

The chemotherapy-induced senescence-associated secretome promotes cell detachment and metastatic dissemination through metabolic reprogramming

Corresponding Author: Dr Katherine Aird

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

This manuscript addresses an important question regarding the role of fructose metabolism and the senescent secretome in ovarian metastasis. The work links fructose, cholesterol metabolism, and ovarian metastatic progression. The fructose-to-cholesterol mechanism is well explored. However, some mechanistic aspects remain insufficiently supported by the current data. In particular, a GLUT-5 knockdown in their ovarian line would bolster their fructose implications. Additionally, clarification of the relative contributions of the secretome versus direct senescent cell interactions are needed to strengthen the conclusions. Specific points that should be addressed are listed below.

1. The authors provide convincing evidence that fructose modulates cholesterol metabolism and promotes ovarian metastasis; however, to directly establish that fructose is the key (or major) driver, additional experiments are needed. GLUT5 is the principal fructose transporter in ovarian cancer cells. Without genetic or pharmacologic inhibition of GLUT5 it cannot be concluded that the observed effects depend predominantly on fructose uptake. GLUT5 knockout in ovarian lines followed by repeating the detachment assays using SCM, as well as repeating the in vivo model in Figure 5I with GLUT5-deficient cells, would be essential. Likewise, repeating the in vivo experiments in Figures 1B and 1E with GLUT5 knockdown in proliferating cells would clarify whether fructose uptake is also required. I think this is the most critical comment to be addressed as it would rule out other mechanisms such as conversion of glucose to fructose via the polyol pathway.
2. The authors claim that the senescence secretome drives metastatic spread and Figure 1E/F shows it is sufficient to increase tumor nodules. to show this is mediated through NDUFA, Figure 2M should use SCM as used in Figure 1E/F.
3. In Figure 3E, if SREBP is driving cholesterol synthesis via complex I-dependent NAD⁺ production, it is unclear why SREBPi does not alter detachment in the control groups. If SREBP1 is sufficient, SREBPi should increase detachment compared to control. This raises the possibility of alternative mechanisms of detachment or that the assay is saturated. If the assay is saturated, this would explain the lack of change. Alternatively, a ChIP assay targeting additional cholesterol-related transcription factors (e.g., LXR, RXR, ATF4) would strengthen the mechanistic conclusions, especially since the manuscript emphasizes SREBP.
4. The authors should provide PFS data for the mouse experiments. If mice are fed fructose versus glucose, does this impact PFS?

Reviewer #2

(Remarks to the Author)

In the paper "The chemotherapy-induced senescence-associated secretome promotes cell detachment and metastatic dissemination through metabolic reprogramming" Cole et al. demonstrate how senescent ovarian cancer cells can promote metastatic dissemination. The paper is well designed and of interest. The mechanistic description of fructose as a novel SASP component and how secreted fructose from senescent ovarian cancer cells can fuel is nicely demonstrated. Furthermore, some translational value is added by validating the finding in human ovarian patient material.

- The fructose concentration for the cell culture experiments is not reported, neither the absolute concentration in SAS. The fold change between control and SAS is 1.5 fold. Are the concentrations the authors use in their experiment physiologically relevant?

- The authors propose a conversion of fructose to DHAP. They should silence ketohexokinase (KHK) and aldolase B.
- The authors supplement NA to increase NAD⁺ in cells. They need to confirm the relevance of NAD⁺ by expressing LbNOX
- The authors write that "statins to more generally deplete intracellular cholesterol" which is incorrect because statins block only de novo cholesterol synthesis. Does cholesterol supplementation rescue the effects of statins?
- The key experiments are mainly performed in one cell line and need to be confirmed by at least 1-2 more cell lines.
- The patient data need to be analyzed for co-founding factors
- The claim that the senescent ovarian cancer cell are the driver should be strengthened with a senolytic experiment preferentially in vivo. Especially it was shown before that senolytics are very efficient in killing HGSOc PMID: 31186408. Alternatively, an introduced "suicide gene" such as INK ATTACK construct could be introduced into the senescent cells to eliminate them in an in vivo setting.

Minor comments

- The observation that the fraction below 3kDa secreted from senescent cells can drive dissemination is a novel concept it would be interesting to see if it's the sole driver of dissemination process. As such it would be interesting to see the higher than 3kDa fraction secreted from senescent cells can also drive detachment.
- a second senescent inducer to validate the findings is important to strengthen the claims. Alternatively the authors need to tune down their claims and make clear they are specific to cisplatin
- The authors indicate in Figure S5A a conversion of F1P to F1,6P. Could they please further elaborate on this conversion
- The authors need to confirm that after CIS treatment the cells are senescent using at least one more marker besides b-gal

Reviewer #3

(Remarks to the Author)

In this article entitled "The chemotherapy-induced senescence-associated secretome promotes cell detachment and metastatic dissemination through metabolic reprogramming" by Cole et al., the authors suggest that upon cisplatin treatment, some ovarian cancer cells may enter senescence and then promote dissemination of non-senescent cancer cells in a paracrine manner. This effect may involve the production/release of fructose by senescent cells promoting the detachment of non-senescent cancer cells. This detachment may rely on complex 1 activity, NAD⁺ production, and Sirt1-dependent inactivation of SREBP1-dependent expression of genes involved in cholesterol production.

Major issues:

- 1- Is there any evidence in mouse models and/or human samples that cisplatin treatment induces senescence in some cancer cells? Without this evidence, although interesting, the relevance of the observation will be rather speculative.
- 2- These observations are interesting, but unfortunately made in a unique context, that of ovarian tumors, and from one or two cancer cell lines treated in vitro. The generalizability of these results is not yet established. For example, do non-cancerous senescent cells in the tumor microenvironment, such as fibroblasts, also produce/release fructose and cause cancer cells to detach? Do SAS and fructose cause other types of cancer cells to detach and promote their dissemination? Is this effect specific to cisplatin or can it be observed during other stresses (e.g., other chemotherapies, oxidative stress, telomere shortening, etc.)? All these results will strongly strengthen the argument and allow the results to be placed in the context of aging, which the authors have not yet really done.
- 3- There are mechanistic gaps. Figure 5 and the glucose and fructose treatments suggest, in particular, the specific ability of fructose to induce downstream effects. Do the authors have any mechanistic answers explaining why fructose, and not glucose, impacts the entire pathway? For example, a difference in metabolite production (glycolysis and TCA cycle) and in electron transport chain activity?
- 4- Given the critical role of complex 1 activity and NADH/NAD⁺ levels in the authors' hypotheses, it is essential to measure them in some critical experiments throughout the manuscript.
- 5- The main thrust of the article is based on the results presented in Figure 1G, which aim to demonstrate that senescent cancer cells promote the dissemination of proliferative cells through their SAS. However, the effect of SAS treatment on dissemination is rather weak compared to the co-injection of senescent and proliferative cells (Figure 1G vs. 1D). Can the author optimize the test presented in Figure 1G to fully convince the reader?

Other issues:

- 1- Sometimes the level of information is insufficient:
 - What does Labeled with GFP mean? Is it expression or other methods? Is it stable?
 - Crystal violet staining gives an idea of cell number and density, but not the percentage of proliferation. Please modify or perform specific experiments, such as the EdU incorporation assay.
 - Please provide more complete and easily accessible information on the chronology of the experiments, for example, when IHC against Ki67 was performed.
 - Please indicate the names of the genes in front of the heatmaps and, when a geneset is mentioned, the names of the genes and their differential levels.
- 2- Figure 1K, can the authors try other senescence signatures to confirm the results?
- 3- Can the authors show the mRNA and protein levels across all cholesterol genes differentially expressed under SCM vs PCM, and under NDUF5 loss?
- 4- I don't understand the differences between Filipin's methodology for measuring plasma membrane cholesterol and total cholesterol, please explain.
- 5- The differences between normal senescent cells and cancerous senescent cells need to be discussed.
- 6- Can the authors use a complementary approach to filipin fluorescence intensity to quantify cholesterol?

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

For the most part the authors were able to address most of my concerns and the manuscript is greatly improved and should have a wide appeal to those interested in senescence but also thought from outside the field. I would only make one comment that the authors might consider. We have so many acronyms, I would consider eliminating SAS and just staying with SASP. SASP is not just transcriptionally controlled factors as the authors suggest but also post-transcriptionally controlled factors. I understand this manuscript is chasing metabolites but I am not sure we need more acronyms that encompass SASP and metabolites. If the authors/editor feels a new name is needed, perhaps something specific for metabolites might better serve the field.

Reviewer #2

(Remarks to the Author)

The authors have addressed my questions.

Reviewer #3

(Remarks to the Author)

The authors have addressed my concerns satisfactory.

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RESPONSE TO REVIEWERS

We would like to express our sincere gratitude to the referees and the Editor for their constructive and thoughtful review of our manuscript resubmitted to *Nature Aging*. We are grateful for the acknowledgment from the Editor and all Reviewers regarding the significance and impact of our research, including *“This manuscript addresses an important question regarding the role of fructose metabolism and the senescent secretome in ovarian metastasis” (Reviewer 1)*, *“These observations are interesting...” (Reviewer 3)*, and *“The paper is well designed and of interest. The mechanistic description of fructose as a novel SASP component and how secreted fructose from senescent ovarian cancer cells can fuel is nicely demonstrated.” (Reviewer 2)*.

We truly appreciate the critical role and effort of this set of knowledgeable and helpful reviewers. Our commitment to addressing the Reviewers' comments is reflected in the comprehensive point-by-point response outlined below and **the substantial addition of new data** presented in the revised manuscript. We have highlighted the new sections in red to help facilitate a more efficient review. We believe that these revisions result in a more compelling, robust, cohesive, and scientifically sound manuscript.

Editorial Comments

The reviewers make several useful comments and valuable suggestions for new experiments and analyses, in particular towards providing additional support for your mechanistic hypotheses and to probe generalizability of your findings. Regarding generalizability, editorially we would also encourage you towards testing your main conclusions in ovarian cancer cellular senescence models induced using another platinum-based chemotherapy beyond cisplatin (e.g. carboplatin) as well as and other types of senescence (via classic induction protocols, in additional, non-ovarian cancer cell lines) as these two extensions would provide different but valuable biological information.

Response: We are grateful to the Editor for providing guidance and additional suggestions to improve the generalizability of our findings. Towards this goal, we performed the following experiments:

1. Another platinum-based chemotherapy: We used carboplatin as a second platinum-based therapy as suggested by the Editor. Our new data demonstrate that conditioned media from two carboplatin-induced senescent ovarian cancer cell models increases detachment similar to cisplatin (**new Extended Data Fig. 4b-d**). We also demonstrate that fructose abundance is increased in conditioned media from two carboplatin-induced senescent ovarian cancer cell models (**new Extended Data Fig. 8e**). These data provide evidence that multiple platinum-based agents affect detachment, corresponding to increased fructose secretion.
2. Other types of senescence (via classic induction protocols, in additional, non-ovarian cancer cell lines): We performed **four new experiments** to demonstrate that detachment and fructose production is a generalizable feature of senescent cells, regardless of inducer or cell model. This includes cisplatin-induced senescence of melanoma cells and fibroblasts, gemcitabine-induced senescence of pancreatic cancer cells, and replicative senescence of fibroblasts (**new Extended Data Fig. 4b-c, e-f**). Indeed, conditioned media from these models has increased fructose abundance (**new Extended Data Fig. 8e**) and increases detachment of cancer cells (**new Extended Data Fig. 4d and Extended Data Fig. 4g**).

Together, these new experiments provide different but valuable biological information regarding the generalizability of our findings that senescence increases detachment of bystander cells, corresponding to fructose secretion (**Page 37, Lines 628-633**).

Reviewer 1

We want to thank the reviewer for identifying specific key experiments to clarify and improve this work. In response to these comments, we have provided new experiments validating the role of fructose metabolism in driving SAS-induced detachment and dissemination, providing more rigorous validation of the role of complex I *in vivo*, and clarifying the role of SREBP1 and cholesterol metabolism in our model. The specific experiments and rationales used to address these concerns are detailed below, as are annotations of where these improvements can be found in the revised manuscript.

Comment #1: *The authors provide convincing evidence that fructose modulates cholesterol metabolism and promotes ovarian metastasis; however, to directly establish that fructose is the key (or major) driver, additional experiments are needed. GLUT5 is the principal fructose transporter in ovarian cancer cells. Without genetic or pharmacologic inhibition of GLUT5 it cannot be concluded that the observed effects depend predominantly on fructose uptake. GLUT5 knockout in ovarian lines followed by repeating the detachment assays using SCM, as well as repeating the in vivo model in Figure 5I with GLUT5-deficient cells, would be essential. Likewise, repeating the in vivo experiments in Figures 1B and 1E with GLUT5 knockdown in proliferating cells would clarify whether fructose uptake is also required. I think this is the most critical comment to be addressed as it would rule out other mechanisms such as conversion of glucose to fructose via the polyol pathway.*

Response: We appreciate the reviewer's request for additional experiments to validate the role of fructose in promoting detachment both *in vitro* and *in vivo*. Towards this goal, we performed knock down of GLUT5/SLC2A5 and assessed detachment in SCM. Indeed, knockdown of GLUT5 decreased detachment of human HGSOC cells cultured in SCM (**new Fig. 5d; knock-down confirmed in Extended Data Fig. 8g**), which is consistent with the notion that fructose is imported by HGSOC cells to promote detachment. Unfortunately, the murine cell line used in our *in vivo* model does not highly express GLUT5, although they do express other sugar

transporters that have some affinity for fructose¹ (**Fig. 1A for Reviewers**) and are indeed capable of taking up ¹³C₆-fructose (**Fig. 1B for Reviewers**). Thus, we instead knocked down the fructolysis enzymes ketohexokinase (KHK) and aldolase B (ALDOB)² [as suggested by Reviewer #2]. As expected, knockdown of KHK and ALDOB decreased detachment in HGSO cells cultured in SCM (**new Fig. 5d; knockdown confirmed in Extended Data Fig. 8g**). *In vivo*, Khk knockdown prevented dissemination caused by SCM treatment (**new Fig. 6a-c**) and fructose diet supplementation (**new Fig. 6h-j**). Collectively, these experiments rigorously demonstrate the requirement of fructolysis within bystander cells to drive detachment and dissemination.

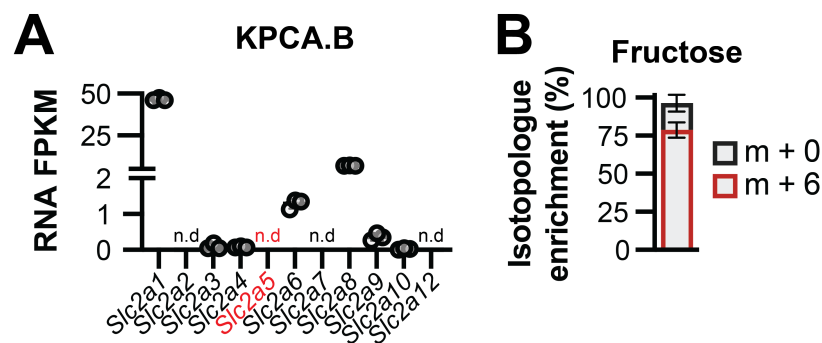


Figure 1 for Reviewers. Slc2a family transporter expression and fructose uptake in murine KPCA.B HGSO cells. (A) mRNA expression of all 11 Slc2a family glucose/fructose transporters in the murine HGSO cell line model KPCA.B. The canonical fructose transporter, Slc2a5, is highlighted in red. **(B)** HGSO cells were cultured in ¹³C₆-fructose for 2 hours and intracellular m+6 fructose was determined by LC/MS.

Comment #2: *The authors claim that the senescence secretome drives metastatic spread and Figure 1E/F shows it is sufficient to increase tumor nodules. To show this is mediated through NDUFA, Figure 2M should use SCM as used in Figure 1E/F.*

Response: We appreciate the reviewer's suggestion to directly investigate the role of NDUFA5 in driving dissemination in response to the secretome of senescent cells. We have now performed the experiment suggested by the reviewer and demonstrate that NDUFA5 knockdown decreases SCM-mediated metastatic spread (**new Fig. 2I-n**). This experiment further

strengthens the conclusion that the senescence secretome drives metastatic spread, which is mediated through NDUFA5.

Comment #3: *In Figure 3E, if SREBP is driving cholesterol synthesis via complex I–dependent NAD⁺ production, it is unclear why SREBPI does not alter detachment in the control groups. If SREBP1 is sufficient, SREBPI should increase detachment compared to control. This raises the possibility of alternative mechanisms of detachment or that the assay is saturated. If the assay is saturated, this would explain the lack of change. Alternatively, a ChIP assay targeting additional cholesterol-related transcription factors (e.g., LXR, RXR, ATF4) would strengthen the mechanistic conclusions, especially since the manuscript emphasizes SREBP.*

Response: We thank the reviewer for highlighting this potentially confusing conclusion from our manuscript. SREBPI does not alter detachment in the sgControl cells cultured in SCM because SREBP1 is not active in these cells [there is little nuclear localization (**Fig. 3h-i**) and no chromatin occupancy of SREBP1 at cholesterol synthesis genes (**Fig. 3j-l**)]. These data indicate that SREBP does not actively promote *de novo* cholesterol synthesis in SCM cultured cells and therefore inhibiting SREBP in this condition does not have any effect on the detachment in these cells.

To address the reviewer's question, we aimed to determine whether SREBP1 inhibition is sufficient for detachment. Indeed, SREBP1 inhibition is sufficient to induce detachment of HGSOC cells cultured in PCM (**new Fig. 3g**). Furthermore, we performed detachment experiments in SCM using serum starvation, which is well-known to increase nuclear SREBP1³. These experiments demonstrate that enforced nuclear SREBP1 suppresses detachment in HGSOC cells cultured in SCM (**new Fig. 3g**). Together, these new experiments add further rigor and demonstrate that SREBP1 activity is necessary and sufficient for detachment. We believe these new

data, along with multiple other lines of evidence (SREBP1 localization **Fig. 3h-i** and ChIP-qPCR **Fig. 3j-l**), demonstrate that SREBP1 is the main transcription factor regulating *de novo* cholesterol synthesis genes in our model. This has been further clarified in the revised Discussion (**Page 39, Lines 688-691**).

Comment #4: *The authors should provide PFS data for the mouse experiments. If mice are fed fructose versus glucose, does this impact PFS?*

Response: We appreciate the reviewer's request for progression-free survival (PFS) data for the mouse experiments. Unfortunately, the cell line model we used is highly aggressive, and all mice reach IACUC endpoints within ~25 days post-implantation. The aggressive nature of this tumor model does not allow us to precisely identify and make conclusions about the effects of the senescence secretome on PFS. Additionally, in order to measure dissemination and metabolites, the primary goals of this study, all mice need to be necropsied at the same time to accurately compare amongst groups. We have noted this as a limitation in the study (**Page 35, Lines 604-605**) and clarified the necessity of this experimental design in the revised Methods (**Page 67, Lines 973-974**).

Reviewer 2

We appreciate the insightful questions asked and experiments proposed by the reviewer to add rigor and strength to our work. We have addressed these comments both with additional experiments, such as validation of the necessity of fructolytic enzymes, the use of LbNOX as an alternative orthogonal approach to NAD⁺ generation, rescuing the effects of statin treatment with cholesterol supplementation, the use of a senolytic as a tool to confirm senescence and demonstrate the reversal of the detachment phenotype, and the inclusion of multiple senescent cell models and induction methods. Moreover, we have clarified the text to provide more accurate details and remove ambiguities. Each of these experiments and edits are explained in depth below with the where new data and revisions to the text can be found in the revised manuscript.

Comment #1: *The fructose concentration for the cell culture experiments is not reported, neither the absolute concentration in SAS. The fold change between control and SAS is 1.5 fold. Are the concentrations the authors use in there experiment physiological relevant?*

Response: We apologize to the reviewer that absolute quantification was not reported. To address the reviewer's question, we now report the absolute quantification of both fructose and glucose in the conditioned media from proliferative and senescent cells normalized to cell number (**new Fig. 5a-b and new Extended Data Fig. 8e**). Physiological plasma fructose concentrations range from tens to hundreds of micromolar⁴. Thus, the concentration used for *in vitro* experiments (500 μ M) is physiologically relevant. This has been noted in the revised text (**Page 29, Line 518**).

Comment #2: *The authors propose a conversion of fructose to DHAP. They should silence ketohexokinase (KHK) and aldolase B.*

Response: We appreciate the reviewer's suggestion to directly investigate the role of fructolysis in driving detachment. As recommended by the reviewer, we knocked down the fructolysis enzymes ketohexokinase (KHK) and aldolase B (ALDOB)². As expected, knockdown of either KHK or ALDOB decreased detachment in both human and murine HGSOC cells cultured in SCM (new Fig. 5d; knockdown confirmation in Extended Data Fig. 8g). *In vivo*, Khk knockdown prevented dissemination caused by SCM treatment (new Fig. 6a-c) and fructose diet supplementation (new Fig. 6h-j). Collectively, these experiments demonstrate the requirement of fructolysis within bystander cells to drive detachment and dissemination.

Comment #3: The authors supplement NA to increase NAD⁺ in cells. They need to confirm the relevance of NAD⁺ by expressing LbNOX.

Response: We thank the reviewer for suggesting rescue of NDUFA5 knockdown by expressing LbNOX to regenerate NAD⁺ from NADH independently of the electron transport chain. Indeed, LbNOX expression rescues the decrease in detachment caused by NDUFA5 loss in Ovar8 cells similarly to nicotinic acid supplementation (new Fig. 2q; validation of LbNOX expression in new Extended Data Fig. 5o). Unfortunately, we were unable to express LbNOX in murine HGSOC cell line model (Figure 2 for Reviewers), preventing us from being able to confirm this in a second cell line. However, we believe the NA supplementation data, which we now show

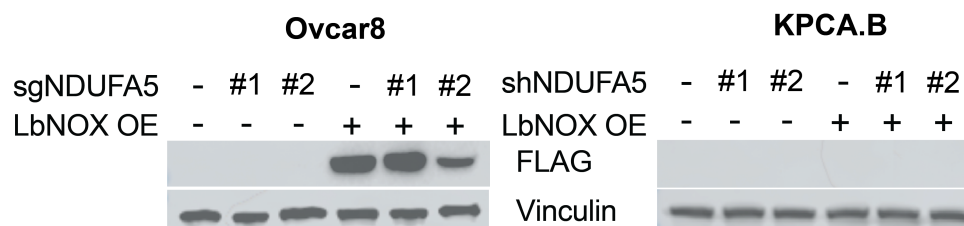


Figure 2 for Reviewers. LbNOX overexpression in human and murine HGSOC cell lines. Ovar8 human and KPCA.B murine cells were transduced with sg/shRNA controls or sg/shRNA targeting NDUFA5 followed by transduction with lentiviral LbNOX-FLAG stable overexpression. FLAG western blot shows that Ovar8 cells were transduced whereas KPCA.B cells were not. This experiment was attempted 3 times in KPCA.B cells.

increases the NAD⁺/NADH ratio (**new Extended Data Fig. 5n**) provide this rigor. These data confirm the relevance of NAD⁺ to the observed detachment phenotype.

Comment #4: *The authors write that “statins to more generally deplete intracellular cholesterol” which is incorrect because statins block only de novo cholesterol synthesis. Does cholesterol supplementation rescue the effects of statins?*

Response: We agree with the reviewer that this statement is incorrect. To clarify, we intended to mean that statins decrease the total cholesterol in the cell through blocking *de novo* synthesis. This in contrast to MβCD, which specifically depletes plasma membrane cholesterol⁵, of specific mechanistic interest to this study. We have revised this sentence in the text (**Page 22, Lines 412-416**).

We appreciate the reviewer's suggestion to assess whether cholesterol supplementation rescues the effects of statins. We now demonstrate that cholesterol supplementation rescues the effects of statin treatment of NDUFA5 knockout bystander cells cultured in SCM (**new Extended Data Fig. 7a-b**). These data further support the claim that decreased *de novo* cholesterol synthesis is required for increased bystander cell detachment.

Comment #5: *The key experiments are mainly performed in one cell line and need to be confirmed by at least 1-2 more cell lines.*

Response: We appreciate the reviewer's request to rigorously confirm key experiments in multiple cell lines and **consulted with the Editor, Dr. Hannah Walters**, to confirm which further experiments are essential to be performed in multiple models. Many of the key experiments have

been performed in two ovarian cancer cell lines: one human (Ovcar8) and one murine (KPCA.B), indicating a biologically conserved mechanism. This includes:

- 1) Experiments to demonstrate induction of senescence by cisplatin
- 2) Experiments to demonstrate that SCM drives detachment *in vitro*
- 3) Experiments to demonstrate the requirement of NDUFA5 for detachment and rescue by NA
- 4) Assessment of cholesterol downstream of SCM, NDUFA5, NA, SREBP1, SIRT, fructose, and glucose
- 5) Assessment of SREBP1 nuclear localization downstream of SCM, NDUFA5, NA, fructose, and glucose
- 6) Assessment of SIRT activity on detachment and cholesterol
- 7) Experiments to demonstrate that fructose drives detachment and that glucose rescues PCM-mediated detachment

For ease of review, we have included all of these data in the table below (**Table 1 for Reviewers**).

Table 1 for Reviewers: Experiments performed within the original manuscript in two HGSOc cell line models (note that the figures reference those in the revised version).

Experiment	Ovcar8	KPCA.B
Induction of senescence by cisplatin	ED Fig. 2b-h	ED Fig. 2b-h
Demonstration that SCM drives detachment <i>in vitro</i>	Fig. 2e and Fig. 2k	Fig. 2e and Fig. 2k
Demonstration of the requirement of complex I and NDUFA5 for detachment	Fig. 2k and ED Fig. 5c	Fig. 2k and Fig. 5c
Changes in basal and maximal OCR	Fig. 2i and ED Fig. 5d	Fig. 2i and ED Fig. 5d
Lack of changes in proliferation, viability, and spheroid formation in SCM vs. PCM	ED Fig. 3d-e and ED Fig. 4a	ED Fig. 3d-e and ED Fig. 4a
Rescue of detachment in SCM by NA	Fig. 2p	Fig. 2p
Decrease of total cell cholesterol by Filipin staining in SCM vs. PCM	ED Fig. 6f-g	ED Fig. 6f-g
Rescue of detachment in NDUFA5 KD/KO cells by MBCD and statins	Fig. 3d-e and ED Fig. 7a-b	Fig. 3d-e and ED Fig. 7a-b
Increased total cell cholesterol by Filipin staining upon KD/KO of NDUFA5, which is rescued upon NA supplementation	ED Fig. 6f-g	ED Fig. 6f-g
Decreased nuclear SREBP1 in SCM vs. PCM, which is increased by NDUFA5 KD/KO cells and rescued upon NA supplementation	Fig. 3h-i	Fig. 3i and ED Fig. 7c
Decreased detachment by SIRT inhibition in NDUFA5 KD/KO cells treated with NA	Fig. 4b	Fig. 4b
Increased total cell cholesterol by Filipin staining in SCM cultured cells upon SIRT inhibition	Fig. 4g	Fig. 4g
No rescue of increased total cell cholesterol by Filipin staining in NDUFA5 KD/KO cells	Fig. 4h	Fig. 4h

Demonstration that fructose drives detachment <i>in vitro</i>	Fig. 5c	Fig. 5c
Demonstration that increased glucose decreased detachment of SCM cultured cells	ED Fig. 8f	ED Fig. 8f
Decreased nuclear SREBP1 in fructose supplemented cells, which is not affected by NDUFA5 KD/KO	Fig. 5e-f	Fig. 5e-f
Decreased cholesterol in fructose supplemented cells, which is not affected by NDUFA5 KD/KO	Fig. 5g-h	Fig. 5g-h
Increased nuclear SREBP1 in SCM cultured cells supplemented with glucose	ED Fig. 9e-f	ED Fig. 9e-f
Increased total cell cholesterol by Filipin staining in SCM cultured cells supplemented with glucose	ED Fig. 9g-h	ED Fig. 9g-h

To further increase the rigor of our experiments, we have performed the time-lapse imaging (new Fig. 2a-c) and determined fructose and glucose media concentrations (new Fig. 5a-b and Extended Data Fig. 8a) in a second cell line. We have also confirmed the effects of the SAS on detachment in an additional cancer model and non-cancer model, using a melanoma cell line, a pancreatic ductal adenocarcinoma cell line, and normal fibroblasts (new Extended Data Fig. 4b-g). Moreover, new experiments in response to reviewers have been performed in the two HGSOC models:

- 1) Senolytic experiment *in vitro* to confirm senescence induction by cisplatin
- 2) Detachment of cells treated with SCM from carboplatin-treated HGSOC cells, cisplatin-treated fibroblasts, or replicative senescent fibroblasts
- 3) Demonstration of the necessity and sufficiency of SREBP on detachment
- 4) Assessment of the NAD⁺/NADH ratio in NA supplemented cells
- 5) RNA expression of *de novo* cholesterol synthesis proteins
- 6) Rescue of stains by cholesterol supplementation
- 7) Assessment of fructose in CM from different senescence inducers and in different cell types
- 8) Demonstration that knockdown of fructolysis genes decreases detachment
- 9) Differential glucose tracing upon supplementation with fructose

For ease of review, we have included all of these data in the table below (Table 2 for Reviewers).

Table 2 for Reviewers: Experiments performed within the revised manuscript in two HGSOC cell line models.

Experiment	Ovcar8	KPCA.B
Senolytic experiment <i>in vitro</i> to confirm senescence induction by cisplatin	ED Fig. 2i	ED Fig. 2i

Demonstration that SCM from other stressors and cells increases detachment	ED Fig. 4b-g	ED Fig. 4b-g
Timelapse imaging to demonstrate that SCM increases detachment from spheroids	Fig. 2c	Fig. 2c
Timelapse imaging to demonstrate that knockdown/out of NDUFA5 decreases in SCM detachment from spheroids	Fig. 2j	Fig. 2j
Demonstration of the necessity and sufficiency of SREBP on detachment	Fig. 3g	Fig. 3g
Increased NAD ⁺ /NADH ratio in NA supplemented cells	ED Fig. 5n	ED Fig. 5n
Decreased RNA expression of <i>de novo</i> cholesterol synthesis proteins in SCM cultured cells	ED Fig. 6d	ED Fig. 6d
Decreased cholesterol in SCM rescued by knockdown/out of NDUFA5 measured by mass spectrometry	ED Fig. 6e	ED Fig. 6e
Increased fructose in SCM from carboplatin induction	ED Fig. 8e	ED Fig. 8e
Demonstration that knockdown of fructolysis genes decreases detachment	Fig. 5d	Fig. 5d
Differential glucose tracing upon supplementation with fructose	ED Fig. 9d	ED Fig. 9d

We acknowledge that some experiments, especially the extensive *in vivo* models, have only been performed in one cell line. We have noted this in the text as a limitation (**Pages 35-36, Lines 605-606**).

Comment #6: *The patient data need to be analyzed for co-founding factors.*

Response: We thank the reviewer for inquiring about patient data and requesting more information. The most relevant confounding factors for analyzing ovarian cancer stage in the context of senescence are treatment status and age. All the patient stage information used are from treatment-naïve patients at diagnosis, removing treatment as a confounding factor. This has been clarified in the revised text (**Page 16, Line 324**). We worked with a biostatistician to understand the role of age as a confounding factor and performed statistical analysis on the stage distributions presented in the manuscript (**Fig. 2h, Fig. 6g, and Extended Data Fig. 5b**). These data support the conclusions that metabolic changes correlate with disseminated disease in patients, regardless of age.

Comment #7: *The claim that the senescent ovarian cancer cell are the driver should be strengthened with a senolytic experiment preferentially in vivo. Especially it was shown before that senolytics are very efficient in killing HGSOV PMID: 31186408. Alternatively, an introduced “suicide gene” such as INK ATTACK construct could be introduced into the senescent cells to eliminate them in an in vivo setting.*

Response: We thank the reviewer for the suggested experiment and reference for rescuing the effects of senescent cells using senolytic drug treatment. We confirmed that cells induced to senesce are more sensitive to ABT-263, a Bcl2 family inhibitor senolytic (**Extended Data Fig. 2i and shown here again as Fig. 3A for Reviewers**). We have given careful thought to the reviewer’s proposed experiment, and consulted the Editor, Dr. Hannah Walters. We believe the use of senolytics *in vivo* introduces confounding factors that would make interpretation of the results of this experiment challenging, and Dr. Walters agreed. Senolysis of co-injected senescent cells would result in cell death, as seen *in vitro* (**Fig. 3B for Reviewers**). While we are able to remove dead cells after senolytic treatment *in vitro* before conditioning media, we would be unable to do so *in vivo* regardless of whether we use a pharmacological or genetic approach. This increased cell death would alter the microenvironment and potentially change the immune compartment, which would hinder our ability to deconvolute the effects of senescence vs. senolysis. Additionally, while senescent cells show an increased sensitivity to a pharmacological approach, the proliferative cells are affected by ABT-263 treatment, especially at higher doses (**Fig. 3A for Reviewers**). Nonetheless, in an attempt to answer the reviewer’s question, we performed an *in vitro* experiment using the well-known senolytic navitoclax (ABT-263) (**Fig. 3C for Reviewers**). As expected, conditioned media from ABT-263 treated cisplatin-induced senescent cells completely rescued detachment (**Fig. 3D for Reviewers**), likely because very few cells remain during the media conditioning period of the experiment (**Fig. 3C for Reviewers**). Unexpectedly, conditioned media from ABT-263 treated control cells had a small but measurable

effect on bystander cell detachment in both HGSOC cell lines (**Fig. 3D for Reviewers**). Given these data, we believe that the *in vivo* conditioned media treatment model (**Fig. 1g-i**), which bypasses the need for senescent cells to be present *in vivo*, demonstrates the direct sufficiency of the secretome of senescent cells to drive dissemination without needing a senolytic approach.

We appreciate that a limitation of our work is that we have not used systemic chemotherapy to induce senescence *in vivo*, which would then necessitate this senolytic experiment. We have included this as a limitation in the revised Discussion (**Page 41, 730-732**).

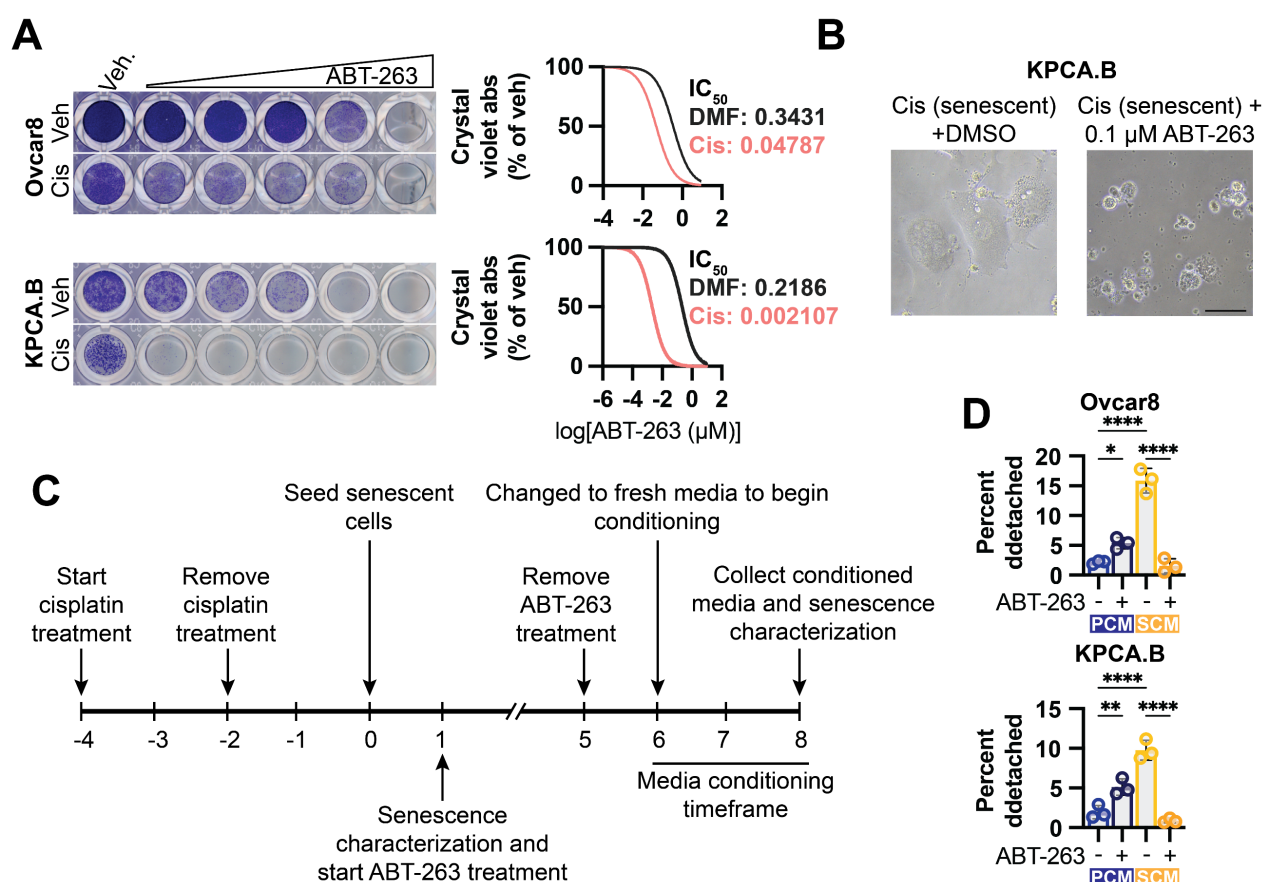


Figure 3 for Reviewers. Cisplatin therapy-induced senescent cells are more sensitive to the senolytic navitoclax (ABT-263). (A) After senescence induction using cisplatin, senescent or proliferative HGSOC cells were treated with increasing concentrations of navitoclax (ABT-263) for 96 hours. Cells were fixed and stained with crystal violet. (B) Representative images of senescent cells treated with ABT-263 demonstrating senolysis in cisplatin-induced senescent cells. (C) Timeline of senolytic treatment of cisplatin-induced senescent cells indicating when cells were treated with cisplatin to induce senescence, treated with the senolytic ABT-263, when media was changed, and when conditioned media was harvested and senescence was characterized. (D) Conditioned media was collected from cells after senescence induction with cisplatin (SCM) or proliferative control (PCM) with or without senolytic treatment with ABT-263. The indicated naive malignant bystander cells were cultured in the indicated conditioned medias for 72h, after which detachment was assessed by the controlled trypsin assay. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$

Minor Comment #1: *The observation that the fraction below 3kDa secreted from senescent cells can drive dissemination is a novel concept it would be interesting to see if it's the sole driver of dissemination process. As such it would be interesting to see the higher than 3kDa fraction secreted from senescent cells can also drive detachment.*

Response: We appreciate the suggestion of the reviewer to see if the isolated proteins from the SAS are sufficient to drive detachment. We have performed this experiment and show that the >3kDa fraction also increases detachment, albeit to a slightly lesser extent than the small molecule fraction (**Figure 4 for Reviewers**). As the reviewer notes, the novelty of this work is that the small molecule and metabolites in the SAS are *sufficient* to drive dissemination. The role of SAS proteins in affecting cell adhesion and detachment is well described through direct matrix remodeling or bystander cell signaling effects⁶⁻⁹, and this has now been included in the revised Discussion (**Page 35, Lines 589-593**).

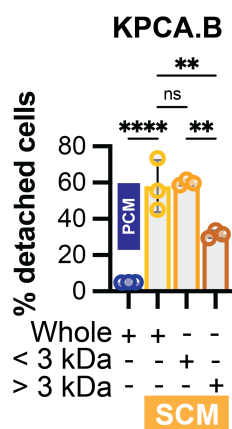


Figure 4 for Reviewers. Contribution of different SAS components to cell detachment. Conditioned media from proliferative (PCM) or cisplatin therapy-induced senescent (SCM) cells was fractionated using a 3kDa cutoff column where <3 kDa is the small molecule fraction and the >3 kDa is the protein fraction. Murine KPCA.B HGSOC cells were cultured in whole conditioned media or the different fractions for 72 hours, after which detachment was assessed by the controlled trypsin assay. ns = not significant, ** p < 0.01, **** p < 0.0001

Minor Comment #2: *A second senescent inducer to validate the findings is important to strengthen the claims. Alternatively the authors need to tune down their claims and make clear they are specific to cisplatin.*

Response: We appreciate the reviewer's suggestion to enhance the rigor of our study by broadening the generalizability with multiple senescence inducers. This is echoed by the requests of both the **Editor** and **Reviewer #3**. We have now increased the generalizability of our findings by validating the sufficiency of SAS to increase detachment. We have also found that there is an increase in fructose in the SAS from multiple additional senescence models. This has been discussed in the revised manuscript (**Page 37, Lines 628-633**).

- 1) As requested by the Editor, we induced senescence with carboplatin and verified the effects on bystander cell detachment and SAS fructose concentration (**new Extended Data Fig. 4b-d and new Extended Data Fig. Fig. 8e**).
- 2) We induced replicative senescence by serial passaging of normal fibroblasts (**new Extended Data Fig. 4e-f**). The SAS from these replicative induced senescent cells also promoted detachment and increased fructose abundance (**new Extended Data Fig. 4g and new Extended Data Fig. 8e, respectively**), demonstrating these effects are also features of senescence accumulation during the aging process.
- 3) Finally, we replicated this data with conditioned media from normal fibroblasts or a non-ovarian cancer cell line induced to senesce with cisplatin or a third chemotherapy treatment, gemcitabine (**new Extended Data Fig. 4b-g and new Extended Data Fig. 8e**). This supports the conclusion that the SAS from therapeutically different induced senescent cell types drives detachment and fructose abundance.

Minor comment #3: *The authors indicate in Figure S5A a conversion of F1P to F1,6P. Could they please further elaborate on this conversion.*

Response: We thank the reviewer for highlighting an error in our schematic – fructose-1-phosphate is converted to DHAP by aldolase, after which it can be converted to either fructose-1,6-phosphate or glyceraldehyde-3-phosphate in reversible reactions. We have updated our schematic to correct this error (**Extended Data Fig. 8b**).

Minor comment #4: *The authors need to confirm that after CIS treatment the cells are senescent using at least one more marker besides b-gal.*

Response: We apologize for the lack of clarity around the confirmation of senescence induction after cisplatin treatment. As senescence experts¹⁰⁻²⁶, we fully agree that b-gal is not sufficient to confirm senescence. After cisplatin treatment and conditioned media generation (timeline **Extended Data Fig. 2a**), senescence was confirmed by lack of proliferation (**Extended Data Fig. 2b**), increased senescence associated β -galactosidase activity (**Extended Data Fig. 2c**), decreased lamin B1 expression (**Extended Data Fig. 2e**), and induction of canonical SAS proteins by mRNA expression (**Extended Data Fig. 2g, h**) and protein level by cytokine array (**Extended Data Fig. 2f**). In the revised manuscript, we have also added a measurement of active cell proliferation by BrdU incorporation (**new Extended Data Fig. 2d**) and confirmed a sensitivity to the senolytic ABT-263 (**new Extended Data Fig. 2i**). We have updated the text to clarify that senescence was confirmed with multiple markers (**Pages 9-10, Lines 210-216**).

Reviewer 3

We thank the reviewer for their thoughtful questions and valuable experimental suggestions, which have helped enhance the rigor and robustness of our study. We have included additional experiments to address major questions about the role fructose plays in driving NAD⁺ generation by complex I and the role of the SAS *in vivo*. We have also addressed the clinical relevance and generalizability of our study with an in depth bioinformatic analysis to provide evidence for senescence induction in patients and the use of multiple cell models and induction models of senescence. Finally, we have edited the manuscript text to provide more clarity and details about our methodology and conclusions. Each of these revisions and the locations for new included data or manuscript text changes are described in depth under the appropriate comment below.

Comment #1: *Is there any evidence in mouse models and/or human samples that cisplatin treatment induces senescence in some cancer cells? Without this evidence, although interesting, the relevance of the observation will be rather speculative.*

Response: We thank the reviewer for inquiring about the physiological relevance of senescence induction in cancer cells during the course of cisplatin treatment in mouse and human models. Clinical studies have identified subsets of senescent cells in lung cancer patients treated with platinum (either cisplatin²⁷, carboplatin²⁸, or a non-specified platinum based therapy regimen²⁹). More generally, cytotoxic DNA damaging chemotherapy have commonly been shown to induce senescence in human patient tumors in breast cancer³⁰⁻³³, and therapy induced senescence has also been reported in gastrointestinal cancers³⁴. In ovarian cancer, therapy induced senescent cells were found and characterized in murine HGSOc tumors *in vivo* following cisplatin treatment³⁵. To address whether platinum-based chemotherapy has the same effect in human HGSOc, we analyzed three publicly available datasets containing matched patient samples before and after platinum-based treatment to identify transcriptional reprogramming indicative of

senescence, namely an increase in transcription of SenMayo³⁶ genes concurrent with downregulation of DNA replication genes³⁷⁻³⁹ and *LMNB1*⁴⁰. We found robust evidence of senescence signatures in HGSOC patients after platinum chemotherapy (**new Fig. 1a and Supplementary Tables 1-3**). Similar upregulation in senescence-associated genes was also observed in two publicly available NanoString datasets of matched neoadjuvant chemotherapy (NACT)-treated tumors (**new Extended Data Fig. 1a**). Together, these data support multiple previous reports that cisplatin induces senescence and a senescence-associated secretome (SAS)^{27-29,41,42}. To further investigate the intratumoral senescence response to chemotherapy, we applied a senescence score derived from these signatures to publicly available single cell RNA-Seq data of matched pre- and post- NACT-treated patient tumors⁴³. As expected, multiple cell types show an increase in the senescence signature, including malignant epithelial cells and other stromal cells (**new Extended Data Fig. 1b**). Interestingly, there was a heterogeneous senescence induction in malignant epithelial cell populations (**new Fig. 1b and Supplementary Table 4**), suggesting a post-chemotherapy tumor microenvironment (TME) comprised of both therapy-induced senescent (TIS) malignant cells and proliferative malignant “bystander” cells (**new Fig. 1c**). While TIS has been frequently reported²⁷⁻³⁵, and the SAS from multiple senescent cell sources are known to affect cancer progression⁴⁴⁻⁵³, the contribution of the malignant cell SAS on dissemination and tumor progression of malignant bystander cells remains unclear. Given the complexity of senescence induction *in vivo*, here we aimed to determine the paracrine effects of the cisplatin-induced SAS from malignant cells on HGSOC bystander cells. This has been clarified in the revised text (**Pages 7-9, Lines 184-207**).

Together, these data provide evidence that senescence is induced in the course of clinical HGSOC therapy, strongly supporting the relevance of our work.

Comment #2: *These observations are interesting, but unfortunately made in a unique context, that of ovarian tumors, and from one or two cancer cell lines treated in vitro. The generalizability of these results is not yet established. For example, do non-cancerous senescent cells in the tumor microenvironment, such as fibroblasts, also produce/release fructose and cause cancer cells to detach? Do SAS and fructose cause other types of cancer cells to detach and promote their dissemination? Is this effect specific to cisplatin or can it be observed during other stresses (e.g., other chemotherapies, oxidative stress, telomere shortening, etc.)? All these results will strongly strengthen the argument and allow the results to be placed in the context of aging, which the authors have not yet really done.*

Response: We appreciate the reviewers question regarding the generalizability of this study.

We have experimentally addressed these comments in multiple ways:

- 1) To address the issue of other cells in the microenvironment, we have induced senescence in fibroblasts using cisplatin (**new Extended Data Fig. 4e-f**). We show that the SAS from these cells is fructose enriched (**new Extended Data Fig. 8e**) and sufficient to drive detachment (**new Extended Data Fig. 4g**).
- 2) To address the generalizability of this phenotype outside of ovarian cancer, we used both a melanoma cell line model and a pancreatic ductal adenocarcinoma cell line model to show that the SAS from other malignant cell types can induce detachment of naïve bystander malignant cells (**new Extended Data Fig. 4b-d**) and is enriched in fructose (**new Extended Data Fig. 8e**).
- 3) As requested by the Editor, we used carboplatin as a secondary platinum-based chemotherapy as additional senescence inducer (**new Extended Data Fig. 4b-c**). Again, we show that the SAS from carboplatin-induced senescent cells is enriched in fructose (**new Extended Data Fig. 8e**) and promotes bystander cell detachment (**new Extended Data Fig. 3d**).
- 4) Finally, to assess senescence outside of chemotherapy, we used a replicative senescence model to mimic the effects of senescence accumulation during aging (**new Extended Data**

Fig. 4e-f). Consistent with the previous models, we confirmed the presence of fructose in the SAS from aged fibroblasts (**new Extended Data Fig. 8e**) and the ability of the SAS to promote detachment in bystander cells (**new Extended Data Fig. 4g**).

Taken together, these models strongly indicate that fructose is a universal component of the SAS and that senescent cells increase bystander cell detachment regardless of specific cell or cancer type or senescence-inducing stressor (**Page 37, Lines 628-633**).

Comment #3: *There are mechanistic gaps. Figure 5 and the glucose and fructose treatments suggest, in particular, the specific ability of fructose to induce downstream effects. Do the authors have any mechanistic answers explaining why fructose, and not glucose, impacts the entire pathway? For example, a difference in metabolite production (glycolysis and TCA cycle) and in electron transport chain activity?*

Response: We thank the reviewer for highlighting a potential weakness of our manuscript, and for the suggestion on how to address this gap. We determined that fructose induces glucose flux through glycolysis and into the TCA cycle (**new Extended Data Fig. 9c-d**). A recent study found that mitochondrial respiration correlates with carbon retention in the canonical TCA cycle⁵⁴, which may explain our results. Additionally, KHK-dependent fructolysis has been shown to increase mitochondrial respiration through activating AMPK signaling⁵⁵. To investigate if the same signaling mechanism was active in our bystander cells, we found that fructose increased AMPK phosphorylation upstream of complex I activity (**new Extended Data Fig. 9a-b**). Together, these data provide multiple pathways that fructose induces changes in mitochondrial metabolism. We acknowledge that a more definitive mechanism to deeply interrogate differences in fructose vs. glucose utilization, in addition to how fructose is directly inducing complex I activity, are lacking,

although we believe this is beyond the scope of the current study. We have added this as a limitation to our revised Discussion (**Page 38, Lines 659-666**).

Comment #4: *Given the critical role of complex 1 activity and NADH/NAD⁺ levels in the authors' hypotheses, it is essential to measure them in some critical experiments throughout the manuscript.*

Response: We acknowledge the importance of quantifying NAD⁺ levels given the importance in our proposed mechanism. This point was also raised by **Reviewer 2 (Comment #3)**. Indeed, we confirmed the increase in NAD⁺:NADH ratio after nicotinic acid supplementation (**new Extended Data Fig. 5n**). Moreover, we performed new experiments to assess whether expressing LbNOX to regenerate NAD⁺ from NADH independently of the electron transport chain rescues NDUFA5 knockdown abrogation of detachment. Indeed, LbNOX expression rescues the decrease in detachment caused by NDUFA5 loss in Ovarc8 cells similarly to nicotinic acid supplementation (**new Fig. 2q; confirmation of LbNOX expression in new Extended Data Fig. 5o**). These orthogonal approaches further support the claim of complex I activity to produce NAD⁺ as being necessary for bystander cell detachment.

Comment #5: *The main thrust of the article is based on the results presented in Figure 1G, which aim to demonstrate that senescent cancer cells promote the dissemination of proliferative cells through their SAS. However, the effect of SAS treatment on dissemination is rather weak compared to the co-injection of senescent and proliferative cells (Figure 1G vs. 1D). Can the author optimize the test presented in Figure 1G to fully convince the reader?*

Response: We thank the reviewer for recognizing the strength of the co-injection model, which demonstrates that senescent cancer cells promote the dissemination of proliferative cells. We have now fully optimized this experiment and demonstrate the injection of SCM increases total

tumor nodules with particularly strong tropism to the intestines and more distal peritoneal organs (new Fig. 1g-i).

Minor comment #1: *Sometimes the level of information is insufficient:*

- *What does Labeled with GFP mean? Is it expression or other methods? Is it stable?*

Response: We apologize for the lack of clarity regarding experimental procedures. GFP was expressed by stable lentiviral transduction. We have updated the revised Methods for clarity (Page 67, Lines 970-972).

Minor comment #2: *Crystal violet staining gives an idea of cell number and density, but not the percentage of proliferation. Please modify or perform specific experiments, such as the EdU incorporation assay.*

Response: We appreciate the request for more clarity and rigorous characterization of the senescence induction. We performed a BrdU incorporation assay as suggested by the reviewer (new Extended Data Fig. 2d). This supports the conclusion that the treated cells are no longer synthesizing new DNA and, along with additional supporting experiments, are no longer proliferating.

Minor comment #3: *Please provide more complete and easily accessible information on the chronology of the experiments, for example, when IHC against KI67 was performed.*

Response: We appreciate the request for more clarity regarding the *in vivo* experiments. We have provided timelines of all mouse experiments in the to show when cells were implanted,

how long tumors were allowed to develop, and when mice were necropsied and tissues were taken for analysis.

Minor comment #4: *Please indicate the names of the genes in front of the heatmaps and, when a geneset is mentioned, the names of the genes and their differential levels.*

Response: We apologize for any lack of clarity in the heat map data due to space constraint. We have provided all the source data for the reviewer and readers to see individual gene names and their levels. We have also included additional supplementary tables with the geneset lists in addition to the names of the genes and their differential levels (**Supplementary Table 1, 2, and 8**).

Minor comment #5: *Figure 1K, can the authors try other senescence signatures to confirm the results?*

Response: We thank the reviewer for the suggestion. After running more stringent statistical analyses and accounting for confounding factors of the correlations between stage and different senescence gene signatures available in bulk RNA-sequencing, we have decided to remove this piece of data. Instead, we now provide data in **new Fig. 1a-b and new Extended Data Fig. 1a** to demonstrate that senescence induction (determined by multiple gene sets) occurs in patients after treatment with platinum-based agents, consistent with prior reports²⁷⁻²⁹.

Minor comment #6: *Can the authors show the mRNA and protein levels across all cholesterol genes differentially expressed under SCM vs PCM, and under NDUFA5 loss?*

Response: We thank the reviewer for the suggestion. We have included the mRNA expression of specific cholesterol synthesis genes identified in our RNA-seq results (**new Extended Data**

Fig. 6d) as well as protein expression of cholesterol synthesis genes shown to have decreased SREBP binding at their gene promotor sites by ChIP-PCR (**new Extended Data Fig. 7d**).

Minor comment #7: *I don't understand the differences between Filipin's methodology for measuring plasma membrane cholesterol and total cholesterol, please explain.*

Response: We apologize for any lack of clarity regarding the measurements of cholesterol. Filipin is a fluorescent probe that binds cholesterol; therefore, we are able to use microscopy imaging techniques to image and measure the probes intensity as a proxy for the amount of cholesterol present. By using total internal reflection fluorescence (TIRF) imaging⁵⁶, we measured the filipin intensity specifically at the cell membrane. In contrast, widefield microscopy was used measure the filipin intensity of the entire cell. We have revised the text to make the differences in the techniques and our interpretations of the results clearer (**Page 19, Lines 400-401; Pages 21-22, Lines 406-410**).

Minor comment #8: *The differences between normal senescent cells and cancerous senescent cells need to be discussed.*

Response: We thank the reviewer for the suggestion and have included data obtained from **Comment #1**, where we showed that SAS produced from senescent fibroblasts is consistent with the SAS from therapy induced malignant cells. We have also added to the Discussion to highlight the roles of both malignant and normal cells undergoing therapy induced senescence (**Pages 40-41, Lines 715-722**).

Minor comment #9: *Can the authors use a complementary approach to filipin fluorescence intensity to quantify cholesterol?*

Response: We appreciate the reviewer requesting a complementary approach to quantify the cholesterol content in bystander cells. As an orthogonal approach, we used mass spectroscopy to measure non-esterified cholesterol in bystander Ovar8 and KPCA.B cells and demonstrate that SCM decreases cholesterol, which is rescued by NDUFA5 knockdown/knockout in both our cell line models (**new Extended Data Fig. 6e**). These data support the conclusion that cholesterol is depleted in bystander cells cultured in SCM and provide absolute cholesterol levels in the bystander cells.

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Point-by-Point Response

Below we have addressed the final comment from Reviewer #1.

Reviewer #1:

For the most part the authors were able to address most of my concerns and the manuscript is greatly improved and should have a wide appeal to those interested in senescence but also thought from outside the field. I would only make one comment that the authors might consider. We have so many acronyms, I would consider eliminating SAS and just staying with SASP. SASP is not just transcriptionally controlled factors as the authors suggest but also post-transcriptionally controlled factors. I understand this manuscript is chasing metabolites but I am not sure we need more acronyms that encompass SASP and metabolites. If the authors/editor feels a new name is needed, perhaps something specific for metabolites might better serve the field.

Response:

We thank the reviewer for this comment. Accordingly, we have edited the revised text and figures to read “SASP” instead of “SAS”.