

Senescent cells cluster CTCF on nuclear speckles to instruct an alternative splicing program

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This manuscript has been previously reviewed at another journal. This document only contains information relating to versions considered at Nature Aging.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors made a recognized effort to answer our queries.

Fig. 1a – at the end of page 3, it is stated that HMGB2 is depleted. According to the figure, this is not entirely accurate, as we only see a decrease in protein levels. Consider updating the figure to reflect the actual reduction.

End of page 4 – BANF1 reduction – no quantification. Did the authors verify BANF1 knockdown in cells that formed SICC?

May be possible to estimate the minimal levels of BANF1 required for generation of SICC.

Top of page 5- the claim is that Tautomycin dispersed many speckles; however, this is not apparent in Extended Data Fig. 4a. Additionally, Tautomycin is not mentioned in the Methods section. Speckle dispersion by Tautomycin is concentration-dependent. Testing higher concentrations might effectively disperse nuclear speckles and potentially allow SICC formation.

Fig. 2C,D – please include the words "with SICC" to % of cells in the graphs, and anywhere else if needed. It will be much clearer like that.

Page 8: "1,6-hexan treatment dissolved SICC, but not nuclear speckles (Extended Data Fig. 3g)." This should be corrected to Extended Data Fig. 5g.

Fig. 3e – what is the difference between the two nuclei shown?; write under the zoomed images the protein name. In addition, suggest to add the separated channels as it is very hard to see the green signal.

Why don't the authors connect the AS paragraph (page 9) to the findings of interactions with many splicing factors (page 6).

Figure R2 does not need to be added to figure 2.

Reviewer #2

(Remarks to the Author)

The authors have adequately addressed my questions. They also have expanded the relevance of their findings to senescence and ageing contexts.

Reviewer #3

(Remarks to the Author)

I want to thank the authors for the prompt sharing of many of the source images generated in this study. Upon in-depth investigation, I've found a number of anomalies that need to be scrutinized before the manuscript can be considered for publication. I have uploaded a pdf that present the issues in more detail.

Major issues:

1. Figure 2C: the BANF1 panel in the siBANF2 condition is a cut-out (jpg) that is completely black, with the exception of a single dot of signal that becomes visible upon strong boosting of contrast. This is different from the widefield source file (tiff): here a weak but noticeable background and a number of brighter puncta are visible (page 1 and 4 in the pdf). It seems unlikely that the jpg was extracted from the widefield tiff, unless the authors selected a different region or used very different contrast settings as compared to other panels in the manuscript. The authors need to trace back how the jpg file was obtained from the source tiff file.

The same issue may be present in Figure S2D-F of the supplemental data: the signal for the ATXN2, SQSTM1 and FMR1 panels in the siRNA-treated cells appears different from other panels. The source files for these panels were not shared and therefore this issue remains to be confirmed.

2. Figure 2D: the BANF1-Venus channel is nearly completely black in both the cut-out (jpg) and widefield soured file (tiff). Upon strong boosting of contrast, very weak background signal becomes visible in the tiff file, but this is absent within the cells (page 2 and 5 in the pdf). The jpg cut-out instead is evenly black, with the exception of some minor stripes, which may be a jpg compression artifact.

This signal pattern is highly unlike, as the Venus fluorescent protein emits light within a range that is quite close to the Alexa fluor A488 fluorophore that was used to visualize the CTCF protein (see pdf). Using a conventional widefield microscope, as used in this study, a minor bleedthrough of the A488 signal is expected in the Venus channel. The authors need to confirm that the Venus protein was visualized using the correct laser and filter.

The same issue may be present in Figure S6B of the supplemental data: a certain amount of bleedthrough is expected in the SRRM2 RBD-Venus panels. The source files for these panels were not shared and therefore this issue remains to be confirmed.

Minor issue:

Figure 2C: the visualized cell in the siCTRL condition is flipped and resized along the X-axis, which is undisclosed (page 1 and 3 in the pdf). Although this does not change the conclusions, such modifications relative to the source data are not allowed.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

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Response to editorial requests:

[...] For the study to be a good fit at Nature Aging, as discussed, we would require you to validate your key findings in the context of replicative senescence.

The emergence of (replicative) senescence-induced CTCF clusters across different cells types has already been documented in our previous work (Zirkel et al, *Mol Cell* 2018; PMID 29706538), but we exemplify this again for primary endothelial cells (HUVECs) in **Extended Data Fig 12d**. In addition, we now show that BANF1- and SRRM2-knockdown averts CTCF clustering also in replicatively senescent IMR90 (**Extended Data Fig 3b**), which in turn delays their commitment to senescence significantly (**Fig 7h**). Last, we analysed alternative splicing changes in replicative senescence from both IMR90 and HUVECs, as well as from HUVECs upon *HMGB2*-knockdown (which induces SICCs), and found significant correlation to the changes already documented using ICM (**Extended Data Fig 12a-f**). Therefore, all this new data and analyses consolidate the validity of our original findings in the ICM-induced senescence model.

We would additionally strongly encourage you to expand the physiological relevance of the work, in the context of aging.

We are thankful for this suggestion, because it motivated us to analyse alternative splicing changes in chronologically aged human cells. We used RNA-seq data from primary skin fibroblasts (in which SICCs we could show that also form; see **Extended Data Fig 12g**) and whole blood derived from young or old individuals. Despite their diverging cell identities, aged human cells displayed substantial overlap in their splicing changes and affected pathways compared to senescent IMR90 (when it comes to skin) and HUVECs (when it comes to whole blood). All these analyses have now been added to **Extended Data Fig 12g-m** and indeed expand the relevance of our findings in the context of senescence to human aging.

Point-by-point Response to the Reviewers' comments (shown *italicised*):

Reviewer #1: *This study sets out to show, using a wide range of experimental approaches, what are the nuclear formations of CTCF under senescence, and finds they are connected to nuclear speckles through the SRRM2 and BANF1 proteins. Examining 3D organization and alternative splicing patterns the authors suggest that under senescence there is reorganization of genes in the 3D space with respect to nuclear speckles and this suggests changing alternative splicing patterns. The study encompasses a very large body of work, however, while this reviewer found the first part clear to follow, once the large amount of sequencing and genome organization data appeared, with far too many details, the study became very hard to follow and therefore to fully see how all the parts come together.*

We appreciate the Reviewer's comment on the "wide range of experimental approaches" we used, as well as on the "very large body of work" in our study. We also acknowledge that the second half of the manuscript would benefit from rewriting and weeding through the many data and figure panels to streamline its main message. Nevertheless, this message is precisely as the Reviewer describes: "nuclear formations of CTCF ...connected to nuclear speckles" ultimately lead to a "reorganization of genes in the 3D space with respect to nuclear speckles ...changing alternative splicing patterns". We hope that with our clarifications (below) and the respective changes and additions to the manuscript, this message does come together more clearly now. Please also note that we added new analyses that confirm our key findings in replicative senescence of both HUVECs and IMR90, as well as in chronologically aged human skin fibroblasts and whole blood samples (see **Extended Data Fig 12**).

Fig. 1a – top row has small nuclei, bottom row the nucleus is huge, yet there is only one scale bar – does that mean the nuclei grow under ICM treatment? If not, then show at same scale, and for the treatment show a field of cells, not just one. Also, comparing to Fig. 3a, in 3a the CTCF looks like nuclear speckles indeed, while in Fig. 1a its less typical.

Yes, nuclei are shown at the same scale (hence the single scale bar); IMR90 typically become more flat and larger upon ICM treatment, which is very much what also happens upon replicative senescence via the serial passaging of cells. These effects were already described in our Palikyras et al (2024) *Aging Cell* paper, where the ICM-induced senescence system is presented in detail and is accompanied by a large resource of biochemical and genomics data for the community to use (see PMID 38196311).

Now, regarding the comparison between **Figs 1a** and **3a** (and generally between the many figure panels showing CTCF clustering), there is indeed some heterogeneity which is (also) reflected in the shape of these clusters – this is to be expected of senescence. We deliberately chose not to show only cells with uniformly round SICC so that readers can appreciate this heterogeneity. Nevertheless, CTCF clusters will invariably overlap nuclear speckles.

More biochemical measurements of HMGB2 are required to show the reduction in total HMGB2. While under DMSO treatment HMGB2 looks more diffuse in the nucleus, adding ICM lowers the diffuse portion, but the nucleolus remains predominant. Is there a connection between HMGB2 relocation to nucleolus and SICC appearance?

We have documented this senescence-associated reduction of HMGB2 levels (both protein and RNA) in different primary human cell lines via different measurements (western blots, mass spectrometry, RNA-seq, RT-qPCR, ELISA) in our previous work on replicative senescence (Zirkel et al, *Mol Cell* 2018; PMID 29706538), as well as in our recent paper describing the ICM-induced senescence model (Palikyras et

al, *Aging Cell* 2024; PMID 38196311). We therefore did not feel we had to reiterate this here. However, we now make this more explicit in the relevant part of the **Results** (see pg. 3).

Regarding the nucleolar HMGB2 signal: this is a known side-effect of formaldehyde fixation (in response to which HMGB2 displays a peculiar behaviour likely due to the nature of its DNA-binding domain; Pallier et al, *Mol Cell Biol*; PMID 12925773), whereby some protein “sticks” to the nucleolus. In senescence, whatever residual HMGB2 is left will tend to appear nucleolar upon fixation. This is also documented for its sister protein, HMGB1, by us (Sofiadis et al, *Mol Syst Biol* 2021; PMID 34166567) and others (Mensah et al, *Nature* 2023; PMID 36755093), but does not influence quantification of total nuclear signal. We are happy to highlight this in the **Methods** section for the sake of clarity (see pg. 30), and thank the Reviewer for pointing this out.

Fig. S1b – large field, few, and very small nuclei.

Senescent IMR90 generally grow sparse; we now provide a zoomed-in inset in this figure.

Fig. 1b – mark BANF1 and SRRM2.

This is a good suggestion; both proteins are now demarcated in the plot.

In some images there is a decrease (Fig. 1a) in HMG while in others there is a disappearance (Fig. 1d). Can the difference be explained?

Although ICM produces largely homogeneous IMR90 populations within 6 days of treatment (please see detailed quantification at the single cell-level in Palikyras et al, *Aging Cell* 2024; PMID 38196311), some heterogeneity will still exist. Thus, some cells show more of a decrease in HMGB levels than others.

ICM does not result in DNA damage (Fig. 1c) – please provide evidence.

The experimental evidence for this is shown and quantified in **Extended Data Fig 1d**.

Fig. 2a – any idea why CTCF has 2 bands after ICM?

In western blots from proliferating IMR90, CTCF will typically appear as a single 130-kDa band, although its presumed molecular weight based on its open reading frame is ~70 kDa. This discrepancy is primarily because CTCF is post-translationally modified via poly(ADP)-ribosylation (Farrar et al, *Methods Mol Biol* 2011; PMID 21870268). We have now discovered that CTCF becomes hypo-PARYlated in senescence, regardless of whether this is induced by ICM or by serial passaging; hence this second band in the blot of **Fig 2a**. We have started investigating this, and have validated that it is poly(ADP)-ribosylation-specific by the use of PARP inhibitors that also lead to the formation of SICCs (see **Fig R1, below**)—but we feel that this biochemical dissection is beyond the scope of our (admittedly already large) present study.

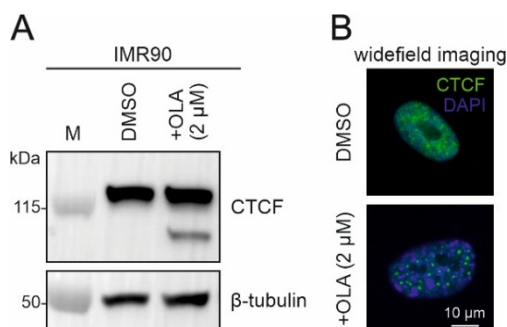


Fig R1: **A**, Western blots showing the emergence of a second lower CTCF band upon treatment of IMR90 with Olaparib (2 μM for 24 h). **B**, Typical images of IMR90 nuclei treated or not with Olaparib, immunostained for CTCF (green), and counter-stained with DAPI. CTCF clusters emerge upon treatment.

Fig. 2d – not sure why SC35 needs to appear here.

Thank you for pointing this out; it does not, and we have now removed this sub-panel.

Would BANF1 overexpression lower the time or concentration of ICM treatment? Is there a correlation between the amount of BANF1 protein and the appearance of SICC, inferring that overexpression of BANF1 fastens senescence/SICC formation.

We have not explicitly tested whether BANF1 overexpression renders ICM treatment more efficient, mainly because heterogeneity in expression and ICM responsiveness can blur small effects during live-cell monitoring of IMR90 proliferation. However, we have done the converse: assessed BANF1 levels in relation to the appearance of SICCs. Indeed, there seems to be a correlation between BANF1 availability in the nucleus and the emergence (or not) of SICCs (see **Fig R2**, below) in line with the Reviewer's train of thought. We are happy to add this data to **Fig 2**, if requested.

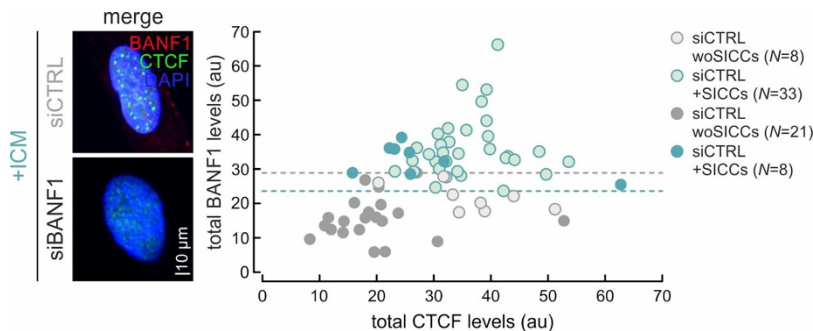


Fig R2: Left: Typical images of IMR90 treated with ICM and with control or BANF1-targeting siRNAs. Right: Scatter plot quantifying total BANF1 and CTCF intensity in cells carrying (green) or not SICCs (grey). Dotted lines represent the bottom and top values in each subset.

The authors state that SICCs colocalize to nuclear speckles. Could it not be possible that with ICM, CTCF enters the NS and does not generate a different body? Perhaps SRRM2 connects CTCF to the NS, which is claim to be seen in Fig. 3c.

We think that CTCF most likely forms a different body by clustering on nuclear speckles (perhaps even inside its outer-most layer; see Fei et al, *J Cell Sci* 2017; PMID 29133588) and is connected to them via SRRM2. Hence, SRRM2 depletion leading to a complete SICC dissolution (**Fig 3c**). This is what our super-resolution STED images also suggest (**Fig 3e**; the CTCF signal is somewhat offset with respect to the bulk of the speckle signal). We now back our postulation up by lines scan (**Fig 3e**) and colocalization analyses (**Extended Data Fig 4h**), and make it more explicit in our revised main text (see pg. 5).

Note this publication – PMID: 33095160, stating that the main target of SC35 mAb is SRRM2, which means when SRRM2 is knocked down and NS are labeled with SC35 then this suggest that the knockdown wasn't very good (need to add a SRRM2 staining for KD validation like with BANF1). Suggest to add SON staining, as it's the 2nd core protein of the NS aside from SRRM2, for NS marking.

We were aware of this issue, and chose the mouse monoclonal antibody anti-SC35 (NB100-1774) from Novus Biologicals because it was *not* one of those flagged in Ilik et al. (2020; PMID 33095160) as cross-reactive with SRRM2. In fact, this antibody does not recognise any of the two parts of the SRRM2 protein that we overexpressed (i.e., RBD aa 197-259 or C-terminal IDR aa 1633-1695, the latter also included in the part of SRRM2 that Ilik et al. reported as the recognition region for SC-35 antibodies) as evidenced from **Fig 3g,h**. Also, upon ~80% SRRM2-knockdown, SC-35 staining persists (see **Extended Data Fig 4e**). Nevertheless, we agree with the Reviewer that SON staining would resolve this, and we already provided an example of this in **Extended Data Fig 4d**.

Short term treatment of 1,6 hexanediol dissolves liquid but not solid condensates (PMID: 38659924), maybe this can help differentiate between SICCs and NS. There are ways to disassemble nuclear speckles. What we see is that the short (2-min) treatment with 6% 1,6-HD will dissolve SICCs, but not dramatically compromise nuclear speckles (see **Extended Data Fig 4f**), thus decoupling the two entities, much like

the Reviewer suggests. However, once we begin disassembling nuclear speckles via the knock-down of SRRM2, SICCs will readily dissolve highlighting their interdependence.

Verify that it's clear in all the figures and images which channel is which in the colored merge. For example, in Fig 2c, DMSO, we don't know which is green and which is red. If CTCF is red (like +ICM) where did it go in DMSO?

We acknowledge this oversight, and we have now amended all relevant figure legends to display which channel corresponds to which staining. For **Fig 2c** specifically, the Reviewer is correct—CTCF was shown inadvertently in the green channel in control cells, but in the red in ICM-treated cells. We have redrawn this figure and checked all other for consistent data presentation. We now also provide zoomed-in insets of representative regions of interest from all channels in our IF figures. Thank you for noticing this.

Fig. S2c-e – state that the cells were treated with ICM in both figure and legends.

Now corrected, as suggested.

Fig. 3a – show the unmerged channels separately.

We could not do this due to space limitations in the crowded **Fig. 3**, but are happy to provide this below (see **Fig. R3**). These IFs do not differ to any other example of CTCF/SC-35 IFs provided throughout the manuscript, but to follow the Reviewer's suggestion we provide zoomed-in views of one SICC in all three relevant channels (CTCF, SC-35, and merge) as insets in **Fig. 3a**.

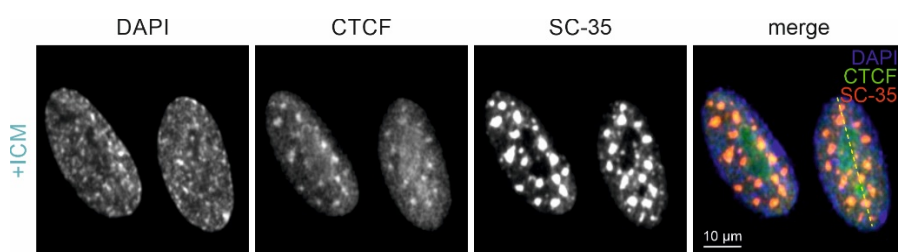


Fig R3: A typical image of IMR90 treated with ICM, immunostained with SC-35 (red) and CTCF (green), and counter-stained by DAPI.

Fig. S3a – “Tautomycin dispersed speckles and SICCs into smaller formations that are more elliptical (Supplementary Fig 3a,b)” – don't see this in the images.

We show a characteristic field of view with three nuclei (**Extended Data Fig 4a**), but now also provide zoomed-in insets to exemplify the changes that the quantification of the radius and eccentricity of 70 nuclei revealed (**Extended Data Fig 4b**).

“Cantharidin caused them to fuse into more round, larger structures (Supplementary Fig 3c,d).” – just one nucleus.

We have now replaced this image, but again show insets and quantification of radius and eccentricity in >50 nuclei alongside this (**Extended Data Fig 4a,b**).

Fig. 3c,d – “This was not due to a reduction in CTCF levels, but rather due to its redistribution (Fig 3c,d)” – would need a Western blot to show whether there is no reduction.

We now also provide a western blot following SRRM2-knockdown in ICM-treated cells to confirm that little reduction in CTCF protein levels occurs (added to **Extended Data Fig 4c**, and agreeing with the IF quantification in **Fig 3d**).

Fig. 3e – the text is somewhat vague – on the one hand it says the signals are overlapping, on the other hand it says they are offset to each other. Perhaps some kind of colocalization analysis could help make this observation more precise.

Across all our conventional (e.g., Fig 3a) and super-resolution images (Fig 3e) there is clear overlap of CTCF (SICCs) with SC-35 foci (nuclear speckles). However, super-resolution gSTED imaging allows us to notice a small but noticeable offset in CTCF peak signal intensity relative to the center-of-mass of the nuclear speckles these colocalize with. We also provide additional line scan analysis across a typical SICC in our gSTED images (Fig 3e; see also Fig R4 below), as well as colocalization analysis in all gSTED images that indeed verifies this offset positioning of SICCs relative to speckles (see Extended Data Fig 4h). We comment on this new analysis on pg. 5 of the Results.

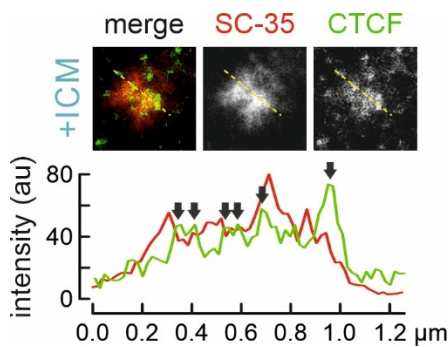


Fig R4: Line scan analysis of one characteristic SICC (CTCF; green) in ICM-treated IMR90 overlapping a nuclear speckle (SC-35; red). The line crosses consecutive CTCF signal maxima (black arrows), most of which are offset to the SC-35 signal maxima.

Also, the CTCF signal appears quite weak and resembles background compared to the supplementary image (Fig. S3h) and other figures. Suggest replacing the images in Fig. 3e.

There is a small, yet measurable decrease in CTCF of 15% protein levels in senescence (Palikyras et al, *Aging Cell* 2024; PMID 38196311), so such weaker signal is not an exposure artefact (compared to that in a proliferating cell in Supplementary Fig 3h), but rather the result of dramatic relocalization within a thin optical section.

Different cells throughout the manuscript show varying numbers of CTCF foci. Is there any correlation between nuclear speckles numbers and CTCF foci?

Yes, there is a clear correlation – for example, when we manipulate speckles pharmacologically either with cantharidin or with tautomycin to fuse or disperse, respectively, the CTCF foci follow, which also highlights their interconnection (see Supplementary Fig 3a-d).

Fig. S4a,b – where are the controls showing the RNase/Dnase treatments worked?

We have lysed IMR90 cells and quantified residual RNA or DNA many times after such treatment. Please see Fig R5 below for a reference on RNase A degradation.

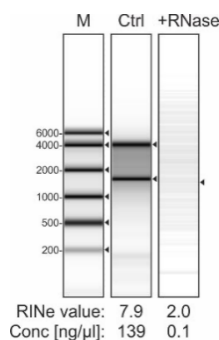


Fig R5: Tapestation profiles of RNA isolated from mock- (Ctrl) and RNase A-treated IMR90 (+RNase) following *in situ* permeabilization as for immunofluorescence assays.

Fig. 3g – don't really see nuclear speckles-like staining in the CTCF channel when the RBD is added; compare for instance to Fig. 3c. Therefore, the conclusion at the end of this section is not based on very strong evidence.

In **Fig 3g**, the SRRM2-RBD-Venus signal overlaps the SC-35 foci shown for that same nucleus. However, there is an important consideration here that explains the Reviewer's observation: compared to **Fig 3c** (and to any other image of a senescent nucleus), the SRRM2-RBD initiates CTCF clustering in the context of a still-proliferating cell that is not permissive of SICCs. Still, focal CTCF signal becomes obvious (please compare the two cells in the field of view: the left-hand-side one expresses the RBD, but the right-hand-side one does not and has no SICCs). If one looks at the same experiment performed in senescent cells pre-depleted of full-length SRRM2, the RBD overexpression effect is essentially the same, but visually more pronounced (see **Extended Data Fig 6b**). To make this even clearer, we have now added insets of magnified SICC foci in **Fig 3g** and a textual explanation on pg. 6.

Fig. 4b – whats going on the top Venus image?

This image shows expression of GFP alone (as a control) via the same Dox-inducible construct used in all panels; GFP expression is strong and not restricted to the nucleus, as expected. We have seen similar images in overexpression experiments before (see Zirkel et al, *Mol Cell* 2018; PMID 29706538).

Page 5 third line- RBD instead of RDB.

Thank you for noticing this, it has now been corrected.

Fig. 4j – not sure this panel is referred to in the text, and not clear what one is seeing or what it tells us (and Fig. S8).

This figure panel, together with data in **Extended Data Fig 8**, is based on whole-genome coarse-grained simulation via the Chrom3D framework that allows us to ask some questions on CTCF clustering at the levels of larger chromatin domains that are difficult to address experimentally. As mentioned in the text, these simulations of our actual experimental data further “supported the notion that active, CTCF- and HMGB2-rich domains reside in spatial proximity to nuclear speckles” and reorganise dramatically upon entry into senescence. However, panel **4j** was wrongly referred to as **4i**, which is now corrected.

The explanation of how SICC formation alters alternative splicing patterns is missing.

We agree with the Reviewer that we should have been more explicit with our model of how SICCs may work to alter splicing patterns upon senescence entry. In brief, the reorganization of CTCF loops will now position different genes (e.g., the three genes from chr5 shown in **Fig 7g**) at specific distances to nuclear speckles thereby affecting their splicing patterns. This is also evidenced by data that show that loci with altered AS patterns in senescence are enriched for TSA-seq signal (indicating speckle proximity; **Fig 7f**) and show exon-intron ‘GC signatures’ expected of speckle associated genes (**Extended Data Fig 11i**; signatures obtained from Tammer et al, *Mol Cell* 2022; PMID 35182478). We have added an expanded version of this explanation in our revised **Discussion** (see pg. 13). We have also added a graphical model in **Fig 7i** to help readers visualize this concept (see also **Fig R6** in response to Reviewer #2, below).

The author should provide supplementary data demonstrating that treatment of fetal lung fibroblasts with ICM induces senescence.

As also mentioned above, the full description of the ICM model for replicative-like senescence in IMR90 is detailed and accompanied by a large resource of biochemical and genomics data in our recent paper (Palikyras et al, *Aging Cell* 2024; PMID 38196311). We now explicitly direct readers to this (see pg. 3-4).

Reviewer #2: *In this manuscript, Palikyras et al. provide an in-depth characterisation of SICC formation in unique culture models. Using a proteomics approach, they identified BANF1 and speckles' components, particularly SRRM2, as key regulators of SICC formation. In addition, using multimodal omics approaches and computational simulation, they show evidence suggesting a negative correlation between HMGB2 and CTCF-Speckles interaction as well as formation of longer-range, nested CTCF loops. At the single-cell level, they employed 3D DNA tracing, validating some of these findings. Further introducing CLIP/APEX2 analyses suggests that alternative splicing events are associated with SICC formation. Interestingly, they show that disruption of SICC integrity by depleting SRRM2, BANF1 or CTCF alleviates proliferative arrest in ICM-treated cells. This study potentially identifies important mechanisms for the spatial clustering of SICC and its functional relevance. The dynamic interaction between HMGB2, CTCF, and speckles, as well as RNA, is interesting, but the data integration is quite complex: the manuscript could benefit from the inclusion of a model illustration.*

We are happy to see that the Reviewer finds that we provide an “in-depth characterisation of SICC formation in unique culture models” in a study that “potentially identifies important mechanisms for the spatial clustering of SICC and its functional relevance”, where the “dynamic interaction between HMGB2, CTCF, and speckles, as well as RNA, is interesting”. We also agree that the overall integration of different types of data, especially the link between biochemistry and cell biology with 3D genomics, can become quite complex. To remedy this, we have now revisited figures and text to streamline our main message, but also provide a model as suggested (see **Fig 7i** and **Fig R6** below) to make this more comprehensible. Please also note that we added new analyses that confirm our key findings in replicative senescence of both HUVECs and IMR90, as well as in chronologically aged human skin fibroblasts and whole blood samples (see **Extended Data Fig 12**).

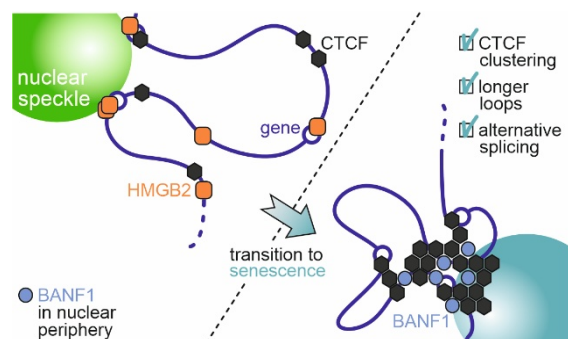


Fig R6: Graphical model showing 3D reorganization of CTCF-bound chromatin with respect to speckles via BANF1 and SRRM2 that ultimately leads to senescence-associated splicing patterns on the genes involved in these clusters.

Major points:

1. *The role of RNA in SICC formation on speckles: Their data suggest that overall RNA inhibits CTCF-speckles interaction and RNA-unbound CTCF contributes to CTCF clustering. Yet, CTCF eCLIP shows increased peaks during senescence (supple fig. 4c). Do they mean that those CTCF CLIP peaks represent the non-SICC fraction of CTCF?*

We agree with the Reviewer that our data suggest that RNA likely acts in a refractory manner for the CTCF-speckle interplay, which is in line with their stronger interaction and ultimately CTCF clustering on speckles in senescent cells, which anyway produce less RNA overall (see Palikyras et al, *Aging Cell* 2024; PMID 38196311). But how is this reflected in our eCLIP data? Our CTCF eCLIP from proliferating IMR90 show minimal (if any) specific association of CTCF with RNA (just 74 targets can be identified; **Extended Data Fig 13d**). This is fully in line with a recent careful biochemical dissection by the Guttman lab (see Guo et al, *Mol Cell* 2024; PMID 38387462) according to which CTCF does not show specific RNA binding capacity. However, the number of eCLIP peaks increases in senescence as CTCF is positioned on/around

RNA-rich speckles due to SICC formation (we now identify >250 target RNAs; **Extended Data Fig 13d**). We believe that these associations simply reflect persistent CTCF proximity to RNA in speckles, and when crossed with our APEX2-SRSF7 data, we find measurable overlap of co-associated genes. Still, we cannot formally rule out that some of the CTCF::RNA associations occur outside of SICCs, and now acknowledge this on pg. 5 of our revised **Results** section.

2. Similarly, they show that overexpression of SRRM2-RBD is sufficient for CTCF clustering, where SRRM2-RBD and CTCF clusters mostly colocalise. Could the authors clarify how they interpret the data? Do the data imply that the speckles-mediated 'focal' enrichment of RNA is critical for CTCF clustering, while the enzymatic (global) depletion of RNA does not compromise SICC formation? If so, can the authors test this by using an ectopic expression of an RNA-binding-deficient mutant (if available) of SRRM2-RBD? Together with the CTCF data, I wonder if the following interpretation is in line with the authors' - while speckle-mediated RNA recruitment may be important for CTCF clustering, CTCF-RNA binding primarily occurs outside of CTCF clusters.

We thank the Reviewer for this insight—it is a plausible scenario and our take on it is the following. First, the test that the Reviewer is asking for, we have essentially already performed by overexpressing a disordered region from the C-terminal part of SRRM2 that does not encompass its presumed RNA-binding domain; this overexpression cannot induce SICC formation (**Fig 3h**). Unfortunately, since the SRRM2 RNA-binding domain has not been experimentally dissected (but rather *in silico* annotated in UniProt; <https://www.uniprot.org/uniprotkb/Q9UQ35/>), we would not know where to perform targeted mutations or deletions in order to confidently produce a binding-deficient SRRM2-RBD.

However, there are two striking results from our SRRM2-RBD overexpression experiments. On the one hand, SRRM2-RBD overexpression already induces SICCs (**Fig 3g**) despite the presence of more RNA and HMGB2 (the factor that needs to be depleted in order for SICCs to appear in senescence; see Zirkel et al, *Mol Cell* 2018; PMID 29706538). On the other hand, introduction of this short SRRM2-RBD peptide in senescent cells depleted of full-length SRRM2 with siRNAs, and thus also depleted of SICCs, suffices for *de novo* CTCF clustering (although these clusters are somewhat smaller; **Extended Data Fig 6b**). Thus, we think that it is rather protein-protein interactions that drive these effects, and that CTCF RNA-binding is at best secondary. As the Reviewer suggests, this might mean that crosslinking of CTCF outside SICCs might produce these associations or it might be that these RNA associations simply reflect the persistent CTCF proximity to RNA in speckles via SICCs. Although we favor the latter (since the majority of CTCF is found on chromatin at any given time; see **Fig 2a**), we cannot formally rule out the former. Accordingly, we amended the respective section of the **Results** (on pg. 5) to acknowledge this.

3. What could be the functional relevance of the longer-range, nested CTCF loops that emerge during senescence? Or more generally, can individual alternative splicing events be linked to differential loops at these loci?

This is precisely what we are proposing on the basis of our 3D genomics (Micro-C and LoopTrace) data and mRNA splicing analysis. To elaborate a bit, we postulate that this reorganization of CTCF loops will now position many genes (e.g., the genes from chr5 shown in **Fig 7g**) at specific distances to nuclear speckles thereby affecting their splicing patterns. This is also evidenced by data in **Fig 7f** showing loci with altered AS patterns in senescence being focally enriched for TSA-seq signal (indicative of speckle proximity), and in **Extended Data Fig 11i** showing exon-intron 'GC signatures' expected of speckle-associated genes (signatures obtained from Tammer et al, *Mol Cell* 2022; PMID 35182478). We have

added an expanded version of this explanation in our revised **Discussion**, which is also reflected in the graphical model added to **Fig 7i** following the Reviewer's suggestion.

4. *Suppl fig. 11i: HMGB2-binding mRNAs substantially overlap with ICM-induced AS genes. Probably I miss some points, but I wonder how we interpret this. What is the relationship between these 287 HMGB2-binding/AS mRNAs and APEX2-SRSF7 mRNA?*

We added the HMGB2 sCLIP data to the manuscript for the sake of completion, but our explanation should have been more detailed. As the Reviewer correctly points out, there exists substantial overlap between HMGB2-bound mRNAs and ICM-induced AS genes (**Extended Data Fig 13a-c**). There also exists substantial overlap between ICM-induced and HMGB2-KD-induced AS genes (**Extended Data Fig 11g**), and many individual AS events on mRNAs occur at positions bound by HMGB2 prior to senescence entry (**Fig 7e**). Thus, we propose that the depletion of HMGB2 from senescent cell nuclei underlies these AS changes due to its loss from its cognate sites on both chromatin and RNA.

As regards the overlap with the APEX2-SRSF7-captured mRNAs: we have now looked into this and >700 (i.e., 57%) of all HMGB2-bound sCLIP targets are speckle-associated RNAs (given a 4-fold enrichment cutoff in APEX2-seq data), while ~1/4 of all HMGB2-bound sCLIP targets are significantly depleted from speckles in senescence (but none are enriched). Moreover, of the 287 HMGB2-bound AS mRNAs, 79 qualify as 'speckle-depleted' and 188 as speckle-associated. We have now added this new information on pg. 11—and we thank the Reviewer for motivating this.

5. *How do the alternative splicing events in this model compare to alternative splicing previously reported in other or similar senescence models (PMID 39118304)?*

We thank the Reviewer for pointing us to the work by Deschênes *et al* which we had missed. The authors make a number of interesting observations, including that senescence downregulates multiple splicing factors (which we also see in our data from Palikyras *et al*, *Aging Cell* 2024; PMID 38196311), that the pharmacological inhibition of splicing alone can induce senescence and senescence-related AS changes or that splicing defects (elicited via *SF3B1*-knockdown) may also induce senescence signatures. Thus, we reanalysed their RNA-seq data (from early-passage and senescent BJFs) and found significant overlap to the AS changes in our ICM model, but moderate overall correlation (which should be expected given the different cell types). We provide this analysis below (see **Fig R7**), but we have now also included in the manuscript a comprehensive analysis comparing AS events in ICM-induced senescence with those in replicative senescence of IMR90 and endothelial cells (HUVECs), as well as with data from late-age individuals (whole blood and skin fibroblast transcriptomes; see **Extended Data Fig 12**). This new data highlights the broad relevance of our findings for senescence and its ageing-related implications, which we discuss alongside the Deschênes *et al* observations (which we cite on pg. 10).

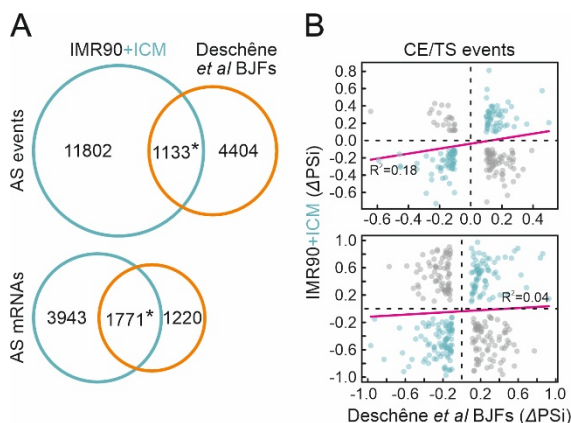


Fig R7: A, Venn diagrams showing the overlap of individual alternative splicing (AS) events (top) or AS mRNAs (bottom) following Whippet analysis of our data (IMR90+ICM) and those in Deschênes *et al* (using BJ fibroblasts). *: more than expected by chance. **B**, Correlation of ΔPSi values of individual core exon (CE; top) or transcription start site AS events (TS; bottom) from the two datasets.

Minor points:

6. “.... are not a result of accumulating DNA damage (Supplementary Fig 1c).” Do they mean acute DNA damage or DNA damage-induced senescence?

Our wording was not ideal here. To rephrase: cells committing to senescence following 6 days of ICM treatment do not accumulate DNA damage (therefore, they would not resemble DNA damage-induced senescence). We now clarify this in the main text (see pg. 4).

7. The enzymatic (RNase A) depletion of RNA promotes CTCF-SON interaction (supple fig. 4b). Do they see increased recruitment of speckles components to SICCs? This is not obvious in Supple fig. 4a (bottom).

Essentially, we see little change in immunofluorescence images following RNase A treatment. However, there likely are changes to speckle properties that we have not quantified here, hence our statement only pertains to how RNA affects the phenotype in reference to **Extended Data Fig 5a,b**.

8. “.... (Fig 4c), suggesting that its disordered tail alone interferes with CTCF-BANF1-SRRM2 interactions” The imaging data alone (Fig. 4c), without additional assays like co-IP, wouldn’t support this statement.

Our imaging data in **Fig 4c** show that overexpression of the disordered C-terminal tail of HMGB2 alone suffices for dissolution of SICCs. This likely involves the same mechanism as the rescue via the full-length HMGB2 protein, which does interact with speckle components (see **Fig 4d**). Thus, it is not unreasonable to propose that this occurs via its disordered C-tail. Nevertheless, we performed a new co-IP experiment in IMR90 expressing the HMGB2 C-tail and find that it indeed interacts with multiple speckle-associated components as suggested (e.g., U2AF1, SF3B2, SRSF1,-2, and-9), as well as with BANF1 and SRRM2 (data presented in **Extended Data Table 2**).

Reviewer #2 Remarks on Protocols: Apart from comment #6, I did not identify any major issues regarding methods availability.

We apologise for the oversight and have added these treatment details in the revised figure legend.

Reviewer #3: *In this study, Palikyras and colleagues continue the characterization of SICCs formation in senescence, addressing their mechanisms of formation and function. For the former, they report that the clustering of CTCF during senescence entry, previously reported to depend on depletion of HMGB2, also requires on a repurposing of the SRRM2 and BANF1 protein. For the latter, they report that SICCs associate with nuclear speckles, which changes the RNA splicing program.*

I consider the functional characterization (Figures 4-7) interesting, although some findings may be considered somewhat incremental. The characterization of SICCs formation (Figures 1-3) instead has a range of issues: incomplete characterization, tunnel vision and inconsistencies with previous work from the same group. Based on my evaluation, I advise against publication in Nature Cell Biology.

We are happy to see that the Reviewer finds the functional characterization of SICCs (spanning **Figs 4-7**) “interesting”. We also understand where the issues with the characterization of SICC formation (**Figs 1-3**) may come from and have made changes to remedy this on the basis of the Reviewer’s comments. Please also note that we added new analyses that confirm our key findings in replicative senescence of both HUVECs and IMR90, as well as in chronologically aged human skin fibroblasts and whole blood samples (see **Extended Data Fig 12**).

Major issues:

1. *The SICCs formation part of the study alludes regularly to phase separation / condensate formation, particularly through its computational focus on IDRs and experiments with 1,6-hexanediol. As reported by many groups, the confirmation of phase separation effects requires careful experimental validation (see e.g. <https://pubmed.ncbi.nlm.nih.gov/30682370/>). Due to its ubiquitous effect on nuclear and cellular processes, 1,6-hexanediol experiments require careful follow up, for instance in-vitro reconstitution of condensate formation or mutations / truncations of IDRs. This study does not include such experiments, rendering any conclusions premature and speculative (ie. discussion, page 12).*

We fully agree with the Reviewer here and are aware that we are not in a position to formally comment on the “phase separation” nature of SICCs based on the data in our manuscript. We also agree the 1,6-HD treatments do not provide a decisive readout due to pleiotropic effects. Nevertheless, the lack of further experiments in the direct of “phase separation” is not intentional, but rather due to various aspects pertaining to our system of study. First, IMR90 (as most primary human cells) are not amenable to genome editing within their replicative lifespan (i.e., one cannot obtain pure genome-edited clones before cells senesce or start dying); this does not allow us to test the exact nature of SICCs via FRAP, because we cannot tag endogenous CTCF (and ectopic overexpression of CTCF-GFP in most primary cells, including IMR90, leads to immediate cell cycle defects). Second, the propensity of CTCF to form condensates *in vitro* has already been demonstrated (Zhou et al, *iScience* 2022; PMID 36117989). Third, nuclear speckles represent *bona fide* phase separated entities that are well studied (e.g., Greig et al, *Mol Cell* 2020; PMID 32048997). These aspects make it almost impossible to discuss the formation of SICCs on speckles without alluding to “phase separation”, although we acknowledge that we offer no biophysical characterisation of the nature of SICCs (due to the aforementioned technical limitations).

Our intention was to discover molecular partners that can control CTCF clustering in senescence (which we did in BANF1 and SRRM2), and to assess how SICC formation might be related to senescence commitment (which we also did by connecting 3D genome refolding to splicing patterns, and by showing that SICC dissolution delays commitment to senescence in both ICM-treated and replicatively senescent cells). Thus, the Reviewer’s remark is not at odds with our intention, and we have carefully reworded the whole manuscript to reflect these caveats and removed this part from the revised **Discussion**.

2. Other work, including citation 49 and notably the work of Karsten Rippe and colleagues, has shown that other mechanisms can explain the formation of nuclear foci. The study does not report any efforts to exclude alternative mechanisms beyond phase separation, nor does it give a rationale why such avenues were not explored. The finding that SICCs formation is sensitive to DNase treatment (Supplementary Fig. 4b) provides compelling evidence that phase separation is not involved in SICCs formation. This possibility should be addressed or excluded prior to publication.

We assume that the Reviewer is referring to LLPS (liquid-liquid phase separation) here, which is indeed not compatible with chromatin dependence (and, thus, not compatible with sensitivity to DNase I). We are in full agreement and now explicitly state this in our revised **Results section** (on pg. 5). We should clarify though that, when it comes to condensates associated with chromatin, “phase separation” is still regularly used as a term that loosely implies a phase transition without specifying an exact biophysical mechanism (we actually discuss this issue in a recent review we co-wrote with Karsten Rippe; Papantonis and Rippe, *Nat Rev Genetics* 2025; PMID 40537661). Thus, taking into account the technical limitations discussed in response to comment #1 above, we now do state that the data in **Extended Data Fig 5b** essentially exclude LLPS for SICCs (see pg. 5). This makes the manuscript much clearer with respect to the “phase separation” aspect of the work, and we thank the Reviewer for motivating this.

3. The study claims that SICCs formation critically relies on functional repurposing of SRRM2 and BANF1. These proteins localize differently in the nucleus (SRRM2 at speckles, mostly centrally located and BANF1 mostly at the nuclear periphery) and both proteins are studied independently from each other. In the discussion (page 12), the authors claim “a subset BANF1 molecules apparently move from the periphery to the nuclear interior”, but any proof for such behavior is missing. Without characterization of a functional interplay between both factors (MS upon senescence entry?), the dual dependence of SICCs formation on both factors remains poorly explained.

First, we want to state that our attempt for an explanation on pg. 13 is within the limits of the **Discussion** of our results, and does not claim any more direct evidence than that provided in the figures. We agree with the Reviewer that one might expect SRRM2 and BANF1 to co-associate in senescence, especially since (i) CTCF co-IP/MS show it interacts with both proteins in senescence, and (ii) that the knockdown of either protein prevents efficient SICC formation. To this end, we have performed a co-IP between the two factors to find that they indeed interact with one another (data added to **Extended Data Fig 5b**).

4. Based on imaging, Fig. 1a claims a drastic (~90%) reduction in HMGB2 upon ICM treatment, but using the same experimental setup and a more direct measurement of protein amounts (Western blotting; Fig. 1h in ref. 38), the authors previously reported a much more moderate difference. Similarly, the reduction in CTCF amounts upon ICM treatment were more moderate in ref. 38 (Western blotting, Fig. 1h) as compared to this study (Western blotting, total amount in Fig. 2a). This discrepancy raises questions about the reproducibility of the cell system that is used in this study, which remains unexplained.

We understand where the Reviewer comes from here. The reduction in HMGB2 levels in **Fig 1a** of this manuscript is 6-fold (i.e., ~83%) by immunofluorescence, whereas in western blots shown in Palikyras et al (*Aging Cell* 2024; PMID 38196311) it is in the order of ~60%. A similar trend applies to CTCF when one compares **Fig 2a** here with **Fig 1h** in Palikyras et al (2024), although the blots in **Fig 2a** were exposed separately and might not be fully comparable (as we wanted chromatin bands to not oversaturate). To reassure the Reviewer, we are showing below independent replicates of western blots using whole cell and fractionated extracts from IMR90 treated with ICM for 0-6 days (see **Fig R8**). Nevertheless, in our hands (including hundreds of ICM treatments performed over the last 5 years) HMGB2 and CTCF are

consistently reduced, SICCs readily form, and the senescence gene expression program is initiated. Thus, although some heterogeneity arises, the phenotype of cells is robust and reproducible, and offers two key advantages in the study of replicative senescence. First, it drastically reduces the time required to obtain senescent cell populations (6 days by ICM compared to >2 months by serial passaging); second, it produces an almost uniformly senescent IMR90 populations rendering comparisons cleaner. We hope the Reviewer acknowledges these advantages especially for genomics approaches that require multiple replicates and large numbers of cells.

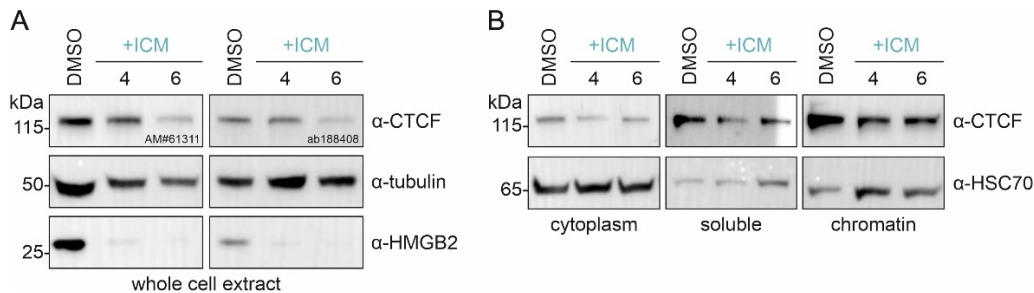


Fig R8: A, Western on whole cell extracts from IMR90 treated with DMSO or ICM for 4 or 6 days; please note that CTCF is detected by two different antibodies, while tubulin levels provide a loading control. **B**, As in panel A, but from fractionated IMR90 using HSC70 as a loading control.

Minor issues:

- Page 3: *“All five proteins had a biophysical profile similar to that of CTCF”. What do the authors mean by biophysical profile?*

In hindsight, we agree that the word “profile” might not best reflect what is analysed in **Extended Data Fig 2a**. We meant to refer to the biophysical properties predictive or not of the condensation behaviour of proteins with intrinsically disordered domains as computed via the CIDER framework from the Pappu lab (Holehouse et al, *Biophys J* 2017; PMID 28076807). We have now changed this to read “protein state properties computed via CIDER” as per the authors’ nomenclature (see pg. 4).

- Page 4: *“CTCF and SC-35 immunostaining revealed a strong co-localization between SICCs and speckles (Fig 3a)”. How was strength of co-localization determined? The green signal appears generally enriched away from the nuclear periphery, whereas the red signal has peaks and valleys. In Fig. 3a: are the red and green lines the opposite from the color code in the image? How were arrows positioned in the graph: most but not all peaks are marked by an arrow, but an arrow is present within a dip as well.*

The line scan transverses 6 apparent SICCs (albeit not always directly across their center, which explains the variability in CTCF signal intensity). These are the ones highlighted by arrows in the graph. For clarity, we have now added zoomed-in insets of two characteristic SICCs under the line scan (black rectangles), as well insets with all acquisition channels of another typical SICCs (white rectangle).

- Page 4: *discussion of results from Supplementary Fig. 3a-d: despite being significantly different, the actual differences are minor as compared to the diversity of speckles within the same group. These results should be presented in a more toned-down manner.*

We agree with the Reviewer that the differences between treatments and controls are not dramatic, and we have now toned this down in the relevant part of the **Results** (see pg. 5).

- Page 4: *“Thus, RNA likely competes with CTCF or repels it from clustering on speckles.” Based on the presented results, I don’t understand how the authors come to this conclusion.*

This is based on a number of observations. First, **Extended Data Fig 5a** shows that RNase A treatment of senescent IMR90 does not compromise SICCs. Second, **Extended Data Fig 5b** shows that RNase A treatment, in contrast to DNase I treatment, produces a much stronger CTCF-SON interaction. Third, we know that senescent cells produce less RNA overall (Palikyras et al, *Aging Cell* 2024; PMID 38196311). Fourth, our eCLIP data show that CTCF shows little RNA binding capacity in proliferating cells (**Extended Data Fig 5c**) in line with recent biochemical evidence published by the Guttman lab (Guo et al, *Mol Cell* 2024; PMID 38387462). Together, these observations suggest that higher RNA levels likely repel CTCF interactions with an RNA-rich body like the speckles. But this changes dramatically in senescence, where our APEX2-seq data show strong depletion of RNAs from nuclear speckles (**Extended Data Table 9**).

- Page 5: “*inherent affinity for speckle-enriched proteins*”. What result shows that this effect is inherent? Again, we acknowledge that the wording is not ideal and have reworded this to simply read “...affinity for speckle-enriched proteins”. We thank the Reviewer for pointing this out.

- Page 5 and Fig. 4e: “*Venn diagram showing the overlap between the HMGB2 (green circle) and CTCF interactomes (grey circle) from proliferating and senescent IMR90, respectively*”. The figure purportedly shows two different data sets, but only a single Venn diagram is shown.

There was a miswording here that causes confusion; we apologise. **Fig 4e** shows the overlap between proteins interacting with HMGB2 (which is only expressed in proliferating cells), all proteins that CTCF co-immunoprecipitates with, and the TSA-MS catalogue of nuclear speckle components (from Dopie et al, *J Cell Biol* 2020; PMID 32609799). This was meant to show that some speckle-enriched interactors of HMGB2 in proliferating cells, like SRRM2 may be “handed over” to CTCF in senescence, when HMGB2 is depleted from cells. We have adjusted the figure legend and figure panel, and clarify this on pg. 6.

- Page 5-8 and Figs 5 and 6: *ChIPmentation results are illustrated with browser views and averaged enrichment plots. This provides little information about genome wide behavior of peak signal or overlap between data sets. Addition or replacement by Venn diagrams and aligned heatmaps would be preferred.*

The information conveyed by such Venn diagrams was already in the **Results** text on pg. 7, and we prefer this due to space limitations in a rather crowded figure. The aligned heatmaps we can of course provide, and they expand what the average profile plots show in **Figs 4** and **5** (see **Fig R9**, below). We have now added these heatmaps to **Extended Data Fig 7f**.

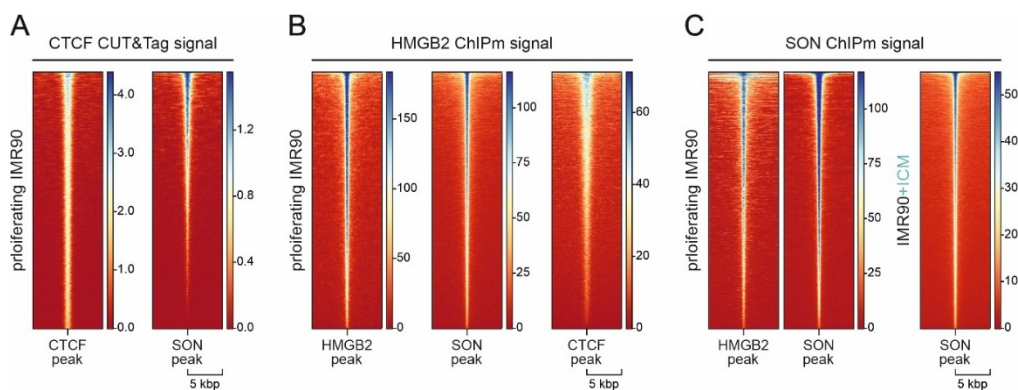


Fig R9: A, Heatmaps of CTCF CUT&Tag data in the 10 kbp around CTCF or SON peaks from proliferating IMR90 that match the average profile plots shown in Fig 4g. **B**, As in panel A, but for HMGB2 ChIPmentation data that match the average profile plots shown in Fig 4h. **C**, As in panel A, but for SON ChIPmentation data from proliferating and ICM-treated IMR90 that match the average profile plots shown in Figs 4h and 5b.

- Page 7: section from “interestingly, >25% of HMGB2 peaks fell within”. Unless I’m mistaken, the mentioned HMGB2 peaks are those in proliferative cells (HMGB2 data upon ICM treatment is not shown). Intersecting those with loop anchors in senescent cells is not optimal. The authors should consider modifying this analysis and/or rephrase the discussion (for instance focusing on SON, which maintains its genomic position upon HMGB2 degradation).

This is indeed that comparison we made, but there is scope to it (which we did not make very clear in the text). Just like the Reviewer suggests, HMGB2 produces robust peaks only in proliferating cells, as it is quantitatively depleted from senescent nuclei. We intersected this HMGB2 peak list with loop anchors from both proliferating and senescent cells. This helps reveal that many loop anchors previously bound by HMGB2 are affected at the onset of senescence, with >500 being rewired to form senescent-specific loops anchored at new genomic positions. We now explain this more clearly (see pg. 8).

- Page 7: “while loops with both anchors bound by HMGB2 were the smallest in size and essentially dissolved”. Signal and enrichment for these loops remains clearly visible in Fig. 7d and the absolute reduction is in a similar range as the other categories. The authors should more carefully interpret this result.

We agree with the Reviewer and have rephrased this to reflect signal reduction rather than dissolution.

- Page 7: “Last, we generated Micro-C data after 1,6- hexanediol treatment”. As mentioned above, the effect of 1,6- hexanediol is highly ubiquitous and aspecific. Without additional validation, the authors should not interpret the changes relative to SICCs structure or function.

We do not disagree with the Reviewer on this. Indeed, 1,6-HD treatment will have pleiotropic effects, and work by the Maeshima lab showed that it may also condense chromatin and reduce its overall mobility (assessed by live-cell imaging in Itoh et al, *Life Sci Alliance* 2021; PMID 33536240). At the same time though, we hope that the Reviewer acknowledges that it is not by sheer coincidence that the loops lost upon 1,6-HD treatment are predominantly those that were gained upon senescence entry (**Fig 5j,k**). Thus, we are happy to tone down this part of the **Results** even more, and also point out the pleiotropic effects of this crude treatment (see pg. 8). Still, we would rather keep this data in the manuscript as (to our knowledge) it is the only Micro-C dataset generated using such a treatment and might also serve as a resource for others wanting to analyse 1,6-HD effects on 3D chromatin folding.

- Page 8: I appreciate the results from the LoopTrace studies. The authors should explain though why the three selected regions were chosen and where CTCF, HMGB2 and SON bind in these regions in proliferative and senescent cells.

The Ellenberg lab had a small collection of readily-available probes targeting 1-Mbp regions on different chromosomes at the highest (12-kbp) resolution. We screened this collection for regions containing transcriptionally-active genes mapping to the A1 subcompartment (harboring most speckle-associated chromosomal loci; see Ahanger et al, *Nat Neurosci* 2021; PMID 34239128). The 1-Mbp region on chr5 studied here was chosen because it met these requirements; the lower resolution probe sets used alongside this one serve as internal controls for the LoopTrace experiment (i.e., were used to confirm expected 3D genome folding at 0.2- and 1-Mbp resolution; see **Extended Data Fig 7a-c**). We now explain this on pg. 9 of the revised **Results** section, and we provide browser views of the HMGB2 and SON tracks under the existing CTCF tracks in revised **Fig 6c** as requested.

- At several places in the manuscript, words are used that convey an opposite message over what is reported: (1) title: “sustain” -> SICCs formation upon senescence entry instead appears to instruct a

different splicing program, (2) “rescue” on page 5: “we could rescue SICCs” and in the title of Fig. 4 -> HMGB2 reintroduction perturbs SICCs formation; do the authors mean “revert” instead?

We thank the Reviewer for pointing these issues out; we have now reworded all of these and combed the manuscript to correct any other mistakes. We are especially happy with the suggestions about the manuscript’s title and now use “instruct” instead of “sustain”.

Point-by-point Response to the Reviewers' comments (*italicized in blue*):

Reviewer #1:

The authors made a recognized effort to answer our queries.

We are happy to see the Reviewer acknowledging our effort; we have now also addressed all remaining remarks (below).

Fig. 1a – at the end of page 3, it is stated that HMGB2 is depleted. According to the figure, this is not entirely accurate, as we only see a decrease in protein levels. Consider updating the figure to reflect the actual reduction.

This is correct, the depletion is not complete; the actual extent of HMGB2 reduction is reflected in the signal quantification presented via the violin plots of **Fig 1a** (~70%). We now mention this number at the bottom of pg. 3 of the revised **Results** section.

End of page 4 – BANF1 reduction – no quantification. Did the authors verify BANF1 knockdown in cells that formed SICCs? May be possible to estimate the minimal levels of BANF1 required for generation of SICCs.

We have now added the percent of BANF1 reduction (~69%) on pg. 4 of the main text. The knockdown of BANF1 was indeed validated (see RT-qPCR in **Fig 2b**) and, as also explained in our previous rebuttal, we did assess BANF1 levels in relation to the appearance of SICCs, and there seems to be a threshold of BANF1 availability below which SICCs emerge (see **Fig R2**, below). This is the same figure the Reviewer refers to in their last comment (see next page), advising us not to add it to the manuscript.

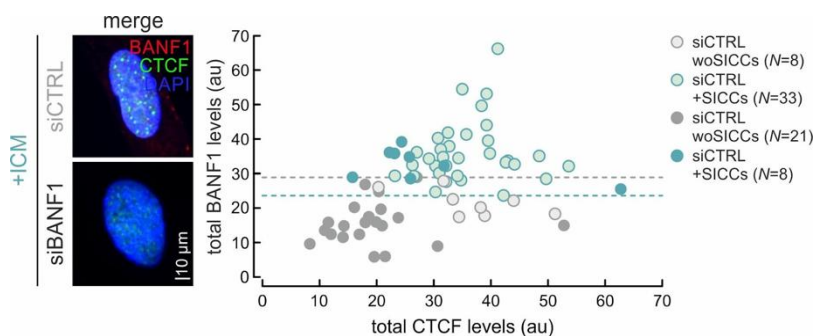


Fig R2: Left: Typical images of IMR90 treated with ICM and with control or BANF1-targeting siRNAs. Right: Scatter plot quantifying total BANF1 and CTCF intensity in cells carrying (green) or not SICCs (grey). Dotted lines represent the bottom and top values in each subset.

Top of page 5- the claim is that Tautomycin dispersed many speckles; however, this is not apparent in Extended Data Fig. 4a. Additionally, Tautomycin is not mentioned in the Methods section. Speckle dispersion by Tautomycin is concentration-dependent. Testing higher concentrations might effectively disperse nuclear speckles and potentially allow SICC formation.

We agree that this may not be so apparent to the naked eye but, as already explained in our previous rebuttal, we quantified the radius and eccentricity of speckles in 70 cells treated with tautomycin plus ICM, and in 64 cells treated with ICM alone; this analysis showed significantly reduced speckle radii and a trend towards higher eccentricity upon treatment with this drug (**Extended Data Fig 4b**).

Apologies for omitting the treatment details. We have now added these for both tautomycin and cantharidin in the first paragraph of the revised **Methods** (on pg. 24). Finally, we indeed tested different concentrations (0.1-4 μM) and time points (1 or 4 hours) based on existing literature (by the Lawrence lab; see Hall et al, *Anat Rec A Discov Mol Cell Evol Biol*, 2006), but had to be cautious of toxicity effects to our primary cells; hence the final decision to use 2 μM for 4 hours (that is certainly at the higher end of the concentrations and time points tested).

Fig. 2C,D – please include the words "with SICCs" to % of cells in the graphs, and anywhere else if needed. It will be much clearer like that.

These bar plots quantify the percent of cells with and without SICCs (and they are used throughout the manuscript). As they are meant to present both outcomes ("with SICCs" and "without SICCs"), the y-axis should be labeled as "% cells". Moreover, we had already colour-coded these plots and labels ("with SICCs" in green and "without SICCs" in grey) to make interpretation of the graphs intuitive.

Page 8: "1,6-hexan treatment dissolved SICCs, but not nuclear speckles (Extended Data Fig. 3g)." This should be corrected to Extended Data Fig. 5g.

We thank the Reviewer for noticing this mistake, which we have now corrected (it should actually refer to **Extended Data Fig 4f** in this final version of the manuscript).

Fig. 3e – what is the difference between the two nuclei shown? write under the zoomed images the protein name. In addition, suggest to add the separated channels as it is very hard to see the green signal.

There is no difference, we just wanted to show two examples instead of one. The protein names that were missing under the zoom-ins of the top image have now been added. Unfortunately, the figure has not got enough space to add separate channels for the whole-cell view, but we already present each channel imaged for the three representative SICC zoom-ins, which we think provide good examples of the signal structure in either channel.

Why don't the authors connect the AS paragraph (page 9) to the findings of interactions with many splicing factors (page 6).

This is a good suggestion; we have now added a sentence in the paragraph opening this section (see pg. 10) that now reads: "Given that [...] both CTCF and HMGB2 interact with numerous splicing factors, we hypothesized that alternative splicing (AS) might constitute a programmed driver of senescence entry."

Figure R2 does not need to be added to figure 2.

Thank you, we have not added it.

Reviewer #2: *The authors have adequately addressed my questions. They also have expanded the relevance of their findings to senescence and ageing contexts.*

We thank the Reviewer for this endorsement.

Reviewer #3: *I want to thank the authors for the prompt sharing of many of the source images generated in this study. Upon in-depth investigation, I've found a number of anomalies that need to be scrutinized before the manuscript can be considered for publication. I have uploaded a pdf that present the issues in more detail.*

We want to thank the Reviewer for pointing to these issues, which we address below.

Major issues:

1. Figure 2C: the BANF1 panel in the siBANF2 condition is a cut-out (jpg) that is completely black, with the exception of a single dot of signal that becomes visible upon strong boosting of contrast. This is different from the widefield source file (tiff): here a weak but noticeable background and a number of brighter puncta are visible (page 1 and 4 in the pdf). It seems unlikely that the jpg was extracted from the widefield tiff, unless the authors selected a different region or used very different contrast settings as compared to other panels in the manuscript. The authors need to trace back how the jpg file was obtained from the source tiff file.

The same issue may be present in Figure S2D-F of the supplemental data: the signal for the ATXN2, SQSTM1 and FMR1 panels in the siRNA-treated cells appears different from other panels. The source files for these panels were not shared and therefore this issue remains to be confirmed.

The BANF1 panel of the 'siBANF1' condition should indeed show the very weak signal that the Reviewer refers to. For the sake of clarity, we can explain how any microscopy panel in our figures is created: The raw image .LIF file from our Leica widefield microscope is opened in ImageJ (Fiji) and each channel is extracted in .TIFF format using default parameters. Then, this .TIFF file is "saved as" .JPEG (again under default settings) and imported into CorelDRAW to crop and present. For every set of images in a given figure panel, we will concertedly enhance contract by 5-10% (as typically advised to improve printing of the figures). We cannot say for certain if this process (with three different CorelDRAW versions used to date on these figures) might have somehow compressed the signal here, but we have recompiled the figure panel ensuring that the weak BANF1 signal is visible. Besides, no knockdown is 100% efficient (as seen in BANF1 RT-qPCR data in **Fig 2b**). Nevertheless, this does not at all affect data interpretation or the message of this figure panel.

2. Figure 2D: the BANF1-Venus channel is nearly completely black in both the cut-out (jpg) and widefield sourced file (tiff). Upon strong boosting of contrast, very weak background signal becomes visible in the tiff file, but this is absent within the cells (page 2 and 5 in the pdf). The jpg cut-out instead is evenly black, with the exception of some minor stripes, which may be a jpg compression artifact.

This signal pattern is highly unlike, as the Venus fluorescent protein emits light within a range that is quite close to the Alexa fluor A488 fluorophore that was used to visualize the CTCF protein (see pdf). Using a conventional widefield microscope, as used in this study, a minor bleedthrough of the A488 signal is expected in the Venus channel. The authors need to confirm that the Venus protein was visualized using the correct laser and filter.

The same issue may be present in Figure S6B of the supplemental data: a certain amount of bleedthrough is expected in the SRRM2 RBD-Venus panels. The source files for these panels were not shared and therefore this issue remains to be confirmed.

We think that there is some misunderstanding here. The Reviewer says that the BANF1-Venus image in the '-Dox' condition shows "very weak background signal". We absolutely agree; this is the uninduced control, where no BANF1-Venus is supposed to be expressed and only background-level signal is to be expected (as Dox-inducible promoters can be slightly leaky). Based on our explanation above, this might

be a *JPEG* compression artefact, as the Reviewer also assumes. We have also recompiled this panel just to be absolutely sure of the source of the cut-out (which we anyway provided to the Reviewers).

Regarding “bleed-through”: If we interpret the Reviewer correctly, they assume that CTCF is imaged using an Alexa488 secondary antibody, and this is based on us not reporting the use of other secondaries in the **Methods** section. However, we feel that it would be apparent by the broad use of multi-colour IFs across the manuscript (e.g., **Extended Data Figs 1d, 2d-f, 4a,e,f, 5a,g**) that we would never image two protein targets in the same colour channel. In this particular case, CTCF was imaged in the red channel using a goat anti-rabbit-Cy3 secondary (#ab6939). In addition, should bleed-through occur, one would expect the two images to have similar signal patterns, which is not the case here. Nonetheless, this comment made us realise that we had forgotten to mention the additional secondary antibodies in the **Methods** section, which we have now corrected (see pg. 23-24).

Minor issue:

Figure 2C: the visualized cell in the siCTRL condition is flipped and resized along the X-axis, which is undisclosed (page 1 and 3 in the pdf). Although this does not change the conclusions, such modifications relative to the source data are not allowed.

Looking at the Reviewer’s detailed analysis for the cell shown under ‘siCTRL’, we agree that resizing along the x-axis did occur, most likely during alignment of the figure panel elements. We thank the Reviewer for noticing this, since we wouldn’t have noticed resizing in the order of a few pixels. Both flipping and resizing have been corrected.

Point-by-point Response to the Reviewers' comments (shown *italicised*):

Reviewer #1: *This study sets out to show, using a wide range of experimental approaches, what are the nuclear formations of CTCF under senescence, and finds they are connected to nuclear speckles through the SRRM2 and BANF1 proteins. Examining 3D organization and alternative splicing patterns the authors suggest that under senescence there is reorganization of genes in the 3D space with respect to nuclear speckles and this suggests changing alternative splicing patterns. The study encompasses a very large body of work, however, while this reviewer found the first part clear to follow, once the large amount of sequencing and genome organization data appeared, with far too many details, the study became very hard to follow and therefore to fully see how all the parts come together.*

We appreciate the Reviewer's comment on the "wide range of experimental approaches" we used, as well as on the "very large body of work" in our study. We also acknowledge that the second half of the manuscript would benefit from rewriting and weeding through the many data and figure panels to streamline its main message. Nevertheless, this message is precisely as the Reviewer describes: "nuclear formations of CTCF ...connected to nuclear speckles" which ultimately lead to "reorganization of genes in the 3D space with respect to nuclear speckles ...changing alternative splicing patterns". We hope that with our clarifications (below) and the respective changes to the manuscript, this message does come together more clearly now.

Fig. 1a – top row has small nuclei, bottom row the nucleus is huge, yet there is only one scale bar – does that mean the nuclei grow under ICM treatment? If not, then show at same scale, and for the treatment show a field of cells, not just one. Also, comparing to Fig. 3a, in 3a the CTCF looks like nuclear speckles indeed, while in Fig. 1a its less typical.

Yes, nuclei are shown at the same scale (hence the single scale bar); they should become more flat and larger upon ICM treatment, which is very much what happens under induction of replicative senescence via serial passaging of cells. These effects were already described in our Palikyras et al (2024) *Aging Cell* paper, where the ICM-induced senescence system is presented in detail and is accompanied by a large resource of biochemical and genomics data for the community to use (see PMID 38196311).

Now, regarding the comparison between **Figs 1a** and **3a** (and generally between the many figure panels showing CTCF clustering), there is indeed some heterogeneity which is (also) reflected on the shape of these clusters – this is to be expected of senescence. We deliberately chose not to show only cells with uniformly round SICC's so that readers have an appreciation of this heterogeneity. We are of course happy to show more images, if needed.

More biochemical measurements of HMGB2 are required to show the reduction in total HMGB2. While under DMSO treatment HMGB2 looks more diffuse in the nucleus, adding ICM lowers the diffuse portion, but the nucleolus remains predominant. Is there a connection between HMGB2 relocation to nucleolus and SICC appearance?

We have documented this senescence-associated reduction of HMGB2 levels (both protein and RNA) in different primary human cell lines via different measurements (western blots, mass spectrometry, RNA-seq, RT-qPCR, ELISA) in our previous work on replicative senescence (Zirkel et al, *Mol Cell* 2018; PMID 29706538), as well as in our recent paper describing the ICM-induced senescence model (Palikyras et al, *Aging Cell* 2024; PMID 38196311). We therefore did not feel we had to reiterate this here. However, we now make this more explicit in the relevant part of the results.

Regarding the nucleolar HMGB2 staining: this is a known side-effect of formaldehyde fixation (to which HMGB2 displays a peculiar behaviour likely due its DNA-binding domain; Pallier et al, *Mol Cell Biol*; PMID 12925773), where any residual protein will “stick” to the nucleolus. This is now also documented for its sister protein, HMGB1, by us (Sofiadis et al, *Mol Syst Biol* 2021; PMID 34166567) and others (Mensah et al, *Nature* 2023; PMID 36755093), but does not influence quantification of total nuclear signal. We are happy to add these details in the **Methods** section for clarity, and thank the Reviewer for pointing this out.

Fig. S1b – large field, few, and very small nuclei.

Thank you for pointing this out; we now provide a more zoomed-in version.

Fig. 1b – mark BANF1 and SRRM2.

Thank you for suggesting this; now both proteins are demarcated in the plot.

In some images there is a decrease (Fig. 1a) in HMG while in others there is a disappearance (Fig. 1d). Can the difference be explained?

Although ICM produces largely homogeneous IMR90 populations within 6 days of treatment (please see detailed quantification at the single cell-level in Palikyras et al, *Aging Cell* 2024; PMID 38196311), some heterogeneity will still exist. Thus, some cells show more of a decrease in HMGB levels than others.

ICM does not result in DNA damage (Fig. 1c) – please provide evidence.

The experimental evidence for this is shown and quantified in **Supplementary Fig 1c**.

Fig. 2a – any idea why CTCF has 2 bands after ICM?

In western blots from proliferating IMR90, CTCF will typically appear as a single band of ~130 kDa, but the presumed molecular weight of CTCF based on its open reading frame is ~70 kDa. This discrepancy is because CTCF is post-translationally modified, primarily poly(ADP)-ribosylated (Farrar et al, *Methods Mol Biol* 2011; PMID 21870268). We have now discovered that CTCF is hypo-PARylated in senescence, regardless of whether this is induced by ICM or by serial passaging; hence this second band in the blot of **Fig 2a**. We have started investigating this, and have validated that is poly(ADP)-ribosylation-specific by the use of PARP inhibitor—but we feel that this is beyond the scope of this (already large) study.

Fig. 2d – not sure why SC35 needs to appear here.

Thank you for pointing this out; it does not, and we have now removed this sub-panel.

Would BANF1 overexpression lower the time or concentration of ICM treatment? Is there a correlation between the amount of BANF1 protein and the appearance of SICC, inferring that overexpression of BANF1 fastens senescence/SICC formation.

We have not explicitly tested whether BANF1 overexpression renders ICM treatment more efficient, mainly because primary cells like IMR90 are not easy to transfect with high efficiency, and it becomes difficult to obtain homogeneous populations overexpressing a given factor—and this required for live-cell monitoring of cell proliferation. However, we do have the data on BANF1 amounts that the Reviewer is referring to. Indeed there seems to a correlation between the levels of BANF1 in a given nucleus and the appearance (or not) of SICCs (please see **Fig R1**, below). We are happy to add this data to **Fig 2**, if requested.

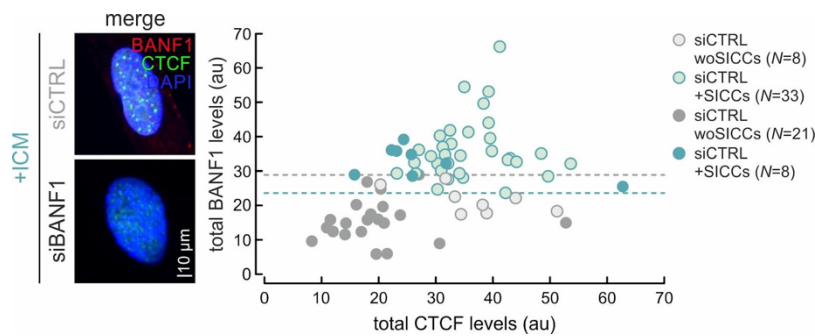


Fig R1: *Left:* Merged IF images of IMR90 treated with ICM and with control (top) or BANF1-targeting siRNAs (bottom). *Right:* Scatter plot quantifying total BANF1 and CTCF intensity from cells carrying (green) or not SICCs (grey). Dotted lines represent the bottom and top values for each subset.

The authors state that SICCs colocalize to nuclear speckles. Could it not be possible that with ICM, CTCF enters the NS and does not generate a different body? Perhaps SRRM2 connects CTCF to the NS, which is claim to be seen in Fig. 3c.

We agree with the Reviewer that CTCF most likely does not form a different body, but rather clusters on nuclear speckles (and perhaps inside its outer-most layer; see Fei et al, *J Cell Sci* 2017; PMID 29133588) and is connected to them via SRRM2. Hence, SRRM2 depletion leads to SICC dissolution (**Fig 3c**). This is what our super-resolution STED images would also suggest (**Fig 3e**; with the CTCF signal somewhat offset with respect to the bulk of the speckle signal. We now makes this postulation more explicit in our revised main text (see pg. 4).

Note this publication – PMID: 33095160, stating that the main target of SC35 mAb is SRRM2, which means when SRRM2 is knocked down and NS are labeled with SC35 then this suggest that the knockdown wasn't very good (need to add a SRRM2 staining for KD validation like with BANF1). Suggest to add SON staining, as it's the 2nd core protein of the NS aside from SRRM2, for NS marking.

We were aware of this issue, and chose the mouse monoclonal antibody anti-SC35 (NB100-1774) from Novus Biologicals because it was *not* one of those flagged in Ilik et al. (2020; PMID 33095160) to cross-react with SRRM2. In fact, this antibody does not recognise any of the two parts of the SRRM2 protein that we overexpress (i.e., RBD aa 197-259 or C-terminal IDR aa 1633-1695, the latter also included in the part of SRRM2 that Ilik et al. reported as the recognition region for SC-35 antibodies) as evidenced from **Fig 3g,h**. Also, upon ~80% SRRM2-knockdown, SC-35 staining persists (see **Supplementary Fig 3f**). Nevertheless, we agree with the Reviewer that SON staining would resolve this, but we provided this in **Supplementary Fig 3e**.

Short term treatment of 1,6 hexanediol dissolves liquid but not solid condensates (PMID: 38659924), maybe this can help differentiate between SICCs and NS. There are ways to disassemble nuclear speckles. What we see is that the short (2-min) treatment with 1,6-HD will dissolve SICCs, but will not dramatically compromise nuclear speckles (see **Supplementary Fig 3g**), thus somewhat decoupling the two entities as the Reviewer suggests

Verify that it's clear in all the figures and images which channel is which in the colored merge. For example, in Fig 2c, DMSO, we don't know which is green and which is red. If CTCF is red (like +ICM) where did it go in DMSO?

We acknowledge this oversight, and we have now amended all relevant figure legends to display which channel corresponds to which staining. For **Fig 2c** specifically, the Reviewer is correct—inadvertently, CTCF is shown in the green channel in cells, but in the red in ICM-treated cells. We have redrawn this figure and checked all other for consistent data presentation. Thank you for noticing this.

Fig. S2c-e – state that the cells were treated with ICM in both figure and legends.

Now corrected, as suggested.

Fig. 3a – show the unmerged channels separately.

Now shown, as suggested.

Fig. S3a – “Tautomycetin dispersed speckles and SICCs into smaller formations that are more elliptical (Supplementary Fig 3a,b)” – don’t see this in the images.

We show a characteristic field of view with one larger speckle upon treatment and many smaller ones; our statement is based on quantification of the radius, eccentricity and SICC formation in >60 nuclei as shown alongside this image.

“Cantharidin caused them to fuse into more round, larger structures (Supplementary Fig 3c,d).” – just one nucleus.

We showed one representative nucleus, but show quantification of >40 nuclei alongside this. We are happy to provide more examples, but—as also pointed out by this Reviewer—the paper is very data dense already and we thought that the quantification would suffice.

Fig. 3c,d – “This was not due to a reduction in CTCF levels, but rather due to its redistribution (Fig 3c,d)” – would need a Western blot to show whether there is no reduction.

We now also a western blot following SRRM2-knockdown in ICM-treated cells to show that no reduction in CTCF levels occurs (added to revised **Fig 3**).

Fig. 3e – the text is somewhat vague – on the one hand it says the signals are overlapping, on the other hand it says they are offset to each other. Perhaps some kind of colocalization analysis could help make this observation more precise.

There is clear overlap of the signals (also in non-super-resolution images like **Fig 3a**), and we now back our claim of (offset signal between the speckle and the CTCF stainings) via a line scan plot, as proposed.

Also, the CTCF signal appears quite weak and resembles background compared to the supplementary image (Fig. S3h) and other figures. Suggest replacing the images in Fig. 3e.

There is a small, yet measurable decrease in CTCF of 15% protein levels in senescence (Palikyras et al, *Aging Cell* 2024; PMID 38196311), so such weaker signal is not an exposure artefact (compared to that in a proliferating cell in **Supplementary Fig 3h**), but rather the result of dramatic relocalization within a thin optical section.

Different cells throughout the manuscript show varying numbers of CTCF foci. Is there any correlation between nuclear speckles numbers and CTCF foci?

Yes, there is a clear correlation – for example, when we manipulate speckles pharmacologically either with cantharidin or with tautomycetin to fuse or disperse, respectively, the CTCF foci follow, which also highlights their interconnection (see **Supplementary Fig 3a-d**).

Fig. S4a,b – where are the controls showing the Rnase/Dnase treatments worked?

We have lysed IMR90 cells and quantified residual RNA or DNA many times after such treatment. Please see **Fig R2** below for a reference on RNase A degradation.

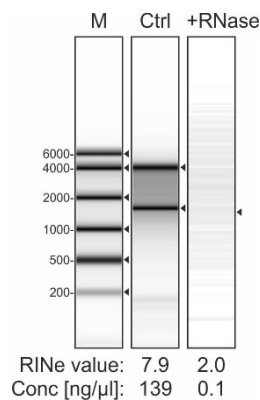


Fig R2: Tapestation profiles of RNA isolated from mock- (Ctrl) and RNase A-treated IMR90 (+RNase) following *in situ* permeabilization as for immunofluorescence assays.

Fig. 3g – don't really see nuclear speckles-like staining in the CTCF channel when the RBD is added; compare for instance to Fig. 3c. Therefore, the conclusion at the end of this section is not based on very strong evidence.

In **Fig 3g**, the SRRM2-RBD-Venus signal overlaps the SC-35 foci shown for that same nucleus. However, there is an important consideration here that explains the Reviewer's observation: compared to **Fig 3c** (and to any other image of a senescent nucleus), the SRRM2-RBD initiates CTCF clustering in the context of a still-proliferating cell that is not permissive of SICCs. Still, focal CTCF signal becomes obvious (please compare the two cells in the field of view: the left-hand-side one expresses the RBD, but the right-hand-side one does not and has no CTCF foci). If one looks at the same experiment performed in senescent cells pre-depleted of full-length SRRM2, the RBD overexpression effect is essentially the same, but more pronounced phenotypically (see **Supplementary Fig 5b**).

Fig. 4b – whats going on the top Venus image?

The expression of piggybac overexpression constructs can have varying output levels; here, HMGB2 is expressed quite highly and also spills out into the cytoplasm of these expanded senescent IMR90. This is not unusual, and is merely a reiteration of an experiment performed previously (i.e., that full-length HMGB2 rescue most SICC formation in senescent cells; see Zirkel et al, *Mol Cell* 2018; PMID 29706538).

Page 5 third line- RBD instead of RDB.

Thank you for noticing this, it has now been corrected.

Fig. 4j – not sure this panel is referred to in the text, and not clear what one is seeing or what it tells us (and Fig. S8).

This figure panel, together with data in **Supplementary Fig 8**, is based on whole-genome coarse-grained simulation via the Chrom3D framework that allows us to ask some questions on CTCF clustering at the levels of larger chromatin domains that are difficult to address experimentally. As mentioned in the text, these simulations of our actual experimental data further "supported the notion that active, CTCF- and HMGB2-rich domains reside in spatial proximity to nuclear speckles" and reorganise dramatically upon entry into senescence. However, panel **4j** was wrongly referred to as **4i** on pg. 6, but is now corrected.

The explanation of how SICC formation alters alternative splicing patterns is missing.

We agree with the Reviewer that we should have been more explicit with our model of how SICCs may work to alter splicing patterns upon senescence entry. In brief, the reorganization of CTCF loops will now position different genes (e.g., the three genes from chr5 presented in **Fig 7h**) at specific distances to nuclear speckles thereby affecting their splicing patterns. This is also evidenced by data in **Fig 7f,g** that

show that loci with altered AS patterns in senescence are enriched for TSA-seq signal (indicating speckle proximity) and show exon-intron 'GC signatures' expected of speckle associated genes (the signatures are obtained from Tammer et al, *Mol Cell* 2022; PMID 35182478). We have added an expanded version of this explanation in our revised **Discussion**. We have also added a graphical model to revised **Fig 7** to help readers visualize this concept (see also **Fig R3**, below).

The author should provide supplementary data demonstrating that treatment of fetal lung fibroblasts with ICM induces senescence.

As also mentioned above, the full description of the ICM model for replicative-like senescence in IMR90 is detailed and accompanied by a large resource of biochemical and genomics data in our recent paper (Palikyras et al, *Aging Cell* 2024; PMID 38196311). We now explicitly direct readers to this.

Reviewer #2: *In this manuscript, Palikyras et al. provide an in-depth characterisation of SICC formation in unique culture models. Using a proteomics approach, they identified BANF1 and speckles' components, particularly SRRM2, as key regulators of SICC formation. In addition, using multimodal omics approaches and computational simulation, they show evidence suggesting a negative correlation between HMGB2 and CTCF-Speckles interaction as well as formation of longer-range, nested CTCF loops. At the single-cell level, they employed 3D DNA tracing, validating some of these findings. Further introducing CLIP/APEX2 analyses suggests that alternative splicing events are associated with SICC formation. Interestingly, they show that disruption of SICC integrity by depleting SRRM2, BANF1 or CTCF alleviates proliferative arrest in ICM-treated cells. This study potentially identifies important mechanisms for the spatial clustering of SICC and its functional relevance. The dynamic interaction between HMGB2, CTCF, and speckles, as well as RNA, is interesting, but the data integration is quite complex: the manuscript could benefit from the inclusion of a model illustration.*

We are happy to see that the Reviewer finds that we provide an “in-depth characterisation of SICC formation in unique culture models” in a study that “potentially identifies important mechanisms for the spatial clustering of SICC and its functional relevance”, where the “dynamic interaction between HMGB2, CTCF, and speckles, as well as RNA, is interesting”. We also agree that the overall integration of different types of data, especially the link between biochemistry and cell biology with 3D genomics, can become quite complex. To remedy this, we have now simplified figures and text to streamline our main message, but also provide a model as suggested (in **Fig 7** and as **Fig R3** below) to make this more comprehensible.

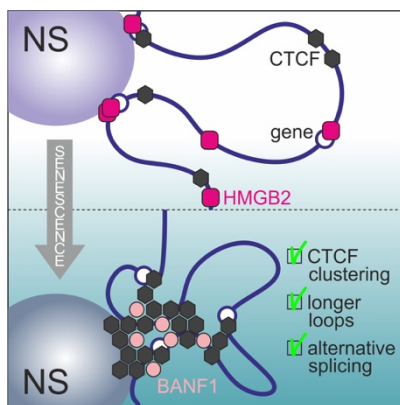


Fig R3: Graphical model showing 3D reorganization of CTCF-bound chromatin with respect to speckles via BANF1 and SRRM2 that ultimately leads to senescence-associated splicing patterns on genes involved in these cluster formations.

Major points:

1. *The role of RNA in SICC formation on speckles: Their data suggest that overall RNA inhibits CTCF-speckles interaction and RNA-unbound CTCF contributes to CTCF clustering. Yet, CTCF eCLIP shows increased peaks during senescence (supple fig. 4c). Do they mean that those CTCF CLIP peaks represent the non-SICC fraction of CTCF?*

We agree with the Reviewer that our data suggest that overall RNA is likely acts in a refractory manner for the CTCF-nuclear speckle interactions, which is in line with stronger interaction and ultimately CTCF clustering on speckles in senescent cells that produce less RNA overall (see Palikyras et al, *Aging Cell* 2024; PMID 38196311). We deduce from our simulations (**Supplementary Fig 6g-i**) and experiments using TPA or $ZnCl_2$ (**Supplementary Fig 4g**) that unbound CTCF (not only from RNA, but primarily from chromatin) will critically contribute to the observed clustering. But how does the CTCF eCLIP data reflect this? They are actually in line with a recent careful biochemical dissection by the Guttman lab (see Guo et al, *Mol Cell* 2024; PMID 38387462) where CTCF does not show specific RNA binding capacity. This also holds true in our data from proliferating IMR90, but the number of eCLIP peaks indeed increases in

senescence as CTCF is positioned on RNA-rich speckles due to SICC formation (**Supplementary Fig 11**). We believe that these RNA associations simply reflect persistent CTCF proximity to RNA in speckles, and when crossed with our APEX2-SRSF7 data, we found measurable overlap of co-associated genes.

2. Similarly, they show that overexpression of SRRM2-RBD is sufficient for CTCF clustering, where SRRM2-RBD and CTCF clusters mostly colocalise. Could the authors clarify how they interpret the data? Do the data imply that the speckles-mediated 'focal' enrichment of RNA is critical for CTCF clustering, while the enzymatic (global) depletion of RNA does not compromise SICC formation? If so, can the authors test this by using an ectopic expression of an RNA-binding-deficient mutant (if available) of SRRM2-RBD? Together with the CTCF data, I wonder if the following interpretation is in line with the authors' - while speckle-mediated RNA recruitment may be important for CTCF clustering, CTCF-RNA binding primarily occurs outside of CTCF clusters.

We thank the Reviewer for this insight—it is a plausible scenario and our take on it is the following. First, the test that the Reviewer is asking for, we have essentially performed by overexpressing a disordered regions from the C-terminal part of SRRM2 that does not encompass its presumed RNA-binding domain; this overexpression cannot induce SICC formation (**Fig 3h**). Unfortunately, since the SRRM2 RNA-binding domain has not been experimentally dissected (but rather annotated *in silico* in the UniProt database; <https://www.uniprot.org/uniprotkb/Q9UQ35/>), we cannot perform targeted mutations or deletions to confidently produce a binding-deficient peptide.

However, there are two striking results from our SRRM2-RBD overexpression experiments. On the one hand, SRRM2-RBD overexpression already induces SICCs (**Fig 3g**) despite the presence of more RNA and HMGB2 (the factor that needs to be depleted to induce SICCs in senescence; see Zirkel et al, *Mol Cell* 2018; PMID 29706538). On the other hand, in senescent cells depleted of full-length SRRM2 with siRNAs, and thus of SICCs, introduction of this short SRRM2-RBD peptide suffices for CTCF clustering (although these clusters look somewhat smaller; **Supplementary Fig 3f**). Thus, we think that it is rather protein-protein interactions that lead to these effects, and that CTCF RNA-binding is at best secondary. As the Reviewer suggests, this might mean that crosslinking of CTCF outside SICCs might produce these associations or it might be that these RNA associations simply reflect persistent CTCF proximity to RNA in speckles via SICCs that also involve chromatin. Although we favor the latter (because the vast majority of CTCF is found in the chromatin fraction at any given time; see **Fig 2a**), we cannot formally rule out the former. Accordingly, we amended the respective section of the **Results** (on pg. 10) to reflect this.

3. What could be the functional relevance of the longer-range, nested CTCF loops that emerge during senescence? Or more generally, can individual alternative splicing events be linked to differential loops at these loci?

This is precisely what we are proposing by combination of our 3D genomics (Micro-C and LoopTrace) data and splicing analysis of RNA-seq. To elaborate a bit, we postulate that this reorganization of CTCF loops will now position different genes (e.g., the three genes from chr5 presented in **Fig 7h**) at specific distances to nuclear speckles thereby affecting their splicing patterns. This is also evidenced by data in **Fig 7f,g** that show that loci with altered AS patterns in senescence are enriched for TSA-seq signal (indicating speckle proximity) and show exon-intron 'GC signatures' expected of speckle associated genes (the signatures are obtained from Tammer et al, *Mol Cell* 2022; PMID 35182478). We have added an expanded version of this explanation in our revised **Discussion**, which can be also visualized via the graphical model added to **Fig 7** (see also **Fig R3**, above) as the Reviewer suggested.

4. *Suppl fig. 11i: HMGB2-binding mRNAs substantially overlap with ICM-induced AS genes. Probably I miss some points, but I wonder how we interpret this. What is the relationship between these 287 HMGB2-binding/AS mRNAs and APEX2-SRSF7 mRNA?*

We added the HMGB2 sCLIP data to the manuscript for the sake of completion, but our explanation should have been more detailed. As the Reviewer correctly points out, there exists substantial overlap between HMGB2-bound mRNAs and ICM-induced AS genes (**Supplementary Fig 11i**). There also exists substantial overlap between ICM-induced AS and HMGB2-KD-induced AS genes (**Supplementary Fig 10g**), and many individual AS events on mRNAs occur at positions bound by HMGB2 prior to senescence entry (see **Fig 7e**). Thus, we propose that the loss of HMGB2 in senescence is linked to these AS changes, which are further associated by their relative positioning relative to the active speckle environment. As regards the overlap with the APEX2-SRSF7-captured mRNAs: we have now looked into this and we have added this information on pg. 9-10, and we thank the Reviewer for motivating this.

5. *How do the alternative splicing events in this model compare to alternative splicing previously reported in other or similar senescence models (PMID 39118304)?*

We are happy to provide this analysis (see **Fig R4**, below), but the work by Deschênes *et al* suggests that changes in splicing may be seen as defects, which might not be entirely true. Our data suggest more of a programmed change to senescence-associated AS patterns implemented 3D genome reorganization.

Minor points:

6. *".... are not a result of accumulating DNA damage (Supplementary Fig 1c)." Do they mean acute DNA damage or DNA damage-induced senescence?*

Maybe our wording was not ideal here. To rephrase: cells committing to senescence following 6 days of ICM treatment are not accumulating DNA damage (therefore, they would not resemble DNA damage-induced senescence). We have now clarified this in the main text.

7. *The enzymatic (RNase A) depletion of RNA promotes CTCF-SON interaction (supple fig. 4b). Do they see increased recruitment of speckles components to SICCs? This is not obvious in Supple fig. 4a (bottom).*

Essentially, we see little change in immunofluorescence images following RNase A treatment. However, there likely are changes to speckle properties that we cannot quantify here, hence our statement only on how RNA affects this interaction in reference to **Supplementary Fig 4a,b**.

8. *".... (Fig 4c), suggesting that its disordered tail alone interferes with CTCF-BANF1-SRRM2 interactions" The imaging data alone (Fig. 4c), without additional assays like co-IP, wouldn't support this statement.*

Our imaging data in **Fig 4c** show that overexpression of the disordered C-terminal tail of HMGB2 alone suffices for dissolution of SICCs. This likely involves the same mechanism as the rescue via the full-length HMGB2 protein, which does interact with speckle components (see **Fig XX**). Thus, it is not unreasonable to propose that this interaction is via its disordered C-tail. Nevertheless, we have now performed a co-IP experiment in IMR90 expressing the disordered HMGB2 tail to show interactions of the HMGB2 tail alone with speckle-associated components as suggested (see new **Supplementary Fig 5d**).

Reviewer #2 Remarks on Protocols: Apart from comment #6, I did not identify any major issues regarding methods availability.

We apologise for the oversight and have added these treatment details in the **Methods** section.

Reviewer #3: *In this study, Palikyras and colleagues continue the characterization of SICCs formation in senescence, addressing their mechanisms of formation and function. For the former, they report that the clustering of CTCF during senescence entry, previously reported to depend on depletion of HMGB2, also requires on a repurposing of the SRRM2 and BANF1 protein. For the latter, they report that SICCs associate with nuclear speckles, which changes the RNA splicing program.*

I consider the functional characterization (Figures 4-7) interesting, although some findings may be considered somewhat incremental. The characterization of SICCs formation (Figures 1-3) instead has a range of issues: incomplete characterization, tunnel vision and inconsistencies with previous work from the same group. Based on my evaluation, I advise against publication in Nature Cell Biology.

We are happy to see that the Reviewer finds the functional characterization of SICCs (spanning **Figs 4-7**) “interesting”. We also understand where the issues with the characterization of SICC formation (**Figs 1-3**) may come from and have made changes to remedy this on the basis of the Reviewer’s comments.

Major issues:

1. *The SICCs formation part of the study alludes regularly to phase separation / condensate formation, particularly through its computational focus on IDRs and experiments with 1,6-hexanediol. As reported by many groups, the confirmation of phase separation effects requires careful experimental validation (see e.g. <https://pubmed.ncbi.nlm.nih.gov/30682370/>). Due to its ubiquitous effect on nuclear and cellular processes, 1,6-hexanediol experiments require careful follow up, for instance in-vitro reconstitution of condensate formation or mutations / truncations of IDRs. This study does not include such experiments, rendering any conclusions premature and speculative (ie. discussion, page 12).*

We fully agree with the Reviewer here and are aware that we are not in a position to formally comment on the “phase separation” nature of SICCs based on the data in the paper. We also agree the 1,6-HD treatments do not provide a decisive readout for this due to pleiotropic effects. However, there are a number of aspects that need to be considered here given our particular system of study. First, IMR90 (and most primary human cells) are not amenable to genome editing within their replicative lifespan (i.e., one cannot really obtain pure genome-edited clones before cells senesce or start dying); this does not allow us to test the liquid or other nature of SICCs via FRAP, because we cannot tag endogenous CTCF (and ectopic overexpression of CTCF-GFP in most primary cells including IMR90 leads to prompt cell cycle defects). Second, the propensity of CTCF to form condensates *in vitro* has already been demonstrated (Zhou et al, *iScience* 2022; PMID 36117989). Third, nuclear speckles represent *bona fide* phase separated entities that are well studied (e.g., Greig et al, *Mol Cell* 2020; PMID 32048997). These aspects make it almost impossible to discuss the formation of SICCs on speckles without any reference to “phase separation”, although we acknowledge that we have no biophysical characterisation of SICCs (mostly due to technical limitations), and that the 1,6-HD treatment is not sufficient.

Our intention still is to discover molecular partners that can control CTCF clustering in senescence (which we find in BANF1 and SRRM2, and offer functional evidence for this), and to then assess how the formation of SICCs on speckles might be related to senescence commitment. Thus, the Reviewer’s remark is not at odds with our intention, and we have now reworded the manuscript to reflect the issues discussed here. Moreover, on pg. 12 of the **Discussion**, we now cautiously refer to this.

2. *Other work, including citation 49 and notably the work of Karsten Rippe and colleagues, has shown that other mechanisms can explain the formation of nuclear foci. The study does not report any efforts to exclude alternative mechanisms beyond phase separation, nor does it give a rationale why such avenues were not explored. The finding that SICCs formation is sensitive to DNase treatment*

(Supplementary Fig. 4b) provides compelling evidence that phase separation is not involved in SICCs formation. This possibility should be addressed or excluded prior to publication.

We assume that the Reviewer is referring to LLPS (liquid-liquid phase separation) here, which is indeed not compatible with chromatin dependence (and, thus, not compatible with sensitivity to Dnase I). We are in full agreement and now explicitly address this in the **Results** (already on pg. 3). We should clarify though that, when it comes to condensates associated with chromatin, “phase separation” has been regularly used as a term that loosely implies a phase transition, without specifying any exact biophysical mechanism (we actually discuss this issue in a recent review we co-wrote with Karsten Rippe; please see Papantonis and Rippe, *Nat Rev Genetics* 2025; PMID 40537661). Thus, and also taking into account the limitations discussed in response to comment #1 above, we now explicitly state that the results in **Supplementary Fig 4b** essentially exclude LLPS for SICCs. Next, in the **Discussion** we refer to chromatin-seeded condensates without claiming that we have addressed their underlying biophysical properties or mechanisms. This makes the manuscript way more clear with respect to the “phase separation” aspect of the work, and we thank the Reviewer for motivating this.

3. The study claims that SICCs formation critically relies on functional repurposing of SRRM2 and BANF1. These proteins localize differently in the nucleus (SRRM2 at speckles, mostly centrally located and BANF1 mostly at the nuclear periphery) and both proteins are studied independently from each other. In the discussion (page 12), the authors claim “a subset BANF1 molecules apparently move from the periphery to the nuclear interior”, but any proof for such behavior is missing. Without characterization of a functional interplay between both factors (MS upon senescence entry?), the dual dependence of SICCs formation on both factors remains poorly explained.

First, we want to state that our attempt for an explanation on pg. 12 is within the limits of the discussion of our results, and does not claim any more direct evidence than that provided in the figures. We agree with the Reviewer that one might expect SRRM2 and BANF1 to co-associate in senescence, especially since (i) CTCF co-IP/MS show it interacts with both proteins in senescence, and (ii) that the knockdown of either protein prevents efficient SICC formation. To this end, we have performed a co-IP between the two factors to find that they do *not* directly interact.

4. Based on imaging, Fig. 1a claims a drastic (~90%) reduction in HMGB2 upon ICM treatment, but using the same experimental setup and a more direct measurement of protein amounts (Western blotting; Fig. 1h in ref. 38), the authors previously reported a much more moderate difference. Similarly, the reduction in CTCF amounts upon ICM treatment were more moderate in ref. 38 (Western blotting, Fig. 1h) as compared to this study (Western blotting, total amount in Fig. 2a). This discrepancy raises questions about the reproducibility of the cell system that is used in this study, which remains unexplained.

We understand where the Reviewer comes from here. The reduction in HMGB2 levels in **Fig 1a** of this manuscript is 6-fold (i.e., ~83%) by immunofluorescence, whereas in western blots shown in Palikyras et al (*Aging Cell* 2024; PMID 38196311) it is in the order of ~60%. A similar effect seems to be documented for CTCF when one compares **Fig 2a** with **Fig 1h** in Palikyras et al (2024), although the blots in **Fig 2a** were exposed separately and might not be fully comparable (as we wanted the chromatin bands to not oversaturate). However, in all cases (including hundreds of ICM treatments we have performed in the last 5 years) HMGB2 is consistently reduced by at least 60%, CTCF readily forms clusters, and relevant senescence markers are differentially regulated. We now know that any discrepancies are simply due to ICM batch purity; when bought “off the shelf” from Sigma, ICM tends to be a bit less potent than the industrial-scale batch that we ordered recently and store in aliquots for future use. Thus, although some

discrepancies arise, the overall phenotype of the system is robust and reproducible, and offers two key advantages in the study of replicative senescence. First, it drastically reduces the time one has to wait in order to obtain senescent cell populations (6 days via ICM compared to >2 months via serial passaging); second, it produces an almost uniformly senescent population which renders comparisons cleaner. We hope that the Reviewer acknowledges these advantages when studying molecular mechanisms via such approaches as genomics that require multiple replicates and large cell numbers.

Minor issues:

- Page 3: *“All five proteins had a biophysical profile similar to that of CTCF”. What do the authors mean by biophysical profile?*

In hindsight, we agree that the word “profile” might not best reflect what we show in **Supplementary Fig 2a**. We meant to refer to the biophysical properties predictive or not of the condensation behaviour of proteins with intrinsically disordered domains as computed via the CIDER framework from the Pappu lab (Holehouse et al, *Biophys J* 2017; PMID 28076807). We have now changed this to read “computed protein state properties” as per the Pappu’s lab nomenclature.

- Page 4: *“CTCF and SC-35 immunostaining revealed a strong co-localization between SICCs and speckles (Fig 3a)”. How was strength of co-localization determined? The green signal appears generally enriched away from the nuclear periphery, whereas the red signal has peaks and valleys. In Fig. 3a: are the red and green lines the opposite from the color code in the image? How were arrows positioned in the graph: most but not all peaks are marked by an arrow, but an arrow is present within a dip as well.*

The line scan transverses 5 apparent SICCs (albeit not always directly across their center, which explains the variability in CTCF signal intensity). These are the ones highlighted by arrows in the graph. We have now added the same arrows also on the image for clarity, and have replaced the word “strong” with “apparent” as we indeed do not measure strength here, just qualitative overlap.

- Page 4: *discussion of results from Supplementary Fig. 3a-d: despite being significantly different, the actual differences are minor as compared to the diversity of speckles within the same group. These results should be presented in a more toned-down manner.*

We agree with the Reviewer that the differences between treatments and controls are not dramatic, and we have now toned this down in the relevant **Results** section (see pg. 4).

- Page 4: *“Thus, RNA likely competes with CTCF or repels it from clustering on speckles.” Based on the presented results, I don’t understand how the authors come to this conclusion.*

This is based on a number of observations. First, **Supplementary Fig 4a** shows that RNase A treatment of senescent IMR90 does not compromise SICCs. Second, **Supplementary Fig 4b** shows that RNase A treatment, in contrast to DNase I treatment, produces a much stronger CTCF-SON interaction. Third, we now that senescent cells produce less RNA overall (see Palikyras et al, *Aging Cell* 2024; PMID 38196311). Fourth, our eCLIP data show that CTCF does not show specific RNA binding capacity in proliferating cells (**Supplementary Fig 4c**) in line with recent biochemical evidence published by the Guttman lab (see Guo et al, *Mol Cell* 2024; PMID 38387462). Together, these observations would suggest that strong RNA presence likely repels CTCF interactions with an RNA-rich body like the speckles, but this changes dramatically in senescence, where our APEX2-capture experiment showed strong depletion of mRNAs from nuclear speckles (**Supplementary Table 9**).

- Page 5: *“inherent affinity for speckle-enriched proteins”. What result shows that this effect is inherent?*

Again, we acknowledge that the wording is not ideal and have reworded this to simply read "...affinity for speckle-enriched proteins". We thank the Reviewer for pointing this out.

- Page 5 and Fig. 4e: *"Venn diagram showing the overlap between the HMGB2 (green circle) and CTCF interactomes (grey circle) from proliferating and senescent IMR90, respectively". The figure purportedly shows two different data sets, but only a single Venn diagram is shown.*

This is a misunderstanding; **Fig 4e** shows the overlap between the proteins interacting with HMGB2 (as it is only expressed in proliferating cells) to the proteins that CTCF co-immunoprecipitates with in ICM-treated cells (when CTCF now associates strongly with speckles). This overlap between conditions is needed to show that speckle-enriched interactors of HMGB2 in proliferating cells are taken over by CTCF in senescence, when HMGB2 is absent/reduced in the cell nucleus. We now clarify this.

- Page 5-8 and Figs 5 and 6: *ChIPmentation results are illustrated with browser views and averaged enrichment plots. This provides little information about genome wide behavior of peak signal or overlap between data sets. Addition or replacement by Venn diagrams and aligned heatmaps would be preferred.*

The information conveyed by Venn diagrams is already in the **Results** text on pg. 7, and we opted for this due to space limitations in a rather crowded image. The aligned heatmaps we can of course provide, and they expand what the average plots in **Figs 5** and **6** show.

- Page 7: section from *"interestingly, >25% of HMGB2 peaks fell within". Unless I'm mistaken, the mentioned HMGB2 peaks are those in proliferative cells (HMGB2 data upon ICM treatment is not shown). Intersecting those with loop anchors in senescent cells is not optimal. The authors should consider modifying this analysis and/or rephrase the discussion (for instance focusing on SON, which maintains its genomic position upon HMGB2 degradation).*

There is scope to this comparison, but we failed to make it clear in the text. Indeed, HMGB2 produces robust peaks only in proliferating cells, as it is quantitatively depleted from senescent nuclei. Here, we interested this HMGB2 peak list with loop anchors found in both proliferating and senescent cells. This was meant to reveal that many HMGB2-bound loop anchors are remodeled in senescence, and that the rest are rewired to form senescent-specific loops. We now explain this more clearly.

- Page 7: *"while loops with both anchors bound by HMGB2 were the smallest in size and essentially dissolved". Signal and enrichment for these loops remains clearly visible in Fig. 7d and the absolute reduction is in a similar range as the other categories. The authors should more carefully interpret this result.*

We agree with the Reviewer and have rephrased this to reflect signal reduction rather than dissolution.

- Page 7: *"Last, we generated Micro-C data after 1,6- hexanediol treatment". As mentioned above, the effect of 1,6- hexanediol is highly ubiquitous and aspecific. Without additional validation, the authors should not interpret the changes relative to SICCs structure or function.*

We do not disagree with the Reviewer here. Indeed, 1,6-HD treatment will have pleiotropic effects, and work by the Maeshima lab showed that it may also condense chromatin and reduce its overall mobility (as assessed by live-cell imaging in Itoh et al, *Life Sci Alliance* 2021; PMID 33536240). At the same time though, we hope that the Reviewer acknowledges that it is not by sheer coincidence that the loops lost upon 1,6-HD treatment are predominantly those that were gained upon senescence entry of cells (**Fig 5j,k**). Thus, we are happy to significantly tone down this part of the Results, while also pointing out the

uncertain pleiotropic effects of this crude treatment, but we would rather keep this data in the paper as it is (to our knowledge) the only Micro-C dataset generated under such a treatment and might also serve as a resource for others studying 1,6-HD effects on 3D chromatin structure.

- Page 8: I appreciate the results from the LoopTrace studies. The authors should explain though why the three selected regions were chosen and where CTCF, HMGB2 and SON bind in these regions in proliferative and senescent cells.

The Ellenberg lab had a small collection of probes that targeted various domains on different human chromosomes at the highest (12-kbp) resolution. We screened this collection for probes that contained transcriptionally active genes also mapping to the A1 subcompartment (that was shown to contain most speckle-associated chromosomal parts (Ahanger et al, *Nat Neurosci* 2021; PMID 34239128). The 1-Mbp region on chr5 studied here matched these requirements and was thus chosen; the lower resolution probe sets used alongside this serve as internal controls for the LoopTrace experiment. Finally, we provide a browser view of the HMGB2 and SON tracks under the existing CTCF ones in this chr5 region from both proliferative and senescent IMR90 (in revised **Fig 6c**).

- At several places in the manuscript, words are used that convey an opposite message over what is reported: (1) title: "sustain" -> SICCs formation upon senescence entry instead appears to instruct a different splicing program, (2) "rescue" on page 5: "we could rescue SICCs" and in the title of Fig. 4 -> HMGB2 reintroduction perturbs SICCs formation; do the authors mean "revert" instead?

We thank the Reviewer for pointing these mistakes out; we have now reworded all of these and combed the manuscript to correct language throughout.