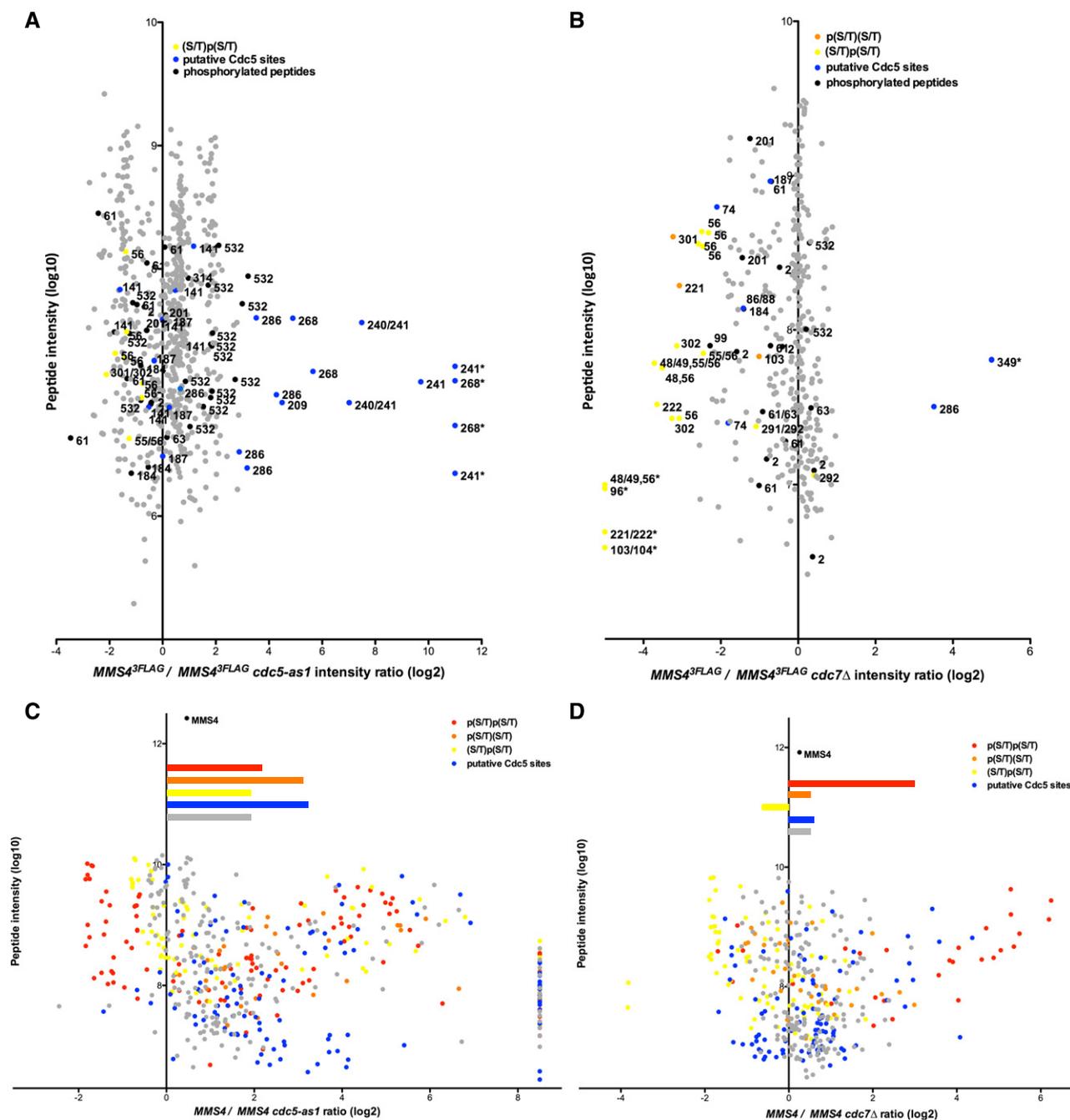
**Figure 3.  Analysis of Mms4 phosphorylation sites reveals Cdc5 and DDK target sites, as well as the interdependence between the two.**

Changes of the abundance of phosphorylated Mms4 peptides after Cdc5 inhibition (as in Fig 2C) (A, C) or in the absence of Cdc7 (B, D) in mitotically arrested cells.

A, B   Depicted are SILAC-based intensity ratios of individual MS evidences for peptides of endogenously expressed Mms4. Evidences of non-phosphorylated Mms4 peptides are shown in grey; evidences of phosphorylated peptides are shown in black, yellow, orange or blue. Blue colour indicates putative Cdc5 phosphorylation as defined by the (D/E/N)X(S/T) consensus (and additionally S268, which was also very strongly deregulated upon Cdc5 inhibition). Yellow or orange colours mark singly phosphorylated (S/T)(S/T) motifs, with orange marking p(S/T)(S/T) and yellow marking (S/T)p(S/T). Numbers indicate the phosphorylated residue in the depicted peptide. An asterisk marks peptide evidences that contained measured intensity values exclusively in the heavy or light sample. For doubly phosphorylated peptides, the two phospho-sites are separated by a comma. For singly phosphorylated (S/T)(S/T) motifs, peptide ion fragmentation was in some cases unable to unambiguously identify the phosphorylated residue. In these cases, possible phosphorylation sites are indicated as "a/b". Note that doubly phosphorylated (S/T)(S/T) sites were not reproducibly identified under conditions of endogenous Mus81-Mms4 expression.

C, D   As in panels (A, B) but using Mus81-Mms4 expressed from a high-copy promoter. Depicted are SILAC-based H/L ratios of individual MS evidences for phosphorylated peptides only. Peptides were sorted into categories according to their phosphorylation status: putative DDK target sites ((S/T)(S/T) motifs) were differentiated into the categories p(S/T)p(S/T) (red), p(S/T)(S/T) (orange) or (S/T)p(S/T) (yellow). Phosphorylated peptides matching the Cdc5 consensus site are coloured in blue. All other phosphorylated peptides are marked in grey. Bars depict the mean of the ratios of the respective category. Overall, Mms4 H/L ratio is shown on top.
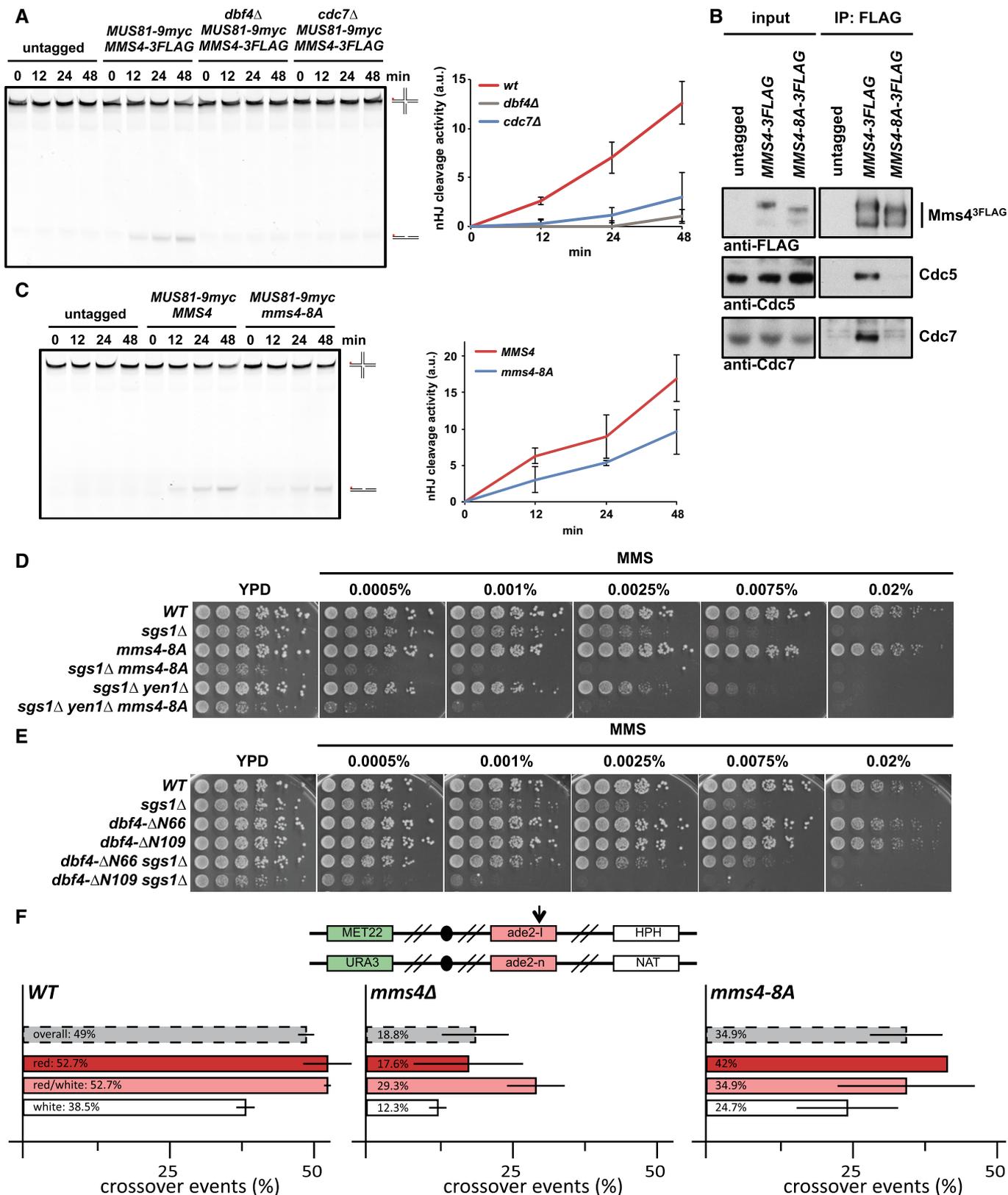
Figure 4.

S4F). These data thus indicate that DDK targets Mus81-Mms4 and that (S/T)(S/T) phosphorylation events are essential for full activation of Mus81 in mitosis.

Additionally, we investigated the relevance of the *mms4-8A* mutation *in vivo*. In comparison with *mus81Δ* or *mms4Δ* mutants, the *mms4-8A* mutant showed a hypomorphic phenotype. For

**Figure 4.  DDK phosphorylation controls activation of Mus81-Mms4 resolvase activity in mitosis.**

A     DDK is required for mitotic activation of Mus81-Mms4. Resolution assay using a nicked Holliday junction (nHJ) substrate and Mus81$^{9myc}$-Mms4$^{3FLAG}$ purified from mitotically arrested *bob1-1* (DDK-WT$^+$), *bob1-1 dbf4Δ* and *bob1-1 cdc7Δ* strains or untagged control cells. Right panel: quantification of cleavage products. See Appendix Fig S4D for Western blots samples of anti-myc IPs. Left panel: representative gel image.

B     A defect in the phosphorylation of Mms4 (S/T)(S/T) sites causes reduced association of Cdc5 and DDK with Mus81-Mms4 and reduced phosphorylation of Mms4. Mms4$^{3FLAG}$ pull down as in Fig 1A, but using mitotically arrested WT and *mms4-8A* mutant cells, which harbour 8 serine to alanine exchanges at (S/T)(S/T) motifs (detailed in Appendix Fig S3).

C     Reduced (S/T)(S/T) phosphorylation of Mms4 generates a defect in Mus81-Mms4 activity. Resolution assay as in (A), but comparing mitotic Mus81-Mms4 from untagged, WT and *mms4-8A* strains (see Appendix Fig S4F for Western blot samples of anti-myc IPs).

D, E     The *mms4-8A* mutation and lack of the Cdc5-DDK interaction (*dbf4-ΔN109*) lead to hypersensitivity towards MMS specifically in the *sgs1Δ* background. Shown is the growth of indicated strains in fivefold serial dilution on plates containing MMS at indicated concentrations after 2 days at 30°C.

F     The *mms4-8A* mutant leads to a reduction in crossover formation. Recombination assay between heterologous *ade2* alleles in diploid cells as described in Ho *et al* (2010). The top panel indicates markers on both copies of chromosome XV that are used to determine genetic outcomes of DSB repair. Arrow indicates the I-SceI cut site. Bottom panel indicates rates of crossover events (%) overall (grey) and in the individual classes (red, red/white, white) that differ in gene conversion tract length. Error bars indicate standard deviation of two independent experiments, each scoring 400–600 colonies per strain.

Data information: (A, C) Depicted are means from three independent experiments, error bars correspond to standard deviation.

example, it did neither significantly increase the MMS hypersensitivity of a *yen1Δ* mutant, nor did it confer synthetic lethality with mutants defective in STR function, such as *sgs1Δ*, even though the *mms4-8A sgs1Δ* double mutant displayed a slow growth phenotype (Figs 4D and EV2A). Importantly, however, we did observe a strongly increased hypersensitivity towards MMS, when we tested an *mms4-8A sgs1Δ* double mutant and compared it to an *sgs1Δ* single mutant (Fig 4D). The *mms4-8A* mutation thus leads to a phenotype that is very similar to other activation-deficient *MMS4* mutants in budding and fission yeast (Gallo-Fernández *et al*, 2012; Dehé *et al*, 2013; Matos *et al*, 2013). Remarkably, the MMS hypersensitivity phenotype of the *mms4-8A* mutant was highly similar to that of the Cdc5 binding-deficient *dbf4-ΔN109* mutant (Figs 4E and EV2B), which also showed reduced survival when combined with *sgs1Δ* (Fig 4E). These data are therefore consistent with DDK functioning to stimulate JM resolution via Mms4 hyperphosphorylation.

It is likely that the *mms4-8A* mutant is only partially deficient in DDK phosphorylation, since Mms4 contains overall 15 (S/T)(S/T) sites and DDK may phosphorylate the protein on non-(S/T)(S/T) sites as well. We therefore note that an *mms4-12A* mutant, harbouring four additional S to A exchanges on (S/T)(S/T) motifs, showed further increased MMS sensitivity in the *mms4-12A sgs1Δ* mutant, when compared to the *mms4-8A sgs1Δ* mutant, even though there were only minor additional effects on either the Mms4 mitotic phosphorylation shift or JM resolution activity (Fig EV2C–E).

In order to directly assess whether DDK phosphorylation was required for Mus81 function during JM resolution, we tested the influence of the *mms4-8A* mutant in a genetic crossover assay (Ho *et al*, 2010). In this system, a site-specific DSB is induced in diploid cells and repair products can be measured by the arrangement of markers and colony sectoring (Fig 4F, upper panel). In this assay, *mus81Δ* and *mms4Δ* mutants show a reduction in CO products and a proportional increase in NCO products (Fig 4F; Ho *et al*, 2010), as would be expected from a defect in JM resolution and the accompanying shift of repair pathways towards JM dissolution. The *mms4-8A* mutant shows a similar, albeit weaker defect in the formation of CO products (Fig 4F), suggesting that the defect in Mus81 activation in mitosis results in an overall defect in JM resolution. We therefore conclude that DDK—in conjunction with Cdc5—acts directly on Mms4 and that these phosphorylation events are required for efficient Mus81-dependent JM resolution in mitosis.

## The Dpb11-Mms4 interaction is not required for DDK-Cdc5-dependent activation of Mus81-Mms4

It is noteworthy that the association of DDK and Cdc5 with Mus81-Mms4 coincides with the formation of the Mus81-Mms4 complex with scaffold proteins such as Slx4, Dpb11 and Rtt107, which come together in mitosis (Fig 2A). Therefore, we asked whether the scaffold proteins Dpb11, Slx4 or Rtt107 would be required to target DDK and Cdc5 to Mus81-Mms4. In order to investigate the influence of Dpb11, we searched for an *MMS4* mutant that was deficient in the interaction with Dpb11. When we used a two-hybrid approach to map the Dpb11 interaction site on Mms4, we found that Mms4 constructs comprising aa 1–212 or 101–230 interacted with Dpb11, while constructs comprising aa 1–195 or 176–230 showed no or reduced interaction (Appendix Fig S5A). This suggested that the Dpb11 binding site may be located between aa 101–212 of Mms4. Consistently, we observed that the Mms4-S201A mutation abolished binding to Dpb11 in yeast two-hybrid and co-IP (Fig 5A and B), while the Mms4-S184A mutation reduced it (Fig 5A). Serine 201 and 184 are therefore likely candidates for phospho-sites bound and read by Dpb11. Serine 201 matches the full CDK consensus motif (S/T)PxK, while serine 184 matches the minimal CDK consensus motif (S/T)P. Indeed, we find that CDK inhibition reduced the Dpb11 interaction with Mus81-Mms4 (Appendix Fig S5B) consistent with a requirement of CDK phosphorylation for a robust interaction between Dpb11 and Mms4.

When we investigated the phenotype of the *mms4-SS184,201AA* mutant, we found that it showed enhanced hypersensitivity to MMS specifically in the *sgs1Δ* mutant background, consistent with a role of Dpb11 in JM resolution after MMS damage (Fig 5C). We also noted that the phenotype of this *MMS4* variant differed from that induced by Dpb11 binding-deficient version of Slx4 [*slx4-S486A* (Gritenaite *et al*, 2014; Ohouo *et al*, 2012)]. This could suggest that these mutants are able to separate different Dpb11 functions such as a mitotic function in conjunction with Mus81-Mms4 and an S-phase function, which Slx4 and Dpb11 might have independently of Mus81-Mms4 (Ohouo *et al*, 2012; Gritenaite *et al*, 2014; Cussiol *et al*, 2015; Princz *et al*, 2015). However, it also needs to be considered that Slx4 and Mus81-Mms4 may be connected by more than one scaffold protein (see below).

Importantly, however, we did not observe a defect in the association of DDK or Cdc5 with Mus81-Mms4, when we performed
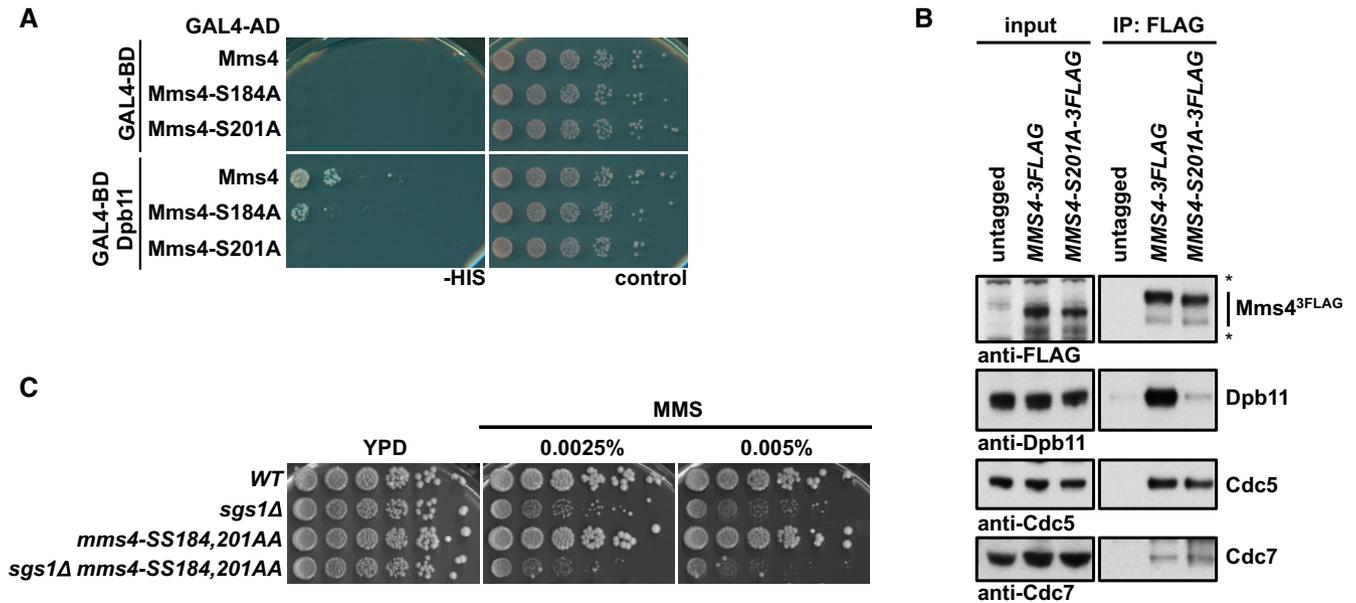
**Figure 5.  The interaction between Mms4 and Dpb11 is dispensable for binding of Cdc5 and DDK and mitotic Mus81-Mms4 activation.**

A, B   Serine 201 of Mms4 is required for Dpb11 binding, but not for interaction with DDK and Cdc5. (A) Two-hybrid interaction analysis using Gal4-BD-Dpb11 with Gal4-AD-Mms4, Gal4-AD-Mms4-S184A and Gal4-AD-Mms4-S201A constructs. (B) Mms4^3FLAG pull downs from mitotically arrested cells as in Fig 1A, but using WT or *S201A* variants of Mms4^3FLAG. Asterisks mark cross-reactive bands.

C     The Dpb11 binding-deficient allele *mms4-SS184,201AA* leads to a MMS hypersensitivity specifically in the *sgs1Δ* background. Spotting assay as in Fig 4D.

Mms4-S201A^3FLAG pull downs and compared them to a WT Mms4^3FLAG pull down (Fig 5B). Furthermore, we only observed a very minor defect in the *in vitro* resolution of nHJ substrates, when we purified Mus81-Mms4 from mitotically arrested *mms4-S201A* cells (Appendix Fig S5C). We therefore reason that Dpb11 is most likely not involved in promoting Mms4 phosphorylation or DDK-Cdc5-dependent activation of Mus81-Mms4.

**The Rtt107 scaffold recruits DDK and Cdc5 to Mus81-Mms4**

Having excluded a role of Dpb11 in the recruitment of DDK and Cdc5, we next tested a possible involvement of the Rtt107 scaffold protein. Indeed, when we used an *rtt107Δ* mutant in IP and SILAC-based IP-MS experiments, we observed that DDK and Cdc5 binding to Mus81-Mms4 was strongly reduced (Fig 6A and Appendix Fig S6A). Interestingly, Rtt107 bound to DDK and Cdc5 even under conditions where Rtt107 binding to Mus81-Mms4 was abolished (*mus81Δ*, Appendix Fig S6B). This suggests that Rtt107 may form a subcomplex with DDK and Cdc5. Consistently, we found that Rtt107 bound to Cdc7 in a two-hybrid assay (Fig 6B). These data therefore suggest that Rtt107 mediates binding of DDK and Cdc5 to the Mus81-Mms4 complex, most likely via a Cdc7 interaction site on Rtt107.

During our co-IP studies, we furthermore found that the location of Rtt107 in the mitotic Mus81-Mms4 complex was different than expected. Given that Slx4 was required to bridge between Rtt107 and Dpb11 (Ohouo *et al*, 2010) and that Mms4 and Dpb11 seemingly interact directly (Gritenaite *et al*, 2014 and Fig 5A and B), we initially expected that Slx4 and Dpb11 would be required to mediate the interaction between Rtt107 and Mus81-Mms4. Surprisingly, we found that an *slx4Δ* mutant did not influence DDK or Cdc5 binding

to Mus81-Mms4 and thereby differed from *rtt107Δ* (Fig 6A). Therefore, we tested if Rtt107 could bind to Mus81-Mms4 independently of Slx4 or Dpb11. Indeed, we found that the Mus81-Mms4 interaction to Rtt107 was not influenced by the *slx4Δ* mutant (Fig 6C) or the Dpb11 binding-deficient *mms4-S201A* allele (Fig 6D), indicating that Rtt107 binding to the Mus81-Mms4 complex occurs independently of the other scaffold proteins. In contrast, our data also show that its binding is strongly dependent on kinases and Mms4 phosphorylation, since Rtt107 binding was strongly reduced in the absence of DDK (Fig 2E), after Cdc5 inhibition (Appendix Fig S2A) or in the *mms4-8A* phosphorylation site mutant (Fig EV3).

Therefore, these data provide novel insight into the role of Rtt107 in Mus81-Mms4 regulation. First, it shows that Rtt107 mediates the association of DDK and Cdc5 kinases with Mus81-Mms4. Second, it also suggests that Rtt107 may bind directly to Mus81-Mms4 and that this binding is dependent on Mms4 phosphorylation and the cell cycle kinases DDK and Cdc5, although an alternative model whereby Rtt107 indirectly promotes DDK and Cdc5 to tightly associate with Mus81-Mms4 cannot be ruled out entirely. The fact that Rtt107 promotes the interaction of Mus81-Mms4 with the kinases, yet in turn requires the kinases and Mms4 phosphorylation for interaction, suggests that Rtt107 may be acting after initial Mms4 phosphorylation has occurred and at this late stage tethers the kinases, thus promoting phosphorylation of otherwise inefficiently phosphorylated sites.

**Rtt107 stimulates Mms4 hyperphosphorylation in order to enhance Mus81-Mms4 activity in mitosis**

Given Rtt107's involvement in tethering DDK and Cdc5 to the Mus81-Mms4 complex, we asked whether Rtt107 would mediate
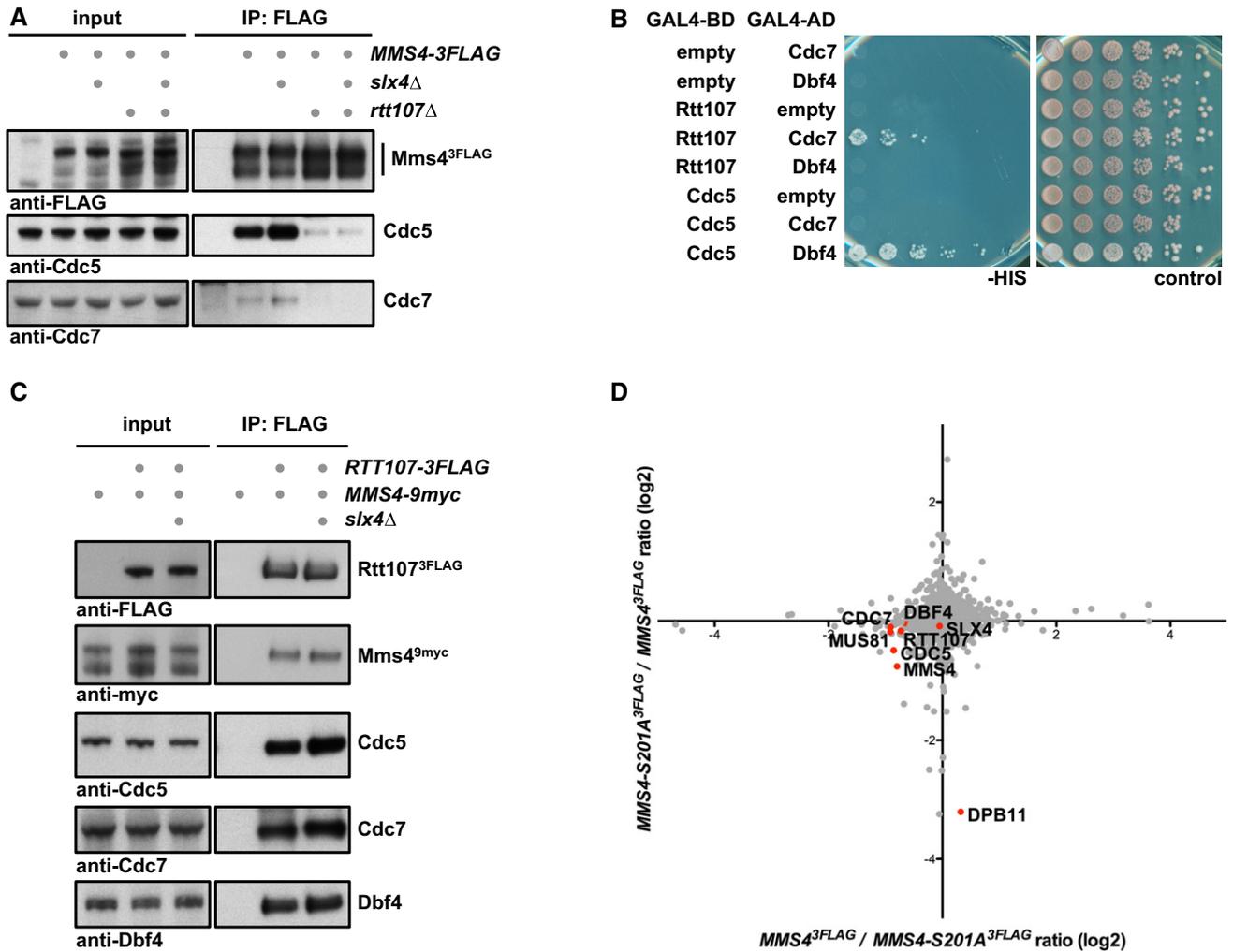
**Figure 6.** The Rtt107 scaffold tethers DDK and Cdc5 to Mus81-Mms4 independently of Slx4 and Dpb11.

A   Rtt107, but not Slx4, is required for DDK and Cdc5 interaction with Mus81-Mms4. Mms4$^{3FLAG}$ pull downs from mitotically arrested cells as in Fig 1A, but specifically comparing interactions of Mus81-Mms4 in WT, *slx4Δ*, *rtt107Δ* and *slx4Δ rtt107Δ* mutant backgrounds.

B   Rtt107 interacts with Cdc7. Two-hybrid interaction was tested using Gal4-BD-Rtt107 constructs and Gal4-AD-Cdc7 or Gal4-AD-Dbf4 constructs. Interaction between Gal4-BD-Cdc5 and Gal4-AD-Dbf4 serves as positive control.

C   Rtt107 interacts with Mus81-Mms4, DDK and Cdc5 independently of Slx4. Rtt107$^{3FLAG}$ co-IPs from untagged control, WT or *slx4Δ* cells arrested in mitosis were probed for indicated proteins.

D   Rtt107 interacts with Mus81-Mms4 independently of the Mms4-Dpb11 interaction. SILAC-based Mms4$^{3FLAG}$ pull down in WT and *mms4-S201A* cells reveals changes in the Dpb11 association, but not in Rtt107, Slx4, Cdc5 or DDK binding. Plotted are the H/L ratios of two experiments including label switch.

mitotic hyperphosphorylation of Mms4 and concomitant activation of the Mus81 nuclease. We observed only a minor effect on the mitotic phospho-shift of Mms4 when using *rtt107Δ* mutants (Fig 6A and Appendix Fig S2C). However, as it is still unclear which phosphorylation sites contribute to the Mms4 phospho-shift, we investigated the effect of *rtt107Δ* on individual phosphorylation sites in our mass spectrometry data. Appendix Fig S7A and B shows SILAC-based comparisons of Mms4 phosphorylation sites in WT and *rtt107Δ* cells, expressing Mus81-Mms4 from endogenous (Appendix Fig S7A) or high-copy promoters (Appendix Fig S7B). The overexpression set-up allowed us to quantify phosphorylation at (S/T)(S/T) motifs, and we found that double phosphorylation of several of these sites was reduced (Appendix Fig S7B), although the change was much smaller compared to cells lacking DDK. On the

other hand, while we could not detect higher order phosphorylated Mms4 peptides using endogenous Mus81-Mms4, we could detect an effect of Rtt107 on several other sites (T209, S241 and S268, and to a lesser extent S286; Appendix Fig S7A), which were also deregulated after Cdc5 inhibition (Fig 3A and C). These data are thus consistent with Rtt107 promoting efficient DDK and Cdc5 phosphorylation of Mms4.

Therefore, we tested whether Rtt107 would affect the mitotic activation of Mus81-Mms4. We immunopurified Mus81$^{9myc}$-Mms4$^{3FLAG}$ from WT and *rtt107Δ* cells that were arrested in mitosis and found that Mus81-Mms4 activity on a nHJ substrate was reduced in the *rtt107Δ* background (Fig 7A and Appendix Fig S7C). Furthermore, in the background of deficient DDK (*cdc7Δ bob1-1*), additional mutation of *rtt107Δ* did not lead to a further defect in

Mus81-mediated cleavage (Appendix Fig S7D). Therefore, we conclude that Rtt107 is required for full mitotic activation of Mus81-Mms4 and that it works at least in part through cell cycle kinases such as DDK.

In order to test whether such a defect in Mus81-Mms4 activation would translate into a shifted balance of JM removal pathways, we measured rates of crossover and non-crossover formation in the absence of Rtt107. We observed a reduction in crossover rates in the *rtt107Δ* mutant indicating a shift in the balance of JM removal pathways (Fig 7B). The decrease was mostly visible in one class of recombinants (Fig 7B, "red") and is smaller compared to the phenotype of a *mus81Δ* or a *mms4-8A* mutant (Ho *et al*, 2010; Fig 4F), consistent with a stimulatory but non-essential role of the Rtt107

scaffold in Mus81-Mms4 function. These data thus provide the first mechanistic insight of how the interaction of the mitotic Mus81-Mms4 complex with the scaffold proteins influences Mus81 function, as Rtt107 facilitates DDK and Cdc5 tethering, full mitotic phosphorylation of Mms4 and activation of Mus81-Mms4.

## Discussion

Activation of Mus81-Mms4 during mitosis is critical for the response to DNA damage, in particular to process repair intermediates that may arise from DSBs and stalled replication forks (Matos *et al*, 2011, 2013; Gallo-Fernández *et al*, 2012; Saugar *et al*, 2013; Szakal
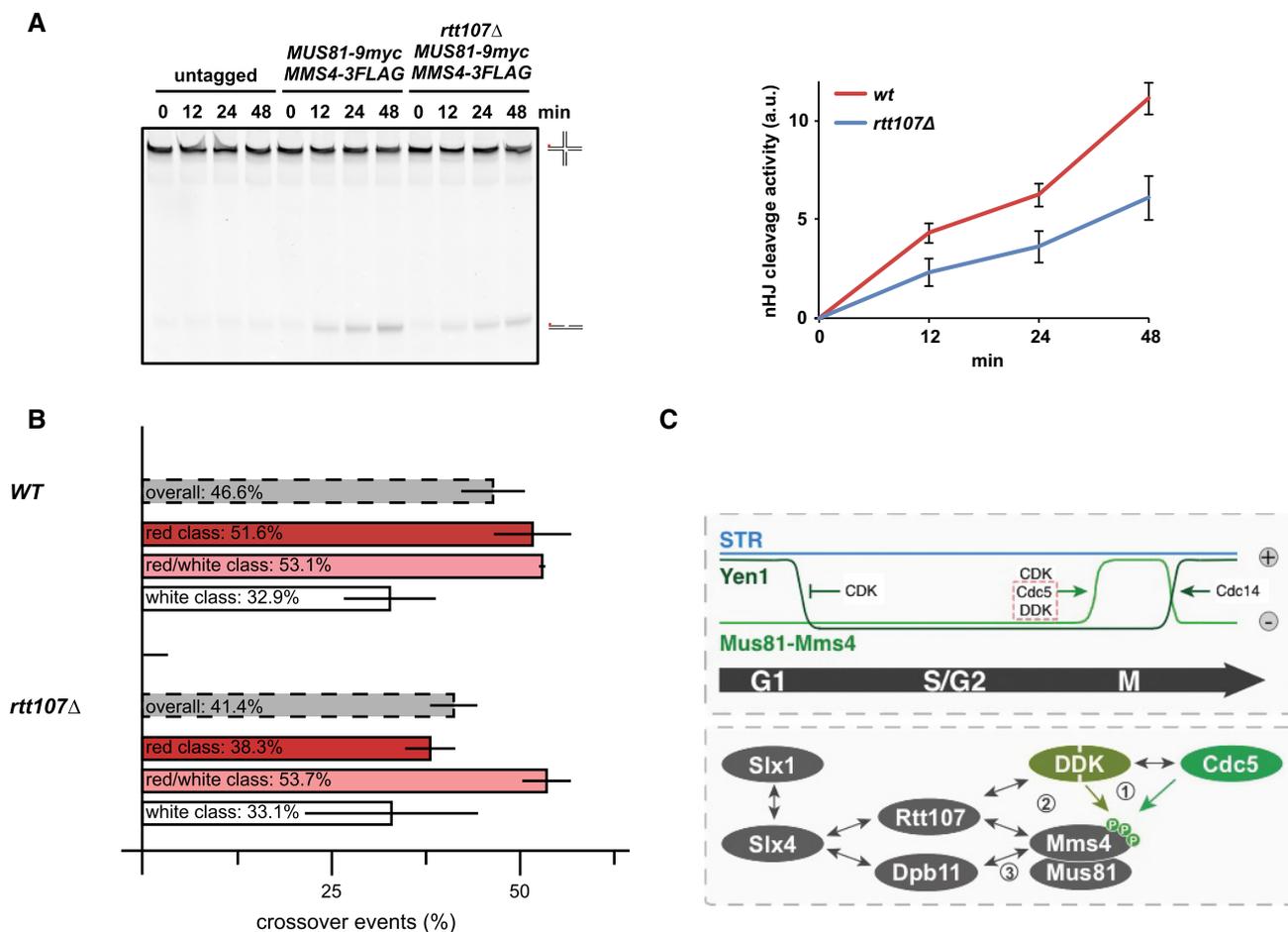


**Figure 7. Rtt107 is required for efficient Mus81-Mms4 activation in mitosis.**

A   Mus81-Mms4 purified from mitotic *rtt107Δ* cells is less active compared to Mus81-Mms4 from WT cells. *In vitro* resolution activity of Mus81[9myc]-Mms4[3FLAG] purified from WT or *rtt107Δ* cells is tested on a nHJ substrate (see Appendix Fig S7C for control Western blot). Right panel: quantification of cleavage products from three independent experiments (mean ± SD). Left panel: representative gel picture.

B   The *rtt107Δ* mutant leads to a reduction in crossover formation. Recombination assay as in Fig 4F. Note that the *rtt107Δ* mutant particularly affects crossover formation in the red class (long conversion tracts), while no significant defect could be observed in the red/white and white class (mean ± SD).

C   Hypothetical model of Mus81-based JM resolution. Upper panel: cell cycle regulation of JM removal pathways, indicating Mus81 activation in mitosis. Lower panel: physical interactions of Mus81-Mms4 and its regulatory complex in mitotic cells. Grey arrows indicate physical interactions; green arrows specifically indicate kinase–substrate interactions. Genetic data indicate a hierarchy of molecular events leading to Mus81 activation. (1) DDK, Cdc5 and CDK (not shown) phosphorylate Mms4. (2) Rtt107 binds to DDK and Cdc5 and—in a phosphorylation-dependent manner—associates with Mus81-Mms4. This interaction is either direct or could potentially depend on bridging effects by DDK and Cdc5. Rtt107 promotes the stable interaction of DDK and Cdc5 with Mus81-Mms4 and thus full phosphorylation of Mms4 and Mus81 activation. (3) Upon Mms4 phosphorylation, two scaffold proteins, Rtt107 and Dpb11, bind independently to Mus81-Mms4. Both proteins can also bind to Slx4 enabling two alternative connections of Slx4 with Mus81-Mms4.

& Branzei, 2013). Previously, this regulation was shown to critically depend on phosphorylation by the cell cycle kinases CDK and Cdc5 (Matos *et al*, 2011, 2013; Gallo-Fernández *et al*, 2012; Saugar *et al*, 2013; Szakal & Branzei, 2013), but also involve the formation of a multi-protein complex comprising several scaffold proteins (Gritenaite *et al*, 2014). Here, we not only identify a new cell cycle kinase to be crucial for this regulation—DDK—but moreover show that the two regulatory pathways—cell cycle kinase phosphorylation and scaffold complex formation—are connected by Rtt107 (see Fig 7C for a hypothetical model). Rtt107 association depends on active cell cycle kinases and Mms4 phosphorylation, but in turn Rtt107 is required for stable DDK and Cdc5 association with the Mus81-Mms4 complex, as well as full phosphorylation of Mms4 and mitotic activation of Mus81. This study thus extends our mechanistic understanding of the regulatory framework that controls cell cycle-regulated JM resolution.

Interestingly, our work shows that for its function as a regulator of Mus81-Mms4 DDK must act interdependently and as a complex with Cdc5. DDK and Cdc5 have been shown to interact physically (Miller *et al*, 2009; Chen & Weinreich, 2010), but until now DDK was viewed to antagonize mitotic functions of Cdc5 (Miller *et al*, 2009). In contrast, in meiosis I DDK and Cdc5 are known to cooperate in order to promote chromosome segregation and jointly phosphorylate the monopolin and cohesin subunits Lrs4 and Rec8, respectively, as well as the meiotic regulator Spo13 (Matos *et al*, 2008). We now provide the first example for a joint DDK and Cdc5 substrate in the mitotic cell cycle, suggesting that cooperation between DDK and Cdc5 could be a more widespread phenomenon than previously anticipated. The apparent antagonism between DDK and Cdc5 in the regulation of mitotic exit (Miller *et al*, 2009), a canonical Cdc5 function, could be explained if DDK targeted Cdc5 to a specialized subset of substrates rather than to substrates involved in mitotic exit. It is also interesting to note that we could detect significant DDK binding to Mus81-Mms4 only after cells finished S phase (Fig 2A). Therefore, the role of DDK in Mms4 phosphorylation is clearly post-replicative and further challenges a simplified view of DDK as an S-phase kinase (Matos *et al*, 2008). It will therefore be interesting to see whether additional DDK substrates during mitosis can be identified and whether DDK collaborates with Cdc5 for their phosphorylation as well.

Mus81-Mms4 has previously been shown to be cell cycle-regulated and Mms4 to be a critical CDK and Cdc5 phosphorylation target (Matos *et al*, 2011; Gallo-Fernández *et al*, 2012). We add DDK to this already complex regulation. Our data clearly show that phosphorylation of (S/T)(S/T) motifs is critical for Mus81-Mms4 function. The hypomorphic phenotype of the *mms4-8A* mutant (Fig 4C, D and F) is likely due to additional DDK phosphorylation sites either on Mms4 or perhaps even on Mus81. Importantly, DDK does not appear to establish the timing of Mms4 phosphorylation in mitosis, as Cdc5 still seems to be the limiting factor for this temporal control in undisturbed cell cycles (Fig EV1B). However, the fact that activation of Mus81-Mms4 depends on the activity of several kinases makes it a coincidence detector that integrates the activity of several cell cycle regulators. Therefore, it can be envisioned that there are specific cellular conditions under which DDK activity becomes limiting for Mus81-Mms4 activation. Notably, DNA damage checkpoint kinases are known to phosphorylate DDK and counteract its function during S phase (Weinreich & Stillman, 1999; Lopez-Mosqueda *et al*, 2010; Zegerman & Diffley, 2010). Therefore,

it can be speculated that the checkpoint acts as a negative regulator of Mus81-Mms4 activation via inhibition of DDK. Such regulation could therefore explain how the presence of DNA damage restricts Mus81 activity towards replication intermediates (Matos *et al*, 2011, 2013; Saugar *et al*, 2013; Szakal & Branzei, 2013; Gritenaite *et al*, 2014), suggesting that cell cycle and checkpoint pathways converge in the regulation of Mus81.

A second layer of Mus81 regulation relies on the formation of a multi-protein complex, which assembles specifically in mitosis and contains Mus81-Mms4, DDK, Cdc5 and Slx4 as well as the scaffold proteins Dpb11 and Rtt107 (Gritenaite *et al*, 2014). We are only beginning to understand the mechanism whereby this scaffold complex influences Mus81 function. Here, we show that Rtt107, but not Dpb11 or Slx4, promotes the stable association of DDK and Cdc5 with Mus81-Mms4 (Fig 6), suggesting that one function of the multi-protein complex is to promote efficient Mus81-Mms4 phosphorylation. Conversely, our new data as well as our previous work (Gritenaite *et al*, 2014) show that phosphorylation by cell cycle kinases also regulates the formation of the multi-protein complex. In particular, Rtt107 association with Mus81-Mms4 depends strongly on DDK and Cdc5 (Fig 2E and Appendix Fig S2A). A direct interaction of Rtt107 with Mus81-Mms4 seems the most plausible interpretation of our data, although we currently cannot exclude that Rtt107 may facilitate the interaction of DDK and Cdc5 with Mus81-Mms4 without a direct interaction. A possible phosphorylation dependence of Rtt107 binding to the complex could thus originate from Mms4 phosphorylation generating a binding site for Rtt107 [e.g. for Rtt107 BRCT domains (Li *et al*, 2012)].

Importantly, Rtt107 is in turn required for stable binding of DDK and Cdc5 (Fig 6A and Appendix Fig S6A). Via tethering the kinases, Rtt107 regulates the phosphorylation of specific Mms4 sites and is required for full Mus81 activation (Fig 7A and Appendix Fig S7A and B). The interdependence between Rtt107 and Cdc5/DDK phosphorylation therefore suggests that Rtt107 may be part of a signal amplification mechanism, which ensures efficient Mus81-Mms4 phosphorylation and activation. Mechanistically, Rtt107-dependent stimulation of Mms4 phosphorylation thus resembles a kinase priming mechanism. It is entirely possible that other kinase priming mechanisms for either Cdc5 or DDK are at work in the Mms4 phosphorylation cascade, although the *in vitro* kinase assays with full-length proteins did not provide support for such a mechanism (Fig 1B, and Appendix Fig S1C and D). Altogether, it seems plausible to speculate that Rtt107-dependent and Rtt107-independent amplification mechanisms are involved in generating a switch-like activation of Mus81 in mitosis.

Furthermore, Rtt107 can also bind to Slx4 (Ohouo *et al*, 2010). There are thus two BRCT-containing scaffold proteins—Dpb11 (Gritenaite *et al*, 2014) and Rtt107—that could bridge between Mus81-Mms4 and Slx4. Interestingly, our data with different *mms4* mutants suggest that either one of these BRCT scaffold proteins is sufficient to connect Slx4 and Mus81-Mms4 [Figs 6D and EV3; note that the *rtt107Δ* mutant (Appendix Fig S6A) is difficult to interpret in this regard as it also leads to defects in Slx4 phosphorylation and the Slx4-Dpb11 interaction (Ohouo *et al*, 2010)]. This redundancy may thus explain the modest phenotype of the *mms4-S201A* mutant that is deficient in the Mms4-Dpb11 interaction (Fig 5C).

Several aspects of Mus81-Mms4 regulation are conserved throughout eukaryotic evolution. The HJ resolution activity of

Mus81-Eme1 in mammalian cells is cell cycle-regulated (Matos *et al*, 2011; Wyatt *et al*, 2013). Mus81-Eme1 furthermore binds to Slx4 and forms multi-protein complexes (Fekairi *et al*, 2009; Muñoz *et al*, 2009; Svendsen *et al*, 2009; Castor *et al*, 2013; Wyatt *et al*, 2013), albeit these complexes may have a different organization to that in yeast. Therefore, it will be interesting to explore in the future if in human cells DDK is also required for activation of Mus81-Eme1 and if this mechanism may contribute to the anti-tumorigenic activity of DDK inhibitors (Montagnoli *et al*, 2008).

## Materials and Methods

All yeast strains are based on W303 and were constructed using standard methods. Plasmids were constructed using the In-Fusion HD cloning kit (Clontech Laboratories), and mutations were introduced by site-directed mutagenesis. A summary of all yeast strains used in this study can be found in the Appendix Table S2.

Cell cycle synchronization was achieved using alpha-factor (G1), hydroxyurea (S), or nocodazole (mitosis). DNA content was measured by flow cytometry with a BD FACSCalibur system using SYTOX green to stain DNA.

Co-immunoprecipitations of yeast extracts were performed on anti-FLAG agarose resin (Sigma) for 2 h with head-over-tail rotation at 4°C as previously described (Gritenaite *et al*, 2014). After bead washing, proteins were eluted by 3X FLAG-peptide (Sigma), precipitated and separated on 4–12% Bis-Tris gels. For SILAC-based mass spectrometry, cells were labelled with heavy-isotope-labelled lysine (Lys6 or Lys8), and proteins were digested with Lys-C. Mass spectrometry data were analysed using MaxQuant (Cox & Mann, 2008).

Yeast two-hybrid assays, genetic interaction assays, *in vitro* kinase assays and peptide binding assays were performed as described previously (Pfander & Diffley, 2011; Gritenaite *et al*, 2014).

Nuclease assays were done as described (Matos *et al*, 2011, 2013). Briefly, Mus81[9myc] was immunopurified from mitotically arrested cells and mixed with 5′-Cy3-end-labelled nicked Holliday junctions. After incubation at 30°C for the indicated times, the reaction was stopped by proteinase K and SDS for 1 h at 37°C. Products were separated by 10% PAGE, and cleavage efficiency was normalized to the level of immunoprecipitated Mus81[9myc]. Unspecific nHJ cleavage in untagged controls was subtracted in the quantifications.

DSB-induced recombination assays were performed as described (Ho *et al*, 2010). Diploids harbouring I-SceI under the control of the *GAL* promoter were grown in adenine-rich raffinose medium and arrested in mitosis. Nuclease expression was induced by addition of galactose for 2.5 h. Cells were plated on YPAD and replica plated on YPAD + Hyg + Nat, YPAD + Hyg, YPAD + Nat, SC-Met, SC-Ura and SCR-ADE + Gal media after 3–4 days to classify recombination events.

Detailed experimental procedures are available in the Appendix.

### Data availability

Mass spectrometric datasets are available at EBI PRIDE. DDK and the Rtt107 scaffold promote Mus81-Mms4 resolvase activation during mitosis (2015). PXD005356.

**Expanded View** for this article is available online.

## Author contributions

PW and JM performed *in vitro* resolution assays of Figs 4A and C, 7A, and EV2E, and Appendix Figs S4A, C, E, and S7D and analysed the data. FJA and MGB provided recombinant purified Mus81-Mms4 used in Fig 1B, and Appendix Figs S1C and D, and S4A. All other experiments were performed and analysed by LNP, JB and BP. BP wrote the paper and all authors commented on the manuscript.

## Conflict of interest

The authors declare that they have no conflict of interest.

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