Circadian PERIOD complex regulates TC-DSB repair through anchoring to the nuclear envelope.

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Running title: Circadian regulation of TC-DSB repair
Abstract

Repair of DNA Double-Strand Breaks (DSBs) produced in transcriptionally active chromatin occurs through a poorly characterized pathway called Transcription-Coupled DSB repair (TC-DSBR). Here, using a screening approach scoring multiple outputs in human cells, we identified the PER complex, a key module ensuring circadian oscillations, as a novel TC-DSBR player. Circadian rhythm has been involved in repair of UV-induced DNA damage but very little is known about its contribution to DSB repair pathways. We show that the PER complex is recruited at DSBs occurring in transcribed loci and we further found that the core protein PER2 contributes to targeting TC-DSBs at the nuclear envelope (NE) and to foster RAD51-mediated repair. PER2 deficiency triggers decreased DSB anchoring to the NE, resulting in an increase of DSB clustering and translocation frequency. In agreement, we found that the circadian clock also regulates DSB anchoring to the NE and RAD51 assembly. In conclusion, our study provides a direct link between the circadian rhythm and the response to DSBs occurring in active genes, opening new therapeutic strategies for chemotherapies based on topoisomerase poisons that mostly induce DSBs in active loci.
Main

DNA Double-Strand breaks (DSBs) are among the most toxic lesions that occur on the genome, given their potential to elicit large chromosomal rearrangements. Genome-wide mapping of DSBs established that endogenous DSBs mostly appear in transcriptionally active chromatin (TC-DSBs)\(^1\)–\(^4\). While global genome DSB repair (GG-DSBR) mechanisms have been well characterized, a transcription-coupled arm of DSB repair (TC-DSBR) has only recently been identified\(^5\). TC-DSBR entails the transcriptional repression of the damaged genes via ATM and DNA-PK dependent mechanisms\(^5\)–\(^6\) which is necessary for the initiation of resection. In G2, TC-DSBs are further channeled to repair by HR thanks to transcription-associated chromatin marks such as H3K36 trimethylation and H4 acetylation for instance\(^7\)–\(^9\), in a manner that also depends on Senataxin (SETX)-dependent removal of RNA:DNA hybrids that accumulate at TC-DSBs. In G1, TC-DSB repair is delayed\(^10\) and may rely on CSB and RAD52\(^11\). The persistent TC-DSBs in G1 are prone to cluster in large subnuclear structures\(^10\), that we recently identified as a novel, DSB-induced, chromatin compartment, called the D-compartment\(^12\) and which enhances translocation frequency. Additionally, when occurring on human ribosomal DNA, TC-DSBs are physically relocated at the nucleolar periphery where they can contact invaginations of the Nuclear Envelope (NE)\(^13\). Whether this also occurs at RNAPII-transcribed damaged loci is yet unknown, although such NE targeting of persistent DSBs has been previously reported in *Drosophila* and *S. Cerevisiae*\(^14\)–\(^17\).

Multi-output screen to identify new TC-DSBR factors

In search of novel TC-DSBR players, we performed a siRNA library screen scoring multiple outputs (Fig. 1a) in the well-characterized human DIvA cell line, where 174 DSBs are induced in a temporally controlled manner at annotated positions, a large fraction of which
occur in RNAPII-enriched (transcribed) loci\textsuperscript{18,19}. The siRNA library comprised of 130 candidate proteins, previously found to be preferentially associated with the SMC1 cohesin subunit post-DSB induction\textsuperscript{13}. The screen was designed to quantify the effect of candidate protein depletion on 1) γH2AX foci intensity using high-content imaging, 2) cell survival using a cell proliferation assay, 3) chromosome rearrangement frequency using quantitative PCR and 4) DSB clustering using high content microscopy (Fig. 1a, Extended Data Fig. 1a). A positive control siRNA against SETX, previously involved in TC-DSBR\textsuperscript{20,21}, triggered as expected increased γH2AX foci intensity, translocation frequency and DSB clustering as well as decreased cell proliferation (Fig. 1b-e, red bars). We further validated our screening dataset with the candidate protein Periphilin (PPHLN1, Fig. 1b-e, green bars), an H3K9me3 reader of the HUSH complex that we previously involved in ribosomal DNA DSB repair\textsuperscript{13}. Similar to the screen results, depletion of PPHLN1 triggered an increase in γH2AX foci intensity and translocation frequency, and a decreased clonogenic potential (Extended Data Fig. 1b-e).

Importantly, two-parameter correlations established that γH2AX intensity was inversely correlated with cell survival but positively correlated with translocation frequency (Fig. 1f-g), consistent with a decreased repair capacity respectively impairing cell proliferation and promoting genome rearrangement. Moreover, increased DSB clustering positively correlated with translocation frequency (Fig. 1h), and increased translocation inversely correlated with cell survival (Fig. 1i) in agreement with previous work\textsuperscript{12,22}. Altogether, results from our siRNA screen establish that TC-DSBR deficiency severely affects cell survival and further confirm the relationship between DSB clustering and genome rearrangement as recently reported\textsuperscript{12,22}.

The circadian clock PERIOD complex contributes to TC-DSB repair.
Interestingly, among the candidate proteins whose depletion gave similar phenotypes to SETX, we found NONO, SFPQ and DDX5 which all belong to the same complex, the mammalian PERIOD (PER) complex involved in the regulation of the circadian rhythm\textsuperscript{23–26}. SETX itself belongs to the PER complex\textsuperscript{25} and similar outputs were also seen for another candidate, the DDX5-paralog DDX17, both DDX5 and DDX17 being human orthologs of the \textit{Neurospora} PRD1 protein involved in circadian rhythmicity\textsuperscript{27} and existing as a heterodimer\textsuperscript{28}.

Our screen indicated that the depletion of five distinct proteins within the PER complex triggered increased $\gamma$H2AX staining, decreased cell survival, increased chromosomal rearrangement and increased DSB clustering (Fig. 1b-e, blue bars). The circadian clock is molecularly controlled by the BMAL1/CLOCK transcriptional activator complex, which regulates the transcription of thousands of genes. Among those, the PER-CRY transcriptional repressor can downregulate BMAL1/CLOCK activity, creating a negative transcriptional feedback loop that ensures circadian oscillations\textsuperscript{29}. In order to directly investigate whether the core clock components of the PER complex play a role in TC-DSBR, we depleted PER1 and PER2 proteins (Extended Data Fig. 2a). As found for DDX17, NONO, DDX5 and SETX (Fig. 1b), PER1 and PER2 knockdown increased $\gamma$H2AX foci intensity following DSB induction in DIvA cells (Fig. 2a) with no major changes in cell cycle distribution (Extended Data Fig. 2b). In agreement, PER2-depleted cells also displayed elevated $\gamma$H2AX levels detected by chromatin immunoprecipitation (ChIP) around DSBs (Fig. 2b). Moreover, PER1 and PER2 depletion increased translocation frequency (Fig. 2c) and impaired cell survival following induction of TC-DSBs in DIvA cells (Fig. 2d, Extended Data Fig. 2c) as similarly found for DDX17, NONO, SETX and DDX5 (Fig. 1c-d; Fig. 2c-d; Extended Data Fig. 2d). In contrast, depletion of the BMAL1 transcriptional activator had no effect on translocation frequency or cell survival post DSB induction (Fig. 2c-d, Extended Data Fig. 2d). Altogether, these data show that the
PERIOD proteins PER1 and PER2, as well as other members of the PER complex, contribute in the response to DSBs induced in transcribed loci.

In order to determine whether the function of the PER complex is direct, we assessed the recruitment of PER complex proteins at DSBs using ChIP. PER2, DDX5 and DDX17 all showed significant recruitment at a TC-DSB induced in DlVVA cells, while this was not the case for BMAL1 (Fig. 3a). We further performed ChIP-seq against PER2 and BMAL1 in damaged and undamaged DlVVA cells. Both proteins displayed the expected pattern on the genome (see examples Extended Data Fig. 3a). Indeed, in absence of DSB induction, BMAL1 was enriched on genomic loci previously identified by BMAL1 ChIP-seq (Extended Data Fig. 3b) and PER2 accumulated at promoters (Extended Data Fig. 3c) as expected for a transcriptional repressor and from previous studies. Moreover, BMAL1-bound genes displayed significant enrichment for the “rhythmic process” GO term (Extended Data Fig. 3d). Post-DSB induction, we interestingly observed that PER2 accumulated at TC-DSBs (see an example Fig. 3b top panel, purple track) but not at a silent locus (Fig. 3b bottom panel), despite equivalent cleavage (BLESS tracks). In contrast, BMAL1 showed no accumulation at DSBs (Fig. 3b, blue tracks). On average, PER2 (left panel) was significantly targeted on ~2kb around DSBs, while BMAL1 (right panel) was not (Fig. 3c, Extended Data Fig. 3e).

We previously identified a subset of equivalently cleaved DSBs in DlVVA cells with two distinct repair behaviors: the Non-Homologous End-Joining (NHEJ)-prone DSBs and the HR-prone DSBs with the latter having a tendency to locate within transcriptionally active loci (i.e. TC-DSBs). Importantly, here we found that PER2 preferentially accumulates at HR-prone TC-DSBs compared to NHEJ-prone DSBs (i.e. occurring in non-transcribed regions) (Fig. 3d).

Thus, in order to establish whether PER2 contributes to repair at HR-prone DSBs, we further investigated the effect of PER1/2 depletion on end-resection, by quantification of single-
stranded DNA at various distances from DSBs (Fig. 3e left panel). siRNA depletion of both
PER1 and PER2 triggered a moderate, yet significant, decrease of resection (Fig. 3e, right
panel), although less pronounced than upon CtIP depletion. Moreover, PER2 depletion
triggered a significant decrease in RAD51 binding at TC-DSBs (Fig. 3f). One of the first steps
of TC-DSB repair is the transcriptional repression of nearby genes (including the damaged gene
itself) which is essential for the proper execution of resection and HR repair. Given that the
PER complex is a transcriptional repressor, we envisaged that the PER complex could promote
HR repair at TC-DSBs via a role in transcriptional repression. However, RT-qPCR analyses
revealed that both PER2 and PER1 are dispensable for this transcriptional repression following
DSB induction (Fig. 3g). Altogether these data show that the PER complex is recruited at TC-
DSBs where it contributes to resection and fosters RAD51 recruitment, suggesting a direct
function in TC-DSBR downstream of transcriptional repression.

TC-DSBs are targeted to the nuclear envelope via a SUN1-dependent mechanism.

The nuclear envelope (NE) contributes to the regulation of the circadian clock and PER
proteins have recently been involved in the physical targeting of clock-regulated genes to the
NE in Drosophila. Of interest, persistent DSBs were previously found to be relocated at the
NE in yeast, as were heterochromatic (HC) DSBs in Drosophila in order to complete
HR. While in mammalian cells, physical relocation of DSBs to the NE has not yet been
documented, we previously observed that ribosomal DNA DSBs can contact NE invaginations
inside the nucleoplasm. Hence, we set out to determine whether TC-DSBs could be targeted
to the NE. As a first approach, we performed fixed and live super-resolution imaging using
Random Illumination Microscopy (RIM). We observed that γH2AX foci can establish close
contact with LaminB1 filaments (Extended data 4a). Live imaging after DSB induction in DIVA
cells expressing 53BP1-GFP and mCherry-LaminB1, showed that such interactions between DSBs and nuclear lamina were rapidly established (<60s) and transient (Fig. 4a and Supplementary Videos 1-4). Contacts between repair foci and the nuclear lamina were further confirmed by Proximity Ligation Assay (PLA) performed between LaminB1 and 53BP1 (Fig. 4b) or between γH2AX and LaminB1 (Extended Data Fig. 4b). In agreement, ChIP against LaminB1 (Extended Data Fig. 4c, top panel) also revealed that, in DIvA cells post-DSB induction, LaminB1 occupancy increased at TC-DSBs in contrast to a DSB induced in a silent locus and repaired by NHEJ (Fig. 4c). Taken together, these data suggest that TC-DSBs can be physically relocated at the NE in human cells.

Interestingly, we and others previously reported a function for SUN domain containing-proteins, which are components of the Inner Nuclear Membrane (INM), in DSB mobility in mammals\textsuperscript{10,37}. Moreover, SUN proteins orthologs in yeast and Drosophila (respectively Mps3, and Koi/Spag4) were also found as DSB anchoring points in the NE\textsuperscript{15-17}. We thus investigated the potential involvement of the two main SUN proteins in mammals, SUN1 and SUN2, in targeting TC-DSBs to the NE. Interestingly, ChIP against SUN proteins (Extended Data Fig. 4c, middle and bottom panels) revealed that, upon damage, SUN1 displayed enrichment at TC-DSBs compared to NHEJ-DSBs (Extended Data Fig. 4d, top panel). Surprisingly, SUN2 rather showed decreased occupancy post-DSB induction (Extended Data Fig. 4d, bottom panel), suggesting that TC-DSBs are specifically interacting with SUN1 and not SUN2. To further investigate SUN protein recruitment at DSBs, we performed ChIP-seq against SUN1 and SUN2 before and after DSB induction in DIvA cells. Visual inspection and peak calling on SUN1 and SUN2 ChIP-seq datasets revealed that in undamaged conditions, both proteins are enriched on genes (Extended Data Fig. 4e-f), accumulated at promoters (Extended Data Fig. 4g) and largely overlapped on the genome (Extended Data Fig. 4h). Of interest, post-DSB induction, SUN1 (purple track), was recruited on approximately 1kb around the break, while SUN2 rather
displayed eviction (blue track) (see an example Fig. 4d top panel, and average profiles at all DSBs Fig. 4e). Both SUN1 recruitment and SUN2 eviction at DSBs were statistically significant when compared to random, undamaged genomic positions (Extended Data Fig. 4i). Of note, as observed for PER2, SUN1 was specifically recruited at a TC-DSB (Fig. 4d top panel) but not at a DSB induced in a silent locus (Fig. 4d bottom panel). We then performed 3D-super resolution imaging by RIM to detect SUN1 and SUN2. Both proteins localized to the NE and displayed punctuated patterns (Extended Data Fig. 4j-k). Co-staining with γH2AX enabled the detection of a significant colocalization (ICQ) with SUN1, but not with SUN2 (Fig. 4f). Van Steensel Cross-correlation function (CCF) analysis showed non-random overlap between γH2AX foci and SUN1 while non-random exclusion was observed between γH2AX foci and SUN2 (Fig. 4g, see examples on left panels, and quantification on the right panels).

Altogether these data suggest that in DlvA cells, TC-DSBs are physically targeted to the NE through an interaction with SUN1 and not SUN2. In order to determine whether NE-targeting of TC-DSBs is functionally relevant, we further determined the consequence of the depletion of SUN-domain proteins (Extended Data Fig. 4l) on cell survival and translocations frequency. Interestingly, depletion of SUN1 decreased cell survival post-DSB induction which correlated with an increased translocation rate (Fig. 4h-i). Of note, this was not the case when depleting SUN2, in agreement with the absence of SUN2 recruitment at DSBs. SUN2 depletion actually triggered an opposite behaviour, decreasing translocation frequency and increasing cell survival post DNA damage (Fig. 4H-I), suggesting that SUN1 and SUN2 competition for inclusion in the NE may regulate TC-DSB docking at the NE. Taken altogether, these data indicate that, similarly to HC breaks in Drosophila and persistent breaks in yeast, TC-DSBs also display targeting to the NE in mammalian cells, in a manner that depends on SUN1.
PER2 contributes in targeting TC-DSBs to the nuclear envelope

In order to evaluate the consequence of PER2 depletion on TC-DSB targeting at the NE, we performed LaminB1 ChIP in DIvA cells transfected with a PER2 siRNA. Of interest PER2 depletion triggered a decrease in LaminB1 recruitment at TC-DSBs (Fig. 5a). Additionally, PER1 or PER2-depleted cells also displayed decreased 53BP1/LMNB1 PLA signal post-DSB induction when compared to PER1/2-proficient cells (siRNA CTRL) (Fig. 5b, Extended Data Fig. 5a). In order to investigate a potential cell cycle dependency for PER2-mediated DSB anchoring to the NE, we then monitored γH2AX/LaminB1 PLA signal combined with EdU incorporation by quantitative high throughput microscopy (Extended Data Fig. 5b). PER2 depletion triggered a decreased in PLA foci in G1, S and G2 cells (Fig. 5c). Given our above data on SUN1 recruitment at DSBs (Fig. 4d-g) and the consequences of SUN1 depletion on cell survival (Fig. 4h), we wondered whether SUN1 and PER1/2 would act in the same pathway. Importantly co-depletion of both SUN1 and PER1 or PER2 did not exacerbate the impaired survival observed upon depletion of PER1 or PER2 proteins independently (Fig. 5d). Taken together, these data suggest that the PER core clock components contribute in anchoring TC-DSBs to the NE-embedded SUN1 protein, in human cells.

PER2 depletion triggers increased DSB clustering and DSB induced-compartment formation.

In mammalian nuclei, Hi-C experiments revealed that chromatin compartmentalizes to form the so-called “A” and “B” compartments, with the B-compartment corresponding to microscopically visible heterochromatin foci\(^{38}\). Of interest, both experimental data and computational modeling of chromosome behaviour showed that targeting at the nuclear lamina prevents heterochromatin to coalesce into internal larger nuclear bodies\(^{39}\). The current model
postulates that upon detachment of chromatin from the NE, phase separation allows multiple
HC foci to cluster thus increasing B-compartmentalization. Importantly, we recently reported
that upon DSB formation, another chromatin compartment forms, so called the D-compartment
(for DSB-induced). This D-compartment, arising via DSB clustering, gathers γH2AX-modified
topologically associated domains (TADs) as well as additional undamaged loci, including a
subclass of DSB-activated genes\textsuperscript{12}.

Of interest, our siRNA screen indicated that the depletion of NONO, SETX and DDX17 all
increased clustering (Fig. 1e) and we found that PER2 contributes in targeting DSBs to the NE
(Fig. 5a-c). Hence, we set out to investigate whether PER2 depletion could also increase DSB
clustering as a result of DSB detachment from the NE. To address this point, we performed Hi-
C experiments before and after DSB induction in PER2-proficient and deficient cells. PER2
depletion did not drastically affect chromosome organization in undamaged cells (Extended
Data Fig. 5c), nor the overall distribution of A and B compartments (Extended Data Fig. 5d).
However, PER2 depletion triggered increased compartmentalization as visualized by increased
A-A and B-B interaction, as well as decreased A-B interaction (Extended Data Fig. 5e). This is
in agreement with the previously reported role of the PER complex in chromatin anchoring at
the NE which would predict an increased compartmentalization upon NE detachment in PER-
depleted cells. Upon DSB induction, we could recapitulate our previous finding showing DSB
clustering (Extended Data Fig. 5f, arrows). Importantly, PER2 depletion increased DSB
clustering as shown on individual events (Fig. 5e, Extended Data Fig. 5f), average aggregate
peak analysis (APA) plots of inter-chromosomal or intra-chromosomal DSB-DSB contacts
centered on all 80 best-induced DSBs (Fig. 5f, Extended Data Fig. 5g) and box plots quantifying
the interactions between DSBs (Fig. 5g). Given previous findings showing that DSB clustering
increases translocation rate\textsuperscript{12,22,40}, these data also agree with the increased translocation
frequency observed upon depletion of the PER complex proteins (Fig. 2c).
We further computed Chromosomal Eigen Vectors (CEV) using the first PC of Principal Component Analysis (PCA) on differential Hi-C matrices to identify the D-compartment as previously described\(^{12}\). As expected, we could recapitulate D-compartment formation upon damage in DIVA cells transfected with a control siRNA (Extended Data Fig. 5h). PER2 deficiency did not alter D-compartment detection on chromosomes previously found to display D-compartment (Extended Data Fig. 5h). Of interest, we could observe D-compartment formation on additional chromosomes upon PER2 depletion (Fig. 5h), in agreement with an increased DSB clustering. Taken altogether our data support a model whereby the PER proteins contribute in targeting TC-DSBs to the nuclear lamina, thereby preventing DSB clustering and D-compartment formation.

The circadian clock regulates the response to TC-DSBs and their anchoring to the NE.

Given that the PER complex is a core component of the circadian clock, we set out to investigate whether the circadian clock could also regulate the response to TC-DSBs. Dexamethasone treatment synchronized the circadian clock in both U20S and DIVA cells as expected, as assessed by the cycling expression of PER2, BMAL1 and CRY2 (Extended Data Fig. 6a-b). We further monitored γH2AX foci formation in different cell cycle phases, at different time points after dexamethasone-mediated circadian clock synchronization by combining γH2AX staining and EdU labelling, using quantitative high throughput microscopy in DIVA cells. In line with the results obtained with the depletion of PER2 by siRNA, our data indicate that γH2AX foci intensity was increased post-DSB induction during the low-PER2 circadian phase (Fig. 6a, white bars) as compared to the high-PER2 phase (Fig. 6a blue bars). This was independent of the cell cycle phase. Moreover, increased accumulation of γH2AX at TC-DSBs during the low-PER2 phase, was further confirmed by ChIP (Fig. 6b). Of importance the same
holds true in S and G2 phases when DSBs were induced using etoposide, a topoisomerase II poison which preferentially induces DSBs in transcribing loci (Extended Data Fig. 6c, top panel). In contrast, the low-PER2 phase was not associated with enhanced γH2AX foci detection when DSBs were induced using bleomycin, a radiomimetic inducing DSBs throughout the genome with no specificity for active loci (Extended Data Fig. 6c, bottom panel). To further reinforce the notion that the low-PER2 phase of the circadian clock impacts TC-DSB repair, we performed circadian clock synchronization and analyzed several outputs that were affected by the depletion of PER2.

First, given that our data indicated a role of PER2 in RAD51 assembly at DSBs (Fig. 3f), we assessed RAD51 recruitment using ChIP (Fig. 6c) and RAD51 foci formation using high throughput microscopy (Extended Data Fig. 6e) at different time points after dexamethasone treatment. Both experiments revealed impaired RAD51 recruitment during the low-PER2 phase. Second, the circadian clock did not alter transcriptional repression in cis to DSBs (Extended Data Fig. 6f) as expected (Fig. 3g). Third, as found using PER2 depletion (Fig. 5a-c), the low-PER2 circadian phase reduced the recruitment of LaminB1 (Fig. 6d) and SUN1 (Fig. 6e) to TC-DSBs when monitored by ChIP-qPCR, suggesting that DSB anchoring at the NE is defective when the PER1/2 proteins are expressed at low levels. Fourth, quantitative high throughput microscopy further showed an increase in γH2AX foci area during the low-PER2 phase which is in agreement with enhanced DSB clustering (Fig. 6f).

We previously reported that, in addition to γH2AX domains, the DSB-induced chromosome compartment (D-compartment) also physically attracts additional loci including some, but not all, DNA damage responsive (DDR) genes, in order to potentiate their activation post-DSB induction\textsuperscript{12}. Of interest we found that RNF19B, an upregulated DDR gene found within the D-compartment, was significantly more activated post-DSB during the low-PER2 phase when compared to the high-PER2 phase (Fig. 6g). This is in agreement with an increased D-
compartment formation in absence of PER2 (Fig. 5e-h). Of importance, this was not the case for other DDR genes which are not targeted to the D-compartment (PPM1D, SLC9A1), or for other non-induced genes (LPHN2, UTP18). Altogether these data indicate that the circadian rhythm regulates the repair and signaling of TC-DSBs by controlling their PER2-dependent anchoring to the NE and, therefore, the formation of the D-compartment, further fine-tuning the response to DSBs.

Discussion

In this manuscript, we uncovered that the repair of DSBs occurring in transcriptionally active chromatin requires their anchoring to the NE, a process that is under the direct control of the PERIOD complex and is therefore sensitive to circadian oscillations (Fig. 6h).

Previous work uncovered that the maintenance of genomic integrity and cancer progression are tightly linked to the circadian clock. In general, genes involved in DNA repair pathways are transcriptionally regulated by the circadian clock. Here we found that in human cells, the PERIOD complex is directly targeted at DSBs. In contrast to a very recent report, we did not observe BMAL1 binding at TC-DSBs, although BMAL1 distribution on the genome behaved as expected. This difference may arise from analyzing overexpressed versus endogenous (our study) BMAL1, or from the different DSB-inducible system used in both studies. We found that TC-DSBs occurring in RNAPII-transcribed loci are relocated to the NE, showing for the first time the repositioning of DSBs to the nuclear lamina in humans and expanding the list of DSBs that display this behaviour. This suggests that targeting persistent DSBs to this specific nuclear microenvironment is evolutionary conserved. Here we involved SUN1 as being the TC-DSB docking site at the NE and interestingly SUN1 also displays direct interaction with the Nuclear Pore Complex (NPC), as opposed to SUN2. Hence, whether
this specific involvement of SUN1 reflects a function for the NPC as a docking point for TC-DSBs will be worth investigating.

We found that upon PER1/2 depletion, as well as during the low-PER2 phase, γH2AX level were increased while RAD51 assembly was reduced suggesting a decreased repair capacity. Of interest, in the accompanying paper, the Huertas lab, reports that CRY1 is also recruited at radiation-induced DSBs, where it directly represses resection and HR. Altogether our studies suggest a two-arm control of HR repair during the 24h circadian cycle, with minimum HR occurring at night. Indeed, large transcriptomic surveys, performed in baboon\(^4^4\) and very recently in human\(^5^3\), allowed to carefully establish the transcriptional pattern of core clock regulators during the day. PERIOD genes \(Per1\), \(Per2\) and \(Cry2\) display a maximum expression level around noon, while \(Cry1\) expression peaks at sunset, in agreement with a PERIOD complex-independent function of CRY1. Altogether this allows us to propose the following model (Extended Data Fig. 6g): In the morning, the presence of the PERIOD complex (PER1/2 and CRY2) combined with the absence of CRY1, establishes an environment that favors HR repair of TC-DSBs by allowing resection to occur, DSB anchoring to the NE and RAD51 loading. Subsequently, the gradual increase of CRY1 during the afternoon causes minimum resection at night fall. The absence of PERIOD complex expression early at night establishes a second layer of HR repression during the night. The gradual decrease of CRY1, combined with the re-expression of PER proteins, then allows HR to resume the next morning. Such a tight regulation of HR at TC-DSBs during the night may be functionally relevant for handling the repair of TC-DSBs produced in some specific contexts, such as in the brain following neuronal activity\(^5^4,5^5\). Sleep was found to regulate the repair of DSBs in neurons\(^5^6\). Hence circadian regulation may fine-tune the response to this specific class of endogenous DSBs.
In summary, our findings have implication for diseases etiology and treatment. Indeed, evidence suggest that a dysregulation of the circadian clock contribute to the progression of neurodegenerative disorders\textsuperscript{57} and cancer initiation and progression\textsuperscript{41,43}, both types of diseases being tightly coupled to DSB repair mechanisms and the maintenance of genome integrity. Moreover, importantly, topoisomerase II poison-based chemotherapies are first-line treatment against a number of cancers and given that these poisons mainly trigger DSBs in active chromatin, our finding that circadian rhythm regulates the repair of TC-DSBs may be an important feature to take into account for chronochemotherapy.

Data and Code availability

Code is available at https://github.com/LegubeDNAREPAIR/CircadianClock. High throughput data are available at Array Express (E-MTAB-12712) using the link https://www.ebi.ac.uk/biostudies/arrayexpress/studies/E-MTAB-12712?key=2f8af589-d077-466a-a563-725b33761cda

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Authors contributions

B.L.B, L.G.S, E.G, C.P, C.A, A.G, A.L.F and N.P, performed and analyzed experiments. S.C and V.R performed bioinformatic analyses of all high-throughput sequencing datasets. T.M performed RIM acquisition and analysis. G.L. and N.P. supervised the work. G.L. wrote the manuscript. All authors commented, edited and approved the manuscript.

Conflict of interest

The authors declare no competing interest

References


Figure Legends

Figure 1: A multi-output screen for the identification of new TC-DSBR factors.

a. Experimental scheme: DIvA cells allow to temporally induce, following treatment with 4-hydroxytamoxifen (4-OHT), DSBs at annotated positions on the human genome, among which >50% fall in active loci (TC-DSBs). TC-DSB potential interacting partners were identified through a proteomic approach\textsuperscript{13}. 130 proteins were further subjected to a multi output screen in AID-DIvA cells using a siRNA library, to score γH2AX foci intensity, cell survival, chromosome rearrangement and DSB clustering after DSB induction by 4-OHT. Data are expressed normalized to the Control siRNA (CTRL, black). A positive control (SETX) was included in the experiment (red bar). PPHLN1 and PER complex siRNAs are highlighted in green and blue respectively.

b. γH2AX foci intensity was scored in siRNA transfected cells, using quantitative high throughput imaging.

c. Cell survival of siRNA transfected cells was quantified eight days after DSB induction, using a colorimetric assay based on mitochondrial dehydrogenase activity in viable cells.

d. DSB-induced rearrangement frequency \(t(MIS12;TRIM37)\) was analyzed by qPCR following DSB induction and repair.

e. γH2AX foci mean area was scored in siRNA transfected cells, using quantitative high throughput imaging.

f-i. Scatter plots showing the correlations for each siRNA between γH2AX intensity and cell survival (f); γH2AX intensity and translocations (g); translocations and DSB clustering (h) and
cell survival and translocations (i). The 95% confidence intervals and the coefficient of correlation (r, Pearson) are indicated.

**Figure 2.** PER proteins regulate γH2AX foci formation, translocation, and cell survival upon DSB induction

a. γH2AX staining performed in control (CTRL), PER1 or PER2-siRNA transfected DIvA cells, before (-DSB) and after (+DSB) DSB induction. Quantification is shown on the bottom panel (>7000 nuclei, from a representative experiment). Center line: median; box limits: 1st and 3rd quartiles; whiskers: maximum and minimum without outliers. *P*, unpaired t-test.

b. γH2AX ChIP performed in DIvA cells transfected with siRNA control (CTRL) or PER2 before and after DSB induction and analyzed by qPCR at two DSBs (DSB1-2). Data are normalized to a control location devoid of a DSB. Mean and SEM are shown for n =4 biological replicates. *P*, paired t-test (two-sided).

c. *t*(MIS12:TRIM37) rejoining frequencies after DSB induction measured by qPCR in AID-DIvA cells transfected with siRNA CTRL, PER1 PER2, DDX17, SETX, NONO, DDX5 and BMAL1. Mean and SD (n ≥4 biological replicates) are shown. *P*, paired t-test (two-sided).

d. Clonogenic assays in AID-DIvA cells transfected with siRNA CTRL, PER1, PER2, DDX17, NONO, DDX5 or BMAL1. Left and right panels show the mean and SEM after 4-OHT treatment (+DSB) and after IAA treatment (+DSB+Repair) respectively (n ≥4 biological replicates). *P*, paired t-test (two-sided).

**Figure 3.** PER2 is recruited at DSBs and promotes Homologous Recombination repair
a. PER2, DDX5, DDX17 and BMAL1 ChIP efficiency (expressed as % of input immunoprecipitated) before (−DSB) and after (+DSB) DSB induction, at a control genomic locus devoid of DSB, a promoter (BTG2 or PER1 as indicated), and DSB1 or DSB-4. Mean and SEM are shown for n ≥10 replicates. P, paired t-test (two-sided).

b. Genomic tracks showing RNA Polymerase II before DSB induction (RNAPII, green), BLESS after DSB induction (black), as well as BMAL1 (blue) and PER2 (purple) (log2(+DSB/−DSB)) at a TC-DSB (upper panel; chr21:460221789) and a DSB induced in a silent locus (lower panel; chr12:130091880). DSBs are indicated by arrows.

c. Average PER2 (left) and BMAL1 (right) ChIP-seq profiles on a ±5 kb window centered on the eighty best-induced AsiSI-DSBs (top) or eighty random sites (bottom, no DSB). Data are presented in log2(+DSB/-DSB).

d. Box plots representing Log2 (+DSB/-DSB) PER2 ChIP-seq count on a ±2kb at HR-DSB, NHEJ-DSB or random sites (30 in each category). Center line: median; box limits: 1st and 3rd quartiles; whiskers: maximum and minimum without outliers. P-values, Wilcoxon test.

e. Resection assay. (Left) Schematic representation. (Right) quantification of single-strand DNA in control, PER1, PER2 or CtIP siRNA-depleted DIvA cells at two distances (200bp and 1626bp as indicated) from the DSB-1 and at 1122bp from DSB-7. Mean and SEM are shown for n ≥ 3 biologically independent experiments. *: P<0.05, **: P<0.01, ***: P<0.001, ns: not significant. Paired t-test (two-sided)

f. RAD51 ChIP-qPCR in DIvA cells transfected with control or PER2 siRNA before and after DSB induction, at four TC-DSBs (DSB1-4) and one DSB in a silent locus (DSB5). Data are normalized to a control location devoid of a DSB, and further expressed relative to data obtained at DSB4. Mean and SEM are shown for n=3 biological replicates. P, paired t-test (two-sided).
g. RT-qPCR performed before (−DSB) and after (+DSB) DSB induction in control (CTRL), PER1 or PER2 siRNA-depleted DIvA cells for six genes carrying TC-DSB (relative to untreated sample (-DSB)). Mean and SEM are shown for n=6 biological replicates. P, paired t-test (two-sided).

Figure 4: TC-DSBs are targeted to the nuclear envelope through the Sun-domain protein SUN1

a. Super-resolution Live imaging performed using Random Illumination microscopy (RIM) in DIvA cells expressing 53BP1-GFP and mCherry-LaminB1, starting >1h after DSB induction. Arrows show the establishment of a contact between a focus and nuclear lamina. Images were acquired every 40s. Scale bar 1μM.

b. Proximity Ligation Assay (PLA) performed using either 53BP1 antibody (top panels), Lamin B1 antibody (middle panels) or both 53BP1 and LaminB1 antibodies (bottom panels) before (-DSB) and after DSB (+DSB) induction in DIvA cells. PLA quantification is shown on the right panel. An average of 190 cells were analyzed per condition. Center line: median; box limits: 1st and 3rd quartiles; whiskers: maximum and minimum without outliers. P, non-parametric Wilcoxon test.

c. LaminB1 ChIP-qPCR before (−DSB) and after (+DSB) DSB induction, at TC-DSBs (HR-DSBs, DSB1-4) and one DSB induced in a silent locus and repaired by NHEJ (DSB5). Data are normalized to a control location devoid of a DSB. Mean and SEM are shown for n≥3 biological replicates. P, paired t-test (two-sided).

d. Genomic tracks of SUN1(purple) and SUN2 (dark blue) ChIP-seq (log2(+DSB/-DSB)) at a TC-DSB (upper panel; chr17:57184296) and a DSB induced in a silent locus (lower panel; chr12:130091880).
e. Average profiles of SUN1 (left) and SUN2 (right) on a ±5 kb window centered on the eighty best-induced AsiSI-DSBs (top) or eighty random sites (bottom, no DSB). Data are presented as \( \log_2(+\text{DSB}/-\text{DSB}) \).

f. \( \gamma \text{H2AX} \) staining combined with SUN2 (top panel) or SUN1 (bottom panel) performed in DIvA cells after DSB induction and acquired using 3D RIM Super-resolution microscope. The areas represented in yellow (ICQ > 0.45) correspond to a high non-random colocalization (see methods).

g. Left panels: Magnifications of SUN2 (top panels) and SUN1 (bottom panels) staining in nuclear envelope (dotted line) together with \( \gamma \text{H2AX} \) foci. Arrows indicate sites of colocalization. Scale bar 1.5\( \mu \)M. Right panels: Cross Correlation Function (CCF) plots between \( \gamma \text{H2AX} \) foci and NE-embedded SUN2 (top panel, N=70 \( \gamma \text{H2AX} \) foci) or SUN1 (bottom panel, N=46 \( \gamma \text{H2AX} \) foci). CCF of bicolor 3D RIM images was determined by plotting the value of Pearson’s correlation coefficient \( P[0.1] \) against \( \Delta x \) for each voxel of the image (see methods). The arrows show a non-random exclusion between \( \gamma \text{H2AX} \) and SUN2 (dip at \( \Delta x=0 \)) or a non-random overlap between the signals of SUN1 and \( \gamma \text{H2AX} \) (peak at \( \Delta x=0 \)).

h. Clonogenic assays in AID-DIvA cells transfected with control (CTRL), SUN1 or SUN2-siRNA. Mean and SEM after OHT treatment (+DSB) and after IAA treatment (+DSB+Repair) are shown (n=5 biological replicates). \( P \), paired t-test (two-sided). *: \( P<0.05 \), ns: not significant.

i. \( t(MIS12:TRIM37) \) and \( t(LINC00271:LYRM2) \) rejoining frequencies measured by qPCR in AID-DIvA cells transfected with control (CTRL), SUN1 or SUN2-siRNA. Mean and SEM (n≥4 biological replicates) are shown. \( P \), paired t-test (two-sided). *: \( P<0.05 \), **: \( P<0.01 \).
Figure 5. PER proteins promote DSB nuclear envelope targeting and counteract DSB clustering

a. LaminB1 ChIP-qPCR before (−DSB) and after (+DSB) DSB induction in control and PER2 siRNA-depleted DIvA cells, at TC-DSBs (DSB1-4, HR-DSBs) and a DSB in a silent locus (DSB5, NHEJ-DSBs). Data are normalized to a control location devoid of DSB. Mean and SEM are shown for n=4 biological replicates. P, paired t-test (two-sided).

b. Number of PLA foci (53BP1-LaminB1) in CTRL, PER1 or PER2-siRNA transfected DIvA cells as indicated, before (−DSB) and after (+DSB) DSB induction. Data were normalized to the untreated samples. Mean and SEM (n=3 biological replicates) are shown. P, paired t-test (two-sided).

c. Number of PLA foci (γH2AX-LaminB1) following DSB induction in DIvA cells transfected with CTRL or PER2 siRNA, in G1, S, and G2 cells determined by EdU and DAPI incorporation. >7000 nuclei were acquired in each condition for each biological replicates. Data were normalized to untreated cells (-DSB). Mean and SEM (n=3 biological replicates) are shown. P-values, non-parametric Wilcoxon test.

d. Clonogenic assay in control, PER1, PER2, PER1+SUN1 and PER2+SUN1 siRNA transfected AID-DIvA cells after DSB induction (+DSB, left panel), and after DSB induction and IAA treatment allowing DSB repair (+DSB+repair, right panel). Mean and SEM are shown for n=4 biologically independent experiments. P, paired t-test (two-sided).

e. Differential Hi-C contact matrix [(+DSB)-(−DSB)] on a region located on chromosome 2 at 25kb resolution in DIvA cells transfected with a control siRNA or a siRNA directed against PER2 as indicated. γH2AX ChIP-seq track (+DSB) are also shown (red).
f. Interchromosomal DSBs interaction shown as aggregate peak analysis (APA) plotted on a 200kb window (10kb resolution) before (-DSB) and after DSB (+DSB) induction in control and PER2 siRNA-transfected DIvA cells for the eighty best-induced AsiSI-DSBs (left) or eighty random sites (right, no DSB). FC: Fold change calculated between the central pixel and a square of 3x3 pixels on the bottom left corner of the matrix. APAs show an increased signal at the center (DSB-DSB interaction) after DSBs induction only for DSB sites (FC= 3.49, compared to 0.89 for random sites). The signal increases after PER2 depletion (FC= 3.85).

g. Box plot showing the differential Hi-C read counts (as (log2 +DSB/-DSB)) between the 80 best induced DSBs (+/- 500kb) (left) or 80 random sites (right) in control (CTRL, grey) and PER2 (purple) siRNA-transfected DIvA cells. Center line: median; box limits: 1st and 3rd quartiles; whiskers: maximum and minimum without outliers. P, non-parametric Wilcoxon test.

h. Genomic tracks of γH2AX ChIP-seq after DSB induction(red) and of Chromosomal Eigen vectors (CEV) obtained from PCA analyses performed on differential +DSB/-DSB Hi-C matrices. CEV in siCTRL (grey) and siPER2 (blue). The D-compartment is represented as positive values. siPER2 triggered the appearance of the D-compartment on the chromosome 2 normally devoid of D compartmentalization post-DSB induction in DIvA cells (areas highlighted in light grey).

Figure 6. The Circadian rhythm regulates TC-DSB targeting to nuclear envelope and RAD51 loading

a. γH2AX foci intensity, measured by high content microscopy, in DIvA cells after DSB induction (+DSB 2h), in G1, S and G2 phases at different time points after dexamethasone (Dex) treatment (synchronization of the circadian rhythm). Bars at the different time points are colored according to the PER2 expression level (from highest in blue, to lowest in white). Data
were normalized to the average. Mean and SEM (n = 3 biological replicates, with >3000 nuclei acquired per experiment) are shown.

b. γH2AX ChIP-qPCR performed in DIvA cells before (-DSB) and after DSB (+DSB) induction at 28h and 40h after dexamethasone treatment (Dex) at three TC-DSBs (DSB1-3). Data were normalized to a control location devoid of DSB and expressed relative to DSB4-28h. Mean and SEM for n=5 biological replicates. P, paired t-test (two-sided).

c. RAD51 ChIP-qPCR performed in DIvA cells before (-DSB) and after DSB (+DSB) induction at 28h and 40h after dexamethasone (Dex) treatment at four TC-DSBs (DSB1-4) and two NHEJ-DSBs (DSB5-6). Data were normalized to a control location devoid of DSB and expressed relative to DSB4-28h. Mean and SEM for n=6 biological replicates. P, paired t-test (two-sided).

d. LaminB1 ChIP-qPCR performed in DIvA cells before (-DSB) and after DSB (+DSB) induction at 28h and 40h after dexamethasone (Dex) treatment at four TC-DSBs (DSB1-4) and DSB5 (DSB in silent locus). Data were normalized to a control location devoid of DSB and expressed relative to the undamaged condition. Mean and SEM for n=5 biological replicates. P, paired t-test (two-sided).

e. SUN1 ChIP-qPCR performed in DIvA cells before (-DSB) and after DSB induction (+DSB) at 28h and 40h after dexamethasone (Dex) treatment at four TC-DSBs (DSB1-4) and DSB5. Data are normalized to a control location devoid of DSB and further expressed related to data obtained in the undamaged condition. Mean and SEM for n=3 biological replicates. P, paired t-test (two-sided).

f. γH2AX foci mean area, measured by high content microscopy, in DIvA cells after DSB induction (+DSB 2h), in G1, S and G2 phase at different time points after dexamethasone (Dex)
treatment. Data were normalized to the average. Mean and SEM (n=3 biological replicates) are shown. P, paired t-test (two-sided).

g. DSB responsive genes expression was measured by RT-qPCR before (−DSB) and after (+DSB) DSB induction in DivA cells at 28h and 40h after dexamethasone treatment. cDNA level of a D-compartment DSB responsive gene (RNF19B), two non-D compartment DSB responsive genes (PPM1D and SLC9A1) and two control genes not regulated post DSB induction (LPHN2, UTP18) are shown. Mean and SEM for n=4 biological replicates. P, paired t-test (two-sided).

h. Model. During the day (PER phase), the PERIOD complex is recruited at DSBs occurring in transcribed loci (TC-DSBs). This triggers the targeting of the TC-DSBs to the NE and their tethering to the SUN1 INM protein, further ensuring RAD51 loading and HR repair. During the night (BMAL1 phase), in absence of PER1/2, impaired TC-DSB anchoring to the NE leads to impaired RAD51 loading, and enhanced DSB clustering and D-compartment formation.
Methods

Cell culture and treatment

U2OS, DIvA (AsiSI-ER-U20S)\(^9\), AID-DIvA (AID-AsiSI-ER-U20S)\(^8\) and 53BP1-GFP DIvA\(^8\) cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10%SVF (Invitrogen), antibiotics and either 1µg/mL puromycin (DIvA cells) or 800µg/mL G418 (AID-DIvA cells) or both puromycin and G418 (53BP1-GFP DIvA) at 37°C under a humidified atmosphere with 5%CO\(_2\). The cell lines were regularly checked for mycoplasma contamination. For AsiSI-dependent DSB induction, DIvA cell lines were treated with 300nM 4-hydroxytamoxifen (OHT) (Sigma, H7904) for 4h (unless otherwise indicated). For DSB induction in U2OS, cells were treated with 1µM etoposide (Sigma, E1383) for 2h or with 10µg/mL Bleomycin (Sigma, B8416) for 2h. To stop inducing AsiSI-DSBs, AID-DIvA cells were washed twice in pre-warmed PBS after OHT treatment and further incubated with 500µM auxin (IAA) (Sigma, I5148) for 2h (unless otherwise indicated). For circadian rhythm synchronization, cells were incubated for 1h with 500nM dexamethasone (Dex) Sigma, D4902), then the medium was replaced (time = 0h). Dex treatment was done every 2h during 24h and the DSB induction was started for all synchronized cells at the same time, 24h after the last Dex treatment, to obtain a full period circadian rhythm (analyses are shown from 24h to 46h post-Dex). To induce DSBs, synchronized cells are treated during 2h or 4h surrounding the synchronization timepoint (e.g., from 23h to 25h after Dex for a 2h treatment corresponding at the synchronized time = 24h).

Multi-output screen methodology

Each of the 130 SMARTpool siRNAs from the TC-DSBR-focused siRNA library (Extended Data Table 1) was transfected in 10\(^6\) AID-DIvA cells as described below (siRNA and plasmid transfection section), always including a negative (CTRL) and a positive (SETX)
control siRNAs. Transfected cells were plated (i) in 96-well Cell Carrier Ultra plates (20,000 cells/well in triplicates per condition) for γH2AX foci analysis (intensity and area) with quantitative high throughput imaging as described below (High-Content microscopy and Immunofluorescence sections); (ii) in 96-well plates (1000 cells/well in quadruplicates per condition for cell survival analysis as described below (WST-1 cell survival assay section); and in 10cm diameter dish for genomic DNA extraction and translocation frequency analysis as described below (DSB-induced rearrangement /Translocation assay section). All data from these different outputs post-DSB induction were expressed normalized to the negative control siRNA (CTRL) and compared to the positive control siRNA (SETX).

**siRNA and plasmid transfection**

siRNA transfections were performed using the 4D-Nucleofector and the SE cell line 4D-Nucleofector X kits (Lonza) according to the manufacturer’s instructions. Briefly, 1-10µL of 100µM annealed siRNA was transfected in 1-20x10^6 cells with 20-100µL SE solution in Nucleocuvettes using the U2OS program CM-104. Subsequent cell treatments were performed ~42-48h post-transfection. Most siRNAs used in this study were siGENOME SMARTpools (Dharmacon) which are a mixture of 4 individual siRNAs, see Extended Data Table 1. Individual siRNA sequences (Eurogentec) were used for SUN1, SUN2 or RBBP8/CtIP (Extended Data Table 1). For all experiments, the control siRNA (CTRL) was the siGENOME Non-Targeting Control siRNA Pool #2 (Dharmacon), except for Fig. 4h and Fig. 4i where the control siRNA used with SUN1 and SUN2 siRNAs was Ctrl1 (Extended Data Table 1).

Plasmid transfections were performed using the 4D-Nucleofector and the SE cell line 4D-Nucleofector X S kit (Lonza) according to the manufacturer’s instructions. Briefly, 0.5µg DNA was transfected in 10^6 cells with 20µL SE solution in Nucleocuvette strips using the U2OS program CM-104, and subsequent OHT treatments were performed 48h later. mCherry-
LaminB1-10 plasmid was a gift from Michael Davidson (Addgene plasmid #55069; http://n2t.net/addgene:55069 ; RRID:Addgene_55069).

**Immunofluorescence**

Transfected 53BP1-GFP DivA cells were grown on glass coverslips and fixed with 4% Paraformaldehyde during 15min at room temperature. After, 2 washes with PBS, the permeabilization step was performed by treating cells with 0.5%Triton X-100 in PBS for 10min, then cells were blocked with PBS-BSA3% for 30min. Primary antibody against γH2AX (Extended Data Table 4) was diluted in PBS-BSA3% and incubated with cells overnight at 4°C.

After 3 washes for 5min in PBS-BSA3%, cells were incubated with anti-mouse secondary antibody (conjugated to Alexa594 or Alexa488, Invitrogen), diluted 1:1000 in PBS-BSA3%, for 1h at room temperature. After a Hoechst 33342 (Invitrogen) staining (5µg/ml for 5min at room temperature), Citifluor (AF1-25, Clinisciences) was used for coverslip mounting.

**High content microscopy (QIBC)**

U2OS, DivA (AsiSI-ER-U20S)\(^9\), AID-DivA (AID-AsiSI-ER-Transfected or Dex-synchronized DivA and U20S cells were plated in 96-well Cell Carrier Ultra plates (Perkin Elmer). Prior to the end of OHT, etoposide or bleomycin treatments, cells are treated with 10µM EdU (Invitrogen, C10340) at 37 °C for 15min. For γH2AX staining, cells are fixed and permeabilized as described above. For RAD51 staining, buffer II pre-extraction (20mM NaCl, 0.5% NP-40, 20mM HEPES at pH 7.5, 5mM MgCl\(_2\), 1 mM DTT) was added to cells for 20min on ice followed by fixation in 4% Paraformaldehyde during 15min at room temperature and washed twice with PBS. Then EdU was labeled by Click-iT AlexaFluor 647 reaction (Invitrogen, C10340) according to the manufacturer’s instructions (cells protected from light from now). Cells were washed twice for 5min in PBS-BSA3% and then blocked with PBS-BSA3% for 30min at room temperature. Primary antibodies targeting γH2AX or RAD51...
(Extended Data Table 4) were diluted in PBS-BSA3% and incubated with cells overnight at 4°C. After 3 washes of 3 min in PBS-BSA3%, cells were incubated with anti-mouse Alexa 488 secondary antibody (Invitrogen) diluted 1:1000 in PBS-BSA3%, for 1 h at room temperature.

After 3 washes of 3 min in PBS, nuclei were labeled with 5 μg/ml Hoechst 33342 (Invitrogen) for 30 min at room temperature. The cells were then washed twice with PBS and stored at 4°C.

γH2AX foci were further analyzed with an Operetta CLS High-Content Imaging System (Perkin Elmer) and Harmony software (version 4.9). For quantitative image analysis, ~20-80 fields per well were acquired with a 20X (for γH2AX circadian synchronization) or 40X objective lens to visualize >3000 cells per well in triplicate. Subsequent analyses were performed with Columbus software (version 2.8.2). Briefly, the Hoechst-stained nuclei were selected with B method, and the size and roundness of nuclei were used as parameters to eliminate false positive compounds. Then, the Find Spots D method was used (Detection sensitivity = 0.2-0.4; Splitting coefficient = 1; Background correction = 0.5) to determine the number, mean area and intensity of repair foci in each nucleus. For cell cycle analysis, the sum of the Hoechst intensity and the mean of the EdU intensity were plotted in order to select G1, S, and G2 cells.

**DSB-induced rearrangement/translocation assay**

AID-DIVa cells were treated as indicated and DNA was extracted using the DNeasy kit (Qiagen). Illegitimate joining frequencies between different AsiSI sites, \( t(MIS12;TRIM37) \) and \( t(LINC00217;LYRM2) \), were analyzed by qPCR (in 3-4 replicates) using primers referenced in Extended Data Table 3. Results were normalized using two control regions (Norm1 and Norm17) both far from any AsiSI sites and γH2AX domains (primers in Extended Data Table 3). Normalized translocation frequencies were calculated using the Bio-Rad CFX Manager 3.1 software.
WST-1 cell survival assay

Transfected AID-DIVa cells were plated in 96-well plates for each of the 3 conditions: -DSB, +DSB, +DSB+repair. 48h later, cells were treated or not with OHT for 4h and further incubated or not with IAA for 4h. Eight days after DSB induction, the medium is replaced by a mix of 100µl warmed medium + 10µl WST-1 ready-to-use solution per well and the cells were incubated for 2h at 37°C. The tetrazolium salts from Premix WST-1 Cell Proliferation Assay System (TAKARA) are cleaved by mitochondrial dehydrogenase in viable cells to produce formazan dye, which is quantified by measuring its absorbance at 450nm (Multiskan GO Microplate Spectrophotometer from Thermo Fisher Scientific). The percentage of viable cells is normalized to the undamaged cells and compared to the control condition.

Clonogenic assays

Transfected AID-DIVa cells were seeded, in duplicate, at a clonal density in 10cm diameter dishes and, 48h later, were treated or not with 4OHT for 4h and washed twice in pre-warmed PBS and further incubated with auxin (IAA) for another 4h when indicated. Then, the medium was replaced for all cell dishes. After 8 days at 37°C under a humidified atmosphere with 5%CO₂, cells were washed in PBS and stained with crystal violet (Sigma, V5265). Images of stained colonies were obtained using the White Sample Tray for the ChemiDoc Touch Imaging System (Bio-Rad) and colonies were counted using the Spot Detector plugin of Icy software, Version 2.0.2.0 (icy.exe program). Only colonies containing more than 50 cells were scored.

Western blots

DIVa cells were incubated in RIPA buffer (50mM Tris pH8.0, 150mM NaCl, 0.5% sodium deoxycholate, 1% NP-40, 0.1% SDS) on ice for 20 min. Samples were further incubated
with 250 units of Benzonase (Sigma, E1014) per million of cells for 10 min at room
temperature, then centrifuged at 13,000 rpm for 10 min at 4°C. The supernatants, containing
soluble protein extracts, were then mixed with SDS loading buffer and reducing agent, and
incubated 10 min at 70°C. Electrophoreses were performed using 4–12% NuPAGE Bis-Tris
gels (Invitrogen) and semi-dry western blotting on nitrocellulose membranes (Bio-Rad) was
done with the Trans-Blot Turbo System (Bio-Rad) according to the manufacturer’s instructions.
Membranes were incubated in TBS containing 0.1% Tween 20 (Sigma, P1379) and 4% BSA
during 1h at room temperature for blocking, followed by overnight incubation at 4 °C using
primary antibody diluted in TBS-Tween-4% BSA. The corresponding mouse or rabbit
horseradish peroxidase-coupled secondary antibodies (Sigma, A2554 and A0545) were used at
1:10,000 to reveal the proteins, using a luminol-based enhanced chemiluminescence HRP
substrate (Super Signal West Dura Extended Duration Substrate, ThermoScientific). Picture
acquisition of the membranes was done using the ChemiDoc Touch Imaging System (Bio-Rad)
and pictures were analyzed using Image Lab software (Bio-Rad). All primary antibodies used
in this study are detailed in Extended Data Table 4. Note: although different conditions and
several human PER1 antibodies were tested, none of them resulted in a ~140kDa signal
corresponding to the PER1 protein on the western-blots.

**RT-qPCR**

Total RNA was extracted from U2OS and DIvA cell lines before and after DSB
induction using homogenization with QIAshredder and the RNeasy kit (Qiagen) following the
manufacturer’s instructions. RNA was then reverse transcribed to cDNA using the AMV
reverse transcriptase (Promega, M510F). qPCR experiments were performed to assess the
levels of cDNA using primers referenced in Extended Data Table 3. cDNA levels were then
normalized with GAPDH (Gene ID: 2597), TBP (Gene ID: 6908) and 18S (RNA18SN5, Gene
ID: 100008588) cDNA levels using the CFX Manager 3.1 software (Bio-Rad).
Resection assay

Measure of resection was performed as described in\textsuperscript{59} with the following modifications. Genomic DNA was extracted from transfected DIvA cells just after DSB induction using the DNeasy kit (Qiagen) without any vortexing step. 1 µg of DNA was incubated 20 min at 37°C with 15 units of RNaseH1 (New England Biolabs) and first digested for 2 h at 37°C with 35 units of AsiSI restriction enzyme (New England Biolabs). Then, 250 ng of DNA were further digested overnight at 37°C using 10 units of BanI restriction enzyme, that cuts at 200 bp and 1626 bp from the DSB\textsuperscript{1}, or of BanII restriction enzyme, that cuts at 1122 bp from the DSB\textsuperscript{7} (Extended Data Table 2). Both enzymes were heat inactivated for 20 min at 80°C. Digested and undigested samples were analyzed by qPCR (10 ng/well) using primers in Extended Data Table 3. ssDNA% was calculated with the following equation: Percent of ssDNA=\textsuperscript{1}/((\textsuperscript{2};(Ct Digested − Ct Non Digested) − 1) + 0.5)*100.

Proximity Ligation Assay (PLA)

PLA experiments were performed using Duolink\textsuperscript{®} In Situ Detection assay from Sigma-Aldrich according to the manufacturer’s instructions. Primary antibodies used for PLA are detailed in Extended Data Table 4.

\textit{γH2AX-LaminB1 PLA.} siRNA-transfected or Dexamethasone-synchronized DIvA cells were plated in 96-well Cell Carrier Ultra plates (Perkin Elmer). At the end of 4OHT treatment, cells are treated with 10 µM EdU (Invitrogen, C10340) for 15 min. Cells are fixed with 4% paraformaldehyde during 15 min at room temperature. Permeabilization step was performed by treating cells with 0.5% Triton X-100 in PBS for 5 min. Then EdU was labeled by Click-iT Alexa488 reaction (Invitrogen, C10340) according to the manufacturer’s instructions. Cells were blocked with 1 drop of blocking solution provided with the Duolink\textsuperscript{®} PLA Probes (DUO92002 and DUO92004, Sigma-Aldrich) for 1 h at 37°C in a humid chamber. Primary
antibodies targeting γH2AX and LaminB1 were diluted in the antibody dilution buffer provided with the Duolink® PLA Probes and incubated with cells overnight at 4°C. After two 5min washes in Duolink® In Situ Wash Buffer A (DUO82049, sigma-Aldrich), cells were incubated with anti-mouse plus and anti-rabbit minus probes for 1h at 37°C, under slow rotation. After two washes in wash buffer A for 5min, cells were incubated for 30min at 37°C in the ligation reaction mix from the Duolink® In Situ Detection Reagents Red (DUO92008, Sigma-Aldrich). The cells were washed again twice in wash buffer A for 5min and incubated for 1h40 at 37°C under slow rotation for the amplification step. Finally, after two 10min washes in wash buffer B (DUO82049, sigma-Aldrich), nuclei were stained in the staining solution from the Duolink® In Situ Microplate Nuclear Stain (DUO82064, Sigma-Aldrich) for 30min at 37°C and cells were kept in the Anti-Fade solution (DUO82064, Sigma-Aldrich) until image acquisition. For quantitative image analysis, images were acquired with a 40X objective lens to visualize >3000 cells per well in duplicate. Subsequent analyses were performed with Columbus software (version 2.8.2) to determine the numbers of PLA foci in each nucleus. G1, S and G2 nuclei were selected based on EdU and DAPI staining distribution in all cells.

**53BP1-LaminB1 PLA.** siRNA-transfected cells were seeded on 13mm coverslips and treated for 4h with 4OHT before fixation in 4%PFA. Slides were then processed as before, except that there were no EdU staining, and that at the end of the PLA procedure, after the two 10min washes in wash buffer B, cells were then washed for 1min in 0.01% wash buffer B and mounted on slides with Duolink® In Situ Mounting Medium with DAPI (DUO82040, Sigma-Aldrich). Image acquisition was done on Leica DM6000 at 40X objective lens to visualize >175 cells per experiment in triplicate. Subsequent analyses were performed with Cell Profiler software (version 4.2.4) to determine the numbers of PLA foci in each nucleus.

**3D super resolution using Random Illumination Microscopy (RIM)**
**3D RIM two cameras setup.** The 3D RIM home-made setup is coupled to an inverted microscope (TEi Nikon) equipped with 100x magnification, a 1.49 N.A. objective (CFI SR APO 100XH ON 1.49 NIKON) and two SCMOS cameras (ORCA-Fusion, Hamamatsu) mounted in an industrial apochromatic alignment module (abbelight SA). Fast diode lasers (Oxxius) with wavelengths centered at 488nm (LBX-488-200-CSB) and 561nm (LMX-561L-200-COL) are used for all experiments. The bandpass emission filters in front of the two respective cameras are FF01-514/30-25 for camera 1 and FF01-609/54-25 for camera 2. The binary phase modulator (QXGA fourth dimension) conjugated to the image plane combined with polarization elements are used to generate dynamic speckle on the object plane as described in\textsuperscript{36}. The synchronization of the hardware (Z-platform, cameras, microscope, laser and SLM) is performed by an improved version of the commercial software INSCOPER.

**Fixed 3D RIM acquisition and reconstruction.** Two-color 3D RIM imaging was performed with the 3D RIM system. The acquisition of 40 planes was done sequentially with a 120nm step in the image plane. For each image plane, 400 speckles were used to increase the desired super-resolved 3D resolution (95nm,95nm,200nm). The image reconstructions were performed with the software (ALgoRIMhttps://github.com/teamRIM/tutoRIM). The Wiener filter used is 0.01, the deconvolution parameter is 0.02 and the regularization parameter is 0.02 for the first channel images. The Wiener filter used is 0.02, the deconvolution parameter is 0.03 and the regularization parameter is 0.02 for the second channel images. The misalignment between the two cameras and the residual chromatic aberrations are corrected using the SVI (Scientific Volume Imaging) software.

**3D+t two colors RIM super-resolution acquisition and reconstruction.** 3D+t RIM movies were performed on AID-DiVA cells expressing 53BP1-GFP and mcherry-LaminB1. 3D movies of 47min with 40sec intervals were made (60 times). The whole cell acquisition time
was 6 seconds with 48 random patterns optimized for live cells for each plane. Image reconstructions were performed with the software (ALgoRIMhttps://github.com/teamRIM/tutoRIM). The Wiener filter used is 0.01, the deconvolution parameter is 0.151 and the regularization parameter is 0.08 for the first channel images. The Wiener filter used is 0.1, the deconvolution parameter is 0.25 and the regularization parameter is 0.08 for the second channel images. The misalignment between the two cameras and the residual chromatic aberrations are corrected using the SVI (Scientific Volume Imaging) software.

**3D film editing.** Bleaching correction is performed after RIM reconstruction with the open-source FIJI 546 software (https://imagej.net/software/fiji/) based on an exponential FIT from the background. The FIJI 3D drift correction plugin is performed for 3D registration (https://github.com/fiji/Correct_3D_Drift). Rendering of 3D+t movies was performed with the VTK library implemented in ICY software (https://icy.bioimageanalysis.org/) from 3D CROP on 550 areas of interest. Chromatism correction was performed by Scientific Volume Imaging (SVI) software.

**Colocalization analyses.** The colocalization analyzer from the Scientific Volume Imaging (SVI) software is used on fixed 3D RIM images to quantify the interactions of γH2AX nanofoci with those of SUN1 and SUN2.

**Quantitative immunocolocalization (ICQ).** The intensity correlation quotient was defined in by the following equation for each voxel "i":

\[
(I_i^{D1} - I_{avg}^{D1})(I_i^{D2} - I_{avg}^{D2})
\]

For a random or mixed interaction, this number will tend towards 0, and for a dependent correlation, it will tend towards +0.5. This parameter does not directly use the intensity of each
pair of voxels and has the advantage of eliminating the bias towards particularly high or too low intensities.

Cross-correlation function CCF. The Van Steensel cross-correlation function (VSCF) CCF is used to quantify interactions and has been described in\textsuperscript{61}. It is obtained by calculating the Pearson coefficient after shifting the second camera image over a distance of $\Delta x$ voxels. Thresholding was carefully tailored for each image to reject the 10\textsuperscript{th} percentile of the lowest intensity value for both channels. The CCF was measured with the x-shift set to 940nm without rotation and the resulting three graphs were averaged and plotted with a 95 percentil. The Pearson coefficient is the classic equation below for each voxel "i":

$$P = \frac{\sum (I_i^{D1} - \bar{I}_{avg}^{D1})(I_i^{D2} - \bar{I}_{avg}^{D2})}{\sqrt{\sum (I_i^{D1} - \bar{I}_{avg}^{D1})(I_i^{D2} - \bar{I}_{avg}^{D2})}}$$

with $I_{avg}^{D1}$ and $I_{avg}^{D2}$ the averages of camera 1 and camera 2 of the microscope.

For better visualization of the shape of the CCF function for each condition, we normalized the CCF function between 0 and 1 with the following normalization calculation:

$$P_{(0,1)} = \frac{(P-P_{min})}{(P_{max}-P_{min})}$$

Negative values for $\Delta x$ indicate a shift in nm of the red image to the left, positive values indicate a shift to the right. Non-random overlap results in a peak at $\Delta x=0$ and non-random exclusion results in a dip at $\Delta x=0$. Uncorrelated distributions will not show any clear peak in the CCF.

ChIP-qPCR, ChIP-seq and data analysis
ChIP-qPCR. ChIP experiments were performed in DIvA cells according to the protocol described in\textsuperscript{8,19} with 200μg of chromatin per immunoprecipitation. The quantity of primary antibodies used is detailed in Extended Data Table 4. qPCR experiments were performed on both IP and input samples to assess the percent of input DNA immunoprecipitated, using AsiSI-induced DSBs referenced in Extended Data Table 2 and primers detailed in Extended Data Table 3. When indicated in the figure legends, data are normalized to a control location devoid of DSB (Ctrl genomic locus) and could be further expressed related to data obtained at DSB4 or in undamaged condition.

Library preparation and sequencing. Multiple ChIP experiment samples were pooled and sonicated for 15 cycles (30 sec on, 30 sec off, high setting) with a Bioruptor (Diagenode), then concentrated with a vacuum (Eppendorf). 10ng of purified DNA (average size 250–300bp) was used to prepare sequencing libraries with the Next Ultra II Library Prep Kit for Illumina (New England Biolabs) using the application note for “Low input ChIP-seq”, and subjected to 75bp single-end sequencing using Illumina NextSeq500 at the EMBL Genomics core facility (Heidelberg, Germany).

ChIP-seq data processing. ChIP-seq data processing was performed as described\textsuperscript{18}. In brief, raw sequencing reads were aligned using bwa (https://bio-bwa.sourceforge.net/) to the human reference genome (hg19) and then, were sorted, deduplicated, and indexed using samtools (http://www.htslib.org/). Bigwig coverage tracks were subsequently extracted from the processed bam files using bamCoverage from deepTools (HYPERLINK https://deeptools.readthedocs.io/en/develop/), and were normalized by total read count to be used for downstream analyses.

DSB Average profiles and boxplots. Differential coverage tracks representing the log2 ratio between damaged and undamaged ChIP-seq samples were created using the bamCompare
function of deepTools on default settings. These bigwigs were then used as input for the computeMatrix function of deepTools to calculate the coverage at DSBs using 200 bins covering either 10kb or 1kb as indicated on Figures. These matrices were then processed using a custom script in R with ggplot2.

**Peak calling.** Peak calling for ChIP-seq datasets (BMAL1, SUN1 and SUN2) was performed using MACS2 using -q 0.1 for BMAL1 resulting in 1776 peaks, and -q 0.005 for SUN1 and SUN2 resulting in 27326 and 14571 peaks respectively.

**Gene Ontology Analysis.** Genes for BMAL1 GO analysis were selected if the identified BMAL1 peak was within a gene's body or within its promoter region. The subsequent gene list was used to perform gene ontology analysis using the enrichGO function from clusterprofiler in R.

**Peak annotation.** The genomic locations of SUN1 and SUN2 peaks were identified by using the ChIPseeker package in R, specifically the annotatePeak function to annotate the regions and plotAnnoPie for subsequent visualization. To calculate the coverage of SUN1 at SUN2 regions, the computeMatrix function of deepTools was used using 200 bins over a 10kb window. The matrix was then processed using a custom script in R with ggplot2.

**Hi-C and data analyses**

**Hi-C libraries.** Hi-C experiments were performed in DlvA cells following transfection with CTRL or PER2 siRNAs and with or without DSB induction as described in62. Briefly, 10^6 cells were used per condition. Hi-C libraries were generated using the Arima-HiC+ for HiC (Arima Genomics) by following the manufacturer’s instructions. DNA was sheared using the Covaris S220 to obtain an average fragment size of 350-400pb. Sequencing libraries were prepared on beads using the Next Ultra II DNA Library Prep Kit for Illumina and Next Multiplex Oligos.
for Illumina (New England Biolabs) following instructions from the Arima-HiC+ for HiC (Arima Genomics). Hi-C data were processed as described in\textsuperscript{12,62} see below.

**Hi-C heatmaps.** Hi-C reads were mapped to hg19 and processed with Juicer v2.0 using default settings (https://github.com/aidenlab/juicer). Hi-C count matrices were generated using Juicer at multiple resolutions: 100kb, 50kb, 25kb, 10kb, 5kb and 1kb. Hi-C heatmap screenshots were obtained using Juicebox (https://github.com/aidenlab/Juicebox/wiki/Download).

**Aggregate Peak Analysis (APA).** APA plots of inter- or intra-chromosomal interactions of DSBs and control regions were created using the APA program of Juicer tools (https://github.com/aidenlab/juicer/wiki/APA) with a 10kb resolution +/- 100kb.

**Trans contact quantification.** To determine inter-chromosomal interaction changes (in trans), we built the whole-genome Hi-C matrix for each experiment by merging all chromosome-chromosome interaction matrices using Juicer and R to obtain a genome matrix with 33kx33k bin interactions for 100kb resolution. Interactions between bins inside damaged TADs (240X240 for the 80 best-induced AsiSI DSBs) were extracted and counted for each condition, then log2 ratio was calculated on normalized count (cpm), and plotted as boxplots or heatmaps (https://github.com/LegubeDNAREPAIR/ATMcompD/blob/main/scriptR/Heatmap_D_trans.R).

**A/B compartment.** To identify A and B main chromosomal compartments, the extraction of the first Eigen vector of the correlation matrix (PC1) was done on the Observed/Expected matrix at 500kb resolution using juicer eigenvector command. The resulting values were then correlated with ATAC-seq signal in order to attributes positive and negative values to the A and B compartment, respectively, on each chromosome. The Observed/Expected bins were then arranged based on the PC1 values and aggregated into 21 percentiles, to visualize A-B...
interactions on the siPER2 and siCTRL experiments (saddle plots), and differences were computed between PER2 and control conditions for each percentile.

**D-compartment.** For D-compartment identification, we retrieved the first component (PC1) of a PCA made on the differential observed over expected Hi-C matrix $\log_2 \left( \frac{\text{damaged}}{\text{undamaged}} \right)$ at 100kb resolution. Each matrix was extracted from the .hic files using Juicer and the ratio was computed bin per bin. Pearson Correlation matrices were then computed for each chromosome, and PCA was applied to each matrix. The first component of each PCA was then extracted and correlated with the position of DSBs. A PC1 showing a positive correlation with DSBs was then called D-compartment, and PC1 showing negative correlation with DSBs were multiplied by -1. We were able to extract the D-compartment on several chromosomes for +DSB/-DSB.

D-compartment (first component of the PCA) was converted into a coverage file using rtracklayer R package. For analysis of DSB responsive gene expression by RT-qPCR, we used the previously determined D compartment genes as described in.

**Contact for Reagent and Resource Sharing**

Further requests for reagents and resources should be asked to the lab PI, Gaëlle Legube (gaelle.legube@univ-tlse3.fr). DIvA cell lines are subjected to an MTA with the CNRS.
Fold change

γH2AX Intensity

Survival

γH2AX foci intensity

Cell survival

γH2AX foci formation

DSB clustering

DSB-induced Rearrangement

Translocation t(MIS12;TRIM37)

Translocation t(MIS12;TRIM37)

Translocation t(MIS12;TRIM37)

Translocation t(MIS12;TRIM37)

Translocation t(MIS12;TRIM37)

Translocation t(MIS12;TRIM37)

Translocation t(MIS12;TRIM37)

Translocation t(MIS12;TRIM37)

Translocation t(MIS12;TRIM37)
Figure 2

a) γH2AX stain of cells treated with DSB and +DSB conditions. Top: Merged channels showing γH2AX (red) and DAPI (blue). Bottom: Quantification of γH2AX intensity per cell normalised to DAPI stain. No significant differences were observed between siRNA treatments.

b) ChIP efficiency (normalized) for γH2AX and Mock ChIP. No significant differences were observed between siRNA treatments.

c) Translocation frequency measured by γH2AX ChIP and Mock ChIP (left panel). No significant differences were observed between siRNA treatments. Right panel shows translocation frequency for γH2AX ChIP and Mock ChIP.

d) Cell survival (normalized to CTRL) treated with DSB and +DSB conditions. No significant differences were observed between siRNA treatments.

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**a**

ChIP Lamin B1

- siCTRL
- siPER2

![Bar chart showing Lamin B1 ChIP efficiency (normalized) for different conditions](image)

**b**

Number of PLA foci (Normalized)

- siRNA CTRL
- siRNA PER1
- siRNA PER2

![Bar chart showing number of PLA foci for different conditions](image)

**c**

Number of PLA foci (Normalized)

- siCTRL
- siPER2

![Bar chart showing number of PLA foci for different conditions](image)

**d**

Cell Survival (%)

- siCTRL
- siPER1
- siPER2
- siSUN1

![Bar chart showing cell survival for different conditions](image)

**e**

γH2AX-LaminB1

![Heatmap showing γH2AX-LaminB1 expression](image)

**f**

DSBs (80) and random sites (80)

- siCTRL
- siPER2

![Heatmaps showing DSBs and random sites for different conditions](image)

**g**

Trans interaction of each damaged domains with the other damaged domains (80 DSBs)

- siRNA CTRL
- siRNA PER2

![Bar chart showing trans interaction for different conditions](image)

**h**

γH2AX

![Heatmap showing γH2AX expression](image)