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The DNA-Binding Domain of S. pombe Mrc1 (Claspin) Acts to Enhance Stalling at Replication Barriers

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Abstract

During S-phase replication forks can stall at specific genetic loci. At some loci, the stalling events depend on the replisome components Schizosaccharomyces pombe Swi1 (Saccharomyces cerevisiae Tof1) and Swi3 (S. cerevisiae Csm3) as well as factors that bind DNA in a site-specific manner. Using a new genetic screen we identified Mrc1 (S. cerevisiae Mrc1/metazoan Claspin) as a replisome component involved in replication stalling. Mrc1 is known to form a sub-complex with Swi1 and Swi3 within the replisome and is required for the intra-S phase checkpoint activation. This discovery is surprising as several studies show that S. cerevisiae Mrc1 is not required for replication barrier activity. In contrast, we show that deletion of S. pombe mrc1 leads to an approximately three-fold reduction in barrier activity at several barriers and that Mrc1’s role in replication fork stalling is independent of its role in checkpoint activation. Instead, S. pombe Mrc1 mediated fork stalling requires the presence of a functional copy of its phylogenetically conserved DNA binding domain. Interestingly, this domain is on the sequence level absent from S. cerevisiae Mrc1. Our study indicates that direct interactions between the eukaryotic replisome and the DNA are important for site-specific replication stalling.

Introduction

The process of genome duplication is a significant challenge to the cell as genetic and epigenetic information have to be precisely copied as well as the genome integrity maintained. Interestingly, the replication forks do not progress along the DNA at an uniform rate and can pause or terminate at so called site-specific replication barriers (reviewed in Ref. [1]). Site-
specific barriers exist in two main types; “DNA-binding protein” mediated and “hard to replicate” sequences. The latter being repetitive DNA sequences that can form noncanonical stable secondary structures such as hairpins, cruciforms, triplexes and quadruplexes [2–11]. Protein-mediated barriers generally are thought to act to maintain genomic stability by preventing the collision of the transcription machinery with replication forks and the subsequent formation of dysfunctional replication forks (reviewed in Ref. [1]). However, the process of stalling replication at such barriers can itself lead to DNA instability [12,13] and in fission yeast replication barriers have been shown to mediate a program of cellular differentiation involving DNA rearrangements (see below; reviewed in Ref. [14]).

Protein-mediated replication-stalling events are generally mediated by two types of trans-acting factors. One type moves with the replication fork, while the other type consists of DNA binding proteins, which are statically bound to site-specific cis-acting DNA elements at the barrier loci. S. pombe Swi1 (S. cerevisiae Tof1) and Swi3 (S. cerevisiae Csm3) are factors of the first type [15–21]. In both S. cerevisiae and S. pombe, these two factors have been shown to travel with the replication fork and in S. cerevisiae Tof1 and Csm3 have been shown to be integral parts of the replisome [18–21]. Similarly, the human homologues TIMELESS (Swi1) and TIPIN (Swi3) interact to form a complex, and co-localize with PCNA [22]. Swi1/Tof1 and Swi3/Csm3 mediate stalling of the replication forks at loci where the second type of static barrier proteins are bound. Recently this has been shown also for TIMELESS at the human rDNA barrier [23]. The best studied loci include binding sites of Sap1, Reb1, and Rtf1 in TIPIN (Swi3) interact to form a complex, and co-localize with PCNA [22]. Swi1/Tof1 and Swi3/Csm3 have been shown to be integral parts of the replisome [18–21]. Similarly, the human homologues TIMELESS (Swi1) and TIPIN (Swi3) interact to form a complex, and co-localize with PCNA [22]. Swi1/Tof1 and Swi3/Csm3 mediate stalling of the replication forks at loci where the second type of static barrier proteins are bound. Recently this has been shown also for TIMELESS at the human rDNA barrier [23]. The best studied loci include binding sites of Sap1, Reb1, and Rtf1 in S. pombe as well as Fob1 and kinetochores in S. cerevisiae [24–29]. Importantly, in the absence of Swi1/Tof1 and Swi3/Csm3 there is a complete loss of barrier activity at these genetic loci [24–29]. Swi1/Tof1 has a more complex role at stalled forks at tRNA genes and at sequences that can form stable DNA secondary structures [2,29,30].

Swi1 and Swi3 possess functional activities connected with the control of S-phase progression in addition to their replication barrier activity. Swi1 and Swi3 as well as their S. cerevisiae homologues form a trimeric complex with the S-phase checkpoint mediator Mrc1 [31,32]. In S. cerevisiae this complex of Mrc1, Tof1 (Swi1) and Csm3 (Swi3) can be co-purified with other known replisome components [19,31]. Moreover, Swi1, Swi3, and Mrc1 also act in the checkpoint response, activated by replication stress, from the “sensor kinase” Rad3 (S. cerevisiae Mec1/ Metazoan ATR) to the effector kinase Cds1 (S. cerevisiae Rad53/ Metazoan Chk1) [33–35]. However, it is important to highlight that deletion experiments of mrc1, swi1(tof1) and swi3(csm3) indicate functional differences between the genes. For example, Cds1 phosphorylation observed in cells treated with HU is completely lost in an mrc1 deletion background, while only a reduction in Cds1 phosphorylation is observed when swi1 or swi3 are deleted [20,35,36]. Similarly, while a deletion of tof1, the S. cerevisiae homologue of Swi1, only has a minor effect on the general rate of S-phase progression, deletion of S. cerevisiae mrc1 leads to a significant reduction [29,37,38]. Also, while S. cerevisiae Tof1 is required for replication protein-mediated barrier activity at the Fob1 barrier in the rDNA, and at several tRNA genes and centromeres investigated, a deletion of S. cerevisiae Mrc1 does not affect stalling at these loci [17,29,38,39].

In summary the molecular mechanism that underlies replication-stalling events at natural barriers is not well understood to date and its study is complicated by the fact that several important factors seem to be active in multiple pathways. However, the following recent discoveries have improved our understanding of which factors are involved in replication fork stalling as well as the roles they are playing. Using a new screening tool based on the mating-type switching system of S. pombe the flavine adenine dinucleotide-dependent lysine-specific demethylase enzymes, Lsd1 and Lsd2, were identified as required for replication stalling at several replication barriers [40]. These barriers included the MPS1 and RTS1 elements in the mating-type region as well as the rDNA barrier element. It is not known how Lsd1 and Lsd2
act at these elements, but experiments suggest that both enzymes have structural and catalytic roles in mediating replication fork stalling. It has also been shown in *S. cerevisiae* that Tof1 (*S. pombe* Swi1) and Csm3 (*S. pombe* Swi3) counteract the helicase Rrm3 to mediate replication barrier activity [17]. Rrm3 is a helicase that travels with the replication fork and is required for the efficient removal of non-histone proteins in front of the fork [41]. Furthermore, an amino-acid substitution has been identified in Swi1 that abolishes barrier activity of Rtf1 at the RTS1 element but that does not affect barrier activity of other barriers investigated [15]. This suggests that specific protein-protein interactions between Rtf1 and Swi1 might be important for replication stalling at the RTS1 element. Finally, the *S. pombe* factor Rtf2 has been shown to act to prevent Srs2 mediated replication restart, thus promoting termination, at the RTS1 element [42].

In this study, we utilize the mating-type switching system in a novel genetic screen to identify *S. pombe* Mrc1 (*metazoan* Claspin) as required for efficient replication stalling (Fig 1A). We show that *S. pombe* Mrc1 has a general role mediating efficient stalling at several replication barriers, including MPS1, RTS1, the rDNA barrier and a tRNA gene. This novel function of *S. pombe* Mrc1 is independent of the protein’s checkpoint activity, but dependent on a helix-turn-helix DNA-binding domain. This domain has been shown to bind DNA in a non-site specific way with a preference for branched DNA structures [43]. Importantly, this DNA-binding domain is phylogenetically conserved in a wide range of eukaryotic mrc1 (CLASPIN) genes except in *S. cerevisiae* mrc1. This suggests that there might be specific differences between the mechanism of replication stalling in *S. cerevisiae* and *S. pombe*.

**Results**

**Using mating-type switching as a tool to identify potential factors required for fork stalling**

The mating-type switching system of fission yeast *S. pombe* is an excellent model system to study replication fork stalling, because it is dependent on a replication-coupled recombination event that is established through the involvement of several replication barriers ([15]; Fig 1A) and produces an easily detectable phenotype. The high sporulation levels in colonies of wild-type *h^w^* strains result from a high mating-type switching rate. This strains display a dark staining phenotype when exposed to iodine vapours due to the presence of starch compounds in the spores [44]. Genetic alterations or mutations that lead to a reduced rate of mating-type switching (e.g. by affecting the replication-coupled recombination event by changing replication barrier activity) result in a skewed ratio of M and P cells. The consequences are less frequent mating and a decrease in sporulation levels causing an easily detectable low or speckled staining phenotype.

The replication-coupled recombination event includes the following steps (Fig 1A). Pausing of the replication fork at the MPS1 element located at the mating-type locus *mat1* is required for the introduction of an imprint that consists of two ribonucleotides incorporated into the DNA [45–47]. Experiments suggest that these ribonucleotides originate from the primer of an Okazaki fragment that is laid down in response to the replication pause [48]. This ribonucleotide imprint is maintained in the DNA throughout one generation, and during the next S-phase acts as a barrier for leading-strand replication, inducing a recombination event that leads to mating-type switching (reviewed in Ref. [14]). In addition to MPS1 and the imprint, a replication barrier named RTS1 is present in the mating-type region [49]. RTS1 acts to optimize mating-type switching by ensuring unidirectional replication at the *mat1* locus. The activity of both MPS1 and RTS1 depend on Swi1 and Swi3 [26,48].
Fig 1. The use of mating-type switching of \textit{S. pombe} as a screening tool to identify genes, influencing the stalling of DNA replication forks at the \textit{MPS1} site. (A) Mechanism of mating-type switching in \textit{S. pombe}: \textit{S. pombe} cells can switch between two different mating-types \textit{Plus} (\textit{P}) and \textit{Minus} (\textit{M}). Top line drawing. The mating-type of a cell is determined by the \textit{mat1} locus, which can contain either \textit{P} or \textit{M} information. Switching involves the precise replacement of the mating-type cassette at \textit{mat1} with the opposite mating-type information through a recombination event that utilizes one of two donor-loci, located centromere-distal to \textit{mat1}, \textit{mat2P} or \textit{mat3M}, as donors of the genetic information (top line-drawing). In addition, cells of the two mating-types can either be un-switchable (\textit{M}, \textit{P}) or switchable (\textit{M}\textsuperscript{*}, \textit{P}\textsuperscript{*}); switchable cells carry a ribonucleotide imprint at the \textit{mat1} locus (see below). Importantly, the \textit{mat1} locus is replicated in a uni-directional manner due to the presence of a terminator of replication (the \textit{RTS1} element) on the centromere-proximal (cen) side. Lower line drawings. (I) When DNA replication takes place in S-phase, the replisome replicating \textit{mat1} pauses at the \textit{MPS1} barrier located at the boundary of the \textit{mat1} cassette. This pause leads to the site-specific priming of an Okazaki fragment. (II) The replication fork then progresses on, and the primer from the Okazaki fragment is converted into an imprint consisting of two ribonucleotides incorporated into the DNA. (III) After cell division, this imprint is inherited by one daughter cell (\textit{M}\textsuperscript{*}) making it capable of switching mating type: (IV) In the following S-phase a break is introduced at the site of the imprint, when the leading-strand runs into the imprint present in the template strand, (V) leading to the induction of the recombination event (bold dashed line) that underlies mating-type switching. (B) To identify factors involved in replication pausing at the \textit{MPS1} site, the Bioneer knockout library was crossed with an \textit{h90} strain that had been tagged at the \textit{mat1} locus with a \textit{S. cerevisiae LEU2} genetic marker. The sporulation phenotype of different gene knockouts was examined after selection on YEA+G418+cyclohexamide (the different genes are knocked out with a Kan\textsuperscript{R} cassette, and cyclohexamide kills diploid cells due the recessive cyhr mutation) followed by selection on AA-Leu (\textit{LEU2} is linked with \textit{mat1}). Low and non-sporulating strains were identified by iodine staining of strains grown on sporulation media (PMA+), and these candidate strains were analysed by Southern blot analysis to further assess the level of \textit{mat1} imprinting. \textit{\textDelta mrc1} was identified and verified as a candidate that influenced pausing at the \textit{MPS1} barrier. (C) Sporulation staining phenotype and sporulation levels of \textit{\textDelta mrc1} generated from the Bioneer knockout library, as well as of the \textit{mrc1-A700T} \textit{(K234Stop)} generated according to Holmes, et al. \cite{40}. The low sporulation phenotype of the \textit{\textDelta mrc1} stain can be complemented by the transformation of a plasmid containing a genomic copy of the \textit{mrc1} gene (\textit{pMRC1}). Strain names are given above each panel, and the percentage of spores observed in the colonies by confocal microscopy is given below. Graphs to the right display the level of sporulation observed in the mutant colonies relative to wild-type colonies (100%).

\begin{figure}
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\caption{Fig 1. The use of mating-type switching of \textit{S. pombe} as a screening tool to identify genes, influencing the stalling of DNA replication forks at the \textit{MPS1} site. (A) Mechanism of mating-type switching in \textit{S. pombe}: \textit{S. pombe} cells can switch between two different mating-types \textit{Plus} (\textit{P}) and \textit{Minus} (\textit{M}). Top line drawing. The mating-type of a cell is determined by the \textit{mat1} locus, which can contain either \textit{P} or \textit{M} information. Switching involves the precise replacement of the mating-type cassette at \textit{mat1} with the opposite mating-type information through a recombination event that utilizes one of two donor-loci, located centromere-distal to \textit{mat1}, \textit{mat2P} or \textit{mat3M}, as donors of the genetic information (top line-drawing). In addition, cells of the two mating-types can either be un-switchable (\textit{M}, \textit{P}) or switchable (\textit{M}\textsuperscript{*}, \textit{P}\textsuperscript{*}); switchable cells carry a ribonucleotide imprint at the \textit{mat1} locus (see below). Importantly, the \textit{mat1} locus is replicated in a uni-directional manner due to the presence of a terminator of replication (the \textit{RTS1} element) on the centromere-proximal (cen) side. Lower line drawings. (I) When DNA replication takes place in S-phase, the replisome replicating \textit{mat1} pauses at the \textit{MPS1} barrier located at the boundary of the \textit{mat1} cassette. This pause leads to the site-specific priming of an Okazaki fragment. (II) The replication fork then progresses on, and the primer from the Okazaki fragment is converted into an imprint consisting of two ribonucleotides incorporated into the DNA. (III) After cell division, this imprint is inherited by one daughter cell (\textit{M}\textsuperscript{*}) making it capable of switching mating type: (IV) In the following S-phase a break is introduced at the site of the imprint, when the leading-strand runs into the imprint present in the template strand, (V) leading to the induction of the recombination event (bold dashed line) that underlies mating-type switching. (B) To identify factors involved in replication pausing at the \textit{MPS1} site, the Bioneer knockout library was crossed with an \textit{h90} strain that had been tagged at the \textit{mat1} locus with a \textit{S. cerevisiae LEU2} genetic marker. The sporulation phenotype of different gene knockouts was examined after selection on YEA+G418+cyclohexamide (the different genes are knocked out with a Kan\textsuperscript{R} cassette, and cyclohexamide kills diploid cells due the recessive cyhr mutation) followed by selection on AA-Leu (\textit{LEU2} is linked with \textit{mat1}). Low and non-sporulating strains were identified by iodine staining of strains grown on sporulation media (PMA+), and these candidate strains were analysed by Southern blot analysis to further assess the level of \textit{mat1} imprinting. \textit{\textDelta mrc1} was identified and verified as a candidate that influenced pausing at the \textit{MPS1} barrier. (C) Sporulation staining phenotype and sporulation levels of \textit{\textDelta mrc1} generated from the Bioneer knockout library, as well as of the \textit{mrc1-A700T} \textit{(K234Stop)} generated according to Holmes, et al. \cite{40}. The low sporulation phenotype of the \textit{\textDelta mrc1} stain can be complemented by the transformation of a plasmid containing a genomic copy of the \textit{mrc1} gene (\textit{pMRC1}). Strain names are given above each panel, and the percentage of spores observed in the colonies by confocal microscopy is given below. Graphs to the right display the level of sporulation observed in the mutant colonies relative to wild-type colonies (100%).

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\end{figure}
Consequently, mutations that reduce replication pausing at the MPS1 site cause a sporulation deficient phenotype [15,44]. Therefore, we crossed the non-switchable version 2 S. pombe Bioneer gene deletion library with a strain wild-type for mating-type switching (Fig 1B). The latter strain had been tagged with a S. cerevisiae LEU2 gene in the mating-type locus, allowing us to create a library of genetic segregants that carried both the gene deletions (G418 resistance) and the h^h^ wild-type mating-type locus (leucine prototrophs). Colonies obtained from these deletion strains were stained with iodine vapour, to evaluate the effect of the individual deletions on sporulation. More than 425 deletions affected the efficiency of sporulation. Among these 178 lead to a severe or complete loss of sporulation including several genes already known to be required for efficient mating-type switching, such as swi3 (see S2 Table). This demonstrated the capability of our screening method to identify genes involved in replication fork pausing. It should be noted that a swi1 deletion strain is not present in this Bioneer library.

We identified the mrc1 deletion as our best candidate for being a mutation affecting replication pausing. Firstly, several studies have previously shown that Mrc1 is a component of the replisome in both S. pombe and S. cerevisiae [19,31,32]. Furthermore, independent from our original screen we identified a mrc1 nonsense mutation (mrc1-A700T), in a screen recently described by Holmes et al. that specifically identifies mutants that affect pausing and imprinting [40].

This study focuses on the further characterization of the function of the mrc1 gene. First, we quantified the effect the identified mrc1 mutations have on sporulation efficiency and confirmed the genetic correlation between the mrc1 mutants and the sporulation deficient phenotype. The Δmrc1 mutant strain displayed 12.9% sporulation corresponding to 23% of the wild type levels (Fig 1C; EG250). A slightly greater reduction was observed when an allele of the wild-type mating-type region (lacking the LEU2 gene) was combined with the Δmrc1 allele (Fig 1C; EG256). An experimental comparison of the sporulation of the mrc1 deletion strain to functional null mutations in the swi1 and swi3 genes, showed that while the swi1 and swi3 mutations almost abolish sporulation, the mrc1 mutation only leads to a 3–4 fold reduction in sporulation (Fig 2C). Backcrossing experiments using the mutant mrc1 strain to the parental wild type strain did not detect any crossovers between the low-switching phenotype and the Kan’ marker gene used to delete the mrc1 gene in the 22 tetrads analysed. In addition, the nonsense mutation in mrc1A700T displayed 26.5% sporulation corresponding to 44% of the wild-type levels (Fig 1C; EG260). Finally, the low-sporulation phenotype of the mrc1-A700T mutant could be complemented by a plasmid containing the wild-type genomic allele of mrc1 (Fig 1C; EG55). In summary, these data show that loss of Mrc1 function is correlated in S. pombe with a reduction of the ability to sporulate.

**Mrc1 is required for efficient replication stalling at MPS1**

To determine whether the reduction of sporulation resulting from the Δmrc1 mutation was due to defects in imprinting and replication pausing, the genomic DNA from mutant and control strains was purified using the method described by Dalgaard and Klar [45]. Using this method the imprint at mat1 is efficiently converted into a double strand break (DSB). The analysis of wild type, Δmrc1 and mrc1-A700T strains showed that, while the imprint was easily detectable in the wild-type strain, it was significantly reduced in the Δmrc1 (EG250) and mrc1-A700T (EG16) strains to 40.8% and 33.8% of the wild-type level, respectively (Fig 2A & 2B). A comparison to the swi1-111 and swi3-146 strains showed that while loss of Swi1 and Swi3 function abolishes imprinting, the mrc1 deletion only leads to a reduction in imprinting (Fig 2D).

We then tested whether the loss of Mrc1 affected pausing of the replication fork at MPS1. Comparison of the pausing signal in wild type and Δmrc1 replication intermediates showed that there was an approximately three-fold reduction in the mutant background (Fig 2E). This
Fig 2. Characterization of the mrc1 mutations. (A) Top line-drawing; schematic representation of the mating type region showing the 10.4 kb HindIII fragment containing the mat1 locus. The positions of the imprint-dependent DSB and of the S. cerevisiae LEU2 gene inserted in the mat1 region (strain JZ217) are indicated. (B) Left panel, Southern blot of HindIII-digested DNA probed with a mat1P specific probe. This probe hybridises to the mat1 (10.4 kb), mat2P (6.3 kb) and mat3M (4.2 kb) cassettes as well as to the mat1 DSB products (5.0 and 5.4 kb, DSB). Above the panel, strains names are given. Below the panel the mrc1 alleles (vertical bold text) and the mat1 alleles (horizontal text) are given. To the right a graph displays the mean level of DSBs in the strains relative to the wild-type level (100%). The values are based on two measurements, with values indicated with vertical lines. (C) Comparison of sporulation levels of the Δmrc1 strain to swi1-111 and swi3-146 strains. To the right a graph displays the mean level of sporulation in the strains relative to the wild-type level (100%). See Fig 1C for description. (D) Comparison of imprinting levels between Δmrc1, swi1-111 and swi3-146 strains. For description see panel 1B. (E) Top line-drawing; WT; schematic representation of the 2.7 kb NdeI fragment of the mating-type region used to examine replication fork pausing at the mat1 MPS1. The position of the DSB, the polarity of replication in this region (black arrow), the replication pause site MPS1 and the position of the probe used to hybridise 2D-gels of this region are shown. Middle panels, quantification of replication pausing in wild type and mrc1 strains at the MPS1 site.
is a similar fold reduction to that observed for the level of sporulation and imprinting as seen in Fig 1C. Therefore, we concluded that Mrc1 is required for efficient pausing at the MPS1 barrier. However, as in the case of sporulation efficiency and imprinting, the effect of the mrc1 deletion on pausing was significantly less than that previously observed for the swi1 and swi3 deletions, which lead to a complete loss of the MPS1 barrier signal [15].

Mrc1 is important for fork stalling at several replication barriers

Mrc1 has been shown to form a complex with Swi1 (S. cerevisiae Tof1) and Swi3 (S. cerevisiae Csm3) in both budding and fission yeast [31,50,51]. As mentioned above, the replication proteins Swi1 and Swi3 are required for replication barrier activity at several other genetic loci (see above). Therefore, we tested whether Mrc1 also influences the activity at other barriers.

The RTS1 barrier plays a role in optimising mating-type switching by controlling the direction in which the mat1 locus is replicated [49]. Quantification of the pause and termination signals at RTS1 showed that a deletion of mrc1 reduces both types of barrier signals to 47.5% and 41.4% of the wild-type levels (Fig 3A). To address whether Mrc1 has a role outside the mating-type region we looked at the rDNA replication barrier. This barrier element is located at the 3’-end of the polymerase I transcription unit [16,52]. While we were unable to resolve the sub-barrier elements present at this locus [16,53], our data established that the overall level of barrier signals are reduced to approximately 26.4% of wild-type level in Δmrc1 strains (Fig 3B).

Finally, we looked at barrier activity at a plasmid-borne tRNA gene. This replication barrier has previously been shown to be very weak, only clearly visible in a pfh1-mt/C3 (S. cerevisiae rrm3 and pif1, metazoan PIF1) mutant background [54]. However, by careful comparison of the very weak barrier signal in the wild-type strain with the corresponding position on the Y-arc in the Δmrc1 strain using a phosphorimager we could measure a reproducible reduction in barrier activity (Fig 3C upper panels). This is supported by the clearly visible reducing effect the Δmrc1 mutation has on the tRNA barrier signal enhanced by the pfh1-mt mutation. Here a ~60% reduction in intensity is observed for the barrier signal (Fig 3C lower panels). Thus, our data show that Mrc1, like Swi1 and Swi3, is required for replication barrier activity not only at MPS1 but also at at least three other S. pombe DNA replication barriers. However, while swi1 and swi3 functional-null mutations abolish barrier activity the mrc1 deletion mutation only leads to a reduction in barrier activity. Finally, we would like to point out that the interaction of the replisome with tRNA barriers might be more complex than with other protein mediated barriers. Earlier observations have already shown that tRNA barriers behave differently in the absence of Swi1/Tof1 and Swi3/Csm3 than other barriers [24–30] (see introduction). While our findings clearly show a reduction of tRNA barrier activity in a Δmrc1 pfh1-mt strain compared with a pfh1-mt strain where retained polymerase III complexes form a strong barrier, the apex of the Y-arc of the tRNA barrier appears slightly more intense in the Δmrc1 background than in the presence of Mrc1 in a strain with wild-type pfh1. This could be an indication that Mrc1’s role at tRNA barriers might be more complex and depends on the presence or absence of Pfh1 (e.g. preventing fork stalling at tRNA barriers and ensuring a smooth passage of the replication fork in the presence of Pfh1 versus enhancing fork stalling at tRNA barriers in its presence). However, the signals in the 2D gels in the upper panels of Fig 3C are not strong enough to draw conclusions beyond the fact that Δmrc1 and not pfh1-mt causes the reduction in tRNA barrier activity observed in the lower panels of Fig 3C.
Mrc1 is required for fork stalling at MPS1 in imprinted and unimprinted cells

As outlined in Fig 1A, pausing occurs at the MPS1 barrier both in imprinted and un-imprinted cells. Since we only observed a reduction in MPS1 pausing and not a complete loss, an explanation could be that mrc1 is only required for pausing in one of these two populations. To test if there is a difference in a Δmrc1 background between the two populations, we analysed the pausing signal in a genetic background where the ribonucleotide imprint was absent due to the cis-acting smt0 deletion [55]. We observed a similar reduction of the pause signal compared to a wild type strain as in the Δmrc1 strain. (Fig 4A; lower panel). Thus, Mrc1 is required for efficient pausing at the MPS1 site both in the presence and in the absence of the mat1 ribonucleotide imprint. This is similar to what has been observed for the other four factors known to be required for pausing at MPS1 (Lsd1, Lsd2, Swi1 and Swi3) [15,40].

While the checkpoint function of Mrc1 is not required, the Mrc1 DNA-binding domain is necessary for efficient pausing at MPS1

Next we wanted to check whether the observation that Mrc1 regulates efficient replication barrier activity is correlated with one of its known functions. Since Mrc1 is a target for Rad3-dependent phosphorylation as part of the intra-S phase checkpoint (reviewed in Ref. [56]), we first wanted to see whether the S-phase checkpoint plays a role in Mrc1 mediated replication barrier activity. Mrc1 phosphorylation is required for the full activation of the Cds1 effector kinase (reviewed in Ref. [56]). We therefore first tested whether mutation of the rad3 and cds1 genes affect sporulation. No such effects were observed (Fig 4B). Similar results have previously been published by Roseaulin et al. [57]. We then went on to test whether a checkpoint-inactive allele of mrc1 (mrc1-3A [58]), which carries alanine substitutions of the Rad3 SQ/TQ phosphorylation sites responsible for S-phase checkpoint activation, affected sporulation. Again, no effect was observed (Fig 4B). Finally, a mutation of the Rad3-dependent effector kinase Chk1, which acts in the G2-M checkpoint pathway, did not affect sporulation either (Fig 4B).

Secondly, Mrc1 has been shown to be hyper-phosphorylated by the Hsk1 kinase (S. cerevisiae, human Cdc7) as a response to replication stress ([59]; Fig 4C). Hsk1 is an essential protein required for initiation of replication, which also has a role in the intra-S phase checkpoint [59,60], but recent work has shown that a double-mutant ΔrifΔhsk1 is viable [61]. We therefore investigated whether the Δrif and ΔrifΔhsk1 strains displayed decreased sporulation. While the Δrif mutation did not affect sporulation, the sporulation level of the ΔrifΔhsk1 double mutant was strongly reduced (Fig 4C; lower panel). However, when we quantified the levels of imprinting in these strains, we did not detect any decrease in imprinting in the ΔrifΔhsk1 double-mutant (Fig 4D), showing that the effect of the hsk1 mutation on sporulation was unrelated to replication pausing and imprinting required for mating-type switching. Thus, Δhsk1 must
Mrc1 Enhances Stalling at Replication Barriers

A

Mps1 probe

Msmt0

Msmt0 Δmrc1

Persistence (

Msmt0

Msmt0 Δmrc1

(100%)

(26.3±1.9%)

B

Mrc1-3A

DNA binding domain

with Helix-Loop-Helix motif

SqiTQ phosphorylation

sites are targets for Rad3

and necessary for

checkpoint activation via Cds1

C

Replication stress

RPC

hsk1

Mrc1

WT

Δmrc1

Δrif1

Δhsk1

pRep2-hsk1

Replication

spontaneous

Percentage of WT Spontation

54.5±2.0%

23.0±2.5%

56.0±1.5%

57.9±1.4%

WT

Δmrc1

Δrif1

Δhsk1

pRep2-hsk1

spontaneous

35.3±4.8%

4.2±4.8%

0

100

WT Spontation

D

HindIII

mat1

DSB

mat2p

mat3p

0

25

50

75

100

Percentage of WT DSB

0

25

50

75

100

Percentage of WT Spontation

WT

Δmrc1

Δrif1

Δhsk1

pRep2-hsk1

WT

Δmrc1

Δrif1

Δhsk1

pRep2-hsk1
effects sporulation in a different manner than that through a role in replication pausing and imprinting.

In addition to its role in intra-S phase checkpoint activation, *S. pombe* Mrc1 also possesses a conserved DNA-binding domain of unknown function ([43], Fig 5A). An alignment of the *S. cerevisiae* Mrc1 protein shows that *S. cerevisiae* Mrc1 lacks this DNA binding motif ([Fig 5A]). Mutation of this helix-turn-helix domain either by deletion or by introduction of two point mutations ([Fig 5B]) only has a minor effect on the protein’s role in the cellular response to HU treatment [43]. We tested if the mutation of this domain had a similar effect on sporulation, imprinting and replication pausing as the deletion of *mrc1* and this was indeed the case ([Fig 5C–5E]). Both the domain deletion mutation and the aforementioned two amino acid substitutions reduced the level of sporulation to levels similar to that of the Δ*mrc1* mutation. Furthermore, analysis of imprinting levels by quantification of DSB levels at mat1 confirmed that this result was due to Mrc1’s role in mating-type switching ([Fig 5D]). Finally, a 2D-gel analysis showed that the *mrc1Δ221–284* and *mrc1-K235E,K236E* mutations reduced pausing to similar level as in the Δ*mrc1* ([Fig 5E]).

Furthermore, we investigated whether the intra-S phase checkpoint is functional in the *mrc1-K235E,K236E* genetic background, since the presence of Swi1 and Swi3 at the replication fork is a requirement for this checkpoint. We used a method developed by the Huberman group to test the functionality of the *S. pombe* intra-S phase checkpoint [62]. Log-phase *S. pombe* cells mainly spent time in the G2 part of the cell cycle and cytokinesis takes place not after mitosis but at the end of the following S-phase. Therefore, cells in G1-,G2/M- and early S-phase all show a 2C DNA content when analysed by FACS. Thus, when log phase cultures are analysed by FACS, we observed only one peak with a 2C DNA content ([Fig 6, WT panels “-” MMS). However, if cells with a fully functional intra-S phase checkpoint are exposed to alkylation damage through methyl methanesulfonate (MMS) treatment they will arrest in early S-phase. This allows cytokinesis to take place before S-phase is completed resulting in a peak with a DNA content less than 2C in the FACS analysis ([Fig 6, WT 2h & 4h after treatment with 0.015% MMS). This “less than 2C” peak does not appear in cells carrying a mutation leading to defects in the intra-S phase checkpoint ([Fig 6, swi1-111 and Δ*mrc1*). When we applied this method to the *mrc1-K235E,K236E* mutation strain we observed a wild-type intra-S phase checkpoint response to MMS treatment ([Fig 6, *mrc1-K235E,K236E* 2h & 4h after treatment with 0.015% MMS).

Our experiments could not detect any correlation between Mrc1’s role in replication pausing and its role in intra-S phase checkpoint activation. Vice versa we did not observe any measurable effect of the *mrc1-K235E,K236E* mutations alone on the activation of the intra-S phase checkpoint. However, Zhao et al [43] describe a defect in checkpoint arrest and a “cut” phenotype indicating mis-segregation of genomic DNA, when Δ*chk1 mrc1-K235E,K236E* double mutant is treated with 12 mM HU (hydroxyurea).
Loss of Mrc1 reduces the efficiency of pausing

Finally we wanted to answer the question whether loss of Mrc1 function leads to a decreased efficiency of replication fork pausing at a barrier or whether the effect we see in an unsynchronized population is due to a decrease in the duration of the pause. To address this we analysed replication intermediates from the \textit{MPS1} pause site (Fig 7A) in synchronized cultures at different time-points after release into S-phase. Compared with WT strains, the number of these intermediates in mutant strains should decrease to the same degree at all time-points if replication forks pause less efficiently. In contrast intermediate numbers should drop more strongly at later time-points if the duration of the pause is affected. Cultures of \textit{cdc10-ts} and \textit{cdc10-ts \Delta mrc1} strains were arrested in G1 and released synchronously into S-phase using a program of
Fig 6. Detection of a functional intra-S phase checkpoint in the mrc1-K235E, K236E mutant genetic background. WT, mrc1-K235E, K236E, Δmrc1, swi1-111 strains were grown logarithmic in rich YEA media. Cultures were exposed either to 0%, 0.0075% or 0.015% MMS for 2 and 4 hours. Cells cultures were analysed by FACS as displayed. The concentration of MMS used is given above the panels and the genotypes and strain names below the panels.

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temperature shifts (Fig 7B). Importantly, a comparison of the cdc10-ts and cdc10-ts Δmrc1 strains detected the same effect of the mrc1 mutation on MPS1 pausing for log phase cultures as observed earlier (Fig 7C). This excludes an influence of the cdc10-ts mutation on pausing. Furthermore, when synchronized cultures were analysed we observed the same reduction in barrier activity by the mrc1 mutation at the three time-points a barrier signal was detectable (Fig 7D & 7E). The data suggest that the replication forks are paused with less efficiency and
there is no change in the duration of the \textit{MPS1} pause in \textit{Δmrc1} strains. Finally, we would like to note that we observe a slower S-phase progression by FACS analysis in the \textit{Δmrc1 cdc10} strain compared to the \textit{cdc10} strain (Fig 7D), suggesting that loss of \textit{S. pombe} Mrc1 affects S-phase progression in a similar manner to what has been observed for \textit{S. cerevisiae} (see above).

Discussion

All subunits of the heterotrimeric replisome stabilization complex are involved in efficient stalling of the replisome in \textit{S. pombe} but not in \textit{S. cerevisiae}

In the presented work, we have identified \textit{S. pombe} Mrc1 as a novel factor required for efficient replication pausing at the \textit{MPS1} element (Figs 2 and 7). Moreover, we have also shown that \textit{S. pombe} Mrc1 is necessary for full barrier activity at the \textit{rDNA} barrier, a \textit{tRNA} gene and at the \textit{RTS1} element (Fig 3). Importantly, these barriers are mediated by different cis-acting DNA-binding proteins including an unknown factor (at \textit{MPS1}), Rtf1 (at \textit{RTS1}), Sap1 and Reb1 (at the \textit{rDNA} barrier), and the polymerase III complex (at the \textit{tRNA} barrier) [26,28,30,48,53]. The seemingly global role at DNA-binding protein-mediated replication barriers reflects what is observed for the other two subunits which form a trimeric replisome sub-complex with Mrc1 (see above), Swi1 and Swi3. But while Swi1 and Swi3 are absolutely required for stalling at the first three barriers [15,16,40], the absence of Mrc1 only reduces barrier activity at these elements about three fold (Figs 2 & 3). This suggests that, unlike Swi1 and Swi3, Mrc1’s role in replisome stalling at barriers is supportive rather than essential, reflecting the different roles of these factors in the DNA replication process (see introduction). The discovery of the involvement of \textit{S. pombe} Mrc1 in replication barrier activity is somewhat surprising since previously three laboratories independently have established that \textit{S. cerevisiae} Mrc1 is not required for stalling of replication forks at the Fob1 barrier, several \textit{tRNA} genes and kinetochore binding sites [17,29,38,39]. However, it should be kept in mind that these two organisms are only distantly related [63].

Furthermore, our data suggest a mechanistical explanation for the different role Mrc1 has in replication stalling in the two yeast species. \textit{S. pombe} Mrc1 has been shown to posses a helix-turn-helix domain that is phylogenetically conserved in members of this protein family from \textit{S. pombe} to human but that is absent from \textit{S. cerevisiae} Mrc1 (Fig 5A). This domain has been shown to display an affinity to both double-stranded DNA as well as branched DNA structures [43]. Mutations in the domain only cause a slightly increased sensitivity to HU while the \textit{mrc1} gene-deletion mutant is hypersensitive [43]. Importantly, we demonstrated that loss-of-function point mutations in this Mrc1 DNA-binding domain abolishing DNA binding have the same effect as the complete gene deletion with regards to \textit{mat1} imprinting and \textit{MPS1} pausing (Fig 5). Since Swi1, Swi3 and Mrc1 form a hetero-trimeric complex, one possible explanation for the decrease in replication barrier activity observed in the \textit{mrc1} mutant strains could be that in the absence of a functional Mrc1 DNA binding domain Swi1 and Swi3 are not efficiently loaded onto the replisome. We do not think this explanation is likely. Firstly, because a loss of function mutation in the DNA binding domain does not effect measurably the intra-S phase checkpoint, which is thought to be dependent on Swi1 loading, whereas the checkpoint deficient point mutation \textit{swi1-111} or a complete deletion of \textit{mrc1} abolish the intra-S phase checkpoint (Fig 6). Secondly, it has been shown for \textit{S. cerevisiae} that Tof1 and Csm3 do not require Mrc1 to be loaded onto the replisome, although the opposite is true; Mrc1 needs Tof1 and Csm3 for loading [64]. Thirdly, Shimmoto et al. [32] have shown, that Swi3 interacts at WT levels with an Mrc1 fragment, which lacks the DNA binding domain. Vice versa, we have shown that the checkpoint-inactive allele \textit{mrc1-3A} does not cause the sporulation defect associated with a loss of \textit{MPS1} pausing and \textit{mat1} imprinting, showing that it is the DNA binding
activity and not the intra-S phase checkpoint activity of Mrc1 that is required at replication barriers (Fig 4). This is further supported by the observation that a deletion of hsk1, a gene encoding a kinase which hyperphosphorylates Mrc1 as a response to replication stress [59], does not result in a reduction of mat1 imprinting (Fig 4). Thus, our discovery suggests that S. pombe Mrc1 acts to enhance replication stalling at replication barriers by directly interacting with the DNA via its helix-turn-helix domain, a domain absent from S. cerevisiae Mrc1, rather than via Mrc1’s function in the intra-S phase checkpoint, a function which is conserved for S. cerevisiae Mrc1 (Fig 8). We think it is likely that this function of S. pombe Mrc1 at replication barriers is conserved in other eukaryotes since the DNA binding domain, with the mentioned exception of S. cerevisiae Mrc1, is conserved among the Mrc1/CLASPIN protein family. Further studies in other model organisms are necessary to better determine if indeed Claspin has a function at replication barriers in higher eukaryotes.

**Mrc1 and its role in replication restart**

One of the activities that has been attributed to S. cerevisiae Mrc1 is its role in replication restart [38]. This is reflected by ∆mrc1 cells decreased ability to re-initiate replication after they have been exposed to HU [39]. Since we are looking here at a natural replication pause-site, MPS1, where forks are stalled and restarted, we can conclude that S. pombe Mrc1 is not absolutely required for replication restart. At a natural replication barrier a defect in replication restart would be expected to lead to an increase in the pause signal and the appearance of termination structures, both we do not observe (Fig 2). Nor do we observe slow moving forks as the replication barriers are passed, as previously observed in case of the rtf2-mutation at the RTS1 barrier [42]. Slow moving forks are indicative of a defective or alternative replisome that is restarted after the replication pause. We made similar observations for the RTS1, rDNA and tRNA barriers (Fig 3). However, since we observe residual barrier activity at the barriers analysed, we cannot fully exclude that Mrc1 could both have negative and positive effects on replication re-start processes at all these barriers. Alternative explanations to the absence of an involvement of Mrc1 in replication restart in these experiments, are 1) differences between organisms, (Mrc1 is involved in restart in S. cerevisiae but not in S. pombe Mrc1), or 2) differences between restart of replication forks stalled using HU and at natural barriers. Indeed, there is evidence supporting the latter. Firstly, both S. cerevisiae and S. pombe mrc1 deletion strains are sensitive to transient exposure to HU [43,65]. Secondly, an mrc1 deletion in S. cerevisiae did not show significant changes in the intensity of pause or termination signals at the analysed replication barriers [17,29,38,66]. Thirdly, large single-stranded regions have been detected at replication forks stalled in HU, which are thought to be absent or very short at forks stalled at site-specific barriers [67,68]. Fourthly, it has been shown that there is an uncoupling of the replicative helicase from the site of DNA synthesis, when replication forks are stalled using HU in a ∆mrc1 background [18]. In contrast, pausing of the fork does not lead to replisome disassembly and Cdc45, a component of the replicative helicase, could be localised at the site of a paused replication fork in a S. cerevisiae ∆mrc1 strain [37]. 2D-gel analysis of replication intermediates from both S. cerevisiae and S. pombe cells did not show any evidence for fork collapse at barriers in a ∆mrc1 background ([37], see above). Therefore, so far there is no indication for an uncoupling of replicative helicase and polymerase at replication barriers in a ∆mrc1 background. In conclusion Mrc1 is most likely not required for replication restart at natural replication barriers in both S. cerevisiae and S. pombe.

**Two groups of proteins mediate fork arrest in S. pombe**

The observation that some factors only have supportive roles, rather than essential, is not novel, as for example loss of catalytic activity for the lysine–specific demethylases Lsd1/Lsd2
**Processive Replisome**

WT

mrc1Δ/mrc1-K235E/K236E/mrc1-A700T
or
lsd1Δ/lsd1-ao/lsd1-E918a/lsd1-891A

**Recognition of Active Replication Barrier**

**Formation of Paused Replisome**
complex only leads to a reduction of \textit{MPS1} activity \cite{40}. It is unknown how Lsd1/Lsd2 is recruited to the replication barriers. So far no interaction between the Swi1/Swi3/Mrc1 and Lsd1/Lsd2 complexes has been demonstrated, and it is not known if there might be a mechanistic link between the functions of these protein complexes. Mutations of Swi1 and Swi3, Mrc1’s interaction partners in the replisome stabilization complex, abolish barrier activity at binding sites of Sap1, Reb1 and Rtf1 completely \cite{24,26–28}. This indicates that there are two groups of proteins, which affect barrier activity in \textit{S. pombe} in different ways. Unfortunately, we do not have enough data to definitively assign roles to the involved proteins or propose a specific mechanism. Nevertheless, it is possible to speculate on the basis of the available data and to propose a tentative hypothesis about the function of the different groups of proteins. Firstly, it is known that Lsd1/Lsd2 complexes regulate transcription of target genes and the position of heterochromatin boundaries (i.e. the accessibility of genomic DNA for transcription factors and other DNA binding proteins) by influencing the methylation status of histones \cite{69–72}. As mentioned above, the activity of replication barriers is reduced when the catalytic activity responsible for normal Lsd1/Lsd2 complex function is lost \cite{40}. It is therefore conceivable, that the chromatin at DNA replication barriers has to be in a receptive state for the incoming replisome to recognize the barrier efficiently (Fig 8). Secondly, we have shown in this study that the presence of a functional Mrc1 DNA binding domain is necessary for the replisome to recognize a DNA replication barrier with optimal efficiency (Figs 5 and 7). An interesting idea would be that an interaction between the replisome and chromosomal DNA at receptive barriers via the Mrc1 DNA binding domain is required for an efficient recognition of the DNA replication barrier (Fig 8). Finally, Swi1 and Swi3 are essential for the formation of a paused replication fork at protein-mediated DNA barriers (see above). Genetic evidence based on the study of the \textit{RTSI} barrier suggest, that they are probably involved in the formation of a stably paused replisome-barrier-complex through the interaction with the staticly bound barrier proteins \cite{15,26} (Fig 8).

Re-evaluation of the cellular roles of Mrc1

Finally, we suggest that our findings invite to re-evaluate results obtained in earlier studies, which have been using \textit{Δmrc1} strains and contributed observed effects to the loss of checkpoint function of Mrc1. These effects might in fact have been, at least in part, due to loss of Mrc1 DNA-binding and replication barrier activity. For example, in \textit{C. elegans}, loss of the \textit{mrc1} homologue leads to some embryonic lethality \cite{73,74}. Previously, these defects have been attributed to Mrc1’s role in checkpoint activation. However, our data raise the possibility that the defects observed are due to Mrc1’s role in replication stalling. Replication barriers could potentially play a role in cellular differentiation and development in higher eukaryotes in a manner similar to fission yeast.

Furthermore, rearrangements of the \textit{rDNA} clusters have been found in a variety of cancer patients with lung and colorectal cancer as well as in cell lines derived from Hodgkin’s lymphoma \cite{75,76}. It has been shown that a deletion of \textit{S. pombe mrc1} alone leads to a shortening of the \textit{rDNA} cluster on chromosome III \cite{77}. While this result could be also achieved with a non-phosphorylatable, checkpoint deficient \textit{mrc1-14A} allele, the findings presented in this study raise the question whether this phenomenon also would be observed when the Mrc1
DNA binding activity required for full rDNA barrier activity is affected. This question becomes more relevant since a recent study showed that CLASPIN, the human homologue of Mrc1, is stabilised by the deubiquitinating enzyme USP20 during checkpoint activation and that USP20 suppresses xenograft tumor growth [78]. This effect can be initially explained by the influence USP20 has on checkpoint activation. However, a knockdown of TIMELESS (the human counterpart of Swi1/Tof1) has recently been shown not only to cause reduced barrier activity but also an increased collision rate between the transcription and replication machinery in human rDNA repeats [23]. Therefore, it is necessary to revisit the question whether rearrangements in the rDNA are only indicators of an increased mutation rate due to a failed checkpoint response or whether they are caused by more local effects due to decreased activity of replication barriers.

**Materials and Methods**

**Strains**

Strains used in this study are given in S1 Table. Genetic laboratory procedures are given in Ref. [79].

**Genetic screen using Bioneer library**

Strain EG9, where the wild-type mating-type region had been tagged with a LEU2 marker gene, and the version 2 Bioneer knockout library strains (Fig 1B) were grown up in YEA media and mixed in 96 well plates. After mixing, cells were transferred to ELN media using a frogger and allowed to sporulate at 25°C for 5 days. Using the frogger, sporulating cells were transferred to a 2% glucuronidase solution and incubated overnight at 37°C. This step kills cells, and releases the spores from the asci. Spores were washed with water, resuspended in water and transferred to YE plates using the frogger. Germinated colonies were first replica-plated to YEA + 100 μg/ml G418 (selecting for the segregants that carried the gene deletions) + 100 μg/ml cyclohexamide (counter selecting for diploid cells not killed by the glucuronidase treatment) and incubated overnight at 33°C, and then to AA-leu plates (selecting for segregants that carried the wild-type mating-type locus) followed by incubation overnight at 33°C. To assess the sporulation phenotype, strains were replica-plated to PMA+ media followed by incubation at 30°C for three days. Finally, sporulating colonies were stained with iodine vapour.

**Quantification of sporulation**

Strains were streaked for single colonies on PMA+ solid media. Plates were incubated at 33°C for two days and then moved to 30°C for one day. The percentage sporulation was determined from at least three different colonies for each strain. The percentage sporulation was calculated as the (number of spores divided by two) divided with the ((number of spores divided by two) plus the number of cells). Average values were calculated. The standard deviation from this averages were displayed as error bars.

**Quantification of imprinting**

Strains were grown in 10 ml liquid PMA+ media over night. Cells were spun down, washed with water, re-suspended in 1M Sorbitol and 0.1 M EDTA and treated with zymolase for 2 hours at 37°C. Spheroplasts were spun down and lysed by adding 0.5 ml DNAzol (Invitrogen). The DNA was subsequently precipitated using ethanol, resuspended in 0.5 ml DNAzol and precipitated again. The precipitated DNA was dissolved in 0.5 ml TE, phenol/chloroform extracted and precipitated again using ethanol. The pellet was washed with 70% Ethanol, dried...
and resuspended in TE. The DNA was digested with HindIII and separated on a 1% agarose gel. The 10.4 kb \textit{mat1P} HindIII fragment was used a probe for the Southern analysis. The intensity of individual bands was quantified using a phosphor imager. At least two independent measurements were done for each strain. Average values were calculated. The standard deviation from this averages were displayed as error bars.

**Quantification of replication pausing**

2D-gel electrophoresis was done as described by Brewer and Fangman [52], except the cells were cultivated in rich YEA media. 50 μg/ml of DNA was digested from each sample. Replication intermediates were purified using BND cellulose [80]. The intermediates were separated in gels containing 0.5% agarose for the first dimension and 1.2% for the second dimension. The intensity of the barrier signals and the ascending part of the Y-arc were quantified using a phosphor imager and corrected for the background signals. Each barrier signals was then normalized by dividing it with the obtained signal from the corresponding ascending Y-arc. To determined the effect of the mutations, we determined the mutant barrier signals intensity relative to the wild-type signal. Importantly, in each experiment the intensity for the mutant and wild-type barrier signals were obtained in parallel such that both mutant and wild-type signals were measured from the same membrane. This is true for the data in all figures except for the time course shown in Fig 7E were the wild-type and mutant signals’ intensities were obtained from separate membranes. Subsequently, both the WT and mutant signal intensities were divided by the WT signal and multiplicated with 100%. At least two independent measurements were taken for each replication barrier analysed. The average intensity of the barrier signals for each strain was calculated and the standard deviation of the data from this average reduction was displayed as error bars. In Fig 7E all time points for each of the two strains were blotted on one membrane and the intensity of the barrier signals subtracted the background signals were directly displayed as a function of time.

**Detection of the intra-S Phase checkpoint**

Log-phase cultures, grown in YEA, were exposed to the given concentrations of MMS (Fig 6) [62]. Cells were fixed in 70% ethanol. Flow cytometric analysis was carried out as described in reference [81].

**Time-course experiment**

\textit{cdc10-ts} cell cultures were grown overnight at 25°C, diluted and allowed to recover for 1 h at 25°C [82]. The cultures were then incubated at 37°C for 4 h to arrest them with a 1N DNA content. The cultures were released from the block by shifting the temperature to 25°C. Samples were collected for flow cytometry and 2D-gel analysis at 30-min intervals.

**Supporting Information**

S1 Table. Strainlist.

(DOCX)

S2 Table. List of deletions causing a severe or complete loss of sporulation.

(XLSX)
Acknowledgments

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Author Contributions

Conceived and designed the experiments: JZ ELG JZD. Performed the experiments: JZ ELG. Analyzed the data: JZ ELG JZD. Contributed reagents/materials/analysis tools: JZ ELG HM EH JZD. Wrote the paper: JZ JZD.

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