High-resolution profiling of γ H2AX around DNA double strand breaks in the mammalian genome

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Chromatin acts as a key regulator of DNA-related processes such as DNA damage repair. Although ChIP-chip is a powerful technique to provide high-resolution maps of protein–genome interactions, its use to study DNA double strand break (DSB) repair has been hindered by the limitations of the available damage induction methods. We have developed a human cell line that permits induction of multiple DSBs randomly distributed and unambiguously positioned within the genome. Using this system, we have generated the first genome-wide mapping of γ H2AX around DSBs. We found that all DSBs trigger large γ H2AX domains, which spread out from the DSB in a bidirectional, discontinuous and not necessarily symmetrical manner. The distribution of γ H2AX within domains is influenced by gene transcription, as parallel mappings of RNA Polymerase II and strand-specific expression showed that γ H2AX does not propagate on active genes. In addition, we showed that transcription is accurately maintained within γ H2AX domains, indicating that mechanisms may exist to protect gene transcription from γ H2AX spreading and from the chromatin rearrangements induced by DSBs.

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Introduction

Phosphorylation of H2AX (known as γ H2AX), a histone H2A variant that is present in about 10–15% of mammalian nucleosomes, is by far the most studied chromatin modification induced by double strand breaks (DSBs) (for review see Stucki and Jackson, 2006). Although its function in DNA repair is still poorly understood, H2AX-deficient mice are hypersensitive to irradiation and subject to increased genomic instability (Celeste *et al*, 2002). On DSB induction by

irradiation, γ H2AX rapidly forms large foci in cells, which are believed to be important for the accumulation and retention of DSB repair factors (such as 53BP1 or the MRN complex) around DSBs (Celeste *et al*, 2003; Ward *et al*, 2003). γ H2AX foci formation involves the spreading of γ H2AX (or γ H2A in yeast) from the DSB into very large domains of surrounding chromatin (Rogakou *et al*, 1999; Shroff *et al*, 2004; Berkovich *et al*, 2007; Kim *et al*, 2007). How the spreading of γ H2AX from the DSB to the neighbouring chromatin is achieved is not yet known, and a thorough description of its distribution should help to uncover not only the molecular basis of such spreading, but also its consequences on the activity of the surrounding chromatin. High-resolution chromatin immunoprecipitation (ChIP) studies of γ H2A or γ H2AX distribution, respectively, conducted in yeast (Shroff *et al*, 2004; Kim *et al*, 2007) and mammals (Berkovich *et al*, 2007; Meier *et al*, 2007; Savic *et al*, 2009), have shown that it does not distribute symmetrically around DSBs nor uniformly on chromatin.

The determinants that control γ H2AX distribution are still unknown, mainly because of the intrinsic limitations of available DSB induction methods. Genotoxic drugs and radiation, often used to generate DSBs, induce random breaks throughout the genome (thus heterogeneously across the cell population), which are inappropriate for subsequent ChIP analyses. A few systems have been developed to induce and study localized DSBs in yeast and mammalian cells (Kramer *et al*, 1994; Rouet *et al*, 1994; Wolner *et al*, 2003; Berkovich *et al*, 2007; Savic *et al*, 2009). However, none of them permits precise analysis of γ H2AX spreading around many breaks located in various chromatin contexts. On one hand, the I-SceI-based (Rouet *et al*, 1994) and the HO-mediated (Kramer *et al*, 1994) DSB-inducible systems, as well as the VDJ locus (Savic *et al*, 2009), lead to only one localized DSB, generating insufficient γ H2AX coverage to perform statistical analyses. On the other hand, the I-PpoI-based DSB-inducible system (Berkovich *et al*, 2007) generates several endogenous sequence-specific DSBs. Unfortunately, these breaks are mainly located in ribosomal genes, which reside within unassembled regions of the human genome because of their repetitive sequence, and are not suitable for ChIP-chip analyses. Therefore, we have developed a mammalian system that generates several sequence-specific DSBs, which are randomly distributed and properly annotated across the genome. Using this cell line, we provide here the first high-resolution map of DSB-induced γ H2AX around multiple breaks in the human genome, and have identified gene transcription as one of the major determinants that control γ H2AX distribution.

Results

γ H2AX profiling around DSBs induced by AsiSI-ER

As epigenetic modifications, such as γ H2AX, may occur over very large domains after damage, we chose to induce DSBs by using the AsiSI restriction enzyme, an 8 bp cutter that on

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average generates fragments longer than 1 Mbp in the human genome. To precisely control the induction of DSBs, we fused *AsiSI* to a modified oestrogen receptor hormone-binding domain, which only binds to 4-hydroxy tamoxifen (4OHT) (Littlewood *et al*, 1995; Agger *et al*, 2005), and raised a clonal U2OS cell line that stably expresses this *AsiSI*-ER fusion protein (*AsiSI*-ER-U2OS cells). Treatment with 4OHT induced nuclear localization of *AsiSI*-ER and generated DSBs, as evidenced by γ H2AX staining (Figure 1A). Moreover, 4OHT-induced γ H2AX was detected by ChIP-Q-PCR using primer targeting regions proximal (3.7 kb), but not distal (2 Mb), to an *AsiSI* site (Figure 1B). 4OHT treatment did not trigger γ H2AX induction in parental U2OS cell lines (as detected by immunofluorescence and ChIP, data not shown). Thus, the *AsiSI*-ER-U2OS cell line constitutes a proper model to analyse chromatin modifications and the recruitment of DNA repair complexes around DSBs at a molecular level.

H2AX is believed to be incorporated in about 10% of nucleosomes. However, the precise distribution of H2AX across the human genome has still not been analysed. We thus also performed H2AX ChIP experiment to take into account H2AX occupancy within nucleosomes. We could

see a clear enrichment in H2AX in comparison to mock ChIP at all genomic loci analysed by Q-PCR (Supplementary Figure S1A), indicating that H2AX ChIP functions properly.

To study γ H2AX spreading properties at a high resolution around DNA DSBs, we hybridized γ H2AX and H2AX ChIPs (as well as input samples), obtained before and after 4OHT treatment, to the Affymetrix Human Tiling Array 2.0R A that covers chromosomes 1 and 6 with an average resolution of 35 bp. Together, these two chromosomes possess 146 sequenced *AsiSI* sites, a sufficient number of DSBs to evaluate damage-induced chromatin modifications within various genomic contexts.

Loading of H2AX into chromatin was not completely random, as H2AX accumulated at gene-rich regions and was depleted near centromeres (Supplementary Figure S1B and C). In contrast, the mock ChIP showed a background pattern. The non-random incorporation of H2AX in mammalian chromatin has been suggested by both immunofluorescence and ChIP (Bewersdorf *et al*, 2006; Savic *et al*, 2009). We therefore chose to analyse the γ H2AX ChIP data after normalization against the H2AX data to take into account H2AX occupancy within nucleosomes.

γ H2AX distribution across chromosomes 1 and 6 showed broad regions that were highly enriched in γ H2AX primarily in the samples treated with 4OHT (Figure 2A). Two successive enlargements of normalized data obtained around a single *AsiSI* site are presented in Figure 2B. Two independent ChIP-chip experiments were averaged and these values were analysed using an algorithm (see Materials and methods) to demarcate γ H2AX-enriched regions. We detected 28 enriched domains in 4OHT-treated samples compared with only 4 in the untreated controls (Supplementary Table S1).

As already reported in proliferating fibroblasts (Meier *et al*, 2007), regardless of DNA damage, γ H2AX was enriched at telomeres 1p, 6q and, to a lesser extent, 6p, but not at telomere 1q (Supplementary Table S1; Figure 3). All other regions that showed 4OHT-dependent γ H2AX enrichment were either encompassed or were located in close proximity to annotated *AsiSI* sites. These γ H2AX-enriched regions were not detected before 4OHT treatment, with the exception of one domain, presumably generated by leakage of the inducible system (Supplementary Table S1). Therefore, we can conclude that all 4OHT-induced γ H2AX domains are generated by *AsiSI*-mediated DSBs.

All *AsiSI*-induced DSBs trigger phosphorylation of H2AX

Although all identified γ H2AX-enriched domains contained at least one annotated *AsiSI* site, only 25% of the 146 annotated *AsiSI* sites on chromosomes 1 and 6 were enriched in γ H2AX. This could indicate that some *AsiSI* sites are not efficiently cut *in vivo*. Alternatively, this absence of γ H2AX around some *AsiSI* sites could also indicate that DSBs do not always lead to H2AX phosphorylation of the surrounding nucleosomes. Indeed, it has been already proposed that DSBs located in heterochromatin may not be signalled by γ H2AX (Cowell *et al*, 2007).

To determine the cutting efficiency of *AsiSI* throughout the genome, we developed a technique to trap cleaved sites, using a biotinylated double-stranded oligonucleotide harbouring an *AsiSI* cohesive end. This oligonucleotide was ligated to genomic DNA prepared from 4OHT-treated and -untreated *AsiSI*-ER-U2OS cells. Ligated DNA was

Figure 1 4OHT treatment induces sequence-specific DSB induction in the *AsiSI*-ER U2OS cell line. (A) U2OS cells, which stably express *AsiSI*-ER-HA, were co-stained with DAPI (DNA) after incubation with antibodies against the HA tag, and γ H2AX, before and after 4OHT treatment (4 h). (B) ChIP analysis was performed in *AsiSI*-ER-U2OS cells, before and after a 4 h 4OHT treatment, using an anti γ H2AX antibody (Upstate 07-164) or no antibody (mock), as indicated. γ H2AX enrichment was assessed by real-time Q-PCR amplification using proximal and distal primers located 3.7 kb and 2 Mb away from the *AsiSI* site on chr22 at position 19180307. The mean and standard deviation for four independent experiments are shown.

Figure 2 γ H2AX mapping across two human chromosomes, before and after DSBs induction. (A) γ H2AX (Upstate) and H2AX ChIPs were performed on AsiSI-ER-U2OS cells, before and after 4 h of 4OHT treatment and were hybridized to the Human Tiling Array 2.0R A that covers chromosomes 1 and 6. The γ H2AX/H2AX ratio (linear scale) before (blue, left) and after (red, right) DSB induction is presented. The AsiSI sites positions are also indicated. (B) Detailed views of the log₂ γ H2AX/H2AX ratio across a region from chromosome 1 before (blue, left) and after 4OHT treatment (red, right). AsiSI site positions are indicated by arrows. An absence of signal reflects the lack of probes on the tiling array because of repetitive sequences.

digested with *Eco*RI to generate short DNA fragments and pulled down with streptavidin beads. Cleavage efficiency was determined by Q-PCR analysis of pulled down DNA, using primers proximal to a set of seven AsiSI sites. 4OHT treatment induced a clear increase in AsiSI cleavage at the four sites associated with γ H2AX but not at the three sites that were not γ H2AX enriched, nor at three control genomic sequences lacking AsiSI sites (Figure 4A), supporting the conclusion that AsiSI sites devoid of γ H2AX were not efficiently cleaved. To confirm this observation on a larger scale, we hybridized pulled down DNA, alongside input samples, to the Affymetrix Human Tiling Array 2.0R A. As the DNA was initially fragmented using the *Eco*RI restriction enzyme, we were able to attribute a value representing the cleavage efficiency to each of the 146 AsiSI sites by averaging the probe signals located between the pair of flanking *Eco*RI sites for each AsiSI site (illustrated in Figure 4B). In parallel, we associated a γ H2AX value to each AsiSI site by averaging the γ H2AX signal over a 20 kb window. We observed a strong positive correlation ($r=0.71$) between cleavage efficiency and γ H2AX enrichment (Figure 4C). Importantly, we could

not identify a single AsiSI site that was cleaved (high streptavidin/input ratio) but not enriched in γ H2AX. Therefore, all DSBs triggered by AsiSI induce H2AX phosphorylation. The AsiSI sites that do not show γ H2AX enrichment on 4OHT treatment are not efficiently cut, possibly because of the structure of the surrounding chromatin or to DNA methylation (because the AsiSI enzyme is sensitive to CpG methylation). By comparing the AsiSI annotations with the recently published human DNA methylome mapped in two cell lines, H1 and IMR90 (Lister *et al*, 2009), we found that 87% of the AsiSI sites located on chromosomes 1 and 6, which are not encompassed in a γ H2AX domain (not cleaved), are in a methylated form in both H1 and IMR90 cells, whereas on the contrary 67.5% of the γ H2AX-associated AsiSI sites were unmethylated in these two cell lines.

γ H2AX accumulates unevenly and asymmetrically around DSBs

To uncover determinants that control γ H2AX distribution we performed detailed analyses on our γ H2AX profile. In agreement with previous studies conducted in mammals and yeast

Figure 3 γ H2AX is enriched at telomeres. Detailed views of γ H2AX/H2AX enrichment (expressed as a log2 ratio smoothed using a 500 probes sliding window), across the telomeres of chromosomes 1 and 6. ChIP-chip was performed using chromatin from AsiSI-ER-U2OS cells treated (red) or not (blue) with 4OHT for 4 h. A representative experiment (performed with the Upstate γ H2AX antibody) is shown. The black dotted lines indicate the positions of the boundaries detected by our domain finding algorithm (applied on the average of two independent experiments). γ H2AX clearly accumulates on telomeres 1p and 6q and less on telomere 6p. As already reported (Meier *et al*, 2007), telomere 1q does not show γ H2AX enrichment.

(Shroff *et al*, 2004; Berkovich *et al*, 2007; Kim *et al*, 2007), we found that γ H2AX was depleted in the immediate vicinity of AsiSI sites (Supplementary Figure S2) and that it localized over large domains flanking each break (ranging in size from about 0.5 to up to 1.7 Mb, Supplementary Table S2).

To facilitate domains inspection and visualization, we performed a smoothing of γ H2AX data, using a sliding window of 500 probes. Interestingly, though γ H2AX distribution was always bidirectional, it was asymmetrical at several domains, such as chr1_6 and chr6_5 for example (Figure 5; Supplementary Table S2). Moreover, γ H2AX enrichment within domains was highly discontinuous (Figure 5). The discontinuous distribution was also found when γ H2AX ChIP-chip was normalized against input signal (Supplementary Figure S3) instead of unmodified H2AX, indicating that some DNA and/or chromatin feature(s) influence the establishment or maintenance of H2AX phosphorylation independently of its distribution within chromatin. This uneven profile was confirmed in experiments using two different γ H2AX antibodies (Supplementary Figure S4), indicating that the signal reflects actual changes in γ H2AX distribution.

To analyse whether the γ H2AX landscape observed in AsiSI-ER-U2OS cells reflects a general behaviour of γ H2AX, we also generated another stable cell line, AsiSI-ER-T98G, which was synchronized by serum starvation (data not shown), and used in γ H2AX ChIP-chip analyses at G1 and G2. The γ H2AX profiles under these conditions were very similar to those observed with AsiSI-ER-U2OS and showed a comparable number of domains (data not shown) that were both asymmetrical and unevenly distributed (Supplementary Figure S5).

Active genes are refractory to γ H2AX

When taking into account all γ H2AX domains (on chromosomes 1 and 6), γ H2AX spreads in total over more than 20 Mb

of DNA. This coverage permits us to analyse the nature of the DNA/chromatin features that dictate γ H2AX discontinuity within domains. When visualized linearly, each γ H2AX domain consists of a series of 'peaks' and 'holes'. We precisely demarcated the locations of these regions by re-applying our domain detection algorithm with altered parameters. The borders of peaks/holes from AsiSI-ER-U2OS cells were analysed with respect to previously published ChIP-seq datasets conducted in CD4⁺T cells (Barski *et al*, 2007). Data from Barski *et al* were plotted with respect to border positions from our data over a 10 kb window centred at the peak/hole boundary. The peak/hole borders coincided with major changes in chromatin features associated with active promoters such as H3K4me3 (Barski *et al*, 2007) (Figure 6A). As the chromatin landscape mapped by Barski *et al* (2007) was generated in CD4⁺T cells, and not in U2OS cells, we also compared our γ H2AX distribution with genomic annotations, to analyse in an unbiased manner the potential relationship between γ H2AX and gene position. We selected the genes located in γ H2AX-enriched domains and analysed the distribution of γ H2AX around the transcription start site (TSS). We found that the γ H2AX signal strongly declined at gene promoters (as defined by the RNA Polymerase II (Pol II) peak, retrieved from previous ChIP-seq studies; Barski *et al*, 2007) (Figure 6B). The same pattern was observed when normalizing against input, irrespective of the γ H2AX antibody used (Supplementary Figure S6A–C), and with the γ H2AX ChIP-chip data obtained from AsiSI-ER-T98G cells in both G1 and G2 phases (data not shown). To ensure that the behaviour of γ H2AX around the TSS was not because of general nucleosome loss occurring on promoters, we also performed H3 ChIP-chip experiments. Promoters-associated γ H2AX depletion was clearly observed when normalizing γ H2AX against H3 signal (Supplementary Figure S6D). As this decrease also did not correspond to a change in unmodified H2AX

Figure 4 Cleavage analysis of AsiSI sites. (A) Genomic DNA was extracted before and after 4OHT treatment and assayed for cleavage at AsiSI sites as described in ‘Materials and methods’ section. Pulled down DNA was analysed by Q-PCR amplification to assess cleavage of four γ H2AX-enriched and three γ H2AX-unenriched AsiSI sites as well as three control sequences, as indicated. Data correspond to mean and standard deviation from three independent experiments. (B) Pulled down DNA was amplified and hybridized to the Human Tiling Array 2.0R A. Data obtained for one γ H2AX-enriched (left panel) and one non- γ H2AX-enriched AsiSI site are shown. AsiSI and EcoRI sites are indicated. The meaningful signal is expected between the two EcoRI sites surrounding the AsiSI site. (C) The γ H2AX signal (averaged on a 20 kb window surrounding the AsiSI site) of each of the 146 analysed AsiSI sites is plotted against the cleavage efficiency (as determined by the streptavidin/input signal averaged between the two flanking EcoRI sites). Cleavage efficiency and γ H2AX/H2AX ratios were analysed from two independent experiments.

occupancy at promoters (Supplementary Figure S6E) these data suggest that promoters are refractory to either the establishment or maintenance of H2AX phosphorylation.

The fact that promoters are globally devoid of γ H2AX suggests the existence of a link between transcription and γ H2AX spreading. We thus analysed transcriptional features in our AsiSI-ER-U20S cell line before DSB induction, by performing Pol II ChIP-chip experiments. As expected (Barski *et al*, 2007), Pol II was highly enriched at gene

promoters on a genome-wide scale and was also detected within some gene coding regions, reflecting ongoing transcription (Supplementary Figure S7). We next compared Pol II and γ H2AX distribution. We first observed that within domains, areas lacking γ H2AX (‘holes’) often coincided with Pol II peaks (see examples in Figure 6C and a global analysis in Supplementary Figure S8A). This mutual exclusion between γ H2AX and Pol II binding was confirmed when raw data within domains were plotted against each other

Figure 5 γ H2AX distributes asymmetrically and discontinuously around DSBs. Six γ H2AX-enriched domains are shown as examples. The log2 ratios of γ H2AX/H2AX ChIP-chip from two independent experiments (+ 4OHT) were calculated using a sliding window size of 500 probes. The AsiSI sites are indicated by arrows and domains boundaries (as defined by our domain detection algorithm applied to the average of the two experiments) by black lines.

(Supplementary Figure S8B). Finally, to analyse γ H2AX behaviour related to gene transcription, we assigned an expression level and a γ H2AX enrichment value to each gene located within γ H2AX domains, by averaging Pol II binding and γ H2AX over the transcribed region. The genes highly enriched in Pol II harboured low γ H2AX levels (Figure 6D). Normalizing γ H2AX against input (Supplementary Figure S8C) or total nucleosomes density (using H3 ChIP-chip data, Supplementary Figure S8D) did not affect this result, and therefore led us to conclude that transcribed genes located in γ H2AX domains are refractory to γ H2AX establishment and/or maintenance. To confirm these findings, we also assayed transcription by directly measuring RNA levels. cDNA originating from both DNA strands were hybridized on the same tiling arrays, to generate a high-resolution strand-specific expression map (See Materials and methods). As observed earlier with Pol II binding, the genes within γ H2AX domains, which showed high RNA levels, were less enriched in γ H2AX (Supplementary Figure S9A and S9B).

In conclusion, our combined analyses of Pol II binding and RNA levels in AsiSI-ER-U2OS cells indicate that, though γ H2AX spreads over megabases of chromatin surrounding DSBs, the active genes encompassed in these DSB-induced chromatin domains show low γ H2AX accumulation.

While analysing the dataset generated in AsiSI-ER-T98G cells, we could observe that γ H2AX peaks/holes positions within domains were sometimes different compared with those observed in AsiSI-ER-U2OS cells (Figure 7A). To see whether these differences in γ H2AX distribution could be linked to transcriptional changes between these two cell lines, we analysed by RT-Q-PCR the transcription level of some underlying genes in AsiSI-ER-U2OS and AsiSI-ER-T98G. ARV1, SARS and ZFAND3 genes, located in a γ H2AX 'hole' in T98G compared with U2OS, all showed a higher expression level in T98G than in U2OS (Figure 7B). On the contrary, the CELSR2 gene related to a 'hole' in U2OS, but not in T98G, showed a higher expression level in U2OS. Finally, genes showing relatively similar γ H2AX level in both cell lines show similar transcriptional level (C6ORF129 and AMPD2) (Figure 7B).

Figure 6 Transcription antagonizes γ H2AX enrichment. (A) ‘Holes’, or regions depleted in γ H2AX within a γ H2AX domain, were identified using the algorithm detailed in ‘Materials and methods’ section (applied on the average of two 4OHT-treated γ H2AX/H2AX ChIP-chip analyses). In all, 534 hole borders were aligned and overlaid (right and mirror left borders are combined). Profiles are shown for γ H2AX (upper panel) and H3K4me3 (lower panel), over a 10 kb window centred on the border of the holes and averaged using a 500 base window size. ChIP-seq data for H3K4me3 was retrieved from Barski *et al* (2007). Note the major change in H3K4me3 signal positioned exactly at the border of the γ H2AX holes. (B) The γ H2AX/H2AX signal after 4OHT treatment (in red) smoothed using a 200 bp window was plotted relative to the TSS from all 368 genes located within γ H2AX domains. Also shown is the Pol II distribution data (in grey), plotted in the same manner, obtained from Barski *et al* (2007). (C) Detailed views of two γ H2AX domains, with γ H2AX/H2AX signal in red, Pol II/input signal in grey and *Asi*SI sites indicated by arrows (signals are expressed as log_2 ratios and smoothed using a 500 probes sliding window). Grey boxes indicate peaks of Pol II, which are also depleted in γ H2AX. (D) Average log_2 ratio plots of Pol II/input (x axis) versus γ H2AX/H2AX (y axis) averaged across gene loci from the 368 genes encompassed by γ H2AX domains (from the TSS to the end of the gene). Genes that show a high Pol II value also have a low average γ H2AX level.

Altogether these data indicate that transcription is refractory to γ H2AX accumulation and account at least in part for the uneven distribution of γ H2AX within γ H2AX domains.

Transcription in γ H2AX domains is not affected by DSB induction

The fact that γ H2AX does not spread on genes that were active before DSB induction may indicate that transcription is still ongoing after DSB induction despite the wide chromatin remodelling induced around breaks. However, in human cells, transcription, assessed by *in vivo* 5-bromouridine

triphosphate (BrUTP) incorporation, never co-localizes with γ H2AX foci, leading the authors to conclude that DSB-induced remodelled chromatin is not permissive to transcription (Solovjeva *et al*, 2007). In addition, induction of DNA breaks in the nucleolus leads to a transient repression in Pol I transcription in proximity to DNA DSBs (Kruhlak *et al*, 2007). However, in yeast, induction of an HO-induced DSB triggers a transcriptional inhibition of proximal genes within a time frame that correlates with DNA resection, rather than with chromatin remodelling and γ H2A spreading (Kim *et al*, 2007).

Figure 7 Transcriptional changes account for differences observed between U2OS and T98G γ H2AX profiles. **(A)** Detailed views of three γ H2AX domains that showed differences between *AsiSI*-ER-U2OS (red) and *AsiSI*-ER-T98G in G1 phase (orange). Smoothed γ H2AX/input signal of representative experiments performed with the Epitomics γ H2AX antibody is shown. Grey boxes indicate regions with differences in γ H2AX profiles between the two cell lines and gene symbols are indicated below. **(B)** RNA levels for the genes from the domains shown in **(A)** were analysed with respect to P0 by RT-Q-PCR, in *AsiSI*-ER-U2OS and *AsiSI*-ER-T98G (G1 phase). For each gene, the average ratio and standard deviation of cDNA levels in *AsiSI*-ER-T98G-G1 relative to *AsiSI*-ER-U2OS, obtained by four independent experiments, are shown.

To clarify the effect of γ H2AX spreading on proximal gene transcription, we therefore decided to monitor transcriptional changes induced by 4OHT treatment, by a genome-wide survey of both Pol II binding and RNA levels. It is important to note that the efficiency of *AsiSI* site cleavage in our system is high enough to perform this type of analysis (Supplementary Figure S10).

For the few genes directly cut by *AsiSI*, we observed a decrease in Pol II binding after 4OHT treatment (see the arrow of Figure 8A), which validates the fact that our experimental settings allow to detect transcriptional changes. However, the Pol II occupancy across all the other genes located within γ H2AX-enriched domains was unchanged on addition of 4OHT (Figure 8A). When analysing the 368 genes located within γ H2AX domains, the Pol II signal, averaged over transcribed regions, was not significantly modified by DSB induction (Figure 8B). Analyses of the RNA levels for each of these 368 genes, by strand-specific expression mapping, also showed that expression levels were unchanged on DSB induction (Supplementary Figure S11). Taken together, these results indicate that although chromatin surrounding DSBs undergoes wide H2AX phosphorylation, the genes

located within these remodelled domains are still properly transcribed.

Discussion

In this study, using a new DSB-inducible system, we provide the first high-resolution mapping of γ H2AX around multiple DSBs in the mammalian genome. We analysed 23 DSBs, which collectively induced γ H2AX spreading over more than 20 Mb of DNA.

First, our data indicate that H2AX phosphorylation always occurs over large (from 0.5 to 2 Mb) domains surrounding the DSB. Interestingly, we could not detect γ H2AX 4OHT induction at any chromosomal location devoid in *AsiSI* site, suggesting that γ H2AX mainly spreads in *cis* around DSBs. As it has been suggested that several DSBs could cluster together within the nucleus, the multiple DSBs induced by our system may trigger a γ H2AX spreading that does not reflect the γ H2AX distribution that would occur around specific breaks. However, we consider this possibility quite unlikely because, at least, concerning the range of γ H2AX extent, our data are in agreement with previous studies

Figure 8 Transcription in γ H2AX domains is not affected by DSB induction. **(A)** Detailed view of a cleaved *Asi*SI site (bottom arrow) of the γ H2AX/H2AX signal (red), or the Pol II/input signal obtained with 4OHT (black) or without 4OHT (grey), expressed as log2 ratios and smoothed using a 500 probes sliding window. Note that Pol II binding within γ H2AX domains does not change on 4OHT treatment, except at the immediate vicinity of the *Asi*SI restriction site (angled arrows). **(B)** Pol II-binding signals were averaged over each of the 368 genes encompassed in the γ H2AX domains, with (y axis) or without (x axis) 4OHT treatment.

performed around either a single DSB or several I-PpoI-induced DSBs in mammals (Berkovich *et al*, 2007; Savic *et al*, 2009). Moreover, we could not detect any DSBs devoid of γ H2AX. Taken together, these findings suggest that large amounts of γ H2AX containing nucleosomes may be universally required for the proper recruitment of DNA repair complexes and consequently for efficient DSB repair. However, as some of the *Asi*SI sites were not cleaved in our model system, we cannot exclude that DSBs located in heterochromatin (that may be inaccessible to *Asi*SI) do not recruit γ H2AX, and may be signalled by another pathway, as proposed earlier (Cowell *et al*, 2007).

Second, thanks to the statistical power of our genome-wide analysis, we are able to provide major insights into the molecular basis of γ H2AX distribution.

First of all, the asymmetrical distribution of γ H2AX, as well as its sharp decrease at the ends of some domains, indicates the existence of boundaries that block γ H2AX spreading. Importantly, γ H2AX spread to the same extent in two different cell lines, and in G1 versus G2 cell cycle phase, suggesting that boundaries are likely to be defined either by DNA features or by stable chromatin marks. These domain boundaries (listed in Supplementary Table S1) were analysed with respect to various chromatin features (retrieved from publicly

available ChIP-chip and ChIP-seq datasets), and no significant correlation could be found (data not shown). One hypothesis is that chromatin elements involved in chromosome looping, such as matrix attachment regions, participate in defining domains boundaries (see below). However, these elements are, at present, poorly annotated and therefore cannot be used in correlation analyses.

Next, the presence of holes and peaks within the γ H2AX domains suggests that H2AX phosphorylation levels are affected by chromatin structure. Importantly, we have identified gene transcription as one chromatin feature that has a major role in the control of γ H2AX propagation. Indeed, by analysing transcription either by genome-wide Pol II mapping or by strand-specific expression profiling, we found that transcribed units within domains are depleted in γ H2AX. In addition, the variation in γ H2AX distribution observed between two cell lines correlated with difference in gene transcription, at least for the locations analysed in our Q-PCR analyses.

Finally, we found that within γ H2AX domains, transcription is adequately maintained after breaks induction. This finding was rather unexpected as Pol I transcription seems to be transiently inhibited in the vicinity of DSBs induced in the nucleolus (Kruhlak *et al*, 2007). However, this Pol I inhibition was found to be independent of H2AX (Kruhlak *et al*, 2007). This may indicate that DSBs induced in ribosomal DNA trigger a differential response leading to different effects on surrounding transcription.

As transcription is still ongoing within γ H2AX domains, γ H2AX depletion on active genes could either be a cause or a consequence of transcriptional maintenance.

Indeed, the process of transcription itself could interfere with the presence of γ H2AX through multiple mechanisms.

For example, the lack of γ H2AX on transcribed regions may reflect the extensive nucleosome remodelling required by transcription. Alternatively, the high amount of transcription machineries on active genes may restrain the accessibility of the H2AX kinases.

However, one can also imagine that mechanisms exist to prevent spreading of γ H2AX on active genes to protect their transcriptional status. Along this line, the current model of chromosome organization within the nucleus may account for both the observed uneven γ H2AX distribution and the mechanism that ensures proper gene transcription (Dorman *et al*, 2007). Indeed, this model relies on the existence of chromatin loops of various sizes. 'Large' loops represent a high level of chromosome compaction. The boundaries we observe here could be dictated by the chromatin elements specifying large loops. In addition, the uneven propagation of γ H2AX within domains could reflect the 3D organization existing on this domain before the break into smaller loops, defining functional domains. On one hand, some loops would be pulled out from the γ H2AX foci by the action of specific activities (for example, because they belong to transcription factories; Osborne *et al*, 2004; Sutherland and Bickmore, 2009), and therefore would show low γ H2AX enrichment. On the other hand, some loops, which are linearly further away but within the same chromosomal domain, would be anchored in close proximity to the loop where the break occurred and could therefore be included in γ H2AX foci (see our model in Supplementary Figure S12). We can speculate that active mechanisms exist to physically localize expressed

genes outside γ H2AX foci, that is outside chromatin regions extensively remodelled on DSB induction. Interestingly, this hypothesis is also supported by immunofluorescence data showing that γ H2AX does not colocalize with transcription detected by *in vivo* incorporation of 5-bromouridine triphosphate (BrUTP) (Solovjeva *et al*, 2007).

In conclusion, in this study, we describe a new DSB-inducible system that generates a sufficient amount of endogenous, sequence-specific and unambiguously positioned DSBs to analyse DSB-induced chromatin landscape by ChIP-chip. The number of DSBs generated by our system corresponds to an irradiation of 10–15 Gy, which is a dose classically used for DSB repair studies (Rogakou *et al*, 1999; Celeste *et al*, 2003). This new tool offers the unique opportunity to analyse, at a molecular level, DSB-associated mechanisms at several loci at the same time and therefore permits the discovery of new biological aspects that can only be detected by statistical analysis. As an example, we used this new cell line to provide the first high-resolution profiling of a DSB-induced chromatin modification, that is γ H2AX, which, combined to transcription mapping, showed insights into the global orchestration of DSB repair-associated chromatin remodelling with transcription. This cell line represents an important tool for future analyses of chromatin remodelling during DSB repair, as one will be able to explore events that take place under different chromatin contexts, at unprecedented molecular and statistical levels.

Materials and methods

Construction of the pBabe-AsiSI-ER vector

AsiSI genomic DNA, kindly provided by New England Biolabs (NEB), was amplified using the following primer pair (FW: ATAGATCTCATGGGCGAGTCTATTGATCA, REV: CTCGTCGACTCA CAACATCACCTGGTC). *Bgl*II/*Sall* digested PCR fragments were cloned into *Bam*HI/*Sall* sites of the retroviral pBabe vector, in fusion with the HA-tagged ligand-binding domain of the oestrogen receptor (HA-ER) and a consensus NLS (Escafit *et al*, 2007). Cloning was performed into a modified *Escherichia coli* strain (AsiSI-met), also provided by NEB.

Cell culture and transfection

U2OS cells were cultured in Dulbecco's modified Eagle's medium supplemented with antibiotics and 10% FCS (Invitrogen) at 37°C under a humidified atmosphere with 5% CO₂. T98G were cultured in minimum essential media (MEM) GlutaMAX, supplemented with MEM non-essential amino acid, antibiotics and 10% FCS (Invitrogen). The pBABE HA-AsiSI-ER plasmid was transfected using a standard calcium phosphate protocol and selection was performed using 1 µg/ml puromycin. Cells were treated with 300 nM 4OHT for 4 h (where indicated). Synchronization of AsiSI-ER-T98G cells was achieved by 72 h of serum starvation (0% FBS). Cells were collected in G1 and G2 phases after 10 and 28 h, respectively, of 20% FBS re-induction.

Immunofluorescence

Immunofluorescence of U2OS cells were carried out following a standard protocol, using the anti-HA monoclonal antibody HA11 (Covance) and a rabbit anti- γ H2AX monoclonal antibody (Epitomics). All secondary antibodies were purchased from Molecular Probes. Image acquisition was performed with the MetaVue imaging system (Universal Imaging Corp., West Chester, PA).

Chromatin immunoprecipitation

ChIP assays were carried out according to the protocol described in Tyteca *et al* (2006) with the following modifications. In all, 200 µg of chromatin was immunoprecipitated using 2 µg of rabbit polyclonal anti- γ H2AX (Upstate 07-164, Abcam 2893), 2 µl of rabbit monoclonal anti- γ H2AX (Epitomics 2212-1), 2 µg of anti-H2AX antibody

(Upstate 07-627) or without antibody (mock). In all, 50 and 10 µg of chromatin, respectively, were used for Pol II (CTD4H8 Upstate), and H3 (Abcam ab1791) ChIP. After washing, the bead/chromatin complexes were re-suspended in 200 µl of TE buffer (Tris 10 mM pH8, EDTA 0.5 mM pH8) and formaldehyde crosslink was reversed in the presence of 0.5% SDS at 70°C overnight. Immunoprecipitated and input DNA were purified with phenol/chloroform, precipitated and analysed in duplicate by real-time Q-PCR. The sequences of the primers are supplied in Supplementary Table S3. For ChIP-chip, 8 ng of ChIPs and input were amplified, labelled and hybridized to high-density oligonucleotide tiling arrays covering human chromosomes 1 and 6 (Affymetrix Human Tiling 2.0R A), using the standard Affymetrix procedure, by the GeneCore facility at EMBL Heidelberg. Experiments were performed in duplicate. All microarray data are available through Array Express (accession number E-MEXP-1769).

RNA and RT analyses

RNA was extracted using the RNeasy kit (Qiagen) following manufacturer's instructions. In all, 1 µg of RNA was reverse transcribed using Im-Prom^{II} RT (Promega) with random hexamers. cDNAs were analysed by Q-PCR using primers described in Supplementary Table S3 and normalized against P0 cDNA levels.

Strand-specific expression profiling

Strand-specific expression protocol using tiling arrays has been derived from a strand-specific expression protocol using exon arrays (Ge *et al*, 2008). RNA was purified from AsiSI-ER-U2OS cells, treated or not with 4OHT for 4 h, using the MasterPure RNA purification kit (Epicentre Biotechnologies). To determine transcription on both DNA strands (i.e. in both directions), two different cDNA populations were produced from the total RNA. cDNA1 was synthesized from 250 ng of total RNA (without rRNA reduction) according to the 'GeneChip Whole Transcript Sense Target Labeling Assay' (Affymetrix) and is identical to RNA. cDNA2 was synthesized from 12 µg of total RNA, as previously, but starting from the second cycle of cDNA synthesis, thus skipping the first cycle of cDNA synthesis and the *in vitro* transcription amplification step. Therefore, cDNA2 is complementary to RNA. Fragmentation and labelling of cDNAs were performed according to the instructions of Affymetrix in their detailed manual 'GeneChip Whole Transcript Sense Target Labeling Assay' with the exceptions that 7.5 µg of single-stranded DNA was used instead of 5.5 µg and that UDG and APE1 concentrations were accordingly adjusted. The differential cDNAs were hybridized on the GeneChip Human Tiling 2.0R A arrays (Affymetrix), which contain DNA probes from the forward DNA strand, thus allowing plus and minus strand expression analysis. Hybridization and scanning were performed at the Plateforme Biopuces (INSA). Minus and plus strand expression were monitored by the cDNA1 and cDNA2 experiments, respectively. For each gene, sense or antisense expression was analysed from the cDNA1 or cDNA2 array experiments depending on the gene's orientation.

Microarray data analysis

Scanned array data were normalized using TAS (quantile normalization, scale set to 500). For strand-specific expression, signal intensities from cDNA1 and cDNA2, monitoring minus and plus strand expression, respectively, were calculated using a bandwidth of 300. For each gene, sense expression was analysed from the cDNA1 or cDNA2 array experiments depending on gene's orientation. For ChIP-chip analyses, enrichment values (ChIP/input or γ H2AX ChIP/ γ H2AX ChIP) were calculated with a bandwidth of 300. Then, we developed a 'domain detection algorithm', inspired from Guen *et al* (2008). Domains were determined through a two-step process. The first step defined zones of interest as a contiguous section of 800 probes in which 70% of the probes were positive (>0). These zones were merged together using a base-pair window size of 80 000 and then domains were selected as regions that were enriched in positively selected zones and greater than 100 000 bases in size. A score (representing the average of the log₂ of the γ H2AX/H2AX signal over the entire region) was further attributed to these identified regions, and domains with a score below 0.1 were eliminated. The output of this algorithm is presented in Supplementary Table S1. Further inspection of the regions, which were detected after 4OHT treatment, showed that, because of the discontinuous nature of the γ H2AX signal, some of these domains were detected as two separate domains. Therefore, we further

merged some regions to properly annotate boundaries before further analysis. This allowed the clear characterization of 23 AsiSI-induced γ H2AX domains (Supplementary Table S2).

The domain finding algorithm was applied to the values within domains to demarcate sets of internal peaks. Parameters were altered such that peaks were seeded by contiguous sections of 50 probes in which 70% of the values were above the average score of the parent domain. Seeds within 10 000 bases of each other were merged and no minimum size parameter was used to generate the final set of peaks. Feature correlations were performed by plotting various forms of data using the positions of relative peak boundaries. Positive relative positions represent data within the peaks and negative positions are outside. To plot data with respect to TSSs, gene transcript positions and orientations were obtained from the refFlat table from UCSC (hg18). Data were positioned relative to the TSS with positive positions being located downstream of the TSS. Data within all correlation plots were smoothed by plotting a y axis value that represents the average value of a 200 base window surrounding each point. The same procedure was used to plot data relative to the AsiSI sites.

All genomic coordinates are from the March 2006/NCBI36/hg18 genome assembly, and all annotations flat files were retrieved from the UCSC genome browser <http://genome.ucsc.edu> ftp site. Histone methylation and Pol II profiles are from ChIP-seq studies by Barski et al (2007) (<http://dir.nhlbi.nih.gov/papers/lmi/epigenomes/hgtcell.html>).

Cleavage efficiency assay

Single-stranded oligonucleotides (Supplementary Table S3) were hybridized at a final concentration of 20 μ M in 250 mM Tris pH7.7 by incubation at 95°C for 3 min and 70°C for 1 min followed by a cool down to room temperature (over 2 h). Genomic DNA was extracted from 4OHT-treated or -untreated AsiSI-ER-U20S cells using the DNeasy kit (Qiagen); 1 μ g of DNA was ligated to 1 μ l of 20 μ M biotinylated double-stranded oligonucleotides in 20 μ l at 4°C overnight. T4 ligase was heat inactivated at 65°C for 10 min. DNA was then fragmented by EcoRI digestion at 37°C for 2 h followed by heat inactivation at 70°C for 20 min. DNA was then diluted in 1 ml

of RIPA buffer and pre-cleared with agarose A beads (Sigma) at 4°C for 1 h. After centrifugation, digested DNA was pulled down with streptavidin beads (Sigma) at 4°C overnight, and then washed FIVE times in RIPA buffer and twice in TE. Beads were resuspended in 100 μ l of water and digested with HindIII at 37°C for 4 h. This was followed by phenol/chloroform purification and DNA precipitation. DNA was resuspended in 100 μ l of water, and submitted to Q-PCR, or amplified, labelled and hybridized to Affymetrix Human Tiling 2.0R A as described earlier.

Supplementary data

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

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Authors' contributions. JSI performed all bioinformatic analyses of the ChIP-chip data. PC performed ChIP experiments. IL participated in the generation and validation of the AsiSI-ER-U20S cell line. EN performed strand-specific expression maps. LM optimized AsiSI-ER-T98G culture and synchronization, GL and DT conceived, designed, realized and analysed experiments. GL, DT and JSI wrote the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

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