



Dundee, 21.07.2023

Dear Esther,

I am happy to provide you with our manuscript “Transcription restart promotes and challenges the re-establishment of post-replicated chromatin” by Bandau et al. This is the work I have presented in Heidelberg, and you kindly mentioned that EMBO reports would potentially be interested. We have received 3 reviewer’s comments through Review Commons, and we are pleased to send you our revision plan.

In this study we have combined our expertise in chromatin biology and mass spectrometry to tackle one of the key questions in epigenetics: the mechanisms underlying inheritance of chromatin organization. Using quantitative MS-based proteomics coupled to iPOND, we provide the first comprehensive picture of how transcription restart contributes to chromatin restoration following DNA replication.

We are happy that all reviewers acknowledge the broad interest of our work. The strongest critic comes from Reviewer 1, asking for missing controls (that we provide below), and criticising the normalisation method we used (that we discuss in detail below). Reviewers 2 and 3 ask for additional biological replicates to increase the robustness of our findings, and more mechanistic experiments, such as distinguishing the effect of blocking transcription on DNA replication ahead and behind replisomes. We propose to clarify these important points by performing experiments within the time frame of a 3-month revision. Therefore, please find below our revision plan and a point-by-point response to all reviewer’s comments. We hope you will find them appropriate. If anything is unclear, please let us know.

I am looking forward to hearing from you.

Yours sincerely,

Constance

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I. REVISION PLAN

Comments from reviewers are labelled as followed: **R1.1.1:** Reviewer 1, Comment 1, Section 1. Sections were created to address the multiple points raised by reviewers within one comment.

Description of the planned revisions

- 1) To validate the key findings of this study: use an orthogonal approach, PLA-EdU (R1.2.4)
- 2) To strengthen the iPOND-MS conclusions: increase the number of biological replicates (R2.7, R3.2)
- 3) To distinguish the effect of TPL and DRB ahead and behind replisomes: iPOND-MS in chase experiments (24h, as in Alabert et al. 2015) (R1.2.6, R2.1)
- 4) To measure the proportion of DNA replication independent EdU signal: Quantification of PCNA and EdU foci colocalization (+/- transcription inhibitor) (R1.1.3)
- 5) Test FRAP based prediction: measure RNAPII level on chromatin after short DRB treatment by QIBC (R2.4.1)

Existing Data and information we would like to include in the revised version of the manuscript.

- 1) Effect of DMSO: Analysis of EdU incorporation, cell cycle distribution and DNA damage in +/- DMSO (R1.1.2)
- 2) EdU chase conditions: pulse chase quantification by flow cytometry (R1.1.5)
- 3) Effect of TPL/DRB on DNA repair: Quantification of γ H2AX levels in EdU positive and negative cells (R1.1.3)
- 4) Effect of TPL/DRB on the replication program: Analysis of S phase progression, cell cycle- and S phase-distribution (QIBC) (R1.1.4)
- 5) DNA damage on newly replicated chromatin: PLA experiment between EdU and γ H2AX +/- transcription inhibition as well as the quantification of the H2AFX peptide from iPOND-MS (n=3) (R2.1).
- 6) DNA damage on chromatin: Quantification of γ H2AX +/- transcription inhibition on total chromatin via QIBC and Western Blot (R1.1.2, R2.1)
- 7) Assessment of the reproducibility of the dataset: Clustering and enrichment of selected proteins in each individual biological replicate (R3.2).
- 8) Expand the limitation section (R3.1 and 3.4)

Description of analyses that authors prefer not to carry out.

At this point, we do not consider repeating all the iPOND-MS experiments using a spike-in for external normalisation due to the reasons presented in R1.2.1 (p6).

II. POINT BY POINT RESPONSE TO REVIEWER COMMENTS

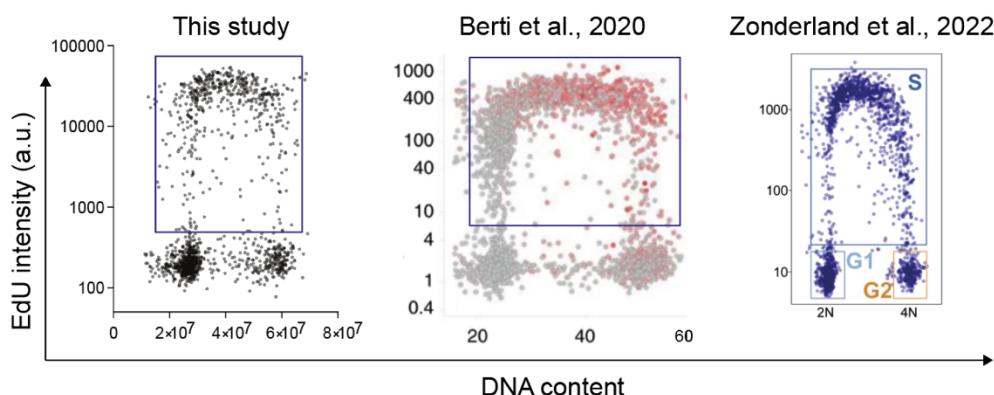
Reviewer #1 (Evidence, reproducibility and clarity (Required)):

The goal was to characterize the changes in the composition of the proteome associated with replicated DNA in conditions of genome-wide inhibition of transcription initiation or transcription elongation. They use iPOND, a MS based technique that identifies proteins specifically associated with replicated DNA labeled with EdU. They use non-synchronized foetal human lung fibroblasts and examine time points immediately after replication (after the 11 min EdU pulse) and 1 and 2 hrs after the Thymidine "chase" later when chromatin has "matured", to assess how the inhibition of transcription influences chromatin maturation and the binding of chromatin associated proteins to replicated DNA. The question is pertinent and is in line with the long-standing interest of the group in chromatin replication dynamics. They conclude that 1. RNAPII loading is necessary for the binding of some TFs, chromatin remodelers and DNA repair factors and 2. RNAPII elongation is needed for H2A.Z incorporation , H3K36me2 restoration and DNA repair factor binding. Transcription is on the other hand not needed for nucleosome assembly, histone acetylation and H3K9me3, H3K27me3 and H4K20me2 incorporation or restoration.

There are two main issues that make the interpretation of their results very difficult and make me question their conclusions:

R1.1.1 They don't provide sufficient evidence that the treatments with TPL and DRB do not interfere with replication. The distributions of EdU intensity per EDU+ cell after treatment in Figures 1D-E and S1A are not sufficient. It is not clear why EdU incorporation is so heterogeneous in the cell population (the range of intensities goes from near 0 to 50000!), which makes me wonder if the DMSO treatment also has an effect on replication. I don't think this heterogeneity can simply be explained by the fact that the cell population is asynchronous.

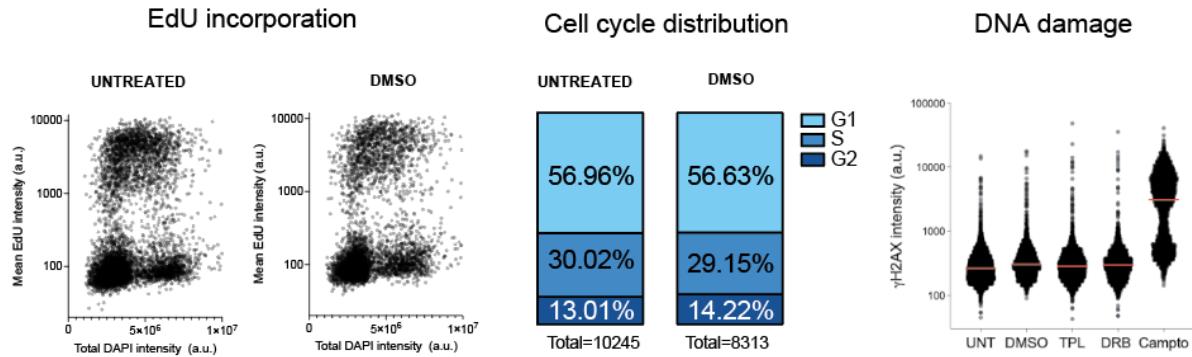
In Figure 1D-E, a linear scale is shown instead of a logarithmic scale to facilitate comparison of EdU incorporation upon DMSO, TPL and DRB treatments. As shown below, the range of EdU intensities is in good accordance with previous publications (see examples from Massimo Lopes and Luis Toledo labs).



Range of EdU intensities. Cells were labelled with EdU and imaged by Quantitative Image-based Cytometry (QIBC). EdU intensities (a.u., arbitrary units) are shown as a function of total DAPI intensities. Each dot represents a nucleus identified based on DAPI intensities. This study, data from Fig. 1D, EdU intensities in S phase cells range from 800 to 50.000. Berti et al. 2020, from 6 to 600. Zonderland et al. 2022, from 30 to 4000.

R1.1.2 They need to show a -DMSO control as well.

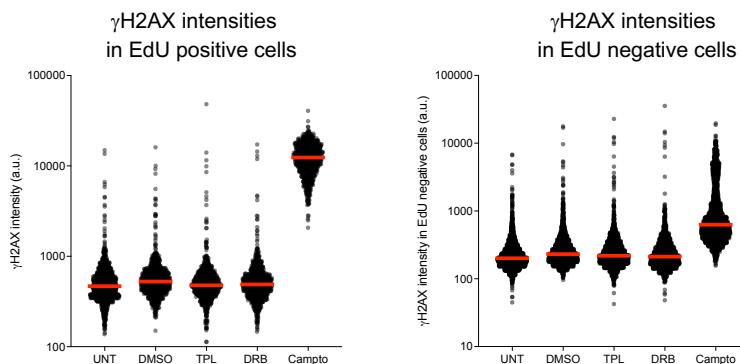
A standard concentration of DMSO was used (0.06%). When comparing DMSO and no DMSO conditions, no defects in EdU incorporation, changes in cell cycle distribution or increases in DNA damage were observed (see panels below). These controls will be added in the revised version of the manuscript.



Comparison of DMSO and no DMSO conditions. Left, EdU incorporation. Cells were labelled with EdU for 20 min and imaged by QIBC. Each dot represents a single cell. Middle, cell cycle distribution. EdU and DAPI were used to gate G1, S and G2 phase cells. Right, quantification of γ -H2AX fluorescence signals in individual cells. Each dot represents a single cell. Median values are shown in red. Cells were treated 3h with Camptothecin (Campto, 1 μ M) as a positive control for DNA damage. >1464 nuclei were analysed per condition.

R1.1.3 Besides since they are only using a positive EdU signal as their criteria for replicating cells, they cannot rule out that some of the EdU signal is coming from DNA repair after replication and depending on how deleterious DMSO/TPL/DRB are to replication the fraction of cells that undergo DNA repair might be significant.

The drug treatments used in this study, DMSO, TPL and DRB, do not increase γ -H2AX signal (see right panel above). γ H2AX levels have also been measured in EdU positive and negative cells (see panels below). It seems therefore unlikely that a significant proportion of EdU signal is coming from DNA repair. These controls will be included in the revised version of the manuscript. Moreover, we propose to quantify by microscopy the percentage of EdU foci colocalising with chromatin bound PCNA in +/- transcription inhibitor conditions, to quantify the proportion of DNA replication independent EdU signal.

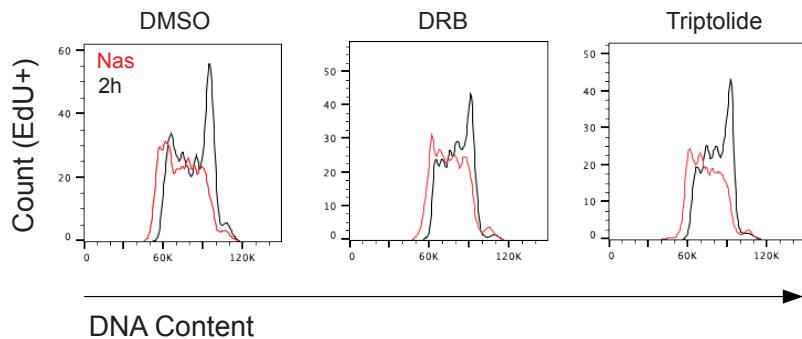


Quantification of γ H2AX signal in EdU positive and negative cells. Quantification of γ H2AX fluorescence signals in individual cells measured by QIBC. Cells were treated with TPL and DRB according to conditions used in the study, and labelled with EdU for the last 20 min. Camptothecin treatment is used as a positive control for DNA damage. >600 nuclei were analysed per condition.

R1.1.4 More importantly, they need to show that the various treatments don't interfere with the replication program, especially since replication is coupled with new nucleosome assembly

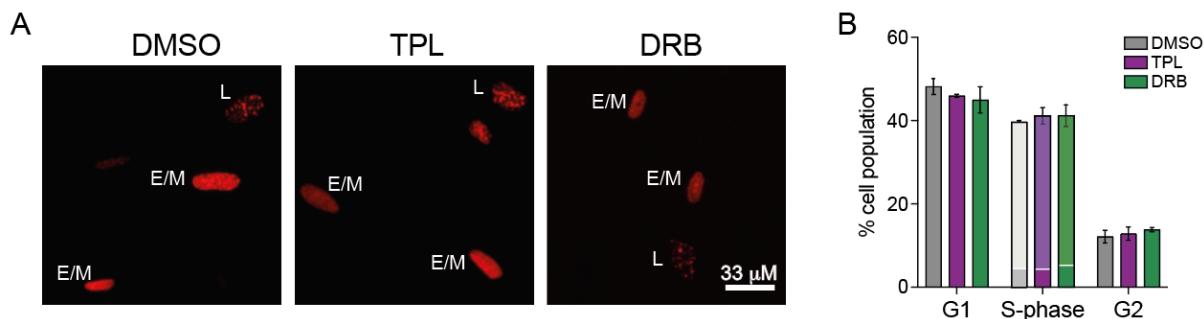
and the transcription of replication dependent histone variants is induced during S-phase. Transcription inhibition could disproportionately affect the replication of some parts of the genome more than others and since there is no evidence to the contrary the differences that they observe between the TPL/DRM treated and DMSO treated proteomes bound to replicated DNA could just be because they were isolated from different genomic loci.

We have carefully examined the impact of TPL and DRB treatments on the replication program using multiple approaches. First, inhibitor treatments minimally affects DNA replication efficiency (Fig. 1D-E) and do not alter S phase progression (see panel below), which is in good accordance with previous published work (Stewart-Morgan et al. 2020).



Analysis of S phase distribution upon DMSO, TPL and DRB treatment. Flow cytometry plots of asynchronous cells treated with DMSO, TPL and DRB. Cells were EdU pulsed and chased according to the conditions defined in the manuscript. Nas, Nascent sample; 2h, Mature sample.

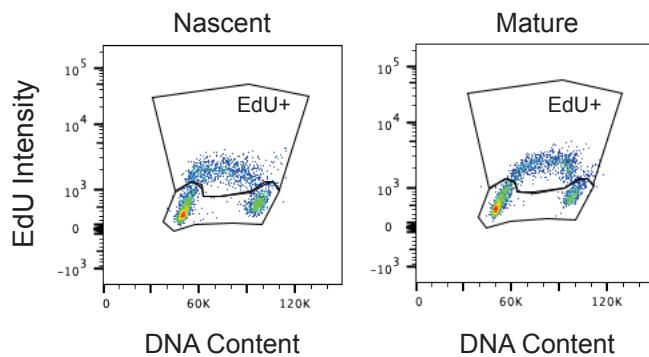
Second, we have inspected replication foci patterns (Dimitrova & Gilbert 1999) and quantified the distribution of the cell population between early/mid and late S phase cells. We did not observe any abnormal patterns, or changes in patterns distribution upon transcription inhibition as observed after DNA damage (Seiler et al. 2007) or upon RIF1 depletion (Yamazaki et al. 2012) (see panel below). Altogether these data support that the replication program is unaffected 2h after transcription inhibition, and to address the point raised by Reviewer 1, we propose to include these controls in the revised version of the manuscript.



Cell cycle and S phase distribution. DMSO, TPL and DRB treated cells are pulsed with EdU for 20 min and analysed by microscopy. A) Representative images of Early/Mid and Late S phase patterns. Early and Mid-S phase cells have a pattern of replication foci distributed throughout the nucleus. Late-S-phase cells would have a small number of large foci within the nucleus. B) Distribution of cells in G1 and G2 phase based on EdU and DAPI intensities. Distribution of cells in Early/Mid (top section) and late S phase (bottom section) based on the distinct EdU patterns shown in A. >1230 nuclei were analysed per condition.

R1.1.5 I am also not convinced that they are able to stop EdU incorporation after 11min with the addition of only equimolar amounts of Thymidine (20 μ M EDU and 20 μ M Thymidine). Equimolar amounts of Thymidine are not sufficient to stop EdU incorporation rapidly. They need to show the kinetics of EdU incorporation in synchronized cells +/- Thymidine.

EdU incorporation has been monitored after thymidine chase in TIG-3 cells and showed that the chase is stopping EdU incorporation (see panel below). This new panel will be added in the revised version of the manuscript to address Reviewer 1's concern.



EdU pulse chase quantification by flow cytometry. Cells were pulsed for 11 min with EdU (20 μ M) (Nascent sample) and chased 2h by 20 μ M of thymidine (Mature sample).

R1.1.6 Without these controls it is impossible to draw any meaningful conclusions from the iPOND data.

We hope that the controls provided above will satisfy Reviewer 1 concerns regarding the experimental conditions of this study.

R1.2.1 The normalization of iPOND and total protein MS data is problematic. It seems that each time point from each treatment was first normalized internally to the median of all protein levels in each dataset and then the relative abundances of each protein were normalized to 100% over all treatments and time points. Internal normalization makes it impossible to directly compare time points and treatments between each other. If the enrichment of a protein goes down from one time point to the next it doesn't mean that there is less of that protein on replicated DNA in absolute terms, it just means that there is less of that protein relative to the median of the whole set of proteins at that time point.

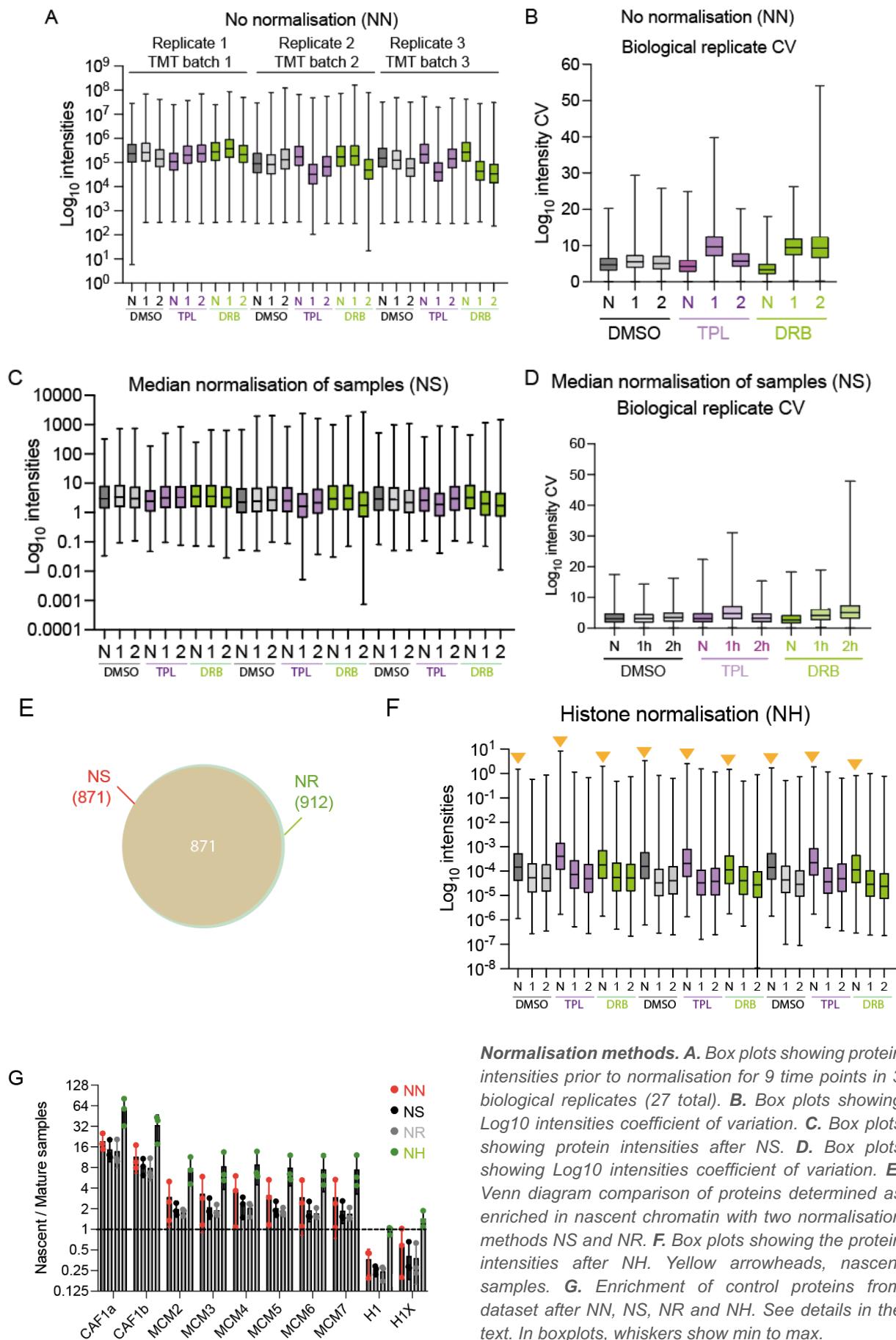
Reviewer 1 is raising an important point. There is no consensus in the field on how iPOND-MS and NCC-MS should be normalised. As a result, four methods have been used: 1) No normalisation (**NN**) (Lopez-Contreras et al. 2013); 2) Normalisation based on histone proteins (**NH**) (Alabert et al. 2014); 3) Each sample is median normalised (**NS**) based on the assumption that most of the proteins in the compared samples have similar abundances and only a relatively small percentage of proteins are differentially abundant (Alvarez et al. 2023); 4) The ratio between two conditions are median normalised (not assuming that proteins in compared samples have similar abundance) (**NR**) (Nakamura et al. 2021); We have tested all four methods on our dataset and selected NS for the reasons listed below:

1. When examining the abundance of the proteins identified, variations between the different batches of TMT (known as the batch effect) seems minor (Panel A-B below).
2. Variations between time points within a batch can on the other hand be observed. This can be attributed to sample preparation variation or to the fact that proteins in different samples have different abundance. In our experience, sample preparation variation is substantial in iPOND, using proteomics or western blot as a readout. We have tested multiple conditions over the years (different phases of the cell cycle, different drug treatments), and the main source of variation arises from variable efficiency of the click-

It reaction and subsequent pulldown. As a result, **NS** seems an appropriate normalisation method (Panel C-D).

4. NS is based on the assumption that most of the proteins in the compared samples have similar abundances and only a relatively small percentage of proteins are differentially abundant. As pointed by Reviewer 1, NS potentially erases biological differences between samples, and to preserve these differences, **NR** should be used instead. Yet, in our datasets such differences have never been observed across biological replicates, whether we use replication stress agents (HU, CPT, ATM), translation inhibitor (CHX), or here transcription inhibitors (TPL, DRB, Panel A-B). Consistent with this, the overlap between the list of proteins selected following NS or NR is very high (Panel E), further supporting the validity of the assumption and that NS is an appropriate normalisation method.
5. Another possible normalisation method is to use histones as protein rulers (NH) (Alabert et al. 2014). This method was successful for NCC-MS (Alabert et al. 2014) but proven to be problematic for iPOND-MS. In NCC (20 min biotin-dUTP labelling), the amount of histone detected on nascent and mature samples moderately changes. In iPOND (11min EdU labelling) it greatly increases (Fig. 1H). Consequently, normalising the iPOND-MS dataset using histones shifted the proteins toward nascent time points (Panel F). This method was therefore considered not appropriate for this dataset.
6. Others have also used no normalisation approach (NN). In that case, whether a protein is a hit or not relies on cut-off and/or the protein been identified as a hit in 2/3 or 5/6 experiments (Lopez-Contreras et al. 2013). We have run such analysis on our data and although it reduces the number of hits, several key findings remain (Panel G).
7. Finally, Reviewer 1 is suggesting an external normalisation method using a “spike in” protein. EdU-labelled DNA from Drosophila S2 cells has been spiked in samples prior to the click-iT reaction and used to normalize read counts in iPOND sequencing experiments (Reveron et al. 2018). However, normalising iPOND mass spectrometry dataset using a ‘spike in’ protein, to allow to correct for sample preparation variation, requires a synthetic peptide or a protein clearly distinguishable from the human homologue and that binds EdU labelled DNA. To our knowledge, there is no known protein that could be used for this purpose, including histones due to the high degree of histone sequence conservation.

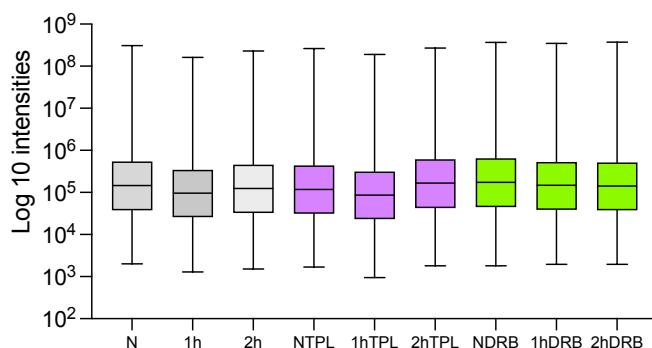
In conclusion, we have tested the four published methods to handle iPOND-MS on our dataset, and as most of our conclusions stands, we do not think the conclusions of this study are normalisation artefacts.



R1.2.2 Their claim that they are comparing iPOND enrichments to total protein abundance is misleading since the data from total protein extracts was also internally normalized so they are comparing relative enrichments in iPOND data to relative enrichments in total cell extracts, which unsurprisingly do not correlate.

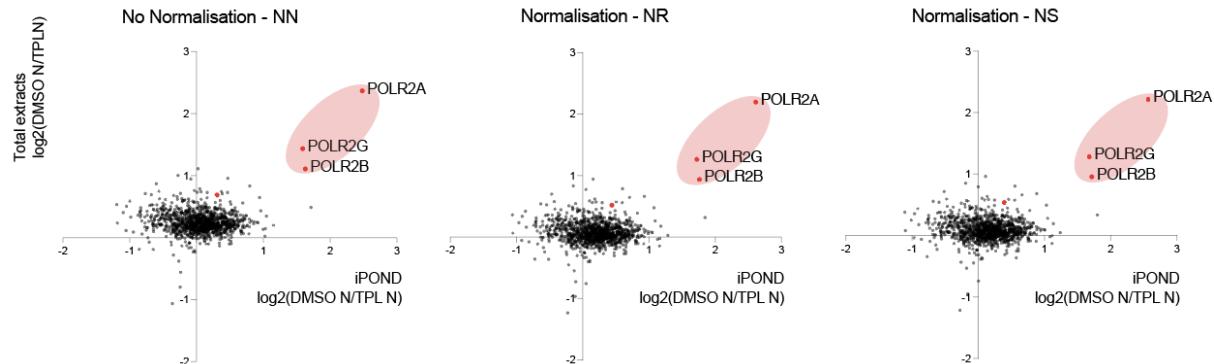
In total extracts, 2 hr after TPL or DRB treatments, most proteins are expected to have similar abundances, while only a small percentage of proteins are differentially abundant (Skalska et al. 2021). As for R1.2.1, analysis of the raw intensities further supports that NS is an appropriate normalisation method (see panel A below). Consistent with this, the correlation between RNAPII change in abundance in total extract and iPOND-MS is conserved following NN, NR and NS (see panels B-D). Of note, this comparison is important to identify changes on replicated chromatin that may be mirrored by changes in total extracts.

A



Comparison of newly replicated chromatin and total extracts. **A.** Box plots showing the protein intensities prior to normalisation for 3 conditions, 3 biological replicates (9 in total). Whiskers show min to max. **B,** ratio of DMSO and TPL intensities, log₂ transformed (NN). X axis, iPOND-MS; Y axis, total extract-MS. The mean of three experiments is shown. **C.** Same as B but following NR. **D.** Same as A but following NS.

B



R1.2.3 It is impossible to make any meaningful conclusions about proteome dynamics using this kind of analysis. They should have used external normalization with a "spiked in" protein to be able to directly compare time points and treatments.

Please see R1.2.1, point 7 above.

R1.2.4 Such as it is right now, their analysis produces some puzzling conclusions that I suspect will turn out to be artefacts of their normalization procedure.

We have tested the no normalization method and the three other normalization strategies on our dataset, and as most of the conclusions stands, we do not think the conclusions of this study are normalisation artefacts. We propose to state in the result section that various normalization strategies has been compared and why NS was chosen. In the discussion we propose to further expand on the importance of normalisation choice. Moreover, we propose

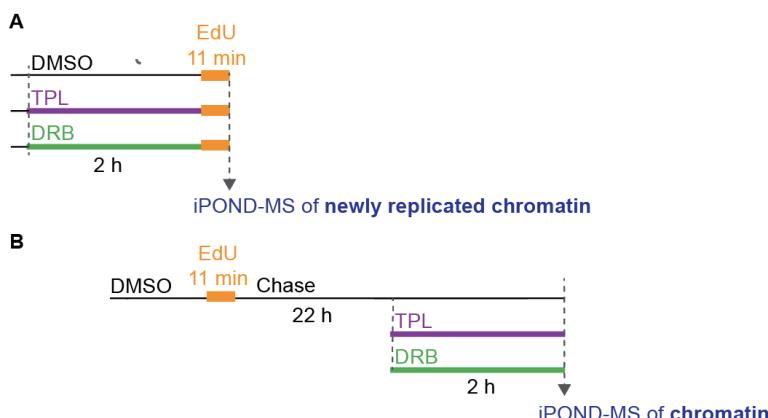
to validate key findings using an orthogonal approach, PLA-EdU (Serebryannyy and Misteli, 2019, Fenstermaker et al. 2023), as previously done in Alvarez et al. 2023, and for the TF ZNF462 in this manuscript (Fig. 3I).

R1.2.5 It is not clear for example why the appearance of histones on replicated DNA would be delayed as they claim: in yeast nucleosomes (new and old recycled ones) are assembled on replicated DNA within minutes of the passage of the replication fork, I don't see why this would not be the case in human cells since the replication machinery is essentially the same in humans and yeast.

Yeast and human are indeed very similar with only ~300 bp of DNA nucleosome free behind each replisome in average. Consequently, even if nucleosome assembly is rapid, when pulling down nascent chromatin, we do expect to pulldown less histones than in mature samples. We, and others (Lopez-Contreras et al. 2013), have detected a similar drop in histone abundance on nascent chromatin compared to mature samples by iPOND-MS, and iPOND-western blot, whether the same amount of protein or number of cells is loaded per well (Fig. 1H).

R1.2.6 It is also puzzling why RNAP2 is enriched in the nascent and 1hr time points but then becomes depleted in the 2hr time point in the DRB treatment since global RNAPII levels don't change in the DRB treatment compared to DMSO (Figure 1C).

This is either an interesting biological difference between chromatin and newly replicated chromatin, or due to the different sensitivity of the two technologies used (High content microscopy for chromatin, iPOND-MS for replicated chromatin). To monitor the effect of transcription inhibition on chromatin using iPOND-MS, we propose to perform an iPOND-MS on cells EdU pulse chased for 24h, as previously done in Alabert et al. 2015 (see below and point R2.1).



Experimental design to compare newly replicated chromatin and chromatin by iPOND-MS. **A.** Experimental design of existing dataset. **B.** Experimental design of the new set of experiment proposed ($n=3$). See details in the text above.

R1.2.7 All the conclusions for PTM restoration/incorporation are essentially meaningless: internal normalization makes it impossible to detect whether PTM levels double at the 2hr time point compared to the Nascent time point in the DMSO treatment, as would be expected for all examined PTMs except for H4K5K12Kac which are marks of new histones.

The histone PTMs data are not internally normalized. For each biological replicate and each time point, for any given peptide identified, the proportion of each modified state of the peptide is calculated as in Alabert et al 2015. For instance, for the peptide histone H4 aa20-24 carrying K20, the proportion of peptides with K20me0, K20me1, K20me2 and K20me3 are calculated.

Moreover, in human, histone methylations are not expected to double within 2h, requiring instead 6h to 20h, depending on the histone residue (Alabert et al. 2015, Reveron et al. 2018). Histone modifications expected to decrease within 2h are H4K5K12ac and are behaving accordingly in this dataset (Fig. 4D). This dataset is therefore in good accordance with published MS-analysis of histone modifications.

R1.2.8 Right now, relative PTM levels are all over the place: only histone acetylations seem to increase, while H3K9me3 and H3K27me3 don't change even though they should also double since heterochromatin should also be restored on both sister chromatids.

H3K9me3 and H3K27me3 are not expected to double within 2h in human cells (see point above).

R1.2.9 They will only be able to accurately assess the impact of transcription inhibition on PTM restoration when they are able to reliably measure the rate of increase of PTM levels during chromatin maturation.

This is what is shown for the first 2h after the passage of the fork. Longer time points (12 and 20h for instance) would be required to complete the histone methylation restoration analysis. However, such experiments will be affected by the known effect of prolonged transcription inhibitors treatments on genome stability (Skalska et al. 2021).

****Referees cross-commenting****

On reviewer's 2 comment on significance:

I think a thorough descriptive analysis of a biological process is extremely valuable and unlike my colleague, I think these types of studies need to be published in high impact journals with a broad readership. Biological processes need to be described first as completely as possible before we can propose meaningful models on how they function and identify the molecular mechanisms that execute and regulate them. As my colleague is surely aware, thorough descriptive studies of any poorly characterized biological process take years (i.e. at least one grant cycle) and comprehensive follow up mechanistic studies can take even longer than the initial descriptive study and can only be done during the following grant cycle, if the authors were lucky enough to obtain funding. Funding agencies however are more likely to award grants to perform these follow up mechanistic studies if the authors (especially if they are junior PIs) have published in higher impact journals in their previous grant cycle. The kind of thinking exhibited by reviewer 2 disproportionately disadvantages junior PIs that work on understudied biological processes. It is a disservice to scientific progress to dismiss excellent descriptive studies and "downgrade" them to lower impact journals where they will be unfairly labeled as a "work of lesser importance". This kind of thinking is also a disservice to the lower impact journals that often publish works whose quality is comparable to articles published in high impact journals. I value more any comprehensive description of a biological process over what most of the time passes for mechanistic insight that is deemed worthy of publication in a high impact journal i.e. a hastily analyzed phenotype of, more often than not, one single mutant tacked on at the end of a descriptive study. This one mutant phenotype then forms the basis of a somewhat "slapdash" model that is often proven wrong by subsequent publications and that the authors would have probably dismissed themselves had they been given more time to develop and test their model in a follow up publication.

I do not think the main issue with the present study is its descriptive nature. As I said in my review, the main issues are technical: the lack of external normalization of MS data and insufficient evidence of the impact of transcription inhibitors on replication dynamics. The study

should not be published in any journal (high or low impact) before those issues are resolved.

on reviewer 2's remarque 4. in major comments:

iPOND identifies proteins bound to 100-300bp fragments labeled with EdU (i.e. after replication or DNA repair). It is by definition identifying proteins bound to chromatin behind the fork, so I don't think that the isolation of RNAPolII bound in front of the fork is a major issue

Reviewer #1 (Significance (Required)):

I am not convinced by their conclusions and I cannot recommend that the study be published at this stage due to normalization issues and insufficient evidence that transcription inhibition does not perturb the replication program (see above). They would need to redo all the iPOND experiments using external "spike in" normalization and monitor replication genome-wide before they can make any meaningful conclusions about the transcription dependent composition of the proteome associated with replicated DNA.

Expertise keywords: Chromatin, Genomics (assay development and bioinformatics analysis) , Replication, Transcription

Reviewer #2 (Evidence, reproducibility and clarity (Required)):

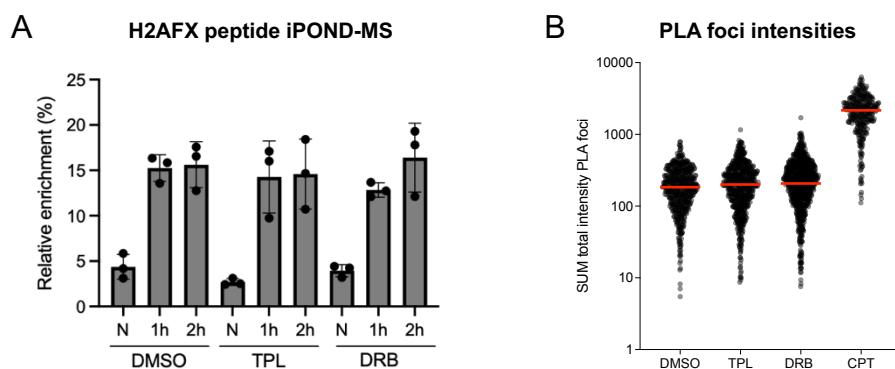
In this manuscript, the authors characterized the re-establishment of chromatin after DNA replication in fibroblasts using iPOND-MS. By using a short pulse of EdU, followed by different length of thymidine-chase, the authors compare the proteome at nascent DNA (just after the EdU pulse) with the proteome on re-established chromatin (1h and 2h post EdU pulse). Moreover, by using two different transcription inhibitors, they investigate the implication of active transcription elongation and of RNAPII binding itself on the reestablishment of chromatin. They show that different transcription factors bind to newly replicated DNA with different kinetics and are affected differentially by transcription inhibition. They also show that upon transcription inhibition by DRB, certain DNA damage repair proteins are depleted, implicating transcription in the recruitment of these factors at nascent DNA. Chromatin remodelers were shown to be enriched on nascent DNA, but triptolide-transcription inhibition reduced their enrichment, implicating RNAPII in the reestablishment of chromatin structure and of steady-state chromatin accessibility. Lastly, the authors show that histone incorporation and histone modification restoration on nascent DNA is mostly uncoupled from transcription with the exceptions of H3.3K36me2 (transcription inhibition by triptolide or DRB drastically reduces restoration) and H4K16ac (DRB treatment increases its incorporation in nascent DNA). Overall, the results and the analysis of the datasets appear robust and well executed. Nevertheless, the work provided by the authors feels mainly descriptive and does not provide further mechanistic insights beyond the current state of the art. Some follow-up experiments to study the functional impact on the different enrichment patterns on nascent DNA or the function of the dependency on RNAPII for the reestablishment of steady-state enrichment on chromatin of some factors would have greatly increased the scientific impact of the manuscript. Nevertheless, the proteome of nascent DNA, its kinetic, and the effect of transcription inhibitors will provide interesting information and a useful resource for research groups in the DNA replication, chromatin, epigenetic, and DNA damage repair fields. Thus, in conclusion, I would recommend this manuscript to be published in its current state in a lower tier journal such as MBoC or PLOS ONE journals. If the authors can provide additional mechanistic insights by

addressing at least a few of the specific points listed below, I think it would become a stronger candidate for a journal with higher impact.

Major comments:

R2.1. At p.7, the authors state: "Altogether, this analysis further confirms that RNAPII's binding and elongation on newly replicated chromatin are a source of genotoxic stress, and identifies dedicated repair factors handling transcription replication conflicts.". I don't think that depleted DNA repair proteins from nascent chromatin upon DRB treatment is enough to claim that the analysis confirms that transcription on nascent DNA is a source of stress. Another possibility could be that transcription helps handling prior DNA damage on nascent DNA without causing the damage. A useful experiment to clarify this point would be the direct quantification of DNA damage markers on nascent chromatin (e.g γ H2AX-EdU colocalization quantification by immunofluorescence). Has the γ H2AX variant been detected in the iPOND MS dataset? Another possible follow-up experiment could be to detect direct physical DNA damage on nascent DNA for example by using a TUNEL assay or similar DSB mapping method. Can the DNA damage be prevented by DRB or TRP addition?

Several DNA repair factors are reduced on nascent chromatin upon DRB and TPL treatments, but as pointed out by Reviewer 2 we have no indication that the DNA damage is reduced on nascent chromatin. Indeed, the variant H2AX (gene name H2AFX) is detected by iPOND-MS, and its abundance is not reduced upon transcription inhibition (Fig. 2F and see panel A below). To further clarify this point, γ H2AX level on nascent chromatin was quantified directly by high content microscopy (QIBC), measuring PLA signal between EdU and γ H2AX. Similar to the iPOND-MS result, γ H2AX levels do not drop on newly replicated chromatin upon transcription inhibition (See panel B below).

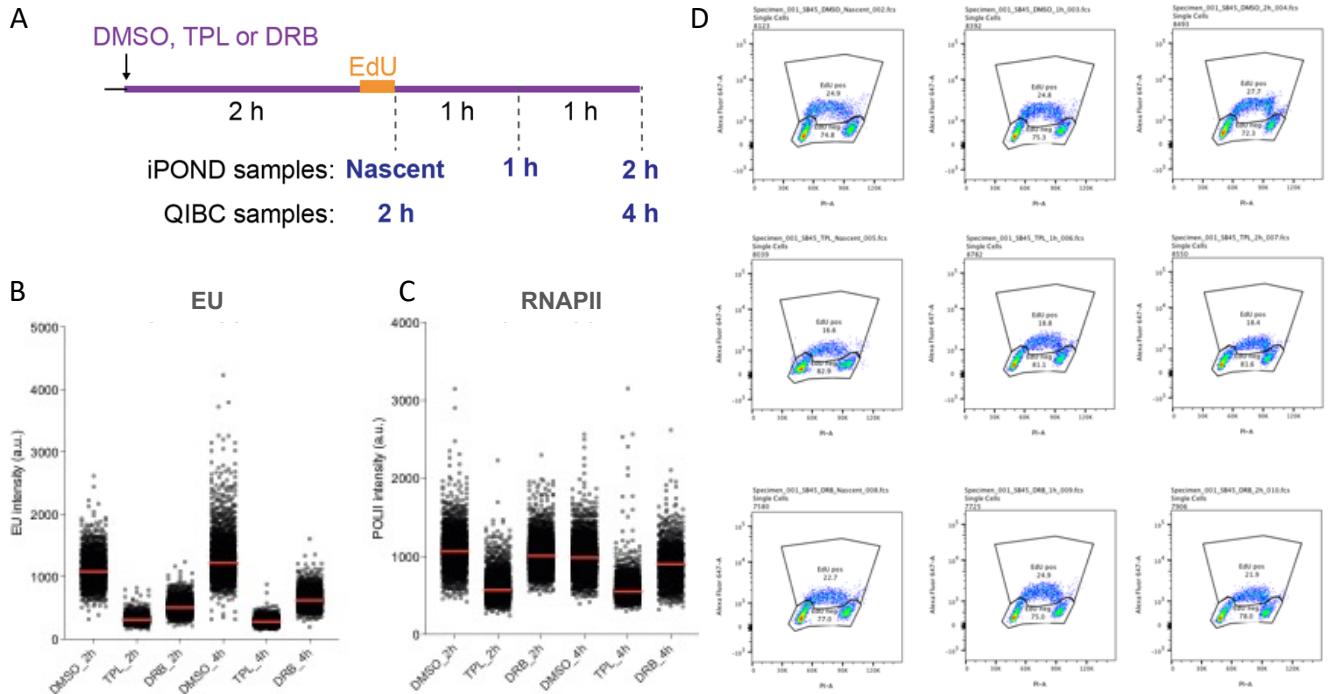


DNA damage assessment on nascent chromatin. **A.** Quantification of the H2AFX peptide from iPOND-MS ($n=3$). Median and standard deviation are shown. Dots, values from each individual experiments. **B.** PLA foci from a PLA between EdU and γ H2AX in cells treated with DMSO, TPL and DRB. CPT is used as a positive control for the assay. >750 nuclei were analysed per condition.

As suggested by Reviewer 2, one possible explanation is that in unperturbed condition, these DNA repair factors are recruited to replisomes due to replicative stress present ahead of the fork. To test this possibility, we propose to isolate chromatin in G1 phase, in cells treated with DMSO, TPL or DRB. To do so we will perform a longer EdU pulse chase experiment (24h, as in Alabert et al. 2015) and collect chromatin by iPOND-MS. Other markers of DNA damage (such as 53BP1) will also be assessed (see R1.2.6).

R2.2. Figure 1B-E: Can the authors also show quantifications of EU, RNAPII and EdU at the 1h and 2h timepoints after the chase?

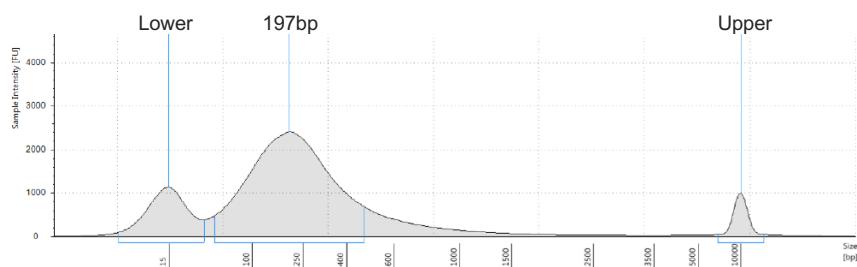
Quantification of EU and RNAPII 2h after the chase are shown below (Panel A-C). We do not have quantification for EU and RNAPII at the 1h chase. Quantifications of EdU in nascent and 1 or 2hr after the chase are shown below (Panel D). We are happy to repeat these experiments to collect the 1h after the chase time point, and add these controls in the revised version of the manuscript.



Quantification of EU, RNAPII and EdU in chase time points. **A.** Experimental design. **B.** EU incorporation quantified by QIBC after 2h and 4h of treatment. **C.** RNAPII level quantified by QIBC after 2h and 4h of treatment. **D.** EdU intensities determined by flow cytometry in EdU pulsed cells (20 μ M) and chased for 1h or 2h with thymidine (20 μ M). Left to right, 11min pulse, 1 and 2h chase. Top to bottom, DMSO, TPL and DRB. >950 nuclei were analysed per condition.

R2.3. The authors state in p.7 that "The other proteins are DNA repair proteins involved in fork quality control and HR as well as transcription replication conflicts (Berti et al., 2020)". I think this gives rise to the question if the effect of DRB treatments on the enrichment of certain proteins at nascent DNA is due to the inhibition of transcription elongation inhibition on nascent DNA or in front of replication forks, affecting the enrichment of proteins implicated in handling transcription-replication conflicts in front of the fork and not on nascent DNA itself. The authors should address the possibility that some of the proteins enriched in the iPOND-MS datasets could be there because they are enriched in front of the replisome instead of on the nascent DNA.

Because DNA fragments generated in iPOND range from 100 to 300bp, isolation of unlabelled DNA is unlikely (see below).



Comparison of genomic DNA fragment size using TapeStation. Example of electropherograms of DNA fragments generated by iPOND protocol.

R2.4.1 On this topic, transcription inhibition is performed for two hours prior to the EdU pulse and iPOND-MS procedures. For the DRB treatment, I would expect RNAPII to be paused/stalled prior to the passage of the replication fork that will replicate the analyzed EdU-labelled nascent DNA. This would mean that replication forks during the EdU pulse will encounter paused/stalled RNAPII, generating potential problems. Such interference would most probably lead to chromatin removal of RNAPII from the chromatin. Surprisingly, the authors show enrichment of RNAPII at nascent DNA.

FRAP experiments show that DRB initially increases the pool of stalled RNAPII onto chromatin, followed by RNAPII unloading (Steurer et al. 2018). By QIBC, 2h after DRB treatment, RNAPII abundance on chromatin did not change (Fig. 2N), suggesting that the cycle of unloading has started. Therefore, in these DRB conditions, at the moment of the EdU pulse, forks will not face a higher number of RNAPII. As FRAP experiments suggest that a shorter DRB treatment should increase RNAPII level on chromatin (Steurer et al. 2018), we are happy to test this prediction by QIBC to reconcile FRAP and QIBC observations.

R2.4.2 How can the author differentiate from accumulation of RNAPII in front of the fork, leading to purification by iPOND, and RNAPII on nascent DNA.

As mentioned above, because of fragment size generated in the iPOND protocol, we do not expect to isolate chromatin ahead of the fork (please see R2.3).

R2.4.3 Also, if the accumulation of RNAPII is on the nascent DNA, do the authors suggests that RNAPII gets loaded more on nascent DNA while under DRB inhibition or that stalled RNAPII are mainly by-passed by replication forks, leading to their enrichment on nascent DNA?

Our data suggest that in DMSO and DRB, RNAPII is loaded to similar extent on newly replicated chromatin. In later time points, while in DMSO, RNAPII remains on replicated chromatin, in DRB, RNAPII is unloaded. This is in good accordance with DRB blocking the elongation step and not the loading step of RNAPII.

R2.5. At p.14, the authors state: "Because they share the same DNA template, transcription is known to challenge replisome progression at high frequency, from RNAPII constituting a roadblock to progressing replisomes, to generate RNA:DNA hybrids (Berti et al., 2020). It is therefore remarkable that behind replisomes, only a handful of DNA repair factors appear to be involved in response to RNAPII binding and elongation.". How does the fact that transcription represents a roadblock in front of the forks makes it remarkable that only a handful of DNA damage repair pathways are involved behind the fork (where they are not a roadblock to any replisome)?

Because of the diverse range of issues generated by transcription - replication conflicts (such as RNA:DNA hybrid, head-on and codirectional collisions and topological stress), we were expecting to detect a greater variety of DNA repair proteins at replisomes.

R2.6. At p.11, the authors states: "As di and tri-methylations require several hours to be re-established following DNA replication (Alabert et al., 2015; Reveron-Gomez et al., 2018), 11 minutes after the passage of the fork, such increase most probably reflects an increase of H4K20me2 and H3K9me3 on recycled parental histones.". Can the authors extend their

interpretation of this result? Do the authors think that DRB treatments increase methylation of histones in G1, prior to replication, or specifically in front of the fork (due to conflicts? DNA damage?), and that those methylated histones get recycled on nascent DNA?

Our data suggest that parental histones are modified ahead of the fork upon DRB treatments and transferred on nascent chromatin. We are happy to extend this section in the revised version of the manuscript.

R2.7. Figure 4: The authors perform the histone PTM analysis under 0h (nascent chromatin) versus 2h (re-established chromatin) timepoints. It would have been insightful to also include a 1h timepoint in this experiment. There appear to be some trends/changes but they do not show statistical significance (e.g. H4K5K12ac or H3K14ac). It might be useful to increase the number of biological replicates (including the 1h timepoint) here, which could improve the confidence in the results and/or discover additional transcription-dependent changes of histone PTM restoration.

We are happy to increase the number of biological replicates to improve the confidence of the results.

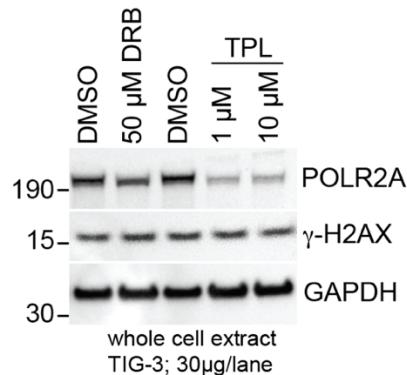
Minor comments:

1. Fig3I: It would be nice to show a TF from the "Restored within 11 min" category as a comparison point.

We agree with Reviewer 3, PLA experiments between EdU and TFs from this category will be included in the revised version of the manuscript.

2. In page 14, th authors state: "However, we did not detect significant signs of DNA damage in DRB treated cells (Fig. 2A, 2B)." Which signs the authors looked at?

We have measured γ H2AX level on chromatin by QIBC (R1.1.2), on nascent chromatin by PLA between EdU and γ H2AX (R2.1) and in total protein extract by westernblot (see below).



γ H2AX level in cells treated with DRB or TPL compared to DMSO. Cells were treated with inhibitors for 3hr according to the concentrations indicated. Total extracts were collected and GAPDH used as a loading control.

3. In the iPOND experiment, which size of DNA fragments is achieved?

DNA fragments are typically 150 to 300bp, see point R2.3.

4. At p.14, the authors state: "Because they share the same DNA template, transcription is known to challenge replisome progression at high frequency, from RNAPII constituting a roadblock to progressing replisomes, to generate RNA:DNA hybrids (Berti et al., 2020)." The paper has not addressed the role of RNA:DNA hybrids in these processes.

We used the antibody that recognize RNA:DNA hybrid (Clone S9.6), and tested by high content microscopy its specificity. In our hands, this antibody was not specific and will therefore require further optimization to explore this pathway.

5. Fig3D: Is there enough datapoints to state a conclusion?

This is something we have considered and unless the additional biological repeat increases the number of TFs included in the final analysis, we will take out this panel.

6. S1A: mistakes in the x axis labels ("no EU" in a EdU quantification graph, "no EdU" in a EU quantification graph).

Thank you for pointing out this mistake, it will be corrected in the revised version of the manuscript.

7. S1F is not sufficiently described in the legend. It took me some time and additional efforts to understand what the right panel of S1F was showing.

Thank you for pointing this out, we will modify the legend accordingly.

8. S2E-F: are the axis wrong? Is it supposed to be Nascent when its comparing total extracts?

Thank you for pointing this out. Total extracts are collected at the nascent and the mature time points, providing information 2, 3 and 4hr after drug treatments. For clarity, axis should be labelled X: DMSO (2hr), Y: TPL (2hr). This will be modified in the revised version of the manuscript.

9. A lot of graphs have non-precise axis labels that needs reading of the manuscript and/or legends to understand. For example: 1K-L (distribution, %), 2L (% of the max), 3B-C-D log2(Nascent/2h), 3G IP/Input, 4C (Inhibitor treatment/DMSO (%)), S2E-F (TPL/DRB Nascent/ DMSO Nascent), S3A (IP/Input), S4A (No Y axis label).

Thank you for pointing this out, it will be corrected in the revised version of the manuscript.

10. FigS4: Assignment of colors in bar graphs of C-J to treatments is not shown. Heatmaps in H and K do not show if these are 0 or 2h. The heatmap in H shows H3 modification and the heatmap in K shows modification in H3.3 but the labels of the modification in K (except the first one) are the names of the modifications of H3, not H3.3. In the legend, GAPDH is written GABDH.

Thank you for pointing this out, it will be corrected in the revised version of the manuscript.

Reviewer #2 (Significance (Required)):

In this manuscript, the authors characterized the re-establishment of chromatin after DNA replication in fibroblasts using iPOND-MS. As mentioned above, the work provided by the authors feels mainly descriptive and incremental and therefore does not provide further mechanistic insights beyond the current state of the art. Some follow-up experiments to study the functional impact on the different enrichment patterns on nascent DNA or the function of the dependency on RNAPII for the reestablishment of steady-state enrichment on chromatin of some factors would have greatly increased the scientific impact of the manuscript. Nevertheless, the proteome of nascent DNA, its kinetic, and the effect of transcription inhibitors will provide interesting information and a useful resource for research groups in the DNA replication, chromatin, epigenetic, and DNA damage repair fields. Thus, in conclusion, I would recommend this manuscript to be published in its current state in a lower tier journal such as

MBoC or PLOS ONE journals. If the authors can provide additional mechanistic insights by addressing at least a few of the specific points, I think it would become a stronger candidate for a journal with higher impact.

Reviewer #3 (Evidence, reproducibility and clarity (Required)):

In this study, the authors used metabolic labeling of newly-replicated or nascent chromatin followed by quantitative Mass spectrometry (iPOND-MS) to characterize protein composition of nascent chromatin at time points after DNA replication: immediately after a short pulse of EdU labeling (nascent), and after 1 and 2 hours of Thymidine chase (maturing chromatin). The iPOND method was established before but in the current manuscript the authors combined this with inhibiting RNA Pol II transcription at distinct stages to determine the effects on transcription and RNA Pol II cycle on chromatin protein dynamics at the wake of DNA replication. The inhibitors they used are Triptolide, which blocks transcription initiation and induces a proteasomal degradation of all chromatin bound RNA PolII, and DRB, which blocks transcription elongation causing an enrichment of paused RNA PolII. The authors compared the relative enrichment of ~1200 proteins on nascent and maturing chromatin and the effects on transcription inhibition on these proteins.

The authors found that RNA PolII does not affect the loading or retention of most histones on nascent chromatin except for the histone variant H2A.Z, which requires RNA PolII loading. However, DRB treatment (no elongation) resulted in stabilization of all histones (which the authors do not seem to catch on). Interesting, unlike the histone, both replication-coupled and -independent histone chaperons seem to be enriched immediately behind the fork and are affected by RNA PolII to different extents. They next look at ATP-dependent remodelers and find that most remodeler families are facilitated by RNA PolII loading, while elongation affects some remodeler families and not others. They see the same trend looking at a wide variety of transcription factors. Interestingly, while RNA PolII loading is required for the establishment of some histone post translational modifications (H3K36me3), some others such as H3K9me3 and H4K20me2 are negatively affected. Finally, the authors find that RNA PolII elongation promotes binding of several DNA repair proteins, and speculate that this is because of DNA damage from replication-transcription conflict.

R3.1 My main concern about this manuscript is that the relative enrichment of most factors show variability across the time points, which make the interpretation of the data difficult. This becomes more concerning when we look at protein complexes such as the ATP-dependent remodelers. Subunits of the same complex which are expected to bind together show different patterns of enrichment. This raises the concern as to how data was normalized.

Proteins that function as part of a stoichiometric protein complex should be captured equally in an iPOND-MS experiment. However, subunits of the same complex often exhibit different enrichments, including subunits of the replisome (See below, figure from David Cortez, Methods in Enzymology, 2017). This has been attributed to the different abundance in tryptic peptides between different subunits. This point will be included in the limitation section of the iPOND-MS method.

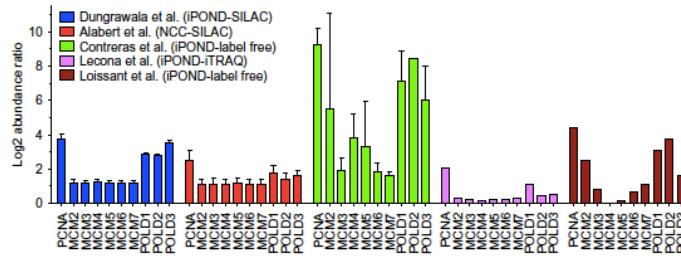
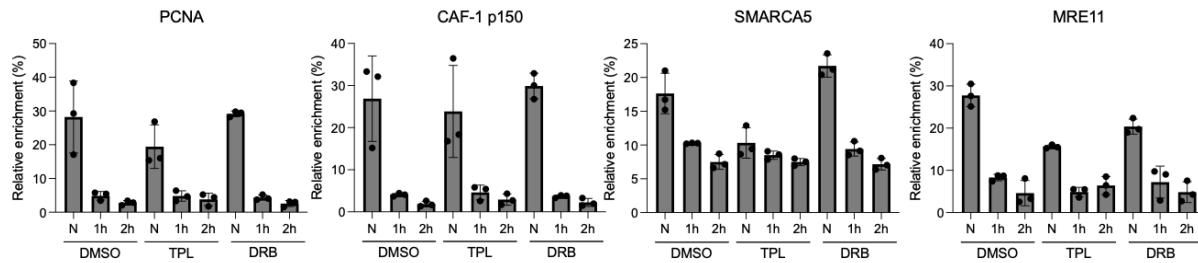


Fig. 5 Comparison of the enrichment of selected replisome proteins at replication forks calculated in five proteomic datasets. A log₂ transformation of the mean enrichment comparing fork/chromatin (pulse/chase) is depicted. Larger positive values indicate increased enrichment at forks compared to bulk chromatin. Error bars were calculated as SEM where possible. (...)

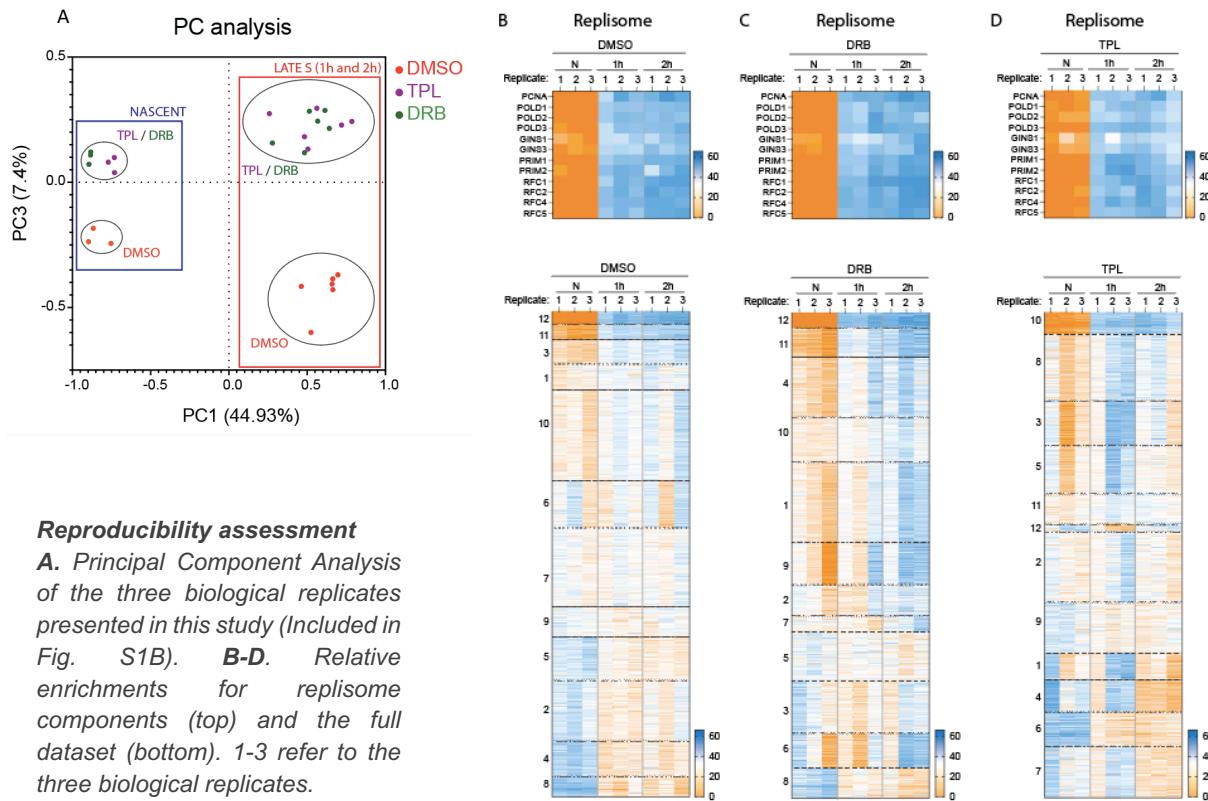
R3.2 Furthermore, how do the replicates compare to each other? The others selected ~1200 proteins which were enriched in all three replicates, but how does their relative enrichment compare in the replicates? The authors need to show some kind of comparison across replicates to confirm that the differential relative enrichments are real and biologically meaningful.

Volcano plots were used to select protein of interest (Fig. 2A-B, and S2A-D), p-values providing a mean to identify statistically reproducible results. To further facilitate the assessment of reproducibility, we are happy to include panels as shown below for the proteins highlighted in the result section in the revised version of the manuscript.



Enrichment for selected proteins in individual biological replicate. Mean of the 3 biological replicates with standard deviation is shown. Dot, value from biological replicate.

For the most direct assessment of reproducibility for the iPOND-MS datasets we provide are Principal Component Analysis and hierarchical clustering (Fig. S1B and see below). In general, mature samples are more variable than nascent samples. To compensate for this variability, we propose to increase the number of biological replicates. This will require 1.5 months total of lab work and analysis.

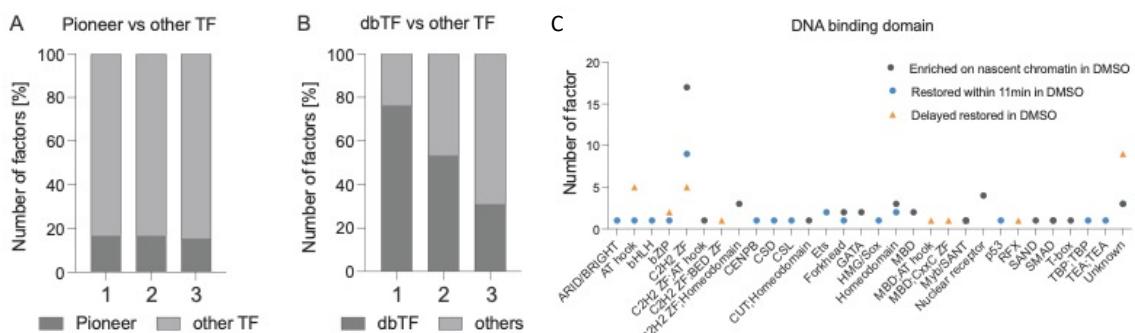


Reproducibility assessment

A. Principal Component Analysis of the three biological replicates presented in this study (Included in Fig. S1B). **B-D.** Relative enrichments for replisome components (top) and the full dataset (bottom). 1-3 refer to the three biological replicates.

R3.3 Also, the TF data is very descriptive. Insightful analysis of similarities/differences between types of TFs would be interesting.

We did not include this analysis in the manuscript as each sub-type of TFs is formed of ~30 TFs, limiting the relevance of the findings. Unless the additional biological replicate increases the number of TFs identified, we will not include these panels.



Analysis of the different types of TF. **A.** Pioneer TFs. **B.** dbTF. **C.** DNA binding domains. 1, Enriched on nascent compared to mature chromatin in DMSO. 2, Restored within 11 min in DMSO. 3, Delayed restoration in DMSO.

R3.4 Minor comment: The formaldehyde cross linking used in iPOND makes it difficult to interpret/distinguish what is actually chromatin bound versus what is enriched due to protein-protein cross linking. The authors should highlight that in the limitations section.

This point will be added in the limitations section of the revised version of the manuscript.

Referees cross-commenting

I agree with most of Reviewer 1's comments about the lack of proper controls and normalization, which make the interpretations difficult. Particularly all of the controls mentioned

under point 1 should not be difficult to perform, and if included, would strengthen the study and the manuscript.

All the controls required by Reviewer 1 are provided page 3-6.

Reviewer 1 makes an important point about normalization, which I totally agree with. Ideally, a spike-in approach would help obtain a much more quantitative and reliable understanding of differential protein enrichment. However, repeating all iPOND experiments with spike-in might be a big ask.

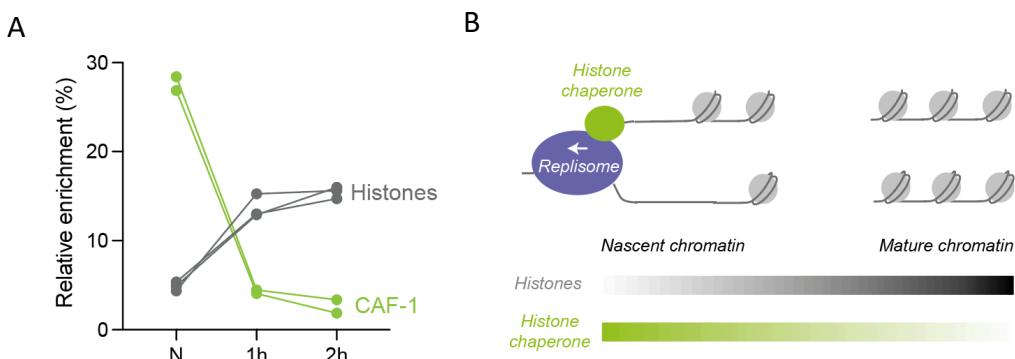
We would like to thank Reviewer 3 for his/her understanding. As mentioned in R1.2.1 point 7, to our knowledge, while spike-in is routinely used to normalize EdU-seq experiments, no such spike-in exist to quantify EdU based proteomic dataset.

What the authors could do at minimum is show how replicates compare with each other. It looks like they pooled all three replicates for analysis, but comparing relative enrichment of all 1257 proteins across replicates would help.

We provide assessment of reproducibility of the dataset (R3.2) that we will include in the revised result section of the manuscript.

The point about delayed histone occupancy is a critical one and difficult to rationalize. To note, histone chaperons are enriched on nascent, but histones are not.

Although histones and histone chaperones form a complex on newly replicated chromatin, their binding kinetic during the maturation process is not expected to be similar. Histone chaperone such as CAF-1 are expected to be enriched on newly replicated chromatin compared to mature samples, mirroring the kinetic of replisome proteins. Histones on the other hand are expected to be low abundant behind replisomes compared to later time points.



Histone and histone chaperones kinetics. **A.** Relative enrichments of histones and replication coupled histone chaperones from iPOND-MS dataset. **B.** Rational of the findings. Changes in relative abundance of the proteins involved along the maturation process are shown.

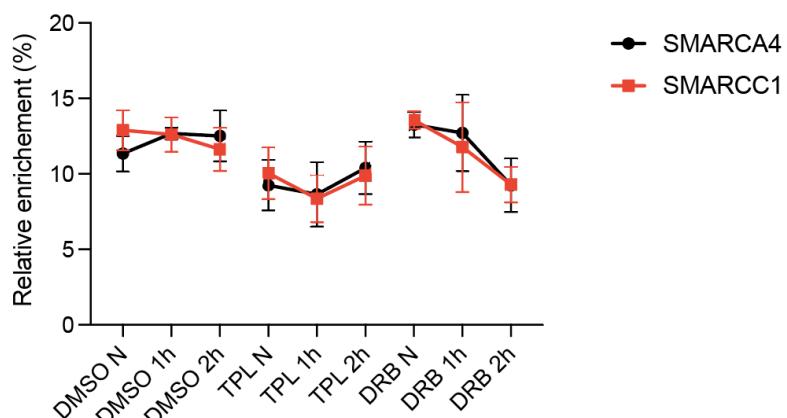
Besides, in the current way that the data is analyzed and presented, there are a lot of fluctuations in protein enrichment across the 1-2 hour timepoints of chromatin maturation, which would be very interesting if real. For e.g., Fig. 1I, Triptolide treatment, most of the cluster I and cluster II proteins show medium-high enrichment on nascent, depleted in 1h, but recover in 2h. If the binding/recruitment of these proteins on newly-replicated chromatin is RNA Pol II dependent, why would they come back after 1h? If this real, this would be very interesting.

We agree with Reviewer 3, and as we have no indication that one experiment should be removed from the analysis, our data support that chromatin maturation is a dynamic process with potential transient binding events. To increase the robustness of this observation, we

propose to perform an additional biological replicate for a total of n=4. This will require 1.5 months total of lab work and analysis.

There are several additional examples of problems with quantification/normalization. As for SWI/SNF subunits, both SMARCA4 and SMARCC1 are core subunits and based on several thorough biochemical studies, cannot be expected to bind separately. However, they show different kinetics in DMSO as well as TPL and DRB.

SMARCA4 and SMARCC1 show slightly different kinetics in DMSO, but these differences are not significant. Consistent with this, both subunits belong to the same cluster, cluster 7.



SMARCA4 and SMARCC1 binding kinetics in DMSO, TPL and DRB. Mean of 3 biological replicates and standard deviation are shown.

Another problem of the assay is that it shows genome-wide average. As Reviewer 1 rightly pointed out, transcription inhibition could disproportionately affect chromatin maturation kinetics in different genomic regions. Perhaps it would be interesting to analyze sets of genomic regions separately, such as highly transcribed and lowly transcribed genes. This might be achieved by adding a purification step using pools of DNA sequence probes before or after the streptavidin enrichment.

We have used replication timing as a mean to compare chromatin dynamic in highly transcribed regions (euchromatin) and low transcribed regions (heterochromatin) (Alvarez et al. 2023, Cell reports, Figure 4). As this strategy requires cell cycle synchronisation or cell sorting, we decided to avoid exposing cells to additional stress. Re-CHIP strategies such as CHOR-seq would be the best approach to tackle this question in a future study.

Additional comment: The formaldehyde cross linking used in iPOND makes it difficult to interpret/distinguish what is actually chromatin bound versus what is enriched due to protein-protein cross linking. The authors should highlight that in the limitations section.

This is an important point, and we will include it in the limitations section of the revised version of the manuscript.

On a positive note, it is a very important and timely study and the manuscript has a lot to consider. Addition of proper controls and normalization/analysis of replicates will make it stronger

We hope that our point-by-point response and the set of new experiments proposed will satisfy Reviewer 3.

Reviewer #3 (Significance (Required)):

Overall, it is a very important and timely study, and the manuscript has a lot to consider. There are several recent papers on the kinetics of chromatin maturation behind the replication fork, and this study adds a very important dimension to this ongoing investigation, and will be of interest to a broad readership in the chromatin and transcription field. We are very happy Reviewer 3 acknowledges the relevance of our work.