

Nuclear Export of R-loop by the DDX1 and XPO1 Complex Promotes Senescence-Associated Secretory Phenotype and Inflammaging

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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 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have clarified the model and some points of confusion. I also think this work is quite appropriate for Nature Aging and have no further suggestions.

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Reviewer #1:

Remarks to the Author:

The authors have clarified the model and some points of confusion. I also think this work is quite appropriate for Nature Aging and have no further suggestions.

Response: We thank the reviewer for the positive comments.

Point-by-point response to reviewers' comments

A point-by-point response to the reviewers' comments is detailed below. Changes that directly address the reviewers' concerns were highlighted [in blue](#) in the revised manuscript.

Reviewer #1 (Remarks to the Author):

I appreciate the effort the authors have put into revising their manuscript. Many of the initial comments have been adequately addressed. The lamin B1 knockdown experiments demonstrate that increased chromatin herniation does not increase cytoplasmic hybrid abundance, and the transfection experiments tracking exogenous hybrids into CCFs are also well executed. The new data clearly demonstrate that XPG is required for CCF-localized cytoplasmic hybrids, and that these hybrids tend to be in the soluble fraction of purified CCFs. These findings make the alternative model previously proposed (that the R-loops are emerging from the CCF chromatin) much less likely and support the author's favoured model in which soluble hybrids localize to CCFs once both components are in the cytoplasm.

A central concern, however, remains unresolved. While this study includes novel mechanistic findings related to the SASP and demonstrates effects at the whole-animal level, the cell biological aspects largely recapitulate mechanisms already established in the literature. Prior studies have shown that nuclear R-loops and RNA-DNA hybrids can be targeted by NER endonucleases and exported to the cytoplasm in an exportin-1-dependent manner where they engage innate immune sensors including cGAS (e.g. Koo et al. J Biol Chem 2015; Cristini et al. Cell Rep 2019; Goulielmaki et al. Nat Commun 2021; Chatzidoukaki et al. Sci Adv 2021; Crossley et al. Nature 2023). The current manuscript converges on this published mechanism, and the authors need to better acknowledge and discuss this prior work in the context of their own work.

Additionally, the new cell biology presented in this paper is focused on the mechanism of soluble hybrid localization to CCFs. As noted in Major Comment #4 of the initial review, the mechanistic basis for this localization remains insufficiently developed and was not addressed by this revision. The major outstanding questions that should be resolved prior to recommending this paper for publication are outlined below.

Response: We thank the reviewer for the recognition of our work. While our data are consistent with previous studies regarding the role of NER endonucleases in R-loop cytoplasmic sensing by cGAS, we revealed a novel DDX1-dependent nuclear export mechanism by which cytoplasmic/CCF localized R-loop drives the SASP and inflamming. As suggested by the Reviewer, we now better acknowledged all the relevant prior work on page 4, paragraph 2 with references 12, 13, 20, 21. We apologize for the confusion related to the mechanism of soluble hybrids localization to CCFs, which we now addressed both experimentally and by clarifications as detailed below.

- New experiments indicate that DDX1 is required to get both S9.6 and cGAS signal in CCFs (Fig. 4E). The authors also see the reciprocal effect, that cGAS knockdown prevents DDX1 and S9.6 signal in CCFs, implying that both cGAS and DDX1 are required for hybrid localization to CCFs (Fig. 5E, 5H). cGAS is found to interact with hybrids in a DDX1-dependent manner (Fig. 4B), but cGAS and DDX1 do not themselves physically associate (Extended Data Fig. 16D, E). Taking all these observations together, what is the proposed mechanism? Are the authors suggesting that one molecule of cGAS and one molecule of DDX1 each bind the same hybrid? If so, why would this interaction be necessary for cGAS to make its way to the CCF, given that

cGAS is a dsDNA-binding protein with no requirement for cofactors (Michalski Nature 2020; Pathare Nature 2020)?

- The authors show that cGAS can bind an R-loop without DDX1 (Fig. 4K). Why, then, is DDX1 required for the cGAS-hybrid association reported in Fig. 4B?

- How does the interaction of cytoplasmic hybrids with CCFs support cGAS activity? Canonically, cGAS must dimerize in a 2:2 ratio (cGAS:dsDNA) to achieve a conformation capable of producing cGAMP (Michalski Nature 2020). If cGAS is associating with DDX1, how are the authors proposing that cGAS is achieving its productive conformation?

These questions underlie the manuscript's main novel contribution – the mechanism by which hybrids become associated with CCFs and enable cGAS signaling. Without sufficient answers to these open mechanistic questions, the current version does not yet provide the level of robust, novel insight needed for publication in NCB.

Response: We sincerely apologize for the confusion caused by our description. Our data support a sequential mechanism at cell biology level. We clearly demonstrated that DDX1 is required for hybrids export from nuclei to cytoplasm and their subsequent localization to CCFs (**Fig. 4e**). Notably, the nuclear DDX1/hybrids complex is not exposed to cGAS that is localized in the cytoplasm. Thus, due to the compartmental distribution (nuclei vs. cytoplasm), the interaction between DDX1 and cGAS can only occur in the presence of hybrid in the cytoplasm because they can each interact with hybrid (**Fig. 4b**), but not themselves directly (**Extended Data Fig. 16d-e**). Once in cytoplasm, the localization of DDX1 and hybrids to CCFs are cGAS dependent because of 1) the binding between cGAS and hybrid, 2) the membraneless nature of CCFs and soluble nature of DDX1 and hybrid; and 3) localized concentration of cGAS at CCFs. This explained cGAS knockdown prevents DDX1 and S9.6 signal in CCFs. Hence, both cGAS and DDX1 are required for hybrid localization to CCFs (**Fig. 5e and 5h**).

Biochemically, cGAS can bind an R-loop without DDX1 (**Fig. 4k**). However, DDX1 is required for the cGAS-hybrid association in the cytoplasm as demonstrated in **Fig. 4b**, which depends on DDX1-dependent nuclear export of hybrids. As detailed above, cGAS does not directly associate with DDX1 and instead it is directly associated with hybrids (**Fig. 4k**). Thus, DDX1 will not affect cGAS's conformation/activity. Indeed, equal molar amounts of DDX1 did not affect cGAS activity toward hybrid as determined by 2'3'-cGAMP production (New Data **Fig. 4l**).

Together, our results support a novel mechanism in which DDX1 is required for nuclear exporting of hybrids, which subsequently in a cGAS-dependent manner localizes to CCFs to enable cGAS signaling to drive SASP. We now clarified the mechanism by including a model in **Fig. 5j** and in discussion section of the revised manuscript on page 17, paragraph 2.

Reviewer #2 (Remarks to the Author):

The authors have addressed my concerns/suggestions which has improved the readability and rigor of the manuscript.

Response: We thank the reviewer for supporting the publication of our manuscript.

Reviewer #3 (Remarks to the Author):

Cellular senescence drives chronic low-grade inflammation through the senescence-associated secretory phenotype (SASP), leading to tissue dysfunction and age-related diseases. Although cytoplasmic chromatin fragments (CCFs) and activation of the cGAS–STING pathway are

known to promote the SASP, the upstream triggers of this inflammatory response are not well understood. R-loops which are made of RNA–DNA hybrids and displaced single-stranded DNA, are known regulators of genome stability and innate immunity, but their role in senescence and inflammaging remains unclear. This study addresses that gap.

The authors show that senescent cells accumulate R-loops derived from the nucleus in the cytoplasm, particularly enriched in alpha-satellite repeat sequences. These cytoplasmic R-loops are not simply byproducts of genomic instability. Instead, they localize to CCFs and actively stimulate the cGAS–STING pathway, increasing SASP production and promoting inflammaging. Mechanistically, the study identifies a previously unrecognized nuclear export pathway for R-loops mediated by XPO1 and DDX1. This demonstrates that R-loop trafficking is an active, regulated process rather than a passive result of nuclear envelope damage. Inhibition of XPO1 with the clinically relevant inhibitor KPT-330 blocks R-loop export, reduces their accumulation in CCFs, suppresses cGAS–STING activation, and attenuates the SASP.

Overall, the study defines R-loops as active pro-inflammatory signaling molecules in senescent cells, extending their role beyond genome instability and cancer. It also reveals an XPO1/DDX1-dependent export pathway and highlights R-loop trafficking as a potential therapeutic target for reducing age-associated chronic inflammation, an important contribution to the field.

The authors have taken my previous comments and suggestions seriously and have thoroughly addressed them through revised text and additional experiments. They have responded to all of my concerns satisfactorily, and I am pleased to recommend the manuscript for publication. I have no further comments.

Response: We thank the reviewer for the positive comments and for supporting the publication of our manuscript.