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High resolution profiling of γH2AX around DNA double strand breaks in the mammalian genome.

Jason Iacovoni, Pierre Caron, Imen Lassadi, Estelle Nicolas, Laurent Massip, Didier Trouche and Gaëlle Legube

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 29 November 2009

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by three referees whose comments to the authors are shown below. As you will see while all three referees consider the study as being an interesting one in principle referee 1 (and to some extent referee 2) raise major concerns regarding the conclusiveness of the data and feel that a number of substantial controls would be required before they can support publication of the study here. Given the interest expressed by the referees in principle we would thus be able to consider a revised version of this manuscript in which you need to address the referees' concerns in an adequate manner by substantial further experimentation and to their satisfaction. I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript as well as on the final assessment by the referees.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

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REFEREE COMMENTS

Referee #1 (Remarks to the Author):

In manuscript EMBOJ-2009-72945 entitled "High resolution profiling of γ-H2AX around DNA double strand breaks in the mammalian genome", Iaconovi and colleagues utilize a ChIP-chip approach to generate genome wide mapping of γ-H2AX around DNA double strand breaks (DSBs). For this purpose, they developed a human US02 cell line expressing the AsiSI restriction enzyme fused to a modified oestrogen receptor-binding domain (AsiSI-ER-U2OS cells) such that DSBs can be induced at numerous AsiSI sites throughout the genome upon treatment with 4OHT, which directs the fusion protein into the nuclease. Iaconovi et al. then conducted γ-H2AX and H2AX ChIP-chip experiments and found all DSBs trigger large γ-H2AX domains that spread from the break in a bidirectional, discontinuous, and often asymmetric manner. The authors found evidence that γ-H2AX is absent where polymerase II binds at active genes and that γ-H2AX formation does not affect gene transcription. This study would be of interest to the readership of EMBO, however many issues should be addressed prior to publication.

Major Criticisms:

1. The authors repeatedly provide incomplete or incorrect references. For example, they treat conclusions of yeast and mammalian studies interchangeably despite the differences of these organisms with respect to the identity and distributions of the histone variants that are modified upon DSBs and the kinases that phosphorylate these histones. Another is they refer to the published V(D)J recombination based studies as being conducted in human cells, not murine lymphocytes. There are far too many others to list here. These referencing errors diminish the reader's confidence in the experimental studies.

2. The authors conduct all of their studies in a single AsiSI-ER-U2OS clone, which raises several concerns. Experimental results generated from only a single derivative clone of a cell line must be cautiously interpreted at best. To address this issue, the authors should conduct parallel experiments on more than one derivative cell line. The parental U2OS cells lines could have deficiencies in mechanisms that regulate γ-H2AX dynamics. To account for this concern, the authors should investigate the functional status of the known H2AX kinases and γ-H2AX phosphatases. Confidence in the generality of the findings would be enhanced by conducting similar experiments in at least one other cell line, possibly one of a different tissue to investigate whether the γ-H2AX/polII findings at active genes occur in other contexts. Finally, results in immortalized and/or transformed cells lines should be substantiated by focused experiments in primary cells. For this purpose, the authors might consider approaches to conduct a subset of their experiments in primary human cells expressing AsiSI-ER-U2OS cells.

3. According to the Methods provided, the authors normalize γ-H2AX ChIP-chip signals to H2AX ChIP-chip signals to account for nucleosome occupancy of genomic regions. The authors fail to realize or consider that phosphorylation of H2AX blocks the immunological epitope to which the anti-H2AX antibody binds, while the anti-γ-H2AX antibody does not recognize H2AX. Consequently, their "normalization" actually skews γ-H2AX signals. Therefore, to account for nucleosomal occupancy, the authors must repeat their experiments by normalizing the γ-H2AX ChIP-chip signals to H3 or H4 ChIP-chip signals. In the absence of this essential control, the authors cannot interpret their data in the manner they are currently.

4. Addition of estrogen could have unanticipated genome wide effects upon transcription, which can cause DSBs and, as suggested by this study, alter H2AX distribution. Thus, the authors also need to conduct parallel γ-H2AX and H3/H4 ChIP-chip experiments on estrogen-treated parental/derivative U20S clones. In the absence of this essential control, the authors cannot interpret their data in the manner they are currently.

5. The authors' findings regarding continued transcription through γ-H2AX domains are intriguing and make biological sense, but they also are opposite of what others have published. This could be due to differences in biological systems, as outlined by Iacovoni et al., or experimental approaches. In this study, the authors assay steady-state mRNA levels as a read-out of transcription, rather than

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directly evaluate transcription rates by nuclear run-on or other techniques. Such experiments should be conducted. An additional concern stems from the high probability that both US02 alleles (or multiple alleles if this cell line exhibits aneuploidy) contain AsiSI restriction sites. Perhaps DSBs are induced in only one allelic copy of the AsiSI sites flanking the active genes in most cells assayed here. If so, transcription might appear unaffected due to an intact allelic copy of the active gene. Thus, the authors need to conduct immuno-FISH based experiments that enable them to monitor γ-H2AX formation and transcription from both alleles within single cells to support their conclusion.

Minor Comments:

1. The authors should either conduct experiments in synchronized cells or comment upon how an asynchronous cellular population would impact their findings.

2. The "high resolution" qualifier is arbitrary and distracting. One could argue that the minor changes and small number of DSBs analyzed do not offer high resolution.

3. The authors should remove mention of γ-H2AX spreading from DSBs since they never conduct kinetic analyses of γ-H2AX signals.

4. The authors mention that because two anti-γ-H2AX antibodies gave the same result, the signals could not be due to antibody accessibility. An explanation of this statement is warranted since it is not apparent how chromatin could be accessible to one antibody, but not to another.

5. The authors fail to consider that γ-H2AX formation in chromatin around DSBs may not simply occur along a broken DNA strand, but also within three-dimensional space around DSBs. In this context, the authors should mention the caveat that their system of inducing multiple breaks within the same cell does not unequivocally allow conclusions regarding γ-H2AX dynamics around particular DSBs.

Referee #2 (Remarks to the Author):

Previous studies have demonstrated that induction of DNA double strand breaks in either yeast or mammalian genomes leads to the rapid phosphorylation of the histone variant, H2AX. In yeast, this modification spreads over ~50 kb of chromatin, whereas in mammalian cells phospho-H2AX spreads for megabases of chromatin. Studies in yeast have been driven by the inducible HO endonuclease which creates a single DSB in the yesat genome, whereas mammalian studies have used the IsceI nuclease or random breaks. In this manuscript, the authors describe a very novel method for introducing multiple, unique location DSBs in a mammalian genome. They fused the AsiSI nuclease to a modified estrogen receptor hormone binding domain, and then created stable cell lines that exprsss this derivative. AsiSI is predicted to cut DNA once every 1 Mbp or so, so in these cell lines, addition of the estrogen analog, 4OHT, leads to the formation of a limited numbers of DSBs at defined positions. The authors then use high density tiling arrays from two chromosomes to map the distribution of phospho-H2AX +/- 4OHT treatment. The results are generally very convincing. They find that only ~25% of the AsiSI sites are cleaved on these chromosomes, but phospho-H2AX domains are found at all DSBs. Furthermore, phospho-H2AX domains are discontinuous, and appear to be interupted primarily by actively transcribed genes. Interestingly, transcription of genes that are located within phospho-H2AX domains is not altered.

In general this was a technically sound study that describes a novel methodology and contains data that should be of broad interest. The finding that transcription is not affected by phospho-H2AX is a bit unexpected, but the data are strong. There are a few weak points in the data that do need to be addressed:

1. I am surprised that the authors have not performed some annotation for the AsiSI sites that were not cleaved effiiciently. Do these map to regions known to be enriched for HP1 or DNA methylation? This data should be out there some where.

2. One big point that the authors try to make is the lack of gamma-H2AX at promoters and in active genes. They try to rule out global effects on nucleosome density by using the H2AX antibody as a

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control. The signal for these ChIPs are not very convincing. This data needs to be strengthened by ChIPs with antibodies to histone H4 or H2A/H2B (even better).

3. The authors argue that the transcription process blocks spreading. This could be tested by performing a limited set of assays in the presence of alpha-amanitin? This should not affect DSB induction or gamma-H2AX induction (shouldn't require new gene transcription).

4. Minor point: end of results say that transcription doesn't change much in gamma-H2AX domains which is surprising given all the "chromatin remodeling events". Actually, only get gamma-H2AX, no evidence for actual "remodeling distal from DSB. Gamma-H2AX is not expected to directly alter chromatin structure.

5. minor point: page 12, last paragraph, first sentence. there is no data to "demonstrate" that H2AX-phos is affected by chromatin structure. In fact, it is not clear if it is the process of transcription per se or the chromatin remodeling during transcription that removes (or prevents) H2AX-phos. I think "suggest" would be more appropriate.

6 I am a little worried that normalization to H2AX values might not be the best here, especially since the values for these ChIPs seem so low. The authors have presented some data for phospho-H2AX/Input values, but I would really like to see the IP/IN H2AX chip data from the figures 4 and 5 as well.

Referee #3 (Remarks to the Author):

This is an outstanding manuscript. It describes a very elegant new approach to study the chromatin fate of double-strand breaks (DSBs) at many genomic locations in mammalian cells. I greatly enjoyed reading it. The experimental approaches are innovative and solid, the data are beautiful, the figures are clear and the manuscript was easy to read. I strongly recommend publication in EMBO Journal a.s.a.p.

Minor comments/ suggestions for improvement:

* page 9: the ChIP data from Barski et al were obtained from a different cell type. This should be pointed out in the text, as it is a potential caveat in the analysis.

* page 10, line 16: "... are kept free of gammaH2AX". This is an overstatement. I agree that gammaH2AX levels are reduced, but certainly not reduced to zero, as the sentence suggests.

* page 12, last sentence: "strongly depleted...". Again an overstatement. The changes are roughly 0.1 log2-unit, which is no doubt significant but not "strong".

* Discussion page 13/14 and Fig 7: The "looping" model is too speculative and somewhat out of place, because no looping assays are done in this manuscript. I strongly suggest to leave out Figure 7 and use the space instead to promote one supplementary figure (e.g. S2 or S3) to main figure.

* Methods: please describe the construction of pBABE HA-AsiSI-ER in detail.

* Figure 1A: brightness/contrast should be improved, because the signals are difficult to see.

* Figure 2A: please add axis with units to the "target sites density" traces.

* Figure 2: I wonder whether the gammaH2AX/H2AX data could be better visualized by using a linear rather than a log2 scale. In this case that seems perfectly valid to me, because only enrichments (not depletions) are expected and found.

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1st Revision - authors' response 05 February 2010

Referee #1

Major Criticisms:

1, The authors repeatedly provide .....experimental studies.

We have thoroughly checked the manuscript for incorrect and misleading references. Instances where references interchange yeast and mammalian studies or mislabel a study in mice as being performed in human have been corrected.

2, The authors conduct all of their studies in a single AsiSI-ER-U2OS clone..... subset of their experiments in primary human cells expressing AsiSI-ER-U2OS cells.

We agree with the referee that conducting these studies in a single cell line (derived from the U20S osteosarcoma cell line) limits the generality of our findings. We now also provide γH2AX distribution data in the AsiSI-ER-T98G stable cell line, derived from the T98G glioblastoma tumor cell line. The γH2AX domains induced by 4OHT treatment were very similar, in both size and boundary positions (Figure S5). Few differences were observed on γH2AX distribution within domains. For the genes tested, we could correlate changes in γH2AX profiles with changes in gene expression as assayed by RT-QPCR in both cell lines (Figure 7).

3. According to the Methods provided, the authors normalize γH2AX ChIP-chip signals to H2AX ChIP-chip signals ..... in the manner they are currently.

We performed the requested experiments (ChIP-chip H3) and we found that the choice of normalization method did not change the results. We now show data normalized to H3 in Figure S6D, S8D, S9B) of the revised manuscript.

4. Addition of estrogen could have unanticipated .... in the manner they are currently.

While the addition of 4OHT may indeed both affect transcription and cause DSBs, in our experiments, 4OHT-induced H2AX phosphorylation always occurred in close proximity to annotated AsiSI sites. We feel that this is evidence that the 4OHT-induced γH2AX domains studied here are indeed linked to an AsiSI-mediated cleavage and not to any potential global or specific transcriptional change induced by 4OHT.
Moreover, to demonstrate univoquely that 4OHT treatment does not induce any detectable H2AX phosphorylation by itself, we have performed immunofluorescence (IF) and γH2AX ChIP with and without 4OHT in U20S parental cells (data not shown in current manuscript, but provided for the reviewers). We could not detect any γH2AX changes induced by 4OHT in U20S parental cells, neither at a global level, as observed by IF, nor by ChIP around two specifics AsiSI sites. The new data confirm that the effects we have observed in our studies are dependent on the presence of AsiSI-ER in the U20S cell line. These new data are now quoted in the revised manuscript as "data not shown".

NOTE FROM MERYL: PLEASE PUT IN THE "DATA NOT SHOWN" FIGURES. THEY ARE "REFEREE ONLY SUPP. MATERIAL" IN 72945R.

5. The authors' findings regarding continued transcription through γH2AX domains....to support their conclusion.

We agree with the referee that these findings were unexpected regarding the previous studies. Unfortunately, immuno-FISH or genome-wide nuclear run-ons (which are both known to be very difficult and time consuming to set up) were beyond our capabilities in light of the other experiment that needed to be performed to complete the revised manuscript. However, we would like to point out several lines of evidence that support our conclusions:
1) Transcription was assayed by analyzing both mRNA steady state levels and PolII binding. We averaged the PolII binding signal over entire genetic loci and not only on promoters (since PolII

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binding on promoter also occurs on genes in a poised, transcriptionally inactive, state, while PolII binding on the gene body rather reflect ongoing transcription). Moreover, while we agree that these two assays do not measure the actual rate of transcription, they do accurately reflect the transcriptional activity of most genes and are only misleading for a particular subset of genes. We conducted these studies on a genome-wide level and thus analysed a large number of genes (and not few selected genes) and therefore are confident that these data support our conclusions related to transcription.

2) With respect to the point that cleavage may be occurring on a single allele from a region containing an AsiSI site, we reiterate our results obtained through the analysis of cleavage efficiency. This assay was performed to measure the amount of DNA containing an AsiSI site that was cleaved with respect to the total amount of DNA encompassing the site. On average, we found that efficiency was greater than 50% and could reach levels upwards of 80%. This finding demonstrates that both alleles are more often cut than not.
Moreover, our experimental settings do allow us to detect transcriptional repression, since, when a gene encompasses an AsiSI site (within its locus), we did observe a decrease in PolII binding after 4OHT treatment (see Figure 8A). This is in contrast to genes within a domain but that do not encompass the cleavage site, where PolII levels are not affected.

Minor Comments:

1. The authors should either conduct experiments in synchronized cells or comment upon how an asynchronous cellular population would impact their findings.

We have conducted our experiments with synchronized T98G cells both in G1 and G2. The results of these analyses completely agree with the findings obtained in the U20S cell line and this has been added to the paper and is also referred to in the discussion.

2. The "high resolution" qualifier is arbitrary and distracting. One could argue that the minor changes and small number of DSBs analyzed do not offer high resolution.

We used the term high resolution to refer to the fact that the Affymetrix arrays have (on average) a probe every 35 bases across the chromosome. We have reduced the number of times that we use the qualifier from the text. However we keep this term in the title, and abstract because it is a general term used in the chromatin field to refer to ChIP-chip/seq studies (Barski et al, 2007; Kugou & Ohta, 2009; Marks et al, 2009; Zhu et al, 2009)

3. The authors should remove mention of γH2AX spreading from DSBs since they never conduct kinetic analyses of γH2AX signals.

We agree with the referee that we did not conduct kinetic studies and we remove mention of γH2AX spreading regarding our results. We however kept this word when describing the literature, since it is the common word used to describe the wide distribution of γH2AX that occurs around DSB.

4. The authors mention that because two anti-γH2AX antibodies gave the same result, the signals could not be due to antibody accessibility. An explanation of this statement is warranted since it is not apparent how chromatin could be accessible to one antibody, but not to another.

We removed this sentence from the manuscript but we keep showing results with the three different antibodies, since one can be more confident in the validity of the data obtained using more than one antibody.

5. The authors fail to consider that γH2AX formation .... γH2AX dynamics around particular DSBs.

We agree with the referee that this is an important point. We now discuss these 3D aspects in the discussion section:

"Interestingly we could not detect γH2AX 4OHT induction at any chromosomal location..... either a single DSB, or several I-PpoI induced DSBs, in mammals (Berkovich et al, 2007; Savic et al, 2009)."

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Referee #2

1. I am surprised that the authors have not performed some annotation for the AsiSI sites that were not cleaved effiiciently. Do these map to regions known to be enriched for HP1 or DNA methylation? This data should be out there some where.

We have now compared the genomic positions of AsiSI with the very recently published DNA methylome mapped in the H1 and IMR90 cell lines, and found that indeed, 87% of the AsiSI sites that are not cut are methylated in these two cell lines. This has been added in the result section of the revised manuscript.
"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 the chromosomes 1 and 6, that are not encompassed in a γH2AX domain (not cleaved) are in a methylated form in both H1 and IMR90 cells, while, on the contrary 67.5% of the γH2AX-associated AsiSI sites were un-methylated in these two cell lines."

2. One big point that ....better).

We performed the requested experiments and we confirmed all our previous findings. The γH2AX depletion observed on promoters when normalizing against H2AX (Figure 6B) was also observed using data normalized against H3 (Figure S6D). Along the same line, the genes showing high PolII binding or high RNA level show low γH2AX/H3 levels (Figure S8B and S9B), as observed using γH2AX /H2AX (Figure 6D and Figure S9B)) and γH2AX /input levels (Figure S8C and Figure S9B)

3. The authors argue that the transcription .....gamma-H2AX induction (shouldn't require new gene transcription).

Indeed we thought of performing these assays using PolII inhibitors such as alpha-amanitin. However the high amount of alpha-amanitin required to inhibit PolII during short treatments (as longer treatments would affect the expression of AsiSI-ER and any DSB signaling and repair proteins) was incompatible with our ChIP experiments that use a large quantity of cells. However we now include datasets obtained in another cell line (AsiSI-ER-T98G in G1 and G2 phase). Few differences were observed on γH2AX distribution within domains. For the genes tested, we could correlate changes in γH2AX profiles with changes in gene expression as assayed by RT-QPCR in both cell lines (Figure 7). We think that these new data provide additional evidence regarding the relationship between γH2AX profile and transcription.

4. Minor point: end of results.....alter chromatin structure.

We have changed the sentence accordingly. "Taken together, these results indicate that although chromatin surrounding DSBs undergoes wide H2AX phosphorylation, the genes located proximally to DSBs are still properly transcribed."

5. minor point: page 12, last paragraph,.....I think "suggest" would be more appropriate.

We have changed the sentence accordingly. "Next, the presence of holes and peaks within the γH2AX domains suggests that H2AX phosphorylation levels are affected by chromatin structure"

6. I am a little worried that normalization to H2AX values might not be the best here, especially since the values for these ChIPs seem so low. The authors have presented some data for phospho-H2AX/Input values, but I would really like to see the IP/IN H2AX chip data from the figures 4 and 5 as well.

For the previous Figure 5 (now Figure 6), we now show all key data normalized to H3 and also against input (Figures S6, S8, S9). H2AX distribution around the TSS is also shown in Figure S6E Concerning the previous Figure 4 (now Figure 5), we show examples of the H2AX/input signal on several domains as data not shown (provided to the reviewer).
In addition, we would like to point that the H2AX ChIP is not low (see the signal in comparison to

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mock in Figure S1A). However, since it is quite uniformly localized on chromatin, there is no peak in ChIP-chips experiments and the signal to input varies not far from the baseline (and thus seems "low"). This is discussed in the legend of Figure S1 "The low enrichment of H2AX observed by ChIP-chip, is not due to low ChIP efficiency (since we could detect high level of H2AX when analysing H2AX ChIP by Q-PCR) but reflects a general incorporation of H2AX along the chromosome arms (as ChIP-chip experiment do not assess absolute level of a protein on chromatin, but its change in distribution along the genome)."

Referee #3 (Remarks to the Author):

This is an outstanding manuscript. It describes a very elegant new approach to study the chromatin fate of double-strand breaks (DSBs) at many genomic locations in mammalian cells. I greatly enjoyed reading it. The experimental approaches are innovative and solid, the data are beautiful, the figures are clear and the manuscript was easy to read. I strongly recommend publication in EMBO Journal a.s.a.p.

Minor comments/ suggestions for improvement:

** page 9: the ChIP data from Barski et al were obtained from a different cell type. This should be pointed out in the text, as it is a potential caveat in the analysis.*

This point has been clarified in the revised manuscript: "Determined γH2AX peak/hole borders in AsiSIER-U20S cells, were next analysed with respect to previously published ChIP-seq datasets using CD4+T cells (Barski et al, 2007). ..... to investigate in an unbiased manner the potential relationship between γH2AX and gene position."

** page 10, line 16: "... are kept free of gammaH2AX". This is an overstatement. I agree that gammaH2AX levels are reduced, but certainly not reduced to zero, as the sentence suggests.*

This sentence has been changed accordingly: "In conclusion, our combined analyses of PolII binding and RNA levels in AsiSIER-U20S cells, indicate that, while γH2AX spreads over megabases of chromatin surrounding DSBs, the active genes encompassed in these DSB-induced chromatin domains, show low γH2AX accumulation. "

** page 12, last sentence: "strongly depleted...". Again an overstatement. The changes are roughly 0.1 log2-unit, which is no doubt significant but not "strong".*

This sentence has been changed accordingly: "Indeed, by investigating transcription either by genome wide Pol II mapping or by strand specific expression profiling, we found that transcribed units within domains are depleted in γH2AX."

** Discussion page 13/14 and Fig 7: The "looping" model is too speculative and somewhat out of place, because no looping assays are done in this manuscript. I strongly suggest to leave out Figure 7 and use the space instead to promote one supplementary figure (e.g. S2 or S3) to main figure.*

The model is now presented as a supplemental data FigS12, and the γH2AX enrichment on telomeres in Fig3

** Methods: please describe the construction of pBABE HA-AsiSI-ER in detail.*

The description of the construction of pBABE HA-AsiSI-ER is now provided in the methods section

** Figure 1A: brightness/contrast should be improved, because the signals are difficult to see.*

Brightness/contrast of Fig1A has been improved

** Figure 2A: please add axis with units to the "target sites density" traces.*

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We changed the Figure 2A, and replaced the target sites density, by the linear signal obtained with these ChIP-chip experiments. We felt that this representation, which directly reflects the raw data, would be easier to understand than the target site density that represents a more processed form of the data.

** Figure 2: I wonder whether the γH2AX/H2AX data could be better visualized by using a linear rather than a log2 scale. In this case that seems perfectly valid to me, because only enrichments (not depletions) are expected and found.*

The linear signals are now represented in Fig2A.

Barski A, Cuddapah S, Cui K, Roh TY, Schones DE, Wang Z, Wei G, Chepelev I, Zhao K (2007) High-resolution profiling of histone methylations in the human genome. Cell 129(4): 823-837

Berkovich E, Monnat RJ, Jr., Kastan MB (2007) Roles of ATM and NBS1 in chromatin structure modulation and DNA double-strand break repair. Nat Cell Biol 9(6): 683-690

Kugou K, Ohta K (2009) Genome-wide high-resolution chromatin immunoprecipitation of meiotic chromosomal proteins in Saccharomyces cerevisiae. Methods Mol Biol 557: 285-304

Lister R, Pelizzola M, Dowen RH, Hawkins RD, Hon G, Tonti-Filippini J, Nery JR, Lee L, Ye Z, Ngo QM, Edsall L, Antosiewicz-Bourget J, Stewart R, Ruotti V, Millar AH, Thomson JA, Ren B, Ecker JR (2009) Human DNA methylomes at base resolution show widespread epigenomic differences. Nature 462(7271): 315-322

Marks H, Chow JC, Denissov S, Francoijs KJ, Brockdorff N, Heard E, Stunnenberg HG (2009) High-resolution analysis of epigenetic changes associated with X inactivation. Genome Res 19(8): 1361-1373

Savic V, Yin B, Maas NL, Bredemeyer AL, Carpenter AC, Helmink BA, Yang-Iott KS, Sleckman BP, Bassing CH (2009) Formation of dynamic gamma-H2AX domains along broken DNA strands is distinctly regulated by ATM and MDC1 and dependent upon H2AX densities in chromatin. Mol Cell 34(3): 298-310

Zhu C, Byers KJ, McCord RP, Shi Z, Berger MF, Newburger DE, Saulrieta K, Smith Z, Shah MV, Radhakrishnan M, Philippakis AA, Hu Y, De Masi F, Pacek M, Rolfs A, Murthy T, Labaer J, Bulyk ML (2009) High-resolution DNA-binding specificity analysis of yeast transcription factors. Genome Res 19(4): 556-566

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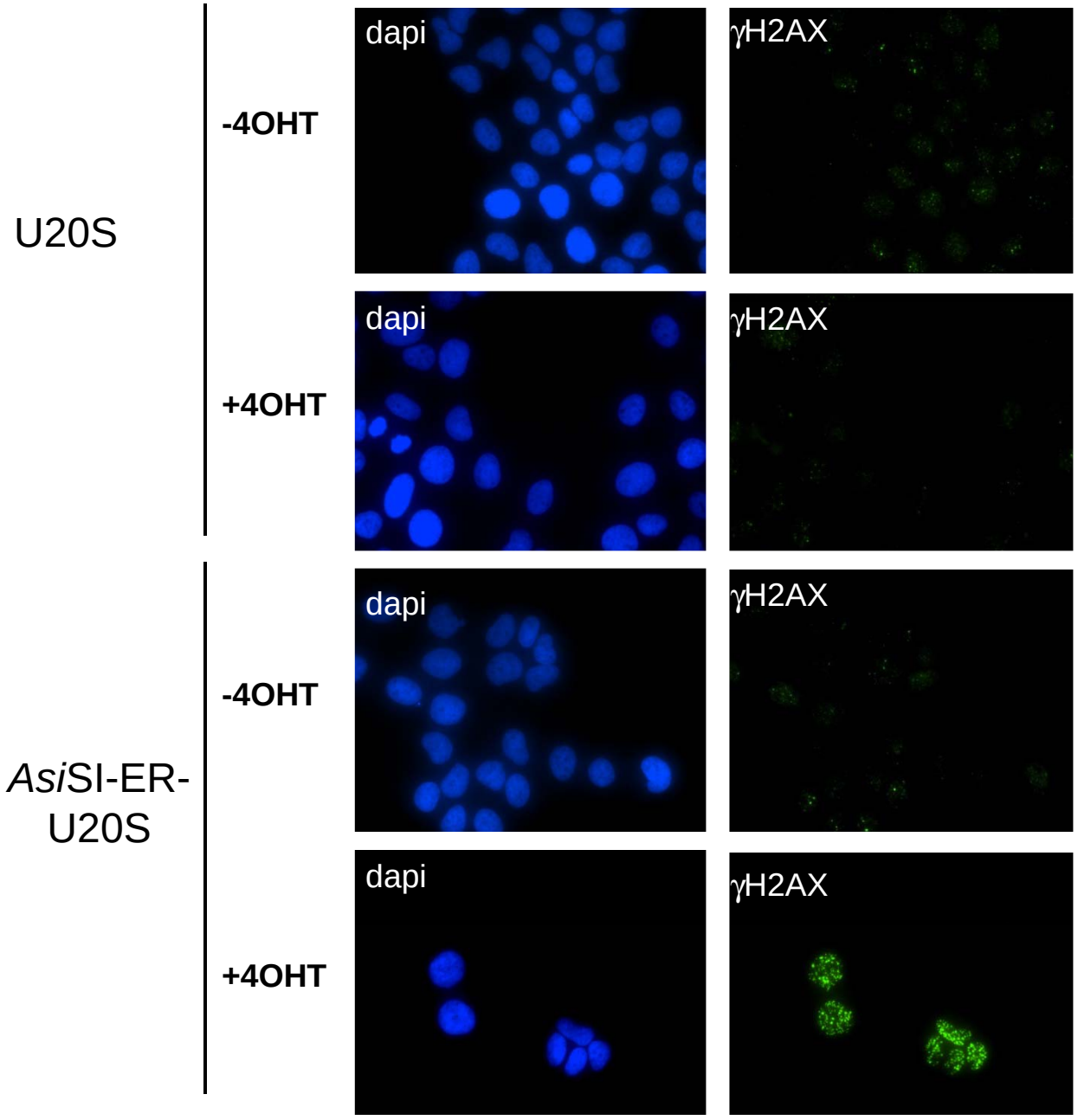
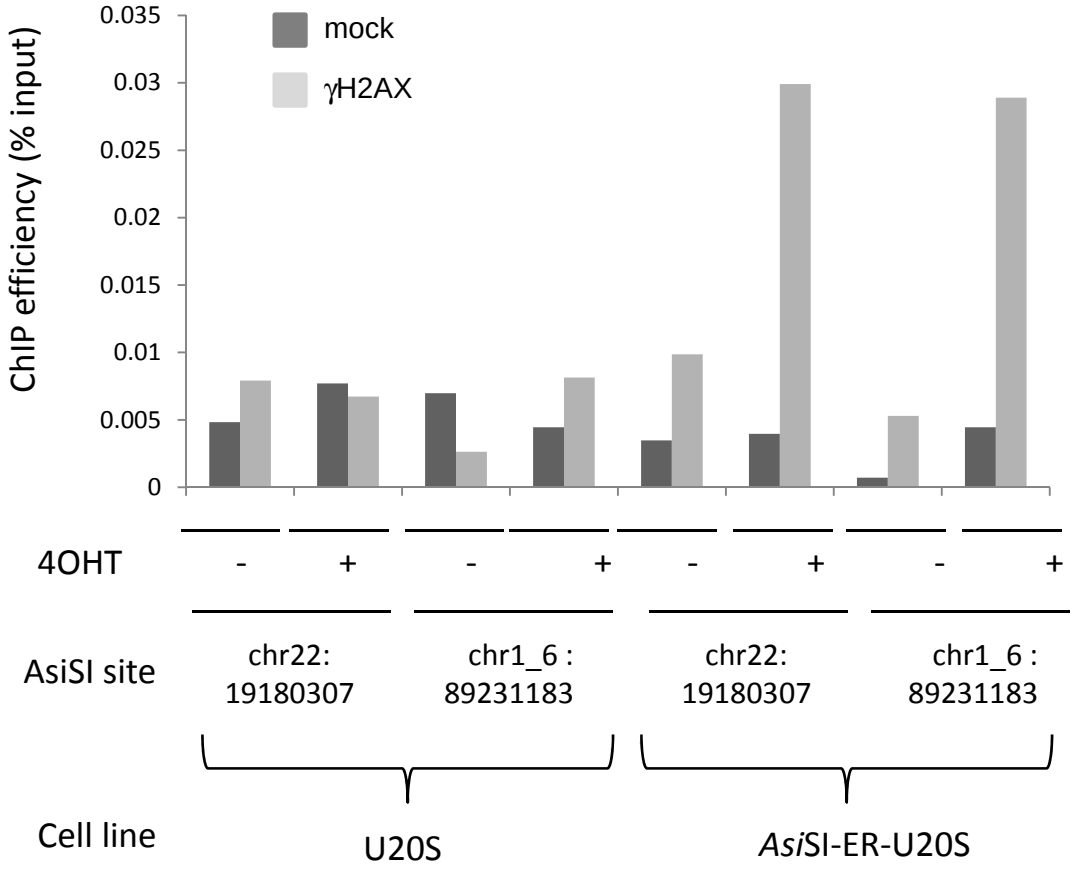


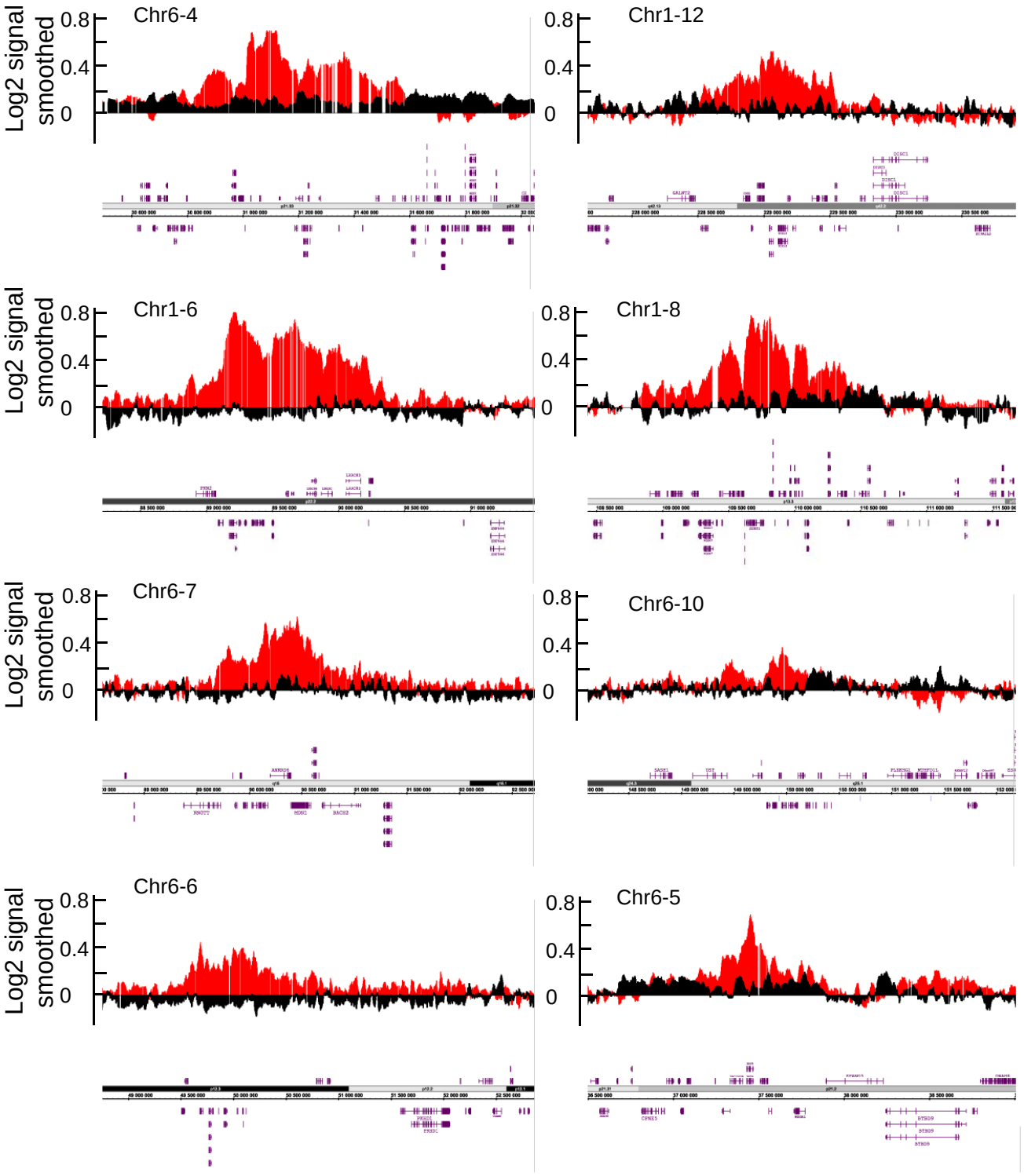
Data not shown 1: 4OHT induces γH2AX foci only in *Asi*SIER-U20S cells and not in parental U20S cell line.

U20S or *Asi*SI-ER-U20S were co-stained with DAPI (DNA) following incubation with an antibody against γH2AX, before and after 4OHT treatment (4 hours). Note that 4OHT treatment does not induce detectable γH2AX foci in the U20S parental cell line.



Data not shown 2: 4OHT induces γH2AX increase at proximity to *Asi*SI site only in *Asi*SIER-U20S cells and not in parental U20S cell line.

Mock and γH2AX ChIPs were performed on chromatin extracted from U20S, and *Asi*SIER-U20S, before and after 4H of 4OHT treatment, and analyzed by Q-PCR using primers proximal to two *Asi*SI sites (at respectively 3.7kb and 200bp).



Data not shown 3: H2AX profiles across 8 differents γH2AX domains.
Detailed views of H2AX/input (in black) and γH2AX/H2AX (in red) distribution across several genomic regions (data expressed as log2 and smoothed using a 500 probes sliding window). Note that the distribution of H2AX does not parallel the one of γH2AX

2nd Editorial Decision 19 February 2010

Thank you for sending us your revised manuscript. Our original referees 1 and 2 have now seen it again, and you will be pleased to learn that in their view you have addressed all criticisms in a satisfactory manner. The paper will now be publishable in The EMBO Journal and you will receive a formal acceptance letter shortly.

Thank you very much again for considering our journal for publication of your work.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

The authors have satisfactorily addressed, either experimentally or with explanation/clarification, the criticisms that I raised over the first draft of this manuscript. Thus, I feel the current manuscript is suitable for publication and of sufficient quality and interest for EMBO.

Referee #2 (Remarks to the Author):

Excellent revision.

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