Nucleosomal organization of replication origins and meiotic recombination hotspots in fission yeast

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In *Schizosaccharomyces pombe*, DNA replication origins (ORIs) and meiotic recombination hotspots lack consensus sequences and show a bias towards mapping to large intergenic regions (IGRs). To explore whether this preference depended on underlying chromatin features, we have generated genome-wide nucleosome profiles during mitosis and meiosis. We have found that meiotic double-strand break sites (DSBs) colocalize with nucleosome-depleted regions (NDRs) and that large IGRs include clusters of NDRs that overlap with almost half of all DSBs. By contrast, ORIs do not colocalize with NDRs and they are regulated independently of DSBs. Physical relocation of NDRs at ectopic loci or modification of their genomic distribution during meiosis was paralleled by the generation of new DSB sites. Over 80% of all meiotic DSBs colocalize with NDRs that are also present during mitosis, indicating that the recombination pattern is largely dependent on constitutive properties of the genome and, to a lesser extent, on the transcriptional profile during meiosis. The organization of ORIs and of DSBs regions in *S. pombe* reveals similarities and differences relative to *Saccharomyces cerevisiae*.

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Introduction

During meiosis, the genome of a diploid cell undergoes replication, after which recombination takes place between homologous chromosomes. This process facilitates chromosome segregation and generates genetic diversity between the resulting haploid gametes or spores. Recombination begins with the introduction of DNA double-strand breaks (DSB) at specific sites along the genome by the proteins

encoded by the *rec12* and the *SPO11* genes in the yeasts *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*, respectively (Keeney *et al.*, 1997; Cervantes *et al.*, 2000), the organisms whose genetics and the molecular biology of meiosis are best understood.

In *S. pombe*, *M26* and *mbs1* (meiosis break site 1) are recombination hotspots that have been characterized in detail through genetic and physical approaches. *M26* was identified as a G to T transversion in the coding region of the *ade6* gene that generates a binding site for the Atf1/Pcr1 transcription factor, which is in turn required for the generation of DSBs and the meiosis-specific chromatin remodelling of that locus (Ponticelli *et al.*, 1988; Kon *et al.*, 1998; Steiner *et al.*, 2002; Yamada *et al.*, 2004). *Mbs1* was initially identified as a region of DSBs in chromosome I (Cervantes *et al.*, 2000; Young *et al.*, 2002) and it adopts a constitutively open chromatin structure independent of Atf1/Pcr1 (Hirota *et al.*, 2007). The size of DSB regions can be estimated by Southern hybridization of DNA samples at different times during meiosis and often extend along 1–2 kb and may include sites of preferential cleavage. For example, DSBs in the *mbs1* locus in *S. pombe* span a 2.1-kb region containing four clusters of sites of enhanced breakage (Cromie *et al.*, 2005). Further examples of DSB regions ranging from a few hundred nucleotides up to over 2 kb have been reported by Steiner and Smith (2005) and by Cromie *et al.* (2007). An expanded consensus of the *M26* sequence has been used successfully to predict the localization of some DSBs, although not all regions harbouring the consensus generate DSBs and most genomic DSBs do not include it (Steiner and Smith, 2005; Cromie *et al.*, 2007). A similar situation applies to other recombination hotspots associated with specific sequences such as *HIS4* in *S. cerevisiae* (White *et al.*, 1993; Mieczkowski *et al.*, 2006) and a degenerate C-rich motif in humans (Myers *et al.*, 2008).

Analyses of specific DSBs have shown that an accessible chromatin context is an important element in the specification of DSBs. In *S. cerevisiae*, an open chromatin structure has been found at specific recombination hotspots (Wu and Lichten, 1994; Fan and Petes, 1996; see Lichten 2008 for review), and it has been very recently reported that this property is a general feature of recombination hotspots in this yeast (Pan *et al.*, 2011). In *S. pombe*, the chromatin at the *tdh1* locus is specifically remodelled during meiosis independently of Atf1/Pcr1 while the *cds1* locus, which lies in a constitutively open chromatin region, requires Atf1/Pcr1 for its maintenance in mitosis and meiosis (Hirota *et al.*, 2007). Also, in the mouse, a low-nucleosome occupancy has been found at four recombination hotspots (Shenkar *et al.*, 1991; Getun *et al.*, 2010). All these studies suggest that accessibility to the DNA molecule through either constitutively or meiotically induced open chromatin is a prerequisite for the generation of DSBs. However, as in the case of sequence-dependent recombination hotspots, an open chromatin structure alone is not sufficient to specify DSBs, and several studies support the notion that specific histone

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modifications also play an important role. For example, deletion of the Set1 H3K4 methyltransferase in *S. cerevisiae* strongly reduces the efficiency of 84% of the recombination hotspots in the genome (Borde *et al*, 2009); *HIM-17* mutants of *Caenorhabditis elegans* have reduced the levels of H3K9 methylation and do not form DSBs (Reddy and Villeneuve, 2004), and in *S. pombe*, deletion of the *gcn5* histone acetyl transferase gene delays chromatin remodelling during meiosis at the *M26* hotspot (Yamada *et al*, 2004). The current view derived from these and other analyses is that meiotic DSBs in *S. pombe* and other eukaryotes are specified by the combined contribution of genetic, structural and epigenetic elements (reviewed by Nishant and Rao, 2005; Kniewel and Keeney, 2009; Wahls and Davidson, 2010). This multiplicity of determinants can explain the variability in the recombination activity of the *M26* hotspot when translocated to ectopic positions in the genome (Ponticelli and Smith, 1992; Virgin and Bailey, 1998).

A major advance in the field of recombination in *S. pombe* was provided by the genome-wide identification of Rec12-DNA covalent linkages by chromatin immunoprecipitation (ChIP) followed by microarray hybridization (Cromie *et al*, 2007; Hyppa *et al*, 2008; Ludin *et al*, 2008). These studies confirmed the lack of sequence consensus elements at DSBs and uncovered a preferential localization in large intergenic regions (IGRs), given that about 50% of all meiotic DSBs mapped to IGRs larger than 3 kb.

A lack of consensus elements and a bias towards localization in larger than average IGRs is also a conspicuous property of replication origins (ORIs) in *S. pombe* (Gómez and Antequera, 1999; Segurado *et al*, 2003; Heichinger *et al*, 2006; Hayashi *et al*, 2007). A significant difference with DSBs, however, is that ORI specification seems to depend mainly, or exclusively, on a high adenine and thymine content to the extent that exogenous A+T-rich sequences without homology to the *S. pombe* genome can initiate chromosomal replication as efficiently as the endogenous ORIs (Cotobal *et al*, 2010).

We have addressed the chromatin organization of DSBs and ORIs in *S. pombe* by generating genome-wide nucleosome profiles during mitosis and meiosis. Our results reveal a significant degree of colocalization of ORIs and DSBs in large IGRs as a consequence of their preference for different features of these regions. We show that nucleosome-depleted regions (NDRs) are, at least so far, the only requirement shared by all meiotic recombination hotspots in *S. pombe* and that the overwhelming majority of them are established at constitutive NDRs already present in mitotic cells. We also show that variations in the distribution of NDRs under different transcriptional regimes during meiosis determine different patterns of recombination.

Results

Colocalization of DSBs and meiotic ORIs

DSB regions and ORIs in *S. pombe* map preferentially to IGRs longer than the genomic average (Gómez and Antequera, 1999; Segurado *et al*, 2003; Cromie *et al*, 2007; Hayashi *et al*, 2007). Given this preference, we wondered whether ORIs and DSBs might tend to share the same IGRs. To address this issue experimentally, we tested ORI activity during the meiotic S-phase in 13 genomic loci previously found to generate DSBs

(Cromie *et al*, 2005; Steiner and Smith 2005) (Figure 1A). Analysis of replication intermediates by two-dimensional gel electrophoresis revealed that 8 out of 13 (60%) DSB regions colocalized with active meiotic ORIs (Figure 1B, see Figure 3B for *mbs1* analysis). To test the degree of colocalization in the opposite direction, we assayed for the presence of DSBs in eight regions encompassing active meiotic ORIs selected at random (Figure 1C). Southern hybridization analyses of DNA at different times during synchronous meiosis in *S. pombe pat1-114* diploid cells detected DSBs in five out of the eight ORI regions tested (60%) (Figure 1D). These DSBs displayed a temporal pattern of appearance and repair similar to *mbs1* (Figure 3C; Cervantes *et al*, 2000) and were absent in *rec12Δ* cells (Figure 1D). This high degree of colocalization between meiotic ORIs and DSBs was much higher than that expected from an independent distribution (χ^2 association test *P*-value < 0.001, see later) and it was not detected in two previous studies on the basis of their predicted genomic localization (Cromie *et al*, 2007; Ludin *et al*, 2008). This was probably due to the higher sensitivity of Southern hybridization for the detection of DSBs and ORIs with respect to ChIP, as has been shown in the case of low-efficiency ORIs (Cotobal *et al*, 2010). For example, DSB sites 1–3 and 3–5 (Figure 1A and B) colocalized with previously undetected ORIs (Segurado *et al*, 2003; Hayashi *et al*, 2007) and ORI AT2112 colocalized with previously undetected DSBs (Figure 1C and D) (Cromie *et al*, 2007).

Genome-wide colocalization of DSBs with NDRs

The high degree of colocalization between ORIs and DSBs, together with their lack of consensus sequences raised the possibility of a shared preference for a specific chromatin organization. To address this matter, we generated genome-wide nucleosome maps from cells during mitosis and at 3 h into meiosis. Briefly, we digested chromatin with micrococcal nuclease (MNase) and used the DNA associated with mono-nucleosomes to hybridize high-density tiling microarrays (see Supplementary Figure S5B and Materials and methods). To validate this approach, we compared the nucleosome profile of the *ade6* gene generated by us with that obtained by Southern hybridization described by Mizuno *et al* (1997) and found that both patterns coincided very precisely (Supplementary Figure S1). We defined NDRs as those longer than 150 nucleotides, with a log₂ ratio below –1 relative to naked genomic DNA (see Materials and methods). Using this definition, we identified a total of 2973 NDRs in the genome of cells in mitosis or meiosis, of which 2928 (98.5%) mapped to IGRs and the remaining 45 (1.5%) colocalized with ORFs. Since in many cases the same IGR included more than one NDR, they were distributed in 1420 IGRs (70%) in which the NDR profile was identical in mitosis and meiosis; 363 IGRs (18%) in which at least one NDR was meiosis specific, and 248 IGRs (12%) in which at least one NDR was mitosis specific (Table I; Supplementary Figure S2).

To explore the correlation between the distribution of nucleosomes and NDRs and the sites of meiotic DSBs, in our map we represented the 243 sites of Rec12-DNA covalent linkages identified by Hyppa *et al* (2008) that have been shown to correlate strictly with sites of DSBs (Cromie *et al*, 2007). Our first observation was that 231 out of 243 DSBs (95%) overlapped with NDRs, indicating that the association between them would be an almost universal feature of DSBs in *S. pombe*. The statistical significance of this correlation had

Figure 1 Colocalization of DSB sites and meiotic origins of replication. (A) Localization of 13 DSB regions (horizontal lines) in the 3 *S. pombe* chromosomes (yellow bars). Green ovals represent centromeres. Red arrowheads indicate DSBs that colocalize with active meiotic ORIs. (B) Analysis of ORI activity by two-dimensional gel electrophoresis of 12 out of the 13 regions containing DSBs in (A). Arrows point to bubble arcs indicative of active ORIs. (C) Activity of eight ORIs associated with the indicated A + T-rich islands during meiosis. (D) Analysis of DSBs by Southern blot of regions encompassing A + T-rich islands 2014, 2091, 2112, 3040 and 3056. Samples were taken at the indicated times during synchronous meiosis in *S. pombe pat1-114* diploid cells (Control) and in *pat1-114 rec12Δ* cells. Major DSBs sites are indicated with arrows.

Table I Distribution of intergenic regions (IGRs), nucleosome-depleted regions (NDRs) and double-strand break regions (DSBs) in *S. pombe*

Total IGRs and NDRs in the genome		IGRs with NDRs	IGRs > 3 kb	IGRs < 3 kb	DSBs
4981 (Total IGRs)		1420 (With constitutive NDRs)	102		79
				1318	112
	2031 (With 2928 NDRs)	363 (With meiosis-specific NDRs)	30		19
2973 (Total NDRs)				333	21
		248 (With mitosis-specific NDRs)	23		0
				225	0
	2950 (Without NDRs)		20		1
				2930	11
Total	4981	2031	175	4806	243

Columns indicate the total number of IGRs and NDRs excluding heterochromatic regions and the rDNA locus; the presence of constitutive, meiosis- and mitotic-specific NDRs; their size classification and the distribution of DSBs in each category. See Supplementary Figure S2 for a diagrammatic representation of these data.

a P -value < 0.001 as estimated by the χ^2 test for association (see Materials and methods). Some examples of this colocalization are shown in Figure 2. The second relevant finding was that 191 out of the 231 DSBs associated with NDRs (83%) mapped to IGRs in which the NDR profile was identical in mitosis and meiosis, while only 40 DSBs (17%) mapped to IGRs harbouring at least one NDR meiosis specific. No DSBs colocalized with any of the NDRs present only during mitosis (Table I; Supplementary Figure S2). These results indicated that the great majority of Rec12-binding sites overlapped constitutive NDRs already present during the vegetative cell cycle. An important conclusion obtained by Cromie *et al* (2007) was that the about 50% of all meiotic DSBs mapped to IGRs larger than 3 kb. In close agreement with their results,

we have found that 98 out of 243 (40%) DSB sites mapped to IGRs longer than 3 kb harbouring NDRs. This represents a strong bias, since there are only 175 IGRs of that size in the single-copy fraction of the genome, which represent 3.5% of all IGRs and account for 6% of the total genome size (excluding heterochromatic regions and the rDNA clusters), and raised the issue of what the reason for this preference was. Our genomic analysis revealed that the 175 IGRs longer than 3 kb often included clusters of closely spaced NDRs (2.5 NDRs on average per IGR longer than 3 kb) that in some cases were associated with non-coding RNA transcripts (Figures 2 and 3A). The colocalization of non-coding RNAs with meiotic recombination hotspots has been previously reported by Wahls *et al* (2008). This suggested that the biased localization

Figure 2 Distribution of NDRs, Rec12, Orc1 and Orc4 binding sites across three regions of the *S. pombe* genome. Mononucleosome DNA isolated from exponential mitotic cells and from cells at 3 h into meiosis was used to hybridize tiling genomic microarrays. NDRs are represented by deep depressions in the nucleosome profile during mitosis (blue) and meiosis (red). Large IGRs in (A, B) include several NDRs. A single NDR at a shorter IGR is shown in (C). Pointed blue rectangles represent annotated genes and ORFs and pointed white rectangles indicate the position of non-coding RNAs as annotated in <http://www.pombase.org>. Brown vertical bars indicate sites of binding of Rec12 (Hyppa *et al*, 2008). The binding profile of the Orc1 (green) and Orc4 (orange) subunits of ORC as detected by ChIP/Chip is also shown. Prominent peaks of colocalization of both proteins indicate the position of ORIs. Data for the entire genome can be accessed from http://genomics.usal.es/cgi-bin/gb2/gbrowse/sp_nrm/.

Figure 3 Functional analysis and chromatin organization of the *mbs1* locus. (A) Nucleosome profile of the *mbs1* region during mitosis (blue) and meiosis (red). Black arrowheads point to DSB sites mapped by Cromie *et al* (2005). The AT1034 AT-rich island is shown by a bracket and brown vertical bars indicate sites of binding of Rec12. Pointed blue and white rectangles indicate the position of ORFs and non-coding RNAs. The Orc1 (green) and Orc4 (orange) subunits of ORC colocalize with the AT-rich island. (B) The AT1034 island colocalizes with an active meiotic ORI in Control *pat1-114* diploid cells, *rec12Δ*, and in cells where the *ura4* gene has replaced AT1034 (*AT1034Δ*) or the DSB (*DSBΔ*) regions bracketed in (A). Arrows indicate bubble replication intermediates. (C) DSBs analysis by Southern blot of the four regions analysed in (B). The distribution of DSBs sites in Control and *AT1034Δ* cells is indicated with brackets.

of DSBs in large IGRs could be due to an enhanced accessibility to DNA provided by the multiple NDRs and not to the size of the IGR alone, as supported by the fact that 19 out of the 20 IGRs larger than 3 kb that lacked NDRs did not bind Rec12 (Table I). Rec12 binds with comparable efficiency to large IGRs harbouring several NDRs and to shorter IGRs with only one NDR (Figure 2A and C). In some cases, the wide peaks of Rec12 binding to IGRs containing several NDRs may result from the combination of the scattering of DSBs (and hence of Rec12 binding), plus the 0.5–1.0 kb average size of the sonicated chromatin fragments used for immunoprecipitation. It is important, however, that the peak of Rec12 binding usually colocalizes with the NDR. Taken together, these results indicated that physical access to DNA seemed to be a strict requisite for the generation of DSBs and this is almost invariably achieved through NDRs, a large proportion of which are constitutive and already present during the mitotic cell cycle.

A list with the genomic position of all constitutive, mitosis- and meiotic-specific NDRs in *S. pombe* is provided in Supplementary Table S3. The genomic distribution of NDRs and the Rec12-binding sites can be accessed from a

searchable genome browser on our web site that also includes data for the ChIP/chip analysis of Orc1 and Orc4 and for the transcription analyses described in the ensuing sections of the manuscript (http://genomics.usal.es/cgi-bin/gb2/gbrowse/sp_nrm/).

ORC binding in vivo does not colocalize with NDRs

Given the strong colocalization of DSBs and NDRs, we next asked whether the same scenario would apply to ORIs. The sequences responsible for ORI activity in *S. pombe* are difficult to determine unambiguously because of their degeneracy and frequent organization in several independent modules across IGRs (Takahashi *et al*, 2003; Cotobal *et al*, 2010). For this reason, and to enhance the resolution of the analysis, we mapped the sites of binding of the origin recognition complex (ORC) across the genome in asynchronous exponential mitotic cells under the same culture conditions used to generate the nucleosome profile. We immunoprecipitated chromatin with antibodies against the Orc1 and Orc4 subunits of ORC, followed by hybridization of high-density tiling microarrays (ChIP/chip).

A previous microarray-based study indicated that the large majority of ORIs in *S. pombe* were equally active in mitosis and meiosis (Heichinger *et al*, 2006). To address this point but using a different approach, we compared the activity of the eight meiotic ORIs in Figure 1C with their activity during mitosis using two-dimensional gel electrophoresis. The results in Supplementary Figure S3 show that their efficiency was very similar in both situations. The consistency between these results and those of Heichinger *et al* (2006) supports the notion that the map of mitotic ORIs can be reliably used to estimate the degree of colocalization with Rec12-binding sites.

We found that the distribution of prominent overlapping peaks of Orc1 and Orc4 largely colocalized with AT-rich islands (Segurado *et al*, 2003) and with sites previously identified by ChIP/chip analysis (Hayashi *et al*, 2007). As regards the colocalization between ORIs and DSBs, we found that 25.3% of Orc1/Orc4 sites shared the same IGR with 37% sites of Rec12 (χ^2 association test *P*-value < 0.001, see Materials and methods). These results are similar to those obtained using the three data sets of the AT-island distribution (Segurado *et al*, 2003), ChIP/Chip of ORC (Hayashi *et al*, 2007) and Rec12 sites (Cromie *et al*, 2005), where 20% of all origins colocalized with 30% of Rec12 sites within the same IGR. It should be stressed, however, that ORC-binding sites did not overlap with NDRs, as illustrated in Figures 2A and B and 3A and Supplementary Figure S4. These examples are representative of the great majority of ORIs since only 14% of all binding sites of ORC in the genome overlap with an NDR. These results suggested that the specification of ORIs and DSBs depended on different sequence and chromatin features, and left open the possibility of a functional link between ORIs and DSBs in the many IGRs where they colocalized. The genomic position of all the Orc1 and Orc4 binding sites and the regions bound by Rec12 are indicated in Supplementary Tables S4 and S5.

Chromatin organization and functional dissection of the *mbs1* locus

The *mbs1* locus of *S. pombe* between the *SPAC4G8.03c* and *SPAC4G8.04* genes is an excellent model of large IGR

harbouring DSBs and a ORI. DSBs associated with this meiotic recombination hotspot have been mapped with great accuracy over a 2.1-kb region (Cromie *et al*, 2005) and the immediately adjacent A+T-rich island 1034 colocalizes with an active meiotic ORI (Figure 3A and B). The microarray profile of MNase accessibility showed that the most prominent feature of this locus was an NDR 400 nucleotides long in mitotic cells that expanded up to 600 nucleotides during meiosis (Figure 3A), in agreement with a previous analysis of this locus by Southern hybridization (Hirota *et al*, 2007). The high resolution of our analysis allowed us to map small meiosis-specific hypersensitive sites that colocalized with the sites of DSBs identified by Cromie *et al* (2005) that, together with the large NDR, overlapped with the peak of highest enrichment of Rec12 binding to this region. In contrast, the A+T-rich island AT1034, which colocalizes with an active ORI during the mitotic and meiotic S-phases, does not overlap with any NDRs, in agreement with the general situation of ORIs described in the previous section.

To test the possibility of a functional dependence between the generation of DSBs and the activity of the AT1034 ORI in the *mbs1* locus, we replaced a 980-bp region encompassing the AT1034 island (bracketed in Figure 3A) by a 1700-bp fragment containing the *ura4* gene as a selectable marker. The resulting strain showed a strong reduction in the activity of this ORI, but DSBs were generated as efficiently as in the control strain (Figure 3B and C; AT1034Δ). This indicated that neither a normal level of ORI activity during the S-phase prior to recombination nor the AT1034 deleted sequences were required for the generation of DSBs. The residual ORI activity observed after the deletion of the A+T-rich island was consistent with the existence of multiple ORI determinants spread over large IGRs harbouring ORIs (Takahashi *et al*, 2003; Cotobal *et al*, 2010).

To test whether the DSB region was required for ORI activity, we replaced a 2480-bp fragment spanning the DSB sites (bracketed in Figure 3A) for the *ura4* gene marker. The results showed that, although DSBs were completely abolished (Figure 3C; DSBΔ), ORI efficiency remained unaffected (Figure 3B; DSBΔ). The activity of this ORI was not affected in *rec12Δ* cells either, which are unable to generate DSBs (Figure 3B and C; *rec12Δ*) (Cervantes *et al*, 2000). Taken together, these results indicated that the sequence and chromatin elements that determine ORI and DSB activity in the *mbs1* locus are not dependent on one another, despite their close physical proximity.

Generation of DSBs in the *mbs1* locus by ectopic sequences

The abrogation of DSBs at *mbs1* as a consequence of being replaced by the *ura4* gene could be due to the loss of specific sequence elements required for the binding of Rec12 or to the removal of the endogenous NDR. To distinguish between these two possibilities, we replaced the DSB region of *mbs1* by two independent IGRs between the *rad17/atp1* genes (R3 fragment, 1500 bp) and between the *cdc25/hxk1* genes (C5 fragment, 1908 bp) (Figure 4A). Both of them encompassed two NDRs of different size upstream from the *atp1* and *cdc25* genes but neither of them colocalized with DSBs in their endogenous loci during meiosis (Figure 4C; R3/end and C5/end). By contrast, when these two fragments

Figure 4 Generation of DSBs at *mbs1* by ectopic sequences. (A) Nucleosome profile across the endogenous R3 and C5 regions during mitosis (blue) and meiosis (red). Black boxes in R3 and C5 indicate the position of NDRs. (B) Diagram of R3 and C5 fragments replacing the DSB region of the *mbs1* locus bracketed in Figure 3A. (C) Absence and presence of DSBs of the R3 and C5 regions in their endogenous loci (R3/end and C5/end) and in the *mbs1* locus (R3/*mbs1* and C5/*mbs1*), respectively, at the indicated times during meiosis. Brackets in (B, C) indicate the extent of the DSB region induced by R3 and C5 after their insertion in *mbs1*.

replaced *mbs1* by homologous recombination, they generated prominent DSBs spanning approximately 0.8 and 1.2 kb that were repaired after 3.5 h of meiosis (Figure 4C; R3/*mbs1* and C5/*mbs1*). The size of the DSBs regions are shown at higher magnification in Supplementary Figure S6. The size of the broken fragments detected by Southern hybridization indicated that the DSB region overlapped the major NDR in R3 and extended about 400 nucleotides into the flanking *mbs1* region (bracket in Figure 4B). In the case of C5, the newly induced DSBs overlapped both NDRs and extended until the end of the fragment (bracket in Figure 4B). A possible interpretation of this result was that the acquired competence of R3 and C5 to generate DSBs could have been favoured by the propensity of the recipient *mbs1* locus to harbour DSBs in wild-type cells, although insertion of the fragment containing the *ura4* gene and its flanks did not generate DSBs (Figure 3C; DSBΔ). Although this gene does not have a NDR in its promoter region, even when actively transcribed (see nucleosome profile in the genome browser), it has been reported that its insertion at an ectopic site in the genome generated the *ura4A* recombination hotspot (Baur *et al*, 2005) and this raised the question of whether insertion into *mbs1*

Figure 5 Insertion of the *ura4* gene into *mbs1* does not generate a NDR. (A) The NDR (red box) and the sites of preferential DSBs in the wild-type *mbs1* locus are indicated. The bracketed region was replaced by a 1.7-kb fragment containing the *ura4* gene (green) and its flanking sequences (thick black line). The vertical lines and the blue bars represent the *SpeI* restriction sites and the hybridization probes, respectively. (B) Naked DNA and chromatin from cells harbouring either the wild-type or a modified *mbs1* locus as shown in (A) were digested with increasing amounts of MNase (indicated with triangles, see Materials and methods) and subsequently digested with *SpeI*. Samples were electrophoresed, transferred onto a membrane and hybridized. A prominent region of hypersensitivity to MNase, also detected in the nucleosome profile in Figure 3A, was present in the wild-type *mbs1* locus (left panel). Hypersensitivity to MNase was lost and the chromatin adopted an ordered nucleosome organization after replacing the *mbs1* region by the *ura4* gene (right panel). Size markers are indicated in kilobases.

could also generate an NDR that would not be competent to harbour DSBs during meiosis. To test this possibility, we compared the nucleosome profile of the *mbs1* region before and after its replacement by *ura4* (Figure 5A) by MNase analysis (Supplementary Figure S5A). Results showed that the endogenous NDR region of *mbs1* (Figures 3A and 5B, left panel) had been replaced by an ordered nucleosomal array over the *ura4* gene and its flanking sequences (Figure 5B, right panel). The disappearance of the NDR was consistent with the absence of DSBs in this region (Figure 3C; DSBΔ) and further stressed the close association between DSBs and NDRs. At the same time, these results suggested the possibility that the NDRs of the C3 and R5 fragments in their endogenous loci could have been maintained in the new location and could now functionally replace the endogenous *mbs1* NDR.

Identical DSB regions induced by the same NDRs at different ectopic loci

A stringent test to address the above questions would be to ask whether the R3 and C5 fragments might be able to induce

Figure 6 Induction of ectopic DSBs by R3 and C5 in chromosome III. (A) Diagram of the *ura4* gene and flanking sequences (thick black line) on its own or ligated to AT2, R3 and C5 fragments inserted between the *SPCC330.19c* and *SPCC330.03c* genes in chromosome III. Black boxes in R3 and C5 indicate the position of NDRs. (B) Two-dimensional gel electrophoresis analysis during meiosis after the insertion of the *ura4*, AT2, R3 and C5 fragments. (C) Analysis of DSBs in the acceptor region of chromosome III before (IGR/Chr III) or after insertion of the *ura4* marker (*ura4*/Chr III), the AT2 ORI (AT2/Chr III), the R3 (R3/Chr III) and the C5 (C5/Chr III) fragments. The DSB regions are indicated with brackets in (A, C).

DSBs after their integration into a region lacking NDRs and meiotic DSBs in wild-type cells. We selected as a recipient locus having these properties the 1.5-kb region between the *SPCC330.19c* and *SPCC330.03c* genes in chromosome III (Figure 6A and C; IGR/Chr III). Analysis during meiosis in the resulting strains showed that insertion of R3 and C5 induced strong DSBs that followed the usual pattern of appearance and repair (Figure 6C; R3/Chr III and C5/Chr III). In addition, the size of the fragments generated indicated that the DSB region mapped to the same position relative to R3 and C5 as when they were inserted into the *mbs1* locus (brackets in Figure 4B). This was particularly revealing in the case of R3, where DSBs extended approximately 400 nucleotides beyond its boundary into the adjacent region as when integrated in the *mbs1* locus, despite the different sequence and chromosome context (R3 and C5 were not linked to the *ura4* maker in the *mbs1* locus). The size of the DSBs regions generated by R3 and C5 in chromosome III are shown at higher magnification in Supplementary Figure S6.

As a negative control for these experiments, the integration in the same site of chromosome III of an 1.7-kb fragment carrying the *ura4* gene did not generate any DSBs (Figure 6A and C; *ura4*/Chr III). We also tested a 0.4-kb fragment (AT2) with an 83% A + T content that is active as an ORI during the mitotic cell cycle when integrated in this site (Cotobal *et al*, 2010). Figure 6B shows that AT2 is also active during the meiotic S-phase; however, this fragment was unable to generate DSBs (Figure 6C; AT2/Chr III), in keeping with the previous finding that the AT1034 ORI could not generate DSBs by itself when the DSB region of *mbs1* was deleted (Figure 3C). None of the *ura4*, R3 or C5 fragments showed any ORI after integration into this locus (Figure 6B).

Figure 7 DSBs at ectopic positions colocalize with sites of hypersensitivity to MNase. (A) Diagram of R3 and C5 fragments at their endogenous and ectopic loci in chromosome III. Vertical bars indicate sites for digestion with restriction enzymes after treatment with MNase. Blue bars represent the end-terminal hybridization probes. White arrows represent flanking genes. (B) MNase analysis of chromatin and naked DNA. Increasing amounts of the enzyme used in different lines are indicated with triangles (see Materials and methods). Brackets in (A, B) indicate that regions of hypersensitivity to MNase of R3 and C5 are in the same position in their endogenous and ectopic loci.

The coincidence in size and relative position of the DSB regions generated by R3 and C5 after insertion in *mbs1* and in chromosome III (Figures 4B and 6A) suggested that some locally encoded feature in the fragments would be responsible for the generation of the DSBs. Given the overlap between the DSB regions and the sequences where the NDRs of R3 and C5 were present in their endogenous loci (Figure 4A), we asked whether these would have been maintained in their new position. To elucidate this, we digested chromatin with MNase, followed by Southern hybridization, to visualize appropriate restriction fragments using locus-specific terminal probes. The control analyses showed that, as expected, the MNase-hypersensitive regions on the endogenous R3 and C5 fragments (Figure 7A and B) colocalized with the NDRs detected in our genome-wide nucleosome analysis (Figure 4A). The parallel analysis of R3 and C5 after integration in their ectopic location showed that, indeed, the MNase-hypersensitive sites had been maintained exactly at the same position as in the endogenous loci (brackets in Figure 7). These results indicated that the NDRs at R3 and C5, although not competent for generating DSBs in their endogenous loci, did have the potential to induce them from an ectopic position. Why was the new context permissive for the generation of DSBs? Genes flanking the endogenous *mbs1*, R3 and C5 loci and those flanking the region of insertion in chromosome III are expressed at similar levels during meiosis (see transcription data in the genomic browser), suggesting that the induction of DSBs was unlikely to be due to differences in the rate of transcription of adjacent genes. This is consistent with previous work carried out in *S. cerevisiae* and *S. pombe* showing that there is no correlation between the recombination activity and the level of transcription (Kon *et al*, 1997; Cromie *et al*, 2007; Kniewel and Keeney, 2009). It is possible, however, that the binding of specific transcription factors not within reach in their endogenous loci or that

differences in the epigenetic landscape could facilitate the formation of DSBs. The fact that the R3 and C5 IGRs are flanked by larger IGRs in their ectopic location could also contribute to the generation of meiotic DSBs (see Discussion). In any case, the fact that the two ectopically generated DSBs were identical in size and position relative to their NDRs (Figures 4 and 6) suggests that the structure and localization of NDRs plays a determinant role in the structure of the DSB regions.

Modifications in the transcriptional pattern during meiosis determine different DSB profiles

The above results underscored the relevance of NDRs as a prerequisite for the specification of meiotic DSBs and opened the possibility that changes in their distribution induced by differential transcription during meiosis could be paralleled by changes in the genomic profile of DSBs. We tested this hypothesis by two independent approaches whose rationale was to modify the expression of a few genes without disturbing the general dynamics of meiosis. First, we induced parallel meiosis in minimal medium in the presence or absence of thiamine and the analysis of gene expression by genomic microarrays showed that only 15 out of the 5006 annotated *S. pombe* genes (excluding those in heterochromatic regions) were down-regulated by a factor above two-fold (log2) in the presence of thiamine (Supplementary Table S6). Consistent with this, the dynamics of meiosis and the efficiency of spore formation were undistinguishable with or without thiamine as monitored by FACS and microscopy analysis. Two of the genes more severely down-regulated were *nmt1* and *thi2*, as has been previously reported for mitotic cells (Maundrell, 1990; Manetti *et al*, 1994). Genome-wide nucleosome analysis of cells at 3 h into meiosis revealed the appearance of prominent NDRs in the promoter regions of these two genes when they were active in the absence of

Figure 8 Parallel induction of NDRs and DSBs by differential transcription during meiosis. **(A)** Nucleosome profile (red) along the regions encompassing the *nmt1* and *thi2* genes in the presence (+T) or absence (-T) of thiamine during meiosis. The transcriptional analysis using genomic microarrays is represented by vertical green lines, each corresponding to a probe in the microarray. Only the profile of the DNA strand corresponding to genes transcribed to the right of the diagram is shown. Data for both strands are available in the browser in our web site. Brackets indicate the regions where DSBs map, as indicated by their size in the panels on the right (black arrows). A map including the restriction sites and the position of the hybridization probes is shown in Supplementary Figure S7. **(B)** Nucleosome profile along the regions encompassing the *caf5* and *SPBC409.13* genes in meiosis in the absence (-Ox) or presence (+Ox) of mild oxidative stress. Only the large NDR associated with the overexpression of the *caf5* gene colocalizes with a newly induced DSB region (indicated with a bracket).

thiamine (Figure 8A). Southern analysis at different times during meiosis detected intense DSB regions that precisely overlapped the NDRs (indicated with brackets). By contrast, the down-regulation of both genes in the presence of thiamine generated neither NDRs nor DSBs, suggesting a strict functional dependence between them.

To support and extend these results through an independent approach, we subjected cells at 2 h into meiosis to mild oxidative stress under conditions that have been reported not to affect viability during mitosis (Chen *et al*, 2008). Global transcription analysis 30 min later revealed that under these conditions 338 genes were differentially expressed by a factor

above two-fold (203 genes and 135 genes up- or down-regulated, respectively) (Supplementary Table S7). As in the thiamine experiment, the dynamics of meiosis and the formation of spores were identical to the non-stressed control meiosis. Figure 8B shows that the overexpression of the *caf5* and the *SPBC409.13* genes under oxidative stress was accompanied by the induction of NDRs in their promoter regions. In the case of the *caf5* gene, a DSB region was generated that overlapped the newly induced NDR and followed the same profile of appearance and repair during meiosis as all the others shown in this work. By contrast, no DSBs were induced on the NDR of the *SPBC409.13* gene, in agreement

with the fact that not all NDRs, either constitutive or meiosis specific, were associated with DSBs. The size of the DSBs regions generated and their position relative to the *nmf1*, *thi2* and *caf5* genes are shown at higher magnification in Supplementary Figure S7. Together, these results reinforced the link between NDRs and DSBs and suggested that the genomic profile of DSBs, and hence the recombination landscape, varies, depending on the distribution of NDRs under different transcriptional regimes.

Discussion

Chromatin organization of ORIs in *S. pombe*

Our results show that ORIs and DSBs colocalize to large IGRs in *S. pombe*, with a probability significantly higher than would be predicted if they were independently distributed along all IGRs in the genome. However, dissection analyses of the *mbs1* locus showed that recombination and replication initiation are not dependent on each other, even in IGRs where a DSB region and an active ORI lie immediately adjacent (Figure 3). This functional independence suggests that the biased colocalization of ORIs and DSBs could be due to a preference for different features of large IGRs.

In *S. pombe*, ORIs do not depend on specific sequences but on the A+T content (Cotobal *et al*, 2010). In many cases, especially ORIs that map in large IGRs, they are made up of a variable number of independent A+T-rich modules that can recruit ORC through the Orc4 subunit whose 9 AT-hook domains are sufficient to target ORC to A+T-rich sequences (Chuang and Kelly, 1999; Lee *et al*, 2001). The simplest possibility to explain the biased localization of ORIs is that, given that the average A+T content of IGRs is 70%, large IGRs will have a higher probability of including favourable combinations of A+T-rich elements capable of recruiting ORC. This possibility is supported by the fact that we have found that Orc1 and Orc4 colocalize in 91 out of the 175 IGRs larger than 3 kb in the genome, consistent with our results in Figure 1.

In *S. cerevisiae*, ORI specification requires the presence of a T-rich ARS Consensus Sequence that, together with downstream A-rich elements, generates an NDR that facilitates the recruitment of ORC, which upon binding imposes a bidirectional pattern of positioning to the flanking nucleosomes (Eaton *et al*, 2010). By contrast, NDRs in *S. pombe* are not particularly enriched in poly (dA–dT) elements (Lantermann *et al*, 2010). These authors have reported that efficient ORIs were significantly devoid of nucleosomes, although to a lesser extent than NDRs at gene promoters. By contrast, our results show that the great majority (86%) of the Orc1/Orc4 binding sites do not overlap with NDRs, although NDRs and ORIs share many IGRs and often lie in close proximity to each other (Figures 2 and 3; Supplementary Figure S4). A possible explanation for this discrepancy is that we have mapped sites of Orc1/Orc4 binding using high-density tiling microarrays, which contain probes of 25 nucleotides with an average overlap of 5 nucleotides while Lantermann *et al* used a microarray-based map with an average density of one probe/kb (Heichinger *et al*, 2006). It is possible that the proximity between NDRs and ORC at many IGRs could have been interpreted as evidence for overlapping owing to the limited resolution of their origin map and to the large size of the genome windows used in the analysis.

Further evidence for this poor overlap is that the average A+T composition of the 2973 NDRs in the genome (Table I) is 63.4%, which is well below the 70% average A+T composition of all IGRs, and much lower than the A+T content required to specify ORIs in the genome (Segurado *et al*, 2003; Heichinger *et al*, 2006; Hayashi *et al*, 2007; Cotobal *et al*, 2010). It is conceivable that the unique structure of the Orc4 protein could compensate for the need for NDRs like those described in this work to facilitate access to DNA. These domains are absent in the Orc4 subunit of *S. cerevisiae* and the differences in the specification of ORIs between both yeasts raise a note of caution concerning the use of nucleosome positioning models developed in *S. cerevisiae* to predict the organization of ORIs in *S. pombe* (Field *et al*, 2008).

Specification of meiotic DSB sites in *S. pombe*

The localization of 40% of all DSBs in large IGRs is likely to be favoured by the enhanced accessibility to the DNA molecule provided by the frequent clustering of NDRs in them (Figure 2A and B). In 80% of these cases, this organization is present constitutively during mitosis and meiosis and could be a consequence of the potential of the transcription machinery to establish nucleosome arrays that are colinear with transcription in *S. pombe* (Lantermann *et al*, 2010). This implies that nucleosome positioning is asymmetric relative to the transcription initiation sites, and the presence of multiple NDRs at large IGRs could result from local sequence preferences to place or exclude nucleosomes in the absence of the strict positioning discipline imposed by transcription.

The finding that DSBs colocalized almost invariably (95%) with NDRs provides the first structural feature shared by the overwhelming majority of DSBs in *S. pombe*. This genome-wide scenario is consistent with previous descriptions of the colocalization of specific DSBs with sites of open chromatin in *S. pombe*, *S. cerevisiae* and *mouse*, as described in the Introduction, and with the very recent report that NDRs are also a general feature of recombination hotspots in *S. cerevisiae* (Pan *et al*, 2011). The distribution of distances between Rec12-binding sites fits well with an exponential function ($R^2 = 0.948$) (Supplementary Figure S8), which is consistent with the absence of any significant bias or clustering of DSBs along the genome as noted in the original genome-wide mapping of Rec12 by Cromie *et al* (2007) and Hyppa *et al* (2008). A similar distribution has been reported for active ORIs using single DNA molecule analyses (Patel *et al*, 2006).

In *S. cerevisiae*, α and β recombination hotspots represent two different categories, not mutually exclusive, whose activity depends on specific transcription factors or on sequences that exclude nucleosomes, respectively (White *et al*, 1993; Kirkpatrick *et al*, 1999; Petes, 2001). The first α -type hotspot characterized in *S. cerevisiae* mapped upstream from the *HIS4* gene and its activity required the binding of the Bas1, Bas2 and Rap1 transcription factors (White *et al*, 1993). The paradigm of the α -type hotspot in *S. pombe* is M26, which relies on the binding of the Atf1–Pcr1 heterodimer to a heptameric sequence in which single point mutations abolish binding, NDR formation, and the generation of DSBs (Schuchert *et al*, 1991; Wahls and Smith 1994; Kon *et al*, 1997; Steiner *et al*, 2002). Most DSBs in *S. pombe*, however, do not colocalize with the M26 consensus sequence, suggesting that other α -type hotspots could be associated with different transcription factors. This possibility is supported

by the recent identification of sequences capable of generating recombination hotspots, some of which depend on the presence of specific transcription factors (Steiner *et al*, 2009, 2011). The generation of NDRs and DSBs at the *nmt1*, *thi2* and *caf1* promoter regions associated with the expression of the genes (Figure 8) is likely to depend on the binding of factors in response to thiamine depletion and oxidative stress. This opportunistic specification of DSBs also probably applies to the 17% of all DSBs that colocalize with meiosis-specific NDRs (Table I).

The use of sequence motifs as predictors of DSBs in *S. pombe* (Steiner and Smith, 2005), *S. cerevisiae* (Mieczkowski *et al*, 2006) and humans (Myers *et al*, 2008) has usually generated a significant fraction of false positives, indicating that additional chromatin or epigenetic elements are also needed. Our results and those of Pan *et al* (2011) in *S. cerevisiae* suggest that NDRs are an obligatory requirement in the specification of DSBs, such that the incorporation of this feature could improve sequence-based predictions. For example, we have found that all 13 sites harbouring DSBs out of the 15 predicted and tested by Steiner and Smith (2005) on the basis on their proximity to the M26/CRE sequence overlapped with NDRs, while the remaining two DSB-negative sites mapped 5 and 1.6 kb away from the nearest NDR (their localization can be visualized in the genome browser in our web site).

Despite their relevance, however, NDRs alone are insufficient to guarantee the generation of DSBs, as illustrated by the fact that many of the NDRs available in meiotic chromosomes do not colocalize with meiotic DSBs in *S. pombe*. The recent finding that different NDRs depend on the binding of different proteins and epigenetic modifications in *S. pombe* (Garcia *et al*, 2010) and in *S. cerevisiae* (Bai *et al*, 2011) suggests that only some combinations of them would make a NDR competent for recruiting the recombination machinery.

The second class of recombination hotspots, called β , in *S. cerevisiae* were initially described from their association with sequences that intrinsically exclude nucleosomes (Kirkpatrick *et al*, 1999; Petes, 2001). In *S. pombe*, IGRs larger than 3 kb represent only 3.5% of all IGRs in the genome but they harbour almost half of all meiotic DSBs (Table I; Supplementary Figure S2; Cromie *et al*, 2007). It is unlikely that this strong bias would be due to a correct combination of transcription factors and epigenetic modifications in the promoters of the flanking genes while the remaining 59% of DSBs are distributed among the remaining 96.5% IGRs in the genome. Instead, we propose that the combined contribution of closely spaced NDRs at large IGRs would facilitate the access to DNA of the recombination machinery during meiosis and, from this point of view, these regions could be functionally equivalent to β hotspots in *S. cerevisiae*. An example of synergism between NDRs is provided by the M26 hotspot, whose associated NDR lies only 250 bp away from a constitutive NDR 300 bp wide upstream from the *ade6* and *bub1* divergent ORFs (Mizuno *et al*, 1997; Supplementary Figure S1). Rec12 binding overlaps this NDR (Cromie *et al*, 2007; Hyppa *et al*, 2008) and the deletion of 510 bp that included this region abolished M26 activity (Zahn-Zabal *et al*, 1995), suggesting a synergistic effect between both NDRs. The possibility of cooperation between NDRs in the specification of DSBs is supported by the

observation that among the 155 IGRs >3 kb that harbour NDRs, those that colocalize with DSBs (Table I) have 3.0 NDRs on average, while those that do not have 2.19.

In sum, our results indicate that the specification of ORI and DSB regions in *S. pombe* depends on intrinsic properties of its genome such as the elevated A + T content of the IGRs and the accessibility to DNA provided by NDRs, respectively. This organization ensures the provision of a large excess of sites competent for initiating both processes that results in a small frequency of usage of individual ORIs and recombination hotspots in each round of replication and recombination, as revealed by Southern hybridization analyses. Such an excess makes replication and recombination very robust processes able to adapt to different physiological conditions.

Materials and methods

S. pombe strains, growth conditions and meiosis synchronization

Diploid strains were generated by protoplast fusion of the haploid *S. pombe* *pat1-114* ts mutant (Nurse, 1985). Synchronous meiosis of diploid *S. pombe* *pat1-114* cells was induced as described by Cervantes *et al* (2000). The genotype of strains harbouring deletions and insertions across the *mbs1* locus and the IGR in chromosome III between the *SPCC330.19c* and *SPCC330.03c* genes are described in Supplementary Table S1. For the experiments shown in Figure 8A, two identical asynchronous cultures of *pat1-114* diploid cells were grown in minimal medium at 25 °C with or without 5 µg/ml of thiamine for 24 h and were then arrested in G1 by transference to minimal medium without nitrogen for 14 h, maintaining the presence of thiamine in one of them. Synchronous entry into meiosis was induced by shifting the temperature to 34 °C in order to inactivate the Pat1 kinase. Cells from both cultures were collected 3 h after the onset of meiosis to isolate total RNA (for microarray analysis of transcription), mononucleosomal DNA (for genome-wide nucleosome profiling), and total DNA (for DSB analysis). For the oxidative stress experiment in Figure 8B, meiosis was induced under standard conditions, as described in Figure 8A (without thiamine). Hydrogen peroxide at a final concentration of 0.5 mM was added 2 h after the induction of meiosis, and the cells were collected 30 min later to prepare RNA, mononucleosomal DNA and total DNA as in Figure 8A. A sample from cells at 3 h into meiosis without thiamine and without hydrogen peroxide was used as a control.

Two-dimensional electrophoresis and analysis of DSBs

DNA isolation and two-dimensional electrophoresis conditions were as described by Segurado *et al* (2002). DSBs were analysed by Southern hybridization as described by Young *et al* (2002), with the following modifications: samples of 10⁸ cells collected at the indicated times during synchronous meiosis were washed in cold 50 mM EDTA (pH 8.0) and converted to spheroplasts. The washed cells were suspended in 2 ml of Zymo Buffer, pH 5.6 (5 mM EDTA, 40 mM Na₂HPO₄, 20 mM citric acid, 0.9 M sorbitol). Zymolyase (0.5 mg/ml) was added to each sample and the samples were incubated for 30 min at 37 °C. Spheroplasts were collected, mixed with 1% low-melting-point agarose in Zymo Buffer, and the mixture was poured into 0.1 ml plug-molds. After 15 min at 4 °C, the plugs were ejected into 1 ml of NDS/PK buffer (50 mM EDTA, 10 mM Tris-HCl pH 9.5, 1% sarcosyl and 0.5 mg/ml Proteinase K). After incubation overnight at 50 °C, the NDS/PK buffer was replaced with 1 ml of TE (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) with 1 mM PMSF and the agarose plugs were incubated for 1 h at room temperature and then washed 2–3 times with 50 mM EDTA. The plugs were then stored in 50 mM EDTA at 4 °C until digestion with the appropriate restriction enzymes. Digested DNA was electrophoresed in agarose gels, followed by Southern blot hybridization.

The restriction sites and the sizes of the fragments analysed in each case are described in Supplementary Table S2. The sequences and positions of all hybridization probes are available upon request.

Chromatin immunoprecipitation

ChIP was performed as described in Cotobal *et al* (2010). Orc4 was immunoprecipitated using 5 µg of polyclonal α -Orc4 raised against the last 14 carboxy-terminal amino acids of the protein. Orc1-HA was immunoprecipitated with 10 µg of α -HA antibody (12CA5, Roche). Ten microliters of each immunoprecipitate and control DNA from whole-cell extract (WCE) were amplified four times with Sequenase (70775Y, USB) and primer A (GTTTCCCAGTCACG GTC(N)₉), followed by 20 rounds of amplification with Taq polymerase (Biotools) in the presence of 8 mM dUTP and primer B (GTTTCCCAGTCACGGTC). Amplified DNA was fragmented and labelled with the GeneChip WT Double-Stranded DNA Terminal Labeling Kit (900812, Affymetrix). Affymetrix GeneChip *S. pombe* 1.0FR tiling microarrays were hybridized according to the manufacturer's instructions. Labelled WCE and ChIP DNA were hybridized to independent microarrays and the log₂ ratio of the ChIP DNA signal relative to WCE was represented. Microarray signals were smoothed by averaging the value of 10 contiguous probes (bandwidth 250). The Orc4 and Orc1 chip signals shown are average of two independent experiments.

Preparation of mononucleosomal DNA

Mononucleosomal DNA for microarray hybridization from *S. pombe* 972 *h*- and diploid *pat1-114* cells was isolated as described by Lantermann *et al* (2009), except that we used MNase (Fermentas #ENO181) to a final concentration of 180 units/ml for 40 min at 37 °C. In all cases, 40 mg of Zymolyase 20T was added to the cell suspension in 10 ml of sorbitol-Tris buffer, except in cells treated with H₂O₂, where we used 20 mg of Zymolyase. Affymetrix GeneChip *S. pombe* 1.0FR tiling microarrays were hybridized with mononucleosomal DNA following the instructions of the Affymetrix GeneChip whole transcript double-stranded target-labelling assay manual. The quality of the mononucleosomal DNA used for microarray hybridization is shown in Supplementary Figure S5B. We defined NDRs as those longer than 150 nucleotides with a log₂ ratio below -1 relative to naked genomic DNA digested with DNase I to an average size of 50 nucleotides, as described by Lantermann *et al* (2009).

Microarray hybridization, data access and genome browser

Raw microarray signals without smoothing or denoising are shown in Figures 2A-C, 3A, 4A, 8A and B. Affymetrix GeneChip *S. pombe* 1.0FR tiling microarrays were also used for transcriptional analyses. Probes hybridizing to more than one position in the genome were excluded from our analysis, which left out most of the subtelomeric and centromeric regions and the rDNA locus. Target labelling preserving the original polarity of RNAs was performed following the instructions of the GeneChip whole transcript sense target-labelling assay manual from Affymetrix. Quantitative measurement of strand-specific differential transcription was performed as described by Quintales *et al* (2010). Data for Rec12-binding sites for diploid *S. pombe* *pat1-114 rad50+* cells were taken from Hyppa *et al* (2008). We have used the *S. pombe* genome version of 23/08/07 and the annotation of 24/02/11 in (http://www.sanger.ac.uk/Projects/S_pombe/). DNA microarray data are deposited in the Gene Expression Omnibus database under accession number GSE28879. Genomic data described in the manuscript can be accessed from a searchable genome browser at (http://genomics.usal.es/cgi-bin/gb2/gbrowse/sp_nrm/).

Digestion with MNase and indirect end-labelling analyses

Cells from 400 ml cultures were harvested 3 h after the induction of meiosis by centrifugation at 5300 g for 10 min at 20 °C and washed once with 50 ml water. Cell pellets were resuspended in 20 ml of preincubation solution (Lantermann *et al*, 2009), incubated at 30 °C for 10 min under shaking in a water bath, centrifuged at 3200 g

for 5 min at 4 °C, and resuspended in 10 ml Sorbitol/Tris buffer containing 7 µl of freshly added β -mercaptoethanol and 40 mg of zymolyase 20T. The cell suspension was incubated at 30 °C for 30 min under shaking, centrifuged as above, and the resulting spheroplasts were washed once with 40 ml Sorbitol/Tris buffer without β -mercaptoethanol. Spheroplasts were resuspended to a final volume of 6 ml in NP-buffer and split into six fractions of 1 ml. MNase was added to each aliquot to a final concentrations of 0, 15, 30, 60, 120 and 240 units/ml and the samples were incubated at 37 °C for 10 min, after which digestion was stopped by the addition of 125 µl of 5% SDS/50 mM EDTA, pH 8.0. Twenty microliters of Proteinase K stock solution (20 mg/ml) was added and the samples were incubated overnight at 37 °C. Samples were extracted once with two volumes of phenol/chloroform/isoamylalcohol and once with chloroform/isoamylalcohol. DNA was precipitated after the addition of 1 µl of 20 mg/ml glycogen, a 1/25 volume of 5 M NaCl and 0.7 volumes of isopropanol, and the pellet was washed with 70% ethanol. DNA was resuspended in 250 µl TE buffer, treated with 20 µl of a 10-mg/ml DNase-free RNaseA solution for 1 h at 37 °C, reprecipitated with 10 µl 5 M NaCl and 2.5 volumes of ethanol, and resuspended in 50 µl TE buffer. In all, 7 µl of this solution was digested with *Hind*III, electrophoresed, blotted and hybridized to specific end-terminal probes. A typical profile of chromatin digestion with increasing amounts of MNase is shown in Supplementary Figure S5A.

χ^2 Test for association

χ^2 Test for association with one degree of freedom using Yates's correction were used. The analysis of the expected independent distribution was restricted to the IGRs without taking their size into account. The contingency table for the association between Rec12 sites and NDRs considered that 231 Rec12 sites (out of 243) colocalized with the same number of IGRs harbouring NDRs (out of 2973). The χ^2 value was 131.32, which corresponded to a *P*-value <0.001. In the case of the association between Rec12 and Orc1/Orc4 sites, we considered that 90 Rec12 sites (out of 243) colocalized with the same number of IGRs harbouring Orc1/Orc4 sites (out of 356). The χ^2 value was 339.19, which corresponded to a *P*-value <0.001.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Author contributions: EC, IS, LM and RS designed the experiments and carried out the experimental work. LQ performed the bioinformatic and computational analyses and constructed the genome browser. FA designed the experiments, supervised the general strategy of the work and wrote the article. All authors analysed the data, discussed the results and approved the final version of the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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