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CA191277

TITLE: Dissecting the Biology and Therapeutic Vulnerabilities of RB1-Mutant Osteosarcoma Using RB iPSCs

PRINCIPAL INVESTIGATOR: Dandan Zhu

CONTRACTING ORGANIZATION: The University of Texas Health Science Center at Houston, Houston, TX

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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Organization Name: The University of Texas Health Science Center at Houston, Houston, TX.
Address: 7000 Fannin Street, Houston, TX. 77030.

8. PERFORMING ORGANIZATION REPORT NUMBER

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14. ABSTRACT

RB1 mutations are found in more than 30% of clinical osteosarcoma specimens, highlighting the crucial role of RB1 in preventing bone malignancy. Patients with hereditary retinoblastoma (RB), an inherited autosomal dominant cancer disorder caused by germline mutations/deletions in the RB1 tumor suppressor gene, have a >400-fold increased incidence of osteosarcoma, suggesting a strong mechanistic link between RB1 loss and osteosarcomagenesis.

Our purpose are 1) To elucidate how loss of RB1 contributes to the upregulated FOXM1 transcriptional program; 2) To evaluate the therapeutic potential of FOXM1 inhibitors for RB1-mutant osteosarcoma treatment. We have reprogrammed RB patient's fibroblasts to iPSCs. Then, we further applied CRISPR/Cas9 technology to target the RB1 genomic locus to correct the RB1 mutation in RB iPSCs and created corrected RB iPSCs (cRB) to serve as isogenic controls. We subsequently differentiated wild type, RB, and cRB iPSCs to OBs. Then we conduct ChIP-seq using an anti-RB1 antibody to map genome-wide RB1-binding sites among these iPSC-derived OBs. We found E2F3a but not E2F1 or E2F2 expression is enriched in RB and cRB OBs. RB1 and E2F3a ChIP-seq and ATAC-seq in RB and cRB OBs revealed that both RB1 and E2F3 bind the FOXM1 promoter and that RB OBs contain more open chromatin at FOXM1 promoter region than cRB OBs. Also, our studies of E2F3a and FOXM1 expression in the TCGA SARC database revealed the positive correlation between E2F3a and FOXM1 and high FOXM1 expression is correlated with poor prognosis. Meanwhile, our in vitro competition assay showed that RB OBs display increased sensitivity to Thiostrepton, suggesting that pharmacological inhibition of FOXM1 has potential in treating RB1-mutant cells.

15. SUBJECT TERMS

RB1, Osteosarcoma, iPSCs, FOXM1, Therapeutic Volunerability

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
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TABLE OF CONTENTS

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

RB1 mutations are found in more than 30% of clinical osteosarcoma specimens, highlighting the crucial role of RB1 in preventing bone malignancy. Patients with hereditary retinoblastoma (RB), an inherited autosomal dominant cancer disorder caused by germline mutations/deletions in the RB1 tumor suppressor gene, have a >400 fold increased incidence of osteosarcoma, suggesting a strong mechanistic link between RB1 loss and osteosarcomagenesis. We found that a significant decrease of FOXM1 transcription and its downstream targets during osteogenic differentiation but there is an elevated FOXM1 expression in RB OBs with RB1 mutation. Here, we plan to elucidate how loss of RB1 contributes to upregulated FOXM1 transcriptional program. Moreover, we evaluate the therapeutic potential of FOXM1 inhibitors for RB1-mutant osteosarcoma.

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

RB1, Osteosarcoma, iPSCs, FOXM1, therapeutic vulnerability

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.

The major goals of this project as stated in the approved SOW are as following.

Major goal 1. Elucidate how loss of RB1 contributes to the upregulated FOXM1 transcriptional program

Subtask 1: Conduct ChIP-seq assay to comprehensively identify preferential binding of RB1 and E2F3a to specific promoters in OBs. Timeline: 1-12 months. Progress: 100% completed at May 14, 2021.

Subtask 2: Study the expression of RB1, FOXM1 and E2F3a in human primary osteosarcoma specimens (US Biomax, OS408 osteosarcoma tissue array) by immunohistochemical (IHC) staining antibodies. Timeline: 13-18 months. Progress: 10% completed at May 14, 2021.

Subtask 3: Investigate the clinical relevance of RB1, E2F3a and FOXM1 using public TARGET and TCGA databases. Timeline: 19-24 months. Progress: 30% completed at May 14, 2021.

Major goal 2: Evaluate the therapeutic potential of FOXM1 inhibitors for RB1-mutant osteosarcoma treatment.

Subtask 1: Perform an *in vitro* competition assay to determine if upregulated FOXM1 is required for cell viability of RB1 mutant cells. Timeline: 6-12 months. Progress: 100% completed at May 14, 2021.

Subtask 2: Perform CFSE cell proliferation and Annexin V assays to assess apoptosis of RB1-mutant OBs. Timeline: 13-18 months. Progress: 10% completed at May 14, 2021.

Subtask 3: Examine the therapeutic effect of FOXM1 inhibitor Thiostrepton to treat RB1-mutant cells. Timeline: 19-24 months. Progress: N/A.

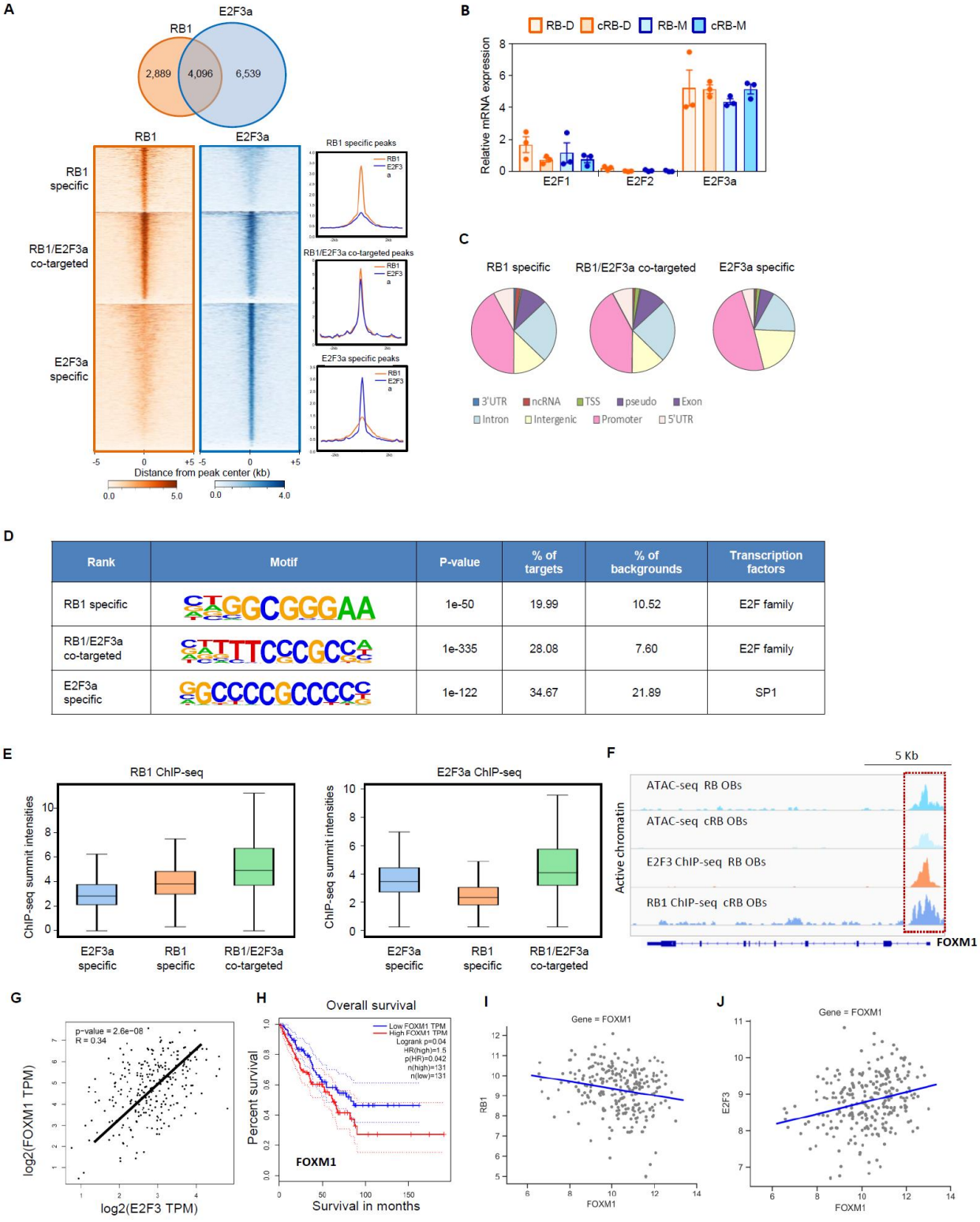
For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include 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 activity 1. Apply ChIP-seq to provide a pathological mechanism to link RB1 loss with aberrant FOXM upregulation.

Subtask 1: To comprehensively identify preferential binding of RB1 to specific promoters in OBs, we conducted ChIP-seq using an anti-RB1 antibody to map genome-wide RB1-binding sites among RB and WT iPSC-derived OBs. In Figure 1A, (Top)Venn diagram depicts the overlap between RB1 and E2F3a binding peaks in cRB_OBs defines RB1 specific (2,889), RB1/E2F3a co-targeted (4,096), and E2F3a specific (6,539) loci. Heat map (Bottom left) depicts RB1 and E2F3a binding to the 5kb genomic loci surrounding identified ChIP-seq peaks, grouped by cluster. Composite plots (Bottom right) show average binding of RB1 and E2F3a to the RB1-specific, RB1/E2F3a co-targeted, and E2F3a-specific loci. As showed in Figure 1B, we examined the genome wide binding of RB1 and E2F3a in OBs. E2F3a but not E2F1 or E2F2 expression is enriched in RB and cRB OBs. (n=3 biological replicates; error bars represent ±SEM; statistical significance is examined by two-tailed Student t-test; *$p< 0.05$; **$p< 0.01$; ***$p< 0.001$). Pie chart showing genomic distribution of RB1 specific, RB1 and E2F3a co-targeted, and E2F3a specific binding sites for known RefSeq genes. Most binding sites locate in gene promoters (Figure 1C). Motif analyses report the top-scoring binding motif for RB1-specific, RB1/E2F3a co-occupied, and E2F3a-specific genomic binding peaks in cRB OBs (Figure 1D). ChIP-seq summit peak intensities of total RB1 ChIP-seq signals at RB1-specific, RB1/E2F3a co-targeted, E2F3a-specific sites (Figure 1E, Left panel). (Right Panel) ChIP-seq summit peak intensities of total E2F3a ChIP-seq signals at RB1-specific, RB1/E2F3a co-targeted, E2F3a-specific sites (Figure 1E, Right panel). Encouragingly, our studies of RB1 and E2F3a ChIP-seq and ATAC-seq in RB and cRB OBs revealed that both RB1 and E2F3a bind to the FOXM1 promoter and that RB OBs contain more open chromatin in FOXM1 promoter region than cRB OBs (Figure 1F).

Subtask 2: Study the expression of RB1, FOXM1 and E2F3a in human primary osteosarcoma specimens (US Biomax, OS408 osteosarcoma tissue array) by immunohistochemical (IHC) staining with specific antibodies. To examine whether our findings from the RB iPSC disease model translate generally to human primary tumors, we are now studying the expression of RB1, FOXM1 and E2F3a in human primary osteosarcoma specimens (US Biomax, OS408 osteosarcoma tissue array) by immunohistochemical (IHC) staining with specific antibodies. Now, we are testing the suitability of RB, FOXM1 antibodies for IHC staining.

Subtask 3: Investigate the clinical relevance of RB1, E2F3a and FOXM1 using public TARGET and TCGA databases. We studied E2F3a and FOXM1 expression in the TCGA SARC database and revealed the positive correlation between E2F3a and FOXM1 and high FOXM1 expression is correlated with poor prognosis (Fig. 1G-J). Currently, we start to analyze TARGET osteosarcoma database.



Rank	Motif	P-value	% of targets	% of backgrounds	Transcription factors
RB1 specific		1e-50	19.99	10.52	E2F family
RB1/E2F3a co-targeted		1e-335	28.08	7.60	E2F family
E2F3a specific		1e-122	34.67	21.89	SP1

Figure 1. ChIP-seq provides a pathological mechanism to link RB1 loss to aberrant FOXM upregulation. (A) RB1 and E2F3a directly co-target spliceosomal genes. Top, Venn diagram depicts the overlap between RB1 and E2F3a binding peaks in cRB OBs defines RB1 specific (2,889), RB1/E2F3a co-targeted (4,096), and E2F3a specific (6,539) loci. Bottom left, Heat map depicts RB1 and E2F3a binding to the 5kb genomic loci surrounding identified ChIP-seq peaks, grouped by cluster. Bottom right, Composite plots show average binding of RB1 and E2F3a to the RB1-specific, RB1/E2F3a co-targeted, and E2F3a-specific loci. (B) Genome wide binding examination of the expression of RB1-interacting E2F1, E2F2, and E2F3a in cRB OBs. E2F3a but not E2F1 or E2F2 expression is enriched in RB and cRB OBs. n=3 biological replicates; error bars represent ±SEM; statistical significance is examined by two-tailed Student t-test; *p< 0.05; **p< 0.01; ***p< 0.001. (C) Pie chart showing genomic distribution of RB1 specific, RB1 and E2F3a co-targeted, and E2F3a specific binding sites for known RefSeq genes. Most binding sites locate in gene promoters. (D) Motif analyses report the top-scoring binding motif for RB1-specific, RB1/E2F3a co-occupied, and E2F3a-specific genomic binding peaks in cRB OBs. (E) Left panel, ChIP-seq summit peak sites, intensities of total RB1 ChIP-seq signals at RB1-specific, RB1/E2F3a co-targeted, E2F3a-specific. Right Panel, ChIP-seq summit peak intensities of total E2F3a ChIP-seq signals at RB1-specific, RB1/E2F3a co-targeted, E2F3a-specific sites. (F) ATAC-seq and ChIP-seq analyses. RB1/E2F3a binds the FOXM1 promoter in RB and cRB OBs, and that RB OBs contain more open chromatin in the FOXM1 promoter regions than cRB Obs. (G-J) Analysis of TCGA SARC. A positive correlation between E2F3a and FOXM1 (p=2.6e-08; R=0.34). High expression of FOXM1 is correlated to poor patient survival.

Major activity 2. Evaluate the therapeutic potential of FOXM1 inhibitors for RB1-mutant osteosarcoma treatment. Timeline: 6-24 months, 50% completed.

Subtask 1: We performed an in vitro competition assay to determine if upregulated FOXM1 is required for cell viability of RB1-mutant cells. We observed upregulation of FOXM1 in RB OBs, RB1-knockdown and RB1-null iPSC-derived OBs (Fig. 2A). In contrast, ectopic expression of RB1 led to FOXM1 downregulation (Fig. 2B). Based on these findings, we conclude that one of the tumor suppressor functions of RB1 is mediated through controlling FOXM1-mediated gene expression. To determine whether RB1-mutant cells are more sensitive to perturbation of FOXM1-regulated G2/M transition machinery, we performed an in vitro competition assay and found upregulated FOXM1 is required for cell viability of RB1-mutant cells. This fluorescence- and proliferation-based assay will reveal compromised cell viability in its readout of a decreasing GFP+ /GFP− ratio during co-culture of GFP+ FOXM1-depleted OBs and GFP− control cells. Encouragingly, our in vitro competition assay showed that RB OBs display increased sensitivity to Thiostrepton (Fig. 2C), suggesting that pharmacological inhibition of FOXM1 has potential in treating RB1-mutant cells.

Subtask 2: Perform CFSE cell proliferation and Annexin V assays to assess apoptosis of RB1-mutant OBs. We will apply CellTrace CFSE cell proliferation and Annexin V (Invitrogen) assays to examine if partial depletion of FOXM1 will significantly trigger RB1-mutant OBs to lose their viability and increase apoptosis. Currently, we designed and constructed the FOXM1 shRNA plasmids, and are testing them for the deletion efficiency of FOXM1 in osteoblasts.

Subtask 3: Examine the therapeutic effect of FOXM1 inhibitor Thiostrepton to treat RB1-mutant cells. Currently, we start to evaluate if RB1-mutant osteosarcomas are sensitive to FOXM1 inhibition.

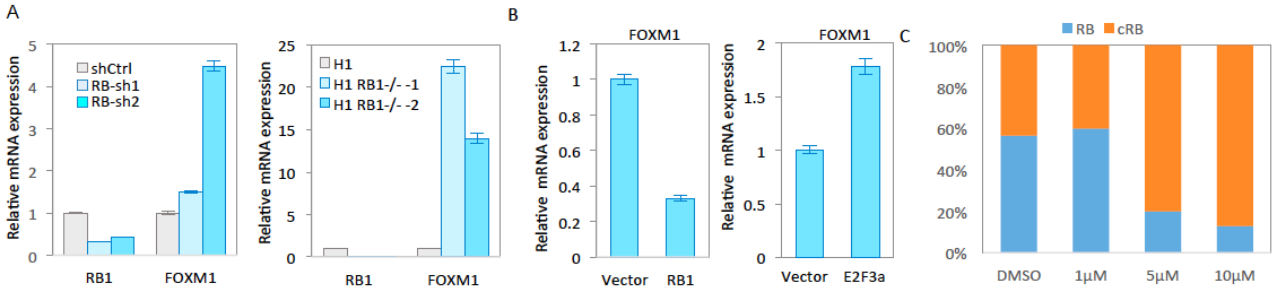


Figure 2. Evaluation of the therapeutic potential of FOXM1 inhibitors for RB1-mutant osteosarcoma. **(A)** Upregulation of FOXM1 in RB, RB1-knockdown, and RB-knockout OBs. **(B)** RB1 inhibits FOXM1 expression through the negative regulation of E2F3a. **(C)** In vitro perturbation of FOXM1 function impairs RB1-deficient cell growth.

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.

With the promising project and the mentor's outstanding guidance, I got many opportunities for training and professional development.

First, considering the scientific writing is an essential skill for postdocs to develop their careers, I start to write the manuscript and Dr. Lee gives me many suggestions and guidance on the manuscript writing in every detail.

Second, to accomplish my project "Dissecting the Biology and Therapeutic Vulnerabilities of RB1-mutant Osteosarcoma Using RB iPSCs", my mentor Dr. Lee already arranged interactions for me with the experts from UTHealth, MDACC and the greater stem cell, cancer biology and computational biology fields, whose members Dr. Lee knows very well. In fact, the PI has already collaborated with Dr. Jason Yustein (an expert in translation research in Baylor College of Medicine) and Dr. Lisa L. Wang (an osteosarcoma expert in Texas Children's Hospital) for her research project. With their involvements, I have the opportunities to learn how to do primary culture of osteosarcoma cells from patients. In regards of the computational biology field, I have learned how to use TCGA and GEO database. In addition, to elucidate how loss of RB1 contributes to upregulated FOXM1 transcriptional program, I learned how to perform ChIP-seq and ATAC-seq as well as analysis using different bioinformatics softwares.

Moreover, my mentor always encourages me to attend weekly lab meeting, to join department journal club, IBP and BMB departmental seminars, Medical School postdoc program activities as well as research-related talks in other departments and institutions within the medical center such as Bone club meeting hosted by BCM and Sarcoma meeting hosted by Texas Children's Hospital. For example, I have attended our Department Seminar Series "Osteosarcoma Epidemiology and Inherited Genetic Susceptibility" given by Lisa Mirabello (NIH) on April 26, 2021. Recently, I just attended Bone Disease Program of Texas "The challenge of Osteoporosis Treatment" given by Robert A. Adler (Hunter Holmes McGuire Veterans Affairs Medical Center) on May 7, 2021.

Finally, UTHealth locates in Texas Medical Center that is the greatest and well-known medical center in the world. During last year, I had many training opportunities and professional development activities that provided by this project.

What do you plan to do during the next reporting period to accomplish the goals?
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.

As stated in SOW, I plan to accomplish the following tasks during the next reporting period.

Major goal 1. Elucidate how loss of RB1 contributes to upregulated FOXM1 transcriptional program.
Subtask 2: To examine whether our findings from the RB iPSC disease model translate generally to human primary tumors, we will study the expression of RB1, FOXM1 and E2F3a in human primary osteosarcoma specimens (US Biomax, OS408 osteosarcoma tissue array) by immunohistochemical (IHC) staining with specific antibodies. We will further compare individual expressions of these genes and variants in osteosarcoma tissues, and reveal their correlation.

Major goal 2: Evaluate the therapeutic potential of FOXM1 inhibitors for RB1-mutant osteosarcoma treatment.
Subtask 2: Perform CFSE cell proliferation and Annexin V assays to assess apoptosis of RB1-mutant OBs. We will examine if partial depletion of FOXM1 will significantly trigger RB1-mutant OBs to lose their viability and increase apoptosis.

4. **IMPACT:** D*escribe 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).

Cancer starts when cells in the body begin to grow out of control. Cells in nearly any part of the body can become cancer, and then spread to other parts of the body. Osteosarcoma is the most common type of bone cancer, comprises almost 60% of bone tumors. RB1 is a protein controls normal cell growth. The loss of RB1 due to its gene mutations or deletions are found in more than 30% of clinical osteosarcoma specimens. Patients with hereditary retinoblastoma (RB), an inherited autosomal dominant cancer disorder, have more than 400-fold increased incidence of osteosarcoma, suggesting a strong mechanistic link between RB1 loss and the initiation of bone cancer. Although mice offer many advantages when conducting cancer research, unlike humans, Rb1 knockout mice do not develop OS, suggesting the urgent requirement for alternative disease models to understand how RB1 mutation leads to the bone cancer initiation.

Taking advantage of state-of-the-art induced pluripotent stem cells (iPSCs) and genome-editing technologies, such as CRISPR/Cas9 methodologies, we generated first human RB iPSCs from RB patient. iPSCs are stem cells derived from skin cells that can be transformed into any cell in

the body. We applied these RB iPSCs to elucidate the pathological mechanisms of osteosarcoma-genesis caused by RB1 mutation. Meanwhile, we evaluated the therapeutic potential of a drug (Thiostrepton) to treat RB1-mutant osteosarcoma. This primary exploration in the lab are the first step to explore the potential of these inhibitors for clinical trials in treating bone cancer with RB mutations in the future.

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.

RB1 mutations have been identified in a spectrum of human cancers. We expect our research outcomes will not only benefit osteosarcoma patients, but also other cancer patients such as breast cancer, bladder cancer, and small cell lung cancer with RB mutations. Since osteosarcoma occurs not only in children, but also in young adults and older adults, our research will definitely benefit all active duty military service members, their families, veterans, and other military beneficiaries and will improve qualities of life for patients and their families.

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:*

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

Nothing to Report

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

Nothing to Report

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.

Nothing to Report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

Nothing to Report

Significant changes in use or care of vertebrate animals

Nothing to Report

Significant changes in use of biohazards and/or select agents

Nothing to Report

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

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

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

A Xu, MF Huang, **D Zhu**, JA Gingold, DA Bazer, B Chang, D Wang, CC Lai, IR Lemischka, R Zhao, DF Lee. LncRNA H19 Suppresses Osteosarcomagenesis by Regulating snoRNAs and DNA Repair Protein Complexes. Front Genet. 2021 Jan 15;11:611823. PMID: 33519915.

  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.*

Nothing to Report

- **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.

Our expertise in iPSC technology enables us to optimize a model system to recapture RB1-associated bone malignancies and explore therapeutic interventions. We established the first iPSC platform to delineate the pathological mechanisms of RB1mutation in tumorigenesis. This platform could be shared by cancer, bone as well as other research community.

- **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.*

(1) This is the first iPSC platform to delineate the pathological mechanisms of RB1 mutation in tumorigenesis.
(2) This research will lay the groundwork for future application of FOXM1 inhibitors (like Thiostrepton) to selectively target RB1-mutant osteosarcoma.

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:	*Dandan Zhu*
Project Role:	*Principle Investigator*
Name:	*Dung-Fang Lee*
Project Role:	*Mentor*

No change

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

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

No change

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

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

Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

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

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

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BRIEF RESEARCH REPORT
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LncRNA H19 Suppresses Osteosarcomagenesis by Regulating snoRNAs and DNA Repair Protein Complexes

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Osteosarcoma is one of the most frequent common primary malignant tumors in childhood and adolescence. Long non-coding RNAs (lncRNAs) have been reported to regulate the initiation and progression of tumors. However, the exact molecular mechanisms involving lncRNA in osteosarcomagenesis remain largely unknown. Li-Fraumeni syndrome (LFS) is a familial cancer syndrome caused by germline p53 mutation. We investigated the tumor suppressor function of lncRNA H19 in LFS-associated osteosarcoma. Analyzing H19-induced transcriptome alterations in LFS induced pluripotent stem cell (iPSC)-derived osteoblasts, we unexpectedly discovered a large group of snoRNAs whose expression was significantly affected by H19. We identified SNORA7A among the H19-suppressed snoRNAs. SNORA7A restoration impairs H19-mediated osteogenesis and tumor suppression, indicating an oncogenic role of SNORA7A. TCGA analysis indicated that SNORA7A expression is associated with activation of oncogenic signaling and poor survival in cancer patients. Using an optimized streptavidin-binding RNA aptamer designed from H19 lncRNA, we revealed that H19-tethered protein complexes include proteins critical for DNA damage response and repair, confirming H19's tumor suppressor role. In summary, our findings demonstrate a critical role of H19-modulated SNORA7A expression in LFS-associated osteosarcomas.

Keywords: osteosarcoma, H19 lncRNA, iPSCs, Li-Fraumeni syndrome, snoRNA, p53

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INTRODUCTION

Li-Fraumeni syndrome (LFS) (OMIM #151623) is a rare familial cancer syndrome characterized by early onset of various tumors, soft-tissue sarcomas, osteosarcomas, breast cancers, brain tumors, adrenocortical carcinomas, and leukemia (Zhou et al., 2017). Germline mutations in the p53 tumor suppressor gene are responsible for LFS. Patient-derived iPSCs have been used to model various diseases (Lee et al., 2009b; Carvajal-Vergara et al., 2010; Itzhaki et al., 2011; Yagi et al., 2011; Mulero-Navarro et al., 2015; Gingold et al., 2016; Liu et al., 2018; Zhu et al., 2018). The development of various refined differentiation protocols utilizing induced pluripotent stem cells (iPSCs) has enabled the production of large quantities of differentiated cells from individual patients. In our previous studies, using a LFS patient-derived iPSC model, we demonstrated the tumor suppressor role of lncRNA H19 and the oncogenic role of SFRP2 during the formation of osteosarcoma in LFS patients (Lee et al., 2015; Lin et al., 2017b; Kim et al., 2018; Zhou et al., 2018). Although H19-mediated tumor suppression has been demonstrated in LFS-associated osteosarcomagenesis, the underlying mechanisms of its tumor suppressor activity remain unclear.

Long non-coding RNAs (lncRNAs) represent a class of nonprotein-coding RNAs longer than 200 nucleotides (Sanchez Calle et al., 2018). Despite not being translated into proteins, lncRNAs are important regulators of diverse biological processes and pathologies. LncRNAs are proposed to function via multiple mechanisms, including co-transcriptional regulation, bridging proteins and chromatin, offering cytoplasmic scaffolding, pairing with other RNAs, and serving as molecular decoys (Ulitsky and Bartel, 2013).

One of the most studied lncRNAs is the imprinted lncRNA H19, located on human chromosome 11 and expressed exclusively from the maternal allele (Gabory et al., 2009). Numerous functional studies have assessed the role of H19 in the pathogenesis of human cancers and yielded conflicting results. On one hand, H19 is a precursor of miR-675 (Keniry et al., 2012) as well as a "molecular sponge" for soaking up microRNAs let-7 (Kallen et al., 2013; Li et al., 2020) and miR-138 (Liang et al., 2015), supporting a role in promoting cancer cell proliferation and migration. On the other hand, *in vivo* mouse (Yoshimizu et al., 2008) and LFS iPSC-derived osteoblast (Lee et al., 2015) studies demonstrated that H19 displays a tumor-suppressive effect. Evidence of numerous human tumors displaying either overexpression or lack of expression of H19 (Hao et al., 1993; Lustig-Yariv et al., 1997; Yoshimizu et al., 2008; Lee et al., 2015; Li et al., 2020) suggests the possibility of context-dependent oncogenic and tumor-suppresive roles.

Small nucleolar RNAs (snoRNAs) are small non-coding RNAs 60–300 nucleotides in length, primarily located in the nucleolus. The main functions of snoRNAs are to assist with post-transcriptional modification and maturation of ribosomal RNAs (rRNAs), small nuclear RNA (snRNAs), and other cellular RNAs. SnoRNAs can interact with RNA binding proteins to form small nuclear ribonucleoproteins. SnoRNAs are divided into two classes according to their catalytic activity: C/D box snoRNAs, catalyzing 2-O-ribose methylation; and H/ACA box snoRNAs, catalyzing pseudouridylation (Bachellerie et al., 2002). Emerging evidence indicates that snoRNAs are widely involved in various cancer-related signaling pathways. For example, SNORA42 is overexpressed in non-small cell lung cancer (NSCLC) and plays an oncogenic role through suppressing p53 function and/or expression (Mei et al., 2012). SNORD76 leads to the activation of the WNT/β-Catenin pathway to promote hepatocellular carcinoma (HCC) tumorigenicity (Wu et al., 2018).

The DNA damage/repair response plays a key role in maintaining genome integrity and stability, and its dysfunction girds the development and progression of various cancer types. Recently, studies revealed that lncRNA also regulates DNA damage response and DNA repair through the ATM/ATR (Wan et al., 2013), p53 regulatory network (Hu et al., 2018), and DNA double-strand break repair pathway (Gazy et al., 2015). However, it remains unclear whether H19 is actively involved in DNA damage/repair response.

Our current study reveals that H19 functions as a tumor suppressor through negatively regulating the expression of oncogenic SNORA7A in the osteoblast context. Furthermore, by exploring its interactions with multiple essential DNA damage/repair response factors, we demonstrate how H19 executes its tumor-suppressive role.

MATERIALS AND METHODS

Cell Cultures

U2OS cells were maintained in DMEM (Invitrogen, USA) supplemented with 10% FBS and 1% penicillin/streptomycin. iPSCs were maintained on Matrigel (Corning, USA)-coated plates in StemMACS™ iPS-Brew XF medium (MiltenyiBiotec, USA). All cells were cultured in a humidified incubator at 37°C and 5% CO2 and were tested to exclude mycoplasma contamination.

Differentiation of iPSCs to Mesenchymal Stem Cells (MSCs) and Then to Osteoblasts

In vitro differentiation of LFS iPSCs to MSCs was performed by a PDGF-AB-based method described previously (Lian et al., 2007; Zhou et al., 2018). Appropriately differentiated CD105+/CD166+/CD24- MSCs were plated in a 6-well plate at a density of 2×10^4 cells per well and cultured in an osteogenic differentiation medium (α-MEM supplemented with 10% FBS, 10 mM β-glycerol phosphate, 200 μM ascorbic acid, and 0.1 μM dexamethasone) to induce osteogenic differentiation as previously described (Lee et al., 2015).

RNA Isolation and RNA-Sequencing

LFS osteoblasts with ectopic H19 expression and vector control osteoblasts were collected and compared for H19-mediated tumor suppressor effects. Total mRNA was isolated using TRIzol reagent (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. All RNA sample preparation and RNA-sequencing (RNA-seq) data analyses were performed as described previously (Lee et al., 2015). RNA was aggregated

from biological triplicate experiments prior to running as a single sample for RNA-seq. The FPKM (fragments per kilobase of exon model per million reads mapped) of genes, lncRNAs, snoRNAs, and miRNAs were calculated and summarized in **Supplementary Table 1**.

Enrichr Analysis

GO Biological Process (GO_BP) and Wikipathway analyses were performed using Enrichr (https://amp.pharm.mssm.edu/Enrichr/) (Chen et al., 2013; Kuleshov et al., 2016) to identify enriched biological processes and pathways in H19-expressing LFS osteoblasts. Processes and pathways enriched with a $p < 0.05$ were considered significant. These genes were analyzed in Enrichr using Wikipathway and GO_BP pathway datasets to identify enriched GO terms, pathways, and functions. The TRANSFAC and JASPAR PWMs databases of transcription factors were used in Enrichr to identify transcription factors positively and negatively correlated with SNORA7A expression.

snoRNA Analysis

To determine pathological functions in osteosarcomagenesis significantly associated with SNORA7A, we identified genes positively and negatively correlated with SNORA7A expression in the TCGA-SARC dataset from SNORic (snoRNA in cancers; http://bioinfo.life.hust.edu.cn/SNORic/basic/) (Gong et al., 2017). Correlation of SNORA7A and mRNA expression of significant genes was run at default settings. Clinical analysis of 5-year survival in different cancers was analyzed from TCGA.

Plasmid Construction

A modified 1x streptavidin-binding RNA aptamer (S1m) (Leppek and Stoecklin, 2014) was synthesized (Integrated DNA Technologies, USA) from DNA oligo pairs (S1m_Forward: AATTGgtagaaaATGCGGCCGCCGACCAGAATCATGCAAGTGCGTAAGATAGTCGCGGGTCGGCGGCCGCATctgctgggG; S1m_Reverse: AATTCcccagcagATGCGGCCGCCGACCCGCGACTATCTTACGCACTTGCATGATTCTGGTCGGCGGCCGCATtttctacC), annealed, and ligated into the multiple cloning sites of the tetracycline-inducible TetO-FUW vector (Lee et al., 2015) 4 times sequentially. The TetO-FUW vector containing 4×streptavidin-binding RNA aptamer was named TetO-FUW-4S1m. H19 was then cloned into the N terminal side of 4S1m to form TetO-FUW-4S1m-H19. For the SNORA7A expression construct, the synthesized full-length SNORA7A (Ensembl Transcript ID: ENST00000384765.1) was cloned into the pLKO.pig plasmid (Lee et al., 2012a,b) within EcoRI-AgeI cloning sites and confirmed by Sanger sequencing.

qRT-PCR

Total RNA was isolated using TRIzol reagent (Thermo Fisher Scientific, USA) and snoRNA was isolated using mirVana miRNA isolation kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. The qRT-PCR primers for GAPDH (internal control), BGLAP, MEPE, FGF23, H19, and SNORA7A were described previously (Lee et al., 2015; Zhang et al., 2017).

Virus Packaging and Infection

TetO-FUW-4S1m or TetO-FUW-4S1m-H19 plasmids were co-transfected with packaging plasmids psPAX2 (Addgene, plasmid # 12260) and pMD2.G (Addgene, plasmid # 12259) into HEK-293T cells, and the virus collected from the cell culture medium 48 hours later. Osteosarcoma cell line U2OS was infected with viral particles together with the M2rtTA virus (Addgene, plasmid # 20342), which was similarly produced in HEK-293T cells, in the presence of 8ug/ml polybrene (Sigma-Aldrich, USA). Thirty-six hours post-infection, U2OS cells were treated with 1μg/ml doxycycline (Sigma-Aldrich, USA) for 24 hours to induce the expression of 4S1m or 4S1m-H9.

In vitro Anchorage-Independent Growth (AIG) Assay

AIG assay was performed as described previously (Lee et al., 2015). Briefly, LFS MSCs were transduced with H19 and/or SNORA7A. 10,000 LFS MSCs were mixed with 0.5% UltraPure low-melting-point agarose (Thermo Fisher Scientific, USA) and cultured in an osteogenic differentiation medium for 1 month. Colonies (≥50 μm) were counted under a Leica microscope DMi8.

H19-Interacting Protein Complex Purification and Mass Spectrometry Analysis

H19-interacting proteins were purified via the streptavidin-binding aptamers S1m as described previously (Leppek and Stoecklin, 2014). Briefly, S1m-H19 expressing U2OS cells were resuspended in 500 μl ice-cold lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1.5 mM $MgCl_2$, 2 mM DTT, 2 mM RNase inhibitor, and complete protease inhibitors cocktail). Lysates were subjected to centrifugation for 5 min at 12,000 rpm at 4°C. The supernatants were incubated with streptavidin agarose beads (Thermo Fisher Scientific, USA) overnight at 4°C under rotation. The streptavidin agarose beads were then washed five times for 5 min at 4°C with wash buffer [20 mM Tris-HCl (pH 7.5), 300 mM NaCl, 5 mM$MgCl_2$, and 2 mM DTT]. The 4S1m-H19 associated protein complex samples were subjected to SDS-PAGE electrophoresis and micro-liquid chromatography/tandem mass spectrometry as described previously (Lee et al., 2007, 2009a, 2012a). Mass spectrometry analysis results are summarized in **Supplementary Table 2**.

Statistical Analyses

Results were presented as mean ± standard error of the mean (SEM). Error bars in figures represent SEM. Differences between two groups were examined by the two-tailed unpaired Student *t*-test. $*p < 0.05$; $**p < 0.01$; and $***p < 0.001$.

Data Availability

The data supporting the findings are available within the manuscript text, figures, and **Supplementary Tables 1, 2**. The RNA-seq data are available at the sequencing read archive (SRA) under accession number PRJNA673185.

RESULTS

H19 Modulates snoRNA Expression in LFS Osteoblasts

To explore the suppressive effects of H19 on osteosarcomagenesis, we analyzed the genome-wide transcriptomes of LFS iPSC-derived osteoblasts by RNA-seq following ectopic overexpression of H19. The RNA-seq experiment was performed with either vector- or H19-expressing LFS osteoblasts ($n = 1$) after pooling RNA from biological triplicate experiments. Transcriptome analysis confirmed ectopic H19 overexpression and identified a set of significantly altered genes [107 upregulated genes (FPKM $\geq$1 and fold change $\geq$5) and 81 downregulated genes (FPKM $\geq$1 and fold change $\geq$5)]. These H19-regulated genes were analyzed for enriched Gene Ontology biological processes (GO_BP) and Wikipathways using the comprehensive gene set enrichment analysis web tool Enrichr. GO_BP analyses showed that the GO_BPs enriched in H19-expressing LFS osteoblasts compared with vector-expressing (H19-depleted) LFS osteoblasts included ketone pathway, regulation of complement activation, skeletal system development, and positive regulation of cell death. In contrast, H19-depleted LFS osteoblasts demonstrated enrichment of GO_BPs including pre-mRNA cleavage required for polyadenylation, negative regulation of the cellular process, negative regulation of transport, and extracellular matrix organization (**Figure 1A**). Wikipathway analyses indicated that H19-overexpressing LFS osteoblasts were enriched for genes in the complement and coagulation cascade, complement activation, oxidative damage, prostaglandin synthesis, and regulation, TYROBP causal network, benzopyrene metabolism, apoptosis, and CCK2R signaling. In contrast, genes enriched in H19-depleted LFS osteoblasts were involving in methylation pathways, biogenic amine synthesis, estrogen metabolism, and hypertrophy model (**Figure 1B**).

In analyzing the transcriptome data, we classified RNA alterations into three groups by their fold-change: less than 2, between 2 and 5, and higher than 5. We unexpectedly found that a much larger share of miRNAs and snoRNAs increased in expression following H19 expression in LFS osteoblasts compared with protein-coding mRNAs, lncRNAs, etc. (i.e all transcripts except miRNAs and snoRNAs). H19 restoration in LFS osteoblasts led to a more than 5-fold change (increased or decreased) in expression in 0.96% of protein-coding mRNAs, lncRNAs, etc. with the majority of mRNAs and lncRNAs changing <2-fold (FPKM<1) (**Figure 1C**). In contrast, 16% of miRNAs and 28.4% of snoRNAs changed greater than 5-fold in expression (**Figures 1D,E**). Scatter plots comparing miRNA and snoRNA expression between H19-expressing and vector control LFS osteoblasts showed that miRNAs (e.g., MIR570, MIR574, MIR943, MIR635, MIR7-1, and MIR145) and snoRNAs (e.g., SNORA6, SNORA7A, SNORA9, SNORA15B, SNORA19B, and SNORA36C) were significantly altered in H19-expressing LFS osteoblasts (**Figures 1D,E**). These findings suggest that H19 suppresses osteosarcomagenesis in LFS patients by primarily altering the expression of miRNAs and snoRNAs rather than mRNAs.

SnoRA7A Is an onco-snoRNA Involved in Tumor Progression and Associated With Poor Prognosis

Among the identified H19-regulated miRNAs and snoRNAs, SNORA7A was chosen for further study in light of its previously recognized role in controlling the self-renewal of MSCs (Zhang et al., 2017). To validate our transcriptome results, we ectopically expressed H19 in LFS iPSC-derived osteoblasts and found that H19 significantly inhibits SNORA7A expression (**Figure 2A**). H19-induced osteogenic gene expression (e.g., BGLAP, MEPE, and FGF23) was inhibited by SNORA7A (**Figure 2B**), indicating that SNORA7A inhibits osteogenesis. To investigate whether the inhibition of SNORA7A plays a role in H19-mediated tumor suppression, we performed an *in vitro* AIG assay and found retarded clonal growth of H19-transduced LFS osteoblasts upon SNORA7A ectopic expression (**Figure 2C**). These results suggest that SNORA7A is negatively regulated by H19, and that SNORA7A functions as an onco-snoRNA by antagonizing H19 tumor suppressor function.

To further explore the role of SNORA7A in tumorigenesis, we analyzed the TCGA-SARC dataset and identified 780 and 945 protein-coding genes whose mRNA levels were positively and negatively correlated with SNORA7A expression, respectively. Wikipathway analysis indicated that genes positively correlated with SNORA7A expression are mainly involved in cytoplasmic ribosomal protein function and cell metabolism (e.g., pentose phosphate metabolism, Cori cycle, pyrimidine metabolism, and glycolysis and gluconeogenesis), while genes negatively correlated with SNORA7A expression are associated with focal adhesion, integrin-mediated cell adhesion, and G protein signaling pathways, the latter of which is involved in osteoblast differentiation (Wu et al., 2010) (**Figure 2D**).

Similarly, GO_BP analysis demonstrated that genes positively correlated with SNORA7A expression are involved in rRNA metabolic process and processing, ribosome biogenesis, translation, glyceraldehyde-3-phosphate metabolic process, and ribose phosphate metabolic process, while genes negatively correlated with SNORA7A expression are associated with glycogen metabolic process and cytosolic calcium ion transport (**Figure 2E**). The molecules controlling pentose phosphate metabolism (G6PD), protein translation (RPS9), and cell cycle progression (CDK4) were positively correlated with SNORA7A expression. In contrast, cyclin-dependent kinase inhibitor (CDKN1B), adenylyl cyclase (ADCY9), and downstream effector of Rho (ROCK1) were negatively correlated with SNORA7A expression (**Figure 2F**). These systems analyses suggest that SNORA7A may transcriptionally and/or post-transcriptionally regulate multiple oncogenic features, including cellular metabolism, ribosome biogenesis, cell cycle, etc., culminating in osteosarcoma development in LFS patients.

The regulation of RNA splicing is increasingly recognized to be an essential mechanism underlying cancer development, and dysregulation of RNA splicing machinery has been found to contribute to tumorigenesis in various human cancers, including glioma, lymphoma, and breast cancer (David et al., 2010; Hsu et al., 2015; Koh et al., 2015). Given the well-recognized

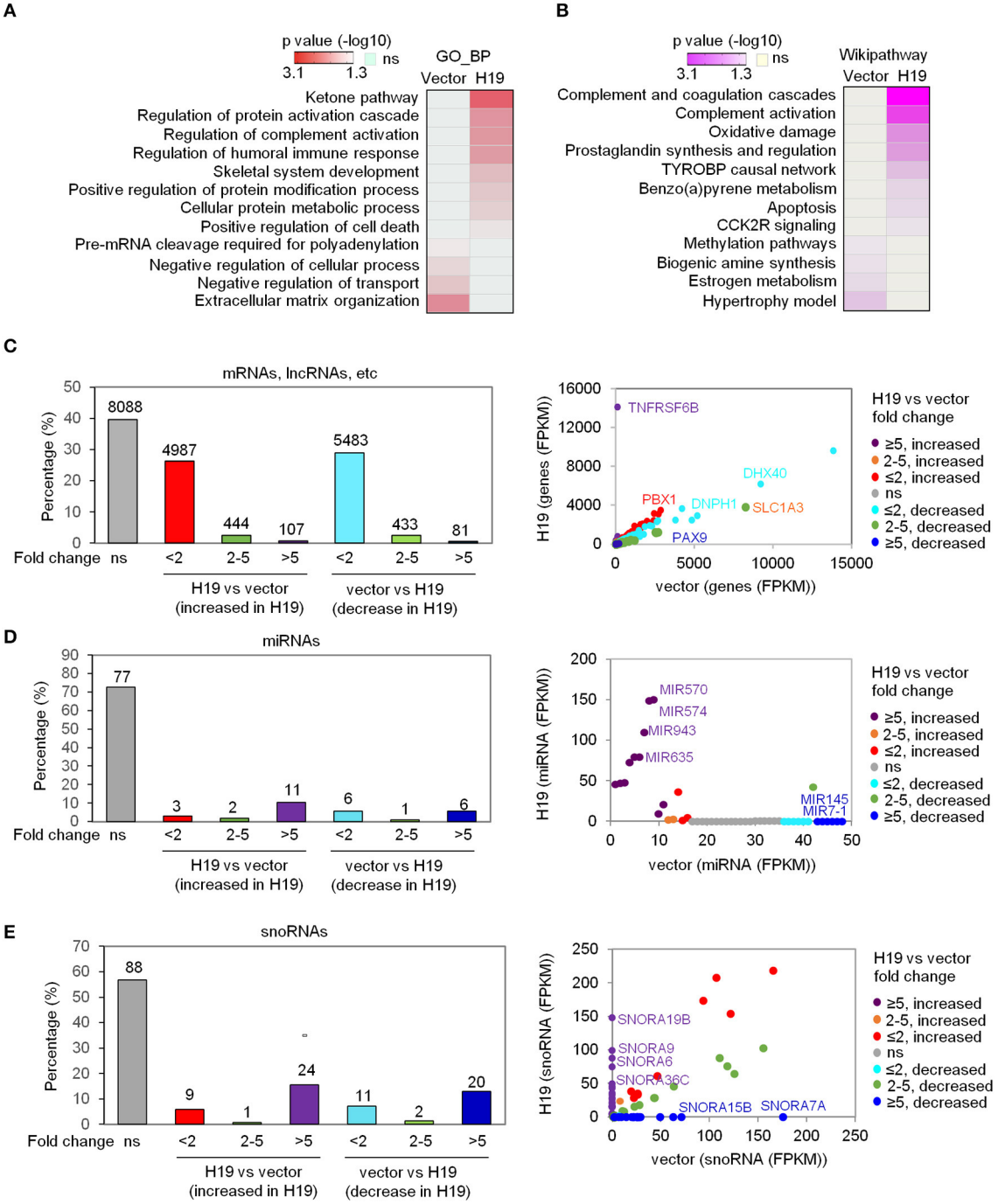

FIGURE 1 | H19 significantly affects miRNA and snoRNA expression. **(A,B)** Heatmaps show the GO_BP **(A)** and Wikipathway **(B)** categories enriched in either H19-expressing LFS osteoblasts (right column) or vector control LFS osteoblasts (left column) compared with each other based on mRNA expression of respective samples. Colors represent *p* values of enrichment. **(C-E)** H19 overexpression induces small changes in overall mRNA expression but large changes in miRNA and snoRNA expression in LFS osteoblasts. The percentage of mRNAs, lncRNAs, etc. **(C)**, miRNAs **(D)**, and snoRNAs **(E)** with different fold-changes (<2, 2-5, and >5 increase or decrease) are shown in bar plots. RNA expression between H19-overexpressing and vector control cells is presented in a scatter plot (right). Dot colors are used to represent the fold change of RNA expression. ns (not significant) indicates mRNAs, lncRNAs, miRNAs, snoRNAs, etc. with fold-change <2 and/or FPKM<1. The RNA-seq experiment was done with one sample pooled from 3 biological replicates. miRNAs and snoRNAs are defined based on the miRbase and snoDB database, respectively. "mRNA, lncRNA, etc." indicates all transcripts except miRNAs and snoRNAs.

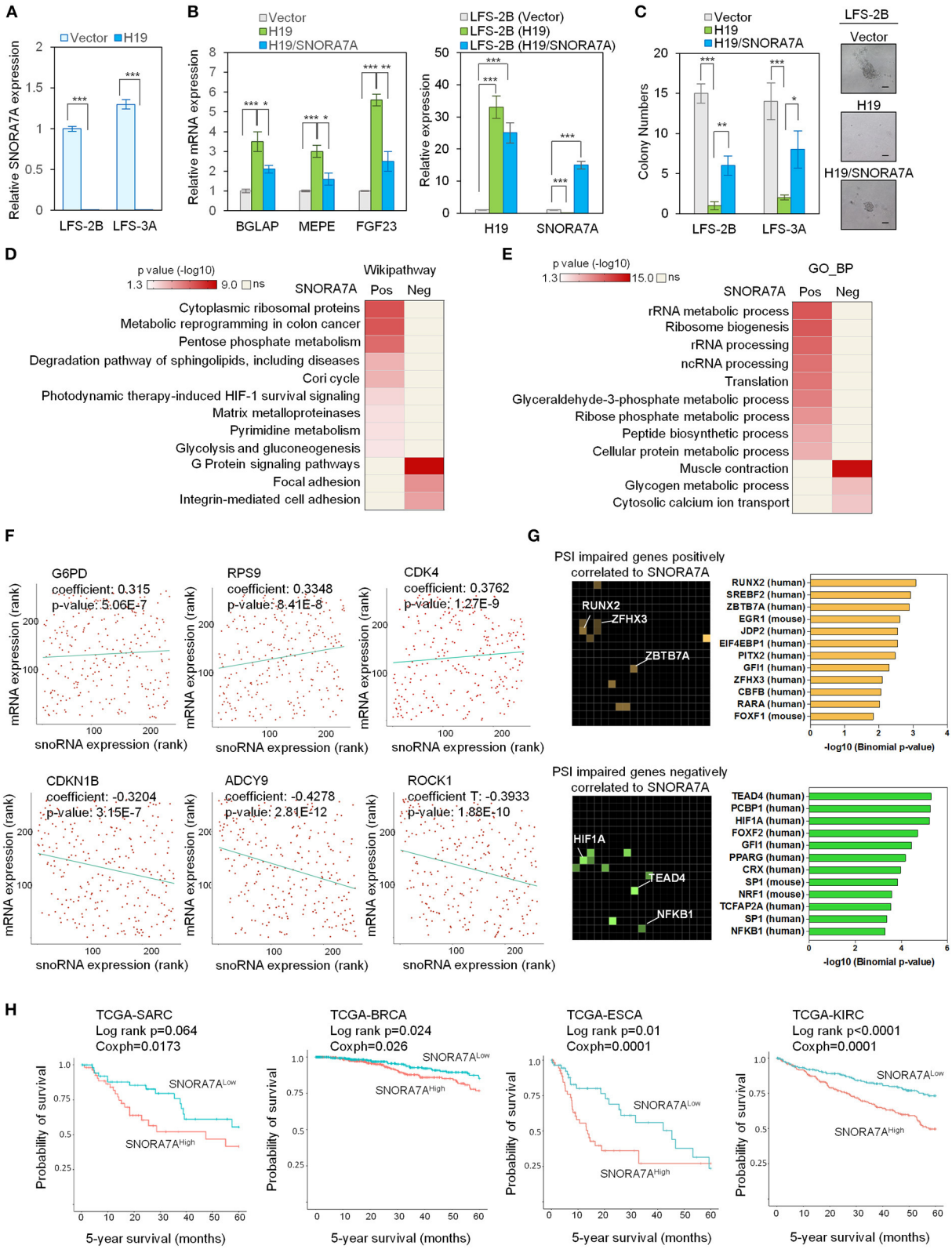

FIGURE 2 | Systems analyses of the oncogenic role of SNORA7A from TCGA datasets. **(A)** Ectopic expression of H19 downregulates SNORA7A expression in LFS osteoblasts. qRT-PCR data are represented as mean ±SEM; $n = 3$ biological replicates; statistical significance is determined using two-tailed Student's *t*-test;

(Continued)

FIGURE 2 | ***$p < 0.001$. **(B)** Left panel, ectopic expression of H19 increases osteogenic gene expression in LFS osteoblasts; in contrast, restoration of SNORA7A expression impairs H19-upregulated osteogenic gene expression in H19-transduced LFS osteoblasts. Right panel, qRT-PCR results demonstrate the expression of H19 and SNORA7A upon their ectopic expression for assays in the left panel. qRT-PCR data are represented as mean ± SEM; $n = 3$ biological replicates; statistical significance is determined using two-tailed Student's *t*-test; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. **(C)** AIG assay for *in vitro* tumorigenicity demonstrates that H19 impairs the colony numbers of LFS osteoblasts and SNORA7A expression rescues the H19-suppressed tumorigenicity of LFS osteoblasts. H19 or H19/SNORA7A-transduced LFS osteoblasts were grown for 1 month and then assayed. Positive colonies are considered those larger than 50μm diameter. Data are represented as mean ± SEM; $n = 6$ biological replicates; statistical significance is determined using two-tailed Student's *t*-test; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. **(D,E)** Genes positively ($n = 780$) and negatively ($n = 945$) correlated with SNORA7A expression in TCGA-SARC are identified. Pathway analysis by Wikipathway **(A)** and GO_BP **(B)** is performed on these gene sets using Enrichr to identify pathways significantly enriched or depleted ($p \leq 0.05$) in association with SNORA7A expression. **(F)** Scatterplots of G6PD, RPS9, CDK4, CDKN1B, ADCY9, and ROCK1 mRNA expression correlation with SNORA7A expression. **(G)** Genes whose abnormal splicing (including exon skip, mutually exclusive splicing, or intron retention) by PSI was positively ($n = 91$, upper panel) or negatively ($n = 182$, lower panel) correlated with SNORA7A expression in sarcomas are identified by TRANSFAC and JASPAR PWWMs in Enrichr. PSI: percent spliced in index. **(H)** High SNORA7A expression is associated with poor cancer survival. Five-year overall survival is analyzed according to SNORA7A expression from TCGA in various cancers. SARC: sarcoma. BRCA: breast invasive carcinoma. ESCA: esophageal carcinoma. KIRC: kidney renal clear cell carcinoma.

potential for snoRNAs to regulate pre-mRNA splicing (Falaleeva et al., 2016; Liang et al., 2019), we investigated the effects of SNORA7A levels toward potentially pathological mRNA splicing events including skipped exons, mutually exclusive alternative splicing, and intron retention. The percent spliced in index (PSI) was calculated for all identified genes in the TCGA-SARC dataset to identify genes whose alternative splicing was correlated with SNORA7A expression. These genes were then mapped to transcription factors using TRANSFAC and JASPAR analysis. Genes whose abnormal splicing was positively correlated to SNORA7A expression were mainly downstream targets of transcription factors such as the osteoblastic lineage regulator RUNX2 (Komori, 2019) and the tumor suppressors ZBTB7A (Liu et al., 2014) and ZFHX3 (Hu et al., 2019). Moreover, genes whose abnormal splicing was negatively correlated to SNORA7A expression were downstream targets of transcription factors such as the tumor angiogenesis regulator HIF1A (Koukourakis et al., 2002), the Hippo pathway transcription factor TEAD4 (Lin et al., 2017a; Shi et al., 2017) and the tumorigenic transcription factor NF-KB1 (Concetti and Wilson, 2018) (**Figure 2G**). These results imply that oncogenic effects of SNORA7A occur through regulation of RNA splicing of tumor suppressor genes and oncogenes.

We next analyzed the expression of SNORA7A in multiple cancers using TCGA datasets and correlated its expression with patient survival data. The five-year overall survival analyses showed that high SNORA7A levels were significantly associated with poorer survival in multiple cancer types including sarcoma (SARC, Log-rank $p = 0.064$, coxph $= 0.017$), breast cancer (BRCA, Log-rank $p = 0.024$, coxph $= 0.026$), esophageal carcinoma (ESCA, Log-rank $p = 0.01$, coxph $= 0.0001$), and kidney carcinoma (KIRC, Log-rank $p \leq 0.0001$, coxph $= 0.0001$) (**Figure 2H**). Taken together, these results suggest a potential role of SNORA7A in promoting cancer progression and emphasize that H19 tumor suppressor functions may be mediated through the repression of SNORA7A expression.

H19 Potentially Interacts With DNA Damage/Repair Response Protein Complexes

Previous studies revealed that H19 is capable of interacting with EZH2 (Luo et al., 2013) to promote WNT/β-Catenin activation and subsequent downregulation of E-cadherin and that H19 also associates with KSRP (Giovarelli et al., 2014) to control mRNA decay. However, the protein complexes tethered by H19 in osteosarcoma remain largely unknown. To elucidate H19-associated protein complexes and gain insights into the potential biological functions controlled by the H19 interactome, we ligated H19 with an optimized 4×streptavidin-binding RNA aptamer (S1m) and expressed H19-S1m in U2OS cells. H19-bound protein complexes could then be pulled down with streptavidin beads and identified by mass spectrometry (**Supplementary Table 2**). Compared with the S1m vector control, S1m-H19 pull-down identified 15 putatively interacting cellular proteins: TUBA1A, KHSRP, ALDH18A1, HIST1H1A, GANAB, TARS2, LRRFIP1, GPANK1, ZNF326, BLM, ABCC12, DMD, CLCN5, RBBP8, and OR8U1 (**Figure 3A**, left panel).

We applied the Biogrid human database to identify proteins previously demonstrated to bind to at least two of these 15 potential H19-binding proteins. This bioinformatics approach yielded 47 proteins strongly suspected to be connected to the 15 H19-associated proteins (**Figure 3A**, right panel). Wikipathway and GO_BP analyses revealed that these putative H19-associated protein complexes mainly function in DNA damage response, DNA repair, the ATM/ATR signaling pathway, the WNT signaling pathway, and the cell cycle (**Figures 3B,C**), emphasizing the potential role of H19 in regulating DNA damage response and repair. Particularly, BLM, an ATP-dependent DNA helicase, participates in DNA replication and DNA repair by modulating DNA double-strand break resection (Gravel et al., 2008). Mutations in the BLM gene are associated with Bloom syndrome, which carries a greatly increased risk of cancers including squamous cell carcinoma, leukemia, lymphoma, and gastrointestinal cancer (Lin et al., 2017b). Another H19-interacting protein is RBBP8 (also known as CtIP), an endonuclease that cooperates with the MRE11-RAD50-NBN (MRN) complex in DNA-end resection, the first step of homologous recombination (HR)-mediated double-strand break repair. RBBP8 mutations are commonly detected in various human cancer cell lines (You and Bailis, 2010). Inactivation of one *RBBP8* allele predisposed mice to multiple types of cancers (e.g., lymphoma) suggesting that RBBP8 functions as a tumor suppressor (Chen et al., 2005). Based on these findings, we speculate that H19 tethers DNA repair

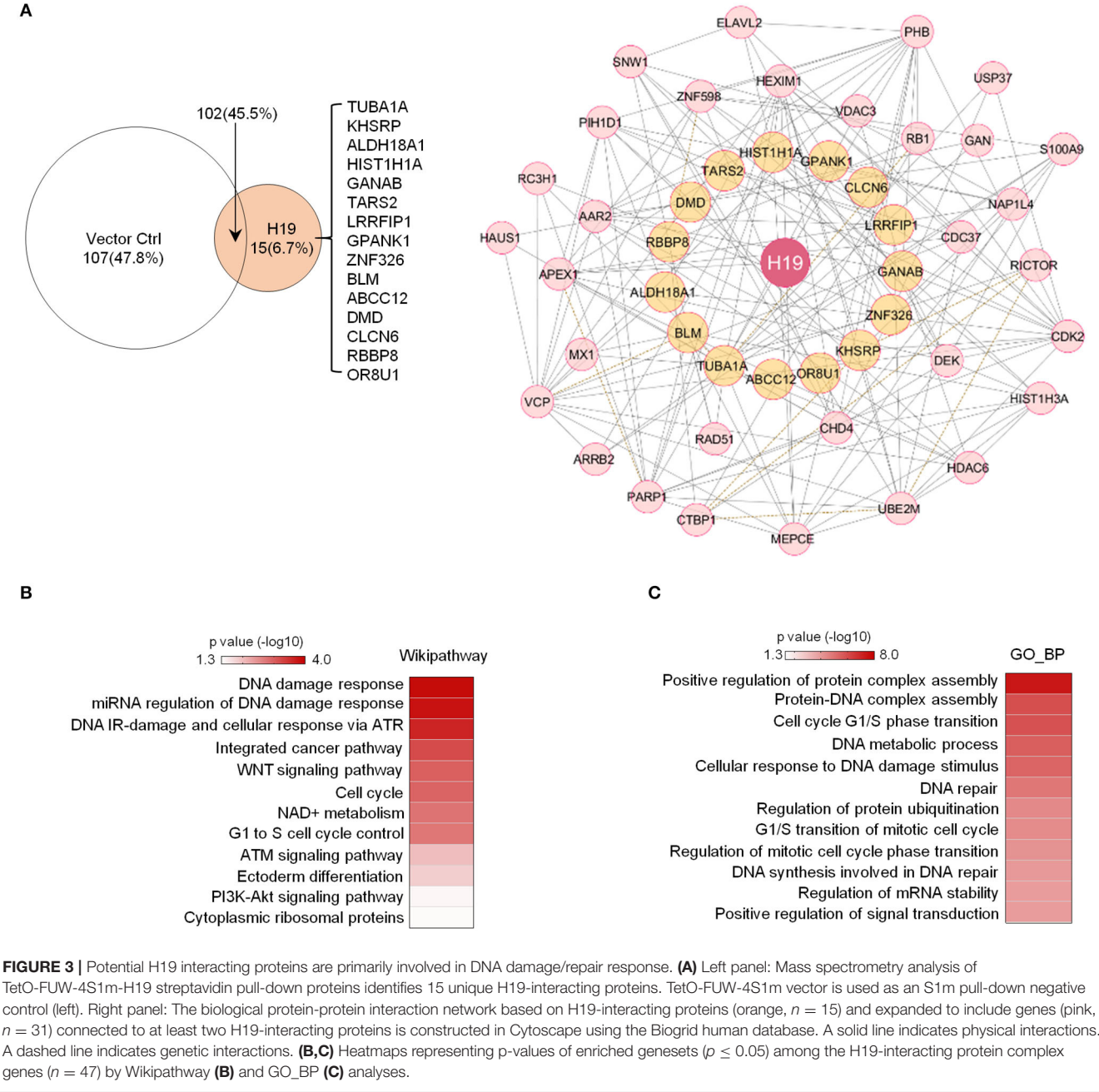

FIGURE 3 | Potential H19 interacting proteins are primarily involved in DNA damage/repair response. **(A)** Left panel: Mass spectrometry analysis of TetO-FUW-4S1m-H19 streptavidin pull-down proteins identifies 15 unique H19-interacting proteins. TetO-FUW-4S1m vector is used as an S1m pull-down negative control (left). Right panel: The biological protein-protein interaction network based on H19-interacting proteins (orange, $n = 15$) and expanded to include genes (pink, $n = 31$) connected to at least two H19-interacting proteins is constructed in Cytoscape using the Biogrid human database. A solid line indicates physical interactions. A dashed line indicates genetic interactions. **(B,C)** Heatmaps representing p-values of enriched genesets ($p \leq 0.05$) among the H19-interacting protein complex genes ($n = 47$) by Wikipathway **(B)** and GO_BP **(C)** analyses.

proteins to other functional factors and helps maintain genomic integrity by suppressing oncogenic events such as DNA double-stranded breaks.

DISCUSSION

The essential biological functions of non-coding RNAs (e.g., miRNAs, snoRNAs, and lncRNAs) and their pathologic roles in tumorigenesis are becoming increasingly appreciated. In our previous work, we reported that H19, a lncRNA, is downregulated by mutant p53s (mutp53s) in LFS iPSC-derived osteoblasts and modulates osteoblastic differentiation and oncogenic repression through the imprinted gene network (IGN) (Gabory et al., 2009; Lee et al., 2015). While H19 has been demonstrated in various contexts to act as either a tumor suppressor or an oncogene, our current work further demonstrates the important regulatory role of H19 in osteosarcoma initiation and progression.

We discovered considerable H19-mediated regulation of snoRNAs. We confirmed that SNORA7A, a snoRNA known to promote MSC proliferation and self-renewal (Zhang et al.,

2017), is suppressed by H19 and that restoration of SNORA7A expression impaired H19-mediated osteogenesis and tumor suppression. These findings suggest that SNORA7A functions as an onco-snoRNA in LFS-associated osteosarcoma. SNORNA7 expression in TCGA datasets is associated with numerous oncogenic pathways (ribosome biogenesis, pentose phosphate pathway, glycolysis, and HIF signaling) and RNA splicing events, suggesting a link between snoRNA expression and oncogenic events in clinical tumors.

p53 mutations have been widely linked to increased tumor oncogenesis through both gain-of-function interactions, for example by promoting HIF1A-regulated angiogenic genes (Amelio et al., 2018), and loss-of-function mutations, for example by abrogating wild-type p53 repression of FBL expression, culminating in increased ribosome biogenesis (Marcel et al., 2013). Upregulation of SNORA7A by mutp53-mediated H19 suppression provides yet another mechanism for mutp53 downstream pathways to cooperate in promoting bone malignancies.

In addition, mass spectrometry analysis of potential H19-interacting proteins revealed multiple DNA damage response and repair molecules directly associated with H19. H19 was previously proposed to regulate DNA damage response (Zheng et al., 2016; Zhu et al., 2017; Ma et al., 2018; Cheng et al., 2019), but the underlying mechanisms have yet to be defined. Our identification of potential interactions between H19 and BLM as well as RBBP8, both of which are recognized to regulate DNA damage response and repair, may provide another angle to elucidate these mechanisms. Importantly, gene mutations in both BLM and RBBP8 are associated with increased risks of tumor formation, including osteosarcoma. The evidence of physical interactions between H19 and DNA damage response genes leads us to speculate that H19 indirectly maintains genomic integrity, explaining its tumor-suppressor activity.

Finally, H19 expression was significantly increased in cancer cells treated with chemotherapeutic drugs (e.g., doxorubicin and cisplatin) (Zheng et al., 2016; Zhu et al., 2017). We speculate that upregulation of H19 following DNA damage events occurs in order to accelerate the repair process and prevent oncogenic effects of DNA damage.

In conclusion, our study indicated that lncRNA H19 plays a vital regulatory role in inhibiting osteosarcomagenesis and provides mechanistic insights for improving our understanding of H19-mediated tumor suppression in LFS patient-associated osteosarcomas.

DATA AVAILABILITY STATEMENT

The transcriptome data has been deposited into the sequencing read archive (SRA; PRJNA673185).

AUTHOR CONTRIBUTIONS

AX, M-FH, DZ, BC, DW, C-CL, and RZ conducted all experiments. AX, M-FH, DZ, DW, C-CL, IL, RZ, and D-FL conceived and designed the study and interpreted results. AX, M-FH, DZ, JG, DB, RZ, and D-FL wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2020.611823/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.