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14. ABSTRACT Sister chromatids are held together by cohesin, a tripartite ring with a peripheral SA1/2 subunit, where SA1 is required for telomere cohesion and SA2 for centromere cohesion. The STAG2 gene encoding SA2 is often inactivated in human cancer, but not in a manner associated with aneuploidy. Thus, how these tumors maintain chromosomal cohesion and how STAG2 loss contributes to tumorigenesis remain open questions. Here we show that, despite a loss in centromere cohesion, sister chromatids in STAG2 mutant tumor cells maintain cohesion in mitosis at chromosome arms and telomeres. Telomere maintenance in STAG2 mutant tumor cells occurred by either telomere recombination or telomerase activation mechanisms. Notably, these cells were refractory to telomerase inhibitors, indicating recombination can provide an alternative means of telomere maintenance. STAG2 silencing in normal human cells which lack telomerase led to increased recombination at telomeres, delayed telomere shortening and postponed senescence onset. Insofar as telomere shortening and replicative senescence prevent genomic instability and cancer by limiting the number of cell divisions, our findings suggest that extending the lifespan of normal human cells due to inactivation of STAG2 could promote tumorigenesis by extending the period during which tumor-driving mutations occur.					
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TABLE OF CONTENTS

	<u>Page</u>
1. Introduction.....	4
2. Keywords.....	4
3. Accomplishments.....	4
4. Impact.....	7
5. Changes/Problems.....	9
6. Products.....	10
7. Participants & Other Collaborating Organizations.....	13
8. Special Reporting Requirements.....	14
9. Appendices.....	14

1. **INTRODUCTION:** Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

Cancer impacts the quality of life for active duty service members, their families, and the American public. Due to exposure to ionizing radiation, chemicals, and environmental carcinogens, military personnel are at particularly high risk for DNA damage that can promote cancer formation. One of the hallmarks of cancer is the presence of gene mutations that may be central to cancer formation and development. Technological advancements have made it possible to use genomic analysis to identify genes frequently mutated in human cancers. A recent analysis identified the cohesin subunit STAG2 as one of twelve genes mutated in four or more tumor types including melanoma, pancreatic cancer, glioblastoma, colorectal adenocarcinoma, bladder, and acute myeloid leukemia. STAG2 encodes the protein SA2, a component of the cohesin complex that holds sister chromatids together. To date it is unknown why STAG2 is frequently mutated in cancer and how STAG2 mutations may contribute to cancer formation and progression. This research projects aims to understand how STAG2 mutation contributes to tumorigenesis.

2. **KEYWORDS:** Provide a brief list of keywords (limit to 20 words).

Cohesion, telomere, telomerase, ALT, STAG2

3. **ACCOMPLISHMENTS:** The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction.

What were the major goals of the project?

List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

Goals:

1. Do STAG2 tumors resemble ALT tumors? (completion rates: 100%)
2. Is telomerase required for STAG2 tumor telomere maintenance and growth? (100%)
3. Is recombination required for STAG2 tumor telomere maintenance and growth? (60%)
4. Determine how to kill STAG2 tumor cells. (50%)

Reporting period: 09/01/2016 – 31/08/2017

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

1) Major activities

Obtained a panel of 10 STAG2 tumor lines and showed that in mitosis they have persistent telomere cohesion by using a 16p subtelomere FISH (fluorescent *in situ* hybridization) probe and have a defective (loss) in centromere cohesion using a 10cen centromere probe. Interestingly, STAG2 tumors also had increased persistent arm cohesion in mitosis (Appendix 1, Figure 1&2). Since persistent telomere cohesion tracks with ALT (alternative lengthening of telomeres) we wondered if STAG2 tumors are ALT tumors. STAG2 tumors had a high levels of telomere recombination determined by CO-FISH (chromosome orientation-FISH). and 4/10 have long and heterogeneous telomeres like ALT cells (Appendix 1, Figure 3). ALT tumors are telomerase negative. Unexpectedly STAG2 tumors were telomerase positive (Appendix 1, Figure 4). In conclusion, we established that STAG2 tumors resemble ALT tumors (**Goal 1**).

2) Specific objectives

Since we discovered that STAG2 tumor cell lines have both telomerase and telomere recombination we wondered: a) if STAG2 have other features of ALT (c-circles, APBs, ATRX, DAXX) and b) if STAG2 tumors can utilize both recombination and telomerase for telomere maintenance.

3) Significant results

By using several different assays, we discovered that STAG2 tumors have high levels on telomere recombination and they are telomerase positive. However, they were different than ALT tumors because they lacked most features of ALT. STAG2 tumors had no c-circles, no or low levels of APBs, were positive for ATRX, DAXX and telomerase (Appendix 1, Figure 4).

To investigate if STAG2 tumors can utilize both telomerase and telomere recombination for telomere maintenance, we inhibited telomerase with a small molecule inhibitor BIBR 1532 and passaged the cells. We found that the control cells HT1080 shortened their telomeres when treated with BIBR 1532. STAG2 tumors showed minimal or no shortening of their telomeres upon treatment with BIBR 1532, suggesting that telomere recombination could provide alternative means of telomere maintenance upon telomerase inhibition (Appendix 1, Figure 5). Based on those findings we concluded that STAG2 tumors do not depend on telomerase for telomere maintenance and growth (**Goal 2**).

We tried inhibiting several proteins involved in telomere recombination but we could not successfully inhibit telomere recombination. Alternatively, we depleted STAG2 in primary human cells with an shRNA and observed an increased level of recombination at telomeres, longer replicative lifespan, delayed senescence and telomere shortening (Appendix 1, Figure 6). Those results suggested that STAG2 mutation in normal human cells may extend their replicative lifespan and give a window of opportunities during which tumor driving mutations may occur. In summary, although we could not inhibit telomere recombination in STAG2 tumors we could deplete STAG2 in normal human cells and show that it can extend their replicative lifespan and potentially contribute to tumorigenesis (**Goal 3**).

4) Other achievements

To understand the role of centromere cohesion on ploidy levels we stably knocked down STAG2 in BJ primary fibroblast human cell. Knock down of STAG2 resulted in a loss of centromere and persistent telomere cohesion in BJ cells. We performed dual 10 and 6 cen FISH and scored monosomy and trisomy to determine aneuploidy for each chromosome at early and late population doublings (PDs). We observed a small but significant increase in aneuploidy in STAG2-depleted cells compared to vector cells at early PDs.

Discussion on goals that were not met.

In order to kill the STAG2 tumor cell lines we tried to inhibit telomere recombination by using a small molecule inhibitor against Rad51, RI-1. However, inhibition of Rad51 ceased the growth of STAG2 tumors as well as STAG2 wild type tumors and normal human cells, suggesting that Rad51 is required for growth of normal human and tumor cells and cannot be used to treat STAG2 tumors specifically. In the future, one could consider treating the cells with inhibitors that target other proteins involved in telomere recombination, like MRE11.

We tried overexpressing Tankyrase1 and tested if the release of persistent telomere cohesion and recombination would sensitize STAG2 tumors. We found that Tankyrase1 overexpression has modest effect on the STAG2 tumor growth. This aspect of the project needs to further be explored. Perhaps the levels of Tankyrase1 overexpression can be modulated to obtain a better response. Probably other factors regulating telomere cohesion like the proteins TIN2 or SA1 could be taken into account. One would predict that loss of TIN2 or SA1 could potentially lead to slow growth/death of STAG2 tumors. In summary, we could not find a way to effectively kill STAG2 tumors cells but there are alternative strategies that we can attempt in the future (**Goal 4**).

Selected Methods

Chromosome Specific FISH

Briefly, cells were isolated by mitotic shake-off, fixed twice in methanol:acetic acid (3:1) for 15 min, cytospun (Shandon Cytospin) at 2000 rpm for 2 min onto slides, rehydrated in 2X SSC at 37°C for 2 min, and dehydrated in an ethanol series of 70%, 80% and 95% for 2 min each. Cells were denatured at 75°C for 2 min and hybridized overnight at 37°C with subtelomeric FITC-conjugated (16ptelo, 13qtelo, or 4ptelo) and TRITC-conjugated 13q14.3 deletion (arm) and 10 cen (centromere) probes from Cytocell. Cells were washed in 0.4X SSC at 72°C for 2 min, and in 2X SSC with 0.05% Tween 20 at RT for 30 s. DNA was stained with 0.2 µg/ml DAPI. Mitotic cells were scored as having telomeres cohered if 50% or more of their loci appeared as singlets, i.e. one out of two or two out of three.

What opportunities for training and professional development has the project provided?

If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Training opportunities:

Zharko Daniloski, PhD candidate, had weekly one-on-one meetings with his mentor Prof. Susan Smith which fostered his training and project progress. He also had 2 thesis committee meetings where he got input and advice from 4 thesis committee members.

Professional developments:

Zharko Daniloski presented his work at multiple meetings and conferences including: an oral presentation at the Skirball-NYULMC Retreat, Cranwell Resort, Oct 2016, an oral presentation at the NY Academy of Sciences, April 2017, an oral presentation at the Telomeres and Telomerase meeting, Cold Spring Harbor Laboratories, May 2017.

How were the results disseminated to communities of interest?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

In order to disseminate the findings of the supported work, multiple presentations were given at a local, national and international level including:

- Skirball-NYULMC Retreat, Cranwell Resort, Oct 2016, an oral presentation;
- NY Academy of Sciences, April 2017, an oral presentation;
- Telomeres and telomerase meeting, Cold Spring Harbor Laboratories, May 2017, an oral presentation.

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

Nothing to report.

This award is given for 1 year, so this is the annual and final report. No further reports are required.

- 4. IMPACT:** Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

What was the impact on the development of the principal discipline(s) of the project?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

This project described how STAG2 mutations contribute to tumorigenesis. This finding can benefit future work that aims at development of tumor treatment therapies.

What was the impact on other disciplines?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Nothing to report.

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to report.

What was the impact on society beyond science and technology?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to report.

5. **CHANGES/PROBLEMS:** The Project Director/Principal Investigator (PD/PI) is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, “Nothing to Report,” if applicable:

Changes in approach and reasons for change

Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

Nothing to report.

Actual or anticipated problems or delays and actions or plans to resolve them

Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

Nothing to report.

Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Nothing to report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution

committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

Nothing to report.

Significant changes in use or care of vertebrate animals.

Nothing to report.

Significant changes in use of biohazards and/or select agents

Nothing to report.

6. PRODUCTS: List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state “Nothing to Report.”

• **Publications, conference papers, and presentations**

Report only the major publication(s) resulting from the work under this award.

Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Daniloski Z., and Smith S.; Loss of tumor suppressor STAG2 promotes telomere recombination and extends the replicative of normal human cells.; Cancer Research; 2017; page number 13; accepted; acknowledgement of federal support – yes.

Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: Author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to report.

Other publications, conference papers, and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

Oral Presentations at:

- Skirball-NYULMC Retreat, Cranwell Resort, Oct 2016;
- NY Academy of Sciences, April 2017;
- Telomeres and telomerase meeting, Cold Spring Harbor Laboratories, May 2017.

- **Website(s) or other Internet site(s)**

List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to report.

- **Technologies or techniques**

Identify technologies or techniques that resulted from the research activities. In addition to a description of the technologies or techniques, describe how they will be shared.

Nothing to report.

- **Inventions, patent applications, and/or licenses**

Identify inventions, patent applications with date, and/or licenses that have resulted from the research. State whether an application is provisional or non-provisional and indicate the application number. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to report.

- **Other Products**

Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment, and/or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:

- *data or databases;*
- *biospecimen collections;*
- *audio or video products;*
- *software;*
- *models;*
- *educational aids or curricula;*
- *instruments or equipment;*
- *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
- *clinical interventions;*
- *new business creation; and*
- *other.*

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate “no change.”

Example:

Name: Mary Smith
Project Role: Graduate Student
Researcher Identifier (e.g. ORCID ID): 1234567
Nearest person month worked: 5

Contribution to Project: Ms. Smith has performed work in the area of combined error-control and constrained coding.
Funding Support: The Ford Foundation (Complete only if the funding support is provided from other than this award).

No change.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Nothing to report.

What other organizations were involved as partners?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:

Organization Name:

Location of Organization: (if foreign location list country)

Partner’s contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner’s facilities for project activities);*
- *Collaboration (e.g., partner’s staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner’s staff use each other’s facilities, work at each other’s site); and*
- *Other.*

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: For collaborative awards, independent reports are required from BOTH the Initiating PI and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to <https://ers.amedd.army.mil> for each unique award.

QUAD CHARTS: If applicable, the Quad Chart (available on <https://www.usamraa.army.mil>) should be updated and submitted with attachments.

9. APPENDICES: Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

Appendix 1: Daniloski Z., and Smith S.; Loss of tumor suppressor STAG2 promotes telomere recombination and extends the replicative of normal human cells.; Cancer Research; 2017.

Loss of Tumor Suppressor *STAG2* Promotes Telomere Recombination and Extends the Replicative Lifespan of Normal Human Cells

Zharko Daniloski and Susan Smith



Abstract

Sister chromatids are held together by cohesin, a tripartite ring with a peripheral SA1/2 subunit, where SA1 is required for telomere cohesion and SA2 for centromere cohesion. The *STAG2* gene encoding SA2 is often inactivated in human cancer, but not in a manner associated with aneuploidy. Thus, how these tumors maintain chromosomal cohesion and how *STAG2* loss contributes to tumorigenesis remain open questions. Here we show that, despite a loss in centromere cohesion, sister chromatids in *STAG2* mutant tumor cells maintain cohesion in mitosis at chromosome arms and telomeres. Telomere maintenance in *STAG2* mutant tumor cells occurred by either telomere recombination or telomerase

activation mechanisms. Notably, these cells were refractory to telomerase inhibitors, indicating recombination can provide an alternative means of telomere maintenance. *STAG2* silencing in normal human cells that lack telomerase led to increased recombination at telomeres, delayed telomere shortening, and postponed senescence onset. Insofar as telomere shortening and replicative senescence prevent genomic instability and cancer by limiting the number of cell divisions, our findings suggest that extending the lifespan of normal human cells due to inactivation of *STAG2* could promote tumorigenesis by extending the period during which tumor-driving mutations occur. *Cancer Res*; 77(20); 1–13. ©2017 AACR.

Introduction

Telomeres, the specialized structures at chromosome ends, are composed of TTAGGG repeats and the shelterin protein complex (1). Because of the end replication problem and nucleolytic processing that occurs with each cell division, telomeres shorten to a limited threshold that signals checkpoint-dependent entry into senescence, a state of permanent growth arrest (2). However, if checkpoint function is compromised cells will continue to proliferate. This continued proliferation leads to crisis and cell death, unless cells can counteract the progressive loss of telomeric DNA. Eighty-five percent of human cancers achieve this by upregulating telomerase (3, 4). The remaining 15% of cancers activate alternative lengthening of telomeres (ALT; ref. 5), a recombination-based mechanism marked by high rates of telomere sister chromatid exchange (T-SCE; refs. 6, 7). ALT cells exhibit defective (persistent) sister telomere cohesion into mitosis that contributes to the high level of T-SCE (8).

Sister chromatid cohesion is established in S phase during DNA replication to keep sisters in proximity for recombination and repair (9). Cohesion is removed in mitosis in a two-step process.

During G₂ and early mitosis cohesin is removed from telomeres and arms by the prophase pathway (10). A small amount of cohesin is protected from removal and remains at centromeres holding sister chromatids together (against the spindle forces) until the metaphase to anaphase transition. Centromere cohesion is essential for the faithful distribution of sister chromatids and defects can lead to chromosomal missegregation and aneuploidy (11). Cohesion is mediated by the cohesin ring, a tripartite structure composed of SMC1, SMC3, and SCC1, and a peripheral SA subunit found as two isoforms SA1 and SA2, which are required for telomere and centromere cohesion, respectively (12, 13). SA1 is distinguished by a unique N-terminal 72 amino acid domain that contains a DNA-binding AT-hook motif and binds the shelterin subunit TRF1 (14), which facilitates its association with telomeric DNA (15).

The gene encoding SA2 (*STAG2*) is frequently mutated in human cancer, whereas mutation of the gene encoding SA1 (*STAG1*) is rare (16, 17). *STAG2* mutations are most common in bladder cancer, but are also found in Ewing sarcoma, melanoma, glioblastoma, and other cancers. In fact, *STAG2* is one of only 12 genes found to be significantly mutated in four or more cancer types (18). Approximately 85% of *STAG2* mutations are truncating and often result in loss of expression, indicating *STAG2* as a tumor suppressor gene (16). However, it is not known how loss of SA2 promotes tumorigenesis. The initial report identifying *STAG2* mutations in cancer showed (using isogenic human cultured cell systems) that *STAG2* mutations can lead to aneuploidy (17). However, subsequent studies on naturally occurring tumors showed limited correlation between *STAG2* mutations and aneuploidy (19). Here we set out to determine how *STAG2* tumors maintain sister chromatid cohesion and how *STAG2* inactivation contributes to tumorigenesis.

Department of Pathology, Kimmel Center for Biology and Medicine at the Skirball Institute, New York University School of Medicine, New York, New York.

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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Materials and Methods

Cell lines

VM-CUB-3 (20), SK-ES-1, SK-NEP-1, TC-32, H4, 42MGBA, 42MGB *STAG2* knock-in, and HCT116 *STAG2* knockout (17) were obtained from Dr. Todd Waldman, Georgetown Medical School (Washington, DC) in 2015. UM-UC-3 (20), SK-N-MC (21), and U138MG (17) were obtained from ATCC in 2014. LOX IMVI (17) was obtained from Frederick National Laboratory in 2014. HCT116, HEK293T, and BJ were obtained from ATCC. SuperHeLa (22) was obtained from Dr. Joachim Lingner, EPFL Lausanne (Lausanne, Switzerland) in 2006. HeLa1.2.11 (23) and HTC75 (24) were obtained from Dr. Titia de Lange, Rockefeller University (New York City, NY), in 1999 and tested for mycoplasma (Invitrogen Testing Kit). Cells were stored in liquid nitrogen, thawed, and passaged for a few population doublings prior to use. Cells were grown under standard conditions. Where indicated cells were grown in the presence or absence of the telomerase inhibitor BIBR 1532 (Selleckchem) at a final concentration of 20 μ mol/L. Cells were passaged twice every 7 days and BIBR 1532 was freshly added at each passage.

Chromosome-specific FISH

Cells were fixed and processed as described previously (25). Briefly, cells were isolated by mitotic shake-off, fixed twice in methanol:acetic acid (3:1) for 15 minutes, cytospun (Shandon Cytospin) at 2,000 rpm for 2 minutes onto slides, rehydrated in $2\times$ SSC at 37°C for 2 minutes, and dehydrated in an ethanol series of 70%, 80%, and 95% for 2 minutes each. Cells were denatured at 75°C for 2 minutes and hybridized overnight at 37°C with subtelomeric FITC-conjugated (16ptelo, 13qtelo, or 4ptelo) and TRITC-conjugated 13q14.3 deletion (arm) and 10 cen (centromere) probes from Cytocell. Cells were washed in $0.4\times$ SSC at 72°C for 2 minutes, and in $2\times$ SSC with 0.05% Tween 20 at room temperature for 30 seconds. DNA was stained with 0.2 μ g/mL DAPI. BJ cells were incubated in 30 ng/mL nocodazole (Sigma) for 16 hours prior to shake-off. Mitotic cells were scored as having telomeres cohered if 50% or more of their loci appeared as singlets, that is, one out of two or two out of three. Quantification of FISH analyses is the average of two independent experiments ($n =$ approximately 50 cells each; range 33–78 cells) \pm SEM.

Chromosome orientation FISH

Chromosome orientation FISH (CO-FISH) was performed as described previously (26). Briefly, cells were treated with 10 μ mol/L BrdUrd:BrdC (3:1) for 24 hours prior to harvest and colcemide (0.5 μ g/mL final concentration) was added 8 hours prior to harvest. Cells were harvested by trypsinization, hypotonicity swollen in 10 mmol/L Tris, pH 7.4, 10 mmol/L NaCl, and 5 mmol/L MgCl₂ for 10 minutes at 37°C, and fixed twice for 15 minutes in methanol/acetic acid (3:1). Metaphase spreads were prepared by dropping fixed cells on coverslips followed by centrifugation at 1,000 rpm for 10 seconds in an Eppendorf 5810R centrifuge. Coverslips were air-dried overnight and were rehydrated in PBS for 5 minutes, treated with RNase A (0.5 μ g/mL in PBS) for 10 minutes at 37°C, stained with Hoechst 33258 (0.5 μ g/mL in $2\times$ SSC) for 10 minutes at room temperature and exposed to 365-nm UV light (Stratalinker 1800 UV irradiator) for 45 minutes. The BrdUrd/dC substituted DNA strand was digested with Exonuclease III (10 U/mL) for 30 minutes at 37°C. The slides were dehydrated through an ethanol series (75%, 95%, and 100%) and hybridized with TAMRA-OO-(TTAGGG)₃ PNA probe in hybridization solu-

tion for 2 hours at room temperature. The slides were washed for few seconds with 70% formamide/10 mmol/L Tris-HCl pH 7.2, 0.1% BSA, and incubated with FITC-OO-(CCCTAA)₃ PNA probe in hybridization solution for 2 hours at room temperature. The slides were washed twice for 30 minutes with 70% formamide, 10 mmol/L Tris, pH 7.5, and 0.1% BSA, and were washed three times for 5 minutes in 0.1 M Tris, pH 7.5, 0.15 M NaCl, and 0.08% Tween 20. After the washes, cells were dehydrated in an ethanol series of 70%, 95%, and 100%, and DNA was counterstained with 4,6-diamino-2-phenylindole (DAPI) 0.5 μ g/mL. CO-FISH on HCT116 WT or SA2KO cells was performed as described above, but the spreads were hybridized with only one probe Cy3-conjugated (CCCTAA)₃ PNA probe (PNAbio).

Preparation of cell extracts

Cells were resuspended in four volumes of TNE buffer [10 mmol/L Tris (pH 7.8), 1% Nonidet P-40, 0.15 M NaCl, 1 mmol/L EDTA, and 2.5% protease inhibitor cocktail (Sigma)] and incubated for 1 hour on ice. Suspensions were pelleted at 8,000 \times g for 15 minutes. Equal amounts of supernatant proteins (determined by Bio-Rad protein assay) were fractionated by SDS-PAGE and analyzed by immunoblotting.

Immunoblot analysis

Immunoblots were incubated separately with the following primary antibodies: goat anti-SA1 C-term A300-157A (0.5 μ g/mL; Bethyl Laboratories, Inc.); goat anti-SA1 N-term A300-156A (0.5 μ g/mL; Bethyl Laboratories, Inc.); rabbit anti-SA1 172 (0.5 μ g/mL; ref. 8); rabbit anti-SA2 N-term A300-580A (0.20 μ g/mL; Bethyl Laboratories, Inc.); goat anti-SA2 C-term A300-158A (0.20 μ g/mL; Bethyl Laboratories, Inc.); rabbit anti-SMC3 – ChIP grade ab9263 (0.5 μ g/mL; Abcam); rabbit anti-SMC1 A300-055A-T (1.0 μ g/mL; Bethyl Laboratories, Inc.); rabbit anti-Rad21 A300-080A (0.5 μ g/mL; Bethyl Laboratories, Inc.); rabbit anti-ATRX sc15408 (0.5 μ g/mL; Santa Cruz); mouse anti-DAXX VMA00318 (1:1,000; Bio-Rad); and mouse anti- α -tubulin (1:10,000; Sigma Aldrich, T5768), followed by horseradish peroxidase-conjugated donkey anti-rabbit or anti-mouse IgG (Amersham) or donkey anti-goat (Bethyl Laboratories, Inc.; 1:2,500). Bound antibody was detected with Super Signal West Pico (Thermo Scientific).

Immunoprecipitation

Cells were lysed as above and supernatants precleared with protein G-Sepharose rotating at 4°C for 30 minutes. Nonspecific protein aggregates were removed by centrifugation and the supernatant was used for immunoprecipitation analysis or fractionated directly on SDS-PAGE. For immunoprecipitation of endogenous SMC3 from HCT116 (WT or SA2KO and HeLa1.2.11 (shvec or shSA2)), cells lysis was performed in 0.7 mL (per one 10 cm dish) of TNE buffer. Equal amounts of supernatant proteins (determined by Bio-Rad protein assay) were used as starting material. Ten micrograms of the supernatant was used as input and the rest was incubated with a 3 μ g of SMC3 antibody or rabbit IgG for 3 hours. Protein G beads were added for 2 hours and washed five times with 1 mL TNE buffer. Samples were fractionated by SDS-PAGE and analyzed by immunoblotting.

Indirect immunofluorescence

Cells were fixed in 2% paraformaldehyde in PBS for 10 minutes at room temperature, permeabilized in 0.5% NP-40/

PBS for 10 minutes at room temperature, blocked in 1% BSA/PBS, and incubated with mouse anti- γ H2AX #05-636 (0.2 μ g/mL; Millipore) and rabbit anti-53BP1 NB 100-304 (0.1 μ g/mL; Bovus Biologicals) or mouse anti-PML sc966 (2.0 μ g/mL; Santa Cruz) and rabbit anti-TRF1 415 serum (1:1,000). For staining RAD51 foci, cells were permeabilized in Triton X-100 buffer (0.5% Triton X-100, 20 mmol/L Hepes-KOH at pH 7.9, 50 mmol/L NaCl, 3 mmol/L MgCl₂, 300 mmol/L sucrose) for 5 minutes at room temperature, fixed in 3% paraformaldehyde (in PBS, 2% sucrose) for 10 minutes at room temperature, permeabilized in Triton X-100 buffer for 10 minutes room temperature, blocked in 1% BSA/PBS, and incubated with rabbit anti-RAD51 (4 μ g/mL; Santa Cruz Biotechnology, 8349) for 2 hours. Primary antibodies were detected with FITC-conjugated or TRITC-conjugated donkey anti-rabbit or anti-mouse antibodies (1:100; Jackson Laboratories). DNA was stained with DAPI (0.2 μ g/mL).

Indirect immunofluorescence + PNA-FISH

To detect RAD51 foci colocalization with telomeres, immunofluorescence for RAD51 was performed as described above. After the incubation with secondary antibody, the samples were fixed with 2% paraformaldehyde in PBS for 10 minutes at room temperature, then washed six times with PBS and dehydrated in 70%, 95%, and 100% ethanol for 5 min each. Samples were air dried and then denatured at 80°C for 3 minutes in hybridization mix [0.5 μ mol/L of a Cy3-conjugated (CCCTAA)₃ PNA probe (PNAbio) in 10 mmol/L NaHPO₄, 10 mmol/L NaCl, 20 mmol/L Tris pH 7.5, 70% formamide, 1 \times Denhardtts, 0.1 mg/mL tRNA, 0.1 mg/mL herring sperm DNA]. Cells were hybridized for 2 hours at room temperature, then washed three times for 10 minutes with 70% formamide, 10 mmol/L Tris, pH 7.2. Cells were washed three times with PBS and DNA was stained with DAPI (0.2 μ g/mL).

Telomere restriction fragment analysis

Genomic DNA was isolated from *STAG2* and control tumor cell lines and digested with *HinfI*, *AluI*, *MboI*, and *RsaI*. Approximately 3 μ g of the digested DNA was fractionated on 1% agarose gels using pulsed-field gel electrophoresis. Telomeres were detected by hybridization to a ³²P end-labeled (TTAGGG)₄ oligonucleotide probe as described (25). The same procedure was used for the BIBR 1532-treated cell lines. For BJ cells, DNA was isolated and digested as described above. DNA was fractionated on a 0.8% agarose gels. G-overhang was detected under native conditions by incubating the gel with a ³²P end-labeled (CCCTAA)₃ oligonucleotide probe overnight at 43°C. The gel was denatured and reprobbed overnight at 55°C to detect the total G-strand telomeric DNA. The mean telomere length was determined using Telometric (Fox Chase Cancer Center).

C-circle assay

The C-circle assay was performed as described (27). Genomic DNA was prepared and digested as described above and its concentration determined using a Nanodrop spectrophotometer. Fifty nanograms of DNA was combined with 0.2 mg/mL BSA, 0.1% Tween, 1 mmol/L each dATP, dGTP, and dTTP, 1 \times ϕ 29 Buffer, and 7.5 U ϕ 29 DNA polymerase (NEB) in 20 μ L final volume and incubated at 30°C for 8 hours, then 65°C for 20 minutes. The reaction products were diluted with 60 μ L 2 \times SCC and dot blotted onto a 2 \times SCC-soaked Amersham Hybond-XL membrane (GE Healthcare). Membranes

were UV-cross-linked and hybridized with end-labeled ³²P-(CCCTAA)₃ probe. The membranes were washed (2 \times 5 min with 2 \times SCC at room temperature), exposed to PhosphorImager screens, and scanned using a Typhoon PhosphorImager. To detect the genomic DNA, the membrane was stripped and denatured using a denaturation buffer (1.5 M NaCl, 0.5 M NaOH) for 2 \times 10 minutes in a 42°C shaker and neutralized for 10 minutes at room temperature in neutralization buffer (3 M NaCl, 0.5 M Tris-HCl, pH 7.0). The membrane was reprobbed with ³²P end-labeled α -satellite probe (5'-ATGTGTGCATTCAACTCACAGAGTTGAAC-3'; ref. 28).

TRAP assay

The TRAP assay was performed as described previously (4). To generate cell extracts, 1 \times 10⁶ cells were washed with ice-cold PBS, resuspended in 200 μ L CHAPS buffer, incubated on ice for 30 minutes, and then centrifuged at 12,000 \times g for 20 minutes at 4°C. The assay was performed with 2,500, 5,000, and 10,000 cell equivalents for tumor cell lines and 5,000 and 10,000 cell equivalents for single cell clones. As a control, extracts were heat-inactivated at 80°C for 10 minutes. Point-one microgram of TS primer was incubated with the cell extract for 25 minutes at 30°C, followed by PCR with the reverse (CX) primer. PCR products were fractionated on 10% acrylamide gels and visualized with SYBR Green (1:10,000; Thermo Fisher Scientific).

SA β -galactosidase assay

For the SA β -galactosidase assay (29), cells were fixed in 2% formaldehyde and 0.2% glutaraldehyde in PBS for 5 minutes, washed three times in PBS, and stained for 4 hours at 37°C in staining solution (1 mg/mL X-gal, 150 mmol/L NaCl, 2 mmol/L MgCl₂, 5 mmol/L K₃Fe[CN]₆, 5 mmol/L K₄Fe[CN]₆, and 40 mmol/L NaPi, pH 6.0).

Image acquisition

Images were acquired using a microscope (Axioplan 2; Carl Zeiss, Inc.) with a Plan Apochrome 63 \times NA 1.4 oil immersion lens (Carl Zeiss, Inc.) and a digital camera (C4742-95; Hamamatsu Photonics). Images were acquired and processed using Openlab software (Perkin Elmer). For chromosome specific FISH, if foci fell in more than one optical plane of focus, multiple planes were merged using Openlab software.

Statistical analysis

Statistical analysis was performed using Prism 7 software. Data are shown as mean \pm SEM. Student unpaired *t* test was applied. *P* < 0.05 values were considered significant: *, *P* \leq 0.05; **, *P* \leq 0.01; ***, *P* \leq 0.0001; ****, *P* \leq 0.0001; ns, not significant.

Results

STAG2 tumor cells exhibit persistent sister telomere and arm cohesion in mitosis

Previous examination of *STAG2* tumor cell lines by chromosome spread analysis showed defects in centromere cohesion (17). Despite defective centromere cohesion, *STAG2* tumors do not associate with aneuploidy, suggesting that other mechanisms might be acting to hold sister chromatids together. We sought to determine the status of cohesion along chromosome arms and

telomeres. Because arm/telomere cohesion is not maintained during chromosome spread preparation, we measured sister chromatid cohesion using chromosome specific FISH (25, 30). In this procedure mitotic cells are isolated by "shake-off" from asynchronous cultures, fixed immediately to preserve cohesion, and analyzed with fluorescent chromosome-specific probes.

We obtained a panel of 10 *STAG2* tumor lines that were either reported (or predicted) to show loss of SA2 expression across a range of tumor types (Supplementary Fig. S1A; refs. 17, 20, 21). Immunoblot analysis with antibodies directed against the N- or C-terminal domains of SA2 showed complete loss of SA2 protein in all 10 *STAG2* lines, whereas SA1 exhibited a range of expression levels from one-tenth to two-fold, the levels found in control HeLa.2.11 cells (a clonal HeLa line with long telomeres; ref. 23; Fig. 1A and Supplementary Fig. S1B). Analysis of centromere cohesion using a 10 cen probe in two *STAG2* tumor lines (LOX-IMVI and UM-UC-3) revealed (as expected) premature loss of centromere cohesion (doublets in mitosis) that was rescued by transient expression of SA2 (singlets in mitosis; Fig. 1B–D). FISH analysis with a dual probe against the 13q subtelomere and arm revealed (unexpectedly) persistent cohesion (singlets in mitosis) at telomeres and arms that was rescued by transient expression of SA2 (doublets in mitosis; Fig. 1E and F). We extended the analysis to additional *STAG2* tumor lines using a 16p subtelomere probe. Although non-*STAG2* tumor lines (HTC75 and HeLa) show limited persistent cohesion (10% of mitotic cells), *STAG2* tumor lines show excessive persistent cohesion (50%–60% of mitotic cells; Fig. 1G and H). Together, these data indicate an abnormal pattern of sister chromatid cohesion in mitotic *STAG2* tumor cells, where centromeres are prematurely separated, but telomeres and arms are persistently cohered (Fig. 1I).

Because *STAG2* tumors contain a number of additional mutations (16), we sought to determine if loss of *STAG2* was (in and of itself) sufficient to induce persistent telomere cohesion. We used lentiviral infection to generate HeLa.1.2.11 cell lines stably expressing two different SA2 shRNAs (SA2-3 and SA2-4) and found that SA2 depletion-induced persistent telomere cohesion (Fig. 1J–L). We next wanted to determine if increased cohesion was due to a change in the level or association of cohesin subunits. Immunoblot analysis showed that the levels of SMC1, SMC3, and SCC1 were unaffected by depletion of SA2, whereas SA1 was slightly increased (Fig. 1M). Immunoprecipitation analysis showed that the ring was intact; SMC1, SCC1, and SA1 coimmunoprecipitated with SMC3 (Fig. 1M), consistent with previous results in *STAG2* tumor cells (31). We obtained similar results in a HCT116 SA2 KO cell line (ref. 17 and Fig. 1N). Thus, despite normal levels of cohesin subunits and formation of the cohesin ring, loss of SA2 induces defective cohesion.

SA2 is the major SA subunit in somatic cells. There is 12- to 15-fold more SA2-cohesin than SA1-cohesin in HeLa cells (32). We considered that upon loss of SA2, even if there is a two-fold increase in SA1, which was the maximum that we observed in our panel of *STAG2* tumor lines (see Fig. 1A and Supplementary Fig. S1B), most of the cohesin rings would be without an SA subunit. We reasoned that the tripartite ring alone may be unable to establish or maintain centromere cohesion and at the same time could contribute to persistent cohesion at telomeres and arms. We thus asked if SA2 rescue of defective cohesion depends on its ability to bind the cohesin ring. We introduced wild-type SA2 or SA2.D793K (a ring

binding-deficient point mutant; ref. 33) into HTC116 *STAG2* KO cells. We found that wild-type SA2, but not SA2.D793K, rescued the defective telomere (Fig. 2A–C) and centromere (Fig. 2D and E) cohesion. Finally, consistent with a role for the ring in persistent cohesion, we found that depletion of the SCC1 ring subunit rescued persistent telomere cohesion in HCT116 *STAG2* KO cells (Fig. 2F–H) and it rescued persistent arm/telomere cohesion in LOX-IMVI and UM-UC-3 *STAG2* tumor cells (Fig. 2I–K).

***STAG2* tumor cells exhibit high rates of telomere recombination**

We considered that keeping sister telomeres in close proximity through G₂ into mitosis might promote increased sister telomere recombination, as it does in ALT cells (8). To measure the rate of T-SCE in *STAG2* tumor cells we performed CO-FISH analysis (26). For controls we measured the rate in non-ALT super-telomerase HeLa cells (a HeLa line overexpressing telomerase that has very long telomeres; ref. 22) and observed a low rate of T-SCE and in the ALT cell line U2OS and observed a high rate of T-SCE (Fig. 3A and B). *STAG2* tumor cells (SK-N-MC, U138MG, LOX-IMVI, and 42MGBA) all exhibited high rates of T-SCE (Fig. 3A and B). Knock-in of *STAG2* in 42MGBA *STAG2* tumor cells (17) reduced T-SCE levels (Fig. 3B), suggesting that the high rate was due to loss of *STAG2*. To determine if depletion of SA2 in non-*STAG2* tumor cells was sufficient to induce T-SCE, we performed CO-FISH on HeLa.2.11 cell lines stably expressing *STAG2* shRNAs and observed a significant increase in T-SCE in SA2-depleted cells (Fig. 3C and D). Similarly, knockout of SA2 in HCT116 cells led to increased levels of T-SCE (Supplementary Fig. S2A and S2B). Together, these data suggest that persistent cohesion promotes T-SCE. To further test this, we asked if the increased T-SCE in HCT116 SA2 KO cells was dependent on the cohesin ring. As shown in Supplementary Fig. S2C and S2D, depletion of the ring subunit SCC1 rescued the T-SCE in SA2 KO cells. Finally, SA2 KO did not induce a genome wide increase in SCE as determined by Giemsa staining (Supplementary Fig. S2E and S2F).

ALT cells exhibit high rates of T-SCE and have long heterogeneous telomeres. To analyze the telomeres in *STAG2* tumor lines, we performed telomere restriction fragment analysis (Fig. 3E). Telomerase positive non-ALT cell lines typically have short (~4–7 kb) homogeneous telomeres, as shown for HEK293T and HTC75 cells. *STAG2* tumor cell lines showed a range of telomere lengths. Four lines had relatively long telomeres; SK-N-MC, U138MG, and 42MGBA were between 12 and 18 kb and LOX-IMVI ranged from 14 kb to extremely long, similar to the U2OS ALT line. Telomeres of the remaining *STAG2* lines were between 4 and 6 kb, similar to telomerase positive tumor cells. We observed no correlation between the ploidy level and telomere length in *STAG2* tumor cell lines (Supplementary Fig. S2G).

***STAG2* cells lack hallmarks of ALT cells**

Because *STAG2* tumor cells exhibited some features of ALT: high levels of T-SCE and in some cases long telomeres, we examined other properties of ALT in our *STAG2* tumor lines. Hallmark features of ALT cells include the loss of ATRX and DAXX expression (34, 35), the presence of ALT-associated promyelocytic leukemia (PML) bodies (APB; ref. 36) and partially single-stranded telomeric extrachromosomal (CCCTAA) DNA circles (C-circles; ref. 27), and the absence of telomerase activity (37). We measured ATRX and DAXX levels in our panel of 10

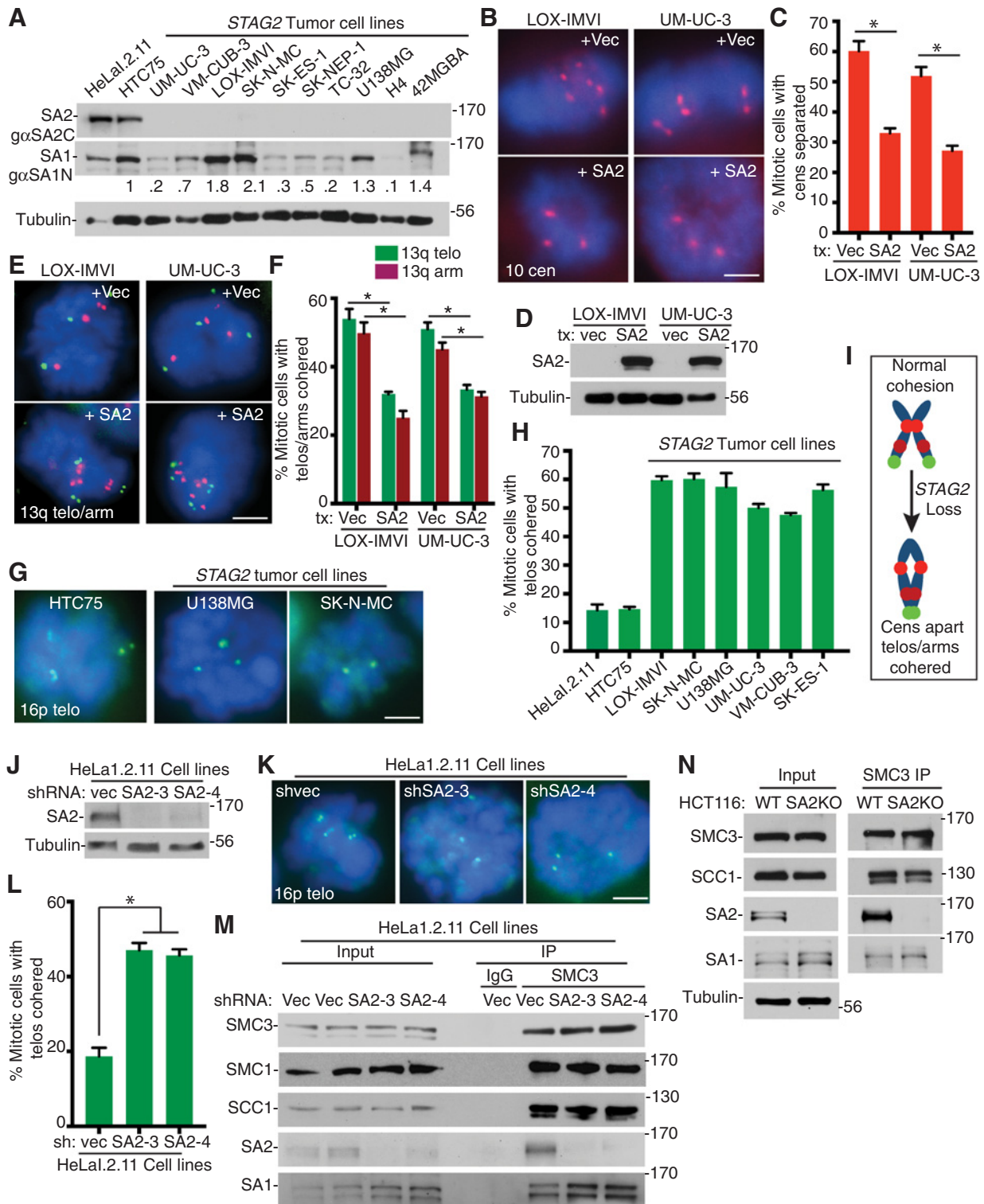
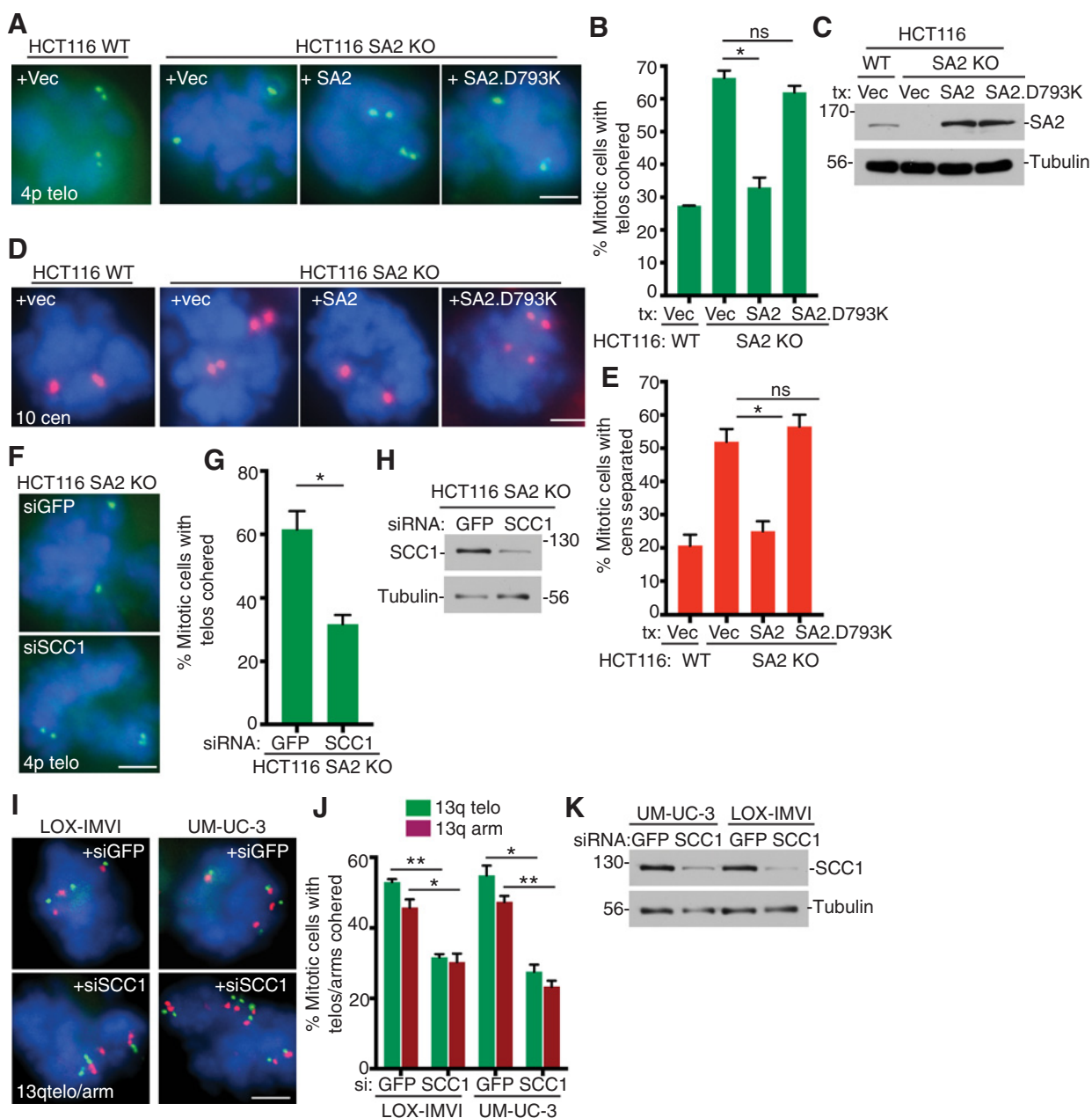


Figure 1.

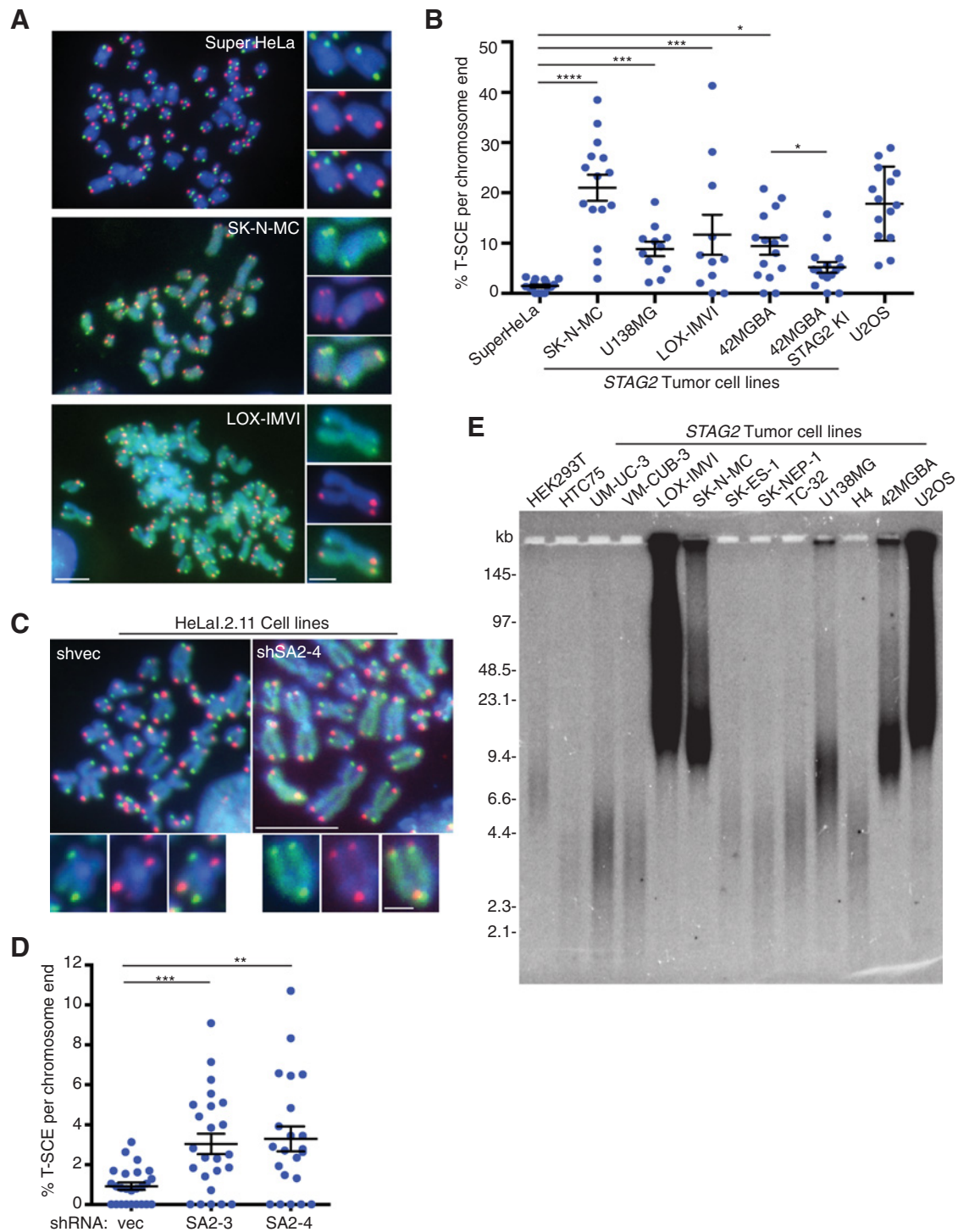
STAG2 tumor cells exhibit persistent sister telomere and arm cohesion in mitosis. **A**, Immunoblot analysis of *STAG2* tumor cell lines. **B**, FISH analysis of mitotic *STAG2* tumor cells using a 10 cen (red) probe. **C**, Quantification of results in **B**. **D**, Immunoblot analysis of *STAG2* tumor cell lines. **E**, FISH analysis of mitotic *STAG2* tumor cells using a 13q arm (red) and 13q telo (green) probe. **F**, Quantification of results in **E**. **G**, FISH analysis of mitotic cells using a 16p telo (green) probe. **H**, Quantification of the frequency of mitotic cells from the indicated cell lines with cohered telomeres. **I**, Schematic of sister chromatid cohesion in wild-type and *STAG2* cells. **J**, Immunoblot analysis of HeLa1.2.11 cell lines. **K**, FISH analysis of mitotic HeLa1.2.11 cells using a 16p telo (green) probe. **L**, Quantification of results in **K**. **M** and **N**, Immunoblot analysis of HeLa1.2.11 (**M**) and HCT116 (**N**) cell lines following immunoprecipitation with anti-SMC3 antibody. **B**, **E**, **G**, and **K**, DNA was stained with DAPI. Scale bar, 5 μ m.

**Figure 2.**

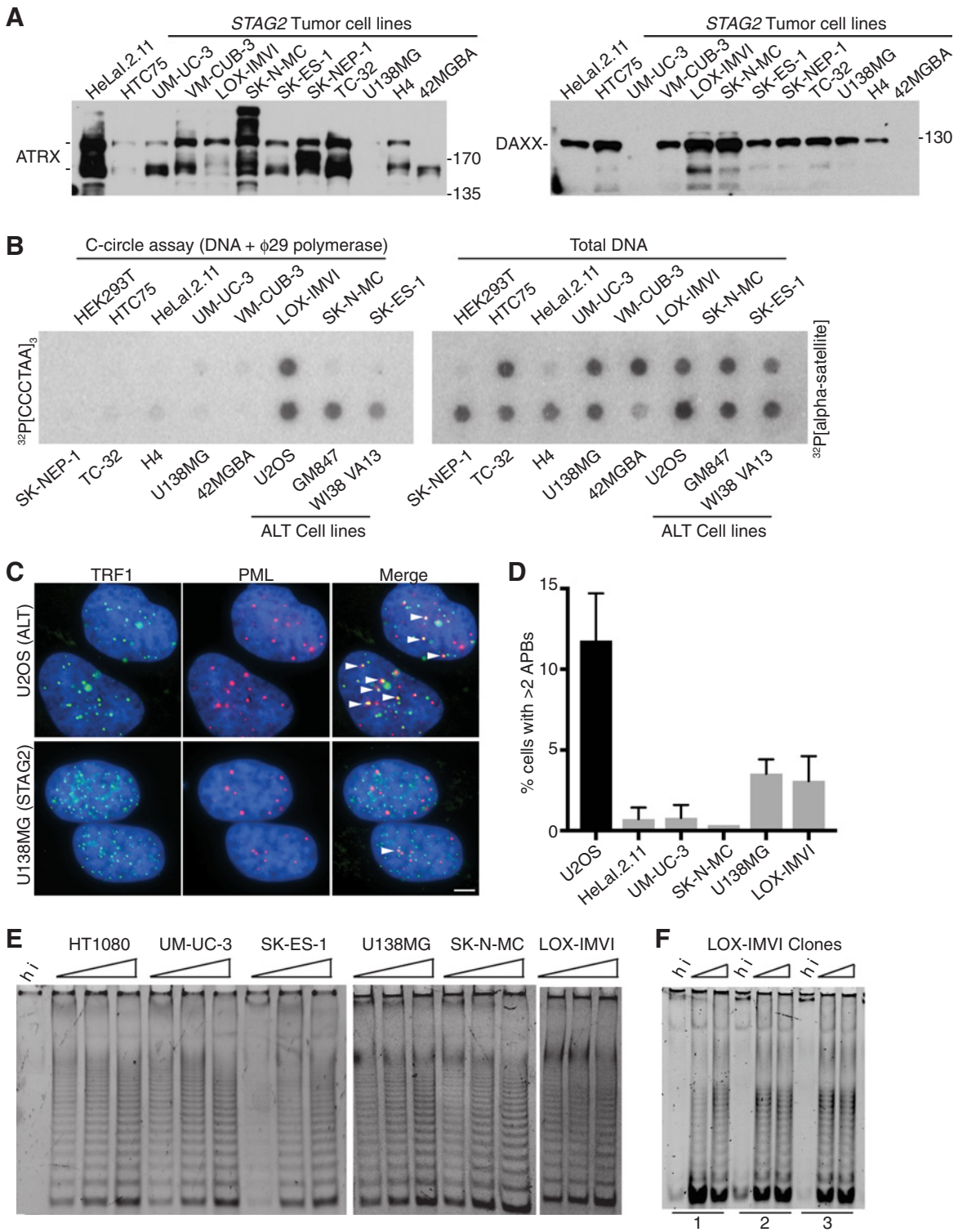
The cohesin ring is required for persistent telomere and arm cohesion in mitosis. **A**, FISH analysis of mitotic HCT116 cells using a 4p telo (green) probe. **B**, Quantification of results in **A**. **C**, Immunoblot analysis of HCT116 cell lines. **D**, FISH analysis of mitotic HCT116 cells using a 10 cen (red) probe. **E**, Quantification of results in **D**. **F**, FISH analysis of mitotic HCT116 cells using a 4p telo (green) probe. **G**, Quantification of results in **F**. **H**, Immunoblot analysis of HCT116 SA2 KO cells. **I**, FISH analysis of mitotic *STAG2* tumor cells using a 13q arm (red) and 13q telo (green) probe. **J**, Quantification of results in **I**. **K**, Immunoblot analysis of *STAG2* tumor cell lines. **A**, **D**, **F**, and **I**, DNA was stained with DAPI. Scale bar, 5 μ m.

STAG2 cell lines using immunoblot analysis and found that most expressed ATRX and DAXX (Fig. 4A). We detected little or no C-circles in most *STAG2* cell lines, compared to robust levels for ALT cells (Fig. 4B). One exception was LOX-IMVI cells, which showed similar C-circle levels as ALT cells. This could be due to the exceptionally long telomeres in that cell line (see Fig. 3E). To determine the frequency of APBs, we performed immunofluores-

cence analysis and measured colocalization of the shelterin subunit TRF1 with PML. As shown in Fig. 4C and D, APBs were minimally or not at all detected in *STAG2* tumor lines compared to U2OS. Even when APBs were detected in *STAG2* cells, they were much smaller than those in the U2OS ALT cells (see Fig. 4C). Finally, measurement of telomerase activity using the telomere repeat amplification protocol (TRAP) indicated high levels of

**Figure 3.**

STAG2 tumor cells exhibit high rates of telomere recombination and a range of telomere lengths. **A**, CO-FISH analysis of metaphase spreads probed with TTAGGG (red) and CCCTAA (green). **B**, Quantification of the frequency of T-SCE in the indicated cell lines ($n = 638$ – $1,314$ chromosome ends from 11 to 15 metaphase spreads) \pm SEM. **C**, CO-FISH analysis of metaphase spreads probed with TTAGGG (red) and CCCTAA (green). **D**, Quantification of the frequency of T-SCE in the indicated cell lines ($n = 912$ – $2,536$ chromosome ends from 22 to 25 metaphase spreads) \pm SEM. **E**, Analysis of telomere restriction fragments isolated from the indicated tumor cell lines, fractionated by pulsed-field gel electrophoresis, denatured, and hybridized with a ^{32}P -[TTAGGG] $_4$ probe. **A** and **C**, DNA was stained with DAPI. Scale bar, 5 μm . Inset scale bar, 2 μm .

**Figure 4.**

STAG2 tumor cells lack most hallmarks of ALT cells. **A**, Immunoblot analysis of STAG2 tumor cell lines from Fig. 1A probed with anti-ATRX (left) and anti-DAXX (right) antibodies. **B**, Dot blot analysis of C-circle assays hybridized with a ^{32}P -(CCCTAA)₃ oligo probe to detect C-circles (left) and stripped and probed with a ^{32}P - α -satellite oligo probe to detect total DNA (right). **C**, Immunofluorescence analysis using TRF1 (green) and PML (red) antibodies to measure APBs. DNA was stained with DAPI. Scale bar, 5 μm . Arrowheads, colocalizing foci; APBs. **D**, Quantification of APBs in the indicated cell lines. Average of two independent experiments ($n = 63\text{--}79$ cells each). None were detected in SK-N-MC cells. **E** and **F**, TRAP assay measuring telomerase activity in tumor cell lines (**E**) and LOX-IMVI clones (**F**). hi, heat-inactivated.

telomerase activity in all *STAG2* tumor lines tested (UM-UC-3, SK-ES-1, UM138G, SK-N-MC, and LOX-IMVI) similar to telomerase positive HT1080 tumor cells (Fig. 4E). To rule out the possibility that telomerase activity resulted from a mixed population of cells in the tumor, we isolated single cell clones from LOX-IMVI cells and performed TRAP analysis. As shown in Fig. 4F, each clone displayed telomerase activity. Taken together, these data indicate that despite high levels of T-SCE and longer than average telomeres, *STAG2* tumors are not ALT.

STAG2 tumors are refractory to telomerase inhibition

Our studies indicate that *STAG2* tumor cells have two mechanism of telomere maintenance available to them: recombination and telomerase. We cannot inhibit recombination during long-term growth, because it inhibits cell growth, but we can inhibit telomerase using the nontoxic selective small molecule inhibitor BIBR 1532 (38, 39). Previous studies showed that continuous treatment of HT1080 cells with 10 $\mu\text{mol/L}$ BIBR had no effect on proliferation for 150 population doublings (PD) and led to telomere shortening (38). We used the TRAP assay to confirm that BIBR 1532 inhibited telomerase in HT1080 cells and in our *STAG2* tumor lines in a dose-dependent manner (Fig. 5A). We passaged cells for 120 to 160 PD in

the absence or presence of 20 $\mu\text{mol/L}$ BIBR 1532; cells were passaged twice every 7 days and BIBR 1532 was freshly added at each passage. We then used telomere restriction fragment analysis to measure telomere length. As shown in Fig. 5B, as expected, telomeres in HT1080 cells shorten. Measurement of the mean telomere lengths (Fig. 5C) indicates that telomeres shorten at a rate of 21 bp/PD (Fig. 5D), similar to previous studies (38). By contrast, the *STAG2* cell lines exhibited minimal or no shortening with rates ranging from 6 bp/PD (UM-UC-3) to 0 bp/PD (U138MG; Fig. 5B–D). Together, these data indicate that the *STAG2* cells (unlike typical telomerase positive tumor cells) do not rely exclusively on telomerase for telomere length maintenance.

STAG2 depletion extends lifespan of normal human cells

We considered that having two pathways of telomere maintenance might provide cells with a growth advantage. We did not detect an obvious effect on cell growth in telomerase-positive HeLa cells depleted of SA2 or in HCT116 *STAG2* KO cells. But what about normal (telomerase negative) human cells? Considering that continued passage of normal human cells leads to replicative senescence due to telomere shortening, we reasoned that SA2-depletion (in this case) might provide a growth

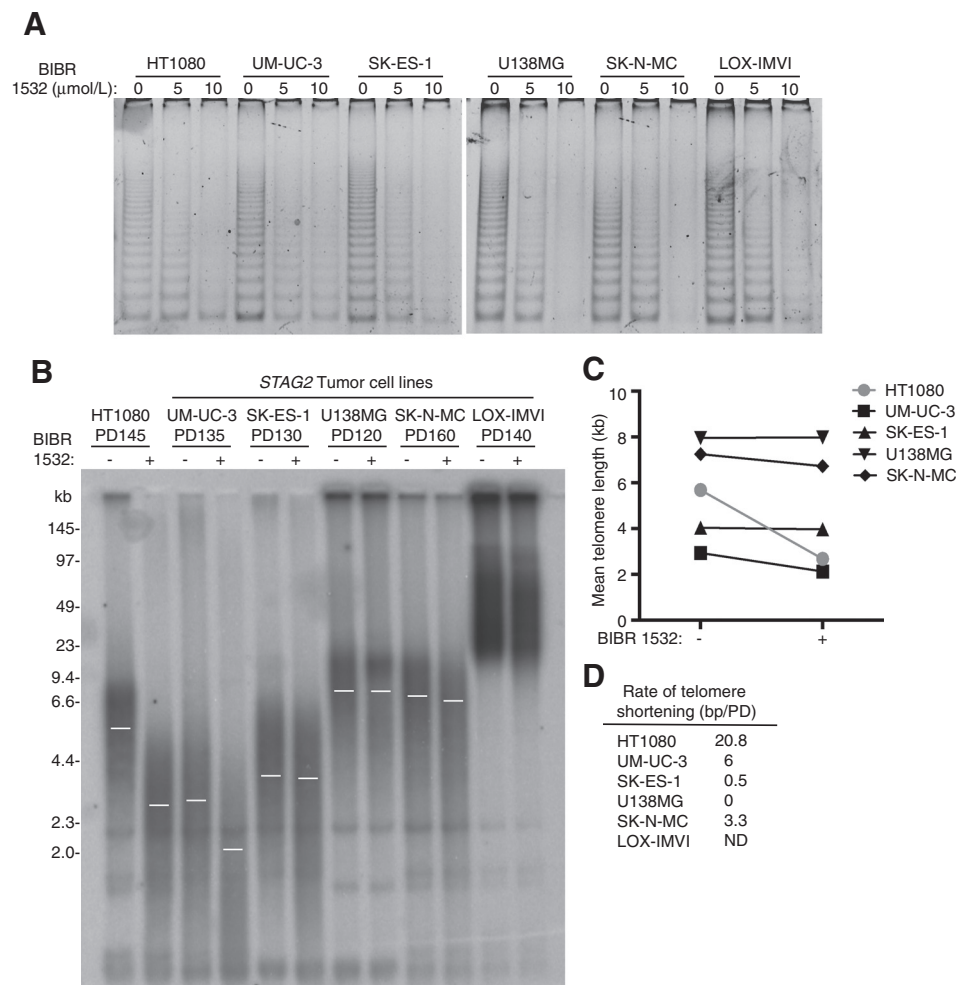


Figure 5.

STAG2 tumor cells are refractory to telomerase inhibition. **A**, TRAP assay measuring telomerase activity in the presence of BIBR 1523. **B**, Analysis of telomere restriction fragments isolated from the indicated cell lines, fractionated by pulsed-field gel electrophoresis, denatured, and hybridized with a ^{32}P -(TTAGGG) $_4$ probe. **C**, Graphical representation of mean telomere length determined using Telometric (Fox Chase Cancer Center), indicated by white lines in **B**. **D**, Summary of the rates of telomere shortening. ND, not determined.

advantage by promoting recombination and extending critically short telomeres. We infected BJ fibroblasts at an early PD (PD 28) with lentiviruses expressing two different SA2 shRNAs (SA2-3 and SA2-4) to generate SA2-depleted BJ cell lines (BJ-2). Immunoblot analysis confirmed depletion of SA2 (Supplementary Fig. S3A) and FISH analysis showed induction of persistent telomere cohesion (Supplementary Fig. S3B and S3C). We performed CO-FISH analysis and observed a small, but significant increase in T-SCE in SA2-depleted BJ cells (Supplementary Fig. S3D). As another way to measure recombination, we determined the frequency of RAD51 foci, a marker of homologous recombination that appears in G₂ phase of the cell cycle. As shown in Fig. 6A and B, we observed a significant increase in RAD51 foci upon depletion of SA2. To determine if these foci coincided with telomeres, we performed dual staining for RAD51 with telomeres using TTAGGG-PNA FISH. As shown in Fig. 6C and D, the RAD51 foci were frequently at or near telomeres in SA2-depleted cells, indicating increased recombination at telomeres.

To determine the impact of SA2 depletion on growth, the BJ-2 cell lines were carried for multiple population doublings. As shown in Fig. 6E, initially at early PDs (from PD 30 to 45; 0 to 60 days in culture), both vector control and SA2-depleted cells grew at the same rate. However, at late PDs (after PD 45; after 60 days in culture), the growth rate for control, but not for SA2-depleted cells, slowed. SA2-depleted cells continued to grow at the same rate for several PDs before slowing. Ultimately, SA2-depletion enabled BJ cells to grow for an additional seven to eight cell divisions up to PD 59 to 60 compared to PD 52 for vector control cells (Fig. 6E). Aging human cells accumulate DNA damage that signals senescence (40). We thus asked if the DNA damage signal was attenuated in SA2-depleted cells. We measured the frequency of DNA damage foci at early (day 25) and late (day 84) PD. As shown in Fig. 6F and G, we observed a significant decrease in the levels of γ H2AX/p53BP1 DNA damage foci in SA2-depleted late PD cells. To measure senescence, we subjected the cells to the senescence associated β -gal assay (29) at early (day 25) and late (day 84) PD. As shown in Fig. 6H and I, we observed a significant decrease in β -gal positive cells in SA2-depleted late PD cells. We independently generated a second set of SA2-depleted BJ cell lines (BJ-1) and confirmed that SA2-depletion extended lifespan, reduced DNA damage foci, and delayed induction of senescence (Supplementary Fig. S3E–S3G).

Our results, showing that SA2-depleted cells have reduced DNA damage signaling and delayed senescence at late PDs, suggested the possibility that telomere shortening was attenuated in these cells. We used telomere restriction fragment analysis under denaturing conditions using a CCCATT telomere probe to analyze telomere length in late PD BJ-2 cells (day 74) and observed longer telomeres in the SA2-depleted cell lines than the vector control (Fig. 6J, right). Telomere shortening in primary cells results from the end replication problem and nucleolytic processing of the 5' C-strand to generate the single-stranded 3' G-strand overhang (41). Reduced telomere shortening could result from reduced C-strand degradation. However, probing of the native telomeric DNA (prior to denaturing) showed that the amount of G-strand overhang was not diminished in SA2-depleted cells (Fig. 6J, left). We repeated these analyses in late PD BJ-2 cells (day 66) and obtained similar results (Supplementary Fig. S3H).

Finally, we measured the impact of SA2-depletion in BJ cells on aneuploidy. We performed dual 10 and 6 cen FISH and scored

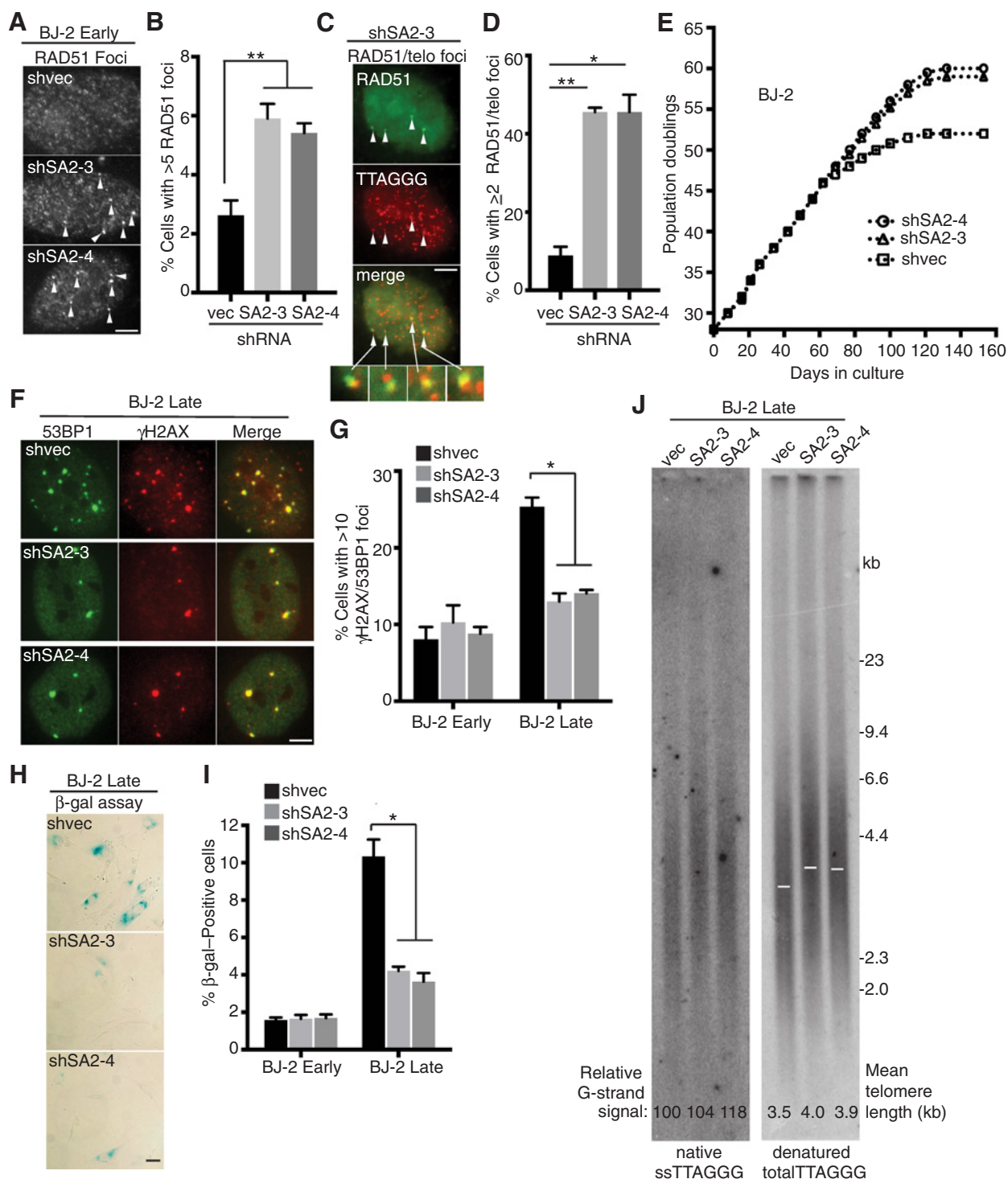
monosomy and trisomy to determine aneuploidy for each chromosome at early and late PD. As shown in Supplementary Fig. S3I and S3J, we observed a small but significant increase in aneuploidy in SA2-depleted cells compared to the vector control at early PD. At late PD, both vector and SA2-depleted cells showed an increase, consistent with a general increase in aneuploidy in aging cells (42). These results are consistent with previous studies showing that loss of *STAG2* in human cultured systems can lead to aneuploidy (17, 20).

Discussion

We have found that *STAG2* tumor cells exhibit a dramatic change in the pattern of sister chromatid cohesion in mitosis. Normally, cohesin is removed from telomeres and arms by the prophase pathway, but remains at centromeres until the metaphase to anaphase transition. In *STAG2* tumors, cohesion is maintained at telomeres and arms, but is lost from centromeres. We show that this pattern can be rescued by reintroducing SA2 and further that rescue depends on SA2 binding to the cohesin ring. Although it is not surprising that SA2 loss leads to a centromere cohesion defect, the observed persistent arm and telomere cohesion was unexpected. Normally, SA2-cohesin is the predominant species; it is 12- to 15-fold more abundant than SA1-cohesin (32). Thus, loss of SA2, without sufficient compensation by SA1 would mean that the majority of cohesin rings would lack an SA subunit. Indeed, in *STAG2* tumors SA2 is lost, SA1 varies only slightly (up or down), and the ring subunit levels remain the same. Thus, the bulk of cohesin in *STAG2* tumors is comprised of just the tripartite ring. One possibility is that rings lacking a SA subunit are not removed efficiently from arms and telomeres by the prophase pathway. The resulting persistent arm/telomere cohesion would keep sisters cohered in mitosis, thereby compensating for the lack of centromere cohesion.

We found that *STAG2* tumors undergo high rates of sister telomere recombination, a feature that it shares with ALT cells. In addition, some *STAG2* tumors show long heterogeneous telomeres like ALT cells. However, *STAG2* tumors do not exhibit most of the hallmarks of ALT cells including APBs, C-circles, and loss of ATRX. In addition, *STAG2* tumors are telomerase positive. Telomerase positive tumors generally rely on telomerase for cell growth and exhibit telomere shortening upon inhibition of telomerase (43). However, *STAG2* tumors exhibited minimal telomere loss during long-term growth in the presence of a telomerase inhibitor. The capacity for telomere recombination may permit *STAG2* tumor cells to maintain their telomeres when telomerase is inhibited. Such a property would be an important consideration for anticancer therapy. Telomerase is an attractive target for cancer therapeutics under active investigation (44). Based on our studies, *STAG2* tumors would not be good candidates for telomerase anticancer therapy. Along these lines, it will be important to determine if other (non-*STAG2*) classes of telomerase positive tumors have a capacity for recombination.

Our data are consistent with the notion that persistent telomere cohesion promotes T-SCE. We showed that depletion of SA2 alone was sufficient to induce T-SCE in non-*STAG2* tumor cells, that reintroduction of *STAG2* into *STAG2* tumor cells rescued T-SCE, and that depletion of the ring subunit SCC1 rescued T-SCE in SA2 KO cells. How might persistent telomere cohesion promote recombination? Studies in mouse

**Figure 6.**

STAG2 depletion diminishes telomere shortening and extends lifespan of normal human cells. **A**, Detection of RAD51 foci (indicated by arrowheads) by immunofluorescence analysis of early BJ-2 cell lines (day 42). **B**, Quantification of results in **A**. Average of three independent experiments ($n = 58-63$ cells each) \pm SEM. **C**, Detection of RAD51 foci colocalizing with telomeres (indicated by arrowheads) using immunofluorescence analysis of RAD51 (green) and TTAGGG PNA-FISH (red) on shSA2-3 early BJ-2 cells (day 42). **D**, Quantification of RAD51/telomere colocalizing foci in early BJ-2 cell lines. Average of two independent experiments ($n = 15-27$ cells each) \pm SEM. **E**, Growth curve analysis of BJ-2 cell lines, generated by lentiviral infection at PD 28. **F**, Detection of 53BP1 and γ H2AX foci by immunofluorescence analysis of late BJ-2 cell lines (day 84). **G**, Quantification of γ H2AX/53BP1 colocalizing foci in early (day 25) and late (day 84) BJ-2 cell lines. Average of two independent experiments ($n = 47-67$ cells each) \pm SEM. **H**, Detection of senescent cells by β -galactosidase assay on late BJ-2 cell lines (day 84). **I**, Quantification of β -galactosidase-positive cells in early (day 25) and late (day 84) BJ-2 cell lines. Average of two independent experiments ($n = 955-1079$ cells each) \pm SEM. **J**, Analysis of telomere restriction fragments isolated from late BJ-2 cell lines (day 74), fractionated on 0.8% agarose gel, and hybridized under native conditions with a 32 P-[CCCTAA]₃ probe to detect the G-strand overhang, and then, denatured and reprobred to detect the total G-strand telomeric DNA. **A**, **C**, and **F**, Scale bar, 5 μ m. **H**, Scale bar, 10 μ m.

suggest that telomeres are highly susceptible to homologous recombination and that this is normally repressed by shelterin (45). Here we suggest that timely resolution of telomere cohesion contributes to repression of recombination and that the persistent cohesion at telomeres (induced by SA2 loss) drives the increase in T-SCE. Homologous recombination between sister telomeres can only occur in a small window of the cell cycle: between post-replication in S phase and separation in mitosis. Resolution of telomere cohesion normally occurs in late S/G₂ and is complete by prophase (14). By contrast, in *STAG2*-depleted cells telomeres remain cohered throughout G₂ and into mitosis. This several fold increase in the window of time that sisters are cohered could underlie the increase in T-SCE.

Mutational inactivation of *STAG2* is an early event in tumorigenesis (16), whereas telomerase activation is late (44). Thus, it was pertinent to determine the impact of SA2 depletion on telomerase negative normal human cells. We observed persistent telomere cohesion and increased recombination at telomeres. We did not detect an effect on cell growth, at least at early PDs. However, at later PDs upon senescence onset, the advantage of SA2-depletion was striking. Cells showed reduced DNA damage signaling and diminished telomere shortening that resulted in delayed senescence. How might increased sister chromatid cohesion and recombination slow the rate of telomere attrition? As telomeres shorten in the absence of telomerase, the shortest telomeres activate a DNA damage response (40). Short telomeres may be subject to increased processing and resection and/or incomplete replication, which would lead to length asymmetry of sister chromatids (46). Here, a recombination-based mechanism using the sister chromatid as a copy template could allow lengthening of a shortened strand, delaying the DNA damage signal and extending lifespan. Ultimately, the telomere reserve is depleted and cells senesce, but not before the critical period prior to senescence is extended. In normal human cells, telomere shortening serves to limit the number of cell divisions to prevent

genomic instability and cancer (47). A recent report indicates that a majority of mutations in cancer arise from replication errors and further, that stem cells with longer replicative lifespan have a higher number of mutations and increased incidence of cancer (48). Lifespan extension of normal human cells by inactivation of *STAG2* could promote tumorigenesis by extending the time period during which tumor-driving mutations can occur.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: Z. Daniloski, S. Smith

Development of methodology: Z. Daniloski, S. Smith

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Z. Daniloski, S. Smith

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Z. Daniloski, S. Smith

Writing, review, and/or revision of the manuscript: Z. Daniloski, S. Smith

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Z. Daniloski, S. Smith

Study supervision: S. Smith

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Loss of Tumor Suppressor *STAG2* Promotes Telomere Recombination and Extends the Replicative Lifespan of Normal Human Cells

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