Dot1-Dependent Histone H3K79 Methylation Promotes Activation of the Mek1 Meiotic Checkpoint Effector Kinase by Regulating the Hop1 Adaptor

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Abstract

During meiosis, accurate chromosome segregation relies on the proper interaction between homologous chromosomes, including synapsis and recombination. The meiotic recombination checkpoint is a quality control mechanism that monitors those crucial events. In response to defects in synapsis and/or recombination, this checkpoint blocks or delays progression of meiosis, preventing the formation of aberrant gametes. Meiotic recombination occurs in the context of chromatin and histone modifications, which play crucial roles in the maintenance of genomic integrity. Here, we unveil the role of Dot1-dependent histone H3 methylation at lysine 79 (H3K79me) in this meiotic surveillance mechanism. We demonstrate that the meiotic checkpoint function of Dot1 relies on H3K79me because, like the dot1 deletion, H3-K79A or H3-K79R mutations suppress the checkpoint-imposed meiotic delay of a synapsis-defective zip1 mutant. Moreover, by genetically manipulating Dot1 catalytic activity, we find that the status of H3K79me modulates the meiotic checkpoint response. We also define the phosphorylation events involving activation of the meiotic checkpoint effector Mek1 kinase. Dot1 is required for Mek1 autophosphorylation, but not for its Mec1/Tel1-dependent phosphorylation. Dot1-dependent H3K79me also promotes Hop1 activation and its proper distribution along zip1 meiotic chromosomes, at least in part, by regulating Pch2 localization. Furthermore, HOP1 overexpression bypasses the Dot1 requirement for checkpoint activation. We propose that chromatin remodeling resulting from unrepaired meiotic DSBs and/or faulty interhomolog interactions allows Dot1-mediated H3K79me to exclude Pch2 from the chromosomes, thus driving localization of Hop1 along chromosome axes and enabling Mek1 full activation to trigger downstream responses, such as meiotic arrest.

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Introduction

During the specialized meiotic cell cycle, two rounds of chromosome segregation follow a single phase of DNA replication dividing the number of chromosomes by half to generate haploid gametes. One of the hallmarks of meiosis is the complex interaction between homologous chromosomes (homologs) involving synapsis and recombination. During meiotic prophase I, homologs find each other, get aligned and finally closely associate along their entire length (synapsis) in the context of the synaptonemal complex (SC). The SC is a tripartite structure composed of two lateral elements (LEs), connected by transverse filaments, which constitute the central region. The chromatin of both sister chromatids of each homolog is organized in loops attached at their base to each of the LEs [1,2]. In budding yeast, the Red1 and Hop1 proteins localize to the LEs [3], whereas the Zip1 protein is a major component of the SC central region [4,5]. Concomitant with SC development, meiotic recombination takes place. Meiotic recombination initiates with programmed double-strand breaks (DSBs) introduced by Spo11 and accessory proteins [6]. Meiotic DSBs are preferentially repaired using an intact non-sister chromatid resulting in physical connections between homologs (chiasmata), which promote proper chromosome segregation.

Accurate distribution of chromosomes to the progeny is essential for generation of functional gametes; thus, meiotic cells are endowed with a meiosis-specific surveillance mechanism, the so-called pachytene checkpoint or meiotic recombination checkpoint, which contributes to faithful chromosome segregation. In response to defects in meiotic recombination and/or chromosome synapsis, the pachytene checkpoint is triggered and blocks or delays exit from prophase of meiosis I to prevent aberrant chromosome segregation and the formation of aneuploid meiotic products [7,8].

This evolutionary-conserved quality-control mechanism operates from yeast to mammals. In S. cerevisiae, the meiotic recombination checkpoint responding to unrepaired resected DSBs shares the same sensors with the DNA damage checkpoint operating in vegetative cells, including the Mec1/Ddc2 kinase, Rad24 and the 9-1-1 complex [9–11]. However, the Rad9 adaptor and the Rad53 checkpoint kinase are dispensable for this meiotic checkpoint. On the contrary, given the special chromosomal...
Budding yeast meiotic mutants, such as progression, including the cyclin-dependent kinase Cdc28, the delay is imposed by inhibition of crucial regulators of meiosis I upon checkpoint activation [12,13]. In turn, the meiotic cell cycle meiotic effector kinase, which, like Rad53, is hyperphosphorylated specific axial chromosomal components Red1 and Hop1 act as relieves the meiotic prophase arrest of recombination intermediates by the checkpoint machinery occur essential for meiotic checkpoint function. Mutation of DOT1, is largely dispensable for unperturbed meiosis, but is linked to meiotic DSB formation [21,22]. On the other hand, modifications are expected to play important roles on these crossover formation) or dmc1 methylation in yeast cells and the DOT1L mammalian homolog also plays resulting in defective meiotic products [26]. Dot1 is also involved in lower protein levels (Figure 1D; [31]). Analysis of H3K79-me1, -me2 and -me3 levels in meiotic cells confirmed a gradually reduced H3K79-me3 and, conversely, progressively increased dot1-G401A (Figure 1C), suggesting that H3K79me is dispensable in unperturbed meiosis. However, similar to zip1 dot1, spore viability was strongly reduced in zip1 H3-K79R and zip1 H3-K79A mutants, in which the lysine 79 targeted by Dot1 cannot be methylated (Figure 1A). Importantly, like dot1, both methylation-site mutants suppressed the pronounced checkpoint-imposed meiotic delay of the zip1 mutant (Figure 1B). In an otherwise wild-type background, DOT1 deletion has no or little meiotic effects and spore viability is high [26,30]; likewise, the H3-K79R and H3-K79A single mutants showed wild-type levels of spore viability (Figure 1C), suggesting that H3K79me is dispensable in unperturbed meiosis. However, similar to zip1 dot1, spore viability was strongly reduced in zip1 H3-K79R and zip1 H3-K79A (Figure 1C), indicating that the defects conferred by zip1 persist in the double mutants despite their wild-type kinetics of meiotic progression. Thus, Dot1-dependent H3K79me is essential for meiotic recombination checkpoint function.

To further investigate the role of the meiotic checkpoint function by H3K79me, we monitored checkpoint function in zip1 diploid strains exhibiting gradually decreased Dot1 activity. In order to generate this set of strains, we used the combination of the dot1-G401A allele, which confers partial catalytic activity [28], with the expression of DOT1 (or dot1-G401A) from a plasmid, which results in lower protein levels (Figure 1D; [31]). Analysis of H3K79-me1, -me2 and -me3 levels in meiotic cells confirmed a gradually reduced Dot1 activity following this order: DOT1>dot1-G401A>dot1-G401A>dot1A, as manifested by progressively reduced H3K79-me3 and, conversely, progressively increased H3K79-me1 (Figure 1D). Interestingly, meiotic checkpoint activity, monitored as the ability to impose the zip1 meiotic delay, also showed a gradual decrease mirroring the drop in Dot1 catalytic function (Figure 1E). Quantification of the relative levels of each H3K79 methylation state revealed a marked correlation between H3K79-me3 and checkpoint function (Figure 1F). Thus, the status of H3K79 methylation modulates the meiotic recombination checkpoint, with the H3K79-me3 form being the most relevant to sustain the checkpoint response.

**Author Summary**

In sexually reproducing organisms, meiosis divides the number of chromosomes by half to generate gametes. Meiosis involves a series of interactions between maternal and paternal chromosomes leading to the exchange of genetic material by recombination. Completion of these processes is required for accurate distribution of chromosomes to the gametes. Meiotic cells possess quality-control mechanisms (checkpoints) to monitor those critical events. When failures occur, the checkpoint blocks meiotic progression to prevent the formation of aneuploid gametes. Genetic information is packaged into chromatin; histone modifications regulate multiple aspects of DNA metabolism to maintain genomic integrity. Dot1 is a conserved methyltransferase, responsible for histone H3 methylation at lysine 79, that is required for the meiotic recombination checkpoint. Here we decipher the molecular mechanism underlying Dot1 meiotic checkpoint function. We show that Dot1 catalytic activity correlates with the strength of the checkpoint response. By regulating Pch2 chromatin distribution, Dot1 controls localization of the chromosome axial component Hop1, which, in turn, contributes to activation of Mek1, the major effector kinase of the checkpoint. Our findings suggest that, in response to meiotic defects, the chromatin environment created by a constitutive histone mark orchestrates distribution of structural components of the chromosomes supporting activation of the meiotic checkpoint.
Figure 1. Methylation of H3K79 by Dot1 is essential for meiotic recombination checkpoint function. (A) Western blot analysis of H3K79 methylation in vegetative (VEG) and meiotic (MEI) cells to compare H3K79 methylation levels in different mutant backgrounds. Samples of meiotic
cells were taken 15 h after meiosis induction. Total histone H3 is shown as a loading control. (B) Suppression of zip1 meiotic delay by dot1 or by H3-K79A and H3-K79R mutations. Time course of meiotic nuclear divisions; the percentage of cells containing more than two nuclei is represented. (C) Spermatid viability determined by tetrad dissection. At least 2400 spores were scored for each strain. Means and standard deviations are shown. Strains for (A), (B) and (C) are: DP806 (wild type), DP807 (H3-K79A), DP808 (H3-K79R), DP809 (zip1), DP812 (zip1 dot1), DP810 (zip1 H3-K79A) and DP811 (zip1 H3-K79R). (D) Western blot analysis of H3-K79 methylation in zip1 strains producing different versions of Dot1 either from the endogenous loci (DOT1 and dot1-G401A) or from a centromeric plasmid (p[DOT1] and p[dot1-G401A]). Samples were taken 24 h after meiosis induction. Dot1 levels are also shown. Total histone H3 serves as a loading control. (E) Time course of meiotic nuclear divisions; the percentage of cells containing more than two nuclei is represented. (F) Quantification of the relative levels of H3-K79 mono-, di-, and tri-methylation. The maximum value of each methylation state was considered 100%. Checkpoint activity represents the ability to impose the zip1 meiotic delay according to data in (E). The meiotic nuclear division values for the latest time point (60 h) were considered in the calculations. Maximum checkpoint activity (100%) was assigned to the zip1 strain expressing endogenous wild-type DOT1. Strains for (D), (E) and (F) are: DP421 + pRS315 (wild type), DP555 + pRS315 (zip1 dot1), DP555 + pRS315-DOT1 (zip1 p[DOT1]), DP555 + pFLS4 (zip1 p[dot1-G401A]), DP556 + pRS315 (zip1 DOT1) and DP560 + pRS315 (zip1 dot1-G401A).

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Dot1 is required for activation of the Mek1 effector kinase

Next, we sought to determine where in the meiotic recombination checkpoint pathway Dot1-dependent H3K79me is acting. We first analyzed checkpoint sensor function by monitoring the formation of zip1-induced Ddc2-GFP foci [11]. Formation of Ddc2 foci was not disrupted in the absence of Dot1 (Figure 2A), suggesting that H3K79me is not required for the ability of Mec1-Ddc2 to detect meiotic recombination intermediates. Upon checkpoint activation, the Mek1 effector kinase forms nuclear foci that can be detected both on chromosome spreads [9]; see below) and in live meiotic cells (Figure 2A). Strikingly, we found that the zip1 mutant accumulated multiple discrete Mek1-GFP foci during meiotic prophase, whereas most zip1 dot1 cells displayed a diffuse Mek1 nuclear signal and only occasional foci were observed (Figure 2A) indicating that Dot1 promotes checkpoint-induced association of Mek1 to meiotic chromosomes (see below).

Mek1 is activated by phosphorylation in mutants that trigger the meiotic recombination checkpoint, including zip1 [12,14,32,33]; therefore, we followed Mek1 phosphorylation throughout meiosis in wild-type, zip1 and zip1 dot1 cells using Phos-tag gels (Figure 2B). In the wild type, Mek1 was weakly and transiently activated during the peak of meiotic prophase in this strain background (around 12–15 h). In contrast, Mek1 was hyperactivated in zip1 cells as evidenced by the presence of additional, more persistent, and stronger phosphorylated forms. However, Mek1 hyperactivation was not observed in the zip1 dot1 double mutant; like in wild type, only a weak and transient phosphorylated form was detected. To rule out the possibility that the difference between zip1 and zip1 dot1 were due to their different kinetics of meiotic progression [zip1 exhibits a marked delay that is bypassed in zip1 dot1; Figure 1B], we monitored Mek1 phosphorylation in ndt80 pachytene-arrested cells. As presented in Figure 2C, zip1-induced hyperphosphorylation of Mek1 was severely impaired in the absence of Dot1.

In summary, these results place Dot1 function upstream of Mek1 in the meiotic recombination checkpoint pathway and indicate that, whereas Mec1/Ddc2 act independently of H3K79 methylation to sense meiotic defects, Dot1 is required for checkpoint-induced activation of Mek1.

Autophosphorylation of Mek1 depends on Dot1

In ndt80-arrested cells, using high-resolution Phos-tag gels, we were able to resolve several zip1-induced shifted forms of Mek1 above the basal band (Figure 3A–3D). Phosphatase treatment eliminated all band shifts indicating that they represent distinct phosphorylated forms (Figure 3A). We used different mekl versions carrying specific mutations, as well as mutants in upstream components of the checkpoint pathway, in order to determine the contribution of different phosphorylation events to the observed checkpoint-induced Mek1 forms in zip1 ndt80 cells (Figure 3E). Mek1 phosphorylation was completely abolished in the hop1 mutant, lacking a LE-component meiotic checkpoint adaptor [3,34,35] (Figure 3B) and in the spo11 mutant, which does not initiate recombination [36] (Figure 3C). However, in the absence of Dot1, only the upper phosphorylated bands were eliminated (Figure 3B–3D, white arrowheads), but the form immediately above the basal Mek1 band remained intact (Figure 3B–3D, black arrowhead). Interestingly, this moderately-shifted form was reduced in mekl cells and virtually disappeared in mekl tel1 and rad24 tel1 mutants (Figure 3C and 3D, black arrowhead), suggesting that it arises from Mec1/Tel1-dependent phosphorylation. On the other hand, the kinase-dead zip1 mekl-K199R allele, as well as the autophosphorylation-defective mekl-T327A and mekl-T331A mutants [33], specifically lacked the upper bands displaying the stronger mobility shift, suggesting that they result from Mek1 autophosphorylation (Figure 3D, white arrowheads). In contrast, the Mek1 form immediately above the basal band (i.e., resulting from Mec1/Tel1 action) remained invariable in those mekl mutants (Figure 3D, black arrowhead). Thus, interestingly, the zip1 dot1 mutant showed a similar pattern to that of zip1 mekl-K199R, zip1 mekl-T327A or zip1 mekl-T331A (Figure 3D), strongly suggesting that Dot1 is mainly required for Mek1 autophosphorylation, but not for its Mec1/Tel1-dependent phosphorylation (Figure 3E).

It has been proposed that dimerization of Mek1 promotes its function, likely by facilitating trans autophosphorylation [33,37]. Thus, we hypothesized that Dot1 could be required for Mek1 dimerization. Importantly, we found that GST-driven forced dimerization of Mek1 restored its full phosphorylation even in the absence of Dot1, although Mek1 activation was not maintained at late time points (Figure 2B). Consistently, expression of GST-MEK1 in zip1 dot1 strains conferred a brief, but significant, meiotic delay (Figure 2D). As previously reported, the zip1 GST-MEK1 mutant was completely halted (Figure 2D) [37], and we found that this block was accompanied by the persistent hyperphosphorylation of GST-Mek1 (Figure 2B). The transient or transient arrest conferred by GST-Mek1 in zip1 or zip1 dot1, respectively, was completely relieved when inactive kinase (GST-mek1-K199R) or autophosphorylation-defective (GST-mek1-T327A) versions were introduced (Figure 2D), confirming that in GST-MEK1 strains, meiotic progression was slowed down by forced Mek1 activation and not by another unrelated cause. To further support this conclusion, we monitored another downstream molecular marker of pachytene checkpoint activation, such as the inhibition of the production of the Cdc5 polo-like kinase [14,18]. As expected, whereas induction of Cdc5 was delayed in zip1 cells, the zip1 dot1 double mutant displayed wild-type kinetics of Cdc5 production (Figure 2B). Strikingly, consistent with the kinetics of meiotic progression (Figure 2D), expression of GST-MEK1 in zip1 dot1 cells restored a significant delay in Cdc5 induction. Furthermore, Cdc5 production was severely impaired in the arrested zip1 GST-MEK1 strain (Figure 2B). In summary, these observations indicate that

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Figure 2. Dot1 is required for checkpoint-promoted localization and activation of Mek1. (A) Formation of zip1-induced Mek1 foci is defective in the absence of Dot1. Representative images of Ddc2-GFP and Mek1-GFP foci in zip1 and zip1 dot1 cells after 24 h in meiosis. Strains are DP460 (zip1 DDC2-GFP), DP579 (zip1 dot1 DDC2-GFP), DP582 (zip1 MEK1-GFP) and DP583 (zip1 dot1 MEK1-GFP). All strains are ndt80-arrested at
artificial dimerization of Mek1 partially overcomes Dot1 requirement for Mek1 activation and further supports the conclusion that Dot1 function promotes Mek1 autophosphorylation.

Dot1 is required for localization and activation of the Hop1 meiotic checkpoint adaptor

It has been reported that activated Hop1 promotes Mek1 dimerization via a C-terminal domain [33,38]; therefore, we investigated whether the effect of Dot1 on Mek1 phosphorylation was mediated by Hop1. First, we studied Hop1 localization on chromosome spreads of ndt80-arrested zip1 and zip1 dot1 strains. As previously described [3], Hop1 displayed a predominantly linear staining along the lateral elements of zip1 chromosomes. In contrast, only short stretches of Hop1 could be detected in the zip1 dot1 mutant, which showed a predominating Hop1 punctate pattern (Figure 2A and 2B, left panel). Consistent with our observations in live cells (Figure 2A), we also detected a marked reduction of Mek1 chromosomal foci in zip1 dot1, compared to the zip1 single mutant (Figure 4A and 4B, right panel). In addition, we also analyzed Hop1 localization in zip1 and zip1 dot1 live meiotic cells expressing HOP1-GFP. In line with the aberrant distribution on spreads, we observed that Hop1-GFP signal was weaker and less continuous in zip1 dot1 cells. (Figure 4C and 4D; Video S1). This discontinuous localization of Hop1 does not result from a pronounced alteration of overall chromosome structure, because the SC lateral component Red1 [3] displayed a linear distribution in both zip1 and zip1 dot1 strains (Figure S2). On the other hand, the dot1 single mutant only showed a modest decrease of Hop1-GFP signal compared with the wild type (see Figure 2C and 2D below). Thus, upon zip1-induced checkpoint activation, Dot1 enables proper loading or maintenance of Hop1 onto chromosomes.

Since Mecl1/Tel1-dependent phosphorylation of Hop1 at defined S/T-Q motifs is required for Mek1 activation and localization [34], we examined zip1-induced Hop1 phosphorylation in the absence of Dot1, by monitoring its gel mobility shift. As shown in Figure 4E, the zip1 dot1 mutant displayed a severe defect in Hop1 phosphorylation, similar to the zip1 mecl and zip1 spo11 mutants also analyzed as controls (Figure 4E and Figure S3). Even after long exposure of the gels, only a barely visible phosphorylated form of Hop1 could be detected in the absence of Dot1 (Figure 4E).

These observations suggest that the defect in Mek1 autophosphorylation observed in the absence of Dot1 stems from impaired Hop1 function. To confirm this notion, we overexpressed HOP1 from a high-copy plasmid in zip1 dot1 cells. As shown in Figure 5, whereas the zip1 dot1 mutant transformed with empty vector showed defective Mek1 localization and activation, HOP1 overexpression in zip1 dot1 restored Mek1 chromosomal foci (Figure 3A), Mek1 phosphorylation (Figure 3B), and reestablished a substantial meiotic delay (Figure 3C). We found that Hop1 phosphorylation also conferred a slightly reduced delay in the efficiency of meiotic progression in the wild type (Figure 3C) and further enhanced the zip1 meiotic delay, as expected from the strong hyperphosphorylation of Mek1 (Figure 3B and 3C). Notably, in all cases (wild type, zip1 or zip1 dot1), the further delay in meiotic progression imposed by high levels of Hop1 was suppressed by the absence of Mek1 (Figure 3C), proving that it was caused from amplified pachytene checkpoint signaling and not from an unrelated cause.

H3K79me is required for Mek1 and Hop1 phosphorylation and localization

We have shown that, like dot1, mutation of H3K79 to non-methylatable residues completely bypasses the checkpoint-induced meiotic delay of zip1 (Figure 1B). On the other hand, we have revealed that, in zip1 cells, Dot1 orchestrates Hop1 and Mek1 activation and chromosomal distribution (Figure 1, Figure 2, Figure 3, Figure 4, and Figure 5). To confirm that Hop1 and Mek1 checkpoint functions are also directly regulated by H3K79me, and not by another possible methyltransferase-independent function of Dot1, we examined their phosphorylation and localization in the zip1 H3-K79R and zip1 H3-K79A mutants. We found that, indeed, these histone point mutants phenocopy the dot1 defects in Mek1 foci formation (Figure 6A and 6B) and Mek1 autophosphorylation (Figure 6D; Figure S4). Likewise, the zip1 H3-K79R and zip1 H3-K79A mutants resemble dot1 in the impaired Hop1 chromosomal distribution (Figure 6A and 6C; Figure S5) and checkpoint-induced phosphorylation (Figure 6E, Figure S4).

Thus, taken together, our results indicate that, upon meiotic recombination checkpoint triggering, Dot1-dependent H3K79 methylation promotes proper chromosomal localization and activation of Hop1, which in turn, is required to sustain Mek1 autophosphorylation and the ensuing checkpoint response.

H3K79me partially controls Hop1 chromosomal localization via Pch2

Previous studies have shown that whereas in the zip1 mutant the Pch2 meiotic checkpoint protein is detected only in the nucleolar (rDNA) region, in the zip1 dot1 double mutant Pch2 is distributed throughout all chromatin [26]. To confirm that the regulation of Pch2 localization by Dot1 depends on the histone H3 methyltransferase activity, we analyzed Pch2 distribution on spread meiotic chromosomes of the zip1 H3-K79R and zip1 H3-K79A mutants. Although global Pch2 protein levels remained fairly invariant in the different mutants (Figure S4), we found that, like in zip1 dot1, Pch2 mislocalized to chromatin outside the rDNA in zip1 H3-K79R and zip1 H3-K79A strains (Figure 7A), suggesting that H3K79me excludes Pch2 from chromosomes.

Several lines of evidence support a role for Pch2 in promoting the turn-over of Hop1 from meiotic chromosomes, at least in unperturbed meioses [29,39,40]; therefore, it was possible that the reduced localization of Hop1 in the absence of Dot1 could stem from the action of the Pch2 protein aberrantly present at chromosomal locations removing Hop1 from zip1 chromosomes. To investigate this possibility, we monitored Hop1 localization in zip1 dot1 pch2 strains. Interestingly, we found that deletion of PCH2
Figure 3. Dot1 contributes to Mek1 activation by autophosphorylation. (A) Whole cell extracts (WCE) from a zip1 ndt80 culture at 24 h in meiosis were incubated in the presence (+) or absence (−) of lambda phosphatase (lPPase). (B), (C) and (D) Detection of different phosphorylated forms of Mek1 in ndt80-arrested cells after 24 h in meiosis using high-resolution Phos-tag gels. Basal Mek1 (line) and several phosphorylated forms (black and white arrowheads) are indicated; see text for explanation. PGK or Ponceau S staining were used as loading controls. Asterisk in (D) marks a weak non-specific band. (E) Schematic representation of a model for the sequential phosphorylation events leading to Mek1 activation and the relevant mutations analyzed above. (1) Priming phosphorylation by Mec1/Tel1 (black arrowhead in B, C, D) is followed by (2) autophosphorylation of
Mek1 (white arrowheads in B, C, D) leading to its full activation and (3) the checkpoint response. H3K79 methylation by Dot1 contributes to Mek1 autophosphorylation. Strains were: (A); DP428 (zip1), (B); DP428 (zip1), DP701 (zip1 hop1) and DP655 (zip1 dot1). (C); DP428 (zip1), DP655 (zip1 dot1), DP680 (zip1 mecl), DP681 (zip1 mecl tel1), DP727 (zip1 rad24 tel1), DP728 (zip1 spo1) and DP674 (zip1 mekl). (D); DP885 (zip1), DP890 (zip1 dot1), DP886 (zip1 mekl-T327A), DP887 (zip1 mekl-T331A), DP888 (zip1 mekl-K199R), DP674 (zip1 mekl), DP680 (zip1 mecl) and DP681 (zip1 mecl tel1).
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alleviated to some extent the defective Hop1 localization pattern of zip1 dot1, although it did not fully restore the high and continuous Hop1 levels present in zip1 (Figure 7B–7D). To determine whether the increased abundance of Hop1 along chromosomes in zip1 dot1 pch2 restores the checkpoint-induced delay we analyzed meiotic divisions and Mek1 phosphorylation (Figure 7E,7F). We found that the checkpoint was still impaired in the zip1 dot1 pch2 triple mutant because, like the zip1 dot1 and the zip1 pch2 double mutants, it displayed wild-type kinetics of meiotic progression (Figure 7E) and defective Mek1 activation (Figure 7F), implying a more complex contribution of Pch2’s function to the pachytene checkpoint response (see Discussion).

In summary, these observations indicate that in the zip1 mutant, methylation of H3K79 by Dot1 controls proper chromosomal distribution of Hop1 by maintaining Pch2 confined in the nucleolar region. The fact that Hop1 localization is still partially impaired in the zip1 dot1 pch2 triple mutant suggests that Dot1 may also regulate Hop1 chromosomal recruitment by a Pch2-independent mechanism (Figure 8).

Discussion

Previous studies have shown that Dot1 is important for the pachytene checkpoint, but the molecular mechanism underlying such function remained unclear. Here, we provide evidence that methylation of H3K79 by Dot1 contributes to the meiotic recombination checkpoint response by enabling proper Hop1 chromosomal recruitment, which, in turn is a requisite for Mek1 activation by autophosphorylation.

We demonstrate that the function of Dot1 in the meiotic recombination checkpoint specifically relies on the methylation of H3K79, since the non-methylatable H3-K79A and H3-K79R mutations confer essentially the same meiotic phenotypes as the lack of Dot1. Moreover, by modulating Dot1 catalytic activity, we found that high levels of the H3K79-me3 are required for full checkpoint activation raising the possibility that this methylation state is particularly critical for promoting the proper localization of the Hop1 meiotic checkpoint adaptor (see below).

In mitotic cells, methylated histones are well-known chromatin marks for recognition of DSBs by checkpoint adaptors. In S. cerevisiae, the Rad9 adaptor is recruited to DSB sites by H3K79me [41,42], whereas in S. pombe, which lacks H3K79me, the recruitment of the Crb2 adaptor relies on H4K20me [13]. In mammalian cells, the Rad9 and Crb2 homolog 53BP1 appears to recognize both H3K79me and H4K20me [44–46]. All these DNA damage checkpoint adaptors (Rad9, Crb2 and 53BP1) contain tandem tudor domains that mediate the interaction with the methylated histones. Rad9, Crb2 and 53BP1 also possess BRCT motifs; in fact, the recognition of DSBs by Rad9 and Crb2 in S. cerevisiae and S. pombe, respectively, is also mediated by their binding to phosphorylated histone H2A (hereafter γH2AX) via the BRCT domains [47,48]. However, the Hop1 meiotic checkpoint adaptor lacks either tudor or BRCT motifs and contains a HORMA domain likely involved in protein-protein interactions [49], raising the possibility that its chromosomal recruitment can be mediated by different mechanisms.

As mentioned before, in DNA damaged vegetative cells, Rad9 function depends both on H3K79me and γH2AX [47,50–52]; however, the relevance of both histone modifications appears to be different in meiotic cells. Dot1-dependent H3K79me is crucial for checkpoint function, at least in Zip1-deficient cells, because deletion of DOT1 (or mutation of H3K79) results in complete bypass of the zip1 meiotic block. In contrast, an H2A-S129* mutant, lacking the four C-terminal amino acids of histone H2A including the SQ phosphorylation site [53], has no defect in the zip1-induced checkpoint (Figure S6A). Moreover, like in both single mutants, meiotic progression and spore viability are essentially normal in the dot1 H2A-S129* double mutant (Figure S6A, S6B).

We show here that Dot1 is required for Mek1 and Hop1 activation in meiotically-challenged cells, but in addition to the checkpoint function, Mek1 and Hop1 promote the repair of meiotic DSBs by Dmc1-dependent interhomolog recombination [34,37,38,54]. Consistent with this function, in the absence of Dmc1, Dot1 prevents the repair of DSBs by Rad54-dependent sister-chromatid recombination, which is controlled, at least in part, by inhibitory phosphorylation of Rad54 by Mek1 [26,54]. In principle, it could be possible that impaired Hop1/Mek1 function in the absence of Dot1 could induce an alternative intersister recombination pathway resulting in meiotic progression because of the disappearance of the meiotic defects initially triggering the checkpoint. However, deletion of DOT1 alleviates the meiotic arrest of zip1 rad54 and dmc1 rad54 mutants, where intersister repair is impaired, strongly suggesting that Dot1 performs a bona-fide meiotic checkpoint function [26]. The fact that, unlike Mek1 and Hop1, the Dot1 protein is dispensable in otherwise unperturbed meiosis implies the H3K79me is mostly relevant to signal defects when meiotic chromosome metabolism is disturbed (i.e., zip1 or dmc1 mutants). Consistent with this notion, Hop1 localization on zip1 chromosomes is dramatically altered in the absence of Dot1, but it is only slightly reduced in the dot1 single mutant as compared with the wild type (Figure 7C and 7D).

In other studies, activation of the Mek1 effector meiotic kinase has been monitored either by a slight electrophoretic mobility shift [32,34] or by using an anti-phospho-Ser/Thr Akt substrate antibody, which specifically recognizes phosphorylation of Mek1 at T327 [33,37,55]. However, these assays do not permit one to delineate the different events contributing to Mek1 activation. Here, by using high-resolution Phos-tag gels, we identify several phosphorylated Mek1 conformations that lack full Hop1, suggesting that Hop1 localization at T327 supports the checkpoint response. We found that Dot1 is chiefly involved in this process, preventing the meiotic arrest of zip1 mutants, whereas Dot1 may also affect the meiotic arrest of zip1 mec1 tel1 mutants, where interhomolog recombination is impaired, strongly suggesting that Dot1 performs a bona-fide meiotic checkpoint function [26].
Figure 4. Dot1 is required for zip1-induced localization and activation of the Hop1 meiotic checkpoint adaptor. (A) Immunofluorescence of meiotic chromosome spreads stained with DAPI (blue), anti-Hop1 (green) and anti-myc (red) antibodies. Representative nuclei are shown. The same exposure time was used to capture the signal from the different strains. Spreads were prepared 24 h after meiotic induction of ndt80 cells. Strains are: DP848 (zip1) and DP849 (zip1 dot1). (B) Quantification of the Hop1 staining pattern (left) and the number of Mek1 foci (right) on spread chromosomes analyzed as in (A). 14 and 21 nuclei were scored for zip1 and zip1 dot1, respectively. (C) Representative images of
ndt80-arrested cells expressing HOP1-GFP in zip1 (DP964) and zip1 dot1 (DP965) captured after 24 h in meiosis. (D) Quantification of the Hop1-GFP signal intensity on fluorescence images (a.u., arbitrary units). 300 individual nuclei were analyzed for each strain. (E) Dot1 is required for Hop1 phosphorylation. Western blot analysis of Hop1 in cell extracts obtained 24 h after meiotic induction in ndt80 cells. The middle panel corresponds to an overexposure (OEx) of the blot shown in the upper panel. PGK was used as a loading control. Strains are: DP428 (zip1), DP655 (zip1 dot1), DP680 (zip1 mec1) and DP674 (zip1 mek1). Means, standard deviations and P-values are shown in (B) and (D).

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We found that global levels of H3K79me do not significantly change in response to the meiotic defects of the zip1 mutant, but this methylation is critical for the checkpoint response. The nature of the signal that triggers the meiotic checkpoint in zip1 is still unclear. Like in mammals [56], the existence of a synaptonemal complex in yeast has also been proposed [7,55,58]. Nevertheless, Dot1 is also required for the meiotic cell cycle arrest of the dmc1 mutant that accumulates unrepaired DSBs [26], indicating that H3K79me is also involved in the response to meiotic DSBs. It has been reported that, under certain conditions, DSBs are efficiently repaired in zip1 mutants [37] implying that the signal triggering the checkpoint could be different. However, Ddc2 foci marking the presence of recombination intermediates are detected in zip1 [11] (Figure 2A), consistent with at least some DSBs remaining unrepaired in zip1 mutants [57,59,60] sufficient to induce the checkpoint. Alternatively, or in addition, Mec1-Ddc2 may also sense defects in structural aspects of interhomolog interactions resulting from the lack of the central region of the SC [39]. In any case, independently of the nature of the signal triggering the meiotic checkpoint response(s), the question of how a constitutive histone mark, such as H3K79me, contributes to Hop1-mediated Mecl activation specifically in challenged meiosis remains to be elucidated. In the DNA damage response in vegetative yeast cells or somatic mammalian cells it has been proposed, though never proven, that chromatin remodeling in the vicinity of DNA lesions may locally expose constitutive marks (i.e., H3K79me, H4K20me) supporting the recruitment of DNA damage checkpoint adaptors to activate the checkpoint [44,45]. In meiotic cells, the DSB metabolism is linked to the special architecture of the chromosome axis [61]. Therefore, we envision that unrepaired DSBs and/or defects in interhomolog connections may provoke chromatin conformational changes unmasking H3K79me capable to drive proper Hop1 distribution along the axes, enabling its activation by Mec1 to elicit the downstream checkpoint events including Mecl full activation by autophosphorylation (Figure 8).

Although it is formally possible that H3K79me may directly facilitate Hop1 recruitment to some extent, we provide evidence indicating that the control of Hop1 chromosomal distribution by H3K79me is substantially driven by regulation of the Pch2 protein. Pch2 was initially discovered as a meiotic checkpoint protein required for the zip1-induced meiotic arrest [29], but more recent studies have shown that Pch2 impacts multiple aspects of meiotic chromosome dynamics [55,62-64]. In particular, Pch2 acts as a negative regulator of Hop1 chromosomal abundance [39,40]. In wild-type pachytene chromosomes, Pch2 localizes to the unsynapsed rDNA region (nucleolus) and also along synapsed chromosomes [29,40]. In contrast, Pch2 is solely detectable at the nucleolar region in the zip1 mutant [29]; remarkably, in the absence of H3K79me, Pch2 is redistributed throughout all chromatin of zip1 nuclei (Figure 7A). We hypothesize that, as a consequence of the synopsis defects of zip1, the H3K79me mark becomes exposed functioning as an anti-binding signal for Pch2, thus permitting the extensive Hop1 distribution found on zip1 chromosomes (Figure 8). In the absence of Dot1 (or H3K79me), the presence of chromosomal Pch2 triggers the removal of Hop1 and the consequent defect in Mecl activation. The reduced global levels of Hop1 detected in zip1 dot1 (Figure S3 and Figure 6E) are also consistent with a higher protein turnover.

Interestingly, like in zip1 dot1, the synaptonemal complex is still completely defective in the zip1 dot1 pch2 triple mutant, despite the partial restoration of Hop1 localization. Since the excess of Hop1 induced by other means, such as Hop1 overexpression, but in the presence of Pch2, does confer a meiotic delay in zip1 dot1 and restores Mecl phosphorylation (Figure 5), it is conceivable that nucleolar Pch2 performs an additional downstream function in Mecl activation (Figure 8) and/or that the excess of Hop1 in the absence of Pch2 is not correctly assembled on chromosome axes to support checkpoint activation. In fact, the zip1 pch2 mutant itself is also checkpoint deficient. Future studies will address these intriguing possibilities.

Dot1/DOT1L is structurally conserved throughout evolution from budding yeast to worms, flies, mice and humans; therefore, it is possible that members of the Dot1 family play similar roles in Metazoa. DOT1L is essential in mammals [63] functioning in embryogenesis, hematopoiesis and cardiac development [27]; however, much less is known about the impact of mammalian DOT1L in the DNA damage response. It would be interesting to determine whether, like the yeast counterpart, Dot1 orthologs are involved in meiotic checkpoint control in higher eukaryotes.

Materials and Methods

Yeast strains and plasmids

Yeast strains genotypes are listed in Table 1. All the strains are in the BR1919 background [66]. Gene deletions were made using a PCR-based approach [67,68] except for dot1::URA3, zip1::LIS2 and ndd00::LEU2, which were previously described [19,26,29]. MEK1-13myc, MEK1-GFP and HOP1-GFP were made by a PCR approach [68]. The C-terminally tagged Mecl-13myc and Mecl-GFP proteins are functional because spore viability of homozygous tagged wild-type diploids was similar to that of untagged strains and, in addition, they supported the checkpoint-induced delay of a hop2 mutant. In zip1 Hop1-GFP strains the meiotic block was less tight, but Hop1-GFP displayed a localization pattern indistinguishable from that of the untagged protein (Figure S5); therefore, we used the native GFP fluorescence for quantitation of Hop1 localization. N-terminal tagging of Pch2 with three copies of the HA epitope has been previously described [29]. Strains carrying DOT1 or dot1-G401A at its genomic locus or in the pRS315 vector (plasmids pRS315-DOT1 and pFvL54, respectively) were described [28,31]. The H3-K79A and H3-K79R strains are deleted...
Spreads were prepared and GFP images were taken 24 h after meiotic induction in Hop1 antibody (red). Strains are: DP428 (zip1), DP1053 (zip1 dot1), DP1052 (zip1 H3-K79R) and DP1051 (zip1 H3-K79A). (D–E) The absence of Pch2 partially restores Hop1 chromosomal abundance in zip1 dot1. (B) Immunofluorescence of meiotic chromosome spreads stained with DAPI (blue), anti-HA (red) and anti-Red1 (green) antibodies. Strains are: DP1050 (hht2(K79A)-HHF2 or hht2(K79R)-HHF2) and express different versions of H3 from centromeric plasmids carrying either the hht2(K79A)-HHF2 or hht2(K79R)-HHF2 mutant genes (pFvL87 and pFvL88, respectively). The mek1-T327A, mek1-T331A, mek1-K199R mutations, as well as the GST-MEK1 construct were introduced as described [33], using plasmids kindly provided by N. Hollingsworth (Stony Brook University, NY). The high-copy HOPI plasmid was also described [69]. Strains harboring the hta1-S129* and hta2-S129* mutations lacking the last four amino acids of the C-terminal tail of histone H2A including the serine 129 phosphorylated by Mec1/Tel1 [53] were made using plasmids pJHA16 and pJHA17 (provided by J. Downs, University of Sussex) following a pop-in/pop-out strategy. For meiotic time courses, strains were grown in 2% KAc, resuspended into 2% KAc (10 ml) and incubated for 8 hours. Cells were harvested, washed with 2% potassium acetate (KAc), resuspended into 2% KAc (10 ml) and incubated at 30°C with vigorous shaking to induce meiosis and sporulation. Both YPDA and 2% KAc were supplemented with 20 mM adenine and 10 mM uracil. The culture volumes were scaled-up when needed.

Western blotting and analysis of Mek1 phosphorylation

TCA cell extracts from 5–10 ml of sporulating cultures were processed as described [14]. To resolve the phosphorylated forms of Mek1 or Mek1-GFP, 10% or 7% gels (acrylamide:bisacrylamide 29:1), respectively, containing 37.5 μM Phos-tag (Wako Chemicals) and 75 μM MnCl2 were used. Gels were run on ice at 100 volts in a MiniProtean3 (Bio-Rad) for 3 h. After running, gels were washed with 1 mM EDTA before transfer to PVDF membranes.

For dephosphorylation assays, total TCA cell extracts solubilized in Laemmli buffer were diluted 10 times with phosphatase buffer supplemented with 1 mM MnCl2. Diluted extracts were treated with 2000 units of lambda phosphatase (New England Biolabs) for 30 min at 30°C. As control, a similar aliquot of the diluted extract was incubated under the same conditions but without adding phosphatase. Samples were re-precipitated with 20% TCA, washed with acetone, boiled in Laemmli buffer and loaded in Phostag gels.

The following antibodies were used: rabbit polyclonal anti-Mek1 (1:1000 dilution) [11] and anti-Dot1 (1:2000 dilution) [31]. Rabbit polyclonal anti-H3K79-me1 (ab2886; 1:1000 dilution), anti-H3K79-me2 (ab3594; 1:2000 dilution), anti-H3K79-me3 (ab3595; 1:2000 dilution) were used.

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Figure 7. H3K79me controls Hop1 localization by excluding Pch2 from chromosomes. (A) H3K79me is required to prevent Pch2 localization outside of the rDNA. Immunofluorescence of meiotic chromosome spreads stained with DAPI (blue), anti-HA (red) and anti-Red1 (green) antibodies. Strains are: DP428 (zip1), DP1053 (zip1 dot1) and DP1052 (zip1 H3-K79R) and DP1051 (zip1 H3-K79A). (B–E) The absence of Pch2 partially restores Hop1 chromosomal abundance in zip1 dot1. (B) Immunofluorescence of meiotic chromosome spreads stained with DAPI (blue) and anti-Hop1 antibody (red). Strains are: DP428 (zip1), DP655 (zip1 dot1) and DP1054 (zip1 dot1 pch2). (C) Representative images of cells expressing HOPI-GFP in wild type (DP963), dot1 (DP966), zip1 (DP964), zip1 dot1 (DP965) and zip1 dot1 pch2 (DP1027). (D) Quantification of the Hop1-GFP signal intensity on fluorescence images (a.u., arbitrary units). 300 individual nuclei were analyzed for each strain. Each spot in the plot represents the fluorescence intensity of every nucleus measured. Error bars represent the median with interquartile range. P<0.01 in pairwise comparisons. In all cases (A–C), spreads were prepared and GFP images were taken 24 h after meiotic induction in ndt80 strains. (E, F) The absence of Pch2 does not restore the pachytene checkpoint response in zip1 dot1. (E) Time course of meiotic nuclear divisions; the percentage of cells containing more than two nuclei is represented. Strains are: DP421 (wild type), DP422 (zip1), DP555 (zip1 dot1), DP1029 (zip1 pch2) and DP1041 (zip1 dot1 pch2). (F) Western blot analysis of zip1-induced Mek1 phosphorylation in ndt80 strains. PGK was used as a loading control. The asterisk marks a presumed non-specific band (see Figure 3D). Strains are: DP428 (zip1), DP655 (zip1 dot1), DP881 (zip1 pch2) and DP1054 (zip1 dot1 pch2).

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Figure 8. Model for Dot1 function in the meiotic recombination checkpoint. See text for details.

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Table 1. *Saccharomyces cerevisiae* strains.

| Strain       | Genotype*                                      |
|--------------|------------------------------------------------|
| BR1919-2N    | MATα/MATα leu2-3,112 his4-260 ura3-1 ade2-1 thr1-4 trp1-289 |
| DP409        | BR1919-2N zip1::LEU2                          |
| DP419        | BR1919-2N hta1-S129* hta2-S129*               |
| DP420        | BR1919-2N hta1-S129* hta2-S129* zip1::LEU2    |
| DP421        | BR1919-2N ynl2::Nhel                          |
| DP422        | DP421 zip1::LYS2                              |
| DP424        | DP421 ndt80::LEU2                             |
| DP428        | DP421 zip1::LYS2 ntd80::LEU2                  |
| DP460        | DP421 zip1::LYS2 ndt80::LEU2 DDC2-GFP::TRP1   |
| DP555        | DP421 zip1::LYS2 dot1::kanMX6                 |
| DP556        | DP421 zip1::LYS2 dot1::kanMX6::DOTT1::URA3    |
| DP560        | DP421 zip1::LYS2 dot1::kanMX6:dot1-G401A::URA3|
| DP579        | DP421 zip1::LYS2 ntd80::LEU2 dot1::URA3 DDC2-GFP::TRP1 |
| DP582        | DP421 zip1::LYS2 ntd80::LEU2 MEK1-GFP::kanMX6|
| DP583        | DP421 zip1::LYS2 ntd80::LEU2 MEK1-GFP::kanMX6 dot1::URA3 |
| DP622        | BR1919-2N hta1-S129* hta2-S129* dot1::kanMX6 |
| DP623        | BR1919-2N hta1-S129* hta2-S129* dot1::kanMX6 zip1::LEU2 |
| DP624        | DP421 dot1::URA3                              |
| DP625        | DP421 dot1::kanMX6                            |
| DP655        | DP421 zip1::LYS2 ntd80::LEU2 dot1::kanMX6     |
| DP656        | DP421 zip1::LYS2 ntd80::LEU2 mek1::kanMX6     |
| DP674        | DP421 zip1::LYS2 ntd80::LEU2 smf1::kanMX6 mec1::KIURA3 |
| DP701        | DP421 zip1::LYS2 ntd80::LEU2 hop1::hphpMX4    |
| DP713        | DP421 mek1::kanMX6                            |
| DP714        | DP421 zip1::LYS2 mek1::kanMX6                 |
| DP716        | DP421 zip1::LYS2 mek1::kanMX6 dot1::hphpMX4   |
| DP728        | BR1919-2N zip1::kanMX6 ntd80::LEU2 spo11::hphpMX4 |
| DP783        | DP421 zip1::LYS2 mek1::kanMX6 dot1::hphpMX4 GST-mek1-K199R::URA3 |
| DP784        | DP421 zip1::LYS2 mek1::kanMX6 dot1::hphpMX4 GST-mek1-T327A::URA3 |
| DP785        | DP421 zip1::LYS2 mek1::kanMX6 dot1::hphpMX4 GST-MEK1::URA3 |
| DP790        | DP421 zip1::LYS2 mek1::kanMX6 GST-mek1-K199R::URA3 |
| DP791        | DP421 zip1::LYS2 mek1::kanMX6 GST-mek1-T327A::URA3 |
| DP792        | DP421 zip1::LYS2 mek1::kanMX6 GST-MEK1::URA3 |
| DP806        | DP421 (hht1-hhf1)::kanMX6 (hht2-hhf2)::natMX4 p[HHT2-HHF2]:TRP1 |
| DP807        | DP421 (hht1-hhf1)::kanMX6 (hht2-hhf2)::natMX4 p[Hht2-K79A-HHF2]:TRP1 |
| DP808        | DP421 (hht1-hhf1)::kanMX6 (hht2-hhf2)::natMX4 p[Hht2-K79R-HHF2]:TRP1 |
| DP809        | DP421 (hht1-hhf1)::kanMX6 (hht2-hhf2)::natMX4 p[HHT2-HHF2]:TRP1 zip1::LYS2 |
| DP810        | DP421 (hht1-hhf1)::kanMX6 (hht2-hhf2)::natMX4 p[Hht2-K79A-HHF2]:TRP1 zip1::LYS2 |
| DP811        | DP421 (hht1-hhf1)::kanMX6 (hht2-hhf2)::natMX4 p[Hht2-K79R-HHF2]:TRP1 zip1::LYS2 |
| DP812        | DP421 (hht1-hhf1)::kanMX6 (hht2-hhf2)::natMX4 p[HHT2-HHF2]:TRP1 zip1::LYS2 dot1::hphpMX4 |
| DP848        | DP421 zip1::LYS2 ntd80::LEU2 MEK1-13myc::kanMX6 |
| DP849        | DP421 zip1::LYS2 ntd80::LEU2 MEK1-13myc::kanMX6 dot1::URA3 |
| DP861        | DP421 zip1::LYS2 ntd80::LEU2 smf1::kanMX6 mec1::KIURA3 tel1::hphpMX4 |
| DP877        | DP421 zip1::LYS2 ntd80::LEU2 rad24::TRP1 tel1::hphpMX4 |
| DP881        | DP421 zip1::LYS2 ntd80::LEU2 pch2::TRP1 |
| DP883        | DP421 zip1::LYS2 ntd80::LEU2 rad24::TRP1 |
| DP884        | DP421 zip1::LYS2 ntd80::LEU2 dot1::hphpMX4 MEK1-13myc::kanMX6 |
| DP885        | DP421 zip1::LYS2 ntd80::LEU2 mek1::kanMX6 MEK1::URA3 |
| DP886        | DP421 zip1::LYS2 ntd80::LEU2 mek1::kanMX6 mek1-T327A::URA3 |
Table 1. Cont.

| Strain | Genotype* |
|--------|-----------|
| DP887  | DP421 zip::LYS2 ndt80::LEU2 mek1::kanMX6 mek1::T331A::URA3 |
| DP888  | DP421 zip::LYS2 ndt80::LEU2 mek1::kanMX6 mek1::K199R::URA3 |
| DP90   | DP421 zip::LYS2 ndt80::LEU2 mek1::kanMX6 MEK1::URA3 dot1::chpMX4 |
| DP93   | DP421 ndt80::LEU2 HOPI1-GFP::kanMX6 |
| DP94   | DP421 zip::LYS2 ndt80::LEU2 HOPI1-GFP::kanMX6 |
| DP95   | DP421 zip::LYS2 ndt80::LEU2 HOPI1-GFP::kanMX6 dot1::URA3 |
| DP96   | DP421 ndt80::LEU2 HOPI1-GFP::kanMX6 dot1::URA3 |
| DP1024 | DP421 zip::LYS2 ndt80::LEU2 ddc2::TRP1 smf1::kanMX6 |
| DP1027 | DP421 zip::LYS2 ndt80::LEU2 pch2::TRP1 dot1::URA3 HOPI1-GFP::kanMX6 |
| DP1029 | DP421 zip::LYS2 pch2::TRP1 |
| DP1041 | DP421 zip::LYS2 pch2::TRP1 dot1::URA3 |
| DP1042 | DP421 (hht1-hhf1)::natMX4 (hht2-hhf2)::chpMX4 zip1::LYS2 ndt80::LEU2 HOPI1-GFP::kanMX6 p[HHT2-HHF2]::TRP1 |
| DP1043 | DP421 (hht1-hhf1)::natMX4 (hht2-hhf2)::chpMX4 zip1::LYS2 ndt80::LEU2 HOPI1-GFP::kanMX6 p[tt2-K79A-HHF2]::TRP1 |
| DP1044 | DP421 (hht1-hhf1)::natMX4 (hht2-hhf2)::chpMX4 zip1::LYS2 ndt80::LEU2 HOPI1-GFP::kanMX6 p[tt2-K79R-HHF2]::TRP1 |
| DP1045 | DP421 (hht1-hhf1)::natMX4 (hht2-hhf2)::chpMX4 zip1::LYS2 ndt80::LEU2 dot1::URA3 HOPI1-GFP::kanMX6 p[HHT2-HHF2]::TRP1 |
| DP1046 | DP421 (hht1-hhf1)::natMX4 (hht2-hhf2)::chpMX4 zip1::LYS2 ndt80::LEU2 MEK1::GFP::kanMX6 p[HHT2-HHF2]::TRP1 |
| DP1047 | DP421 (hht1-hhf1)::natMX4 (hht2-hhf2)::chpMX4 zip1::LYS2 ndt80::LEU2 MEK1::GFP::kanMX6 p[tt2-K79A-HHF2]::TRP1 |
| DP1048 | DP421 (hht1-hhf1)::natMX4 (hht2-hhf2)::chpMX4 zip1::LYS2 ndt80::LEU2 MEK1::GFP::kanMX6 p[tt2-K79R-HHF2]::TRP1 |
| DP1049 | DP421 (hht1-hhf1)::natMX4 (hht2-hhf2)::chpMX4 zip1::LYS2 ndt80::LEU2 dot1::URA3 MEK1::GFP::kanMX6 p[HHT2-HHF2]::TRP1 |
| DP1050 | DP421 (hht1-hhf1)::natMX4 (hht2-hhf2)::chpMX4 zip1::LEU2 PCH2-3HA p[HHT2-HHF2]::TRP1 |
| DP1051 | DP421 (hht1-hhf1)::natMX4 (hht2-hhf2)::chpMX4 zip1::LEU2 PCH2-3HA p[tt2-K79A-HHF2]::TRP1 |
| DP1052 | DP421 (hht1-hhf1)::natMX4 (hht2-hhf2)::chpMX4 zip1::LEU2 PCH2-3HA p[tt2-K79R-HHF2]::TRP1 |
| DP1053 | DP421 (hht1-hhf1)::natMX4 (hht2-hhf2)::chpMX4 zip1::LEU2 PCH2-3HA dot1::kanMX6 p[HHT2-HHF2]::TRP1 |
| DP1054 | DP421 zip::LYS2 ndt80::LEU2 pch2::TRP1 dot1::URA3 |

*All strains are isogenic diploids homozygous for the indicated markers.

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(ab2621; 1:2000 dilution), and anti-histone H3 (ab1791; 1:5000) were from Abcam. Rabbit polyclonal anti-Hop1 (1:2000 dilution) [3], was from S. Roeder (Yale University). Anti-Cdc5 (sc-6739; 1:10000 dilution) was from Santa Cruz Biotechnology. Mouse monoclonal anti-HA (12CA5; 1:2000 dilution) was from Roche. Anti-phosphoglycerate kinase (PGK) (A-6457; 1:1000 dilution) was from Molecular Probes. The ECL or ECL Plus reagents were used for detection. The signal was captured on film and/or with a ChemiDoc XRS (Bio-Rad) system and quantified with the Quantity One software (Bio-Rad).

Cytology

Immunofluorescence of chromosome spreads was performed essentially as described [29]. To detect Mek1-myc and Mek1-GFP, mouse monoclonal anti-myc (clone 4A6, Millipore) and mouse monoclonal anti-GFP (JL-8, Clontech) antibodies, respectively, were used at 1:200 dilution. Rabbit polyclonal anti-Red1 and anti-Hop1 antibodies (gifts from S. Roeder) have been previously described [3,5]. Anti-mouse and/or anti-rabbit AF-488 and AF-594 conjugated secondary antibodies (Molecular Probes) were used at 1:200 dilution. Images were captured with a Nikon Eclipse 90i fluorescence microscope controlled with the MetaMorph software and equipped with an Orca-AG (Hamamatsu) CCD camera and a PlanApo VC 100×1.4 NA objective.

Whole cell images were captured with an Olympus IX71 fluorescence microscope equipped with a personal DeltaVision system (Applied Precision), a CoolSnap HQ2 (Photometrics) camera and a 100× UPLSAPO 1.4 NA objective. Exposure times were 800 ms, 400 ms and 300 ms for Ddc2-GFP, Mek1-GFP, and Hop1-GFP, respectively. Stacks of 20 planes at 0.2 μm intervals were captured. Maximum intensity projections of deconvolved images were generated with the SoftWoRx 5.0 software (Applied Precision). Quantification of GFP signals in the projections of individual nuclei was performed with the Image J software (http://rsb.info.nih.gov/ij/). Background signal was subtracted using the Otsu’s or the Renyi’s entropy threshold methods in Image J. To outline the contour of the cells in the representative whole-cell images presented, an overlay of the DIC image with 15–20% transparency over the GFP signal is shown.

Other techniques

To analyze meiotic nuclear divisions, cells were fixed in 70% ethanol, washed in PBS and stained with 1 μg/μl DAPI for 15 minutes at room temperature. At least 300 cells of every strain were scored at each time point. Analyses of meiotic kinetics were repeated several times; representative time courses are shown. Spore viability was determined by tetrad dissection. To calculate the statistical significance of differences a two-tailed Student t-test was used. P-values were calculated using the GraphPad Prism 4.0 software. P<0.01 was considered significant.

Supporting Information

Figure S1 H3K79 is constitutively methylated during meiosis. (A) Western blot analysis of H3K79 methylation dynamics
throughout meiosis. Total histone H3 is shown as a loading control. Strains are: DP421 (wild type), DP625 (dot1), DP422 (zip1) and DP555 (zip1 dot1). (B) H3K79 methylation does not change in other mutants defective in the meiotic recombination checkpoint. Western blot analysis of H3K79me in ndt80-arrested cells at 24 h after meiotic induction. Total histone H3 is shown as a loading control. Strains are: DP428 (zip1), DP728 (zip1 spo11), DP981 (zip1 mec1), DP883 (zip1 rad24) and DP1024 (zip1 ddc2). (TIF)

**Figure S2** Red1 linear localization in zip1 chromosomes is not significantly altered in the absence of Dot1. Immunofluorescence of meiotic chromosome spreads stained with DAPI (blue) and anti-Red1 (green) antibody. Representative nuclei are shown. Spreads were prepared 24 h after meiotic induction of ndt80 cells. Strains are: DP484 (zip1) and DP489 (zip1 dot1). (TIF)

**Figure S3** Dot1 is required for zip1-induced Hop1 phosphorylation. Western blot analysis of Hop1 in cell extracts obtained 24 h after meiotic induction in ndt80 cells. Poncet S staining of the membrane was used as a loading control. Strains are: DP428 (zip1), DP674 (zip1 mdk1), DP655 (zip1 dot1), DP728 (zip1 spo11) and DP680 (zip1 mcm). (TIF)

**Figure S4** Pch2 protein levels do not change in the absence of H3K79me. Western blot analysis of Pch2-HA in cell extracts obtained 15 h after meiotic induction of ndt80 cells. PGK is shown as a loading control. Strains are: DP428 (zip1), DP1050 (zip1), DP1055 (zip1 dot1), DP1052 (zip1 H3-K79R) and DP1051 (zip1 H3-K79A). (TIF)

**Figure S5** Hop1-GFP localization is impaired in the absence of H3K79me. Immunofluorescence of meiotic chromosome spreads stained with DAPI (blue), anti-Red1 (green) and anti-GFP (red) antibodies. Representative nuclei are shown. Spreads were prepared 24 h after meiotic induction of ndt80 cells. Strains are: DP1042 (zip1), DP1045 (zip1 dot1), DP1044 (zip1 H3-K79R) and DP1043 (zip1 H3-K79A). (TIF)

**Figure S6** Analysis of γH2AX meiotic function. (A) Unlike H3K79me, γH2AX is not required for the checkpoint-induced by zip1 because the H2A-S129* mutation does not suppress zip1 meiotic block. Time course of meiotic nuclear divisions; the percentage of cells containing more than two nuclei is represented. Strains are: BR1919-2N (wild type), DP409 (zip1), DP149 (H2A-S129*), DP420 (zip1 H2A-S129*), DP622 (dot1 H2A-S129*) and DP625 (dot1 H2A-S129*). (B) Spore viability is high in the absence of γH2AX and H3K79me, suggesting that both histone modifications are not required in unperturbed meiosis. At least 288 spores were scored for each strain. Means and standard deviations are shown. Strains are: BR1919-2N (wild type), DP419 (H2A-S129*), DP622 (dot1 H2A-S129*) and DP624 (dot1). (TIF)

**Video S1** Hop1 chromosomal distribution is impaired in the absence of Dot1. 3D reconstruction of deconvolved Z-stack images showing Hop1-GFP signal in zip1 and zip1 dot1 cells. Two different nuclei of each strain are shown during the movie. (MOV)

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**Author Contributions**

Conceived and designed the experiments: DO PAS-S. Performed the experiments: DO IA. Analyzed the data: DO IA PAS-S. Contributed reagents/materials/analysis tools: DO IA FvL RF PAS-S. Wrote the paper: PAS-S.

**References**

1. Roeder GS (1997) Meiotic chromosomes: it takes two to tango. Genes Dev 11: 2600–2621.
2. Zickler D, Kleckner N (1999) Meiotic chromosomes: integrating structure and function. Annu Rev Genet 33: 603–754.
3. Smith AV, Roeder GS (1997) The yeast Red1 protein localizes to the cores of meiotic chromosomes. J Cell Biol 136: 957–967.
4. Dong H, Roeder GS (2000) Organization of the yeast Zip1 protein within the central region of the synaptonemal complex. J Cell Biol 148: 417–426.
5. Sym M, Engebrecht JA, Roeder GS (1993) ZIP1 is a synaptonemal complex central region protein. J Cell Biol 124: 2488–2500.
6. Leu JY, Roeder GS (1999) The pachytene checkpoint in S. cerevisiae depends on Swel-mediated phosphorylation of the cyclin-dependent kinase Cde2. Mol Cell 4: 803–814.
7. MacQueen AJ, Hochwagen A (2011) Checkpoint mechanisms: the puppet controlled by mitotic checkpoint genes. Nature 383: 840–843.
8. Roeder GS, Bailis JM (2000) The pachytene checkpoint. Trends Genet 16: 395–403.
9. Hong EJ, Roeder GS (2002) A role for Ddc1 in signaling meiotic double-strand breaks at the pachytene checkpoint. Genes Dev 16: 363–376.
10. Dong H, Roeder GS (2000) Organization of the yeast Zip1 protein within the central region of the synaptonemal complex. J Cell Biol 148: 417–426.
11. Syn M, Engebrecht JA, Roeder GS (1993) ZIP1 is a synaptonemal complex protein required for meiotic chromosome synopsis. Cell 72: 365–378.
12. Keeney S (2001) Mechanism and control of meiotic recombination initiation. Curr Top Dev Biol 52: 1–53.
13. MaQuen AJ, Hochwagen A (2011) Checkpoint mechanisms: the puppet masters of meiotic prophase. Trends Cell Biol 21: 393–400.
14. Roeder GS, Baill JM (2000) The pachytene checkpoint. Trends Genet 16: 395–403.
15. Hong EJ, Roeder GS (2002) A role for Ddc1 in signaling meiotic double-strand breaks at the pachytene checkpoint. Genes Dev 16: 363–376.
16. Feng Q, Wang H, Ng HH, Erdjument-Bromage H, Tempst P, et al. (2002) Meiotic checkpoint control. Mol Biol Cell 11: 3601–3615.
17. Tung KS, Hong EJ, Roeder GS (2000) The pachytene checkpoint prevents accumulation and phosphorylation of the meiosis-specific transcription factor Ndt80. Proc Natl Acad Sci U S A 97: 12167–12192.
18. Brachet E, Sommermeyer V, Bode V (2011) Interplay between modifications of chromatin and meiotic recombination hotspots. Biol Cell 104: 51–69.
19. Baill JM, Baill JM (2000) Pachytene exit controlled by reversal of Mek1-dependent phosphorylation. Cell 101: 211–221.
20. Wu L, de los Santos T, Zhang C, Shokat K, Hollingsworth NM (2004) Mek1 kinase activity functions downstream of RED1 in the regulation of meiotic double strand break repair in budding yeast. Mol Biol Cell 15: 11–23.
21. Acosta I, Ontoso D, San-Segundo PA (2011) The budding yeast polo-like kinase Zip1 forms. Strains are: DP1050 (zip1), DP1055 (zip1 dot1), DP1052 (zip1 H3-K79R) and DP1051 (zip1 H3-K79A). (TIF)
30. Lui DY, Peoples-Holst TL, Mell JC, Wu HY, Dean EW, et al. (2006) Analysis of
29. San-Segundo PA, Roeder GS (1999) Pch2 links chromatin silencing to meiotic
47. Hammet A, Magill C, Heierhorst J, Jackson SP (2007) Rad9 BRCT domain
44. Botuyan MV, Lee J, Ward IM, Kim JE, Thompson JR, et al. (2006) Structural
41. Grenon M, Costelloe T, Jimeno S, O’Shaughnessy A, Fitzgerald J, et al. (2007)
39. Borner GV, Barot A, Kleckner N (2008) Yeast Pch2 promotes domainal axis
38. Niu H, Wan L, Baumgartner B, Schaefer D, Loidl J, et al. (2005) Partner choice
36. Keeney S, Giroux CN, Kleckner N (1997) Meiosis-specific DNA double-strand
35. Woltering D, Baumgartner B, Bagchi S, Larkin B, Loidl J, et al. (2000) Meiotic
34. Carballo JA, Johnson AL, Sedgwick SG, Cha RS (2008) Phosphorylation of the
33. Niu H, Li X, Job E, Park C, Moazed D, et al. (2007) Mek1 kinase is regulated to
32. Cartagena-Lirola H, Guerini I, Viscardi V, Lucchini G, Longhese MP (2006)
31. Conde F, Ontoso D, Acosta I, Gallego-Sanchez A, Bueno A, et al. (2010)
49. Hunter N (2008) Hop1 and the meiotic DNA-damage response. Cell 132: 731–
48. Hochwagen A, Amon A (2006) Checking your breaks: surveillance mechanisms
47. Hammet A, Magill C, Heierhorst J, Jackson SP (2007) Rad9 BRCT domain
46. Zanders S, Alani E (2009) The Meiotic checkpoint role of H3K79me
45. Hunter N, Wan L, Busygina V, Kwon Y, Allen JA, et al. (2007) Regulation of meiotic
44. Botuyan MV, Lee J, Ward IM, Kim JE, Thompson JR, et al. (2006) Structural
43. Carballo JA, Johnson AL, Sedgwick SG, Cha RS (2008) Phosphorylation of the
42. Farmer S, Hong EJ, Leung WK, Argunhan B, Terentyev Y, et al. (2012)
41. Grenon M, Costelloe T, Jimeno S, O’Shaughnessy A, Fitzgerald J, et al. (2007)
40. Hunter N (2008) Hop1 and the meiotic DNA-damage response. Cell 132: 731–
39. Borner GV, Barot A, Kleckner N (2008) Yeast Pch2 promotes domainal axis
38. Niu H, Wan L, Baumgartner B, Schaefer D, Loidl J, et al. (2005) Partner choice
37. Vader G, Blitzblau HG, Tame MA, Falk JE, Curtin L, et al. (2011) Protection of
36. Keeney S, Giroux CN, Kleckner N (1997) Meiosis-specific DNA double-strand
35. Woltering D, Baumgartner B, Bagchi S, Larkin B, Loidl J, et al. (2000) Meiotic
34. Carballo JA, Johnson AL, Sedgwick SG, Cha RS (2008) Phosphorylation of the
33. Niu H, Li X, Job E, Park C, Moazed D, et al. (2007) Mek1 kinase is regulated to
32. Cartagena-Lirola H, Guerini I, Viscardi V, Lucchini G, Longhese MP (2006)
31. Conde F, Ontoso D, Acosta I, Gallego-Sanchez A, Bueno A, et al. (2010)
29. San-Segundo PA, Roeder GS (1999) Pch2 links chromatin silencing to meiotic
28. Heierhorst J, Jackson SP (2007) Rad9 BRCT domain.