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TITLE: Therapeutic Vulnerability of SETD2 mutant RCC

PRINCIPAL INVESTIGATOR: Dr Frank Mason, PhD

CONTRACTING ORGANIZATION: Vanderbilt University Medical Center

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Fort Detrick, Maryland 21702-5012

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14. ABSTRACT Clear cell renal cell carcinoma (RCC) is the most common form of kidney cancer and individual tumors exhibit extensive intratumor genetic heterogeneity. A major driver of this heterogeneity is loss of the tumor suppressor SETD2, which also contributes to worse progression free survival and metastasis. The goal of this project is to determine therapeutic vulnerabilities of cells and tumors lacking SETD2, namely Rigosertib, a Polo-like kinase inhibitor. Our previous work here identified that Rigosertib will not be efficacious <i>in vivo</i> as its activity is inhibited by a physiological waste product called uric acid (UA), which is present in serum and even higher in renal tissues and tumors. This work is currently under review at <i>Journal of Clinical Investigation</i> . We subsequently identified an orthogonal compound (ON-1500) that functions similarly to Rigosertib but is more potent, but we have made little progress with this compound due to availability issues. During these experiments, we identified loss of SETD2 or its catalytic activity leads to formation of isochromosomes, or chromosomes that are mirror images of themselves. We also identified that the activity of RAD52 drives these chromosomal alterations, and this work has been recently accepted at <i>PNAS</i> . Future work will determine whether RAD52 and SETD2 loss are synthetically lethal and/or decrease intratumoral heterogeneity.			
15. SUBJECT TERMS Clear cell renal cell carcinoma, SETD2, intratumor heterogeneity, mitosis, chromatin, dicentric, isochromosome, Polo-like kinase, Rigosertib, uric acid, RAD52			
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1. **INTRODUCTION:** *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

The goal of our proposal is to identify and target therapeutic vulnerabilities inherent in clear cell renal cell carcinoma tumors that lack the function of the tumor suppressor *SETD2*, a major driver of intratumor heterogeneity, metastasis and disease progression. To accomplish this goal, we are utilizing cell biological and genomic analyses, as well as *in vivo* models.

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

Clear cell renal cell carcinoma, SETD2, intratumor heterogeneity, mitosis, chromatin, dicentric, isochromosome, RAD52, Rigosertib, uric acid

3. **ACCOMPLISHMENTS:** *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official 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.

Major Task 1: Determine mechanisms of genome instability due to *SETD2*-PLK synthetic lethality (Months 1-4).

100% complete.

Major Task 2: Determine alterations in signaling and genome organization due to *SETD2*-PLK synthetic lethality (Months 1-6).

90% complete. (Subtask 1- 100%, Subtask 2- 80%)

Major Task 3: Determine karyotypic alterations and DNA damage due to *SETD2*-PLK synthetic lethality (Months 6-9).

100% complete.

Major Task 4: Inject xenografts and treat mice with PLKi (Months 6-24).

15% complete. (Subtask 1- 100%, Subtask 2- 50%, Subtask 3-7 0%)

Major Task 5: IHC and DNA/RNA isolation (Months 24-30).

0% complete.

Major Task 6: Karyotypic analyses for all tumors (Months 24-30)

10% complete. (Subtask 1: 10%, cell lines generated, Subtask 2: 0%)

Major Task 7: Assess PLKi in 786-0 and 786-0Δ*SETD2* cell lines in 3D culture (Months 3-6).

0% complete.

Major Task 8: Establish patient derived organoid (PDO) pipeline using approved IRB protocols (Months 3-33).

10% complete. (Subtask 1- 100%, Subtask 2-7 0%)

Major Task 9: Synthesize data and prepare manuscripts (Months 18-36).

100% complete. (We have submitted 2 manuscripts, but hopeful for a 3rd).

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.

Major Task 2 was to determine whether alterations in signaling and genome organization in *SETD2*-knockout cells treated with Rigosertib. We have since identified that Rigosertib is not synthetically lethal with *SETD2*-mutated cells and that the primary activity of Rigosertib is to depolymerize microtubules, which does not affect cellular signaling pathways beyond mitotic arrest. Therefore, we did not send these cells for RNA sequencing and genomic analyses. However, we have since treated cells with the new lead compound ON-1500, isolated RNA and sent for sequencing and subsequent analyses. We are currently waiting for those results to be finalized.

While Major Task 3 was previously completed, a large portion of these efforts in the past year have focused on building on our results from this task during the previous funding year and thus do not fall under any previous objectives. However, these achievements led to a manuscript that was recently accepted for publication in *PNAS*. During the previous funding year, we identified that loss of *SETD2* in multiple cell lines resulted in the formation of acentric, dicentric and isochromosomes, a highly surprising and unusual result. We prepared a manuscript and initially submitted that in November 2022. The reviewers were overall positive but had several concerns, which required us to develop new models and tools to address these questions.

One question posed by the reviewers was whether the catalytic activity of SETD2 was required to prevent the formation of these chromosomal abnormalities. To address this, we purchased a small molecule inhibitor (EPZ-719) of SETD2 methyltransferase activity. Treatment with this inhibitor (SETD2i) led to a loss of H3K36me3, indicating that catalytic activity is impaired (Fig. 1). Metaphase spreads were isolated from control, *Setd2*-knockout or SETD2i treated cells and scored for formation of dicentric chromosomes. Loss of SETD2 or SETD2i increased the formation of dicentric chromosomes, demonstrating that the catalytic activity of SETD2 is required to prevent the formation of these chromosomal aberrations. To confirm this, we were asked to rescue *SETD2*-knockout cells with a catalytically inactive SETD2 with a R1625C mutation. We expressed SETD2-rescue constructs in *SETD2*-knockout HeLa TetOn-Cas9 cells then scored for the formation of acentric or dicentric chromosomes (Fig. 2). These experiments confirm that catalytic activity of SETD2 is required to prevent karyotypic alterations following SETD2 loss.

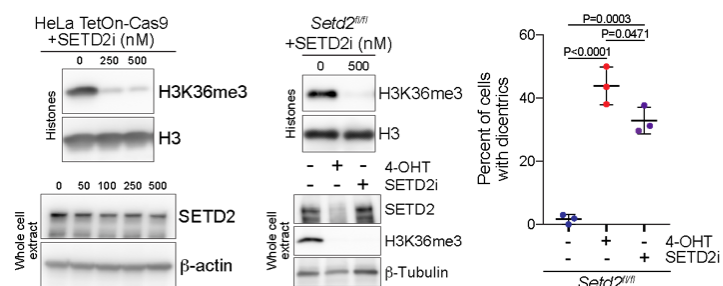
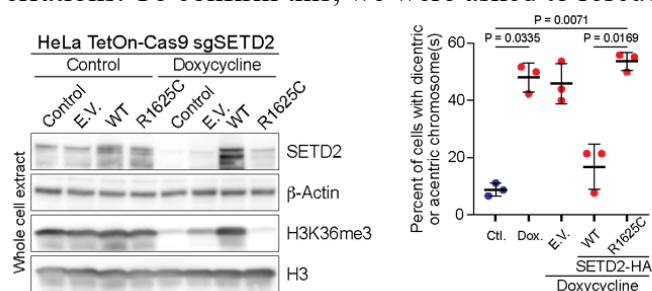


Fig. 1: SETD2i decreases H3K36me3 but does not affect SETD2 levels in HeLa and MEFs. At right, SETD2 loss or SETD2 leads to an increase in dicentric chromosomes.



The reviewers also asked whether these chromosomal aberrations arise during DNA replication or whether they were occurring due to mitotic errors. To address this, we generated a cell line with a degron-tagged SETD2 that would allow us to acutely deplete SETD2 during different phases of the cell cycle. We then synchronized cells at the G1/S transition, depleted SETD2 using the small molecule dTAG47 or inhibited its catalytic activity, released cells into S phase and harvested mitotic cells to analyze metaphases for chromosomal rearrangements, including dicentrics and acentrics (Fig. 3). Here, we found that depletion or catalytic inhibition of SETD2 lead to an increase in the percentage of cells with acentric or dicentric chromosomes. This demonstrates that DNA replication errors result in the formation of acentric, dicentric and isochromosomes following SETD2 loss.

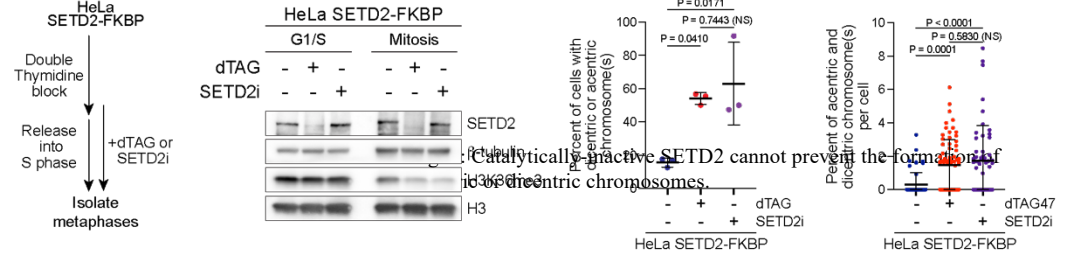
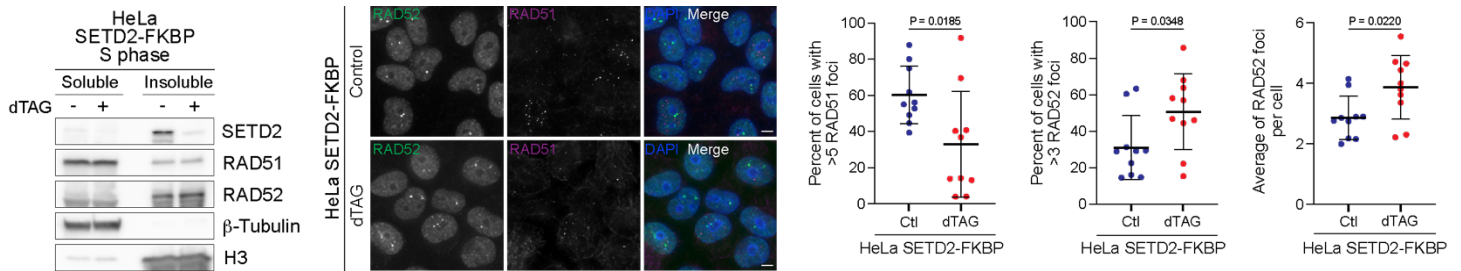


Fig. 3: Depletion or inhibition of SETD2 during DNA replication results in karyotypic alterations.

Catalytically inactive SETD2 cannot prevent the formation of acentric, dicentric and isochromosomes.

The reviewers for the paper required that we identify which pathways are altered during DNA replication to promote the formation of these isochromosomes. We hypothesized they are caused by altered homologous recombination (HR) activity due to loss of RAD51 function and that RAD52 activity is increased to compensate. Inherently, RAD52 is more error prone during HR and has been shown to promote isochromosome formation in yeast. To test whether *SETD2*-mutant cells shift from RAD51 to RAD52 utilization, we synchronized control or



SETD2-depleted cells as described above then released cells into S-phase, and either fractionated chromatin for western blots or immunofluorescence. We then probed for RAD51 and RAD52 accumulation (Fig. 4).

Fig. 4: Chromatin fraction shows that SETD2-depletion does not change loading of RAD51 or RAD52 onto chromatin. However, immunofluorescence indicates there are less RAD51 foci and more RAD52 foci forming, indicating a shift from RAD51 to RAD52 utilization.

To test whether RAD52 is directly required for isochromosome formation, we then knocked-down RAD52 using siRNA then depleted SETD2 and asked whether there was a decrease in the formation of gross chromosomal rearrangements, including acentric and dicentric chromosomes (Fig. 5). We observed a significant decrease in the percentage of acentric or dicentric chromosomes, as well as a decrease in the percentage of cells affected. This demonstrates that SETD2 prevents RAD52 activity during DNA replication to prevent the formation of chromosomal rearrangements.

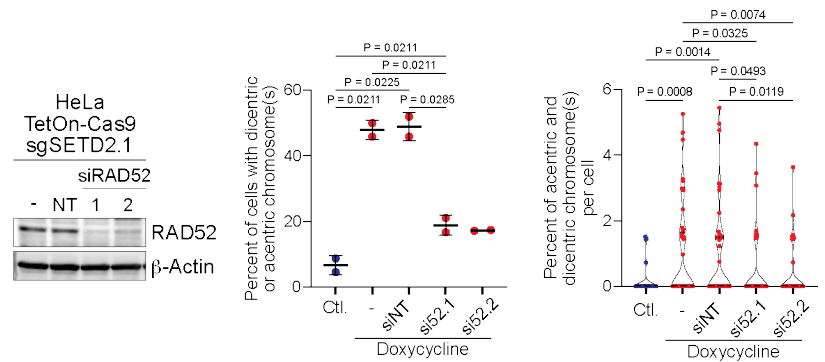


Fig. 5: RAD52 was knocked down using siRNA, and when SETD2 was knocked-out, there was a decrease in the formation of acentric and dicentric chromosomes.

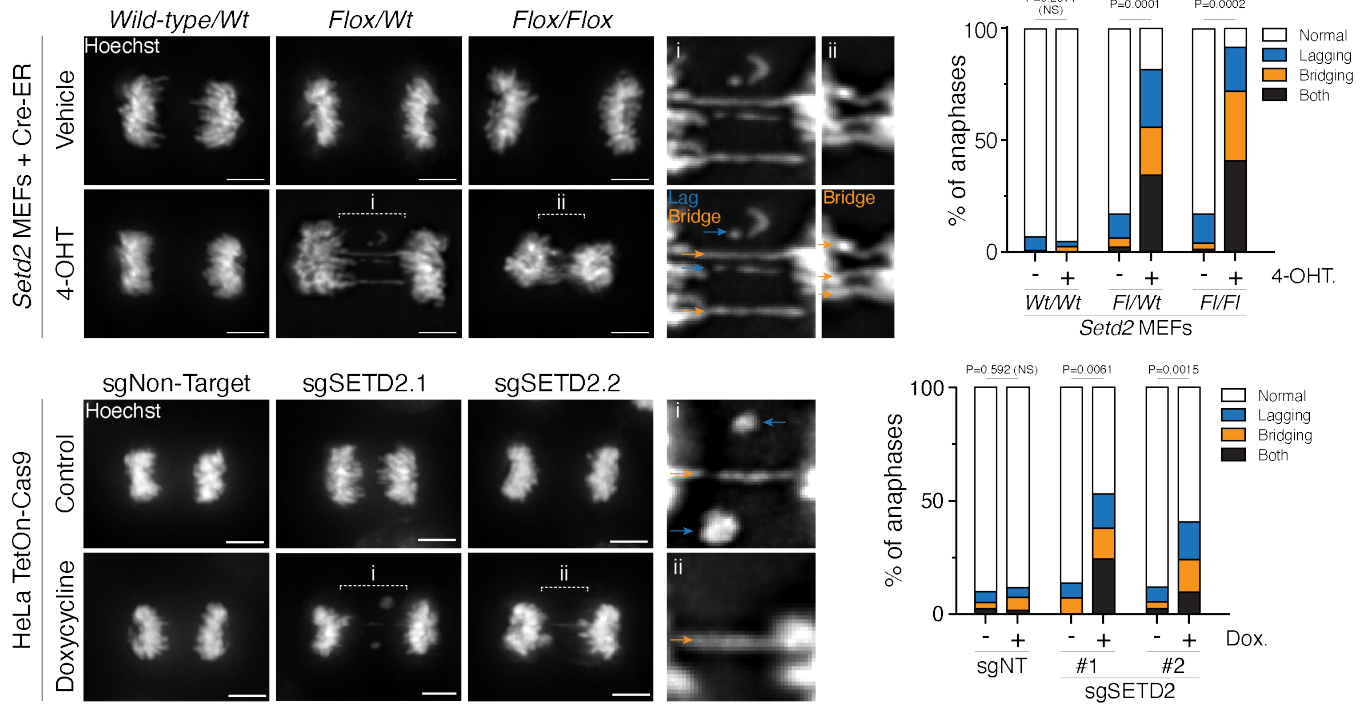


Fig. 6: Loss of *SETD2* leads to chromosome mis-segregation in mouse embryonic fibroblasts (MEFs, top) and HeLa (bottom).

To determine whether the formation of these chromosomal rearrangements leads to chromosome mis-segregation, we fixed and stained control or *SETD2*-knockout cells for DNA and microtubules to determine whether mitotic errors occur and whether there is evidence of chromosome mis-segregation (Fig. 6,7).

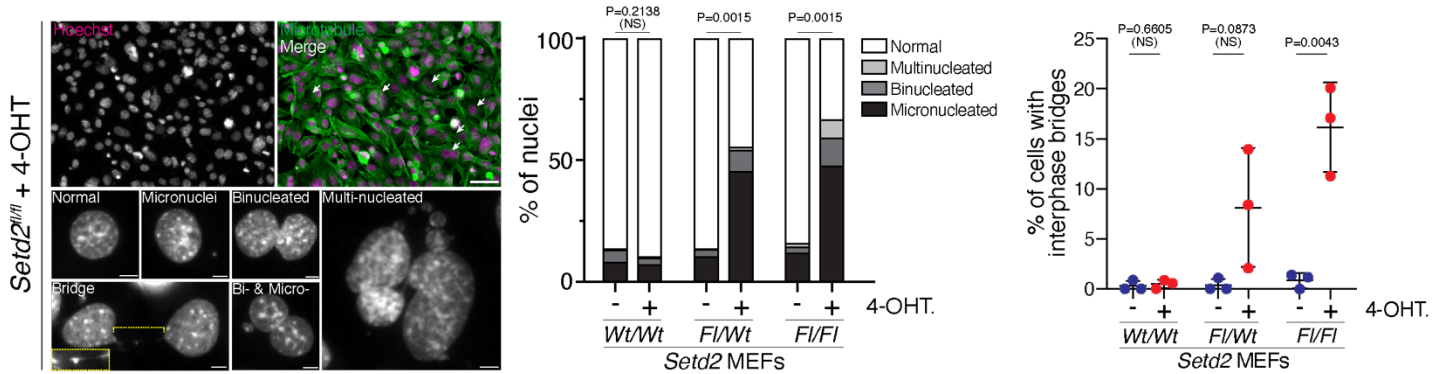


Fig. 7: *Setd2*-mutant cells have hallmarks of mitotic errors and DNA damage.

We observe that loss of *SETD2* and these chromosomal rearrangements do indeed promote chromosome mis-segregation, correlating with altered nuclear organization. The generation of micronuclei are indicative of mitotic mis-segregation. Bi-nucleated cells indicate cytokinesis failure. Chromatin bridges are caused by dicentric chromosomes, and multi-nucleated cells indicate mitotic failure.

Lastly, we were asked to determine whether humans with renal tumors observe hallmarks of these chromosomal rearrangements. We anticipate that if *SETD2*-mutant tumors do indeed form isochromosomes, we would anticipate that we could identify chromosome with asymmetry. Normally, each chromosome should have one p and q arm, however isochromosomes should have 2 p or 2 q arms (Fig. 8). To test this, we utilized the publicly available Pan-Cancer Atlas Whole Genome (PCAWG) dataset and asked whether copy-number variants

(CNVs) are changed among p and q arms in the Renal PCAWG data. Indeed, we do observe an increase in the variance of p and q arms, and we also observe an increase in the amount of total CNVs in renal tumors with *SETD2* mutations.

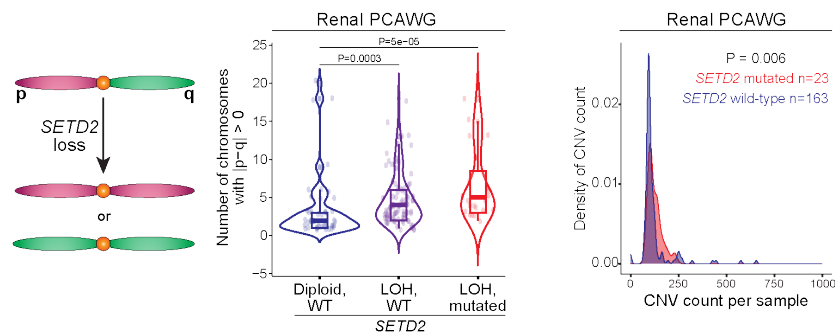


Fig. 8 Human renal tumors with *SETD2* mutations have more chromosomal asymmetry and CNVs.

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 was provided to Anteneh Tebeje (Research assistant) by working in the lab of Jeff Rathmell for supplemental training. Professional development was obtained by Frank Mason (PI) by presenting at two international meetings, the American Association for Cancer Research Special Conference in Cancer Research: Advances in Kidney Cancer Research on June 24-27, 20203 in Austin, Texas and the Mitotic Spindle: From living and synthetic systems to theory on April 16-19, 2023 in Dubrovnik, Croatia. Anteneh Tebeje presented at the Vanderbilt Ingram Cancer Center Annual Scientific Retreat on May 23, 2023 at Vanderbilt University Medical Center.

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.

I prepared a manuscript consisting of the data presented in this and last year’s report around the chromosomal aberrations in *SETD2*-mutated cells. This work was submitted and recently accepted in *PNAS*, and is currently in press. The work discussed in last year’s update was compiled into a manuscript, submitted to *Journal of Clinical Investigation* and is currently out for review.

If this is the final report, state “Nothing to Report.”

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

Within in this next year, we will work to address comments on the paper at JCI and our goal is to publish it. We will also continue to present at regional, national and international meetings. Also, we anticipate that the work we have planned for the upcoming year will result in another manuscript/publication.

- 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).

We have two major findings that should have a significant impact on our understanding of how cancer genomes evolve and why chemotherapeutics work well in preclinical models but fail in practice. First, we identified a previously unidentified mechanism by which renal cell carcinoma tumor cells alter their genomes to drive resistance. Secondly, we have identified that a physiological waste product that is present in humans but absent in cell culture and mouse models inhibits the efficacy of a potent anti-cancer therapy, explaining why this molecule has failed in clinical trials.

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.

What was the impact on technology transfer?

If there is nothing significant to report during this reporting period, state “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 PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official 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:*

There have been no significant changes in the project or its direction from the end of the last reporting period. However, as described in the previous annual report, our initial hypothesis that Rigosertib would be synthetically lethal with *SETD2*-mutated cells did not prove to be true. Additionally, we identified that Rigosertib’s efficacy is inhibited by the physiological waste product, uric acid, which is enriched in kidneys, suggesting that Rigosertib would not be a feasible drug to use in preclinical models that we proposed. Thus, we

decided to utilize a compound that is a degradation product of Rigosertib, called ON-1500, that is not affected by the presence of uric acid. During our previous reporting period, we showed that ON-1500 is a potent cytotoxic compound on a wide range of renal cell carcinoma cell lines, but it is not synthetically lethal with *SETD2*-mutated cells. While we have obtained a small amount of the small molecule for pilot studies from Onconova, the pharmaceutical company that owns the patent, we do not have enough to perform the high-throughput assays or mouse models described. As discussed below, we are currently working to obtain enough compound from outside sources to perform the assays proposed.

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.

There have been no significant changes in approach, but we have had to focus much of our effort over the last year to addressing reviewer comments for our manuscript, the results of which are described in “Accomplishments.” This was a significant amount of work generated during this time, so that has taken away some effort from other major tasks in order to publish the findings from Major Task 3 that led to the publication.

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.

Onconova is not interested in pursuing ON-1500 as a compound, so they will be unable to provide more compound for us to perform the studies we proposed. We have since talked with several companies about synthesizing the compound, who are working on feasibility studies and generating quotes for the production and isolation of ON-1500. This process has taken much longer than anticipated, but we are hopeful to have compound in the next few months.

Secondly, the research assistant working on this project left earlier than anticipated to go to medical school, which slowed progress. We have since hired a new research assistant who is working on the project, but this is his first lab experience. We anticipate he will have significant growth and progress over the next few weeks. The third problem was that our accrual of human patient samples (Major Task 8) has been significantly hampered due to unexpected consequences of COVID protocols (during 2022) and changes in the consenting process at VUMC. We are currently working with the tissue procurement group at VUMC to pinpoint the issues such that we can increase the volume of samples obtained. Additionally, when we have obtained primary human RCC samples, they are too small to perform the experiments proposed. We are currently working with Pathology at VUMC to determine whether the physical sample size can be increased. If not, we will need to adjust our experiments to fit the scope of the size and frequency of samples we obtain.

Changes that had a significant impact on expenditures

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.

We have yet to initiate mouse or work on primary human samples due to reasons described above, so that has decreased our expenditures to date. But we are hoping to start those projects in the upcoming year.

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

We have not had any changes in our use or care of vertebrate animals because we are still waiting to obtain the compound due to the issues described above.

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).*

Rawat V, DeLear P, Prashanth P, Ozgurses ME, **Tebeje A**, Burns PA, Conger KO, Solís C, Hasnain Y, Novikova A, Endress JE, González-Sánchez P, Dong W, Stephanopoulos G, DeNicola GM, Harris IS, Sept D, **Mason FM**, Coloff JL. Drug screening in human physiologic medium identifies uric acid as an inhibitor of rigosertib efficacy. *bioRxiv*. 2023 Jul 28;2023.07.26.550731. doi: 10.1101/2023.07.26.550731. Preprint. Under review at *Journal of Clinical Investigation*.

Mason FM, Kounlavong ES, Tebeje AT, Dahiya R, Guess T, Vlach L, Norris SR, Lovejoy CA, Dere R, Ohi R, Ly P, Walker CL, Rathmell WK. SETD2 safeguards the genome against isochromosome formation. *PNAS*. Accepted, awaiting publication.

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.*

*Mason FM, Kounlavong ES, Tebeje AT, Dahiya R, Guess T, Vlach L, Norris SR, Lovejoy CA, Dere R, Ohi R, Ly P, Walker CL, Rathmell WK. "SETD2 safeguards the genome against isochromosome formation." American Association for Cancer Research Special Conference in Cancer Research: Advances in Kidney Cancer Research on June 24-27, 20203 in Austin, Texas.

*Mason FM, Dahiya R, Guess T, Norris SR, Lovejoy CA, Ohi R, Ly P, Rathmell WK. "SETD2 safeguards the genome against isochromosome formation." Mitotic Spindle: From living and synthetic systems to theory on April 16-19, 2023 in Dubrovnik, Croatia.

- **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. Describe the technologies or techniques were 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. 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;*
- *physical 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.*

We have generated several new cell lines:

HeLa TetOn-Cas9 sgNonTargeting
HeLa TetOn-Cas9 sgSETD2.1 and sgSETD2.2
HeLa TetOn-Cas9 sgSETD2.1 + Halo
HeLa TetOn-Cas9 sgSETD2.1 + SETD2::Halo
HeLa TetOn-Cas9 sgSETD2.1 + SETD2 R1625C::Halo
HeLa SETD2::FKBP::HA

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”.

Name:	Frank Mason, PhD
Project Role:	PI
*Researcher Identifier (e.g. ORCID ID):	0000-0003-1338-494X

**Nearest person month worked:	3.96 month
Contribution to Project:	Oversaw all aspects of the project, performed, and analyzed experiments, compiled data, wrote and submitted manuscripts.
Funding Support:	CDMRP, NCI
Name:	<i>Anteneh T. Tebeje</i>
Project Role:	Research Assistant
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	8.4CM
Contribution to Project:	Performed and analyzed experiments.
Funding Support:	<i>CDMRP</i>
Name:	Luke Cline
Project Role:	Research Assistant
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	3.6CM
Contribution to Project:	Luke Cline joined the team in June 2023 and he is responsible for performing and analyzing experiments.
Funding Support:	CDMRP, NCI

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.

Yes.

Please see attached Other Support documents for:

Dr. Frank Mason, PhD (PI),

Dr. Jeffrey Rathmell, PhD (Key Personnel - Career Guide), and

Dr. Kathryn E. Beckermann, MD, PhD (Key personnel – Collaborator)

with annotated changes since the last time this information has been submitted.

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. No other organizations included.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (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://ebrap.org/eBRAP/public/index.htm> for each unique award.*

QUAD CHARTS: *If applicable, the Quad Chart (available on <https://www.usamraa.army.mil/Pages/Resources.aspx>) 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.*

- 1) Other Support for Frank Mason
- 2) Other Support for Jeffrey Rathmell, PhD
- 3) Other Support for Kathryn E. Beckermann, MD, PhD
- 4) Accepted manuscript for PNAS
- 5) Pre-print of manuscript currently under review at JCI.

CURRENT, PREVIOUS AND PENDING SUPPORT

FRANK MASON, PhD

Current Domestic and Foreign Positions and Scientific Appointments relevant to this grant:

2023-Present	Assistant Professor, Department of Medicine, Vanderbilt University Medical Center, Nashville, TN
2016-2023	Research Assistant Professor, Department of Medicine, Vanderbilt University Medical Center, Nashville, TN
2011-2016	Postdoctoral Associate, Department of Biology, Massachusetts Institute of Technology, Cambridge, MA

CURRENT SUPPORT

Crant ID	5R01CA275082-02 (Rathmell, W. K.)
Title:	<i>Convergent Drivers of Tumor Evolution at the Mitotic Spindle</i>
Effort:	2.40 calendar months
Principal Investigator:	W. K. Rathmell
Supporting Agency:	NIH/NCI
Grants Officer:	Ashley Utter Email: Ashey.utter@nih.gov
Performance Period:	07/01/2022- 06/30/2027
Current Funding Amount:	
Project Goals:	Our Overarching Hypothesis is that VHL and RCC-associated 3p and 5q chromatin remodelers coordinately regulate methylation of spindle microtubules to maintain genomic stability, which when defective, offers unique opportunities for therapeutic intervention.
Specific Aims:	<u><i>Aim 1.</i></u> Test the hypothesis crosstalk between 3p and 5q chromatin remodelers converges at the mitotic spindle. <u><i>Aim 2.</i></u> Test the hypothesis combinatorial defects in 5q chromatin remodelers that occur in ccRCC cause loss of mitotic integrity and genomic stability. <u><i>Aim 3.</i></u> Test the hypothesis that 3p tumor suppressors regulate control modules in mitotic integrity.
Overlap:	None

This RPPR

Crant ID	KC200259/W81XWH2110786
Title:	<i>Therapeutic Vulnerability of SETD2 mutant RCC</i>
Effort:	3.96 calendar months

Principal Investigator:	Frank Mason
Supporting Agency:	Congressionally Directed Medical Research Programs
Grants Officer:	Joshua McKean 820 CHANDLER STREET FORT DETRICK, MD 21702-5014
Performance Period:	08/15/2021- 08/14/2024
Current Funding Amount:	
Project Goals:	The goal of this proposal is to determine the mechanistic connection between SETD2-PLK1 synthetic lethality and, using ccRCC patient-derived organoids (PDOs) and mouse models, establish inhibition of PLK1 as a new therapeutic option for patients with ccRCC.
Specific Aims:	<u>Aim 1.</u> Identify mechanisms of PLKi-mediated cell death. <u>Aim 2.</u> Determine efficacy of PLKi on ccRCC/SETD2 cells using xenograft mouse models. <u>Aim 3.</u> Determine effects of PLKi treatment on ccRCC cell lines in 3D and on primary tumor cells in the context of an intact microenvironment.
Overlap:	None
 <u>PENDING SUPPORT</u>	None
<u>In-Kind</u>	None
<u>Foreign Collaboration</u>	None
 <u>PREVIOUS SUPPORT</u>	
Grant ID	R21-CA184387-05
Title:	<i>Exploiting Notch Inhibition as a Mechanism to Overcome Resistance in ccRCC</i>
Effort:	0.24 calendar months
Role:	Co-Investigator
Principal Investigator:	W. Kimryn Rathmell
Supporting Agency:	NIH/NCI
Grants Officer:	Suzanne Forry National Cancer Institute
	Email: forryscs@mail.nih.gov
Performance Period:	04/01/2015-03/31/2018
Funding Amount:	(NCE)
Project Goals:	Based on proposed activity of DLL4 inhibitor to increase VEGFR2 expression and promote sprouting, we will test the hypothesis that DLL4 inhibition might be most effective

Specific Aims:

following emergent resistance to treatment with a VEGFR2 tyrosine kinase inhibitor.

Aim 1. Determine the effect of VHL mutation on Notch pathway activation in endothelial cells and tumors.

Aim 2. Examine the effect of Notch pathway inhibition on xenograft tumor growth

Aim 3. Determine the efficacy of combination or sequential use of Notch inhibitors with traditional VEGFR targeted therapy.

Overlap:

None

Grant ID:

5R01CA217987-03

Title:

Metabolic Barriers to T Cell Activation in Clear Cell Renal Cell Carcinoma

Effort:

6.00 calendar months/**Effort ended 3.01.2022**

Role:

Investigator

Principal Investigator:

Jeffrey Rathmell

Supporting Agency:

National Institute of Health/NCI

Grants Officer:

Martinson Owusu

Performance Period:

E-mail: owusumo@mail.nih.gov

04/01/2018-03/31/2023

Current Funding Amount:**Project Goals:**

The goal is to examine the hypothesis that metabolic adaptation of CD&T cells to the ccRCC microenvironment impairs glycolysis and leads to accumulation of dysfunctional mitochondria and that improvement of TIL metabolism will enhance the efficacy of T cell immunotherapy.

Specific Aims:

Aim 1. Determine how mitochondrial dynamics are dysregulated to increase ROS generation and impair activation of ccRCC TIL.

Aim 2. Establish how the ccRCC nutrient microenvironment alters TIL metabolic pathways and functions.

Aim 3. Test how PD-1 blockade therapy impacts T cell metabolism and functional populations in ccRCC.

Overlap:

None

Grant ID:

N/A (Mason)

Title:

Therapeutic vulnerability of SETD2 mutant Renal Cell Carcinoma

Effort:

0.12 calendar months

Principal Investigator:

Frank Mason

Supporting Agency:

Kidney Cancer Association

Grants Officer:

Stephanie Shirley
9450 SW Gemini Drive #38269,

Beaverton, Oregon 97008-7105

Phone: (847) 332-1051

Performance Period: 10/01/2020-09/30/2021

Current Funding Amount:

Project Goals:

The goal of this proposal is to determine the mechanistic connection between SETD2-PLK1 synthetic lethality and establish the effect of inhibition of PLK1 in situ as a new therapeutic option for ccRCC-SETD2 using patient-derived organoids (PDOs).

Specific Aims:

Aim 1. Identifying mechanisms of PLK1-inhibitor mediated cell death.

Aim 2. Determining effects of PLKi treatment on ccRCC cell lines in 3D and primary tumor cells in the context of an intact microenvironment.

Overlap:

None

Current Domestic and Foreign Positions and Scientific Appointments relevant to this grant:

- 2018- Professor of Molecular Physiology and Biophysics, Vanderbilt University
- 2017- Co-Director of Graduate Studies, Molecular Pathology and Immunology PhD Program
- 2017- Associate Director, Vanderbilt Institute of Infection, Immunology, and Inflammation (VI4). Position is closely aligned with Directorship of the Vanderbilt Center for Immunobiology (VCI)
- 2015- Cornelius Vanderbilt Professor of Immunobiology, Department of Pathology, Microbiology, and Immunology, Vanderbilt University Medical Center, Nashville, TN
- 2015- Co-Leader, Host-Tumor Interactions Program of the Vanderbilt Ingram Cancer Center
- 2015- Director, Vanderbilt Center for Immunobiology (VCI), Vanderbilt University, Nashville, TN

In-kind/Foreign:

None.

Current

Title: “Exploiting metabolic vulnerabilities of CD4 T cell subsets to control inflammatory disease”
2R01 DK105550-11 (Rathmell, PI)

Time commitments: Principal Investigator (12%, 1.44 calendar months)

Supporting agency: NIH / NIDDK

Procuring Contracting/Grants Officer: Sunshine Wilson, wilsonsa2@mail.nih.gov

Performance period: 03/01/23-01/31/27

Level of funding: Total Costs

Goals: This study will explore metabolic vulnerabilities of inflammatory and regulatory T cells in methionine and nucleotide synthesis pathways that may be exploited to modulate T cell subsets in inflammatory bowel disease and other inflammatory disorders.

Specific Aims:

- 1) Test mechanisms of MTHFD2 as a limiting enzyme in methionine and nucleotide synthesis essential for mTORC1 signaling and effector T cells in IBD.
- 2) Determine how nutrient and microenvironmental factors influence effector and regulatory T cell metabolism and inflammation.

Title: “Metabolic barriers to T cell activation in clear cell renal cell carcinoma” 2R01 CA217987-06
(Rathmell, PI)

Time commitments: Principal Investigator (12%, 1.44 calendar months)

Supporting agency: NIH/NCI

Procuring Contracting/Grants Officer: Mike Steenstra, michael.steenstra@nih.gov

Performance period: 04/01/23-03/31/28

Level of funding: Total Costs

Goals: This study will examine mechanisms and potential approaches to overcome these metabolic barriers to anti-tumor immunity to improve immunotherapy.

Specific Aims:

- 1) Test how nutrients in the ccRCC TME and tumor genetics influence TIL function and metabolism.
- 2) Determine how glucose and glutamine metabolism in the TME promote or suppress anti-tumor immunity.

Title: “Metabolic Networks and Unbiased Genetic Screens to Identify Targets for SLE” 647053 (Rathmell, PI)

Time commitments: Principal Investigator (5%, 0.6 calendar months)

Supporting agency: Lupus Research Alliance

Procuring Contracting/Grants Officer: Diomaris Gonzalez, dgonzalez@lupusresearch.org

Performance period: 03/01/20-02/29/24

Level of funding: Total Costs

Goals: We will test the hypothesis that inflammatory T cell subsets have specific metabolic dependencies including GLS and MTHFD2 that can be inhibited to protect against SLE and that *in vivo* genetic screens will identify additional metabolic targets to selectively modulate immunity and provide potential new SLE therapies.

Specific Aims:

- 1) Test GLS and MTHFD2 as targets to suppress SLE inflammation and autoantibody production.
- 2) Use pooled CRISPR gene targeting in primary cells to identify new metabolic targets in T cell mediated inflammation and SLE.

Title: “Targeting Tfh Cell Metabolic Regulation in SLE and SLE-Associated Atherosclerosis” 5R01 AI153167-04 (Rathmell/Major, Multi PI)

Time commitments: Principal Investigator (8%, 0.96 calendar months)

Supporting agency: NIH

Procuring Contracting/Grants Officer: David Johnson, drjohnson@niaid.nih.gov

Performance period: 05/20/20-04/30/25

Level of funding: Total Costs

Goals: This proposal seeks to determine whether defects in T cell metabolism underpin mechanisms for SLE autoimmunity and resulting cardiovascular disease and test two potential targets to address this unmet medical need.

Specific Aims:

- 1) Establish the role of GLS and ACLY in gene expression, metabolism, and differentiation of Tfh cells.
- 2) Test the effects of GLS or ACLY inhibition on murine SLE and circulating Tfh from SLE patients.
- 3) Determine whether GLS or ACLY inhibition can modulate SLE-accelerated atherosclerosis.

Title: “Cancer Center Support Grant” 5P30 CA068485-27 (Park, PI)

Time commitments: Co-Lead, Host-Tumor Interactions (15%, 1.8 calendar months)

Supporting agency: NCI

Procuring Contracting/Grants Officer: Sonya Roberson, robersos@mail.nih.gov

Performance period: 09/01/20-08/31/25

Level of funding: Total Costs

Goals: Vanderbilt-Ingram Cancer Center (VICC) is a matrix center that integrates all of Vanderbilt’s cancer-related expertise and resources in order to deliver its mission of alleviating cancer death and suffering through pioneering research; innovative patient-centered care; and evidence-based prevention, education and community initiatives. This mission is accomplished through translation of exceptional cancer research into interventions for the prevention, diagnosis and treatment of cancer. The Cancer Center Support Grant provides infrastructure to facilitate multidisciplinary

basic, clinical and population-based research, to advance VICC discoveries to cancer patients and the community, and to educate and train the next generation of cancer investigators and workforce.

Specific Aims:

- 1) To conduct, support and enhance state-of-the-art, multidisciplinary basic, clinical and population-based research.
- 2) To coordinate and integrate cancer-related research and activities across Vanderbilt and to collaborate with our local, regional, national and global partners on initiatives of the highest priority to the National Cancer Program.
- 3) To train and develop the next generation of cancer investigators, cancer leaders and the continuum of cancer care providers.
- 4) To assess and prioritize community needs and to leverage partnerships to address those needs through cancer research, care and control activities.

Title: “Targeting T cell glutamine metabolism in severe asthma” 2R01 HL136664-07 (Rathmell, PI)

Time commitments: (13.5%, 1.62 calendar months)

Supporting agency: NHLBI

Procuring Contracting/Grants Officer: Taylor L. Guardino, taylor.guardino@nih.gov

Performance period: 06/15/21-05/31/25

Level of funding: Total Costs

Goals: This study will test two candidate targets to suppress glutamine metabolism in animal models and patient samples and determine if this pathway may provide new therapeutic targets to overcome severe therapy resistant asthma.

Specific Aims:

- 1) Determine the role and mechanism of GLS and ASCT2-dependent glutamine metabolism to regulate the balance of Th2, Th17, and Treg cells in allergic airway inflammation.
- 2) Test if obesity-induced exacerbation of asthma severity in females is dependent on enhanced T cell glutamine metabolism.

Title: “Metabolic demands and determinants in the RCC tumor microenvironment” W81XWH-22-1-0419 (Rathmell, PI)

Time commitments: Partnering PI (4%, 0.48 calendar months)

Supporting agency: Congressionally Directed Medical Research Programs

Performance period: 09/01/22-08/31/25

Level of funding: Total Costs

Goals: We hypothesize that the distinct metabolic properties of different cell subsets in the ccRCC TME contribute to tumor metabolic heterogeneity (evidenced by mixed PET imaging signals) and that genetic alterations in ccRCC shape the TME and influence the metabolism and function of non-cancer cells present in the tumor space.

Specific Aims:

- 1) To define VHL-myeloid connection
- 2) Characterize metabolic demands of immune cells in human ccRCC
- 3) Single cell approaches to metabolic heterogeneity

Title: “Enhancing Treg Therapeutic Efficacy in GVHD” VUMC104074/R01 HL118979 (Blazar, PI)

Time commitments: Collaborator (4%, 0.48 calendar months)

Supporting agency: NHLBI

Performance period: 05/15/22-03/31/26

Level of funding: Total Costs

Goals: The goal of this proposal is to test the central hypotheses that dual hyperfunctioning Tregs require unique metabolic reprogramming and immunological synapse (IS) signal integration by CD2 to enhance suppression and supramolecular attack participle (SMAP) release.

Title: “Determining the contribution of microbial-derived metabolites to immunosurveillance in obesity-driven cancer risk” VUMC105433/1U01 CA272541-01 (Makowski-Hayes, Liza, PI)

Time commitments: Multi-PI/Subaward PI (10%, 1.2 calendar months)

Supporting agency: NIH/NCI/ University of Tennessee Health Sciences Center

Procuring Contracting/Grants Officer: unassigned

Performance period: 07/01/22-06/30/27

Level of funding: Total Costs

Goals: The overall goal of the project is to determine how obesity mediated-immune changes impact cancer etiology by testing underlying mechanisms in pre-clinical models of obesity and weight loss combined with samples from human subjects.

NEW

Title: “Autoimmune Drivers and Protectors Team Science (ADAPTS)” 1U01 AI0176244-01 (James, Judith; Rathmell, Jeffrey; Subaward PI)

Time commitments: PD/PI-Subaward PI (4%, 0.48 calendar months)

Supporting agency: Oklahoma Medical Research Foundation/NIAID

Performance period: 03/01/2023 - 02/29/2028

Level of funding: Total Costs

Goals: Our aims will assess potentially independent or complementary immune mechanisms of disease progression and restraint by testing the roles of (1) RNA-sensing pathways, (2) age-associated B cells and autoantibody characteristics, (3) T cell lymphopenia, (4) T cell immunometabolism, and (5) predictive models with innate and adaptive immune responses, along with select environmental (microbiome, virome) and genetic (HLA) interactions.

NEW

Title: “Targeting RNA Sensing to Enhance Immunotherapy Responses in Kidney Cancer” KC210092 (Wilson, John, PI)

Time commitments: Co-Investigator (6%, 0.72 calendar months)

Supporting agency: Subaward: DoD/Vanderbilt University

Procuring Contracting/Grants Officer: unassigned

Performance period: 09/01/22-08/31/25

Level of funding: Total Costs

Goals: The objective of the proposed research is to engineer an optimized LNP platform for 3pRNA delivery that maximizes RIG-I activation in the TME and to evaluate the efficacy and safety of this new class of immunotherapy for ccRCC.

Specific Aims:

- 1) Engineer LNP properties to maximize the therapeutic window of RIG-I agonists.
- 2) Investigate the effect of RIG-I signaling on the TME and antitumor immunity in RCC.
- 3) Establish combinatorial immunoregimens for RCC via synergy between RIG-I activation and ICIs.

Pending

Awarded, Contract Pending

Title: “Mechanisms and Therapy of Chronic Graft Vs Host Disease” P01 HL092521 (Blazar, PI)

Time commitments: Co-Investigator (2%, 0.24 calendar months)

Supporting agency: Subaward: NHLBI/University of Minnesota

Procuring Contracting/Grants Officer: unassigned

Performance period: 07/01/22-06/30/27

Level of funding: Total Costs

Goals: Our goal is to develop new therapies for treating chronic GVHD (cGVHD), a devastating, highly debilitating disease and difficult to treat disease, affecting >100 day survivors (adults (60-70%; children 20-50%). CGVHD is the leading cause of late morbidity, impaired quality of life and mortality after allogeneic hematopoietic cell transplantation.

Title: “Inflammatory Drivers of the Obesity-Cancer Connection” Unassigned (Rathmell, PI)

Time commitments: Principal Investigator (12%, 1.44 calendar months)

Supporting agency: The Mark Foundation for Cancer Research

Procuring Contracting/Grants Officer: unassigned

Performance period: 07/01/2023 - 06/30/2026

Level of funding: Total Costs

Goals: Our long-term goals are to establish mechanisms linking adipose and obesity to tumor risk, progression, and responses while promoting training in an open and diverse environment that will increase the reach of our research.

Title: “Vanderbilt Systemic Autoimmune Disease Research Center (V-STAR)” 1P30 AR082743-01 (Crofford, PI)

Time commitments: Co-Core Director (5%, 0.6 calendar months)

Supporting agency: NIAMS

Performance period: 07/01/2023 - 06/30/2028

Level of funding: Total Costs

Goals: The Vanderbilt Systemic Autoimmune Disease Research Center (V-STAR) has an overall goal of bringing together investigators from diverse disciplines who will apply cutting-edge science to rheumatic diseases that advances personalized patient care.

Specific Aims:

- 1) Foster a dynamic, inclusive, and collaborative community of diverse individuals with rheumatic disease-related scientific interests.
- 2) Facilitate novel research ideas and strategies through utilization of cutting-edge research platforms, expert interdisciplinary consultation, and skill-building to achieve highly innovative and impactful science.
- 3) Use a robust Pilot and Feasibility program to attract new ideas and investigators to rheumatic disease-related research across the institution and collaboratively throughout the region.
- 4) Create an enrichment program that promotes training and fosters career development within the research community.
- 5) Collaborate with the Vanderbilt Institute for Clinical and Translational Research (VICTR) and other established centers to support translational research, development of novel therapeutic approaches, and promote personalized medicine.
- 6) Communicate discoveries and activities to stakeholders at local, regional, and national levels.

Title: “The Vanderbilt O’Brien Kidney Center” 1U54 DK137327-01 (Harris, PI)

Time commitments: Co-Investigator (5%, 0.6 calendar months)

Supporting agency: NIDDK

Performance period: 07/01/2023 - 06/30/2028

Level of funding: Total Costs

Goals: The overall theme of The Vanderbilt O’Brien Kidney Center (VOKC) is the investigation of mechanisms underlying progression of chronic kidney injury, with the objective to develop and implement novel and effective modalities for diagnosis and therapy.

Specific Aims:

- 1) Attract new scientific expertise and develop and utilize new tools for the study of chronic kidney disease (CKD) in small animal models and ultimately in humans
- 2) Encourage multidisciplinary research in CKD
- 3) Explore new areas or research with translational potential in the kidney field

Title: “Center for Actionable Systems Biology of Small Cell Lung Cancer (CASB-SCLC)” VUMC U54 (Vito Quaranta, PI (Subaward PI: Rathmell, Jeffrey C))

Time commitments: Co-Investigator (10%, 1.2 calendar months)

Supporting agency: Vanderbilt University/NCI

Performance period: 07/01/2023 - 06/30/2028

Level of funding: Total Costs

Goals: The overarching philosophy of the CASB-SCLC will be that the aggressive properties of a tumor are best understood as an emergent property of cell-state dynamics driven by both cell states defined by gene regulatory networks, and by interactions of cancer cells with the microenvironment.

Title: “Understanding the glycolytic requirements of human T cell responses through the study of glucose-6-phosphatase component 3 (G6PC3) deficiency” 1R01 AI179965-01 (Martinez Barricarte, PI)

Time commitments: Co-Investigator (5%, 0.6 calendar months)

Supporting agency: NIAID

Performance period: 09/01/2023 - 08/31/2028

Level of funding: Total Costs

Goals: To understand the consequences of hexokinase inhibition caused by G6PC3 deficiency in T cell function.

Specific Aims:

- 1) Determine how hexokinase inhibition alters T cell metabolism.
- 2) Determine how hexokinase inhibition leads to impaired T cell function.

Title: “Impact of sex hormone signaling on CD4+ T cell metabolism and inflammation” Uassigned (Newcomb, PI)

Time commitments: Co-Investigator (5%, 0.6 calendar months)

Supporting agency: The Paul G. Allen Family Foundation

Performance period: 09/01/2023 - 08/31/2026

Level of funding: Total Costs

Goals: Specializes in discovering, defining, and delineating how sex hormones modify immune cell mechanisms that contribute to disease pathology and clinical outcomes.

Title: “Single Cell Methods for Bioeffector Discovery and Analysis” 1R01 CA226833-01A1
(Bachmann, PI, Rathmell, Subaward PI)

Time commitments: Co-Investigator (3%, 0.36 calendar months)

Supporting agency: Vanderbilt University/NCI

Performance period: 12/01/2023 - 11/30/2028

Level of funding: Total Costs

Goals: We propose a system to drive natural product discovery via correlation of injury response and regulated cell death pathways to hallmarks of antitumor immunity to discover the next generation of leads for therapeutics capitalizing on chemically induced anti-tumor immunity.

Title: “Integrated analysis of immune metabolic heterogeneity” Uassigned (Mogilenko, PI)

Time commitments: Co-Investigator (5%, 0.6 calendar months)

Supporting agency: T Chan Zuckerberg Initiative

Performance period: 12/01/2023 - 11/30/2025

Level of funding: Total Costs

Goals: This project will create an integrated toolset and multidimensional resource of transcriptional, protein and genetic modalities of CD4 T-cell immunometabolism in tissue inflammation that can be broadly used to reveal single cell metabolic heterogeneity in vivo.

Title: “Hyperglycemia and increased severity to Staphylococcus aureus infections.” 1R01 AI180777-01
(Serezani, PI)

Time commitments: Co-Investigator (5%, 0.6 calendar months)

Supporting agency: NIAID

Performance period: 12/01/2023 - 11/30/2028

Level of funding: Total Costs

Goals: This project will provide new fundamental knowledge regarding regulating inflammatory components, allowing us to take this work further in both basic and translational directions.

Specific Aims:

- 1) Test the hypothesis that hyperglycemia promotes HAT activity, and epigenetic-dependent sustained BLT1 expression during sepsis.
- 2) Test the hypothesis that aberrant BLT1 activation mediates sepsis-associated mortality in diabetic conditions.

Overlap

There is no scientific, budgetary or effort overlap.

Completed (Last 5 years)

Title: “SPORE in Breast Cancer” 5P50 CA098131-20 (Pietenpol/Mayer Multi PI)

Time commitments: Core Director (5%, 0.6 calendar months)

Supporting agency: NCI

Procuring Contracting/Grants Officer: Owusu, Martinson, owusumo@mail.nih.gov

Performance period: 08/07/19-07/31/24

Level of funding: Total Costs

Goals: We will transform how we diagnose and treat individuals with breast cancer through investigation of innovative immunotherapy and molecular targeted therapy approaches; and deepen our understanding of the pathobiology of mammary neoplasia through four translational projects, recruitment of new ideas and investigators, and the integrated collaborative support of four cores.

Specific Aims:

- 1) Mechanisms of resistance to endocrine therapy in ER+ breast cancer.
- 2) Strategies to improve outcomes for triple-negative breast cancer (TNBC) patients integrating subtype-specific genomic and immune-based discoveries.
- 3) Targeting the DNA damage response in breast cancer.

Title: “Phenotype Heterogeneity and Dynamics in SCLC- Rathmell Pilot-“Unbiased Screening of T Cell Immune Checkpoints to Explore Cell Population” VUMC66058 (U54) UCA217450 Vito Quaranta (Subaward PI: Shyr, Yu)

Time commitments: Co-Investigator/Subaward PI (6.5%, 0.72 calendar months)

Supporting agency: Vanderbilt University/NCI

Performance period: 04/01/18-03/31/23

Level of funding: VUMC Total Costs

Goals: This pilot study will perform screening of T Cell Immune Checkpoints to explore cell population dynamics in lung tumors.

Title: “Metabolic barriers to T cell immunity in renal cell carcinoma” No # (Jeffrey Rathmell and W. Kimryn Rathmell, Co-PIs)

Time commitments: Co-PI (3%, 0.36 calendar months)

Supporting agency: Vanderbilt-Incyte Alliance Joint Steering Committee

Procuring Contracting/Grants Officer: Scott Mitchell, glen.s.mitchell@vumc.org

Performance period: 06/01/16-12/31/22

Level of funding: Total Costs

Goals: This proposal addresses the focus area of tumor cell-intrinsic features that modify inflammatory signaling. By its genetic characteristics, ccRCC generates a metabolic microenvironment inhospitable for effector T cells. We aim to understand these metabolic challenges for T cell activation within the tumor, to support better patient selection strategies or develop approaches to overcome metabolic barriers to T cell activation and improve the efficacy and number of patients that respond to immunotherapy.

Specific Aims:

- 1) Test if defects in mitophagy and accumulation of fragmented mitochondria alter ccRCC TIL metabolism.
- 2) Determine if tumor IDO1 expression and activity impairs TIL metabolism.
- 3) Test if modulation of PD-1, Tim-3, GITR, and OX40 alters metabolism and function of ccRCC TIL

Title: “Enhancing Treg Therapeutic Efficacy in GVHD” VUMC65441 R01 HL118979 (Blazar/Hippen, Multi-PI)

Time commitments: Subaward PI (4%, 0.48 calendar months)

Supporting agency: University of Minnesota / NHLBI

Procuring Contracting/Grants Officer: Lori Wallin, l-wall@umn.edu

Performance period: 04/01/18-03/31/22

Level of funding: Total Costs

Goals: Dr. Rathmell will direct metabolomics studies of Treg populations treated with the various conditions throughout the grant using high-resolution non-targeted mass spectrometry and will assist in the analyses of these data.

Title: “Pre-clinical Proof of Concept Studies for the Development of a PET Tracer for Imaging Human CD8+ Cells in Tumors” VUMC73435 (Manning, PI)

Time commitments: Co-Investigator (1%, 0.12 calendar months)

Supporting agency: GE Healthcare

Procuring Contracting/Grants Officer: Tracy Cruze, Tracy.L.Cruse@vumc.org

Performance period: 07/01/19-06/30/21 -effort ended

Level of funding:

Goals: Our goal is to develop and translate a novel PET imaging tracer to quantify activated immune cells in tumors and other organs, before and immediately following therapy.

Title: “Targeting TH17 cell metabolism in steroid resistance,” 5R01 HL136664-04 (Rathmell, PI)

Time commitments: Principal Investigator (16%, 1.92 calendar months)

Supporting agency: NIH/NHLBI

Procuring Contracting/Grants Officer: Eshelman, Lindsey, lindsey.eshelman@nih.gov

Performance period: 04/06/17-03/31/21

Level of funding: Total Costs

Goals: This study will examine the affect of T cell metabolism on glucocorticoid-resistance and the pathogenesis of severe asthma to determine if metabolic regulatory pathways may provide new therapeutic targets to overcome steroid resistance.

Specific Aims:

- 1) Determine T cell metabolic programs and regulatory signals in airway inflammation.
- 2) Test the contribution of T cell metabolism to GC resistance of Th17.

Title: “Cancer Center Support Grant” 5P30 CA068485-24 (Pietenpol, PI)

Time commitments: Co-Investigator (10%, 1.2 calendar months)

Supporting agency: NIH/NCI

Procuring Contracting/Grants Officer: Owusu, Martinson, owusumo@mail.nih.gov

Performance period: 04/30/16-08/31/20

Level of funding: Total Costs

Goals: To identify metabolic vulnerabilities of leukemic T cells by comparing the metabolic phenotypes of proliferating normal T cells with acute leukemic T cells.

Specific Aims:

- 1) To conduct, support and enhance state-of-the-art, multidisciplinary basic, clinical and population-based research.
- 2) To coordinate and integrate cancer-related research and activities at Vanderbilt University and to collaborate with our local, regional, national and global partners on initiatives of the highest priority to the National Cancer Program.
- 3) To train the next generation of cancer scientists and to develop, manage and provide meaningful cancer education programs for the community.

Title: “Proposal to Establish the Role of PPARα Antagonism on T cell Subsets” VUMC65349 (Rathmell, PI)

Time commitments: Principal-Investigator (5%, 0.6 calendar months)

Supporting agency: Tempest Therapeutics, Inc.

Procuring Contracting/Grants Officer: Alicia Levey, alevey@Tempesttx.com

Performance period: 07/06/18-07/05/20

Level of funding: Total Costs

Goals: We propose to test PPAR α antagonists on T cell metabolism and subsets in vitro and in vivo under disease settings. These data will establish the effect of PPAR α antagonism on T cell metabolism and T cell function.

Title: “Immunosuppression in the RCC tumor microenvironment” 18-60-36-RATH (Rathmell, W, PI)

Time commitments: Co-Investigator (2%, 0.24 calendar months)

Supporting agency: American Association for Cancer Research

Procuring Contracting/Grants Officer: Michael K. Stewart, Chief Financial Officer, 615 Chestnut St.,
Philadelphia, PA 19106

Performance period: 07/01/18-06/30/20 *end date extended per COVID.*

Level of funding: Total Costs

Goals: We hypothesize that multiple factors in the tumor microenvironment contribute to the blunted responsiveness of tumor infiltrating immune cells, and that manipulations of immune cell metabolism can promote enhanced functionality in response to checkpoint inhibition.

Title: “Glut1 Inhibitor in T Cell Transfer IBD Model” VUMC63810 (Rathmell, PI)

Time commitments: Principal-Investigator (2%, 0.24 calendar months)

Supporting agency: Kadmon Corporation

Procuring Contracting/Grants Officer: Masha Poyurovsky, masha@kadmon.com

Performance period: 01/18/18-01/31/20

Level of funding: Total Costs

Goals: Glut1^{-/-} T cells fail to induce IBD and we will use KL-03352 to test if inhibition of Glut1 and Glut3 can protect against Inflammatory Bowel Disease in a prophylactic model.

Title: “New approaches to cardiothoracic tolerance induction,” P01 HL018646 (Madsen, PI)

Time commitments: Consortium PI (5%, 0.6 calendar months)

Supporting agency: Massachusetts General Hospital / NHLBI

Procuring Contracting/Grants Officer: Unknown

Performance period: 11/01/15-10/31/18

Level of funding:

Goal: The goal of this project is to establish the role of PTEN in control of regulatory T cell function and metabolism. The Rathmell lab will perform metabolic experiments using PTEN-deficient Treg as part of a subcontract to Project #3, Regulation of Treg Function and Stability via P13K (Project Leader: Dr. Laurence Turka)

PREVIOUS/CURRENT/PENDING SUPPORT

Kathryn Eby Beckermann, MD, PhD

Current Domestic and Foreign Positions and Scientific Appointments relevant to this grant:

2020-Present	Assistant Professor, Division of Hematology and Oncology, Department of Internal Medicine, Vanderbilt University Medical Center
2018-2020	Clinical Instructor, Division of Hematology and Oncology, Department of Internal Medicine, Vanderbilt University Medical Center
2016-2018	Chief Academic Fellow, Division of Hematology and Oncology, Department of Internal Medicine, Vanderbilt University Medical Center
2015-2018	Postdoctoral Fellow, Division of Hematology and Oncology, Department of Internal Medicine, Vanderbilt University, Nashville, TN
2014-2018	Clinical Fellow, Division of Hematology and Oncology, Department of Internal Medicine, Vanderbilt University, Nashville, TN
2012-2014	Resident, Department of Internal Medicine, Vanderbilt University, Nashville, TN

PREVIOUS

Grant ID: N/A

Title: *Metabolic Barriers to T cell Function and Immunotherapy in Renal Cell Carcinoma*

Time Commitments: 9.6 calendar months

Role: Principal Investigator

Supporting Agency: Merck-Cancer Research Institute

Grant Officer: Ryan Godfrey

Cancer Research Institute

29 Broadway, 4th Floor

New York, NY 10006-3111

Performance Period: 03/01/2016-07/01/2018

Level of Funding:

Project Goals: To evaluate how the metabolic driven nature of renal cell carcinoma may alter tumor infiltrating CD8 T cell function.

Specific aims:

Aim 1: Test if RCC TIL have impaired mitophagy which alter metabolic pathways including fatty acid oxidation and reactive oxygen species.

Aim 2: Determine if RCC IDO1 expression and activity impairs TIL metabolism and effector function.

Grant ID: K12CA090625-21

Title: *Vanderbilt Clinical Oncology Research Career Development Award Supplement Directed towards immuno-oncology research*

Time Commitments: 9.0 calendar months/effort ended June 2021

Role: Scholar

Principal Investigator: W. Kimryn Rathmell

Supporting Agency: National Cancer Institute

Grant Officer: Romy Reis

National Cancer Institute

9609 Medical Center Drive

Bethesda, MD 20892-9760

Performance Period: 07/01/2016-06/30/2022 (NCE)

Level of Funding:

Project Goals: To target T cell metabolic dysfunction found in tumor infiltrating lymphocytes of renal cell carcinoma.

Specific aims:

Aim 1: Test if rescuing lack of TIL glycolytic capacity improves effector function through enhanced mitochondrial structure and function.

Aim 2: Determine if targeting co-stimulatory and inhibitory cell surface receptors of RCC TIL can rescue effector function because of alterations in fatty acid oxidation and glycolysis.

Aim 3: Test metabolic effects of treatment with checkpoint inhibitor therapy from patient peripheral blood using single cell analysis.

Overlap: None

This proposal ended

Grant ID: VUMC87276 (W81XWH191082) Subaward Rutgers Cancer Institute

Title: *Endogenous retrovirus expression, chromatin abnormalities and response to immune checkpoint blockade in clear cell renal cell cancer*

Role: Co-Investigator

Principal Investigator: Shridar Ganesan

Time Commitments: .6 calendar months

Support Agency: CDMRP/ Rutgers Cancer Institute

Grant Officer: Richelle Dalere

Rutgers Cancer Institute of New Jersey
Division of Pre-Award Grant Support Services (DGSS)
120 Albany Street, Tower 1, 3rd Floor
New Brunswick, NJ 08901
E-mail: rpd77@cinj.rutgers.edu

Performance Period: 09/30/2019- 09/29/2022 (NCE)

Level of Funding:

Project Goal: This project seeks to investigate the role of endogenous retroviral virus mediated response to immunotherapy in patients with renal cell carcinoma. We will plan to collect patient samples, process, and perform biologic assays including RNA sequencing and DNA sequencing to further elucidate the mechanism behind this biology.

Specific aims:

Aim 1. Determine the relationship between ERV expression and perturbations in DNA methylation, histone modification and mutations in chromatin remodeling genes in ccRCC.

Aim 2. Determine relationships between ERV expression and the immune microenvironment in ccRCC.

Aim 3. Examine the relationship between expression of ERV3.2 and response to immune checkpoint blockade in ccRCC.

CURRENT/ACTIVE

Grant ID: LCFA-IASLC-BMS

Title: *Single cell analysis to understand the biology of response to immunotherapy in Lung Cancer*

Time Commitments: 0.48 calendar months

Role: Principal Investigator

Support Agency: Bristol-Myers Squibb, Lung Cancer Foundation of America, and International Association for the Study of Lung Cancer Young Investigator Immuno-Oncology Scholarship Fund

Grant Officer: Emily Petoskey

13100 E. Cotton Ave, Suite 10
Aurora, Colorado 80011

Performance Period: 04/01/2018-09/01/2023 (NCE)

Level of Funding:

Project Goals: To test if novel immunotherapy combination using PD-1 blockade and VEGF inhibition correlates with clinical response due to changes in the tumor microenvironment and immune milieu.

Specific aims:

Aim 1. To identify if changes in Ki67+ Tcells and MDSC measured by mass cytometry in the peripheral blood, correlates with response to combination therapy with vorolanib and nivolumab.

Aim 2. Using untargeted single cell genomic analysis, we will assess if systemic changes in immune cell populations corresponds with patient response to therapy.

Overlap: None

Grant ID: KC200245

Title: *Kidney Cancer Clinical Trials Consortium*

Role: Co-PI

Principal Investigator: Brian Rini

Time Commitments: 1.80 calendar months

Support Agency: Congressionally Directed Medical Research Programs

Grant Officer: Jennifer Shankle, E-mail: jennifer.e.shankle.civ@mail.mil, Tel: 301-619-2193.
820 CHANDLER STREET
FORT DETRICK, MD 21702-5014

Performance Period: 09/15/2021 - 09/14/2024 (NCE)

Level of Funding:

Project Goal: The goal of the Vanderbilt-Ingram Cancer Center's (VICC) participation in the Kidney Cancer Research Consortium (KCRC) is to meaningfully contribute to Phase I and II clinical research in the context of this multi-institutional collaboration. VICC will contribute intellectual input and translational research expertise to the KCRC as well as robust patient accrual. Participation in the Consortium will provide a critical platform for the co-development of Renal Cell Carcinoma (RCC) clinical trials enhanced by the pooled expertise of the Consortium members.

Specific Aims:

Aim 1. Design innovative, mechanism-based translational clinical trials for the KCRC.

Aim 2. Accrue at least 15 patients per year to KCRC trials.

Aim 3. Perform translational research on samples obtained from KCRC trials.

Grant ID: N/A

Title: Vanderbilt-Arsenal Bio Collaboration

Role: Principal Investigator

Principal Investigator: Kathryn Beckermann

Time Commitments: 0.24 calendar months

Support Agency: Arsenal Biosciences, Inc.

Two Tower Place, Suite 700,
South San Francisco, CA 94080

Grant Officer: Randy Reyes, E-mail: rreyes@arsenalbio.com

Performance Period: 06/01/2022 - 05/30/2024

Level of Funding:

Project Goal: Understand the antigenicity of primary kidney cancer cells.

Specific Aims:

Aim 1. Leverage complementary expertise at Vanderbilt U and ArsenalBio to assess functionality of engineered T cells in patient-derived tumor models of clear cell renal cell carcinoma (ccRCC).

Aim 2. Identify novel mechanisms of tumor-mediated T cell suppression in ccRCC and engineer resistant T cells for future therapeutic applications.

Overlap: None

Grant ID: N/A

Title: Vanderbilt-Aravive Research Collaboration

Role: Principal Investigator

Principal Investigator: Kathryn Beckermann

Time Commitments: 0.24 calendar months

Support Agency: Aravive Inc.

3730 Kirby Drive, Suite 1200

Houston, Texas 77098

Grant Officer: Amy Franke, E-mail: Contracts@aravive.com

Performance Period: 08/01/2022 - 07/31/2023

Level of Funding:

Project Goal: Understand if a soluble GAS6/AXL peripheral blood liquid biopsy correlates with response on cabozantinib.

Specific Aims:

Aim 1. Collect peripheral blood prior to treatment with cabozantinib in patients with metastatic RCC and measure soluble Gas6 and AXL.

Aim 2. Correlate levels of gas6 and axl with response to cabozantinib.

Overlap: None

New

Grant ID: W81XWH-21-KCRP-CA

Title: *Artificial intelligence analysis of histopathology slides to develop biomarkers of response to immunotherapy in kidney cancer*

Role: Co-I

Principal Investigator: Anupama Reddy

Time Commitments: 0.12 calendar months

Support Agency: Congressionally Directed Medical Research Programs

Grant Officer: Jennifer Shankle, E-mail: jennifer.e.shankle.civ@mail.mil

820 CHANDLER STREET

FORT DETRICK, MD 21702-5014

Performance Period: 09/30/2022 - 09/29/2023

Level of Funding:

Project Goal: The goal of this project is to develop biomarkers of immunotherapy response using AI models from H&E-stained histology images

Specific Aims:

Aim 1. AI-based modeling of immune cell types on histological images using mIF data as training labels.

Aim 2. Interpretation of latent features in the AI model.

Overlap: None

New

Grant ID: W81XWH-21-KCRP-CTA

Title: *OPTimal Treatment by Invoking biologic Clusters in Renal Cell Carcinoma (OPTIC RCC)*

Role: Co-I

Principal Investigator: Brian Rini

Time Commitments: 1.20 calendar months

Support Agency: Congressionally Directed Medical Research Programs

Grant Officer: Medha Darshan, E-mail: medha.s.darshan.civ@mail.mil,

Performance Period: 09/01/2022 - 08/31/2026

Level of Funding:

Project Goal: The purpose of this study is to test our hypothesis that a new tool that measures the genetic signature of kidney cancer tumors can precisely match a patient to a pure immunotherapy regimen or an immunotherapy/tumor blood vessel poison combination.

Specific Aims: The current proposal will test this hypothesis by prospectively evaluating gene expression in patient tumors, assigning patient tumors to IMmotion 151 clusters, and using this cluster assignment to choose an FDA-approved first line therapy for patients with metastatic clear cell RCC.

Overlap: None

Grant ID: W81XWH-21-KCRP-IDA/KC210158

Title: *RNAseq-Based Biomarkers Identify Targetable Biologic Drivers of Kidney Cancer*

Role: Co-I

Principal Investigator: Brian Rini

Time Commitments: 0.96 calendar months

Support Agency: Congressionally Directed Medical Research Programs

Grant Officer: Medha Darshan, E-mail: medha.s.darshan.civ@mail.mil, Phone: 301.619.7938

Address of Funding Agency: 820 Chandler St, Fort Detrick, MD 21702-5014

Performance Period: 09/30/2022 - 09/29/2025

Level of Funding:

Project Goal: The central hypothesis that RNAseq can be used to identify biologic drivers of ccRCC in individual patients and improve outcomes.

Specific Aims:

Aim 1: Develop methods to prospectively assign individual tumors to IMmotion 151-derived RNAseq clusters in real time.

Aim 2: Evaluate the RNAseq biomarker in a retrospective validation cohort.

Aim 3: Evaluate macrophage infiltration as a biologic driver of cluster 3/6 tumors.

Overlap: None

New

Grant ID: 2R01CA217987-06

Title: Metabolic barriers to T cell activation in clear cell renal cell carcinoma

Role: Co-I

Principal Investigator: Jeffrey Rathmell (Contact), W. Kimryn Rathmell

Time Commitments: 1.2 calendar months

Support Agency: NCI

Grant Officer: Ashley Utter, Phone: 240-276-5635, Email: Ashley.utter@mail.nih.gov

Performance Period: 04/01/2023 - 03/31/2028

Level of Funding:

Project Goal: These studies will establish mechanisms by which glutamine impairs T cell differentiation and test new potential targets to overcome metabolic immune suppression in the TME to improve anti-tumor immunity.

Specific Aims:

Aim 1: Test how nutrients in the ccRCC TME and tumor genetics influence TIL function and metabolism.

Aim 2: Determine how glucose and glutamine metabolism in the TME promote or suppress anti-tumor immunity.

Overlap: None

New - Awarded Effective Date 09.01.2023

Grant ID: KC220067/W81XWH-22-KCRP-IDA

Title: Peripheral Systemic Response Assessment in RCC

Role: PI

Principal Investigator: Kathryn E. Beckermann

Time Commitments: 3.0 calendar months

Support Agency: CDMRP

Grant Officer: Medha Darshan, E-mail: medha.s.darshan.civ@mail.mil, Phone: 301.619.7938

Performance Period: 09/01/2023 - 08/31/2026

Level of Funding: f

Project Goal: We hypothesize that the peripheral blood of patients with RCC have circulating T cells with high mitochondrial metabolism and exhaustion markers which reflect RCC TIL and expand during treatment with checkpoint inhibition.

Specific Aims:

Aim 1: To test if patients with RCC have circulating peripheral blood T cells with high mitochondrial metabolism that reflect RCC TIL metabolism and function.

Aim 2: To test the hypothesis that expansion of T cell subsets with high mitochondrial activity occurs in the peripheral blood of patients undergoing systemic treatment with checkpoint inhibition.

Overlap: None

PENDING

New – Awarded, Start date 10.01.2023

Grant ID: N/A

Title: Inflammatory Drivers of The Obesity-Cancer Connection

Role: Co-I

Principal Investigator: Jeffrey Rathmell

Time Commitments: 0.6 calendar months

Support Agency: The Mark Foundation for Cancer Research
1350 6th Ave, New York, NY 10019

Grant Officer: Ian Lesser, Phone: 646-866-5950, Email: info@themarkfoundation.org

Performance Period: 10/01/2023 – 9/30/2026

Level of Funding:

Project Goal: We propose a collaborative program of cancer, metabolism, and immunology researchers to test the hypothesis that obesity increases risk for multiple cancer types while potentially augmenting responses to checkpoint blockade therapies through indirect effects on inflammation and active immune communication between adipose and tumor tissues.

Specific Aims:

Aim 1: Develop TABS and IMA cores and the team-led CAIR initiative.

Aim 2: Define fundamental mechanisms that drive the obesity-cancer connection and immunemediated links between adipose and tumor tissues.

Overlap: None

IN-KIND

None

Foreign Collaboration:

None



SETD2 safeguards the genome against isochromosome formation

Q:1 Frank M. Mason^{a,1} , Emily S. Kounlavong^a, Anteneh T. Tebeje^a, Rashmi Dahiya^b, Tiffany Guess^c, Abid Khan^d, Logan Vlach^a, Stephen R. Norris^a , Courtney A. Lovejoy^e , Ruhee Dere^f, Brian D. Strahl^d , Ryoma Ohi^g , Peter Ly^b , Cheryl L. Walker^f, and W. Kimryn Rathmell^{a,1}

Edited by Lorraine Symington, Columbia University Irving Medical Center, New York, NY; received March 6, 2023; accepted August 11, 2023

Q:10 **Isochromosomes are mirror-imaged chromosomes with simultaneous duplication and deletion of genetic material which may contain two centromeres to create isodicentric chromosomes. Although isochromosomes commonly occur in cancer and developmental disorders and promote genome instability, mechanisms that prevent isochromosomes are not well understood. We show here that the tumor suppressor and methyltransferase SETD2 is essential to prevent these errors. Using cellular and cytogenetic approaches, we demonstrate that loss of SETD2 or its epigenetic mark, histone H3 lysine 36 trimethylation (H3K36me3), results in the formation of isochromosomes as well as isodicentric and acentric chromosomes. These defects arise during DNA replication and are likely due to faulty homologous recombination by RAD52. These data provide a mechanism for isochromosome generation and demonstrate that SETD2 and H3K36me3 are essential to prevent the formation of this common mutable chromatin structure known to initiate a cascade of genomic instability in cancer.**

chromosome | mitosis | isochromosome | intratumoral heterogeneity | epigenetics

Genome maintenance is one of the highest order functions of a cell. Numerous processes safeguard against errors in replication and segregation of chromosomes during mitosis. Genomic instability and aneuploidy are also hallmarks of cancer and can be initiated by chromosome instability (CIN), defined as a propensity to mis-segregate chromosomes during cell division (1). Mis-segregated chromosomes can become incorporated into micronuclei, the cytoplasmic DNA-containing structures that are subjected to double-stranded breaks (DSBs) and chromothripsis (2–5). Amplification or deletion of genomic material or altered chromosomal structures downstream of these events have potential to be deleterious and oncogenic.

One such structure is the class of abnormal chromosomes known as isochromosomes. These mirror-imaged chromosomes are commonly observed in hematopoietic and solid tumors and genetic disorders such as Turner syndrome (6, 7). Isochromosomes result in duplications of one chromosome arm contained in the isochromosome and deletion of the unincorporated arm. They may also contain two centromeres, generating a dicentric (8). Dicentric chromosomes form chromatin bridges during anaphase, as microtubules from opposite poles attach to each centromere. Following anaphase, dicentric chromosomes may break causing kataegis and chromothripsis, ultimately promoting subclonal heterogeneity within cell populations and tumors (9–11). Yet, while the high prevalence and importance of dicentric and isochromosomes are appreciated, mechanisms accounting for their origin in human disease are poorly understood.

SETD2 is a methyltransferase responsible for the trimethylation of lysine 36 on histone H3 (H3K36me3). SETD2 and this epigenetic mark regulate transcriptional fidelity, RNA splicing, and the DNA damage response (12). *SETD2* is also widely recognized as a tumor suppressor that is frequently mutated or deleted in many tumor types, including clear cell renal cell carcinoma (ccRCC), pediatric acute lymphoblastic leukemia, lung, bladder, and uterine cancer (13). Intriguingly, recent work in ccRCC demonstrated that tumors with *SETD2* mutations exhibit branched, clonal evolution and more intratumoral heterogeneity than tumors with other driver mutations (14). Consistent with a role in genome maintenance, *SETD2* loss has been shown to disrupt mitotic spindle organization during mitosis to promote CIN, which may contribute to the tumor suppressive role for this enzyme (15, 16). However, a mechanism linking mitotic errors to the origin of branched evolution in cells or tumors with mutation or deletion of *SETD2* remains unknown. As chromosome bridging can drive subclonal heterogeneity in cultured cells, we investigated the prevalence of bridging in cells lacking *SETD2*.

Q:11 While investigating the origin of chromosome mis-segregation in cells lacking *SETD2*, we found that SETD2 and H3K36me3 prevent the generation of isochromosomes and isodicentric chromosomes, establishing a critical link between these epigenomic regulators and genomic instability.

Significance

Chromosomal aberrations, such as dicentric or isochromosomes, are prevalent in cancer and developmental syndromes, yet mechanisms promoting their origin remain elusive. Here, we demonstrate that a key epigenetic mark, H3K36me3, and its regulator, the tumor suppressor SETD2, preserve genomic integrity by preventing isochromosome formation. Tumors with SETD2 loss exhibit increased intratumoral heterogeneity and are more resistant to chemotherapy. Our data identify that SETD2 loss causes the formation of isochromosomes, isodicentric and dicentric chromosomes that may be the source of this heterogeneity contributing to therapeutic resistance.

Author contributions: F.M.M., R.D., C.A.L., R.O., P.L., and W.K.R. designed research; F.M.M., E.S.K., A.T.T., R.D., and L.V. performed research; F.M.M., E.S.K., A.T.T., A.K., L.V., S.R.N., R.D., B.D.S., and C.L.W. contributed new reagents/analytic tools; F.M.M., E.S.K., A.T.T., R.D., T.G., C.A.L., P.L., and W.K.R. analyzed data; and F.M.M. and W.K.R. wrote the paper.

The authors declare no competing interest.

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This article contains supporting information online at <https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2303752120/-/DCSupplemental>.

Published XXXX.

Results

Deletion of *SETD2* Leads to Bridging and Lagging Chromosomes in Mitosis. To quantify the frequency of chromosome mis-segregation following *Setd2* loss, mouse embryonic fibroblasts (MEFs) with wild-type (*Wt/Wt*), heterozygous (*Fl/Wt*), and homozygous (*Fl/Fl*) floxed alleles of *Setd2* were treated with vehicle or 4-OHT to activate Cre-ER and acutely delete *Setd2*. Three days after treatment of 4-OHT, monoallelic deletion of *Setd2* resulted in decreases in both *Setd2* and H3K36me3, whereas biallelic loss resulted in complete loss of *Setd2* and H3K36me3 (*SI Appendix, Fig. S1A*). H3K36me3 was also lost across chromosome arms and at subtelomeric and pericentric regions (*SI Appendix, Fig. S1B*). Mono- and biallelic loss of *Setd2* increased the prevalence of mitotic cells with lagging, bridging, or both types of mis-segregated chromosomes during the late anaphase and early telophase (Fig. 1 *A* and *B*). We have

previously posited that lagging chromosomes arise from alterations in chromosome-microtubule attachment, while bridges may be caused by dicentric chromosomes (*SI Appendix, Fig. S1C*) (1, 15, 16). To confirm whether acute deletion of *SETD2* causes both lagging and bridging chromosomes in human cells, HeLa cells expressing tetracycline-inducible Cas9 (HeLa TetOn-Cas9) (17), and either nontargeting (NT) or *SETD2*-specific gRNAs, were treated with doxycycline for 3 d, and chromosome mis-segregation was quantified. Doxycycline treatment caused loss of *SETD2* as well as H3K36me3 along chromosome arms and at subtelomeric and pericentric regions (*SI Appendix, Fig. S1 D and E*), promoting increases in both lagging and bridging chromosomes (Fig. 1 *C* and *D*).

Increased frequency of lagging and bridging chromosomes should lead to an increase in micronuclei and chromatin bridges in interphase (10, 11). Both *Setd2* heterozygous and homozygous-deleted

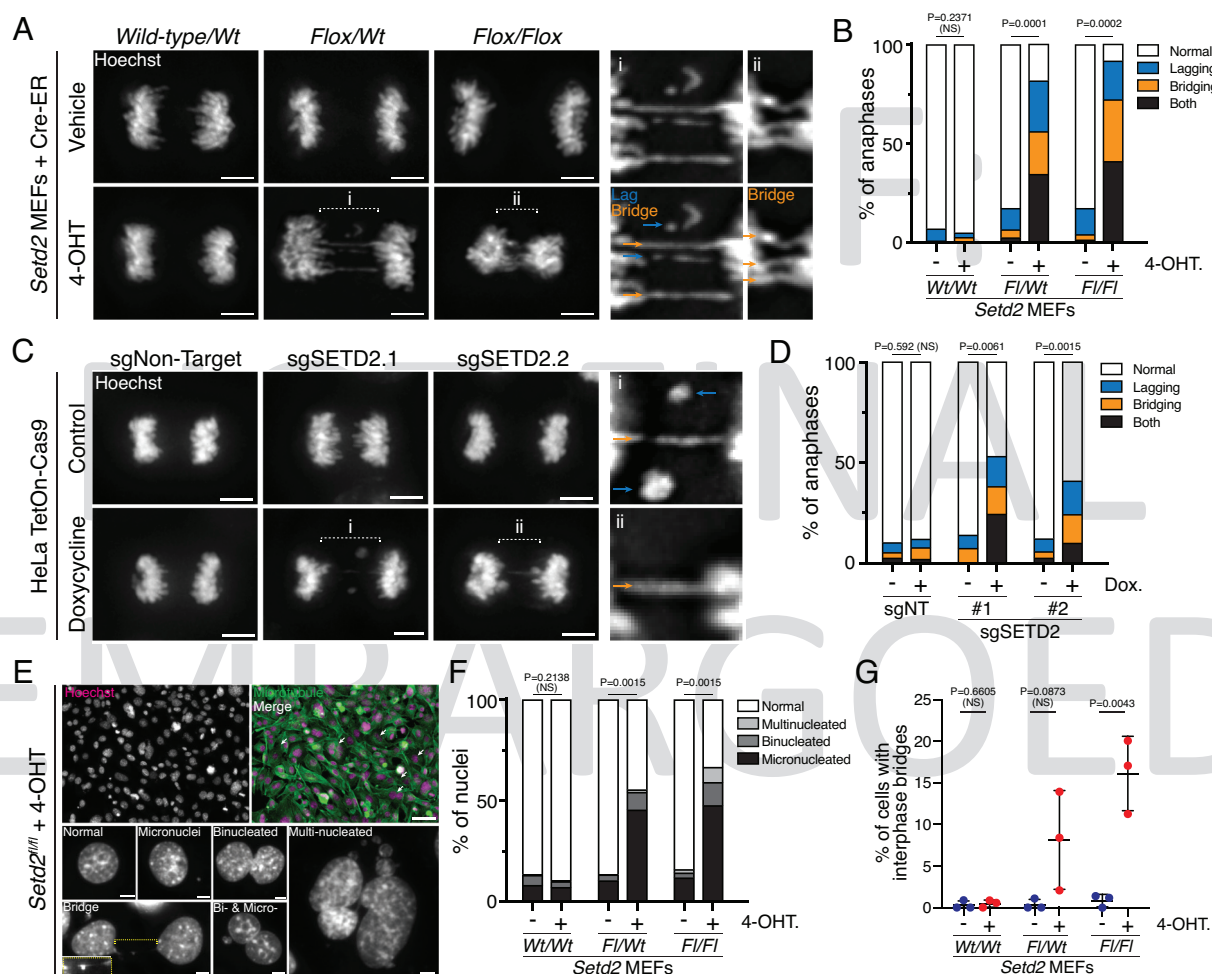


Fig. 1. *Setd2* loss causes chromatin mis-segregation during mitosis. (A) Images of wild-type, heterozygous or homozygous deletion of *Setd2* have lagging (blue arrows) and bridging (orange arrows) chromosomes during the anaphase. MEFs with no, one, or two floxed alleles of *Setd2* (Wild-type/*Wt/Wt*, *Flox/Wt*, and *Flox/Flox*, respectively) were either treated with vehicle (EtOH, control in top panels) or 4-OHT (excise floxed alleles of *Setd2*, bottom panels) for 3 d, fixed, and counterstained with Hoechst (gray). Scale bars are 5 μ m. (B) Quantification of chromosome segregation errors during the anaphase and early telophase in control (vehicle-treated) or 4-OHT-treated MEFs described in (A). $n = 198$ *wt/wt* vehicle, $n = 215$ *wt/wt* 4-OHT, $n = 298$ *fl/wt* vehicle, $n = 227$ *fl/wt* 4-OHT, $n = 258$ *fl/fl* vehicle, $n = 225$ *fl/fl* 4-OHT cells across 2 (*wt/wt*), 4 (*fl/wt*), and 3 (*fl/fl*) biological replicates. P values derived from an unpaired t test of normal cell values between treatment groups for each genotype. (C) Images of anaphases in control (untreated) or doxycycline-treated HeLa cells expressing tetracycline-inducible Cas9 (TetOn-Cas9) and single-guide RNA (sgRNA) that are nontargeting (NT) or specific for *SETD2*. Cells are counterstained with Hoechst (gray). Lagging (blue) and bridging (orange) chromosomes occur in cells lacking *SETD2*. Scale bars are 5 μ m. (D) Quantification of chromosome segregation errors during the anaphase and early telophase in cells described in (C). $n = 183$ sgNT control, $n = 190$ sgNT dox., $n = 198$ sgSETD2.1 control, $n = 240$ sgSETD2.1 dox., $n = 187$ sgSETD2.2 control, $n = 246$ sgSETD2.2 dox. cells across three biological replicates. P values derived from an unpaired t test of normal cell values between treatment groups for each sgRNA background. (E) Image of *Setd2*^{fl/fl} MEFs treated with 4-OHT (at left), showing nuclear defects that occur in interphase after 3 d treatment. Scale bars are 50 μ m (top images) and 5 μ m (grayscale images at the bottom). (F) Quantifications of nuclear phenotypes from images described in (E) for *Setd2* MEFs. $n = 450$ *wt/wt* vehicle, $n = 617$ *wt/wt* 4-OHT, $n = 650$ *fl/wt* vehicle, $n = 509$ *fl/wt* 4-OHT, $n = 736$ *fl/fl* vehicle, $n = 687$ *fl/fl* 4-OHT cells across three biological replicates. P values derived from an unpaired t test of normal cell values between treatment groups each genotype. (G) Quantification of interphase bridges observed in cell images described in (E). P values derived from an unpaired t test of normal cell values between treatment groups each genotype. Error bars are SD.

MEFs, as well as HeLa cells acutely deleted of *SETD2*, exhibited increases in binucleation, micronucleation, and multinucleation (Fig. 1 *E* and *F* and *SI Appendix*, Fig. S2 *A* and *B*). Interphase bridges were identified by accumulation of RPA and cGAS (Fig. 1 *G* and *SI Appendix*, Fig. S2 *C*), markers of single-stranded DNA and cytoplasmic DNA, respectively (10, 18). Thus, either mono- or biallelic loss of *Setd2* is sufficient to promote chromosome segregation defects, which can lead to micronucleation, binucleation, and interphase bridging.

Dicentric Chromosomes Are Prevalent in Cells Lacking *SETD2*. Although lagging chromosomes and defects in spindle morphology could be explained by loss of SETD2-dependent tubulin methylation, the defect(s) underlying anaphase bridges was not clear (1, 19). Chromatin bridges are caused by structural changes in chromosomes and arise from alterations in chromosome decatenation, cohesion, and/or generation of dicentric chromosomes (11). To determine whether loss of *SETD2* leads to aberrations in chromosomal structure, metaphase chromosomes from wild-type, mono- and biallelic *Setd2* floxed MEFs were isolated 3 d after 4-OHT treatment, and chromosomal abnormalities were quantified.

Heterozygous or homozygous loss of *Setd2* resulted in an increase in the percentage of cells that have dicentric chromosomes (Fig. 2 *A* and *B*), as observed by dense DAPI staining, which marks major satellite repeats at pericentric regions of murine chromosomes (20). To account for CIN in *Setd2* deleted cells (15), the number of dicentric chromosome(s) was normalized to chromosome number for each cell, which was confirmed to be increased in *Setd2*-deleted cells (Fig. 2 *C*). 1.1% and 2.0% of chromosomes were dicentric in *Setd2*^{fl/wt} and *Setd2*^{fl/fl} cells treated with 4-OHT or 0.43 and 0.81 chromosomes per diploid murine cell, respectively.

To confirm that dicentric chromosomes have two functional centromeres, metaphase spreads from control or *Setd2*-deleted MEFs were immunostained for CENP-A, the histone H3-variant that specifies centromere identity and activity (21). CENP-A staining verified that 54% of *Setd2*-deleted cells have dicentric chromosomes with CENP-A at two distinct constriction sites on the same chromosome, compared to 6% of controls (Fig. 2 *D* and *E*). The percentage of chromosomes that are dicentric increased from 0.2% in control samples to 2.8% in *Setd2*-knockout cells (Fig. 2 *F*, 0.08 to 1.12 chromosomes per cell). Additionally, the percentage of acentric chromosomes per metaphase was also increased in *Setd2*-deleted cells (Fig. 2 *G*, 0.2 to 3.9%). We also examined dicentric formation in HeLa TetOn-Cas9 cells with *SETD2* deletion, which also displayed an increase in the percentage of cells with dicentrics (1 to 25% for sgSETD2.1 and 3 to 27% for sgSETD2.2, Fig. 2 *H* and *I*). The percentage of dicentric chromosomes per spread, as determined by CENP-A staining, also increased in *SETD2*-knockout cells (0.02 to 0.65% for sgSETD2.1 and 0.06 to 0.72% for sgSETD2.2, Fig. 2 *J*).

For dicentric chromosomes to bridge during mitosis, both centromeres must be active to assemble kinetochores. However, inactivation of either centromere decreases kinetochore assembly, causing the dicentric to segregate like a monocentric chromosome (8). To determine whether dicentric chromosomes assembled kinetochores at both centromeric locations, metaphase spreads from control or *SETD2*-deleted MEFs and HeLa cells were costained for CENP-A and HEC1, the outermost component of the kinetochore that binds microtubules. Additionally, metaphase chromosomes were stained for KNL1 or CENP-T, components of the outer kinetochore and constitutive centromere-associated network, respectively. Both centromeres of dicentric chromosomes contained HEC1, KNL1, and CENP-T and are likely active

(Fig. 2 *K* and *L* and *SI Appendix*, Fig. S3 *A*), indicating that these dicentrics contribute to the elevated frequency of anaphase bridges induced by SETD2 loss.

To determine how rapidly dicentric chromosomes arise following *Setd2* deletion, metaphases were isolated from *Setd2*^{fl/fl} cells, vehicle or 4-OHT treated, starting 1 d after initial treatment. Q:1806
Dicentric chromosome frequency increased up to 3 d after treatment and then waned by 14 d but was still increased relative to controls (*SI Appendix*, Fig. S3 *B* and *C*). This result demonstrates that loss of *Setd2* results in a robust generation of dicentric chromosomes during a discrete time window shortly following deletion. Long-term *Setd2* deletion also resulted in a lower but still significant prevalence of dicentric chromosomes.

Interchromosomal Rearrangements, Telomere Fusion, and Neocentromere Formation Are Not Primary Drivers of Dicentric Chromosomes Following *Setd2* Loss. Dicentric chromosomes are a product of chromosome fusion or neocentromere formation and are known to occur in human diseases and promote genomic instability and tumor evolution (9, 11, 22, 23). A common driver of chromosome fusion is telomere dysfunction, and H3K36me3 is present near telomeric or subtelomeric regions (*SI Appendix*, Fig. S1 *B* and *E*) (10). To test whether telomere attrition occurs following acute *Setd2* loss, quantitative fluorescence in situ hybridization (Q-FISH) was performed to determine telomere length in vehicle- and 4-OHT-treated *Setd2*^{fl/fl} MEFs. There were no changes in telomere length associated with loss of *Setd2* (*SI Appendix*, Fig. S3 *D–F*). Additionally, no telomeric FISH signal was observed between the two centromeres, indicating that telomere fusion was not occurring (*SI Appendix*, Fig. S3 *G* and *H*). Together, these results indicate that telomere dysfunction is not the primary driver of dicentric chromosome formation following acute loss of *Setd2*.

We next examined whether dicentric chromosomes arise due to neocentromere formation or telomere-independent chromosome fusion, which may be downstream of DSBs. If chromosome fusion drives dicentric formation, those chromosomes should harbor an interchromosomal rearrangement between two non-homologous chromosomes. However, if dicentrics lack interchromosomal rearrangements, this may suggest neocentromere formation. To differentiate between these possibilities, multicolor FISH (m-FISH) was performed on metaphase spreads from vehicle- or 4-OHT-treated *Setd2*^{fl/fl} MEFs to determine the genetic composition of the dicentric chromosomes (Fig. 3 *A* and *B*). m-FISH identified that 15.3% of dicentrics have interchromosomal rearrangements, generated from the fusion of either two or more different chromosomes. However, the majority (84.7%) of dicentric chromosomes do not have interchromosomal rearrangements and are composed of the same chromosome (Fig. 3 *C*). Moreover, there is no preference for dicentric formation on specific chromosomes.

Dicentrics may occur by establishing neocentromeres at an ectopic, noncentromeric site (24). To test whether neocentromeres form in *Setd2*-knockout cells, metaphase spreads from vehicle- and 4-OHT-treated *Setd2*^{fl/fl} MEFs were immunostained for CENP-A and CENP-B (Fig. 3 *D* and *E*), the latter of which binds to CENP-B boxes, a 17-base-pair motif sequence located within native α -satellite-containing centromeres for all chromosomes (except the Y-chromosome) (25). Using a complementary approach, spreads were immunostained for CENP-A, followed by FISH using a CENP-B box probe (Fig. 3 *F*). If centromeres form at the correct location, CENP-A should colocalize with CENP-B. If neocentromeres form at ectopic sites, CENP-A would be present at sites lacking CENP-B (Fig. 3 *D*). The percentage of centromeres localizing

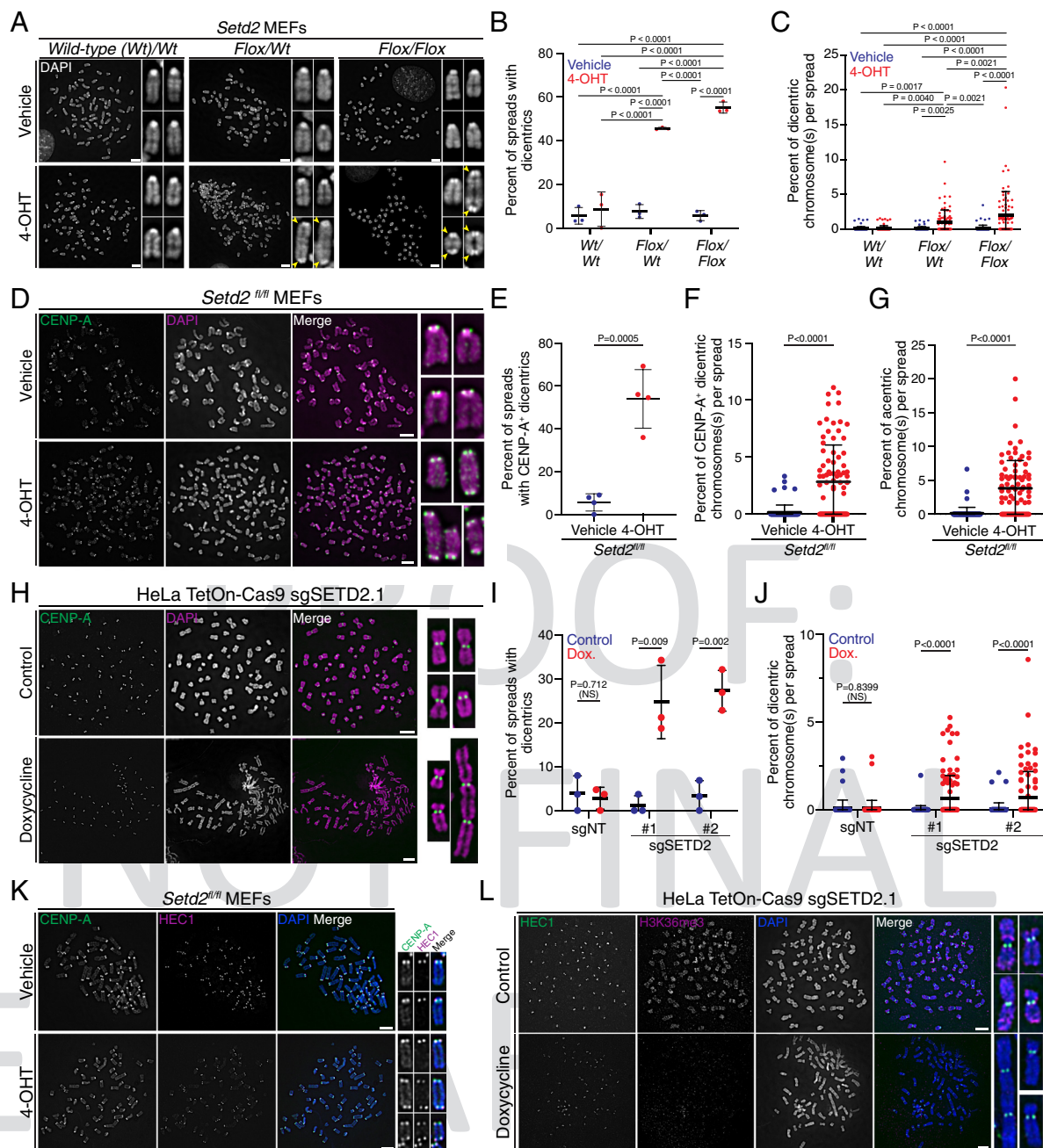


Fig. 2. Dicentric chromosomes arise in cells lacking *Setd2*. (A) Metaphase spread from MEFs with no, one, or two floxed alleles of *Setd2* (*wt/wt*/*wt*, and *fl/fl*, respectively) and treated with vehicle (control, in top panels) or 4-OHT (Bottom) for 3 d. Spreads are counterstained with DAPI (gray). Dicentric chromosomes (yellow arrowheads) are present in cells with homozygous and heterozygous *Setd2* deletion. (B) Quantification of the percentage of metaphase spreads from MEFs described in (A) that have at least one dicentric chromosome from $n = 3$ biological replicates. P values derived from two-way ANOVA with Tukey's multiple comparisons tests. (C) Quantification of the percentage of dicentric chromosome(s) per metaphase spread image from (B). Each point represents an individual image. $n = 88$ *wt/wt* vehicle, $n = 81$ *wt/wt* 4-OHT, $n = 76$ *fl/wt* vehicle, $n = 79$ *fl/wt* 4-OHT, $n = 89$ *fl/fl* vehicle, $n = 85$ *fl/fl* 4-OHT cells. P values derived from two-way ANOVA with Tukey's multiple comparisons tests. (D) Metaphase spread from *Setd2* ^{fl/fl} MEFs treated with vehicle (control, top panels) or 4-OHT (*Setd2* knockout, bottom panels). Spreads were immunostained for CENP-A (green) and counterstained for DAPI (magenta). Dicentric chromosomes in *Setd2*-knockout cells have two CENP-A foci. (E) Quantification of the percentage of metaphase spreads from *Setd2* ^{fl/fl} MEFs treated with vehicle (control) or 4-OHT (*Setd2* knockout) that have dicentric chromosomes containing CENP-A from $n = 4$ biological replicates. P values from an unpaired *t* test between each condition. (F) Quantification of the percentage of chromosomes that are dicentric and contain two CENP-A foci on metaphase spreads from *Setd2* ^{fl/fl} MEFs from (E). $n = 79$ *fl/fl* vehicle, $n = 85$ *fl/fl* 4-OHT cells. P values from an unpaired *t* test between each condition. (G) Quantification of the percentage of chromosomes that are acentric from *Setd2* ^{fl/fl} MEFs treated with vehicle ($n = 79$ cells) or 4-OHT ($n = 85$ cells). P values from an unpaired *t* test between each condition. (H) Metaphase spread isolated from control (untreated) or doxycycline-treated HeLa TetOn-Cas9 cells expressing sgSETD2.1, immunostained for CENP-A (green) and counterstained for DAPI (magenta). Control cells (Top) have monocentric chromosomes, while *SETD2*-knockout cells (Bottom) have mono- and dicentric chromosomes. (I) Quantifications of the percentage of metaphase spreads that have at least one dicentric chromosome from HeLa TetOn-Cas9 cells expressing sgNT or sgSETD2.1 and were untreated (control) or treated with doxycycline and then stained for CENP-A and DAPI. *SETD2*-knockout caused increases in the frequency of cells that have dicentrics in $n = 3$ biological replicates. P values derived from an unpaired *t* test of normal cell values between treatment groups each genotype. (J) Quantifications of the percentage of dicentric chromosomes in each metaphase spread from cells described in (H). *SETD2*-knockout leads to an increase in the percentage of chromosomes that are dicentric in $n = 75$ sgNT control, $n = 75$ sgNT dox., $n = 86$ sgSETD2.1 control, $n = 100$ sgSETD2.1 dox., $n = 85$ sgSETD2.2 control, $n = 89$ sgSETD2.2 dox. cells. P values derived from an unpaired *t* test of normal cell values between treatment groups each genotype. (K) Metaphase spread from *Setd2* ^{fl/fl} MEFs treated with vehicle (control, top) or 4-OHT (*Setd2* knockout, bottom), demonstrating that dicentric chromosomes assemble kinetochores (HEC1, magenta) at both centromeres (CENP-A, green). (L) Metaphase spread from HeLa TetOn-Cas9 cells with sgSETD2, control (Top) or treated with doxycycline (*SETD2*-knockout), demonstrating that dicentric chromosomes assemble kinetochores (HEC1, green) at two locations. Scale bars are 5 μ m. For each plot, error bars are SD.

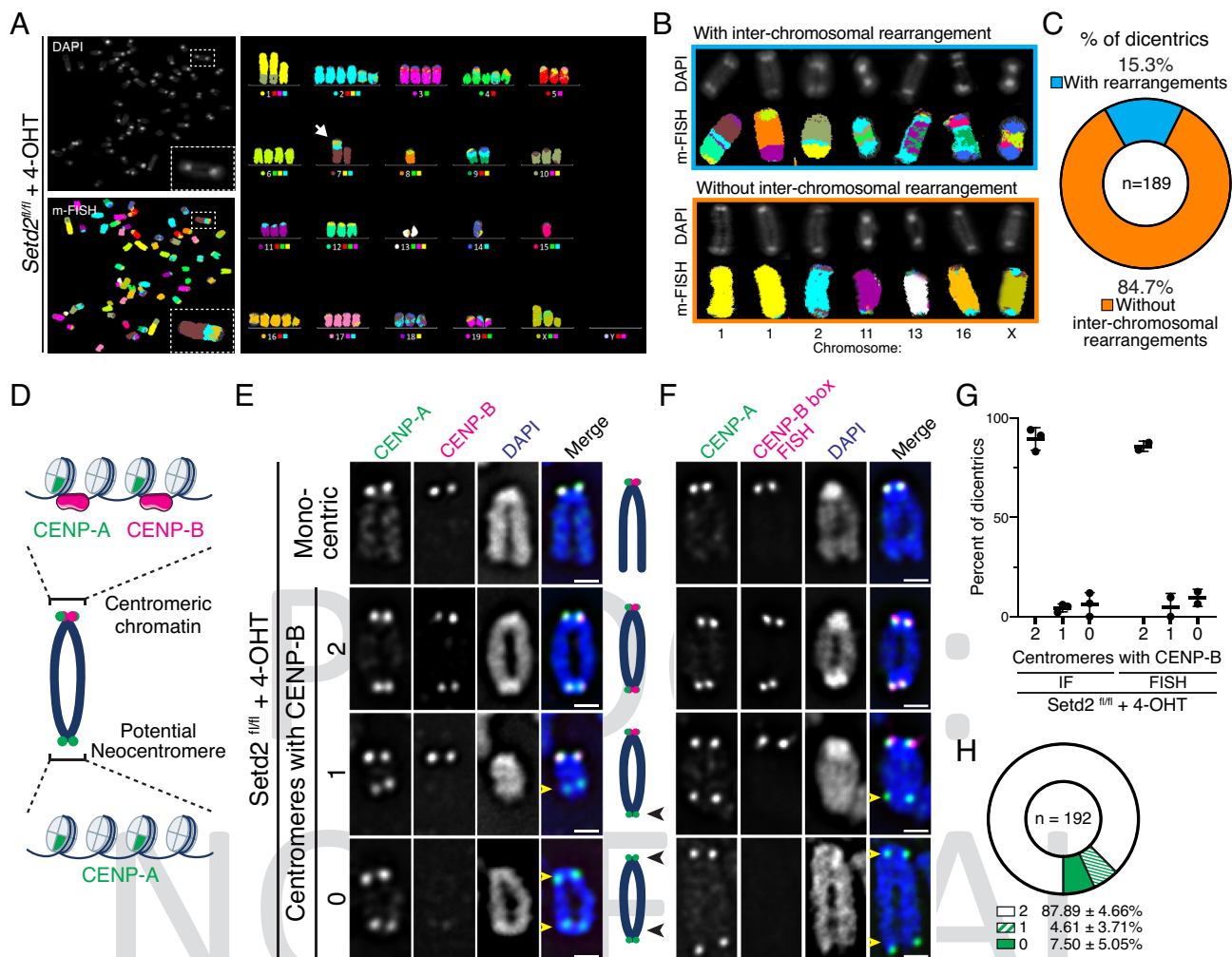


Fig. 3. Dicentric chromosomes in *Setd2*-knockout cells lack interchromosomal rearrangements and neocentromeres. (A) m-FISH image on *Setd2*^{fl/fl} MEFs treated with 4-OHT (*Setd2*-knockout). Arrow indicates a dicentric chromosome. (B) Representative images of dicentric chromosomes with interchromosomal rearrangements (Top) or intrachromosomal rearrangements (Bottom). (C) Quantification of dicentric chromosomes (n = 189 across three biological replicates) with or without interchromosomal rearrangements. The majority of dicentrics lack interchromosomal rearrangements and are composed of genetic material from the same chromosome. (D) Schematic of dicentric chromosomes. CENP-B binds CENP-B box motif on centromeric DNA. Neocentromeres, which contain CENP-A nucleosomes, would lack CENP-B. (E) Representative immunofluorescent images of chromosomes from *Setd2*^{fl/fl} MEFs treated with 4-OHT and stained for CENP-A (green), CENP-B (magenta), and DNA (DAPI, blue). CENP-A localizes with CENP-B at centromeres in monocentric cells and in some dicentrics. Chromosomes lacking CENP-B on one or both centromeres (yellow arrowheads) may be suggestive of neocentromeres. Scale bars are 1 μ m. (F) Representative immunofluorescent and FISH images of chromosomes from *Setd2*^{fl/fl} MEFs treated with 4-OHT and stained for CENP-A (green), CENP-B box FISH probe (magenta), and DNA (DAPI, blue). CENP-A localizes with CENP-B box DNA at centromeres in monocentric cells and in some dicentric cells. Some centromeres contain CENP-A but lack CENP-B box sequences (yellow arrowheads). The scale bar is 1 μ m. (G) Quantifications of percent of dicentrics that have CENP-B colocalizing with CENP-A at 0, 1, or 2 sites across n = 3 (IF) and n = 2 (FISH) biological replicates. Error bars are SD. (H) Quantification of CENP-A colocalization with CENP-B from n = 192 dicentrics described in (G). Mean percentage ($\pm$ SD) across five biological replicates (IF and FISH).

with CENP-B or CENP-B box DNA was quantified, and, using either approach, both centromeres contained CENP-B in 88% of dicentrics, while 12% of dicentrics contained CENP-A at potential noncentromeric sites (Fig. 3 G and H). It should be noted that the *Setd2*^{fl/fl} MEFs were derived from a male mouse, and the Y-chromosome lacks a CENP-B box. Thus, we cannot differentiate between Y-chromosome-derived dicentrics and potential neocentromeres. While these observations indicate that *Setd2* deletion may possibly promote some degree of neocentromere formation, the vast majority of dicentric chromosomes are not formed by this mechanism.

Isodicentric Chromosomes Predominate in *Setd2*-Deleted Cells. We examined the nature of the dicentrics in *Setd2*-deleted cells more carefully. One possibility was that the dicentrics were isodicentric, defined as a derivative chromosome composed of a mirror-imaged duplication that promotes gene amplification

and deletion within an individual chromosome (26). Isodicentrics were suspected because centromeres were exclusively positioned at both chromosome poles in *Setd2*-deleted MEFs, suggesting symmetry (Fig. 4A). Dicentrics in *SETD2*-knockout HeLa cells also appeared symmetrical, often producing abnormally large chromosomes (Fig. 4B). To test whether isodicentric chromosomes occur in *Setd2*-mutant MEFs, metaphase spreads were subjected to Giemsa staining and G-banding analyses (Fig. 4C). This cytogenetic approach identified 75% of dicentrics as bona fide isodicentric chromosomes.

To confirm whether isochromosomes occur in *SETD2*-deleted cells, FISH against nucleolar organizer regions was performed to simultaneously mark the p-arms of the human acrocentric chromosomes (13, 14, 15, 21, and 22, Acro-p) or 45S rDNA of mouse chromosomes (11, 12, 15, 16, 18, and 19). While control HeLa samples showed stereotypic Acro-p FISH staining, *SETD2*-knockout chromosomes exhibited more cells with isochromosomes, including

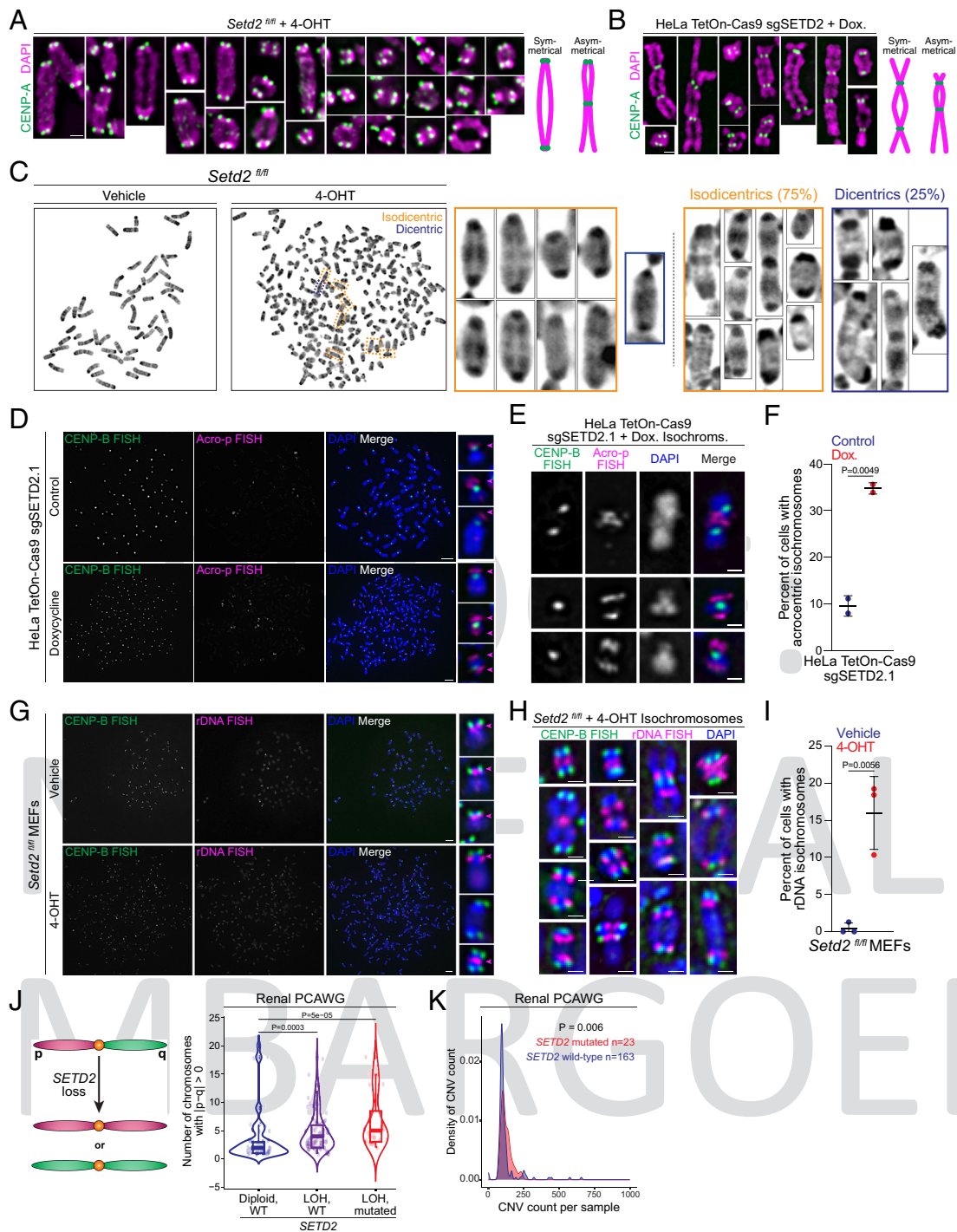


Fig. 4. *Setd2* knockout promotes formation of isodicentric chromosomes. (A) Immunofluorescent images of dicentric metaphase chromosomes from *Setd2*^{null} MEFs treated with 4-OHT (*Setd2*-knockout) and stained for CENP-A (green) and DNA (DAPI, magenta). Nearly all dicentric chromosomes are symmetrical. The scale bar is 1 μ m, and all images are set to the same scale. (B) Representative immunofluorescent images of dicentric metaphase chromosomes from HeLa TetOn-Cas9 cells expressing sgSETD2.1 and treated with doxycycline (*Setd2*-knockout). Chromosomes are stained for CENP-A (green) and DNA (DAPI, magenta). The scale bar is 1 μ m. (C) Images of Giemsa-stained metaphase spreads from *Setd2*^{null} MEFs treated with vehicle (control) or 4-OHT (*Setd2* knockout). Cytogenetic analyses identified that vast majority of dicentrics are isochromosomes (isodicentric, orange boxes). Dicentric chromosomes (blue boxes) occur but the majority are isodicentric. $n = 24$ dicentrics across 18 spreads. (D) Images of FISH for CENP-B box and p-arms of acrocentric chromosomes (Acro-p) on metaphase spreads from control or doxycycline-treated HeLa TetOn-Cas9 cells expressing sgSETD2.1. Acro-p signal should be at short, p-arm (Top); however, *SETD2*-knockout cells have signal on both arms. Scale bars are 5 μ m. (E) Representative images of isochromosomes, including isodicentric, from HeLa TetOn-Cas9 sgSETD2.1 cells treated with doxycycline. Scale bars are 1 μ m. (F) Quantification of the percentage of cells with isochromosomes in control or doxycycline-treated HeLa TetOn-Cas9 sgSETD2.1 cells. $n = 2$ biological replicates. (G) Images of FISH for CENP-B box and rDNA (BAC RP23-225M6) on metaphase spreads from control or 4-OHT-treated *Setd2*^{null} MEFs. rDNA should be present at one location per chromosome, however *Setd2*-knockout leads to isodicentrics (Bottom Right). Scale bars are 5 μ m. (H) Representative images of isochromosomes, including isodicentric, from *Setd2*-knockout MEFs. Scale bars are 1 μ m. (I) Quantification of the percentage of cells with isochromosomes in control (159 spreads) or 4-OHT-treated (241 spreads) *Setd2*^{null} MEFs across $n = 3$ biological replicates. (J) Illustration of normal chromosome with p and q arm, however *SETD2* loss results in isochromosomes with unequal chromosome arms. Quantification of number of chromosomes per sample with unequal p and q arms in Renal PCAWG data with diploid, WT *SETD2* (blue, $n = 47$), heterozygous deletion of *SETD2* (purple, $n = 88$), or deletion and mutation of *SETD2* (red, $n = 23$). P values derived from the Wilcoxon test followed by Holm-Bonferroni to adjust for multiple comparisons. (K) Quantification of distribution of CNV count per sample in wild-type and mutated *SETD2* ($n = 163$ and 23 respectively) from Renal PCAWG data. P values derived from the Wilcoxon signed-rank test. For each plot, error bars are SD and P values derived from an unpaired t test unless stated otherwise.

isodicentric (Fig. 4 D–F). Formation of isochromosomes and isodicentric chromosomes was also observed in *Setd2*-knockout MEFs (Fig. 4 G–I). The generation of isochromosomes, isodicentric, and dicentric should increase copy number variants (CNVs) as well as induce chromosome arm-level changes. To test whether these events occur in tumors with loss or mutation of *SETD2*, we counted the number of chromosomes in each sample with unequal p and q arms in the Renal Cell Carcinoma Analysis of Whole Genomes (PCAWG), excluding sex chromosomes (27). Renal tumors with loss of heterozygosity (LOH) and/or *SETD2* mutation increase the number of chromosomes with unequal p:q arms (Fig. 4J). Concordantly, there is an increase in the number of CNVs in the tumors with *SETD2* mutations (Fig. 4K). While this does not prove that isochromosomes, dicentric, or isodicentric drive CNVs in renal tumors, it is consistent with the consequence of those chromosomal aberrations. To determine whether H3K36me3 or catalytic activity of *SETD2* is required to suppress dicentric chromosome formation,

HeLa or MEFs were treated with a pharmacologic inhibitor of *SETD2* methyltransferase activity (EPZ-719, *SETD2i*) (28). *SETD2i* treatment led to a 97% decrease in H3K36me3 without perturbing *SETD2* protein levels (*SI Appendix*, Fig. S4 A and B). Metaphase spreads were isolated from control, 4-OHT, or *SETD2i* *Setd2*^{+/fl} MEFs, and 33% of *SETD2i*-treated cells displayed dicentric chromosomes, compared to 44% of *Setd2*-knockouts or 1.7% of controls (Fig. 5A). These results demonstrate that the catalytic activity of *SETD2* and/or the presence of H3K36me3 suppresses dicentric chromosome formation. To confirm this, rescue experiments were performed in HeLa TetOn-Cas9 sg*SETD2*.1 expressing cells. Full-length, wild-type or catalytically dead R1625C *SETD2* or an empty vector control under a tetracycline-inducible promoter were introduced in these cells such that doxycycline treatment would simultaneously knock-out and rescue *SETD2* expression (*SI Appendix*, Fig. S4C). We isolated metaphase spreads from these cells and observed that, while wild-type *SETD2* rescues, R1625C mutated-*SETD2* does not, confirming that

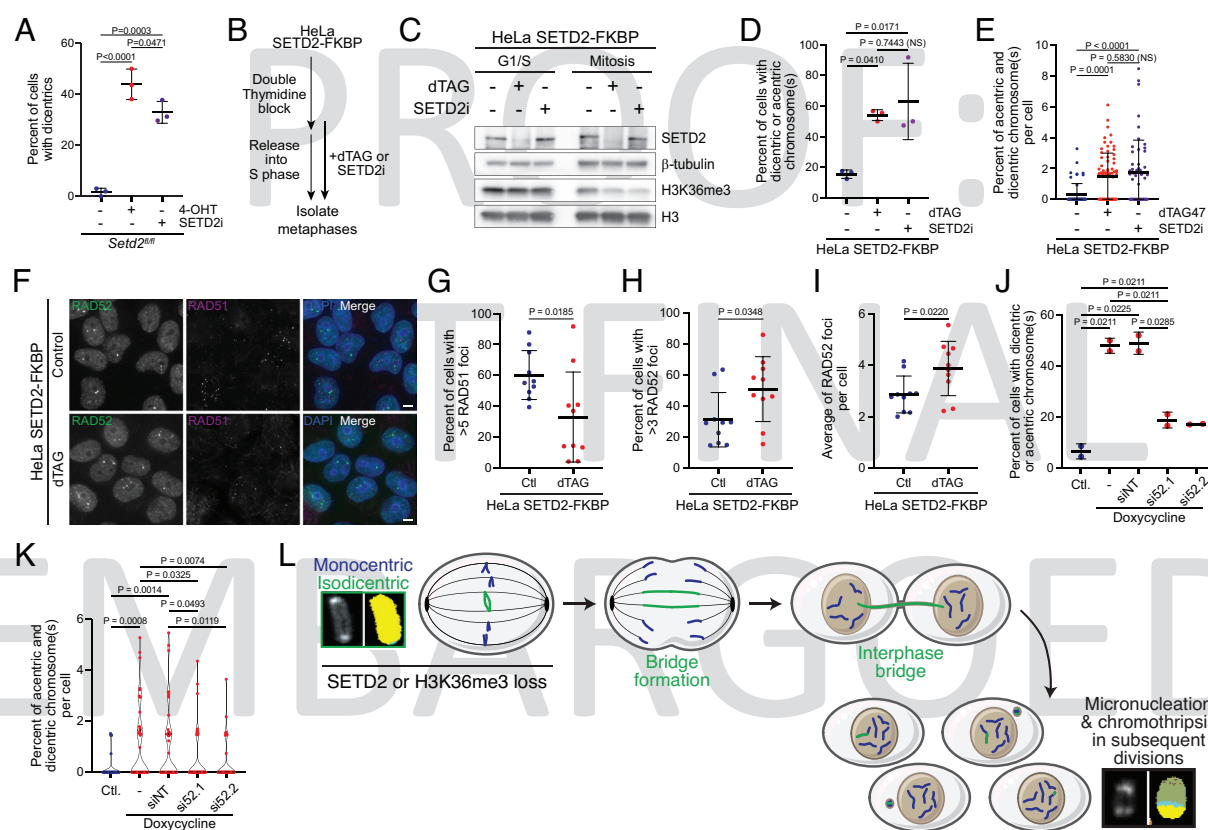


Fig. 5. Loss of *SETD2* catalytic activity during DNA replication promotes formation of dicentric and acentric chromosomes in a RAD52-dependent manner. (A) Quantification of prevalence of dicentric chromosomes in metaphase spreads from *Setd2*^{+/fl} MEFs treated with vehicle (DMSO, control), 4-OHT or 1 μ M *SETD2i*, 3 d after treatment. *Setd2*-knockout or inhibition leads to an increase in dicentric chromosomes from $n = 3$ biological replicates. P values derived from one-way ANOVA with Tukey's multiple comparisons test. (B) Schematic of cell synchronization and *SETD2* inhibition or depletion during DNA replication. (C) Western blot of whole cell extract from HeLa *SETD2*-FKBP cells showing that dTAG treatment degrades *SETD2* prior to S phase release. When arrested at mitosis, *SETD2* is still depleted, and there is a decrease in H3K36me3 in both dTAG- and *SETD2i*-treated cells. (D) Quantification of the percentage of cells with dicentric and/or acentric chromosomes from cells described in (C), using $n = 3$ biological replicates. P values derived from one-way ANOVA with Tukey's multiple comparisons test. (E) Quantification of the percentage of dicentric and acentric chromosomes per cell from cells described in (D). *SETD2* depletion (dTAG, $n = 71$ cells) and *SETD2i* ($n = 51$) had more dicentric or acentric chromosomes than control (DMSO, $n = 51$ cells). P values derived from one-way ANOVA with Tukey's multiple comparisons test. (F) Representative images of HeLa *SETD2*-FKBP cells synchronized then released into S phase following DMSO (control) or dTAG treatment, stained for RAD52 and RAD51. Scale bars are 5 μ m. (G) Quantification of the percentage of cells described in (F) with >5 RAD51 foci during the S phase. $N = 10$ technical replicates for each condition. P values derived from an unpaired t test. (H) Quantification of the percentage of cells described in (F) with >3 RAD52 foci during the S phase. $N = 10$ technical replicates for each condition. P values derived from an unpaired t test. (I) Quantification of average number of RAD52 foci of cells described in (F) during the S phase. $N = 10$ technical replicates for each condition. P values derived from an unpaired t test. (J) Quantification of the percentage of control or doxycycline-treated HeLa TetOn-Cas9 sg*SETD2*.1 cells with dicentric and/or acentric chromosomes with depletion of RAD52 using siRAD52 or siNon-Targeting control (NT) from $n = 2$ biological replicates. siRAD52 decreases the percentage of cells with dicentric or acentric chromosomes. P values derived from one-way ANOVA with Tukey's multiple comparisons test. (K) Quantification of the percentage of dicentric and/or acentric chromosomes per cell described in (J) $n = 49, 48, 49, 62$, and 58 cells across two biological replicates. P values derived from one-way ANOVA with Tukey's multiple comparisons test. (L) Proposed model describing how *SETD2* loss drives genetic heterogeneity. Isodicentric chromosomes initiate gene amplification and deletion, chromosome mis-segregation, and chromothripsis in subsequent divisions to increase clonality.

849 methyltransferase activity is critical for suppressing dicentric and
850 acentric chromosome formation (*SI Appendix, Fig. S4D*).

851 While dicentric chromosomes may arise from faulty DSB repair,
852 telomere attrition, or neocentromere formation (22), mechanisms
853 promoting isodicentrics are less well understood. In yeast,
854 mirror-image dicentric and acentric chromosomes can arise
855 through a DNA replication-dependent, DSB-independent man-
856 ner called faulty template switching or replication template
857 exchange (29, 30). During this process, stalled replication forks
858 restart utilizing RAD52 instead of the recombinase RAD51 and
859 anneal to a nearby inverted repeat, essentially performing
860 “U-turns” to generate palindromic acentric and dicentric chro-
861 mosomes. Loss of H3K36me3 decreases RAD51-dependent
862 homologous recombination (31), so one hypothesis was that cells
863 lacking SETD2 might preferentially utilize RAD52 during DNA
864 replication.

865 To test this, we generated SETD2-FKBP knock-in HeLa cells,
866 synchronized them at G1/S using a double thymidine block, and
867 for the final 2 h of arrest, treated with the SETD2i or dTAG47 to
868 degrade SETD2 (Fig. 5 *B* and *C*). Cells were released and then
869 arrested at metaphase, and spreads were collected and analyzed for
870 dicentric and acentric chromosomes. Degradation or inhibition of
871 SETD2 methyltransferase activity increase the percentage of cells
872 with and percentage of total dicentric and acentric chromosomes
873 (Fig. 5 *D* and *E*). To test whether RAD51 and RAD52 function
874 was altered following SETD2 loss, we again synchronized cells at
875 G1/S then released and performed chromatin fractionation and
876 immunofluorescence. While fractionation and western blot analyses
877 did not reveal a change in loading of RAD51 or RAD52 onto
878 chromatin (*SI Appendix, Fig. S4E*), SETD2-depleted cells displayed
879 a decrease in RAD51 foci and a concordant increase in RAD52
880 foci (Fig. 5 *F–I*). To determine whether the increase in RAD52
881 utilization facilitates the increase in gross chromosomal rearrange-
882 ments, RAD52 was knocked-down using siRNA; then, SETD2
883 was knocked out in HeLa TetOn-Cas9 cells (*SI Appendix, Fig. S4F*).
884 Knockdown of RAD52 was sufficient to decrease the formation of
885 acentric and dicentric chromosomes in SETD2-knockout cells,
886 relative to nontargeting or untreated control cells (Fig. 5 *J* and *K*).
887 These data suggest that cells lacking H3K36me3 and SETD2 may
888 utilize a RAD52-dependent pathway that is more prone toward
889 faulty template switching and isochromosome formation.

890 Discussion

891 SETD2 is the sole source of H3K36 trimethylation in somatic
892 cells and a known tumor suppressor, with roles in mitotic spindle
893 organization, DNA repair, and transcriptional fidelity. Yet, it has
894 been unclear how SETD2 loss causes extensive intratumor hetero-
895 geneity and branched evolution. We propose that in addition to
896 CIN, the formation of isochromosomes following SETD2 and
897 H3K36me3 loss of function provides the mechanism to promote
898 the genomic heterogeneity observed (Fig. 5*L*). This model is con-
899 sistent with previous work demonstrating that a single chromatin
900 bridge is sufficient to promote clonal heterogeneity (9, 11), sug-
901 gesting that this structural intermediate may in fact be the major
902 feature driving genomic instability.

903 Chromosome bridges are subject to cycles of breakage-
904 fusion-bridge (BFBs), which initiate further gross chromosomal
905 rearrangements and chromothripsis in subsequent cell divisions
906 (11, 32). Genomic signatures of BFBs occur in myriad tumor types,
907 and cellular transformation is driven by transient but not chronic
908 formation of dicentric chromosomes (23, 33, 34). Importantly, we
909 observed a robust appearance of dicentric chromosomes following

910 acute SETD2 loss, with a subsequent waning over time. This suggests
911 that a discrete temporal burst of isochromosomes and dicentric chro-
912 mosomes following SETD2 or H3K36me3 loss may be sufficient
913 to promote transformation, gross chromosomal rearrangements, and
914 subclonal heterogeneity. The persistence of isochromosomes and
915 isodicentrics may reflect a low level of ongoing rearrangements or
916 silencing of one centromere such that chromosomes are functionally
917 monocentric (8). Isochromosome persistence may be guided by
918 selective pressures that favor the amplification of genes present in
919 the duplicated chromosome arms.

920 Our findings establish that SETD2 and H3K36me3 prevent
921 isochromosomes and isodicentric chromosomes, potentially by
922 promoting RAD51 activity over the more error-prone RAD52
923 pathway (26, 35). While faulty template switching mediated by
924 RAD52 has been shown to drive “U-turns” in yeast leading to copy
925 number alterations and aneuploidy, this work shows that this path-
926 way may also occur in mammalian cells (29, 30, 36). Nonallelic
927 homologous recombination (HR) between sister chromatids causes
928 isodicentric chromosome formation associated with Turner syn-
929 drome, sex reversal and spermatogenesis defects (6), and fork stall-
930 ing and template switching (FoSTeS), microhomology-mediated
931 break-induced replication or foldback inversions may cause this
932 phenotype following SETD2 loss but future work and sequencing
933 through these inverted repeats will be essential to differentiate
934 between these mechanisms (37, 38).

935 The ability to generate more clones provides tumors with a
936 fitness advantage, and accordingly, a range of tumor types with
937 SETD2 mutations are more resistant to a broad range of thera-
938 peutics (39–42). Notably, ccRCC, where SETD2 was initially
939 established as a tumor suppressor, is among the most resistant
940 cancers to chemotherapy (43). SETD2 mutations commonly
941 occur in hepatosplenic T cell lymphoma (HSTL), a rare yet aggres-
942 sive neoplasm that is also chemoresistant (44). The most common
943 chromosomal abnormality in HSTL is isochromosome 7q [i(7q)],
944 which promotes amplification of genes in 7q and deletion of 7p.
945 Further analyses are needed to assess whether isochromosomes
946 occur in other tumor types with SETD2 mutations. Additionally,
947 our data demonstrate that MEFs and HeLa cells exhibit different
948 frequencies of dicentric and isochromosome formation, suggesting
949 that different genetic or phenotypic backgrounds may influence
950 these rearrangements, which will be the focus of future work.

951 These data demonstrate that loss of the tumor suppressor
952 SETD2 promotes the formation of isochromosomes and isodi-
953 centric chromosomes, leading to chromatin bridges during mito-
954 sis. While these structures have long been appreciated to promote
955 intratumoral heterogeneity, their origin has been elusive. Our
956 findings provide an unexpected and important link between an
957 essential histone methyltransferase and genome maintenance, via
958 an exciting and unique genomic structure that can simultaneously
959 promote copy number alterations, and DNA strand breaks.

960 Materials and Methods

961 **Cell Culture.** All cells were cultured at 37 °C and 5% CO₂. Mouse embryonic
962 fibroblasts (MEFs) were cultured as described previously (15). Briefly, cells
963 were cultured in DMEM, phenol red-free (Gibco, Catalog number 21063-029)
964 supplemented with 1 mM sodium pyruvate (Sigma, S8636-100 mL), GlutaMAX
965 (Gibco, 35050-061), and 10% fetal bovine serum (FBS, GeminiBio, 100-106).
966 HeLa TetOn-Cas9 and 293FTs cells were grown in DMEM (Gibco, 11965-092)
967 supplemented with 1% penicillin/streptomycin (Gibco, 15150122) and 10%
968 tetracycline-free FBS (GeminiBio, 100-800) (17). *Setd2*^{wt/wt}, *Setd2*^{flx/wt}, *Setd2*^{flx/flx}
969 MEFs expressing ER-Cre were treated with 3 μM (Z)-4-hydroxytamoxifen
970 (4-OHT, Millipore Sigma, H7904) or vehicle (Ethanol, 0.1% final volume) for 3 d,
971 unless stated otherwise, and then harvested for the experiment.

Cell Culture Methods, Treatments, and Cell Line Generation. Single guides targeting *SETD2* (sgSETD2.1 sense strand GTGCGGATCAGCAATTGCCG and sgSETD2.2 sense strand GAATGAAGTGGGATTCCGACG) were cloned into plenti-sgRNA (Addgene, 71409) following previous methods (45). Lentivirus was generated using 293FT cells by cotransfection of guide plasmid with psPAX2 and pMD2.G (Addgene, 12260 and 12259, respectively). Conditioned media were harvested, filtered, and stored at -80. HeLa TetOn-Cas9 cells were transduced with 8 μ g/mL polybrene (Millipore Sigma, TR-1003) and viral supernatant for guides targeting SETD2 or nontargeting control sgRNA (Addgene, 80189). Cells were selected using 0.5 μ g/mL puromycin (Millipore Sigma, P9620). To activate expression of Cas9, cells were treated with 1 μ g/mL doxycycline (Millipore Sigma, D5207) daily and harvested after 3 d unless stated otherwise. For SETD2i experiments, EPZ-719 (MedChemExpress, HY-139626) was resuspended in DMSO, and cells were treated and harvested after 72 h.

To generate a stable knock-in SETD2-FKBP, HeLa Kyoto cells were transduced with adeno-associated virus containing homology-directed repair templates (Vectorbuilder), designed using a previously published method (46). After 18 h, cells were nucleofected with SpCas9 2X NLS nuclease loaded with SETD2-targeting sgRNA (GACUCACGGUGUUAUGA with UAU-modified scaffold, Synthego and ThermoFisher Neon Transfection System). Cells were expanded and then selected using Hygromycin. Hygromycin-resistant cells were subjected to single-cell sorting (BD FACSAria) and then screened for homozygous insertion via PCR. The homozygous clone exhibited complete SETD2 depletion after 1.5 h of 1 μ M dTag47 (Tocris).

siRAD52.1 and siRAD52.2 (Dharmacon, J-011760-05 and J-011760-06, respectively) and siNon-targeting (Silencer Select Negative Control No. 2, ThermoFisher, 4390846) were reverse transfected using Lipofectamine RNAiMAX using the manufacturer's protocol. Twenty-four hours after transfection, cells were forward transfected again and treated with doxycycline daily. To generate SETD2 rescue cells, HeLa TetOn-Cas9 sgSETD2.1 cells were transfected with Super piggyBac Transposase (SBI, PB210PA-1) and codon-optimized SETD2 in a PiggyBac expression vector that coexpressed a blasticidin-resistance gene, which was used to select for a polyclonal population (Vectorbuilder).

Immunoblotting. For immunoblots, whole cell extracts were generated by scraping and then collecting cells via centrifugation. Cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed with RIPA buffer (Sigma), supplemented with Halt Phosphatase and Protease inhibitors (Pierce), 1 mM phenylmethylsulfonyl fluoride (PMSF, Sigma), and 25 U/mL Universal Nuclease (Pierce). Extracts were incubated on ice, and protein levels were quantified using the BCA assay (Pierce) and supplemented with 4 $\times$ Laemmli buffer with 2-mercaptoethanol (Bio-Rad). Samples were boiled and sonicated. To generate histone extracts, cells were scraped, centrifuged, and washed twice with ice-cold PBS. Cells were incubated with hypotonic lysis buffer (10 mM Tris-HCl, pH 8.0, 1 mM KCl, 1.5 mM MgCl₂, 1 mM DTT, Halt, and 1 mM PMSF) for 30 min on rotator at 4 °C. Nuclei were pelleted at 10,000 g for 10 min at 4 °C, and histones were extracted from pelleted nuclei using 0.2N HCl at 4 °C overnight on a rocker. Nuclear debris was pelleted at 16,000 g for 10 min, and histone extract was quantified using the BCA assay, neutralized with an equal volume of 1M Tris-HCl, pH8.0, and supplemented with 4 $\times$ Laemmli buffer and then boiled. For chromatin fractionations, cells were resuspended at 10M cells/mL of ice cold Buffer A (100 mM NaCl, 300 mM Sucrose, 3 mM MgCl₂, 10 mM PIPES, 1 mM EGTA, 0.2% TritonX-100, 1 mM PMSF, and Halt) and incubated on ice for 10 min. Lysate was centrifuged at 10,000 g for 10 min, saving supernatant and rinsing pellet in 1 $\times$ volume of Buffer A. Lysate was pelleted again resuspended in an equal volume of Buffer B (50 mM Tris-HCl, pH7.5, 150 mM NaCl, 5 mM EDTA, 1% TritonX-100, 1 mM PMSF, Halt, and Universal Nuclease). Fractions were supplemented with 4 $\times$ Laemmli buffer, boiled, and sonicated. Proteins were run out using 4 to 20% Criterion TGX Precast Gels (Bio-Rad) and then transferred using the Trans-blot Turbo Transfer System (Biorad) for semidry (histones and low-molecular-weight proteins) or Criterion Blotter for wet transfer (high-molecular-weight proteins). Proteins were transferred onto PVDF membranes (ThermoFisher), blocked with 5% BSA in TBS-Tween 0.1% (blocking buffer), and then probed for antibodies in block buffer. SuperSignal West Pico PLUS or Femto Maximum chemiluminescent solution (Pierce, 34580 and 34095) was added, and blots were imaged using either film (HyBlot), Bio-Rad Gel Doc, or Amersham Imager 600. Primary antibodies used were anti-SETD2 (Sigma, HPA042451), H3K36me3 (Cell Signaling,

4909), b-actin (Cell Signaling, 4970), Histone 3 (Cell Signaling, 14269), Vinculin (Abcam, ab129002), b-tubulin (E7, gift of Kristen Verhey), RAD51 (Millipore Sigma, PC130), RAD52 (Santa Cruz Biotechnology, sc-365341), and Cas9 (Active Motif, 61778). Secondary antibodies used were anti-rabbit and anti-mouse HRP conjugates (Pierce, W401B and W402B).

Cell and Metaphase Spread Immunofluorescence and Imaging. Cells were cultured on cleaned glass coverslips (No. 1.5) in multiwell plates or glass-bottom multiwell plates (CellVis). Cells were fixed either by ice-cold 100% methanol for 10 min or 4% paraformaldehyde in Cytoskeleton Buffer (100 mM NaCl, 300 mM Sucrose, 10 mM PIPES pH6.8, 3 mM MgCl₂, and 0.5% TritonX100) for images in Fig. 1 and *SI Appendix, Fig. S2C*. For chromatin extractions (Fig. 5F), cells were incubated in 20 mM HEPES, 50 mM NaCl, 3 mM MgCl₂, 300 mM Sucrose, and 0.5% TritonX-100 for 5 min on ice and then fixed with PFA at 4% final concentration in extraction buffer. Cells were then rinsed three times with PBS and incubated with PBS with 0.1% TritonX100 (PBSTx) for 10 min. Cells were blocked with 1% BSA in PBSTx and then stained with primary and secondary antibodies in blocking buffer. Cells were washed with PBS, counterstained with Hoechst (2.5 μ g/mL), and then mounted with ProLong Antifade Gold or stored in PBS. Primary antibodies used were anti- α Tubulin (12G10, gift of K. Verhey), cGAS (Cell Signaling, 31659), RPA (Cell Signaling, 2208), RAD51 (Millipore Sigma, PC130), and RAD52 (Santa Cruz Biotechnology, sc-365341).

Metaphase spreads were stained as described (47). Briefly, cells were treated with 150 ng/mL of colcemid (KaryoMAX, ThermoFisher) for 1 to 2 h, harvested via shake-off, and then pelleted via centrifugation. Cells were gently resuspended and swelled with a 1:1 ratio of 75 mM KCl:0.9% sodium citrate (MEFs) or 75 mM KCl (HeLa) and prewarmed to 37 °C. Cells were incubated for 5 to 15 min and then cytospun onto poly-lysine coated slides at 1,300 rpm for 10 min. Cells were extracted using KCM buffer (10 mM Tris pH8.0, 120 mM KCl, 20 mM NaCl, 0.5 mM EDTA, and 0.1% TritonX100) for 10 min. Samples were blocked using 1% BSA in KCM, stained with antibodies in 1% BSA in KCM, and then fixed with 4% PFA in KCM for 10 min. Chromosomes were rinsed with PBS, counterstained with DAPI (1 μ g/mL) in PBS, and then mounted with VectaShield (H100) and sealed. Primary antibodies used were CENP-A (Cell Signaling, 2048), CENP-A (Abcam, ab13939), HEC1 (Santa Cruz Biotechnology, sc-135934), HEC1 (GeneTex, GTX70268), H3K36me3 (Abcam, ab9050), CENP-B (Santa Cruz, sc-32285), KNL1 (Abcam, ab222055), CENP-T (Abcam, ab220280), and Centromere Protein Antibody (ACA, Antibodies Inc., 15-234). Secondary antibodies used were anti-mouse, anti-rabbit, or anti-human AlexaFluor conjugates (ThermoFisher, A21121, A11001, A21236, A11032, A32733, A11034, A11037, A21135, and A21445).

Images of mitotic cells and metaphase spreads were acquired on DeltaVision Elite imaging system (GE Healthcare) equipped with a Cool SnapHQ2 charge-coupled device camera (Roper), using a 60 $\times$ 1.4 numerical aperture (NA) or 100 $\times$ 1.4 NA objectives (Olympus) or Nikon MultiExcitation TIRF imaging system, using widefield settings, Andor Zyla 4.2 Plus sCMOS monochrome camera, and 60 $\times$ 1.5NA objective (Olympus). Images were acquired using preset Alexa filter settings, and optical sections were collected at 200-nm intervals and processed using ratio deconvolution in softWoRx (GE Healthcare, version 6.5.2) or NIS Elements. Images were prepared for publication using FIJI (Version 2.1.0/1.53o). Images of mitotic and interphase cells are maximum-intensity projects, and metaphase spreads are single z-slices. Images of interphase cells for quantifying nuclear phenotypes were acquired using Cytation 5 (Biotek) using 20 $\times$ 0.45 NA objective (Olympus) with standard filter sets or Nikon MultiExcitation TIRF imaging system, using widefield settings. Nuclear images and metaphase spreads were manually quantified. Chromatin bridges were defined as DNA structures that were parallel to the axis of poleward elongation and spanned most of the length between the segregated anaphase chromosomes. Lagging chromosomes were scored as DNA that remained in the midzone during anaphase and lacked obvious orientation along the axis of elongation. To count RAD51 and RAD52 foci, nuclei (Hoechst) were thresholded, and foci were identified by finding local maxima and counted per cell using FIJI.

FISH. Cells were treated with KaryoMAX and then harvested via trypsinization. Cells were centrifuged and then gently resuspended in a 1:1 ratio of 75 mM KCl: 0.9% sodium citrate (MEFs) or 75 mM KCl (HeLa) for 5 to 15 min. Cells were partially fixed by addition of 1/10 volume of ice-cold Carnoy's fixative (3:1 methanol:acetic acid). Cells were centrifuged and gently resuspended; then, fixative

was added, dropwise while vortexing. Cells were incubated at least 30 min on ice, spun down, and resuspended in ~0.5 mL fixative and then dropped onto ice-cold slides. Slides were air-dried at room temperature overnight and then stored at 4 °C. Slides were rehydrated in 2× SSC for 2 min, dehydrated in ethanol series (2 min each in 75%, 90%, 100% EtOH), and then air dried. Centromere (PNA Bio, CENPB-AF488 or -647), telomere (PNA, TelC-A647, 1:500), Acro-P (Cytocell, LPE NOR, undiluted), or rDNA (Empire Genomics, RPC123-225M6, 1:5) FISH probes were diluted in hybridization solution (10 mM Tris-HCl pH7.2, 70% formamide, and 0.5% blocking reagent) and then added to the slide. Slides were denatured at 78 °C for 2 to 5 min and then incubated for overnight at 37 °C. Slides were washed in 0.4× SSC and then 2× SSC with 0.5% Tween20 for 2 min each. Slides were washed three times with PBS adding DAPI (1 µg/mL) to second wash. Slides were dehydrated using ethanol series and then mounted with ProLong Antifade Gold (Invitrogen). If spreads were subjected to FISH following immunofluorescence, the immunofluorescence protocol was used, and spreads were dehydrated using an ethanol series prior to FISH protocol. If spreads were stained only with DAPI, the denaturing and hybridization steps were omitted. For G-banding and Q-FISH, samples were prepared, imaged, and analyzed by Creative Bioarray.

Multicolor FISH. *Setd2*^{fllox/fllox} MEFs were treated with vehicle or 3 µM 4-OHT for 3 d, and mitotic cells were enriched by incubation in 100 ng/mL colcemid (KaryoMAX) for ~5 h. To prepare metaphase chromosome spreads, cells were collected by trypsinization, swollen with prewarmed 0.075 M KCl at 37 °C for 6 min, and fixed by washing and resuspending in ice-cold Carnoy fixative. Metaphase spreads were then dropped onto slides and allowed to air dry. For multicolor FISH, 3 µL of 21X Mouse probes (MetaSystems) was applied to the sample and sealed with a coverslip using rubber cement. Samples and probes were codenatured at 75 °C for 2 min followed by incubation at 37 °C overnight in a humidified chamber using the ThermoBrite System (Leica). Slides were then washed for 2 min in 0.4× SSC at 75 °C followed by a 30-s wash in 2× SSC at room temperature. Slides were counterstained with DAPI and mounted with an anti-fade solution. Metaphases were imaged using a Metafer Slide Scanning Platform (MetaSystems). Slides were first scanned to search for metaphase spreads using MSearch and then automatically captured using Autocapt at 63× magnification. Multicolor karyotypes were generated using Isis (v5.8.12, MetaSystems) and adjusted for imaging threshold. Dicentric chromosomes were identified as chromosomes harboring two DAPI-bright staining regions, and interchromosomal rearrangements were assessed by the appearance of different colored FISH probes hybridizing to the same dicentric chromosome.

CNV Association Analysis with SETD2 Mutated Patient Samples. Patient metadata, mutation annotations, and gene-level copy number annotations were obtained from the Pan-Cancer Analysis of Whole Genomes (PCAWG) (27), provided by the cBioPortal (48, 49). Segment-level copy number annotations for the PCAWG-11 dataset were obtained from the UCSC Xena data browser. The data from all sources was processed in R and joined by sample ID and/or Tumor Sample Barcode.

Log2 normalization of segment-level CNV data was performed. We excluded sex chromosomes and only kept chromosomes 1:22. Segments with an initial copy number of 0 were set to -2 in the log2 transformation space. Additionally, we normalized the log2 CNVs by subtracting the mean across each of the patients. This is to normalize samples with whole genome doublings and to look for changes in CNV. To identify samples with *SETD2* LOH, we separated samples by *SETD2* copy number. Samples with a *SETD2* copy number of 0 were neutral, and samples with a copy number of -1 were classified as LOH. Segment level copy number was plotted from our normalized CNV data using the cnSpec() function of the R package GenVisR. P and Q arm annotations were derived from the UCSC hg19 cytoband annotation. Association analysis between *SETD2* mutation status and CNV was performed by computing different metrics from the CNV data: 1) Count of CNV segments: computing the number of segments with a copy number not equal to zero. 2) Count of chromosomes with a difference between in CNV for P and Q arms greater than 0.

Statistics. Statistical analyses were performed in GraphPad Prism 10.0.0, except for PCAWG analyses, which were performed R version 4. All statistical tests are listed in figure legends, along with the number of biological or technical replicates. Statistical significance was considered to be $P < 0.05$, and all significant P values are listed, while nonsignificant values are listed if space permits. All data shown are mean with SD unless otherwise noted.

Data, Materials, and Software Availability. Some study data available [Immunofluorescent image are available upon request due to logistical reasons (size)].

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Q25	Please confirm whether the edited sentence "Setd2wt/wt, Setd2flox/wt, Setd2flox/flox MEFs. . ." conveys the intended meaning.
Q26	Please confirm whether the edited sentence "Cells were. . ." conveys the intended meaning.
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Drug screening in human physiologic medium identifies uric acid as an inhibitor of rigosertib efficacy

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ABSTRACT

The non-physiological nutrient levels found in traditional culture media have been shown to affect numerous aspects of cancer cell physiology, including how cells respond to certain therapeutic agents. Here, we comprehensively evaluated how physiological nutrient levels impact therapeutic response by performing drug screening in human plasma-like medium (HPLM). We observed dramatic nutrient-dependent changes in sensitivity to a variety of FDA-approved and clinically trialed compounds, including rigosertib, an experimental cancer therapeutic that has recently failed in phase 3 clinical trials. Mechanistically, we found that the ability of rigosertib to destabilize microtubules is strongly inhibited by the purine metabolism waste product uric acid, which is uniquely abundant in humans relative to traditional *in vitro* and *in vivo* cancer models. Structural modelling studies suggest that uric acid interacts with the tubulin-rigosertib complex and may act as an uncompetitive inhibitor of rigosertib. These results offer a possible explanation for the failure of rigosertib in clinical trials and demonstrate the utility of physiological media to achieve *in vitro* results that better represent human therapeutic responses.

INTRODUCTION

Tumor growth is influenced by both cell intrinsic and cell extrinsic factors, and nutrient availability is emerging as a critical environmental factor that can shape the metabolic fitness and proliferative capacity of tumors (1–3). Concurrent with these discoveries has been a renewed realization that standard cell culture media were not designed to mimic the nutrient environment found *in vivo*, but were rather designed to provide excess amounts of the minimal nutrients required to sustain cancer cell growth *in vitro* (4–9). As a result, the nutrients present in these traditional culture media do not accurately recapitulate the complexity or abundance of nutrients found *in vivo*. Importantly, it has recently been shown that the highly non-physiological nutrient levels found in culture media can contribute to inconsistencies between *in vitro* and *in vivo* experiments, especially for those directly related to cellular metabolism (10–13). It has also been observed that nutrient availability can affect the response to a variety of cancer therapies(14), including traditional chemotherapies (15, 16), metabolic inhibitors (17, 18), targeted therapies (19, 20), and immunomodulatory checkpoint inhibitors (21).

Because of the importance of nutrient availability in influencing tumor metabolic phenotypes and therapeutic vulnerabilities, there is considerable interest in targeting systemic metabolism either alone or in combination with existing therapies to treat cancer. This includes several dietary interventions that are being investigated as components of cancer therapies (22, 23), including amino acid starvation (24–26), ketogenic diet (27, 28), caloric restriction (29–31), and fasting-mimicking diets (32). The success of dietary intervention studies is critically dependent on mouse models and has led to tremendous

interest in translating these findings to patients. And while mouse models provide an essential platform to study interactions between systemic and tumor metabolism, there are a number of metabolic differences between mice and humans that influence tumor biology (33–36), including how cancer cells respond to cancer therapeutics (15). These issues have motivated the development of novel culture media that specifically mimic the nutrient composition found in human plasma as platforms for studying therapeutic response under more physiological human nutrient conditions (12, 15).

Here, we sought to determine the extent to which nutrient availability impacts the sensitivity of cancer cells to diverse therapeutic agents by utilizing a high-throughput differential sensitivity drug-screening platform to profile therapeutic sensitivity in cancer cells growing in traditional versus physiological human plasma-like medium (HPLM). This screen revealed dramatic nutrient-dependent changes in sensitivity to a wide variety of drugs in cells cultured in HPLM. Among these differences were changes in sensitivity to the experimental therapeutic rigosertib (ON-01910), the efficacy of which was strongly antagonized by the purine degradation product uric acid. Further, our data suggests that uric acid inhibits the microtubule destabilizing function rigosertib by directly binding and weakening the rigosertib:tubulin complex. Importantly, while rigosertib has shown great promise in pre-clinical studies, it has thus far failed in phase 3 clinical trials (37, 38). Given that uric acid levels are uniquely high in humans due to the recent pseudogenization of the uricase gene (39–44), our work offers a potential explanation for the clinical failure of rigosertib. Further, our work suggests that therapies designed to lower uric acid, such as

those used to treat gout and tumor lysis syndrome, may effectively augment rigosertib sensitivity in patients.

RESULTS

Drug screening identifies nutrient-dependent effects on therapeutic response

Commercial culture media such as DMEM and RPMI-1640 (RPMI) contain nutrients at non-physiological levels and lack many critical components present in human plasma (45). Due to these deficiencies, several labs have recently developed media that more accurately mimic physiological nutrient levels found in human circulation (10–12, 15). We have made use human plasma-like medium (HPLM) (15) to culture breast cancer cell lines, where after a two-week adaptation period we observed similar or slightly decreased growth rates (Figure S1A), and consistent remodeling of intracellular metabolite abundance (Figures S1B & S1C). Because of the recent observation of the impact of nutrient availability and cellular metabolism on the response to a variety of cancer therapies (15, 16, 20), we hypothesized that culturing in HPLM would change how cancer cells respond to therapeutic agents on a larger scale. To address this question, we utilized a high-throughput differential sensitivity drug screening platform containing a library of 626 metabolic inhibitors and anticancer compounds arrayed in 10 point dose curves (46). This platform contains compounds targeting a wide variety of cancer-relevant pathways, many of which are FDA-approved or have been evaluated in clinical trials. We screened the triple-negative breast cancer (TNBC) cell line SUM149 growing in RPMI or HPLM media, where we observed dramatic changes in sensitivity to a variety of compounds

(Figure 1A and 1B, Table S1). Interestingly, while very few drugs are more effective in HPLM, a large proportion of drugs are less effective in physiological medium.

Among the drugs most significantly affected by culture in HPLM are four inhibitors of the *de novo* purine biosynthesis pathway – lometrexol, azathioprine, 6-thioguanine (6-TG), and 6-mercaptopurine (6-MP) – all of which are less effective at reducing cell numbers in HPLM (Figures 1A – 1F). We found that both SUM149 cells and another TNBC cell line, HCC1806, are able to proliferate when treated with lometrexol in HPLM, but not RPMI (Figures 1G & 1H). Based on these observations, we investigated the level of purine nucleotides by LC-MS analysis in HCC1806 cells treated with and without lometrexol in both RPMI and HPLM. As expected, we found that lometrexol causes a large drop in the abundance of most purine nucleotides in RPMI; however, this drop is significantly blunted in HPLM (Figure 1I). In addition to *de novo* biosynthesis, cells can acquire purines through the purine salvage pathway (Figure 1J), and the presence of substrates for the purine salvage pathway, such as hypoxanthine, are known to reduce the efficacy of purine synthesis inhibitors (47, 48). While traditional media formulations do not contain salvage substrates, HPLM contains hypoxanthine at 10 μ M as is found in human plasma. Indeed, we found that addition of hypoxanthine to RPMI is sufficient to provide resistance against these compounds (Figure 1K – 1N), and the removal of hypoxanthine from HPLM strongly increases the sensitivity of SUM149 cells to purine biosynthesis inhibitors and (Figure 1O – 1R). While the ability of hypoxanthine to rescue purine synthesis inhibitors is known, these results demonstrate the power and utility of our screening platform to identify physiological nutrients that modify cancer cell sensitivity to therapeutic agents.

Uric acid in HPLM reduces cancer cell sensitivity to rigosertib

Another top hit from our screen was the experimental cancer therapeutic rigosertib, which is significantly less effective against cells growing in HPLM than in RPMI (Figures 1A and 2A). We validated these results by performing rigosertib dose-response analyses in HCC1806 and SUM149 cells, where we observed 2711 and 283-fold increases (respectively) in the IC₅₀ for rigosertib in HPLM (Figures 2B and 2C). Similar results were obtained in two lung cancer cell lines, A549 and Calu6, suggesting that this effect is likely general and not restricted to breast cancer cells (Figures 2D, 2E). Rigosertib's anti-cancer effects have been shown to be mediated by induction of both G2/M cell cycle arrest and cell death (49, 50). Accordingly, we observed that rigosertib treatment induces phosphorylation of histone H3, G2/M cell cycle arrest, and induction of cell death in RPMI but not in HPLM (Figures 2F – 2I).

Next, we sought to determine the component(s) of HPLM that antagonizes rigosertib activity. Like RPMI and other traditional media, HPLM consists of glucose, amino acids, and salts, albeit at different concentrations (15). HPLM also contains 27 additional components that are not found in RPMI but are found in human plasma. Most of these unique ingredients are organized into 11 stock solutions numbered 8 through 18. To determine whether a unique component of HPLM is responsible for the reduced sensitivity to rigosertib, we combined HPLM stocks 8-18 and added them to RPMI and performed dose-curve analyses, where we found that stocks 8-18 were able to recapitulate the effect of HPLM on rigosertib sensitivity (Figures 3A and 3B). We then analyzed stocks 8-18

individually and found that addition of stock 18 alone was sufficient to protect against rigosertib in RPMI (Figure 3C and 3D). Importantly, stock 18 contains only one component: the purine metabolism waste product uric acid, which is present in human plasma and HPLM at 350 μ M. Indeed, we found that removal of uric acid from HPLM was sufficient to dramatically increase sensitivity to rigosertib (Figures 3E). We confirmed the broad protective effects of uric acid by performing rigosertib dose curves on multiple cancer cell lines of different origin, including lung, renal and CML, where we observed the protective effects of uric acid in all cases (Figure S2). To determine whether uric acid protect cells from rigosertib in a dose-dependent manner, we performed a dose curve of uric acid in RPMI in the presence of 80 nM rigosertib. Interestingly, we found that uric acid concentrations as low as 27 μ M are able to partially protect against rigosertib (Figures 3F and 3G). Similar to HPLM, uric acid alone was sufficient to block rigosertib-mediated phosphorylation of histone H3, G2/M cell cycle arrest, and cell death (Figures 3H – 3K).

Uric acid inhibits the microtubule destabilizing activity of pharmaceutical grade rigosertib

While the mechanism of action of rigosertib remains controversial (51–54), several recent reports have demonstrated that rigosertib is a microtubule destabilizing agent that binds tubulin dimers at the colchicine binding site (49, 55, 56). To verify the ability of rigosertib to destabilize microtubules, we performed short-term (4 hr) treatments of HCC1806 and SUM149 cells cultured in RPMI with increasing doses of commercial-grade rigosertib, where we observed increased levels of α -tubulin in the soluble fraction of cell lysates,

suggesting that rigosertib does indeed destabilize microtubules (Figures 4A, 4B, S3A, and S3B). Importantly, however, the ability of rigosertib to destabilize microtubules in cells grown in HPLM is strongly inhibited (Figures 4A, 4B, S3A, and S3B). To determine whether uric acid prevents rigosertib-mediated microtubule destabilization, we treated cells cultured in HPLM with and without 350 μ M uric acid with commercial-grade rigosertib, which results in a dose-dependent increase in the level of soluble α -tubulin only in the absence of uric acid (Figures 4C, 4D, S3C and S3D).

Previous work has shown that the presence of a contaminant in commercial-grade rigosertib may contribute to its anti-cancer effects (57). To determine whether HPLM blocks the effect of rigosertib or a potential contaminant, we made use of pharmaceutical-grade rigosertib that lacks the potentially active contaminant. Similar to commercial-grade rigosertib, culture of cells in HPLM strongly reduces the cellular sensitivity to pharmaceutical-grade rigosertib (Figures 4E and 4F). Importantly, addition of 350 μ M uric acid to RPMI prevented sensitivity to pharmaceutical-grade rigosertib in a panel of renal cancer cell lines (Figures 4G and 4H), indicating that uric acid is protective against rigosertib and not a contaminant found in the commercial-grade compound. Similarly, treatment of 786-O cells with pharmaceutical-grade rigosertib results in a dose dependent decrease in α -tubulin found in the pellet (microtubule fraction) when compared to total tubulin in RPMI, but the addition of uric acid to RPMI prevented rigosertib-mediated destabilization of microtubules (Figures 4I and 4J).

Uric acid binds β -tubulin and weakens the rigosertib: β -tubulin interaction

The acute ability of uric acid to prevent rigosertib-mediated destabilization of microtubules motivated us to explore the molecular effects of rigosertib and uric acid on tubulin structure. As a benchmark, we compared rigosertib to colchicine in our analyses. We started by performing molecular dynamics (MD) simulations of colchicine-bound, rigosertib-bound and apo-tubulin (non-drug bound control). After equilibrating each structure for 0.5 μ s, we then performed four independent simulations of each complex resulting in more than 6 μ s of total simulation time. Using principal component analysis (PCA) to evaluate the large-scale structural differences induced by colchicine and rigosertib, we found that both colchicine and rigosertib produce a similar “kink” in the dimer that likely explains their ability to prevent microtubule polymerization (Figure S4 and Supplementary Movies 1 & 2). In addition, rigosertib induces a unique conformational change that alters the relative orientation of α and β -tubulin (Figure S4, and Supplementary Movies 1 & 2). Specifically, the colchicine-bound simulation features a persistent salt bridge formed between α R221 and β E328 that is directly adjacent to the colchicine binding site (Figure 5A). Since rigosertib alters the intradimer interface and creates a greater distance between α R221 and β E328 (Figure 5B), this salt bridge cannot be formed in rigosertib-tubulin (Figure 5A). Helix H10 in β -tubulin contains E328, and loss of this salt bridge makes H10 more dynamic and creates a pocket between H10 and strand S9 (Figure 5C). Docking studies revealed that uric acid binds within this pocket via hydrogen bonding with residues in both H10 and S9 (Figure 5C). Importantly, S9 also interacts with the carboxyl group of rigosertib. Using free energy calculations we found that the net effect of uric acid binding is to weaken the binding affinity of rigosertib to β -tubulin. This data reveals a potential mechanism by which uric acid antagonizes rigosertib

activity by directly interacting with the rigosertib: β -tubulin structure. Furthermore, because the uric acid binding pocket is not present in colchicine-tubulin and uric acid only binds tubulin when rigosertib is also present, our data suggests that uric acid is potentially acting as an uncompetitive inhibitor of rigosertib.

To experimentally test whether uric acid might impair the ability of rigosertib to bind tubulin, we utilized the cellular thermal shift assay (CETSA) in cells treated with and without rigosertib and uric acid. CETSA can directly assess drug:protein interactions within cells by evaluating shifts in protein melting temperature caused by the binding of small molecules (58–60). Using this assay, we observed denaturation and precipitation of both α and β -tubulin at 60°C that was strongly reduced in the presence of rigosertib, suggesting that rigosertib is capable of binding to tubulin (Figure 5D). However, addition of uric acid to the culture media significantly reduces the stabilization of α and β -tubulin by rigosertib (Figure 5D). These results support the model suggested by our structural data that uric acid reduces the affinity of rigosertib for β -tubulin, thereby inhibiting the ability of rigosertib to destabilize microtubules.

DISCUSSION

Despite promising pre-clinical data and extensive evaluation in early stage studies, rigosertib has thus far failed to improve outcome in the two phase 3 clinical trials in which it has been investigated (37, 38). Importantly, our discovery that uric acid strongly antagonizes the microtubule-destabilizing activity of rigosertib offers a potential

explanation for the relatively poor performance of rigosertib in humans. Most species, including mice and others commonly used in cancer research (e.g., bovine serum), have a functional uricase gene that converts uric acid to the more soluble allantoin, resulting in relatively low circulating uric acid concentrations (Figure 5D). However, due to the evolutionarily recent pseudogenization of the uricase gene in humans and other closely related apes, humans have circulating uric acid levels that are an order of magnitude higher than other mammals (Figure 5D) (39–44). Further, cancer patients, including those with myelodysplastic syndromes where rigosertib has been most thoroughly investigated, often present with hyperuricemia (61, 62), and therefore they may have uric acid levels that are even higher than those found in HPLM. These elevated uric acid levels could cause reduced rigosertib efficacy in humans compared to mouse models and traditional tissue culture where uric acid levels are low. Importantly, given that uric acid is the underlying cause of gout, there are numerous approved therapies to reduce uric acid levels in patients. Our work suggests that such therapies, including a low-purine diet, xanthine oxidase inhibitors (e.g., allopurinol, febuxostat), and uric acid degrading enzymes (e.g., rasburicase, pegloticase) could be utilized in combination with rigosertib to improve therapeutic response (62–65).

As previously mentioned, identifying the precise mechanistic target of rigosertib has been challenging. Rigosertib was initially identified as a PLK1 inhibitor (49, 66–70), and has also been proposed to act as an inhibitor of RAS (71) and PI3K (72). Use of an unbiased CRISPRi/a chemical-genetic approach combined with structural biology studies identified rigosertib as a microtubule destabilizing agent that binds to the colchicine binding site on

β -tubulin (55, 56), a finding that has now been corroborated by other groups (53). Our molecular dynamic simulation studies also suggest that rigosertib binds to the colchicine binding site of β -tubulin and induces conformational changes that are similar to, but distinct from, those induced by colchicine. It is important to note, however, that the crystal structures of tubulin with colchicine or rigosertib are strongly affected by the presence of stathmin, and this limits the interpretation of these structures (55). In addition, docking results suggest that uric acid may act as an uncompetitive inhibitor of rigosertib through interaction with residues in loop S9 – a conclusion that is further confirmed by our CETSA results. Together, these results suggest that the effect of uric acid on rigosertib efficacy is mediated through microtubules and not the other proposed targets of rigosertib.

In vitro tissue culture models offer several advantages over *in vivo* tumor models, including the ability to perform large-scale screening studies. However, there has always been a large bottleneck of promising *in vitro* cancer findings that turn out to be irrelevant in human tumors. While there are many factors that contribute to this bottleneck, our work and that of others has shown that the non-physiological nutrient levels found in traditional culture media can contribute to some *in vitro* and *in vivo* discrepancies. Importantly, unnatural nutrient levels are not an inherent problem of tissue culture, and it is becoming clear that replacement of traditional media with more physiological media can rectify some of the problems with tissue culture systems. And while mouse models will continue to be the gold-standard of pre-clinical cancer studies, it is important to consider that there are also differences between mice and humans that can lead to discrepancies in how cancer cells respond to therapies. Our work demonstrates that use of human physiological media

can lead to clinically relevant findings that otherwise might be missed using traditional tissue culture or mouse models, and we support the notion that use of physiological media is a highly valuable addition to the cancer research pipeline.

METHODS

Cell lines

Cell lines were acquired from the Brugge Lab at Harvard Medical School (HCC1806, SUM149), the Kim Rathmell Lab at Vanderbilt University Medical Center (A498 and Caki2), and the Jiyeon Kim (A549 and Calu6) and Vadim Gaponenko (K562) Labs at the University of Illinois at Chicago. Cell lines were tested for mycoplasma using the MycoAlert Mycoplasma Detection Kit (Lonza) and were authenticated by STR analysis. Cells were grown in human plasma-like medium according to the published formulation (15) with 5% dialyzed fetal bovine serum (FBS) (Sigma) and pen/strep (Invitrogen) at 37°C with 5% CO₂. Media was changed at least every two days. As needed, cells were incubated in RPMI media (Thermo Fischer, 11875-093) with or without uric acid (Sigma) with 5% dialyzed FBS and pen/strep.

Dose curve analysis

To perform dose curve analysis, 2000 cells were seeded in a 96-well plate in corresponding media. The next day cells were treated with the indicated compounds by performing nine-point serial dilutions. The media was removed after 72 hr of drug treatment and cells were fixed with 4% paraformaldehyde (Sigma F8775) for 10 min at room temperature. After fixation cells were washed with PBS and stained with 0.5 µg/ml Hoechst 33342 trihydrochloride (Thermo Fisher, H3570). Cell numbers were determined by imaging and quantifying nuclei using the Celigo imaging cytometer (Nexcelom).

Growth curve analysis

For growth curves 10,000 cells were plated in 12-well plates and were treated with the indicated drugs the following day. Fresh media and drug were added every 2 days. After 5-7 days cells were counted using a Z1 Coulter Particle Counter (Beckman Coulter).

Western blots

Cells were lysed in RIPA buffer (Thermo Fisher, PI89901) containing protease (Thermo Fisher, PI87786) and phosphatase inhibitor (Sigma P572, P0044) cocktails. Protein concentration was determined by BCA assay (Thermo Fisher). Quantified protein samples were separated by electrophoresis on 4–20% ready-made Tris-Glycine gels (Invitrogen) and transferred to PVDF membranes (Millipore). Membranes were blocked with 5% skim milk for 1 hr and incubated overnight with one or more primary antibodies: phospho-Histone H3 (Cell Signaling, 3377S), α -tubulin (Sigma, T9026), β -tubulin (Cell Signaling, 2146S), and β -actin (Sigma, A1978). Western blot quantification was performed using densitometry analysis on ImageJ software.

Intracellular tubulin polymerization assay

50,000 cells per well were plated in a 12 well plate, 24 hours before treatment with increasing concentrations of rigosertib. After drug treatment, the cells were lysed in a hypotonic lysis buffer (1 mM MgCl₂, 2 mM EGTA, 20 mM Tris HCl, pH 6.8, 0.15% IGEPAL, 5 μ M paclitaxel) for 10 minutes at 37°C. The lysates were centrifuged at 15,000 rpm for 10 minutes at room temperature. Equal volumes of the resulting supernatants (S, containing soluble tubulin) and pellet (P, containing microtubules) for each treatment condition were subjected to SDS-PAGE followed by immunoblotting for α -tubulin.

Cell cycle and cell death analysis

For cell cycle analysis cells were treated with 100 nM Rigosertib overnight in corresponding media. The next day cells were washed with PBS, trypsinized, quenched, and washed two times with PBS. After centrifugation, cells were fixed with ethanol at 4°C. Fixed cells were vortexed for 20 min at 4°C, washed 2 times with PBS, and stained with propidium iodide (Thermo Fisher, AAJ66584AB). Stained cells were analyzed using CytoFLEX and Gallios flow cytometers. Data was analyzed using FlowJo software. For the cell death assay cells were treated overnight with 200 nM Rigosertib in corresponding media. Trypsinized cells were suspended in 300 µl FACS buffer and stained with propidium iodide for 30 minutes. Cells were analyzed using a CytoFLEX flow cytometer and data was analyzed using FlowJo software.

Drug screen

The MAPS platform (46) was used to test both a commercial anticancer drug library and a custom-curated metabolic inhibitor drug library. Screen was performed at the ICCB-Longwood Screening Facility (<https://iccb.med.harvard.edu/small-moleculescreening>). SUM149 cells were seeded at a density of 500 cells per well in a final volume of 30 µL per well of 384-well plates. After 24 hr, a Seiko Compound Transfer Robot pin transferred 100 nL of each drug library into wells with plated cells. Following pin-transfer, 20 µL of cell culture medium was added to all wells, resulting in each drug being applied at a final 10-point concentration series ranging from 20 µM to 1 nM. After 72 hr of drug treatment, the cells were washed with PBS, fixed with 4% formaldehyde, and stained with 5 mg/mL

bisbenzimidazole. An Acumen Cellista plate cytometer was used to image plates and determine the cell numbers in individual wells. XY plots were generated comparing relative numbers of surviving RPMI and HPLM cells with concentrations of each drug tested. Area under the curve (AUC) values were calculated for each plot and drugs were ranked based on the difference between the AUCs for RPMI and HPLM cells.

LC-MS metabolite analysis

LC-MS metabolite analysis was performed as previously described (73). Metabolites were extracted using 80% ice cold methanol. A Vanquish UPLC system was coupled to a Q Exactive HF (QE-HF) mass spectrometer equipped with HESI (Thermo Fisher). Chromatographic separation was performed with a SeQuant ZIC-pHILIC LC column, 5 μ m, 150 x 4.6 mm (MilliporeSigma) with a SeQuant ZIC-pHILIC guard column, 20 x 4.6 mm (MilliporeSigma). Mobile phase A was 10 mM $(\text{NH}_4)_2\text{CO}_3$ and 0.05% NH_4OH in H_2O and mobile phase B was 100% ACN. The column chamber temperature was set to 30°C. The mobile phase gradient was as follows: 0-13min: 80% to 20% of mobile phase B, 13-15min: 20% of mobile phase B. ESI ionization was performed in both positive and negative modes. The MS scan range was 60-900 m/z . The mass resolution was 120,000 and the AGC target was 3×10^6 . The capillary voltage was 3.5 KV and the capillary temperature was 320°C. 5 μ L of sample was loaded. LC-MS peaks were manually identified and integrated with EL-Maven (Elucidata) by matching with an in-house library. MetaboAnalyst was used to normalize the peak areas of target metabolites to the median fold change across all identified metabolites, calculate fold changes, and calculate p-values.

GC-MS metabolite analysis

Polar metabolites were prepared for analysis by first drying the samples in individual microcentrifuge tubes, then adding 15 μ L of methoxy amine in pyridine (MOX) (Thermo Fisher) and incubating at 40°C for 90 min. The samples were then further incubated with 20 μ L of N-(tert-butyldimethylsilyl)-N-methyl-trifluoroacetamide with 1% tert-Butyldimethylchlorosilane (TBDMS) (Sigma) at 60 °C for 60 min. The resulting derivatized solution was vortexed briefly, centrifuged, and transferred into polypropylene GC/MS vials (Agilent). Subsequent metabolite abundance analysis was conducted using an Agilent 6890N GC coupled with 5975B Inert XL MS. An Agilent J&W DB-35 ms column was used. Chromatography grade helium (Airgas) was used as the carrier gas, flowing at a rate of 1 mL/min. Depending on sample abundances, either 1 or 2 μ L of samples were injected using either split or splitless modes. The 6890N GC inlet temperature was set to 270 °C, and the oven temperature was initially set to 100 °C, then raised to 300 °C at a rate of 2.5 °C/min. Electron ionization mode with 70 eV was used for 5975B MS measurement. Acquisition was performed using scan mode with a detection range of 150-625 m/z. Mass isotopomer distributions (MIDs) were corrected for natural isotope abundance. Detailed methods are as published (74).

Molecular dynamics simulations

The starting points for our simulations were the pdb structures for colchicine- and rigosertib-bound tubulin (1SA0 (75) and OV7 (55), respectively). We used a single tubulin dimer from each structure, removing the additional proteins that were added to promote

crystallization. As a control, we also removed colchicine from the 1SA0 structure to create apo-tubulin to be used as a reference structure. Each complex had GTP in α -tubulin and GDP in β -tubulin, and we used CGenFF (76) to create initial force-field parameters for both colchicine and rigosertib. Each system was then solvated using TIP3 water with Na⁺ and Cl⁻ added to both neutralize the system charge and set the ionic strength to 50 mM. Simulations were carried out using NAMD(77) using the CHARMM36 (78) force-field. Following heating we performed 0.5 μ s equilibration simulations to remove the effects of the stathmin in the crystal structures. We then performed 4 independent 0.5 μ s simulations of each structure at 300°K in an NpT ensemble with 1 atm pressure. Bonded hydrogens were fixed to allow us to use 2 fs time steps. We employed Particle Mesh Ewald (79) for long-range electrostatics and used a 10 Å cut-off and 8.5 Å switch distance for van der Waals interactions. This resulted in more than 2 μ s simulation data for each of the apo, colchicine and rigosertib systems. Analysis was done using bio3D (80) and images were created using VMD (81).

Molecular docking studies

For uric acid docking studies, we utilized Maestro (Schrödinger LLC, New York). We took a total of 40 different structures from the rigosertib and colchicine simulations (20 from each) for docking of uric acid. The following workflow was used for each structure: 1) the ligand (uric acid) and protein (tubulin-drug complexes) were prepared to be compatible with Maestro applications using LigPrep and ProteinPrep, respectively, 2) generated possible binding sites on the tubulin complex using SiteMap, 3) created receptor grids to be used for docking via Glide, and 4) docked the prepped ligand (uric acid) to the tubulin

complex using ligand docking by Glide. To evaluate the binding free energy of rigosertib with and without uric acid, we utilized the Molecular Mechanics Generalized Born Surface Area methods (MMGBSA) in Prime.

Cellular thermal shift assay (CETSA)

The K562 cells were treated with 40 μ M Rigosertib for 4 hr in the corresponding media. After 4h, cells were washed with 1X PBS. Next, cells were resuspended in PBS containing 1X Halt protease inhibitor cocktail (Thermo Fisher, PI87786) and counted using a Z1 Coulter Particle Counter (Beckman Coulter). Following cell count, 700,000 cells were dispensed in PCR tube and heated at the indicated temperatures for 3 minutes in a thermocycler. After heating, cells were cooled to 20°C and lysed by three cycles of freeze/thaw in liquid nitrogen. Following lysis, denatured proteins were separated by centrifugation at 15,000 rpm for 10 min at 4°C. The lysate was dissolved in 6X loading buffer and run on SDS-PAGE as described in the western blot section.

Statistics

Statistical analyses were performed using GraphPad Prism9 and Microsoft Excel. Unpaired two-tailed t-test was used in most of the experiments. Where applicable one-way ANOVA followed by Tukey's multiple comparisons test and two-way ANOVA was used.

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AUTHOR CONTRIBUTIONS

Conceptualization, J.L.C. and V.R.; methodology, J.L.C., V.R., P.P., M.E.O., P.D., P.G.S, I.H., D.S.; validation, J.L.C., V.R., P.P., A.T, P.D, M.E.O.; formal analysis, J.L.C., V.R., P.P., P.A.B., K.O.C., M.E.O.; investigation, J.L.C., V.R., P.P, M.E.O, A.T., J.E.R., P.D. P.A.B., K.O.C., C.S.,Y.H., A.N., P.G.S., G.M.D., W.K.R., I.H., D.S., F.M.M, G.S., W.D.; resources, J.L.C., V.R, I.H. D.S.; data curation, J.L.C. and V.R.; writing – original draft, J.L.C., V.R., M.E.O., P.P., P.D.; writing, review & editing, J.L.C., V.R., P.P., M.E.O and P.D.; visualization, J.L.C., V.R., and.; supervision, J.L.C.; project administration, J.L.C.; funding acquisition, J.L.C.

FIGURES AND LEGENDS

Figure 1

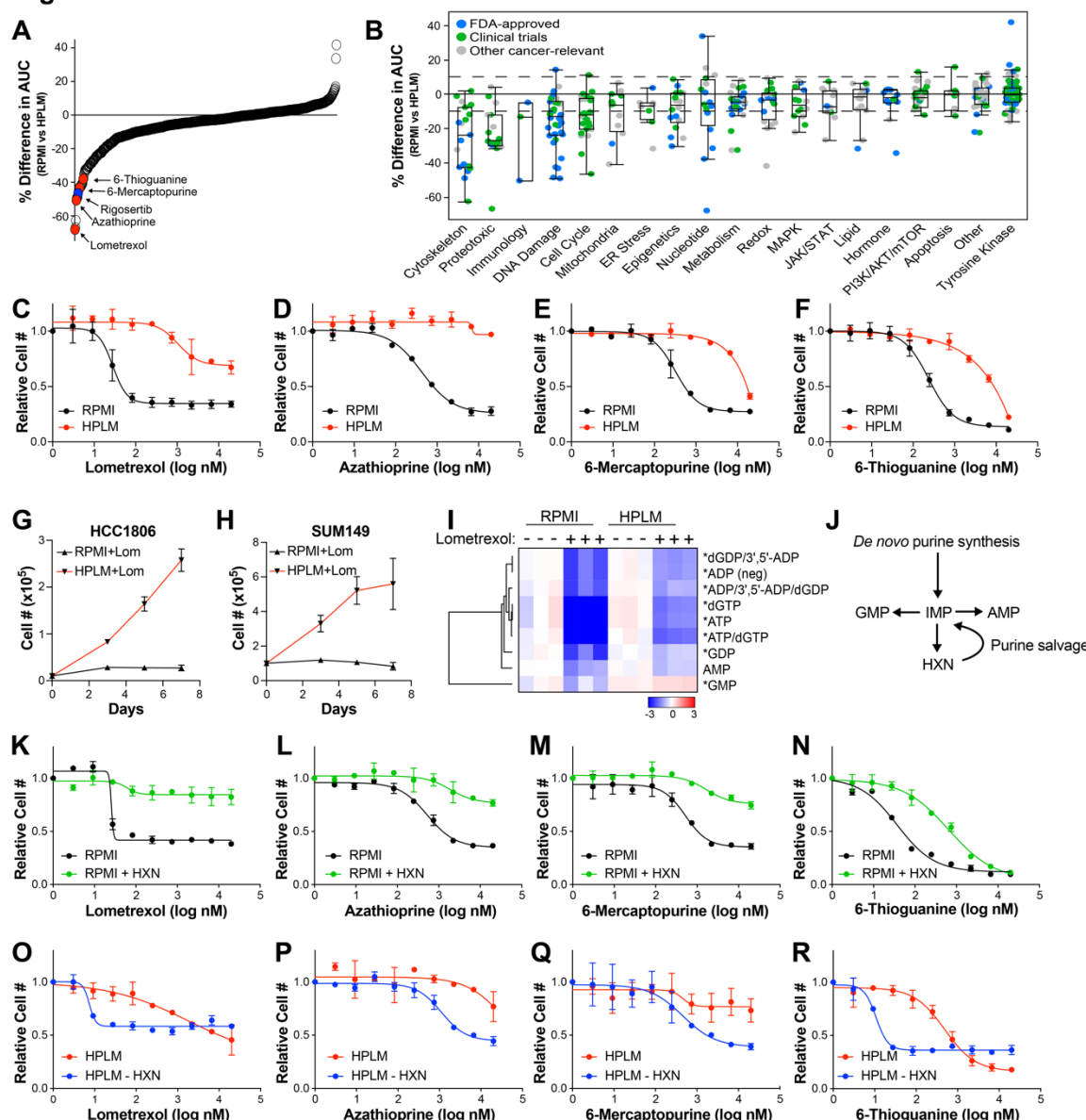


Figure 1. Culture in HPLM changes sensitivity to a variety of therapeutic agents

(A) Percent difference in the area under curve (% Difference in AUC) data for SUM149 cells cultured in either RPMI or HPLM after treatment with anti-cancer and metabolic inhibitor libraries. Only compounds with an Emax >50% in either medium are shown. (B) The same data as in (A) categorized based on target pathway. (C – F) Dose-response curves of the purine biosynthesis inhibitors lometrexol (C), azathioprine (D), 6-mercaptopurine (E), and 6-thioguanine (F) on SUM149 cells growing in RPMI vs HPLM. (G & H) Growth curves of HCC1806 (G) and SUM149 (H) cells treated with lometrexol in RPMI vs HPLM. (I) LC-MS analysis to quantify purine nucleotide abundance in HCC1806 cells treated with lometrexol in RPMI vs HPLM. * indicates $p < 0.05$ for HPLM + lometrexol relative to RPMI + lometrexol (unpaired two-tailed t-test). (J) Schematic representation of purine synthesis and salvage pathways. (K – N) Dose-response curves of the purine

biosynthesis inhibitors lometrexol (K), azathioprine (L), 6-mercaptopurine (M), and 6-thioguanine (N) on SUM149 cells grown in RPMI with and without hypoxanthine (HXN). (O – R) Dose-response curves of the purine biosynthesis inhibitors lometrexol (O), azathioprine (P), 6-mercaptopurine (Q), and 6-thioguanine (R) on SUM149 cells grown in HPLM with and without hypoxanthine (HXN). For all panels data represents the means $\pm$ SD of triplicate samples.

Figure 2

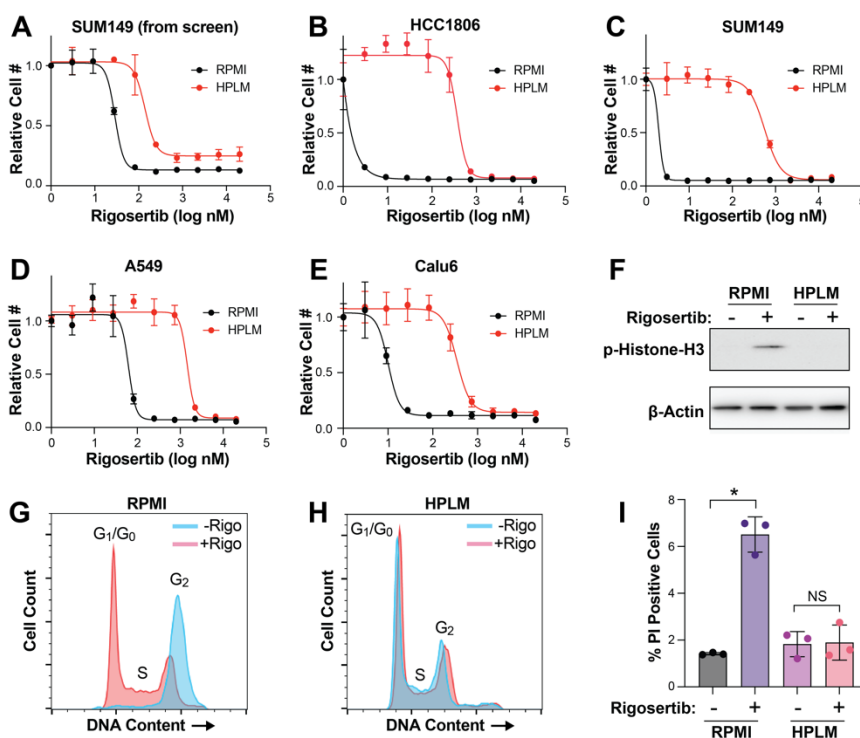


Figure 2. Culture in HPLM reduces sensitivity to rigosertib

(A) Dose response curve of SUM149 cells treated with rigosertib from the high-throughput screen described in Figure 1. Data are the mean $\pm$ SD of triplicate samples. (B – E) Dose response curves for rigosertib treatment of HCC1806 (B), SUM149 (C), A549 (D) and Calu6 (E) cells growing in RPMI vs HPLM. Data are the mean $\pm$ SD of triplicate samples. (F) Representative western blot of phospho-Histone H3 in HCC1806 cells treated with rigosertib in RPMI vs HPLM. (G & H) Cell cycle analysis of HCC1806 cells treated with 150 nM commercial-grade rigosertib in RPMI (G) and HPLM (H). (I) Cell death analysis of HCC1806 cells treated with 200 nM commercial-grade rigosertib in RPMI vs HPLM. Cell death and cell cycle data are the means $\pm$ SD of triplicate samples. * indicates $p < 0.05$ from unpaired two-tailed t-test. NS (not significant) indicates $p > 0.05$.

Figure 3

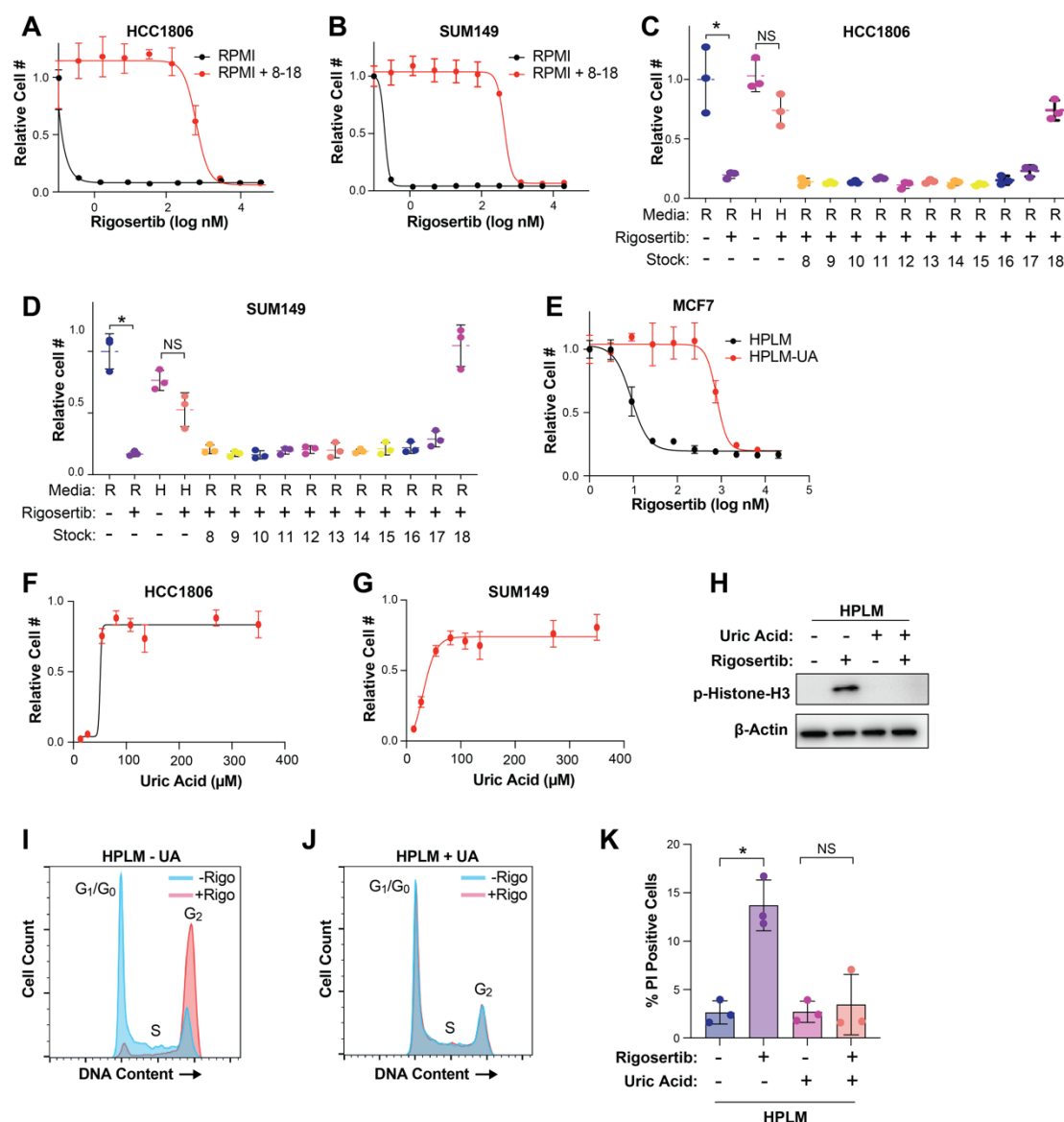


Figure 3. Uric acid prevents the activity of rigosertib

(A & B) Dose response curves of HCC1806 (A) and SUM149 (B) cells treated with rigosertib in RPMI vs RPMI + HPLM stocks 8-18. (C & D) Cell growth assays of HCC1806 (C) and SUM149 (D) cells treated with 80 nM rigosertib in the presence of individual HPLM stocks 8-18. R = RPMI and H = HPLM. (E) Dose response curve of MCF7 cells treated with rigosertib in HPLM vs HPLM - UA. (F & G) Dose response curves of uric acid on HCC1806 (F) and SUM149 (G) cells treated with 80 nM rigosertib. (H) Representative western blot of phospho-Histone H3 in HCC1806 cells treated with 150 nM rigosertib in HPLM vs HPLM - UA. (I & J) Cell cycle analysis of HCC1806 cells treated with 150 nM commercial-grade rigosertib in HPLM (I) and HPLM - UA (J). (K) Cell death analysis of HCC1806 cells treated with 200 nM commercial-grade rigosertib in HPLM and HPLM - UA. For all panels, data is represented as mean $\pm$ SD of triplicate samples. * indicates $p < 0.05$ from unpaired two-tailed t-test. NS (not significant) indicates $p > 0.05$.

Figure 4

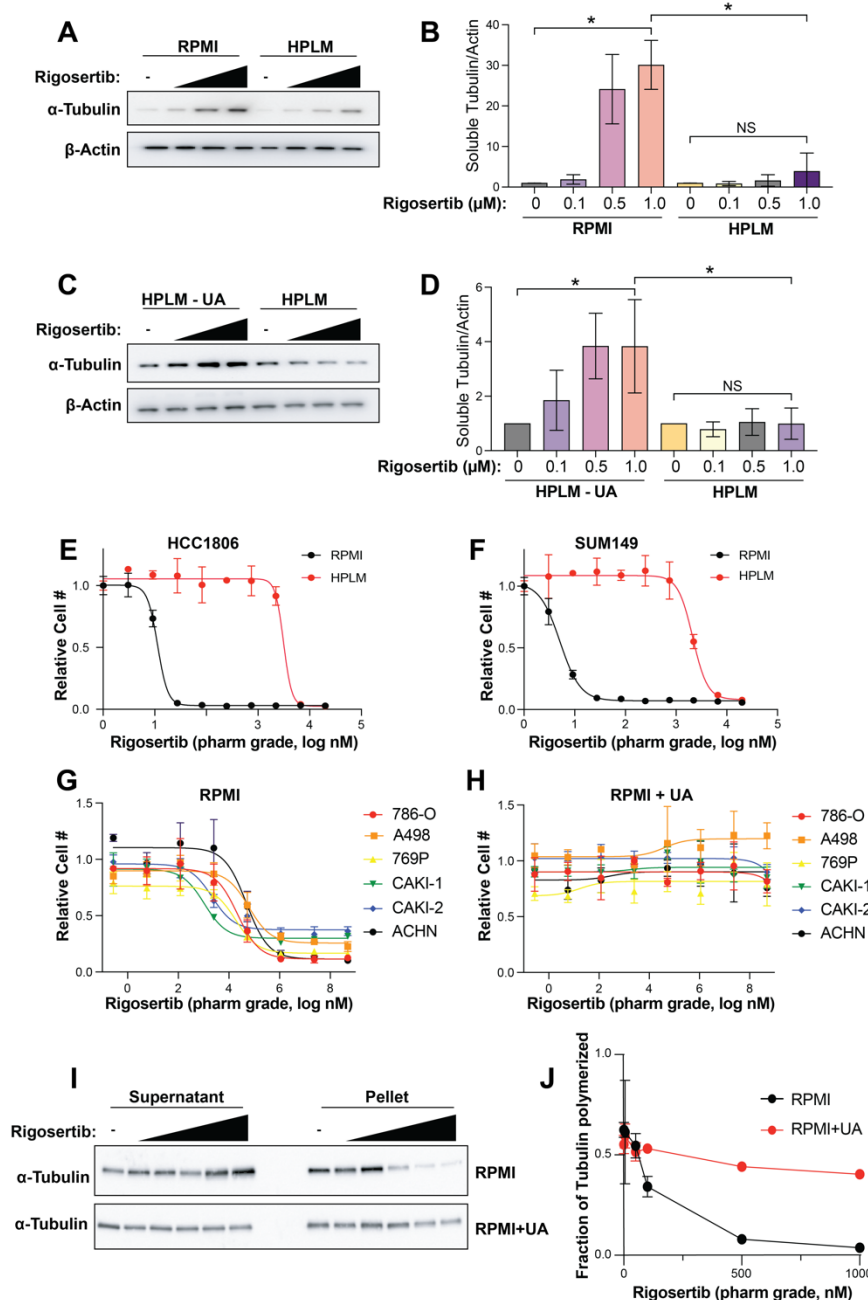


Figure 4. Uric acid inhibits the microtubule destabilizing activity of rigosertib

(A) Western blot of soluble α -tubulin from SUM149 treated with increasing doses of rigosertib (0.1 μ M, 0.5 μ M and 1 μ M) for 4h in RPMI and HPLM. (B) Quantification of western blots from (A). Data is represented as mean $\pm$ SD from three independent experiments. * indicates $p < 0.05$ from one way ANOVA followed by Tukey's multiple comparison test. NS (not significant) indicates $p > 0.05$. (C) Western blot of soluble α -tubulin from SUM149 treated with increasing doses of rigosertib (0.1 μ M, 0.5 μ M and 1 μ M) for 4h in HPLM and HPLM - UA. (D) Quantification of western blots from (C). Data is represented as mean $\pm$ SD from three independent experiments. * indicates $p < 0.05$

from one way ANOVA followed by Tukey's multiple comparison test. NS (not significant) indicates $p > 0.05$. (E & F) Dose response curves of HCC1806 (E) and SUM149 (F) cells treated with pharmaceutical-grade rigosertib in RPMI vs HPLM. (G & H) Dose response curves of a panel of renal cancer cell lines treated with pharmaceutical-grade rigosertib in RPMI (G) vs RPMI + UA (H). (I) Western blot of soluble and pellet α -tubulin from 786-O cells treated with increasing doses (5 nM, 50 nM, 100 nM, 500 nM, 1000 nM) of pharmaceutical-grade rigosertib for 4 hr in RPMI and RPMI + UA. (J) Quantification of western blots from (I). Data is represented as means $\pm$ SD of three independent experiments. * indicates $p < 0.05$ from two-way ANOVA.

Figure 5

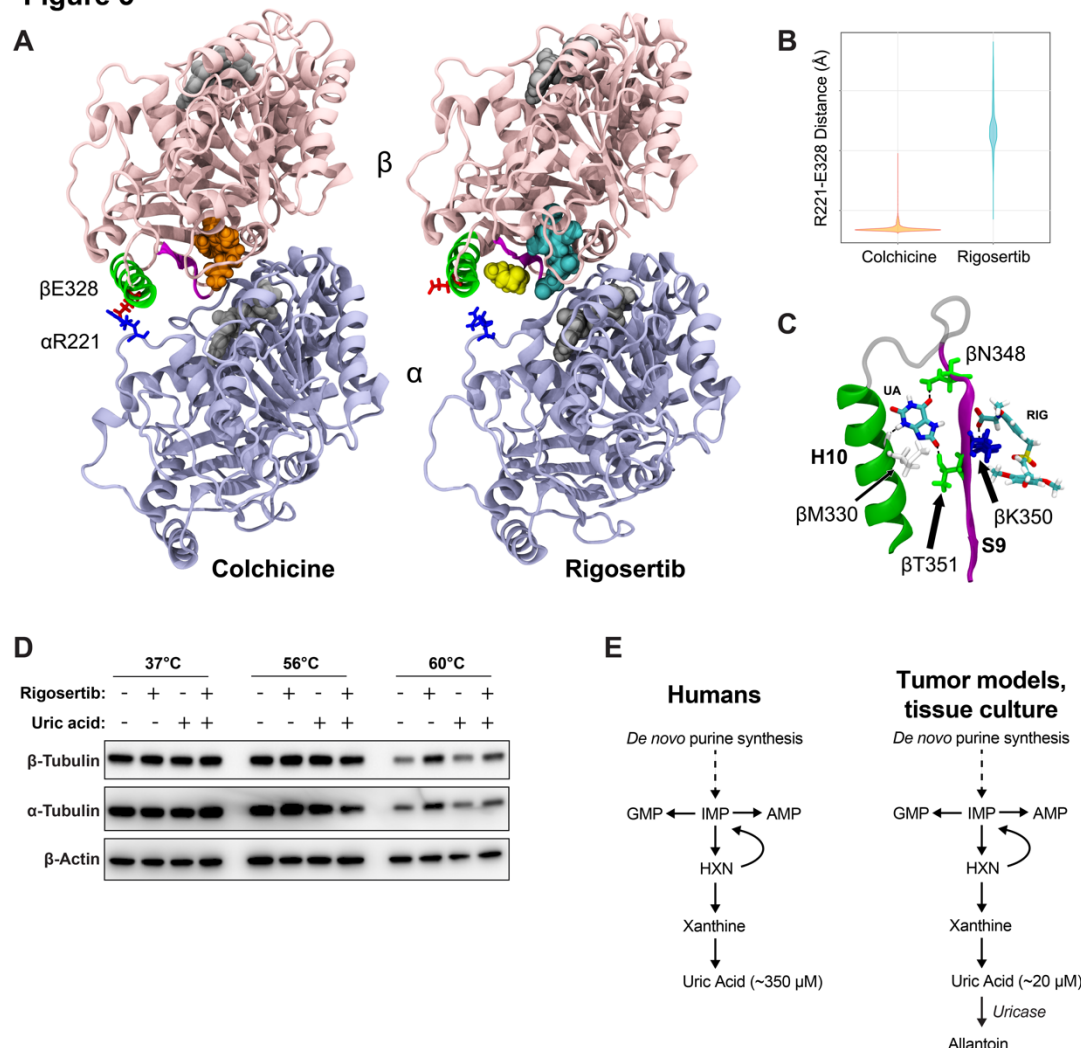
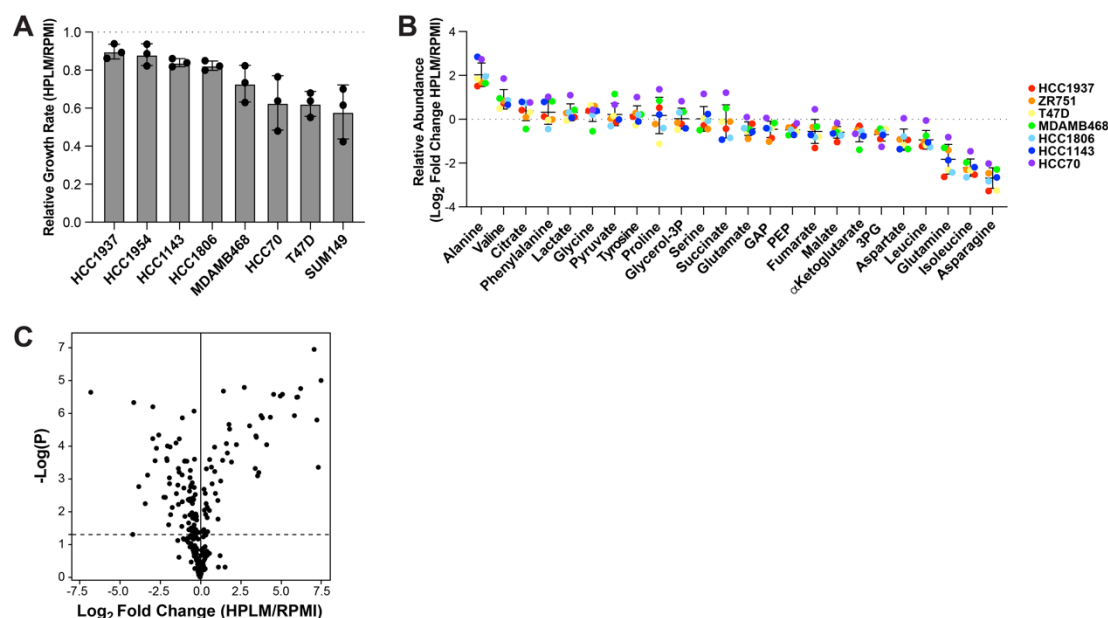


Figure 5. Uric acid inhibits rigosertib activity by reducing the affinity of rigosertib for β-tubulin

(A) Structural comparisons of colchicine-bound and rigosertib-bound tubulin. Colchicine and rigosertib are colored orange and cyan, respectively. The salt bridge between βE328 and αR221 found in colchicine structure is absent in the rigosertib structure, allowing H10 (green) to move away from the dimer body and create a pocket for uric acid (yellow) to bind. (B) Distance between βE328 and αR221 in the colchicine and rigosertib simulations. When this ionic bond is not formed, H10 becomes untethered which creates the binding pocket for uric acid. (C) Molecular details of uric acid binding in the pocket between H10 (green) and S9 (magenta). Residues that form hydrogen-bonds with uric acid are labeled. (D) CETSA analysis of K562 cells treated for 4 hr with 40 μM pharmaceutical-grade rigosertib in RPMI at the indicated temperature. (E) Unlike mice and other model organisms/systems, humans do not express uricase resulting in uniquely high uric acid levels.

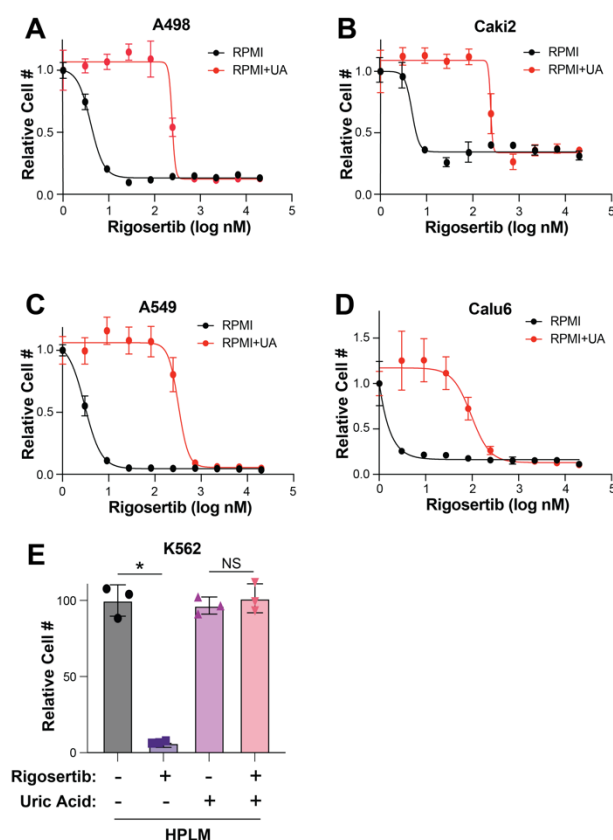
Figure S1



Supplementary Figure 1. Growth of breast cancer cells in HPLM causes remodeling of intracellular metabolite abundance. Related to Figure 1.

(A) Relative growth rate of breast cancer cell lines cultured in HPLM vs RPMI. Data is represented as mean $\pm$ SD from triplicate samples. (B) GC-MS analysis of relative metabolite abundance in breast cancer cells growing in HPLM vs RPMI. Data is represented as the mean of triplicate samples for each cell line. (C) LC-MS analysis of relative metabolite abundance in HCC1806 cells growing in HPLM vs RPMI. Data is represented as the mean of triplicate samples.

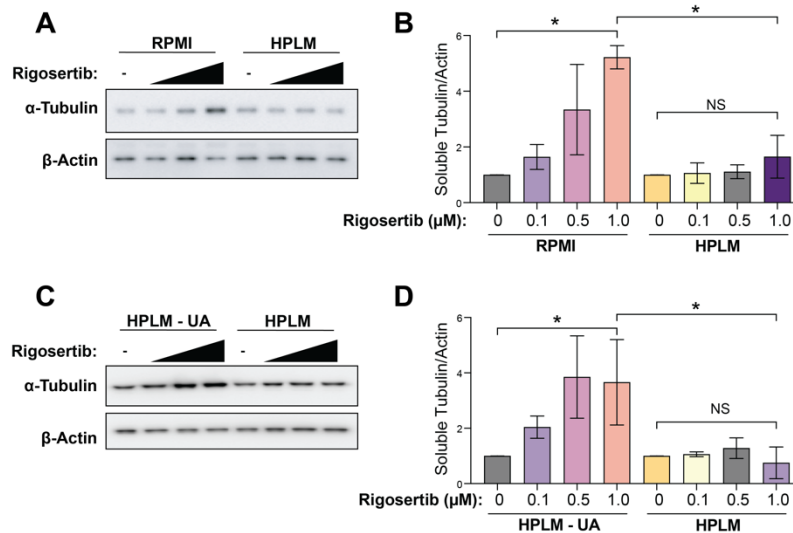
Figure S2



Supplementary Figure 2. Uric acid reduces sensitivity to rigosertib in diverse cell lines. Related to Figure 3.

(A – D) Dose response curves of A498 (A), Caki2 (B), A549 (C), and Calu6 (D) cells treated with rigosertib in RPMI vs RPMI + uric acid. (E) Growth of K562 cells treated with 100 nM rigosertib in HPLM – UA vs HPLM. For all panels, data is represented as mean $\pm$ SD of triplicate samples. * indicates $p < 0.05$ from unpaired two-tailed t-test. NS (not significant) indicates $p > 0.05$.

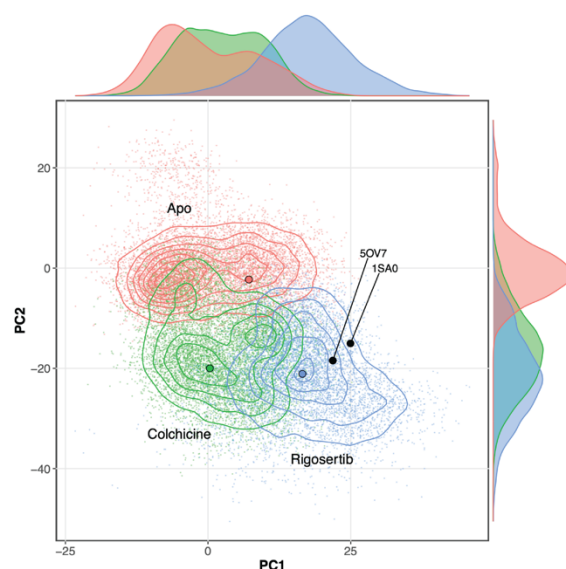
Figure S3



Supplementary Figure 3. Uric acid inhibits the microtubule destabilizing activity rigosertib. Related to figure 4.

(A) Western blot of soluble α -tubulin from HCC1806 treated with increasing doses of rigosertib (0.1 μ M, 0.5 μ M and 1 μ M) for 4 hr in RPMI and HPLM. (B) Quantification of western blots from (A). Data is represented as mean $\pm$ SD from three independent experiments. * indicates $p < 0.05$ from one way ANOVA followed by Tukey's multiple comparison test. NS (not significant) indicates $p > 0.05$. (C) Western blot of soluble α -tubulin from HCC1806 treated with increasing doses of rigosertib (0.1 μ M, 0.5 μ M and 1 μ M) for 4 hr in HPLM and HPLM - UA. (D) Quantification of western blots from (C). Data is represented as mean $\pm$ SD from three independent experiments. * indicates $p < 0.05$ from one way ANOVA followed by Tukey's multiple comparison test. NS (not significant) indicates $p > 0.05$.

Figure S4



Supplementary Figure 4. Rigosertib and colchicine have overlapping effects on tubulin structure.

Comparison of apo (red), colchicine-bound (green), and rigosertib-bound tubulin (blue) using PCA analysis. Crystal structures of rigosertib (5OV7) and colchicine (1SA0) are superimposed on the PCA space, highlighting some of the limited information in the crystal structures. Both colchicine and rigosertib cause a conformational change that makes the dimer non-polymerizable (PC2), but rigosertib induces an additional conformational change that is distinct from colchicine (PC1).

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