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

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14. ABSTRACT The RB1 tumor suppressor has been widely recognized for its role in inhibiting tumor initiation, development, and progression. RB1 functions in negatively regulating cell cycle progression by interacting with E2F1/2/3 transcription factors. In addition to negatively regulating G1/S transition in the cell cycle, it remains incompletely defined if RB1 also contributes to other stages of the cell cycle such as G2/M. RB1 missense mutations and deletions 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. 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 osteosarcomagenesis. Our experience in iPSC technology enables us to perfect a model system for RB-associated bone malignancies and explore therapeutic interventions. We have reprogrammed RB patients' fibroblasts to iPSCs. We also applied CRISPR/Cas9 to correct RB1 mutation in RB iPSCs, named as cRB iPSCs, and use these cells as isogenic controls. Since OBs are derived from multi-potent mesenchymal stem cells (MSCs), we first differentiated WT, RB, and cRB iPSCs to their corresponding MSCs (CD105+/CD166+/CD24-) and then to OBs by our defined MSC and OB lineage differentiation methods. Performing in vivo xenografts, we found potential tumorigenic ability in RB OBs but not WT and cRB OBs. In contrast to the phenotype of no detectable increase in OS incidence in Rb1 knockout mice, our preliminary findings demonstrated multiple OS-related phenotypes (e.g., tumorigenic ability) in RB human iPSC-derived 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 the FOXM1 promoter region than cRB OBs. Also, our studies of E2F3a and FOXM1 expression in the TCGA SARC database revealed a 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 Vulnerability					
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TABLE OF CONTENTS

	<u>Page</u>
1. Introduction	1
2. Keywords	1
3. Accomplishments	1
4. Impact	14
5. Changes/Problems	15
6. Products	16
7. Participants & Other Collaborating Organizations	19
8. Special Reporting Requirements	20
9. Appendices	21

1. INTRODUCTION:

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. In this proposed grant, we 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:

RB1, osteosarcoma, iPSCs, FOXM1, therapeutic vulnerability

3. ACCOMPLISHMENTS:

What were the major goals of the project?

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: 100% completed at May 20, 2022.

Subtask 3: Investigate the clinical relevance of RB1, E2F3a and FOXM1 using public TARGET and TCGA databases. Timeline: 19-24 months. Progress: 100% completed at May 20, 2022.

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: 100% completed at May 20, 2022.

Subtask 3: Examine the therapeutic effect of FOXM1 inhibitor Thiostrepton to treat RB1-mutant cells. Timeline: 19-24 months. Progress: 100% completed at May 20, 2022.

What was accomplished under these goals?

The RB1 tumor suppressor has been widely recognized for its role in inhibiting tumor initiation, development, and progression. RB1 functions in negatively regulating cell cycle progression by interacting with E2F1/2/3 transcription factors (1-5). In addition to negatively regulating G1/S transition in cell cycle, it remains incompletely defined if RB1 also contributes to other stages of the cell cycle such as G2/M. RB1 missense mutations and deletions are found in more than 30% of clinical osteosarcoma specimens (6), 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. 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 osteosarcomagenesis.

FOXM1 regulates a network of proliferation-associated genes involved in G2/M transition, mitotic spindle assembly, chromosome segregation, DNA damage response, and chemoresistance (7-10). Aberrant upregulation of FOXM1 is detected in a broad range of cancer types and implicated to be not only as a tumor initiation factor but also as a key driver of cancer progression (11, 12). Therefore, suppression of FOXM1 activity by pharmacological inhibitors (e.g., Thiostrepton (13, 14), Siomycin A (15-16), and FDI-6 (10)) is considered as an effective strategy for cancer therapy.

Our experience in iPSC technology enables us to perfect a model system for RB-associated bone malignancies and explore therapeutic interventions (17-19). Here, we obtained fibroblasts from 2 affected RB patients and 1 healthy related control subject from the Coriell Institute for Medical Research (**Fig. 1A**) and reprogrammed these fibroblasts to iPSCs by 4 reprogramming factors OCT4, SOX2, KLF4, and MYC. The reprogrammed cells express NANOG and OCT4 pluripotency transcription factors as well as TRA-1-81 and SSEA4 (**Fig. 1B**), show normal karyotype, and are capable of differentiating to the three germ-layers (ectoderm, mesoderm, and endoderm) *in vivo* (data not shown). We also applied CRISPR/Cas9 to correct RB1 mutation in RB iPSCs, named as cRB iPSCs, and use these cells as isogenic controls (**Fig. 1C**).

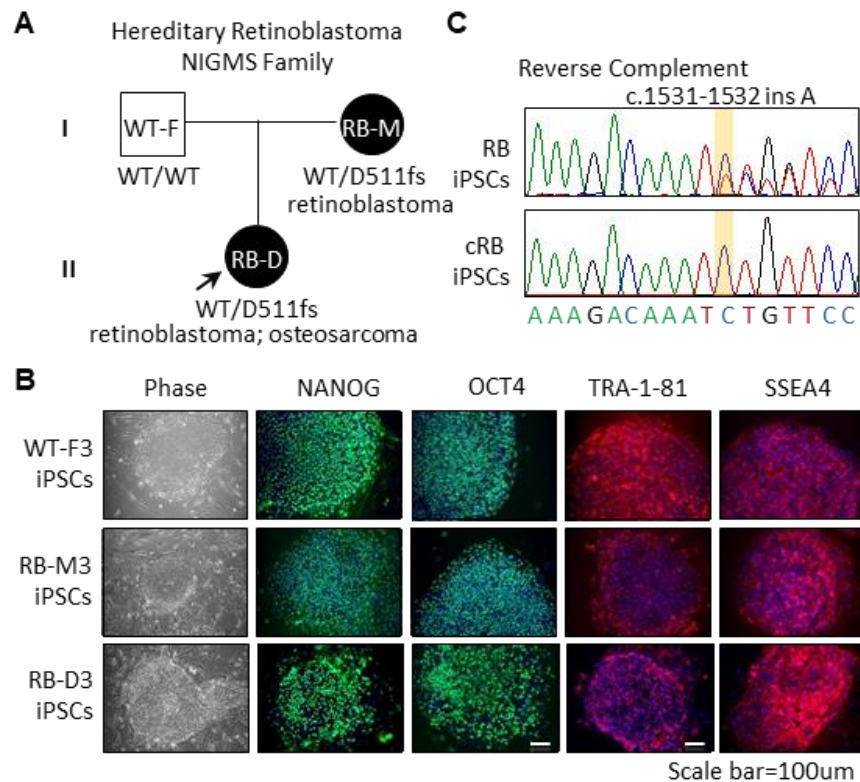


Figure 1. Characterization of RB iPSC. (A) RB family tree. (B) RB iPSC express Hesc pluripotency factors and surface markers. (B)Sanger sequencing indicates c1531-1532 ins A mutation in RB1 exon 17 is corrected in cRB iPSC.

Since OBs are derived from multi-potent mesenchymal stem cells (MSCs), we first differentiated WT, RB, and cRB iPSCs to their corresponding MSCs (CD105⁺/CD166⁺/CD24⁺) and then to OBs by our defined MSC and OB lineage differentiation methods (**Fig. 2A**) (20, 21, 22). Performing *in vivo* xenografts, we found potential tumorigenic ability in RB OBs but not WT and cRB OBs (**Fig. 2B**). In contrast to the phenotype of no detectable increase in OS incidence in *Rb1* knockout mice (23, 24), our demonstrated multiple OS-related phenotypes (e.g., tumorigenic ability) in RB human iPSC-derived OBs.

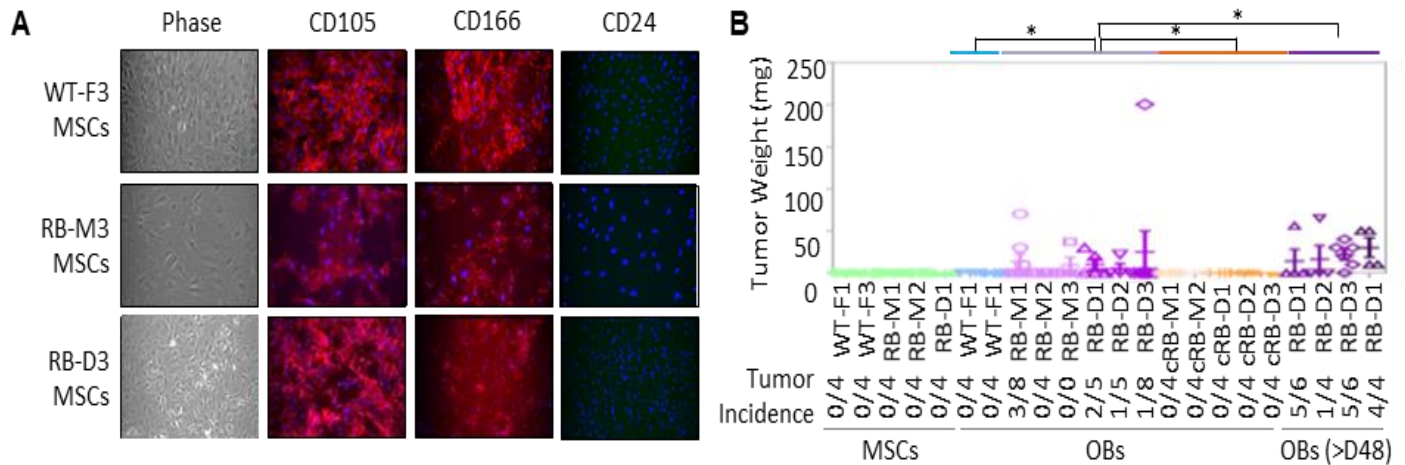


Figure 2. RB OBs show oncogenic properties. (A) RB iPSC-derived MSCs express MSC surface marker CD105 and CD166. CD24 is a pluripotent stem cell marker. (B) The tumor xenograft demonstrates RB OBs recapture *in vivo* tumorigenic ability. *, $p < 0.05$.

In order to gain insights into RB1 loss-associated osteosarcomagenesis, we investigated and compared global transcripts among WT, RB, and cRB OBs. The extracted mRNAs were isolated at three differentiation time points (day 0, MSCs; day 15, pre-OBs; and day 24, OBs) for RNA-seq. Expression profiles of WT, RB, and cRB time course samples analyzed by Spearman's rank correlation demonstrated that gene expression profiles of RB OB samples clustered together but were distinct from WT and cRB OB samples (**Fig. 3A**). Gene set enrichment analysis (GSEA) indicated that OS-associated genes are specifically enriched in RB OBs in comparison with WT/cRB OBs, demonstrating that RB OBs acquire OS characteristics in the absence of additional gene alterations (**Fig. 3B**). Gene Ontology (GO) analysis revealed that genes, including FOXM1, involved in the mitotic cell cycle are enriched in RB OBs, which is consistent with transcription factor target analyses showing the enrichment of FOXM1 bound genes in RB OBs (**Fig. 3C**). We performed RNA-seq analysis of in our RB OBs and WT/cRB OBs. The genome-wide transcriptome analysis of RB OBs showed that there are known G1/S and G2/M targets enriched in RB OBs (for example, PLK1, CCNB1, AURKB, and UBE2C) (**Fig. 3D**). Network visualization of enriched gene sets in RB OBs compared with WT and cRB OBs at day 24 indicates that GO biological processes involved in cell cycle, DNA replication, FOXM1 pathway, and extracellular matrix organization are enriched in RB OBs. And ChEA analysis revealed the top transcription factor involved was FOXM1, E2F7, HOXA2, KDM6A, MYBL1, and E2F4 (**Fig. 3E**). Heatmap of enriched GO biological processes in RB vs. WT and cRB OBs revealed that the top enriched pathways are G2/M cell cycle pathway, DNA replication pathway as well as G1/S cell cycle pathway (**Fig. 3F**). Among the top enriched G2/M cell cycle pathway, they are pathways in cell cycle mitotic, G2/M DNA damage checkpoint, and G2/M checkpoint (**Fig. 3G**).

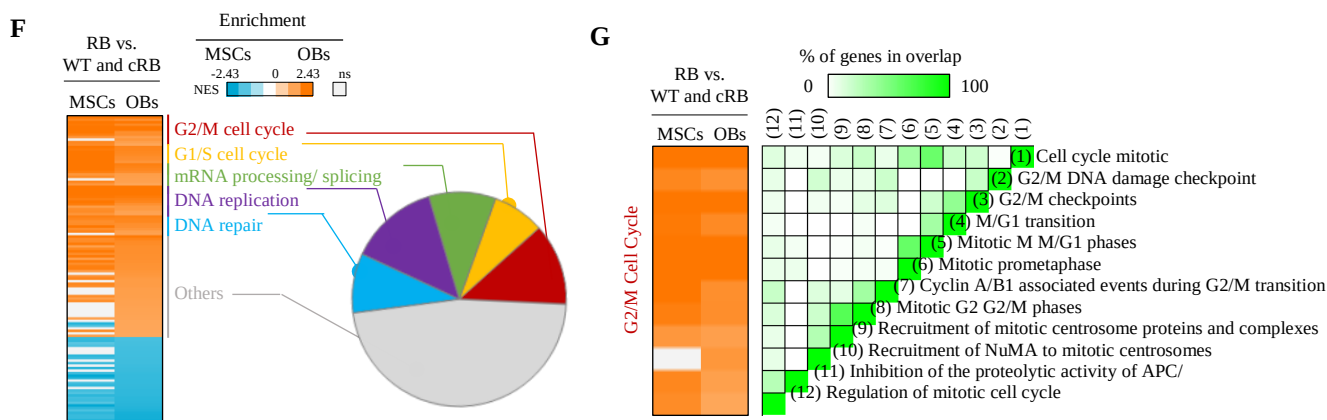
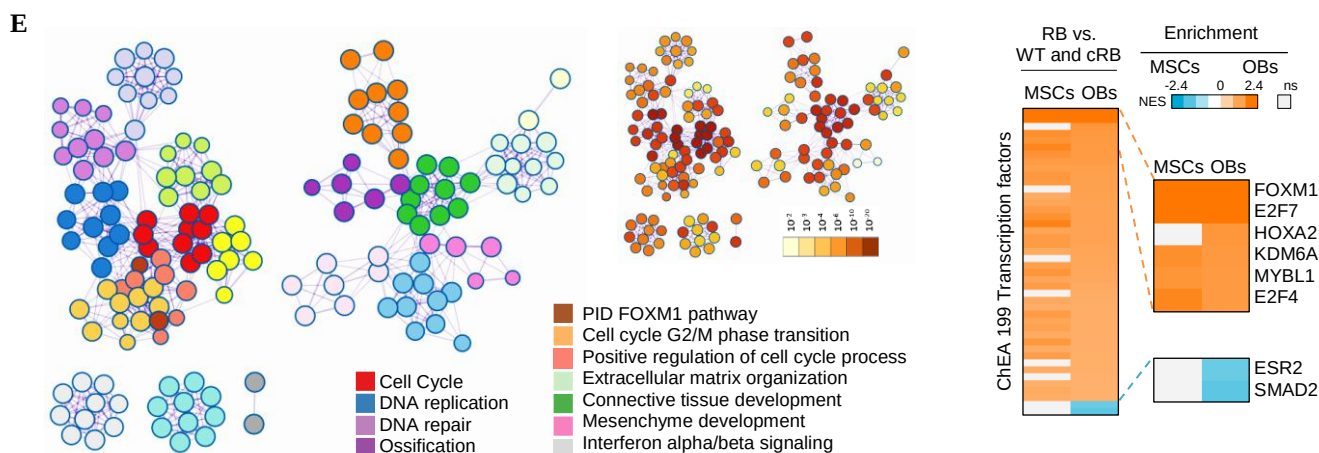
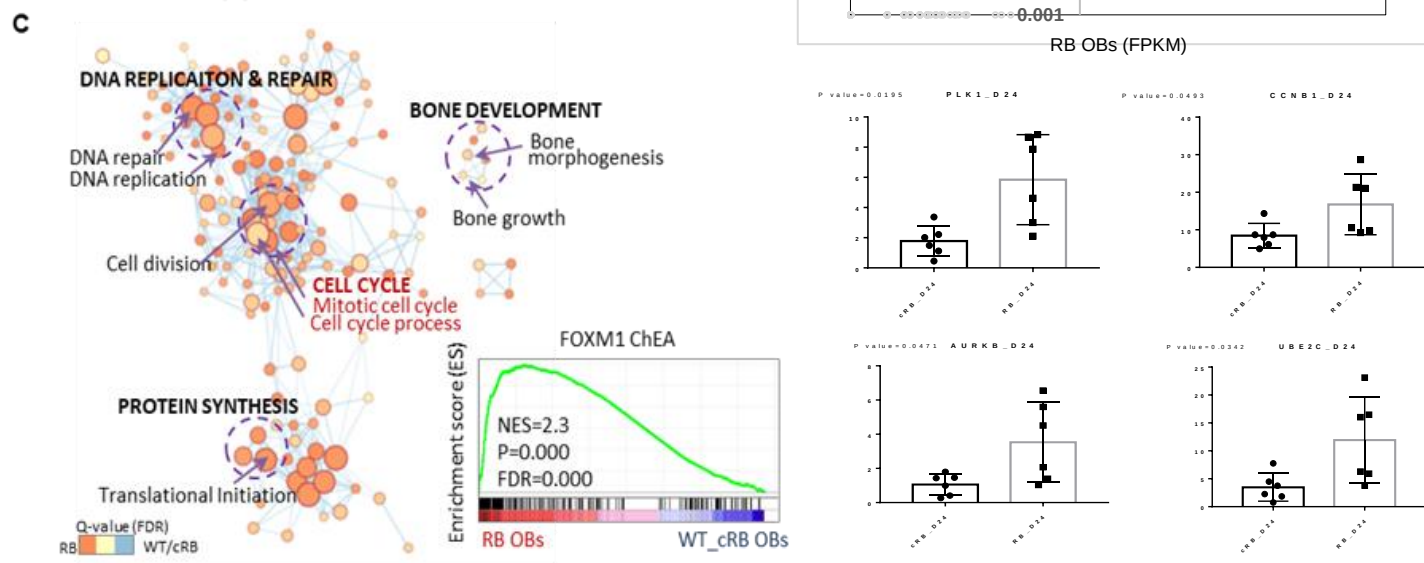
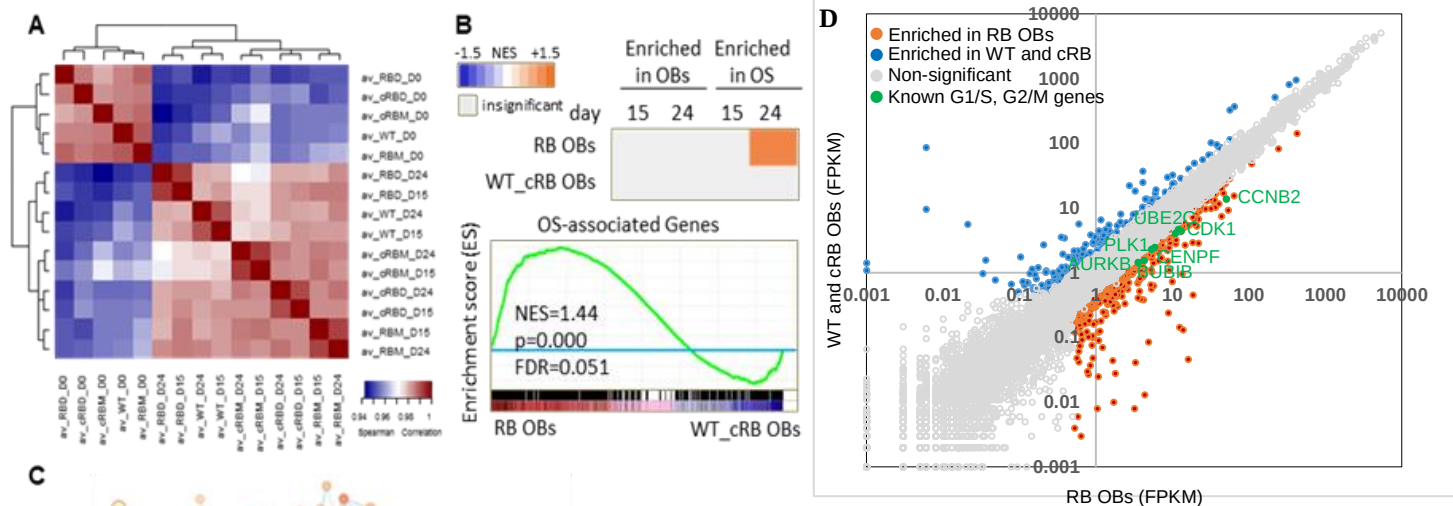


Figure 3. Genome-wide transcriptome analysis of RB OBs. (A) The correlation matrix of WT, RB and cRB RNA-seq time course results. (B) GSEA indicates RB OBs possesses an OS signature. (C) Enrichment of GO mitotic cell cycle and FOXM1 ChIP-targets in RB OBs. (D) Upper. Scatter plot gene expression comparison between RB OBs and unaffected (WT or cRB) OBs. Orange dots indicate genes with higher expression in RB OBs, while blue dots indicate genes with higher expression in WT and cRB OBs. Known G1/S, G2/M targets enriched in RB OBs are labeled with green dots. Lower. FPKM value of PLK1, CCNB1, AURKB and UBE2C in RB OBs vs cRB OBs. (E). Left Enrichment of GO biological processes identified by EnrichmentMap analysis. Network visualization of enriched gene sets in RB OBs compared with WT and cRB OBs at day 24 indicates that GO biological processes involved in cell cycle, DNA replication, FOXM1 pathway, and extracellular matrix organization are enriched in RB OBs. Enriched gene sets in RB OBs are displayed in orange and enriched gene sets in WT and cRB OBs are shown in blue. FDR, false discovery rate. Right. Enriched genes in RB vs. WT and cRB OBs are analyzed by GSEA and summarized by a heatmap. Nonsignificant GOs are shown in white. (F) Enriched GO biological processes in RB vs. WT and cRB OBs are analyzed by GSEA and summarized by a heatmap. (G) Enriched GO biological processes related to G2/M cell cycle in RB vs. WT and cRB OBs.

In agreement with our analysis of public RB1 ChIP-seq data (GSE19899) from growing human diploid MR90 lung fibroblasts (25) revealing RB1 as well as E2F3 binding to the FOXM1 promoter (**Fig. 4A**). Based on these findings, we conclude that one of the tumor suppressor functions of RB1 is mediated through controlling FOXM1-mediated gene expression. To elucidate how the loss of RB1 contributes to upregulated FOXM1 transcriptional program (**Specific Aim 1**), we hypothesize that RB1 negatively modulates FOXM1 transcription through E2F3 (**Fig. 4B**).

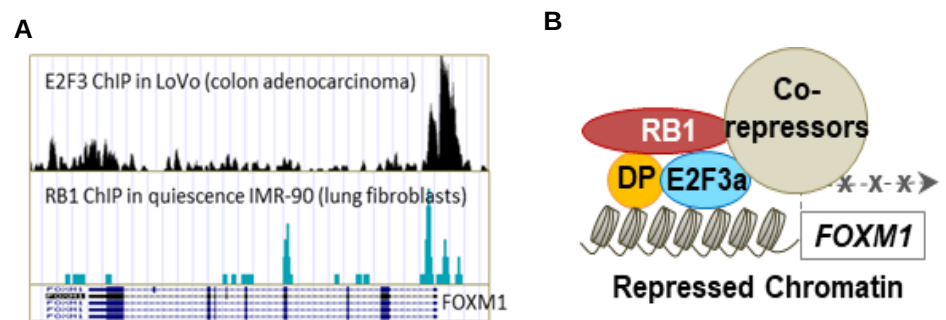


Figure 4. FOXM1 in RB OBs, RB1-knockdown and RB1-null iPSC-derived OBs. (A) ChIP-seq analysis from growing human diploid MR90 lung fibroblasts reveals RB1 and E2F3 bind on the FOXM1 promoter. (B) The proposed model of RB1-repressing FOXM1 gene expression.

We expect to discover that both RB1 and E2F3 preferentially bind to the promoter of FOXM1 in OBs. To comprehensively identify the 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 (**Subtask 1**). 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 (**Fig. 5A**, Top). The heat map (**Fig. 5A**, 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 the average binding of RB1 and E2F3a to the RB1-specific, RB1/E2F3a co-targeted, and E2F3a-specific loci. As shown (**Fig. 5B**), 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 $\pm$ SEM; statistical significance is examined by two-tailed Student t-test; *p< 0.05; **p< 0.01; ***p< 0.001). Pie chart showing the 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 (**Fig. 5C**). Motif analyses report the top-scoring binding motif for RB1-specific, RB1/E2F3a co-occupied, and E2F3a-specific genomic binding peaks in cRB OBs (**Fig. 5D**). ChIP-seq summit peak intensities of total RB1 ChIP-seq signals at RB1-specific, RB1/E2F3a co-targeted, E2F3a-specific sites (**Fig. 5E**, 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 (**Fig. 5E**, 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 (**Fig. 5F**). qRT-PCR indicates increased *FOXM1* mRNA in RB-D OBs compared to RB-M OBs (**Fig. 5G**). Notably, *FOXM1* ChIP targets are found enriched in RB-D OBs (**Fig. 5H**).

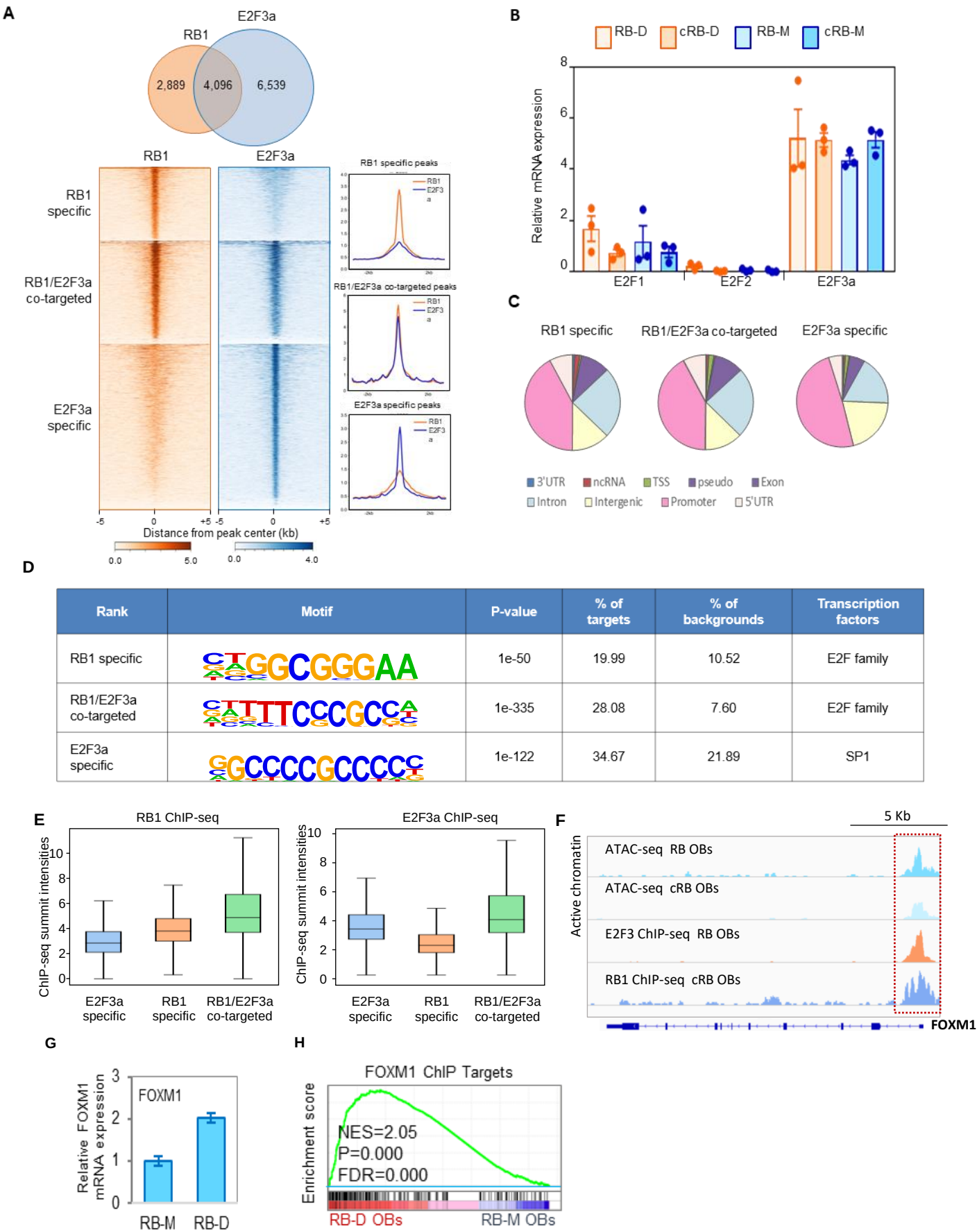


Figure 5. ChIP-seq assay to comprehensively identify preferential binding of RB1 and E2F3 to specific promoters in OBs. (A). RB1 and E2F3a directly co-target spliceosomal genes (A) (Top) Venn diagram depicts the overlap between RB1 and E2F3a binding peaks in cRB OBs defines RB1 specific (2,889), RB1/E2F3a 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 $\pm$ 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 intensities of total RB1 ChIP-seq signals at RB1-specific, RB1/E2F3a co-targeted, E2F3a-specific sites. (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/E2F3 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) qRT-PCR indicates increased *FOXM1* mRNA in RB-D OBs compared to RB-M OBs. (H) FOXM1 ChIP targets are enriched in RB-D OBs.

Study the expression of RB1, FOXM1, and E2F3 in human primary osteosarcoma specimens (US Biomax, OS408 osteosarcoma tissue array) by immunohistochemical (IHC) staining with specific antibodies (**Subtask 2**). All human OS tissue samples were acquired from The Department of Musculoskeletal Oncology, The First Affiliated Hospital, Sun Yat-sen University after obtaining patient or parental informed consent. IHC staining was performed as previously described (26, 27). Antibodies against RB1 (1:800, #9309, Cell Signaling Technology) and E2F3a (1:400, ab50917, Abcam), were used for IHC staining. Differences in RB1 and E2F3a expression among OS specimens were analyzed by Chi-square test, and $p < 0.05$ was considered statistically significant. The levels of IHC staining were evaluated by two independent pathologists. The intensity of staining was ranked into four groups: high, 3; medium, 2; low, 1; and negative, 0. The immunoreactivity score system, generated by incorporating both the proportion of immune-positive cells and the intensity of staining among positive cells, was applied to evaluate the immunoreactivities. The IHC staining cutoff for high or low expression of the indicated protein was calculated according to receiver operating characteristic (ROC) curve analysis. We observed an inverse correlation between RB1 and FOXM1 and a positive correlation between E2F3 and FOXM1 in clinical osteosarcoma specimens (**Fig. 6A**). By analyzing the TARGET database and TCGA database (**Subtask 3**), we find that low RB1, high E2F3 and high FOXM1 gene expression is correlated with poor prognosis in osteosarcoma patients (**Fig. 6B-D**). we find high FOXM1 and it regulated proliferation-associated genes (e.g., G2/M genes) in RB1-low or deficient osteosarcoma samples, with levels correlated with tumor stage and prognosis (**Fig. 6E**). Also, our studies of E2F3 and FOXM1 expression in the TCGA SARC database revealed the positive correlation between E2F3 and FOXM1 and high FOXM1 expression is correlated with poor prognosis (**Fig. 6F-G**). Based on these findings, we conclude that one of the tumor suppressor functions of RB1 is mediated through E2F3 controlling FOXM1-mediated gene expression.

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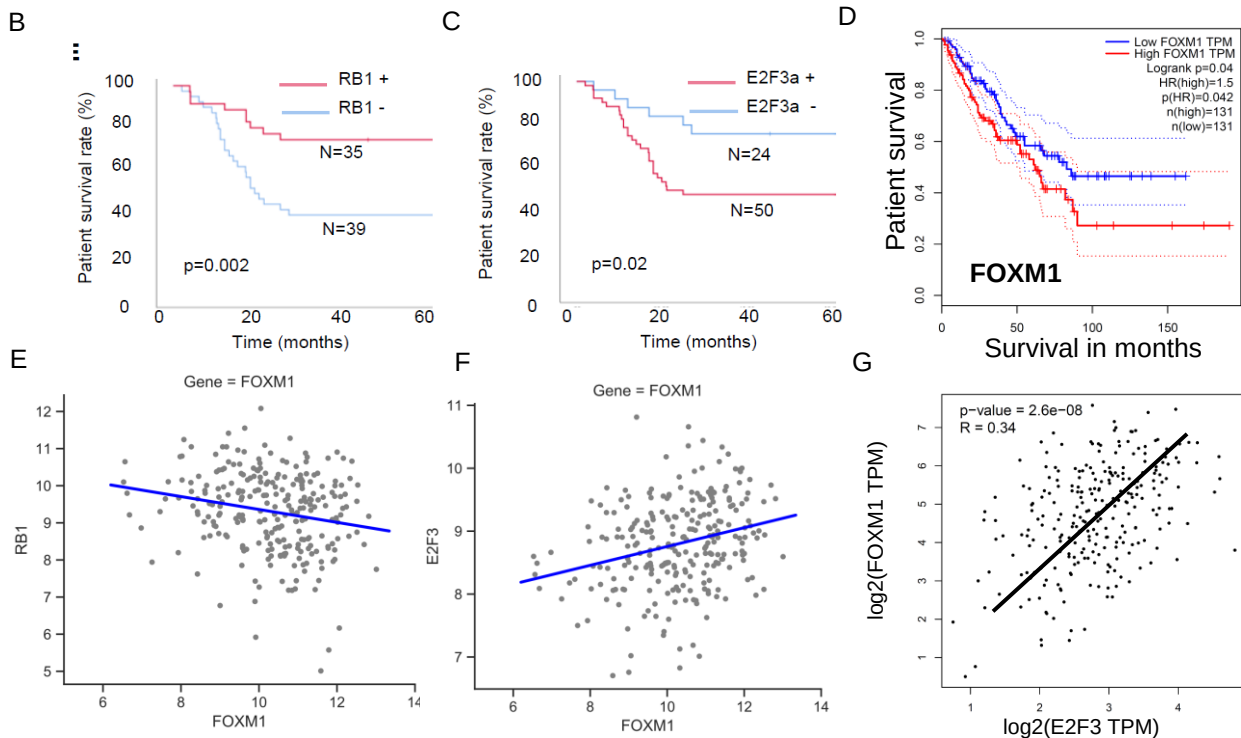
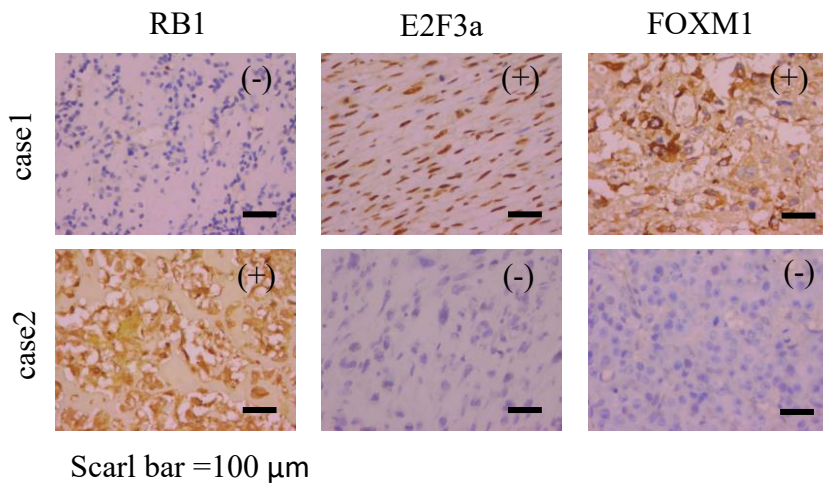


Figure 6. Clinical relevance of RB1, E2F3 and FOXM1. (A) The expression of RB1, FOXM1 and E2F3 in human primary osteosarcoma specimens by immunohistochemical (IHC) staining. (B-D) Kaplan-Meier overall survival curves indicate that decreased RB1 (B) elevated E2F3a, and elevated FOXM1 (C) are associated with a reduction of overall survival among OS cancer patients. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns, $p > 0.05$. (E) A negative correlation between RB1 and FOXM1. (F-G) A positive correlation between E2F3a and FOXM1 ($p=2.6e-08$; $R=0.34$).

To evaluate the therapeutic potential of FOXM1 inhibitors for RB1-mutant osteosarcoma treatment (**Specific Aim 2**). Our hypothesis is that FOXM1 is a therapeutic vulnerability in RB1-mutant osteosarcoma and that inhibition of FOXM1 leads to widespread defects in cell survival. To address this hypothesis, we performed an *in vitro* competition assay (28, 29) to determine if upregulated FOXM1 is required for cell viability of RB1-mutant cells (**Subtask 1**). This fluorescence-based proliferation 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. Depletion of FOXM1 may lead to cell death since FOXM1 is essential for normal cell function. Therefore, we employ a partial depletion strategy (20%-40% knockdown) to ensure that cells retain a moderate level of FOXM1 to execute their normal function.

We observed upregulation of FOXM1 in RB OBs, RB1-knockdown, and RB1-null iPSC-derived OBs (**Fig. 7A**). In contrast, ectopic expression of RB1 led to FOXM1 downregulation (**Fig. 7B**). Encouragingly, our in vitro competition assay showed that RB OBs display increased sensitivity to Thiostrepton in a dose-dependent manner (**Fig. 7C**), suggesting that pharmacological inhibition of FOXM1 has potential in treating RB1-mutant cells. To determine if loss of RB1 leads to increased sensitivity to FOXM1 inhibitors, RB and RB deficient OBs are treated with Thiostrepton (30) or Siomycin A (31). We examine the therapeutic effect of FOXM1 inhibitor Thiostrepton/Siomycin A to RB-mutant cells and observed lower cell viability indicating higher cell apoptosis in RB-mutant cells (**Fig. 7D-E**) (**Subtask 2 and 3**).

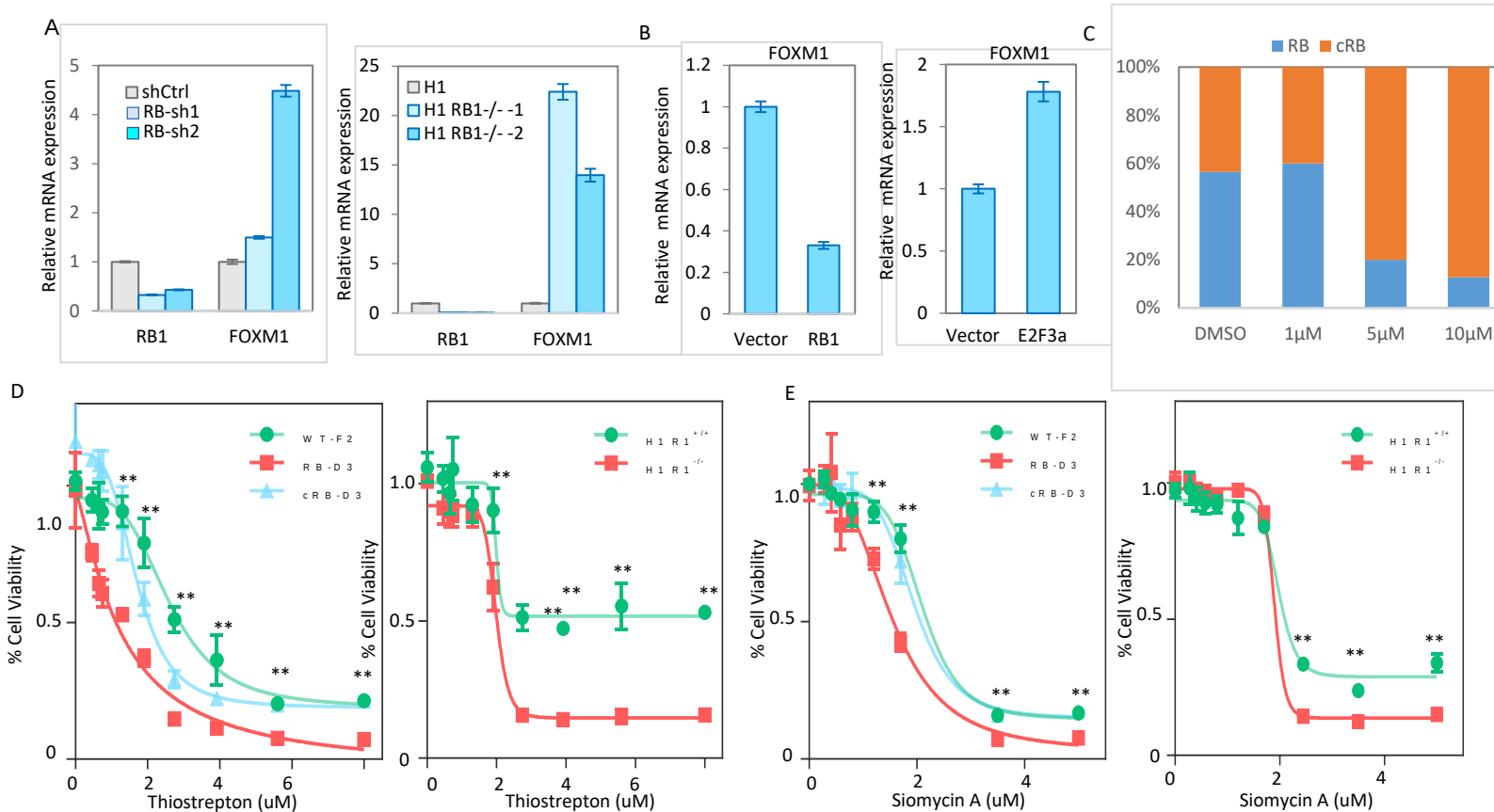


Figure 7. FOXM1 inhibitors treatment. (A). Upregulation of FOXM1 in RB, RB1-knockdown, and RB-knockout OBs. (B). RB1 inhibits FOXM1 expression through the negative regulation of E2F3. (C). In vitro perturbation of FOXM1 function impairs RB1-deficient cell growth. (D) Thiostrepton-treated RB OBs show significantly decreased cell proliferation and lower cell viability. (E) Siomycin-treated RB OBs show significantly decreased cell proliferation and lower cell viability.

Taken together, our findings show that RB1 mutation leads to an increase in FOXM1 culminating through E2F3. Our evaluation of FOXM1 inhibitors in RB-mutant cells indicates that FOXM1 is a therapeutic vulnerability in RB1-mutant osteosarcoma. Our findings imply that FOXM1 inhibitors may be applied for treating osteosarcoma with deficient RB1 expression.

References

1. Grana X, Garriga J, Mayol X. Role of the retinoblastoma protein family, pRB, p107 and p130 in the negative control of cell growth. *Oncogene*. 1998;17(25):3365-83. doi: 10.1038/sj.onc.1202575. PubMed PMID: 9916999.
2. van den Heuvel S, Dyson NJ. Conserved functions of the pRB and E2F families. *Nat Rev Mol Cell Biol*. 2008;9(9):713-24. doi: 10.1038/nrm2469. PubMed PMID: 18719710.

3. Chinnam M, Goodrich DW. RB1, development, and cancer. *Curr Top Dev Biol.* 2011;94:129-69. doi: 10.1016/B978-0-12-380916-2.00005-X. PubMed PMID: 21295686; PMCID: PMC3691055.
4. Dick FA, Rubin SM. Molecular mechanisms underlying RB protein function. *Nat Rev Mol Cell Biol.* 2013;14(5):297-306. doi: 10.1038/nrm3567. PubMed PMID: 23594950; PMCID: PMC4754300.
5. Xu X, Bieda M, Jin VX, Rabinovich A, Oberley MJ, Green R, Farnham PJ. A comprehensive ChIP-chip analysis of E2F1, E2F4, and E2F6 in normal and tumor cells reveals interchangeable roles of E2F family members. *Genome Res.* 2007;17(11):1550-61. doi: 10.1101/gr.6783507. PubMed PMID: 17908821; PMCID: PMC2045138.
6. Knudsen ES, Knudsen KE. Tailoring to RB: tumour suppressor status and therapeutic response. *Nat Rev Cancer.* 2008;8(9):714-24. doi: 10.1038/nrc2401. PubMed PMID: 19143056; PMCID: PMC2914856.
7. Laoukili J, Kooistra MR, Bras A, Kauw J, Kerkhoven RM, Morrison A, Clevers H, Medema RH. FoxM1 is required for execution of the mitotic programme and chromosome stability. *Nat Cell Biol.* 2005;7(2):126-36. doi: 10.1038/ncb1217. PubMed PMID: 15654331.
8. Wonsey DR, Follettie MT. Loss of the forkhead transcription factor FoxM1 causes centrosome amplification and mitotic catastrophe. *Cancer Res.* 2005;65(12):5181-9. doi: 10.1158/0008-5472.CAN-04-4059. PubMed PMID: 15958562.
9. Zona S, Bella L, Burton MJ, Nestal de Moraes G, Lam EW. FOXM1: an emerging master regulator of DNA damage response and genotoxic agent resistance. *Biochim Biophys Acta.* 2014;1839(11):1316-22. doi: 10.1016/j.bbagr.2014.09.016. PubMed PMID: 25287128; PMCID: PMC4316173.
10. Gormally MV, Dexheimer TS, Marsico G, Sanders DA, Lowe C, Matak-Vinkovic D, Michael S, Jadhav A, Rai G, Maloney DJ, Simeonov A, Balasubramanian S. Suppression of the FOXM1 transcriptional programme via novel small molecule inhibition. *Nat Commun.* 2014;5:5165. doi: 10.1038/ncomms6165. PubMed PMID: 25387393; PMCID: PMC4258842.
11. Koo CY, Muir KW, Lam EW. FOXM1: From cancer initiation to progression and treatment. *Biochim Biophys Acta.* 2012;1819(1):28-37. doi: 10.1016/j.bbagr.2011.09.004. PubMed PMID: 21978825.
12. Laoukili J, Stahl M, Medema RH. FoxM1: at the crossroads of ageing and cancer. *Biochim Biophys Acta.* 2007;1775(1):92-102. doi: 10.1016/j.bbcan.2006.08.006. PubMed PMID: 17014965.
13. Gartel AL. Thioestron, proteasome inhibitors and FOXM1. *Cell Cycle.* 2011;10(24):4341-2. doi: 10.4161/cc.10.24.18544. PubMed PMID: 22134246; PMCID: PMC3272263.
14. Gartel AL. A new target for proteasome inhibitors: FoxM1. *Expert Opin Investig Drugs.* 2010;19(2):235-42. doi: 10.1517/13543780903563364. PubMed PMID: 20074015; PMCID: PMC3532816.
15. Bhat UG, Zipfel PA, Tyler DS, Gartel AL. Novel anticancer compounds induce apoptosis in melanoma cells. *Cell Cycle.* 2008;7(12):1851-5. doi: 10.4161/cc.7.12.6032. PubMed PMID: 18583930.
16. Radhakrishnan SK, Bhat UG, Hughes DE, Wang IC, Costa RH, Gartel AL. Identification of a chemical inhibitor of the oncogenic transcription factor forkhead box M1. *Cancer Res.* 2006;66(19):9731-5. doi: 10.1158/0008-5472.CAN-06-1576. PubMed PMID: 17018632.
17. Zhu D, Kong CSL, Gingold JA, Zhao R, Lee DF. Induced Pluripotent Stem Cells and Induced Pluripotent Cancer Cells in Cancer Disease Modeling. *Adv Exp Med Biol.* 2018;1119:169-83. doi: 10.1007/5584_2018_257. PubMed PMID: 30069853.
18. Gingold JA, Zhu D, Lee DF, Kaseb A, Chen J. Genomic Profiling and Metabolic Homeostasis in Primary Liver Cancers. *Trends Mol Med.* 2018;24(4):395-411. doi: 10.1016/j.molmed.2018.02.006. PubMed PMID: 29530485.
19. Su J, Zhu D, Huo Z, Gingold JA, Ang YS, Tu J, Zhou R, Lin Y, Luo H, Yang H, Zhao R, Schaniel C, Moore KA, Lemischka IR, Lee DF. Genomic Integrity Safeguards Self-Renewal in Embryonic Stem Cells. *Cell Rep.* 2019;28(6):1400-9 e4. doi: 10.1016/j.celrep.2019.07.011. PubMed PMID: 31390555; PMCID: PMC6708277.
20. Kim H, Yoo S, Zhou R, Xu A, Bernitz JM, Yuan Y, Gomes AM, Daniel MG, Su J, Demicco EG, Zhu J, Moore KA, Lee DF, Lemischka IR, Schaniel C. Oncogenic role of SFRP2 in p53-mutant osteosarcoma development via autocrine and paracrine mechanism. *Proc Natl Acad Sci U S A.* 2018;115(47):E11128-E37. doi: 10.1073/pnas.1814044115. PubMed PMID: 30385632.
21. Lee DF, Su J, Kim HS, Chang B, Papatsenko D, Zhao R, Yuan Y, Gingold J, Xia W, Darr H, Mirzayans R, Hung MC, Schaniel C, Lemischka IR. Modeling familial cancer with induced pluripotent stem cells. *Cell.*

- 2015;161(2):240-54. Epub 2015/04/11. doi: S0092-8674(15)00246-9 [pii] 10.1016/j.cell.2015.02.045. PubMed PMID: 25860607; PMCID: 4397979.
22. Zhou R, Xu A, Tu J, Liu M, Gingold JA, Zhao R, Lee DF. Modeling Osteosarcoma Using Li-Fraumeni Syndrome Patient-derived Induced Pluripotent Stem Cells. *J Vis Exp*. 2018(136). doi: 10.3791/57664. PubMed PMID: 29985349.
23. Walkley CR, Qudsi R, Sankaran VG, Perry JA, Gostissa M, Roth SI, Rodda SJ, Snay E, Dunning P, Fahey FH, Alt FW, McMahon AP, Orkin SH. Conditional mouse osteosarcoma, dependent on p53 loss and potentiated by loss of Rb, mimics the human disease. *Genes Dev*. 2008;22(12):1662-76. doi: 10.1101/gad.1656808. PubMed PMID: 18559481; PMCID: PMC2428063.
24. Clarke AR, Maandag ER, van Roon M, van der Lugt NM, van der Valk M, Hooper ML, Berns A, te Riele H. Requirement for a functional Rb-1 gene in murine development. *Nature*. 1992;359(6393):328-30. doi: 10.1038/359328a0. PubMed PMID: 1406937.
25. Chicas A, Wang X, Zhang C, McCurrach M, Zhao Z, Mert O, Dickins RA, Narita M, Zhang M, Lowe SW. Dissecting the unique role of the retinoblastoma tumor suppressor during cellular senescence. *Cancer Cell*. 2010;17(4):376-87. doi: 10.1016/j.ccr.2010.01.023. PubMed PMID: 20385362; PMCID: PMC2889489.
26. Lin YH, Jewell BE, Gingold J, Lu L, Zhao R, Wang LL, Lee DF. Osteosarcoma: Molecular Pathogenesis and iPSC Modeling. *Trends Mol Med*. 2017;23(8):737-55. doi: 10.1016/j.molmed.2017.06.004. PubMed PMID: 28735817; PMCID: PMC5558609.
27. Gingold J, Zhou R, Lemischka IR, Lee DF. Modeling Cancer with Pluripotent Stem Cells. *Trends Cancer*. 2016;2(9):485-94. doi: 10.1016/j.trecan.2016.07.007. PubMed PMID: 27722205; PMCID: PMC5050918.
28. Lee DF, Su J, Ang YS, Carvajal-Vergara X, Mulero-Navarro S, Pereira CF, Gingold J, Wang HL, Zhao R, Sevilla A, Darr H, Williamson AJ, Chang B, Niu X, Aguilo F, Flores ER, Sher YP, Hung MC, Whetton AD, Gelb BD, Moore KA, Snoeck HW, Ma'ayan A, Schaniel C, Lemischka IR. Regulation of embryonic and induced pluripotency by aurora kinase-p53 signaling. *Cell stem cell*. 2012;11(2):179-94. Epub 2012/08/07. doi: S1934-5909(12)00339-6 [pii] 10.1016/j.stem.2012.05.020. PubMed PMID: 22862944; PMCID: 3413175.
29. Lee DF, Su J, Sevilla A, Gingold J, Schaniel C, Lemischka IR. Combining competition assays with genetic complementation strategies to dissect mouse embryonic stem cell self-renewal and pluripotency. *Nature protocols*. 2012;7(4):729-48. Epub 2012/03/24. doi: nprot.2012.018 [pii] 10.1038/nprot.2012.018. PubMed PMID: 22441292.
30. Garte AL. Thiostrepton, proteasome inhibitors and FOXM1. *Cell Cycle*. 2011 Dec 15;10(24):4341-2. PMID: 22134246. PMCID: PMC3272263.
31. Klinhom-On N, Seubwai W, Sawanyawisuth K, Obchoei S, Mahalapbutr P, Wongkham S. FOXM1 inhibitor, Siomycin A, synergizes and restores 5-FU cytotoxicity in human cholangiocarcinoma cell lines via targeting thymidylate synthase. *Life Sci*. 2021 Dec 1; 286:120072. PMID: 34688691.

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

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

First, considering that 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 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 translational research at Baylor College of Medicine) and Dr. Lisa L. Wang (an osteosarcoma expert at Texas Children's Hospital) for her research project. With their involvement, I have the opportunity to learn how to do the primary culture of osteosarcoma cells from patients. Regarding the computational biology field, I have learned how to use TCGA and GEO databases. In addition, to elucidate how the 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 software.

Third, my mentor always encourages me to attend weekly lab meetings, join the 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 the Bone club meeting hosted by BCM and Sarcoma meeting hosted by Texas Children's Hospital. For example, I attended our Department Seminar Series "Osteosarcoma Epidemiology and Inherited Genetic Susceptibility" given by Lisa Mirabello (NIH) on April 26, 2021. Recently, I just attended the 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.

Moreover, I attended Texas Bone Disease Program on September 10, 2021. I met with scientists in bone diseases including osteosarcoma. I was selected as Finalist and present my research work as an invited talk. Most importantly, I attend the AACR meeting and did a poster presentation on April 8-12, 2022. The AACR Annual Meeting is the biggest event in the cancer research community. I met with scientists, clinicians, and other health care professionals. I learned history from survivors and patients. It was really a good opportunity for me to learn the latest advances in cancer science and medicine including the bone diseases field.

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

How were the results disseminated to communities of interest?

I submitted abstract to AACR meeting and did poster presentation at April 8-13, 2022. In the AACR Annual Meeting, there were survivors, patients, and advocates gathering. I presented my research work to them thus enhancing public understanding and increasing interest in learning and careers in science, technology, and humanities.

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

Nothing to Report

4. IMPACT:

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

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 and comprises almost 60% of bone tumors. RB1 is a protein that controls normal cell growth. The loss of RB1 due to its gene mutations or deletions is found in more than 30% of clinical osteosarcoma specimens. Patients with hereditary retinoblastoma (RB), an inherited autosomal dominant cancer disorder, have a more than a 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 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 the first human RB iPSCs from RB patients. 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 is the first step to exploring 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?

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?

Nothing to Report

What was the impact on society beyond science and technology?

Nothing to Report

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

Nothing to Report

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

Nothing to Report

Changes that had a significant impact on expenditures

Nothing to Report

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

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:

- **Publications, conference papers, and presentations**

Journal publications.

1. **Zhu D**, Huang MF, Xu A, Gao X, Lu L, Chi TY, Pang LK, Gingold JA, Tu J, Zhou RJ, Bazer DA, Ambrose CG, Shen J, Kameoka J, Yang Z, Zhao R, Lee DF. Genome-wide profiling of human iPSCs/hESC derived osteoblast reveals CROIN as functional surface marker. (PNAS, submitted) *Acknowledgement of federal support (yes)*
2. Tu J, Huo Z, Yu Y, **Zhu D**, Xu A, Wang R, Hu R, Gingold J, Su J, Huang MF, Tsai K, Zhou R, Chen H, Chen S, Webb T, Yang H, Jia P, Zhao Z, Zhao R, Huff C, Shen J, Lee DF. The spliceosome is an exploitable vulnerability in RB1-mutant cancers. *Proc Natl Acad Sci U S A*. 2022 Apr 19;119(16):2117857119. PMID: 35412907. *Acknowledgement of federal support (yes)*
3. Malla S, Bhattarai D, Melguizo-Sanchis, Atanasoai I, Groza P, Román AC, **Zhu D**, Lee DF, Kutter C, Aguilo F. ZFP207 controls pluripotency by multiple post-transcriptional mechanisms. *EMBO Rep*. 2022 Mar 3; 23(3): 53191. PMID: 35037361. *Acknowledgement of federal support (yes)*
4. Jewell B, Xu A, **Zhu D**, Huang MF, Liu M, Lu L, Underwood E, Park J, Gingold J, Zhou R, Tu J, Huo Z, Jin W, Rainusso N, Berg N, Bazer D, Vellano C, Jones P, Eltschig H, Kaiparettu B, Zhao R, Wang L, Lee DF. Patient-derived iPSCs link elevated mitochondrial respiratory complex I function to osteosarcoma in Rothmund-Thomson syndrome. *PLoS Genet*. 2021 Dec 29;17(12):1009971. PMID: 34965247. *Acknowledgement of federal support (yes)*
5. Xu A, Huang MF, **Zhu D**, Gingold JA, Bazer DA, Chang B, Wang D, Lai CC, Lemischka IR, Zhao R, Lee DF. LncRNA H19 Suppresses Osteosarcomagenesis by Regulating snoRNAs and DNA Repair Protein Complexes. *Front Genet*. 2021 Jan 15;11:611823. PMID: 33519915. *Acknowledgement of federal support (yes)*

Books or other non-periodical, one-time publications.

Nothing to Report

Other publications, conference papers and presentations.

1. Pang LK, **Zhu D**, Lee DF. Potential Role of SIX2 in Osteogenic Dysregulation and Osteosarcoma. 2020 CPRIT summer research poster presentation, Houston, TX, 08/10/2020.
2. Yang WL, Huo Z, Chen SY, **Zhu D**, Liu TJ, Lee DF. Monitoring of stem cell differentiation to mature hepatocytes with a machine learning-based AI model. AACR annual meeting 2021, (Virtual Annual Meeting). 4/9-14/2022.
3. **Zhu D**, Tu J, Huo Z, Xu A, Huang MF, Liu Y, Chen YH, Zhao R, Lee DF. Dissecting the Biology and Therapeutic Vulnerabilities of RB1-Mutant Osteosarcoma Using Hereditary Retinoblastoma iPSCs. AACR annual meeting 2022, New Orleans, LA. 4/11/2022.
4. Xu A, Jewell BE, **Zhu D**, Huang MF, Chen YH, Lu L, Zhao R, Wang LL, Lee DF. Patient-derived iPSCs reveal pharmacologic targeting mitochondrial respiratory complex I for treating Rothmund-Thomson syndrome associated osteosarcoma. AACR annual meeting 2022, New Orleans, LA. 4/13/2022.
5. Huang MF, Luo H, Xu A, **Zhu D**, Tsai KL, Lee DF, Zhao R. Mutant p53 confers chemoresistance by activating KMT5B-mediated DNA repair pathway in nasopharyngeal carcinoma. 2022 McGovern Medical School Research Retreat, Houston, TX. 4/28/2022.
6. Huang MF, Luo H, Xu A, **Zhu D**, Tsai KL, Lee DF, Zhao R. Mutant p53 confers chemoresistance by activating KMT5B-mediated DNA repair pathway in nasopharyngeal carcinoma. The University of Texas Health Science Center at Houston, Department of Integrative Biology and Pharmacology Retreat, Houston, TX. 5/4/2022.

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

Nothing to Report

- **Technologies or techniques**

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 RB1 mutation in tumorigenesis. This platform could be shared by cancer, bone as well as other research community.

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

Nothing to Report

- **Other Products**

1. We have generated RB/cRB iPSC model.
2. We have established a model system to recapture RB1-associated bone malignancies and explore therapeutic interventions.
3. We have generated RNA-seq data of RB/cRB iPSCs derived osteoblasts (GSE145235).
4. We have generated ChIP-seq data of RB, E2F3 and FOXM1 (GSE145235).
5. 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?

Example:

Name:

Dandan Zhu

Project Role:

Principle Investigator

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?

Nothing to Report

What other organizations were involved as partners?

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

QUAD CHARTS:

9. APPENDICES:

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EDUCATION

2016 *Ph.D.*, Cancer Biology, **The University of Hong Kong**, Hong Kong, China
2011 *M.S.*, Microbiology & Virology, **Wuhan University**, Wuhan, China
2009 *B.S.*, Biological Science, **Central China Normal University**, Wuhan, China

RESEARCH EXPERIENCE

2020-Present Principal Investigator, The Department of Army DoD Horizon Award
Dissecting the Biology and Therapeutic Vulnerabilities of RB1-Mutant
Osteosarcoma Using RB iPSCs”
Mentor: Dung-Fang Lee
2017-Present Postdoctoral fellow, Laboratory of Dung-Fang Lee, Department of Integrative
Biology and Pharmacology, McGovern Medical School, The University of
Texas Health Science Center at Houston, Houston, TX
Mentor: Dung-Fang Lee
2011-2016 Ph.D. candidate, Laboratory of Tsai-Wah Tsao, School of Biomedical Sciences,
LiKaShing Faculty of Medicine, The University of Hong Kong, Hong Kong,
China. Mentor: Tsai-Wah Tsao
2009-2011 Master student, Laboratory of Zishu Pan, The State Key Laboratory of
Virology, Wuhan University, Wuhan, China
Mentor: Zishu Pan
2006-2009 Bachelor student, Laboratory of Guoxiu Wang, College of Life Science,
Central China Normal University, Wuhan, China
Mentor: Guoxiu Wang

TEACHING EXPERIENCE

2022 Bench mentor of GSBS student Yueh-Ming Shyu. Department of Integrative
Biology and Pharmacology, McGovern Medical School, The University of
Texas Health Science Center at Houston, Houston, TX
2019-2020 Teaching assistant of intern student Bill Lin (Taiwan National Defense
University) Department of Integrative Biology and Pharmacology, McGovern
Medical School, The University of Texas Health Science Center at Houston,
Houston, TX
2018 Teaching assistant of intern student LonKai Pang (Rice University),
Department of Integrative Biology and Pharmacology, McGovern Medical
School, The University of Texas Health Science Center at Houston, Houston,
TX

2016	Teaching assistant of M.D. student Emily Chen (The University of Queensland, Australia). The University of Hong Kong, Hong Kong
2014	Teaching assistant of exchange student Sharmila Velapasamy (Malaysia University, Malaysia). The University of Hong Kong, Hong Kong, China
2011	Teaching Assistant. Molecular Biology course. Wuhan University, China
2010	Teaching Assistant. Cell Biology course. Wuhan University, China

GRANT

2020-Present	The Department of Defense. DoD Horizon Award “Dissecting the Biology and Therapeutic Vulnerabilities of RB1-Mutant Osteosarcoma Using RB iPSCs”
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HONORS AND DISTINCTIONS

2021	Finalist, Rolanette and Berdon Lawrence Bone Disease Program of Texas
2020	DoD Horizon Award, Department of the Defense
2019	Travel Award, 2nd Oversea Young Investigators Symposium of China, New York, NY
2016	2nd prize of Research Proposal Award, 1st Exchange Camp of Biomedical Research Graduates, China
2016	2nd prize of Oral presentation Award, 1st Exchange Camp of Biomedical Research Graduates, China
2011-2016	Ph.D. Scholarship, The University of Hong Kong, Hong Kong, China
2015	International Conference Travel Award, The University of Hong Kong, Hong Kong, China
2013	International Conference Travel Award, The University of Hong Kong, Hong Kong, China
2011	Outstanding Graduates, Wuhan University, China
2010	The First Prize Scholarship Award, Wuhan University, China
2009	The First Prize Scholarship Award, Wuhan University, China
2009	1st Class Undergraduates, Central China Normal University, Wuhan, China
2005-2009	1st Class Fellowship, Central China Normal University, Wuhan, China
2005	Merit Student of Henan Province, China

PROFESSIONAL MEMBERSHIPS

2021-Present	Reviewer, American Journal of Cancer Research
2021	Reviewer, Frontiers in Pharmacology
2018-Present	Reviewer, Journal of Investigative Medicine
2018-Present	Reviewer, Oncotargets and Therapy
2017-Present	Reviewer, Oncotargets
2011-2016	Member, Hong Kong International Cancer Congress, Hong Kong, China
2015-Present	Editorial board member, Translational Cancer Research
2014-Present	Teaching and Learning in Higher Education: Stage 1. The University of Hong Kong
2013-Present	Associate Member, American Association for Cancer Research (AACR)

PRESENTATIONS

Oral Presentations

- 2021 The role of IL1R1 in bone development and formation. Rolanette and Berdon Lawrence Bone Disease Program of Texas Retreat. Houston, TX
- 2019 Characterization and Identification of human osteoblast. Center for Stem Cell and Regenerative Medicine (CSCRM) Seminars. Houston, TX
- 2016 NF- κ B signaling in immortalization of nasopharyngeal epithelial cells. The University of Hong Kong, Hong Kong, China

Poster Presentations

- 2022 Dissecting the Biology and Therapeutic Vulnerabilities of RB1-Mutant Osteosarcoma Using RB iPSCs. AACR meeting, Washington D.C.
- 2016 The role of NF- κ B activation in immortalization of nasopharyngeal epithelial cells. Gordon Research Conference. Hong Kong, China
- 2015 Epstein-Barr Virus encoded LMP1 in immortalization of nasopharyngeal epithelial cells. Cell Metabolism in Cancer and Stromal Cells. Belgium
- 2013 The role of NF- κ B activation in immortalization of nasopharyngeal epithelial cells. American Association for Cancer Research (AACR). Washington D.C.

PUBLICATIONS

1. **Zhu D**, Gao Xue, Huang MF, Xu A, Chen H, Lin T, Dawson C, Lee B, Ambrose C, Zhang Y, Zhao R, Lee DF. Functional osteoblastic IL1R1 is a prerequisite for bone development. (under preparation)
2. **Zhu D**, Huang MF, Xu A, Gao X, Lin T, Chi TY, Dai Y, Pang LK, Gingold JA, Tu J, Zhou R, Huo ZJ, Su J, Bazer A, Ambrose C, Shen J, Kameoka J, Zhao Z, Wang L, Zhang Y, Zhao R, Lee DF. Genome-wide profiling of human osteoblasts reveals surface marker gene CORIN governing human osteoblast identifies. (PNAS, submitted)
3. Tu J, Huo Z, Yu Y, **Zhu D**, Xu A, Wang R, Hu R, Gingold J, Su J, Huang MF, Tsai K, Zhou R, Chen H, Chen S, Webb T, Yang H, Jia P, Zhao Z, Zhao R, Huff C, Shen J, Lee DF. Hereditary retinoblastoma iPSC model reveals aberrant spliceosome function driving bone malignancies. *Proc Natl Acad Sci U S A*. 2022 Apr 19;119(16). PMID: 35412907.
4. Malla S, Bhattarai D, Melguizo-Sanchis, Atanasoai I, Groza P, Román AC, **Zhu D**, Lee DF, Kutter C, Aguilo F. ZFP207 sustains pluripotency by coordinating OCT4 stability, alternative splicing and RNA export. *EMBO Rep*. 2022 Feb 3;23(3). PMID: 35037361.
5. Jewell B, Xu A, **Zhu D**, Huang MF, Liu M, Lu L, Underwood E, Park J, Gingold J, Zhou R, Tu J, Huo Z, Jin W, Rainusso N, Berg N, Bazer D, Vellano C, Jones P, Eltschig H, Kaiparettu B, Zhao R, Wang L, Lee DF. Patient-derived iPSCs link elevated mitochondrial respiratory complex I function to osteosarcoma in Rothmund-Thomson syndrome. *PLoS Genet*. 2021 Dec 29;17(12). PMID: 34965247.
6. Gao X, Ruzbarsky J, Philippon M, Ravuri S, **Zhu D**, Utsunomiya H, Scibetta A, Huard J. The beneficial effect of intra-articular injection of losartan on the microfracture mediated cartilage repair is dose dependent. *Am J Sports Med*. 2021 Jul;49(9):2509-2521. PMID: 34259597.
7. Xu A, Huang MF, **Zhu D**, Gingold J, Bazer D, Chang B, Wang D, Lai CC, Lemischka I, Zhao R, Lee DF. LncRNA H19 suppresses osteosarcomagenesis by regulating snoRNAs and DNA repair protein complexes. *Front Genet*. 2021, 15(11):611823. PMID: 33519915.
8. Huo Z, Tu J, Xu A, Li Y, Wang D, Liu M, Zhou R, **Zhu D**, Lin Y, Gingold JA, Yen CJ, Xiao H, Zhao R. Generation of a heterozygous p53 R249S mutant human embryonic stem cell line by TALEN-mediated genome editing. *Stem Cell Res*. 2019 Jan;34:101360. PMID:

30640060.

9. Su J, **Zhu D**, Huo Z, Gingold JA, Ang YS, Tu J, Zhou R, Lin Y, Luo H, Yang H, Zhao R, Schaniel C, Moore KA, Lemischka IR, Lee DF. Genomic integrity safeguards self-renewal in embryonic stem cells. *Cell Rep*. 2019, 28(6):1400-1409. PMID: 31390555.
10. **Zhu D**, Kong CSL, Gingold JA, Zhao R, Lee DF. Induced pluripotent stem cells and induced pluripotent cancer cells in cancer disease modeling. *Adv Exp Med Biol*. 2018, 1119:169-183. PMID: 30069853.
11. Tu J, Huo Z, Liu M, Wang D, Xu A, Zhou R, **Zhu D**, Gingold J, Shen J, Zhao R, Lee D. Generation of human embryonic stem cell line with heterozygous RB1 deletion by CRIPSR/Cas9 nickase. *Stem Cell Res*. 2018 Apr;28:29-32. PMID: 29414415.
12. Xu A, Zhou R, Tu J, Huo Z, **Zhu D**, Wang D, Gingold JA, Mata H, Rao PH, Liu M, Mohamed AMT, Kong CSL, Jewell BE, Xia W, Zhao R, Hung MC, Lee DF. Establishment of a human embryonic stem cell line with homozygous TP53 R248W mutant by TALEN mediated gene editing. *Stem Cell Res*. 2018 Apr 27;29:215-219. PMID: 29730572.
13. Jewell BE, Liu M, Lu L, Zhou R, Tu J, **Zhu D**, Huo Z, Xu A, Wang D, Mata H, Jin W, Xia W, Rao PH, Zhao R, Hung MC, Wang LL, Lee DF. Generation of an induced pluripotent stem cell line from an individual with a heterozygous RECQL4 mutation. *Stem Cell Res*. 2018 Dec;33:36-40. PMID: 30312871.
14. Gingold JA, **Zhu D**, Lee DF, Kaseb A, Chen J. Genomic Profiling and metabolic homeostasis in primary liver cancers. *Trends Mol Med*. 2018, (4):395-411. PMID: 29530485.
15. Zhou R, Xu A, Wang D, **Zhu D**, Mata H, Huo Z, Tu J, Liu M, Mohamed AMT, Jewell BE, Gingold J, Xia W, Rao PH, Hung MC, Zhao R, Lee DF. A homozygous p53 R282W mutant human embryonic stem cell line generated using TALEN-mediated precise gene editing. *Stem Cell Res*. 2018, 27:131-135. PMID: 29414603.
16. Zhang J, Jia L, Lin W, Yip Y, Lo K, Lau M, **Zhu D**, Tsang C, Zhou Y, Deng W, Lung H, Lung L, Cheung L, Tsao SW. Epstein-Barr virus-encoded latent membrane protein 1 upregulates glucose transporter 1 transcription via the mTORC1/NF- κ B signaling pathways. *J Virol*. 2017 Feb 28;91(6):e02168-16. PMID: 28053105.
17. **Zhu D**, Zhang J, Deng W, Yip YL, Lung HL, Tsang CM, Law WT, Yang J, Lau VM, Shuen WH, Lung ML, Cheung AL, Tsao SW. Significance of NF- κ B activation in immortalization of nasopharyngeal epithelial cells. *Int J Cancer*. 2016,138(5):1175-85. PMID: 26370441.
18. Deng W, Zhou Y, Tiwari A, Su H, Yang J, **Zhu D**, Lau M, Hau P, Yip Y, Cheung L, Guan X, Tsao SW. p21/Cyclin E pathway modulates anticlastogenic function of Bmi - 1 in cancer cells. *Int J Cancer*. 2015,136(6):1361-70. PMID: 25131797.
19. Luo M, Qu X, Pan R, **Zhu D**, Zhang Y, Wu J, Pan Z. The virus-induced signaling adaptor molecule enhances DNA-raised immune protection against H5N1 influenza virus infection in mice. *Vaccine*. 2011, 29(14):2561-7. PMID: 21303708.
20. Tao P, Luo M, **Zhu D**, Qu S, Yang Z, Gao M, Guo D, Pan Z. Virus-like particle vaccine comprised of the HA, NA, and M1 proteins of an avian isolated H5N1 influenza virus induces protective immunity against homologous and heterologous strains in mice. *Viral Immunol*. 2009, 22(4):273-81. PMID: 19594398.

PATENT

1. **Zhu D**, Tao P, Qu X, Pan Z. A method of development of avian influenza virus-like

particles vaccine. 2011. SIPO, Patent No. 201010293705.9

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Hereditary retinoblastoma iPSC model reveals aberrant spliceosome function driving bone malignancies

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The *RB1* gene is frequently mutated in human cancers but its role in tumorigenesis remains incompletely defined. Using an induced pluripotent stem cell (iPSC) model of hereditary retinoblastoma (RB), we report that the spliceosome is an up-regulated target responding to oncogenic stress in *RB1*-mutant cells. By investigating transcriptomes and genome occupancies in RB iPSC-derived osteoblasts (OBs), we discover that both E2F3a, which mediates spliceosomal gene expression, and pRB, which antagonizes E2F3a, coregulate more than one-third of spliceosomal genes by cobinding to their promoters or enhancers. Pharmacological inhibition of the spliceosome in *RB1*-mutant cells leads to global intron retention, decreased cell proliferation, and impaired tumorigenesis. Tumor specimen studies and genome-wide TCGA (The Cancer Genome Atlas) expression profile analyses support the clinical relevance of pRB and E2F3a in modulating spliceosomal gene expression in multiple cancer types including osteosarcoma (OS). High levels of pRB/E2F3a-regulated spliceosomal genes are associated with poor OS patient survival. Collectively, these findings reveal an undiscovered connection between pRB, E2F3a, the spliceosome, and tumorigenesis, pointing to the spliceosomal machinery as a potentially widespread therapeutic vulnerability of pRB-deficient cancers.

hereditary retinoblastoma | iPSCs | pRB | spliceosomal genes | osteosarcoma

The retinoblastoma gene family, also known as pocket proteins, includes three members, *RB1* (p105), *RBL1* (p107), and *RBL2* (p130) (1). In contrast to *RBL1* and *RBL2*, which are very rarely mutated in human cancers, *RB1* mutations are commonly found in various cancer types (2). 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 (OS) (3, 4), suggesting a strong mechanistic link between pRB loss and osteosarcoma-genesis. By regulating E2F1/2/3 function, pRB fine-tunes the cell cycle (1) and cellular senescence (5). In addition to suppressing E2F transcriptional activities, pRB also modulates multiple cellular processes including apoptosis (6), centromeric and pericentromeric structure maintenance (7–9), homologous recombination (10), nonhomologous end joining (11), telomere preservation (12), and silencing of repetitive regions (13). These findings may contribute to some of the oncogenic phenotype associated with pRB loss, though the complete picture of pRB function in tumor suppression remains incompletely defined.

Alternative RNA splicing is a widespread biological process that allows for the expression of multiple RNA and protein isoforms from a single gene, contributing to structural transcript variation and proteome diversity in eukaryotes (14). Cancer cells exploit this process to express remarkable variation in transcriptome and splicing patterns (15, 16), often expressing unique cancer-specific splicing isoforms whose encoded proteins drive cancer progression or contribute to specific oncogenic features of the malignant cells. Expression levels of 261 known splicing factors were significantly more variable across 10 of 11 cancer types compared with normal noncancer controls (15). This altered variance supports the notion that dysregulation of spliceosomal genes is positively selected among cancers.

Several studies have revealed that pRB/E2F signaling regulates and is regulated by spliceosome function. A *Drosophila* study found that SR protein splicing factor B52 controls dE2F2 pre-messenger RNA (mRNA) splicing, suggesting that the spliceosome regulates pRB/E2F-mediated cell cycle control (17). Large-scale RNA interference screening in *Caenorhabditis elegans* revealed that partial inactivation of splicing-related genes overlaps with the pRB inactivation phenotype (18). Consistently, short hairpin RNA (shRNA) and CRISPR-Cas9 screening revealed that knockdown and/or knock-out of RNA splicing factors induce lethal effects in pRB-deficient cancers (19, 20).

Significance

Rare human hereditary disorders provide unequivocal evidence of the role of gene mutations in human disease pathogenesis and offer powerful insights into their influence on human disease development. Using a hereditary retinoblastoma (RB) patient-derived induced pluripotent stem cell (iPSC) platform, we elucidate the role of pRB/E2F3a in regulating spliceosomal gene expression. Pharmacological inhibition of the spliceosome in *RB1*-mutant cells preferentially increases splicing abnormalities of genes involved in cancer-promoting signaling and impairs cell proliferation and tumorigenesis. Expression of pRB/E2F3a-regulated spliceosomal proteins is negatively associated with pRB expression and correlates with poor clinical outcomes of osteosarcoma (OS) patients. Our findings strongly indicate that the spliceosome is an “Achilles’ heel” of *RB1*-mutant OS.

The authors declare no competing interest.

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Investigation of pRB-interacting protein complexes also revealed that pRB physically interacts with RNA-binding proteins, implying that the pRB/E2F pathway may modulate RNA processes through posttranscriptional regulatory machinery (21). Although these studies imply the potential for regulation between pRB/E2F and spliceosome function, the involvement of pRB/E2F as a global regulator of spliceosomal gene expression, culminating in cancer development following pRB loss, has not been previously established.

In this study, we established RB induced pluripotent stem cells (iPSCs) and mutation-corrected isogenic control iPSCs and applied them to elucidate the pathological mechanisms of osteosarcomagenesis caused by *RB1* mutation. By integrating iPSC and cancer cell studies, we unexpectedly find that pRB loss leads to increased expression of numerous spliceosomal genes and that the spliceosome is a therapeutic vulnerability in *RB1*-mutant cancers.

Results

Generation, Characterization, and Mutation Correction of RB iPSCs. To elucidate how *RB1* mutation contributes to tumor initiation and progression, we generated iPSCs from fibroblasts obtained from members of a family including two affected RB patients (mother and daughter) and one unaffected individual (father) (Fig. 1*A*). The mother developed retinoblastoma and the daughter developed both retinoblastoma and OS. Whole-exome sequencing (WES) of these family members revealed that the two affected patients harbor a heterozygous c.1531-1532 ins A that causes a D511fs mutation, which was confirmed by Sanger sequencing (SI Appendix, Fig. S1*A* and *B*). This D511fs mutation results in premature mRNA termination and decreased total pRB protein expression (SI Appendix, Fig. S1*C*). Using nonintegrating Sendai virus (SeV)-based cell reprogramming, we established iPSC clones from the affected and unaffected family members. These iPSC clones demonstrated human embryonic stem cell (hESC) morphology and expressed pluripotency factors (NANOG and OCT4), hESC surface markers (TRA-1-81 and SSEA4), and alkaline phosphatase (AP) (Fig. 1*B*). The lines also showed expression of pluripotency markers at levels comparable to H1 and H9 hESCs by qRT-PCR (SI Appendix, Fig. S1*D*). Consistent with their genotype, RB iPSCs demonstrated lower pRB protein than wild-type (WT) iPSCs (SI Appendix, Fig. S1*E*). We further verified the loss of SeV and exogenous *OCT4*, *SOX2*, *KLF4*, and *MYC* transgenes (SI Appendix, Fig. S1*F*), demonstrating that these WT and RB iPSCs have zero genetic footprints from reprogramming. Both WT and RB iPSC lines were karyotypically normal (SI Appendix, Fig. S1*G*) and demonstrated the capacity to differentiate into all three germ layers in teratomas (SI Appendix, Fig. S1*H*). Together, our results indicate that somatic cells from RB patients can be properly reprogrammed, maintain a pluripotent state, and be effectively differentiated. The characteristics of WT and RB iPSCs are summarized in SI Appendix, Table S1.

In order to generate isogenic controls and correct *RB1* mutation in RB iPSCs, we applied CRISPR-Cas9 nickase constructs targeting the *RB1* genome locus. The CRISPR-Cas9 nickase system minimizes off-target mutations by 50- to 1,500-fold compared with a CRISPR system inducing double-stranded breaks in cultured cells (22). The paired nickase plasmids were designed to target *RB1* intron 16 (c.1531-1532 ins A located in *RB1* exon 17) (Fig. 1*C*). The T7E1 assay validated on-target CRISPR-Cas9 editing events following treatment with CRISPR-Cas9 nickase plus single-guide RNA (sgRNA) pairs

(SI Appendix, Fig. S1*I*). The CRISPR-Cas9 plasmids and donor vector containing an FNF/Neo^R selection cassette (Frt-EM7-Neo^R-Frt) with 1-kb homologous arms were constructed and electroporated into RB iPSCs and selected with G418. Resistant clones were examined for FNF insertion in intron 16 of the *RB1* genomic locus by PCR (SI Appendix, Fig. S1*J*). Southern blot analysis confirmed the unique FNF insertion in the *RB1* genomic locus (SI Appendix, Fig. S1*K*). Once the corrected clones were confirmed by Sanger sequencing, we excised the FNF/Neo^R cassette by Flp recombinase (SI Appendix, Fig. S1*L*). Mutation-corrected iPSC lines (corrected RB [cRB] iPSCs) demonstrated a corrected *RB1* gene sequence (Fig. 1*D*) and expression of the restored pRB protein (Fig. 1*E*). The efficiency of correcting the *RB1* gene mutation with this CRISPR-Cas9 nickase system was 0.5 to 2.3% (SI Appendix, Fig. S1*M*).

RB iPSC-Derived Osteoblasts Demonstrate a Premalignant Phenotype. RB patients are susceptible to developing second cancers such as sarcomas, melanomas, and brain cancers. Among them, OS is the leading cause of death in RB survivors (3, 4). One RB patient in our studied family had already developed OS (Fig. 1*A*). The *RB1* gene is also frequently mutated in OS from patients without *RB1* germline mutations (4). We applied the RB iPSC model to understand the pathogenesis of OS caused by *RB1* mutation. Prior studies have suggested that OS likely arises from the acquisition of malignant features within osteoblasts (OBs) (4, 23). Since human OBs can be induced from multipotent mesenchymal stem cells (MSCs), our iPSC model could be used to assess the effects of *RB1* mutation in OBs. We first differentiated WT, RB, and cRB iPSCs to their corresponding MSCs. These iPSC-derived MSCs expressed the MSC surface markers CD105 and CD166 but not pluripotent marker CD24 (SI Appendix, Fig. S2*A*). In comparison with cRB MSCs, RB MSCs showed higher mRNA expression of numerous pRB/E2F targets *CCNA2*, *CDC6*, *CDK1*, *BRCA1*, and *RAD51* (SI Appendix, Fig. S2*B*) and E2F transcriptional reporter activity (SI Appendix, Fig. S2*C*). These studies demonstrate that RB MSCs maintain comparable cell-surface marker expression to WT and cRB MSCs and that RB MSCs partially lack pRB function while mutation-corrected cRB cells have restored pRB function.

MSCs were then induced to OBs as the osteogenic differentiation process was monitored over time (23–25). Consistent with mouse studies showing enhanced osteogenic differentiation upon loss of pRB (26), RB OBs showed increased bone-associated AP activity at day 6 (SI Appendix, Fig. S2*D*), mineral deposition at days 15 and 24 (SI Appendix, Fig. S2*E*), and expression of skeletal system development genes *ANKH*, *CLEC3B*, and *IGF2* at day 24 (SI Appendix, Fig. S2*F*). We compared expression levels of core transcriptional/epigenetic regulators in RB and cRB cells during osteogenic differentiation. None of the core osteogenic regulators, including ATF4, CEBPD, RUNX2, ZEB1, and ZNF521, showed impaired expression during osteogenic differentiation (SI Appendix, Fig. S2*G*), indicating that loss of pRB has a limited impact on the osteoblastic transcription factor network. To investigate whether RB OBs are able to recapitulate tumorigenic potential, we performed in vitro anchorage-independent growth (AIG) assays and in vivo xenografts. AIG assays showed clonal growth in soft agar by RB OBs but not WT and cRB OBs (Fig. 1*F*). In vivo xenograft assays demonstrated in vivo cell proliferation abilities in RB OBs but not WT and cRB OBs (Fig. 1*G*). The characteristics of WT, RB, and cRB iPSC-derived MSCs and OBs are summarized in SI

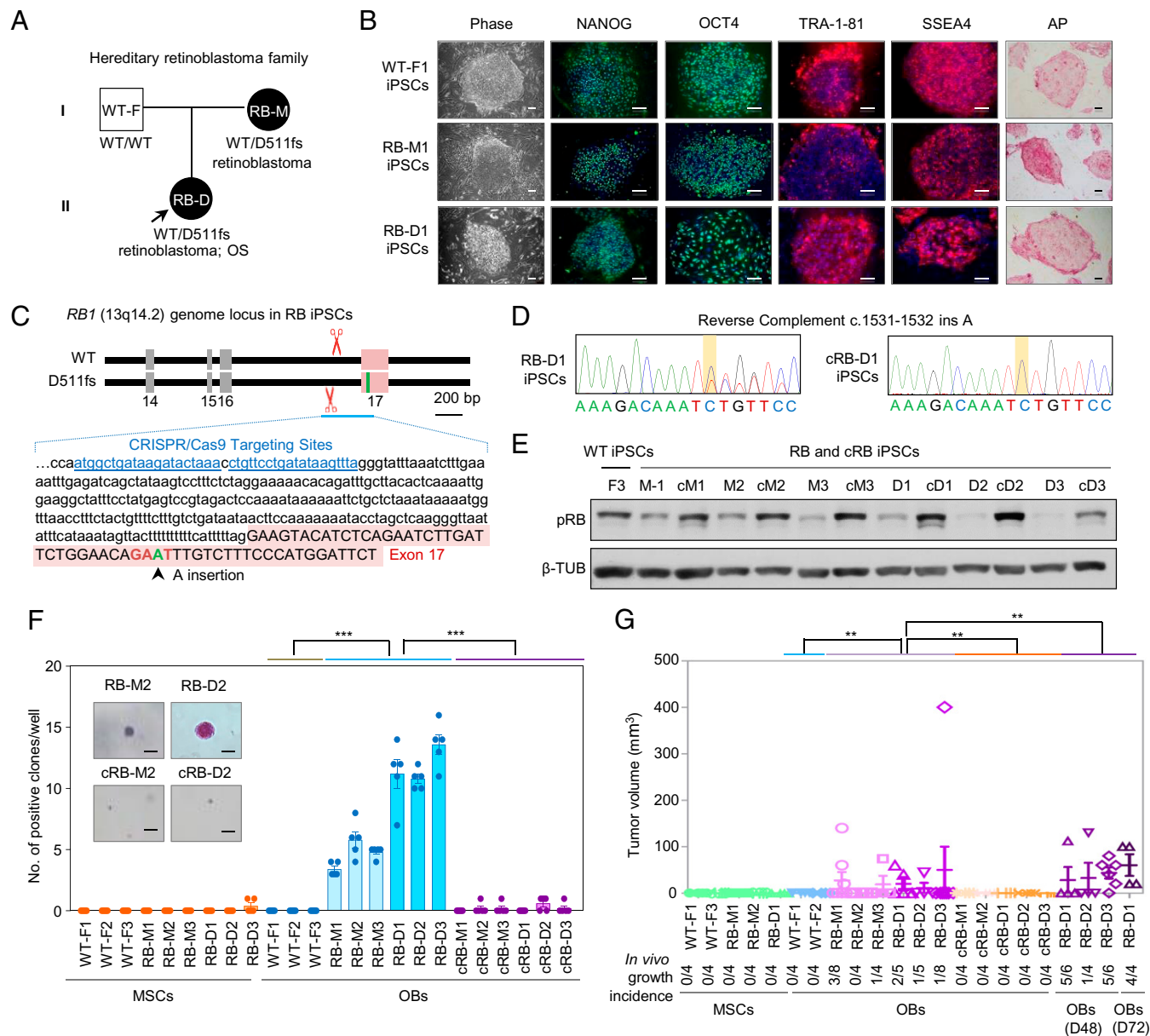


Fig. 1. Generation of a premalignant RB patient-derived iPSC platform. (A) The RB family tree includes two RB patients (mother: RB-M; daughter: RB-D) with a heterozygous pRB(D511fs) mutation and one unaffected relative (father: WT-F). Arrow, proband. (B) SeV-4F (OCT4, SOX2, KLF4, and MYC)-reprogrammed RB and WT iPSCs highly express hESC pluripotency factors (NANOG and OCT4) and hESC surface markers (TRA-1-81 and SSEA4) and have high AP activity. (Scale bars, 100 μ m.) (C) Schematic overview of correcting *RB1* mutation by CRISPR-Cas9 nickase in the *RB1* genomic locus (13q14.2). CRISPR-Cas9 sgRNA target sites are labeled in blue. *RB1* exon 17 is labeled in pink. The c.1531-1532 ins A site is labeled in green and the affected GAT nucleotide encoding aspartic acid (D) is colored red. (D) Sanger sequencing indicates the *RB1* c.1531-1532 ins A mutation is corrected in cRB iPSCs. (E) Western blot reveals comparable pRB protein levels in WT and cRB iPSCs and lower levels in RB iPSCs, indicating restoration of pRB protein upon correction of *RB1* gene mutation. (F) AIG assay demonstrates in vitro tumorigenic ability for RB OBs but not WT and cRB OBs. Colonies larger than 50 μ m after 1 mo of growth are considered positive. (Scale bars, 100 μ m.) Representative photographs show positive colonies from RB-M2 and RB-D2 OBs. (G) Tumor xenograft experiments by subcutaneous transplantation in *NU/NU* mice demonstrate that RB but not WT or cRB OBs recapture in vivo cell proliferation ability. The numbers of xenografts are indicated. Results are expressed as mean $\pm$ SEM. * P < 0.05, ** P < 0.01, *** P < 0.001.

Appendix, Table S1. In summary, our findings demonstrated a premalignant phenotype for RB iPSC-derived OBs.

RB OBs Demonstrate an Up-Regulated RNA Spliceosomal Gene Signature. In order to gain insights into pRB loss-induced oncogenic effects, we investigated and compared global transcripts among WT, RB, and cRB OBs. mRNAs were isolated at three differentiation time points (day 0, MSCs; day 15, pre-OBs; day 24, OBs) and analyzed by RNA sequencing (RNA-seq). Spearman's correlation demonstrated that gene expression profiles from MSCs are distinct from pre-OB and OB samples

(Fig. 2A), suggesting successful osteogenic differentiation in WT, RB, and cRB cells. As expected, levels of numerous known pRB/E2F targets were up-regulated in RB OBs compared with both WT and cRB OBs (SI Appendix, Fig. S3A). Gene set enrichment analysis (GSEA) of transcription factor targets and oncogenic signatures identified E2F chromatin immunoprecipitation (ChIP) targets and a pRB/E2F-associated oncogenic signature as enriched in RB OBs (SI Appendix, Fig. S3B and C). Consistent with loss of pRB facilitating osteogenic differentiation (SI Appendix, Fig. S2D–F), genes up-regulated in RB OBs compared with WT and cRB OBs at day 24 were most highly

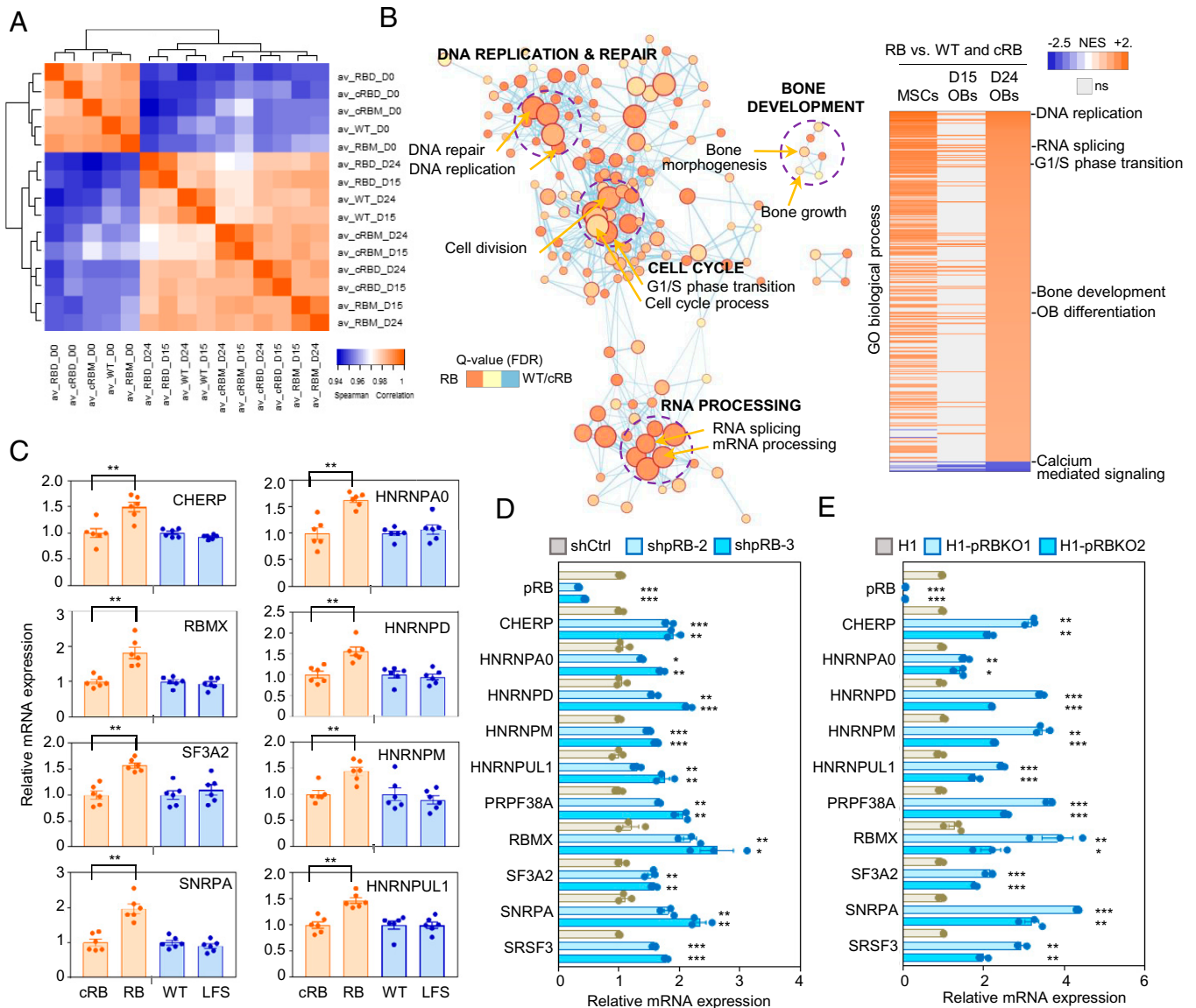


Fig. 2. Enrichment of RNA splicing and spliceosomal genes in RB iPSC-derived OBs. (A) Heatmap demonstrating a pairwise comparison of gene expression levels from WT, RB, and cRB RNA-seq during the time course of differentiation by Spearman's correlation. The pairwise correlation coefficients range from 0.94 (blue) to 1 (orange). (B, Left) Enrichment of GO biological processes identified by EnrichmentMap analysis. Network visualization of enriched gene sets in RB OBs compared with WT and cRB OBs at day 24 indicates that GO biological processes involved in DNA replication and repair, cell cycle, RNA process, and bone development are enriched in RB OBs. Enriched gene sets in RB OBs are displayed in orange and enriched gene sets in WT and cRB OBs are shown in blue. FDR, false discovery rate. (B, Right) Enriched GO biological processes in RB vs. WT and cRB OBs are analyzed by GSEA and summarized by a heatmap. Nonsignificant GOs are shown in light gray. (C) qRT-PCR indicates that expression of spliceosomal genes is enriched in RB iPSC-derived OBs but not LFS iPSC-derived OBs compared with their corresponding WT controls. (D) qRT-PCR demonstrates that depletion of pRB in WT OBs leads to up-regulation of spliceosomal genes. (E) qRT-PCR indicates that H1-pRBKO hESC-derived OBs demonstrate increased spliceosomal gene expression compared with H1 hESC-derived OBs. Results are expressed as mean $\pm$ SEM. * P < 0.05, *** P < 0.01, **** P < 0.001.

expressed in more terminally differentiated (day 21) mouse OB gene profiles as well as in an RB gene-associated cancer signature (SI Appendix, Fig. S3D).

Genomic alterations and rearrangements are commonly found in human OS (27, 28). To investigate if RB iPSC-derived OBs also harbor these rearrangements, we applied in silico cytogenetic region enrichment analysis (CREA) (23) to RB OB samples to identify the potential presence of rearranged regions frequently found in human OS. CREA revealed that chromosomal rearrangements are uncommon in these RB iPSC-derived OBs (SI Appendix, Fig. S3E), suggesting that RB iPSC-derived OBs can model the early stages of OS initiation and progression caused solely by *RB1* mutation prior to the acquisition of secondary genomic alterations. Furthermore, GSEA demonstrated that differentially expressed genes in OS

compared with normal OBs are specifically enriched in RB OBs compared with WT and cRB OBs at day 24 (SI Appendix, Fig. S3F), indicating that RB OBs nonetheless acquire genes associated with an OS signature in the absence of additional gene alterations.

Gene ontology (GO) analyses revealed that RB OBs are enriched for genes involved in DNA replication and repair, cell cycle (e.g., cell cycle process and G1/S phase transition), RNA processing (e.g., RNA splicing and mRNA processing), and bone development (e.g., bone morphogenesis, bone growth, and OB differentiation) (Fig. 2B). Surprisingly, the enrichment of RNA processing pathways was not clearly noted in previous studies. qRT-PCR verified that numerous spliceosomal genes (e.g., *CHERP*, *HNRNPA0*, *HNRNPD*, *HNRNPM*, *HNRNPUL1*, *SF3A2*, *SNRPA*, and *RBMX*) are enriched in RB OBs (Fig. 2C).

The enrichment of spliceosomal genes is unique to RB but not Li–Fraumeni syndrome (LFS) OBs, despite the fact that the hereditary cancer syndrome LFS also carries an OS predisposition, indicating a pRB-specific effect. shRNA-mediated pRB knock-down in WT iPSC-derived OBs led to increased expression of numerous spliceosomal genes (Fig. 2D). To verify if the regulation of spliceosomal genes by pRB is a common phenomenon, we generated pRB-knockout (pRBKO) hESC H1 lines by CRISPR-Cas9 and differentiated these cells to OBs (SI Appendix, Fig. S3G). Compared with H1-derived OBs, H1-pRBKO-derived OBs consistently expressed elevated spliceosomal genes (Fig. 2E). In contrast, overexpression of pRB resulted in the down-regulation of spliceosomal gene expression in RB OBs (SI Appendix, Fig. S3H). In comparison with normal retinal tissues, GSEA of retinoblastoma revealed enrichment of GO biological process RNA splicing and Kyoto Encyclopedia of Genes and Genomes (KEGG) spliceosome (SI Appendix, Fig. S3I). In summary, these findings demonstrate that pRB negatively regulates spliceosomal gene expression across multiple OB and OS lines and tissues and emphasize that spliceosomal dysregulation is involved in pRB loss-induced oncogenesis.

pRB Selectively Binds to E2F3a-Targeted Spliceosomal Genes.

pRB functions as a transcriptional corepressor and many of its tumor-suppression functions have been attributed to its ability to negatively regulate E2F-mediated cell cycle gene programs. Among pRB-interacting E2F family members, E2F3a, but not E2F1 and E2F2, is predominantly expressed in OBs (SI Appendix, Fig. S4A). We hypothesized that pRB binds to E2F3a and negatively modulates E2F3a-regulated spliceosomal gene expression. To address this hypothesis, we carried out pRB and E2F3a chromatin immunoprecipitation followed by next-generation sequencing (ChIP-seq) to determine whether the selective up-regulation of spliceosomal genes in RB OBs is due to preferential binding of pRB and E2F3a to the promoters of these genes. ChIP-seq analyses revealed a striking degree of genome-wide overlap between pRB and E2F3a sites (Fig. 3A). More than one-third of pRB-only (2,889), pRB/E2F3a-cotargeted (4,096), and E2F3a-only (6,539) peaks were detected at promoters and transcription start sites (TSSs) (SI Appendix, Fig. S4B), indicating preferential binding of pRB and E2F3a to gene-proximal regions of transcription initiation. De novo motif analyses revealed that the top motif enriched in pRB/E2F3a-co-occupied sites is identical to the E2F family motif (SI Appendix, Fig. S4C). In addition, pRB ChIP-seq read densities of pRB/E2F3a-co-occupied peaks were significantly higher than those of pRB-only peaks, suggesting that the localization of pRB to chromatin is dependent on E2F3a (SI Appendix, Fig. S4D).

To determine specific functional categories modulated by pRB and E2F3a simultaneously, we performed functional enrichment analysis on the enriched genomic regions co-occupied by pRB and E2F3a. pRB/E2F3a-cotargeted genes were highly enriched for ontologies of processing of capped introns containing pre-mRNA, mRNA splicing, and RNA processing (Fig. 3B). These cotargeted gene ontologies were even better enriched than well-known pRB- and E2F-modulated ontologies such as the cell cycle and telomere maintenance (Fig. 3B). We then analyzed the promoter/TSS regions of 134 critical spliceosomal proteins for potential pRB and E2F3a binding. More than one-third (50 of 134) of spliceosomal gene promoter/TSS regions were cobound by both pRB and E2F3a (Fig. 3C, Upper), and expression of these spliceosomal genes was increased in RB OBs compared with cRB OBs (Fig. 3C, Lower). These findings confirm the

critical function of pRB and E2F3a in regulating global spliceosomal gene expression.

The spliceosomal genes cobound by pRB and E2F3a at the promoter/TSS regions by ChIP-seq included *CHERP*, *HNRNPA0*, *HNRNPD*, *HNRNPM*, *HNRNPUL1*, *PRPF38A*, *SF3A2*, and *SNRPA* (Fig. 3D and SI Appendix, Fig. S4E). ChIP-qPCR validated the binding of pRB and E2F3a to *HNRNPD*, *HNRNPUL1*, *SF3A2*, and *SNRPA* promoter regions but not to regions adjacent to and upstream of the peak regions (Fig. 3E). pRB and E2F3a ChIP specificities were further verified by exogenous Flag-tagged pRB and E2F3a ChIP-qPCR (SI Appendix, Fig. S4F). Furthermore, E2F3a increased the expression of these spliceosomal genes (SI Appendix, Fig. S4G), supporting our hypothesis that pRB negatively regulates spliceosomal gene expression by inhibiting E2F3a. Taken together, these ChIP-seq studies provide a direct mechanistic link between pRB/E2F3a transcriptional regulatory machinery and global spliceosomal gene expression.

pRB and E2F3a Coregulate Distal Enhancers of Spliceosomal Genes.

The analysis of the genomic distribution of pRB/E2F3a-cotargeted binding sites demonstrated that more than half of RB1/E2F3a-cotargeted peaks were not located at promoter or TSS regions (SI Appendix, Fig. S4B). For example, *RBMX* was up-regulated in pRB-deficient cells, but ChIP-seq analysis showed that both pRB and E2F3a barely bind the *RBMX* promoter and TSS regions, suggesting that other regulatory mechanisms (e.g., enhancers) are involved in pRB/E2F3a-regulated *RBMX* gene expression. We found that 31.0% of pRB- and 32.7% of E2F3a-occupied peaks are marked by H3K27ac (a putative enhancer marker) in OBs and multiple OS lines (SI Appendix, Fig. S5A). These findings support a regulatory role for pRB and E2F3a on distal enhancers. Indeed, pRB and E2F3a co-occupy putative enhancer regions of some spliceosomal genes including *RBMX* (SI Appendix, Fig. S5B). ChIP-qPCR validated the binding of pRB and E2F3a to *RBMX* enhancer regions but not upstream of the peak regions (SI Appendix, Fig. S5C). To demonstrate that these putative pRB/E2F3a-bound distal elements represent bona fide enhancers for *RBMX*, we performed CRISPR-Cas9-mediated deletion on this upstream region of *RBMX*. Two sgRNAs were designed to target upstream and downstream of the putative *RBMX* enhancer regions, thus creating a 676-bp deletion (SI Appendix, Fig. S5D). Sanger sequencing of sgRNA-transduced RB OBs demonstrated that CRISPR-Cas9-mediated on-target editing leads to a 676-bp deletion in the targeted pRB/E2F3a-cobound peak region (SI Appendix, Fig. S5E). Deletion of this *RBMX* upstream region indeed reduced expression of *RBMX* (SI Appendix, Fig. S5F), suggesting its function as a distal enhancer. Furthermore, the epigenomic perturbation of the *RBMX* upstream region by CRISPR interference (CRISPRi)-targeted inhibition (29) and CRISPR activation (CRISPRa)-targeted activation (30) demonstrated that CRISPRi suppresses *RBMX* expression (SI Appendix, Fig. S5G) while CRISPRa increases *RBMX* expression (SI Appendix, Fig. S5H). These findings demonstrate that pRB and E2F3a can modulate spliceosomal gene expression through distal regulatory elements. Collectively, our results emphasize that pRB and E2F3a coordinate the expression of spliceosomal genes across multiple transcriptional regulatory layers.

Spliceosome Perturbation in RB OBs Selectively Increases Retained Intron Events and Hampers Cell Proliferation. To further assess the effect of the spliceosomal perturbation on global splicing patterns between RB and cRB OBs, we

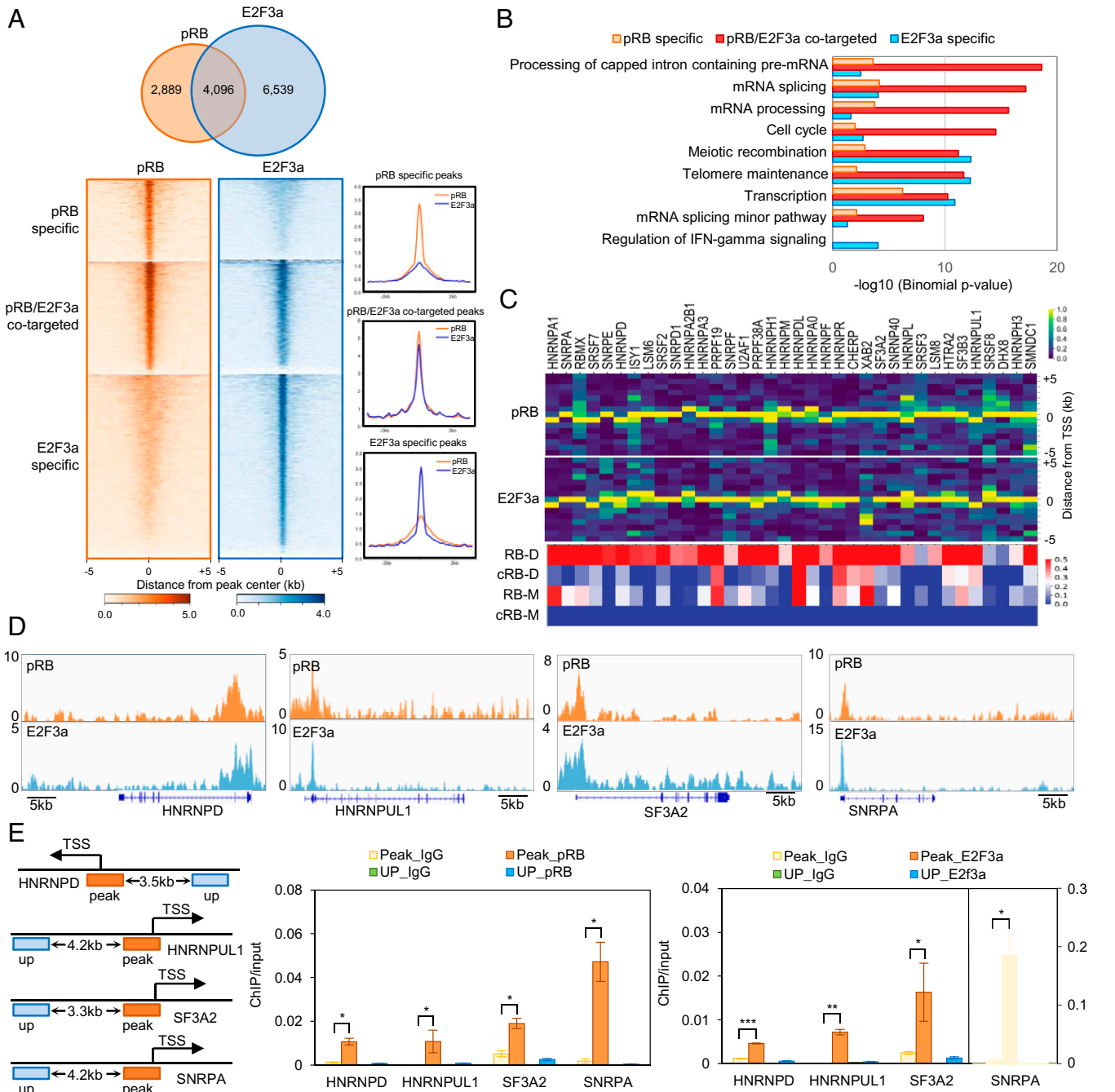


Fig. 3. pRB and E2F3a directly cotarget spliceosomal genes. (A, *Top*) Venn diagram depicts the overlap between pRB and E2F3a binding peaks in cRB OBs and defines pRB-specific (2,889), pRB/E2F3a-cotargeted (4,096), and E2F3a-specific (6,539) loci. (A, *Bottom Left*) Heatmaps depict pRB and E2F3a binding to the 5-kb genomic loci surrounding the identified ChIP-seq peaks, grouped by cluster. (A, *Bottom Right*) Composite plots show the average binding of pRB and E2F3a to the pRB-specific, pRB/E2F3a-cotargeted, and E2F3a-specific loci. (B) GO analyses of pRB-specific, pRB/E2F3a-cotargeted, and E2F3a-specific genomic regions. pRB/E2F3a-cotargeted genes are overrepresented for numerous RNA splicing pathway genes, including those involved in the processing of capped introns containing pre-mRNA, mRNA splicing, mRNA processing, and mRNA splicing minor pathway. (C) Increased expression of pRB/E2F3a-cotargeted spliceosomal genes in RB OBs compared with cRB OBs. (C, *Upper*) Heatmaps of ChIP-seq peak intensities in pRB/E2F3a-cotargeted spliceosomal gene regions (± 5 kb from the TSS). Each box in the heatmap represents a 0.7-kb region. (C, *Lower*) Heatmap of spliceosomal gene expression in RB-D, RB-M, cRB-D, and cRB-M OBs is examined by RNA-seq. (D) Integrative Genomics Viewer snapshot of pRB and E2F3a occupancy over promoter regions of spliceosomal genes *HNRNPD*, *HNRNPUL1*, *SF3A2*, and *SNRPA*. (E) ChIP-qPCR validation of pRB and E2F3a binding peaks at identified spliceosomal genes. (E, *Left*) Schematic of amplicon locations of spliceosomal genes and upstream controls used for ChIP-qPCR validation. (E, *Right*) ChIP-qPCR at spliceosomal TSS peak sites and upstream controls to assess for pRB (*Left*), E2F3a (*Right*), or immunoglobulin G (IgG) enrichment (ChIP/input). ChIP-qPCR confirms specific enrichment of *HNRNPD*, *HNRNPUL1*, *SF3A2*, and *SNRPA* at peak regions in RB OBs. Results are expressed as mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

performed pairwise percent spliced-in (PSI) analyses of exons among spliceosome inhibitor pladienolide B (PB)-treated cells. Different splicing events, including alternative 5' splicing site (A5), alternative 3' splicing site (A3), alternative first exon (AF), alternative last exon (AL), mutually exclusive exon (MX), retained intron (RI), and skipped exon (SE), were examined.

Despite global increases in pRB/E2F3a-mediated gene transcription events in RB OBs, PSI analyses identified no obvious frequency differences in splicing events between RB and cRB OBs (Fig. 4A), implying that the up-regulation of spliceosomal genes following pRB depletion overcomes up-regulated RNA transcription and prevents splicing defects. In contrast,

spliceosomal inhibition by PB increased RI events in RB OBs compared with cRB OBs (Fig. 4 *A* and *B*). Interestingly, aberrant RIs were the primary alternative splicing event that increased following inhibition of spliceosomal function by PB in RB OBs compared with cRB OBs, while other alternative splicing events remained comparable (Fig. 4*B*). RNA-seq demonstrated that PB treatment increased aberrant RIs in tumor-associated genes *METTL2B*, *JAG1*, *ZC3HAV1*, and *BYSL* in RB OBs compared with cRB OBs (Fig. 4*C*). PCR confirmed the increase of RIs on intron 1 in *METTL2B* mRNA, intron 12 in *JAG1* mRNA, intron 3 in *ZC3HAV1* mRNA, and intron 6 in *BYSL* mRNA (Fig. 4*C*). Neither treatment with PB nor presence of *RBI* mutation in OBs was associated with any significant difference in the intron length of transcripts containing RIs or SEs (*SI Appendix*, Fig. S6*A*). PB-induced RI and SE events were broadly observed in all transcripts and independent of transcript lengths (*SI Appendix*, Fig. S6*B*). STREME (sensitive, thorough, rapid, enriched motif elicitation)-enriched motif analysis of the 50-bp intronic sequence from each end of an RI event in PB-treated RB and cRB OBs demonstrated an enriched ACTYAC motif in RI-affected transcripts upon PB treatment (*SI Appendix*, Fig. S6*C*). Suppression of spliceosomal function by PB preferentially enhanced splicing events in pRB and pRB/E2F3a targets (Fig. 4*D*). Transcripts with RIs that were pRB/E2F3a targets were more highly expressed than global transcripts (with or without RIs) (Fig. 4*E*). Together, these results suggest that pRB and pRB/E2F3a targets are relatively vulnerable to spliceosome inhibition.

Interestingly, the transcripts with lower RIs in RB OBs had a significant increase in RIs upon spliceosome inhibition (*SI Appendix*, Fig. S6*D*), indicating that an increase in pRB/E2F3a onco-spliceosome signature (REOSS) gene expression in *RBI*-mutant OBs prevented RI events from occurring in certain transcripts, but that these transcripts showed impaired splicing and increased RIs upon spliceosome inhibition. Examination of 47 previously identified cancer-related alternatively spliced isoforms (15, 16) including MCL1, MDM2, and CD44 revealed that only MDM2 AF decreased in PB-treated RB OBs compared with PB-treated cRB OBs (*SI Appendix*, Fig. S6*E*). Notably, MDM2 alternative splicing isoforms were suggested to convey p53-dependent and independent tumor-promoting abilities (31). Pathway analysis of genes with perturbed splicing events revealed that gene transcripts involved in OS-associated signaling pathways, such as NOTCH, TGF β , α 5 β 3-integrin, and NF- κ B, were markedly more likely to have RIs in RB OBs compared with cRB OBs upon PB treatment (Fig. 4*F*). These results suggest that inhibition of spliceosomal function preferentially impairs RIs and OS-associated oncogenic gene functions in RB OBs. We further examined if pharmacological inhibition of the spliceosome also impaired the cell proliferation potential of RB OBs. Compared with cRB OBs, RB OBs were significantly more sensitive to spliceosome inhibitors SD6 (32) and PB (Fig. 4 *G* and *H*). These findings imply that, in contrast to cRB OBs, premalignant RB OBs confer hyperdependency on spliceosome function for proliferation and survival.

pRB-Deficient OS Lines Are Sensitive to Spliceosome Perturbation.

Since up-regulation of spliceosomal genes in pRB-deficient cells may overcome the final bottleneck to osteosarcomagenesis, we thus investigated whether the spliceosome is an exploitable vulnerability in pRB-deficient cancers. We labeled the 51 spliceosomal genes coregulated by pRB and E2F3a as the REOSS. Knockdown of REOSS genes (*HNRNPUL1*, *RBMX*, *SF3A2*, and *SNRPA*) but not non-REOSS genes (*BUD31*, *SRSF9*, *U2AF2*, and *XAB2*)

selectively reduced cell numbers in HOS-pRBKO cells compared with HOS-Ctrl (control) cells (Fig. 5 *A* and *B*). Ectopic expression of REOSS genes (*HNRNPUL1*, *RBMX*, *SF3A2*, and *SNRPA*) promoted cell proliferation and increased colony-forming activity in HOS cells (Fig. 5 *C* and *D*). These findings indicate that up-regulation of REOSS genes, at least *HNRNPUL1*, *RBMX*, *SF3A2*, and *SNRPA*, plays a role in promoting tumor growth in pRB-deficient OS. Cell viability assay and fluorescence-based competition assay indicated that both SD6 and PB selectively suppress HOS-pRBKO cells compared with HOS-Ctrl cells (Fig. 5*E* and *SI Appendix*, Fig. S7*A*). Consistently, the colony-forming assay demonstrated sensitivity of pRBKO and pRB-knockdown 143B cells to PB-mediated inhibition (*SI Appendix*, Fig. S7 *B* and *C*). Stable transfection of pRB in Saos2 cells led to resistance to spliceosome inhibitors (*SI Appendix*, Fig. S7*D*). Treatment of OS xenografts from the 143B OS line with SD6 potently restrained *in vivo* tumorigenesis in 143B-pRBKO cells but less so in 143B-Ctrl cells, emphasizing that the spliceosome is essential for tumorigenicity of pRB-deficient OS (Fig. 5*F*).

To determine the clinical relevance of the pRB/E2F3a-regulated REOSS in osteosarcomagenesis, we examined the expression of selected REOSS genes (*SNRPA*, *CHERP*, *HNRNP*, and *RBMX*) across 74 human OS specimens by immunohistochemical (IHC) staining. *CHERP* was detected in 15 (38.5%) of 39 specimens with low pRB expression but in only 6 (17.1%) of 35 specimens with high pRB expression, indicating that *CHERP* expression is associated with low levels of pRB ($P < 0.05$; Fig. 5*G*). In contrast, *CHERP* was detected in 18 (36%) of 50 specimens with high E2F3a expression but in only 3 (12.5%) of 24 specimens with low E2F3a expression, suggesting that *CHERP* expression is associated with high levels of E2F3a ($P < 0.05$; Fig. 5*H*). Similarly, *HNRNP*, *SNRPA*, and *RBMX* were also negatively associated with pRB expression (*HNRNP*, $P < 0.05$; *SNRPA*, $P = 0.058$; *RBMX*, $P < 0.05$; Fig. 5*G*) but strongly associated with E2F3a expression (*HNRNP*, $P < 0.05$; *SNRPA*, $P < 0.001$; *RBMX*, $P < 0.001$; Fig. 5*H*). We next analyzed the expression of these REOSS genes in OS tumor tissues and correlated them with reported patient survival data. The Kaplan-Meier overall survival curves showed that low pRB and high E2F3a, *CHERP*, *HNRNP*, *SNRPA*, and *RBMX* levels are associated with poor survival (*SI Appendix*, Fig. S7 *E–J*). Taken together, OS IHC studies suggest that pRB/E2F3a-regulated spliceosomal genes contribute to osteosarcomagenesis and are associated with poor clinical outcomes in OS patients.

pRB/E2F3a Regulate REOSS Gene Expression in Multiple Cancer Types with High *RBI* Mutation Rates.

To determine whether our findings can be generalized to other cancers, we next explored pRB/E2F3a regulation of REOSS gene features in other human cancers by analyzing the expression of REOSS genes across 34 human cancers in The Cancer Genome Atlas (TCGA) pan-cancer datasets. TCGA pan-cancer transcriptome data revealed that expression of a group of REOSS genes is significantly elevated in pRB-low cancers (e.g., TGCT, UVM, DLBC, BRCA, UCS, GBM, LUAD, LUSC, SARC, ACC, OV, BLCA, ESCA, UCEC, CHOL, THCA, PAAD, LIHC, CESC, and HNSC) (*SI Appendix*, Fig. S8*A*) and E2F3-high cancers (e.g., SKCM, UVM, THYM, UCEC, GBM, TGCT, BRCA, COAD, LUSC, OV, LGG, LIHC, READ, STAD, LUAD, LUNG, BLCA, ESCA, PRAD, SARC, CESC, and HNSC) (*SI Appendix*, Fig. S8*B*). These pRB/E2F3a-regulated cancers included multiple cancer types characterized by high

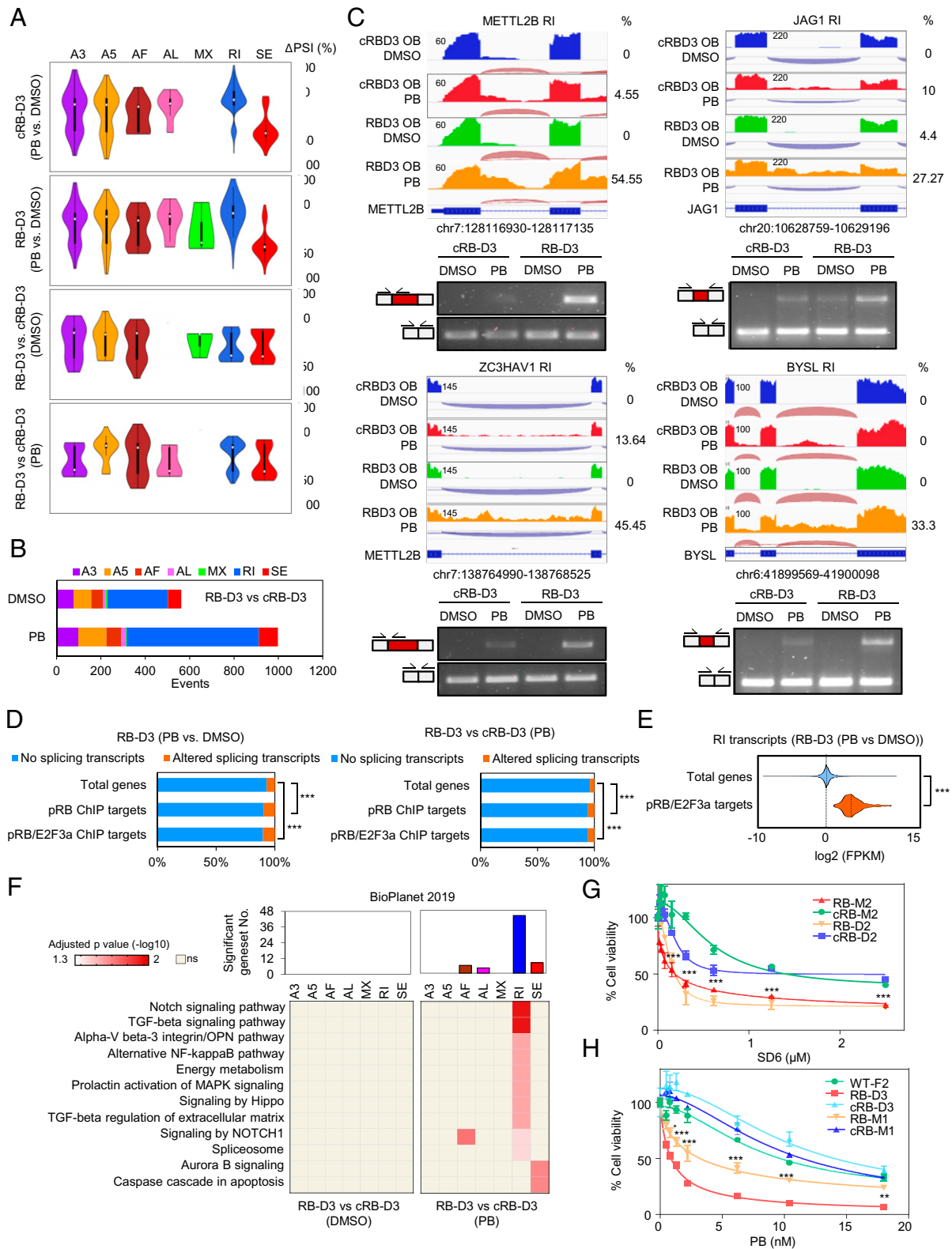


Fig. 4. Spliceosome perturbation influences cell viability and RNA splicing of RB OBs. (A) Violin plots represent distributions of statistically significant Δ PSI values ($P < 0.05$) for different classes of RNA splicing events: A3, A5, AF, AL, MX, RI, and SE. Separate violins are displayed for each pairwise comparison of DMSO- or PB-treated cells, and the percentage of events is presented. Δ PSI values are shown for 1) PB-treated cRB OBs compared with DMSO-treated cRB OBs; 2) PB-treated RB OBs compared with DMSO-treated RB OBs; 3) DMSO-treated RB OBs compared with DMSO-treated cRB OBs; and 4) PB-treated RB OBs compared with PB-treated cRB OBs. (B) Inhibition of spliceosomal function by PB leads to significant increases of aberrant RIs in RB OBs compared with cRB OBs. (C) Visualization of RNA-seq read mappings at *METTL2B*, *JAG1*, *ZC3HAV1*, and *BYSL* in the DMSO- and PB-treated OBs. A sashimi plot displaying the major splice junctions is superimposed. Representative RT-PCR validation of RI events of *METTL2B*, *JAG1*, *ZC3HAV1*, and *BYSL* transcripts. Exons are shown in gray and introns are in red. Half-arrows indicate the locations of primers designed to validate RI events. (D) Inhibition of spliceosomal function by PB preferentially alters RNA splicing events on pRB- and pRB/E2F3a-targeted transcripts. (E) *RB1* mutation-associated RI transcripts are more highly expressed than global transcripts in PB-treated RB OBs. FPKM, fragments per kilobase of exon per million mapped fragments. (F) Unique genes with perturbed splicing events in DMSO- or PB-treated cRB OBs vs. RB OBs are analyzed by BioPlanet 2019 and summarized by heatmaps. (G and H) Both PB and SD6 selectively inhibit the cell proliferation of RB OBs compared with cRB OBs. Results are expressed as mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

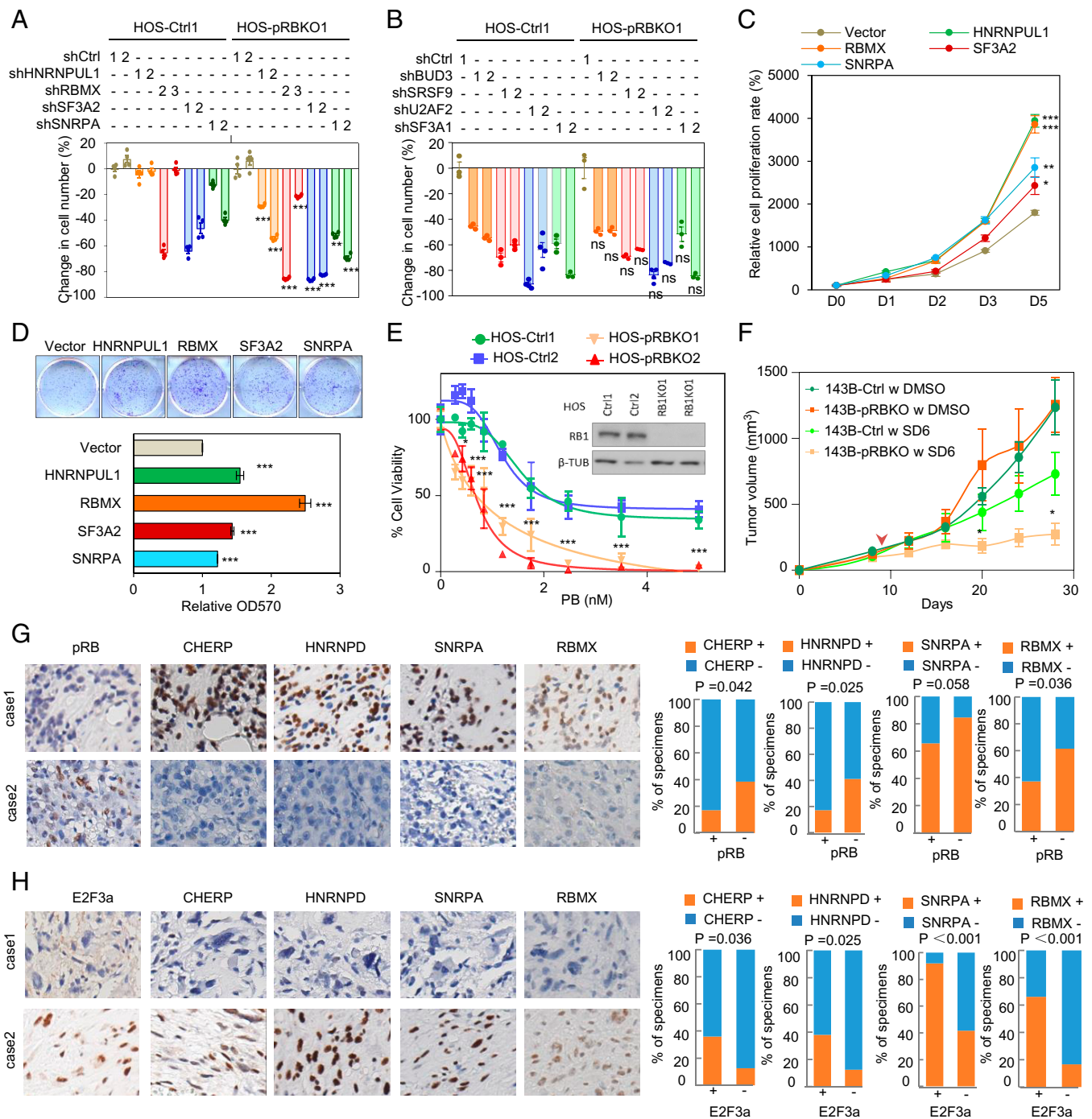


Fig. 5. OS specimens commonly have low pRB, high E2F3a, and high REOSS gene expression, and spliceosome inhibitors selectively impair pRB-deficient OS proliferation. (A) HOS-pRBKO cells are more sensitive to HNRNPUL1, RBMX, SF3A2, or SNRPA knockdown-induced inhibitory effects than HOS-Ctrl cells. (B) Knockdown of BUD31, SRSF9, U2AF2, or XAB2 showed similar inhibitory effects between HOS-Ctrl and HOS-pRBKO cells. (C and D) Ectopic expression of HNRNPUL1, RBMX, SF3A2, or SNRPA promotes HOS cell growth and colony formation. (E) PB selectively suppresses cell growth of HOS-pRBKO compared with HOS-Ctrl. (F) Spliceosome inhibitor SD6 preferentially suppresses pRB-deficient OS growth in vivo. Nude mice inoculated with 143B-Ctrl and 143B-pRBKO cells are injected with DMSO or SD6 (3 mg/kg) for four cycles (3-d constitutive injection and 1-d break) and the tumor growth is measured twice weekly. The arrowhead indicates the day of initial DMSO or SD6 injection. (G) pRB expression is negatively associated with CHERP, HNRNP, SNRPA, and RBMX expression in 74 primary human OS specimens. (G, Left) Two representative specimens. (G, Right) Percentages of specimens with low (–) or high (+) pRB expression in which CHERP, HNRNP, SNRPA, and RBMX are or are not observed. (H) E2F3a expression is positively correlated with CHERP, HNRNP, SNRPA, and RBMX expression in 74 primary human OS specimens. (H, Left) Two representative specimens. (H, Right) Percentages of specimens with low (–) or high (+) E2F3a expression in which CHERP, HNRNP, SNRPA, and RBMX are or are not observed. Results are expressed as mean $\pm$ SEM. ns, nonsignificant; * P < 0.05, ** P < 0.01, *** P < 0.001.

rates of *RB1* gene mutation, including BLCA, BRCA, ESCA, HNSC, LIHC, LUAD, LUNG, LUSC, SARC, and OV (33). These pan-cancer transcriptional analyses using TCGA data further support the notion that the pRB/E2F3a–REOSS regulatory axis is a common pathway underlying cancer development.

Discussion

Recent studies have highlighted that several molecular subtypes of cancers are highly dependent on splicing function for cell survival and that pharmacological inhibition of the spliceosome

is a viable potential oncotherapeutic strategy (34–36). In contrast to most gene mutations, spliceosomal genes are frequently up-regulated in numerous cancers, suggesting that tumors rely on the spliceosome to produce cancer-associated splicing variants and overcome RNA transcriptional stress. The abundant up-regulation of spliceosomal genes observed in pRB-deficient cells likely represents tumor evolution to compensate for pRB loss-induced stress. The preferential lethality of RB OBs and pRB-deficient OS to pRB/E2F3a-regulated spliceosomal gene down-regulation and spliceosome inhibitors PB and SD6 compared with cells with intact pRB underscores the dependence of pRB-deficient tumors on spliceosome function. Our findings strongly suggest that spliceosome inhibitors should be considered in conjunction with current antineoplastic strategies to treat pRB-deficient associated malignancies.

Moreover, systemic chemotherapy and radiation therapy are independent risk factors for developing OS in RB patients (37). Chemotherapy and radiation treatments induce cancer cells to decrease the expression of spliceosomal genes, leading to intron retention in various genes (38). Inhibition of the spliceosome significantly impairs cellular response to DNA damage, which increases the selective lethality of cancer cells to chemotherapy. We suspect that the increase of spliceosomal genes in pRB-deficient cancers provides them with an indispensable advantage to repair chemo- and radiation-induced DNA damage, promote resistance to genotoxic stress, and survive following treatment.

Importantly, our findings show that pRB-deficient cells significantly lose growth and tumor formation potential upon REOSS gene knockdown or pharmacological inhibition of the spliceosome by either PB or SD6. pRB-deficient tumors appear exquisitely dependent on the spliceosomal machinery for growth and survival. In agreement with our findings, spliceosomal factors SRSF1 (39), PRPF6 (40), SRSF6 (41), and BUD31 (42) are either amplified or up-regulated in multiple cancers and function as oncoproteins by promoting tumorigenesis. Genetic or pharmacological inhibition of the spliceosome also selectively suppresses the proliferation and survival of cancers with elevated expression of these splicing factors, highlighting how inhibition of spliceosomal function is a general therapeutic strategy to treat cancers with dysregulated spliceosomal gene expression. Nonetheless, the full antitumor effects of spliceosome inhibitors on pRB-deficient cells may also involve genes not directly up-regulated by *RB1* inactivation. Together, we suggest that a large cohort of cancers driven by pRB deficiency, including OS, lung cancer, retinoblastoma, sarcoma, and breast cancers, are likely dependent on the spliceosome pathway for growth. pRB may also prove to be a useful biomarker for personalized therapeutics using spliceosome inhibitors.

Materials and Methods

Please see *SI Appendix, Materials and Methods* for a detailed description of materials and methods.

RNA-Seq. Time course samples of WT, RB, and cRB iPSC-derived MSCs and OBs were collected on days 0, 15, and 24. RB and cRB OBs were treated with 100 nM PB or dimethyl sulfoxide (DMSO) for 6 h. Cell samples were lysed in TRIzol reagent. RNA sample preparation and RNA-seq data analyses were performed as described previously (23).

ChIP-Seq and ChIP-qPCR. ChIP was performed using modified previous methods (5). For ChIP of pRB and 3×Flag-pRB, cells were prefixed for 30 min in 1.5 mM disuccinimidyl glutarate (Thermo Fisher Scientific, 20593) followed by

fixation in 4% paraformaldehyde (Thermo Fisher Scientific, 28906) at room temperature for 10 min. For ChIP of E2F3a and 3×Flag-E2F3a, cells were cross-linked in 1% formaldehyde at room temperature for 10 min. After glycine quenching, cell pellets were collected and lysed and then subjected to sonication using a Branson Sonifier 450 (sonication conditions: 210 cycles, power 25%, 10 s on and 10 s off for pRB and 3×Flag-pRB; 150 cycles, power 25%, 10 s on and 10 s off for E2F3a and 3×Flag-E2F3a). The supernatant was then diluted in the same sonication buffer, and subjected to immunoprecipitation with corresponding antibodies at 4 °C overnight. The beads were then washed and DNA was reverse-cross-linked and purified. Following ChIP, DNA was quantified by qPCR using standard procedures. Sequencing libraries were prepared using the KAPA HyperPrep Kit (Roche, KK8502). The DNA was sequenced on an Illumina X10 platform using a PE150 Kit. ChIP-qPCR was performed using a CFX96 machine (Bio-Rad Laboratories). The 20-μL PCR solution was composed of 500 ng ChIP product, 1 μL, respectively, of 10 μM forward and reverse PCR primers for amplifying the peak region, 10 μL SYBR Green PCR Master Mix (Bio-Rad Laboratories, 1725124), and 7 μL RT-PCR-grade water. Primer sequences are listed in *SI Appendix, Table S2*.

Quantification and Statistical Analysis. All experiments were performed in at least biological triplicate, and all results are expressed as mean ± SEM. No statistical methods were used to predetermine sample size. Multiple *t* test, Student's *t* test, and one-way ANOVA followed by Student's *t* test (Tukey's multiple-comparison test) were applied to determine statistical significance in the experiments. Excel and GraphPad Prism 7.0 were used for the statistical analysis. ns, nonsignificant; **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

Data Availability. The RNA-seq, ChIP-seq, and WES data reported in this article have been deposited in the Gene Expression Omnibus repository under accession number [GSE145235](#). Previously published data were used for this work (accession nos. [GSE125903](#), [GSE14827](#), and [GSE36001](#)). The data supporting the findings of this study are available within the article and *SI Appendix*.

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1. X. Graña, J. Garriga, X. Mayol, Role of the retinoblastoma protein family, pRB, p107 and p130 in the negative control of cell growth. *Oncogene* **17**, 3365–3383 (1998).
2. E. S. Knudsen, K. E. Knudsen, Tailoring to RB: Tumour suppressor status and therapeutic response. *Nat. Rev. Cancer* **8**, 714–724 (2008).
3. F. L. Wong *et al.*, Cancer incidence after retinoblastoma. Radiation dose and sarcoma risk. *JAMA* **278**, 1262–1267 (1997).
4. Y. H. Lin *et al.*, Osteosarcoma: Molecular pathogenesis and iPSC modeling. *Trends Mol. Med.* **23**, 737–755 (2017).
5. A. Chicas *et al.*, Dissecting the unique role of the retinoblastoma tumor suppressor during cellular senescence. *Cancer Cell* **17**, 376–387 (2010).
6. K. I. Hilgendorf *et al.*, The retinoblastoma protein induces apoptosis directly at the mitochondria. *Genes Dev.* **27**, 1003–1015 (2013).
7. C. H. Coschi *et al.*, Haploinsufficiency of an RB-E2F1-Condensin II complex leads to aberrant replication and aneuploidy. *Cancer Discov.* **4**, 840–853 (2014).
8. A. L. Manning *et al.*, Suppression of genome instability in pRB-deficient cells by enhancement of chromosome cohesion. *Mol. Cell* **53**, 993–1004 (2014).
9. A. L. Manning, M. S. Longworth, N. J. Dyson, Loss of pRB causes centromere dysfunction and chromosomal instability. *Genes Dev.* **24**, 1364–1376 (2010).
10. R. Vélez-Cruz *et al.*, RB localizes to DNA double-strand breaks and promotes DNA end resection and homologous recombination through the recruitment of BRG1. *Genes Dev.* **30**, 2500–2512 (2016).
11. R. Cook *et al.*, Direct involvement of retinoblastoma family proteins in DNA repair by non-homologous end-joining. *Cell Rep.* **10**, 2006–2018 (2015).
12. M. García-Cao, S. Gonzalo, D. Dean, M. A. Blasco, A role for the Rb family of proteins in controlling telomere length. *Nat. Genet.* **32**, 415–419 (2002).
13. C. A. Ishak *et al.*, An RB-EZH2 complex mediates silencing of repetitive DNA sequences. *Mol. Cell* **64**, 1074–1087 (2016).
14. Y. Shi, Mechanistic insights into precursor messenger RNA splicing by the spliceosome. *Nat. Rev. Mol. Cell Biol.* **18**, 655–670 (2017).
15. A. Sveen, S. Kilpinen, A. Ruusulehto, R. A. Lothe, R. I. Skotheim, Aberrant RNA splicing in cancer; expression changes and driver mutations of splicing factor genes. *Oncogene* **35**, 2413–2427 (2016).
16. E. El Marabti, I. Younis, The cancer spliceome: Reprogramming of alternative splicing in cancer. *Front. Mol. Biosci.* **5**, 80 (2018).
17. V. I. Rasheva, D. Knight, P. Bozko, K. Marsh, M. V. Frolov, Specific role of the SR protein splicing factor B52 in cell cycle control in *Drosophila*. *Mol. Cell Biol.* **26**, 3468–3477 (2006).
18. J. Ceron *et al.*, Large-scale RNAi screens identify novel genes that interact with the *C. elegans* retinoblastoma pathway as well as splicing-related components with synMuv B activity. *BMC Dev. Biol.* **7**, 30 (2007).
19. M. G. Oser *et al.*, Cells lacking the *RB1* tumor suppressor gene are hyperdependent on Aurora B kinase for survival. *Cancer Discov.* **9**, 230–247 (2019).
20. R. Brough *et al.*, Identification of highly penetrant Rb-related synthetic lethal interactions in triple negative breast cancer. *Oncogene* **37**, 5701–5718 (2018).
21. J. Ahlander, G. Bosco, The RB/E2F pathway and regulation of RNA processing. *Biochem. Biophys. Res. Commun.* **384**, 280–283 (2009).
22. X. H. Zhang, L. Y. Tee, X. G. Wang, Q. S. Huang, S. H. Yang, Off-target effects in CRISPR/Cas9-mediated genome engineering. *Mol. Ther. Nucleic Acids* **4**, e264 (2015).
23. D. F. Lee *et al.*, Modeling familial cancer with induced pluripotent stem cells. *Cell* **161**, 240–254 (2015).
24. R. Zhou *et al.*, Modeling osteosarcoma using Li-Fraumeni syndrome patient-derived induced pluripotent stem cells. *J. Vis. Exp.* (136), 57664 (2018).
25. H. Kim *et al.*, Oncogenic role of SFRP2 in p53-mutant osteosarcoma development via autocrine and paracrine mechanism. *Proc. Natl. Acad. Sci. U.S.A.* **115**, E11128–E11137 (2018).
26. D. M. Thomas *et al.*, The retinoblastoma protein acts as a transcriptional coactivator required for osteogenic differentiation. *Mol. Cell* **8**, 303–316 (2001).
27. J. R. Batanian *et al.*, Evaluation of paediatric osteosarcomas by classic cytogenetic and CGH analyses. *Mol. Pathol.* **55**, 389–393 (2002).
28. X. Chen *et al.*, St. Jude Children's Research Hospital-Washington University Pediatric Cancer Genome Project, Recurrent somatic structural variations contribute to tumorigenesis in pediatric osteosarcoma. *Cell Rep.* **7**, 104–112 (2014).
29. L. A. Gilbert *et al.*, CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. *Cell* **154**, 442–451 (2013).
30. N. A. Kearns *et al.*, Cas9 effector-mediated regulation of transcription and differentiation in human pluripotent stem cells. *Development* **141**, 219–223 (2014).
31. D. R. Okoro, M. Rosso, J. Bargonetti, Splicing up mdm2 for cancer proteome diversity. *Genes Cancer* **3**, 311–319 (2012).
32. C. Lagiseti *et al.*, Optimization of antitumor modulators of pre-mRNA splicing. *J. Med. Chem.* **56**, 10033–10044 (2013).
33. P. Jia, Z. Zhao, Characterization of tumor-suppressor gene inactivation events in 33 cancer types. *Cell Rep.* **26**, 496–506.e3 (2019).
34. S. C. Lee, O. Abdel-Wahab, Therapeutic targeting of splicing in cancer. *Nat. Med.* **22**, 976–986 (2016).
35. S. Bonnal, L. Vigevari, J. Valcárcel, The spliceosome as a target of novel antitumour drugs. *Nat. Rev. Drug Discov.* **11**, 847–859 (2012).
36. D. P. Steensma *et al.*, Phase I first-in-human dose escalation study of the oral SF3B1 modulator H3B-8800 in myeloid neoplasms. *Leukemia* **35**, 3542–3550 (2021).
37. P. Temming *et al.*, Incidence of second cancers after radiotherapy and systemic chemotherapy in heritable retinoblastoma survivors: A report from the German reference center. *Pediatr. Blood Cancer* **64**, 71–80 (2017).
38. K. S. Anufrieva *et al.*, Therapy-induced stress response is associated with downregulation of pre-mRNA splicing in cancer cells. *Genome Med.* **10**, 49 (2018).
39. R. Karni *et al.*, The gene encoding the splicing factor SF2/ASF is a proto-oncogene. *Nat. Struct. Mol. Biol.* **14**, 185–193 (2007).
40. A. S. Adler *et al.*, An integrative analysis of colon cancer identifies an essential function for PRPF6 in tumor growth. *Genes Dev.* **28**, 1068–1084 (2014).
41. M. Cohen-Elav *et al.*, The splicing factor SRSF6 is amplified and is an oncoprotein in lung and colon cancers. *J. Pathol.* **229**, 630–639 (2013).
42. T. Y. Hsu *et al.*, The spliceosome is a therapeutic vulnerability in MYC-driven cancer. *Nature* **525**, 384–388 (2015).

ZFP207 sustains pluripotency by coordinating OCT4 stability, alternative splicing and RNA export

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Abstract

The pluripotent state is not solely governed by the action of the core transcription factors OCT4, SOX2, and NANOG, but also by a series of co-transcriptional and post-transcriptional events, including alternative splicing (AS) and the interaction of RNA-binding proteins (RBPs) with defined subpopulations of RNAs. Zinc Finger Protein 207 (ZFP207) is an essential transcription factor for mammalian embryonic development. Here, we employ multiple functional analyses to characterize its role in mouse embryonic stem cells (ESCs). We find that ZFP207 plays a pivotal role in ESC maintenance, and silencing of *Zfp207* leads to severe neuroectodermal differentiation defects. In striking contrast to human ESCs, mouse ZFP207 does not transcriptionally regulate neuronal and stem cell-related genes but exerts its effects by controlling AS networks and by acting as an RBP. Our study expands the role of ZFP207 in maintaining ESC identity, and underscores the functional versatility of ZFP207 in regulating neural fate commitment.

Keywords alternative splicing; pluripotency; RNA-binding protein; ZFP207; Zinc finger protein

Subject Categories Post-translational Modifications & Proteolysis; RNA Biology; Stem Cells & Regenerative Medicine

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Introduction

Mouse embryonic stem cells (ESCs) are derived from the inner cell mass of the pre-implantation blastocyst. These cells exhibit unlimited

self-renewal capacity and, under appropriate stimuli, retain the potential to differentiate into the three germ layers (Bradley *et al*, 1984). Mouse ESCs are a useful model to study early mammalian development as their differentiation potential is more robust than that of ESC-like cells derived from other mammals such as humans, which exhibit primed pluripotency and represent a more advanced embryonic stage (Ginis *et al*, 2004; Nichols & Smith, 2009).

Zinc finger-containing proteins (ZFN or ZFP for human or mouse, respectively) are among the largest family of proteins, commonly containing a minimum of one zinc-finger (ZnF) domain, which recognizes DNA sequences with high affinity. This family of transcription factors plays important roles in a variety of cellular processes including development, cellular differentiation, metabolism and oncogenesis (Cassandri *et al*, 2017). Although ZNF/ZFPs were initially classified as transcription factors, several studies have highlighted additional functions of ZNFs. For instance, it has been shown that ZFP217 could recruit the methyltransferase-like 3 (METTL3) into an inactive complex and hence restrict N6-methyladenosine (m⁶A) deposition on pluripotency transcripts (Aguilo *et al*, 2015; Lee *et al*, 2016). In addition, recent studies identified that ZnF domains can bind RNA, and many ZNF/ZFPs act as putative RNA-binding proteins (RBPs) (Brannan *et al*, 2016). Indeed, analysis of quantitative global mRNA–protein interaction approaches identified ZNF207 (also termed BuGZ; Bub3 interacting GLEBS and Zinc finger domain-containing protein) as an RBP, among other ZNFs (Baltz *et al*, 2012; Castello *et al*, 2012). ZNF207 is conserved in eukaryotes. It associates with Bub3 and with spindle microtubules to regulate chromosome alignment (Jiang *et al*, 2014, 2015; Toledo *et al*, 2014). Furthermore, both ZNF207 and Bub3 interact with the spliceosome and are required for interphase RNA splicing (Wan *et al*, 2015), yet its specific molecular role remains elusive.

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In human ESCs, ZNF207 functions as a critical transcription factor by transcriptionally regulating the expression of the pluripotency factor OCT4 (Fang *et al.*, 2018), thereby being implicated in the maintenance of self-renewal and pluripotency. Likewise, ZNF207 has been shown to enhance reprogramming efficiency towards pluripotency (Toh *et al.*, 2016). Alternative splicing (AS), in which splice sites in primary transcripts are differentially selected to produce structurally and functionally distinct mRNAs, plays a critical role in cell fate transitions, development, and disease (Gabut *et al.*, 2011). ZNF207 undergoes AS during somatic cell reprogramming and differentiation of human ESCs, an isoform switch that seems to be required for the generation of induced pluripotent stem cells (iPSCs), and it might also be necessary to maintain ESC self-renewal and to induce proper differentiation programs (Toh *et al.*, 2016; Fang *et al.*, 2018).

Here, we show that ZFP207 plays an important role in the control of mouse ESC identity by a mechanism that differs from the one observed in human ESCs. Specifically, in mouse ESCs, ZFP207 does not regulate *Oct4* transcription but increases OCT4 protein stability by disrupting ubiquitin-dependent proteasomal degradation. In addition, depletion of *Zfp207* results in pluripotency defects and blocks neuroectodermal specification without significant changes in the transcriptome of stem cell and neural genes. ZFP207 regulates the expression of the spliceosome, and silencing of *Zfp207* leads to aberrant AS patterns. We further describe ZFP207 as a novel RNA-binding protein (RBP), which might directly affect RNA fate. Taken together, this study uncovers the versatile species-specific roles of ZFP207 and the link to co- and post-transcriptional pathways that impact cell-fate decisions of mouse ESCs.

Results

Silencing of *Zfp207* impairs proliferation and mouse ESC identity

To explore the function of ZFP207 in mouse ESCs, we analyzed the expression of *Zfp207* in retinoic acid (RA)-induced differentiation

towards the neural lineage (Fig 1A) and in spontaneous differentiation of ESCs into the three germ layers by embryoid body (EB) formation (Fig 1B). Expression of the neuronal differentiation marker *Nestin* (Fig 1A) and of the pluripotency factor *Oct4* (also known as *Pou5f1*; Fig 1A and B) was used to monitor proper cell differentiation. Real-time quantitative reverse transcription PCR (RT-qPCR) revealed that *Zfp207* was significantly enriched in ESCs compared to differentiated cells (Fig 1A and B), and its expression levels gradually decreased along the course of differentiation, correlating with the decrease of ZFP207 and OCT4 protein levels (Fig 1C and D).

To better understand the role of ZFP207 in pluripotency and differentiation, we aimed to generate CRISPR/Cas9-mediated knockout (KO) of *Zfp207* in mouse ESCs. We used two different strategies: (i) one single-guide RNA (sgRNAs #1) targeting exon 3; and (ii) two distinct single-guide RNAs (#1 and #2) targeting the region containing exon 3 and exon 9, which included the microtubule-binding region domain of ZFP207 (Appendix Fig S1A). After picking and expanding individual clones, Western blot analysis and subsequent clone genotyping confirmed that all clones analyzed (94) displayed heterozygosity for *Zfp207* (Appendix Fig S1B–E) suggesting that homozygous deletion of ZFP207 is lethal as previously suggested (Blomen *et al.*, 2015). Next, we conducted loss-of-function assays by using two distinct short-hairpin RNAs (shRNAs) against *Zfp207* (thereafter referred as knockdown 1 and 2 (KD1 and KD2)), to ensure that the observed phenotype is due to *Zfp207* depletion and not due to shRNA off-target effects. Silencing of *Zfp207* gene expression in both KD1 and KD2 ESCs was confirmed by RT-qPCR (Fig 1E) and by Western blot (Fig 1F). Depletion of *Zfp207* led to a reduced proliferation capacity compared to ESCs transduced with scrambled shRNA (thereafter referred as control), although to a lesser degree in KD1 than in KD2 ESCs (Fig 1G; 1.3 and 2-fold, respectively). In addition, KD1 and KD2 ESCs showed a significant 1.5-fold increased apoptotic rate compared to control ESCs (Fig 1H) while no significant differences in the cell cycle profile were detected between the three cell lines (Fig 1I). Overall, our results indicate that ZFP207 is required for the proper proliferation of mouse ESCs.

Figure 1. Depletion of *Zfp207* leads to growth defects of mouse ESCs.

- A, B RT-qPCR analysis of *Zfp207* and *Oct4* in mouse ESCs during (A) retinoic acid (RA)-induced and (B) embryoid body (EB)-mediated differentiation. *Nestin* was used as a neuronal differentiation marker to monitor RA-mediated differentiation. mRNA levels are relative to the expression at day 0.
- C, D Western blot of ZFP207 and OCT4 of mouse ESCs during (C) RA-induced and (D) EB-mediated differentiation.
- E, F (E) RT-qPCR and (F) western blot to monitor the knockdown efficiency of *Zfp207* (sh1 and sh2).
- G Cell proliferation rate of shScr, sh1, and sh2 ESCs assessed over a period of 6 days.
- H Percentage of live (Annexin V[−]) and apoptotic cells (Annexin V⁺) in sh1 and sh2 mouse ESCs compared to shScr.
- I Bar chart displaying the cell cycle distribution in sh1 and sh2 mouse ESCs relative to shScr.
- J, K (J) AP staining of shScr and *Zfp207*-depleted (sh1 and sh2) mouse ESCs. Scale bars, 20 μ M. (K) Percentage of fully differentiated (FD), partially differentiated (PD) and undifferentiated (UN) ESC colonies in shScr, sh1 and sh2.
- L Immunofluorescence analysis of SSEA1 in shScr, sh1, and sh2 ESCs. DAPI was used as the nuclear marker. Scale bars, 20 μ m.
- M, N (M) Western blot of ZFP207, OCT4, NANOG, and SOX2 in shScr, sh1 and sh2 ESCs and (N) RT-qPCR analysis of *Oct4*, *Nanog*, and *Sox2* in shScr, sh1 and sh2 ESCs; data is relative to shScr.
- O, P (O) Western blot of OCT4 during a 9-h cycloheximide (CHX) time course treatment in shScr, sh1 and sh2 ESCs. (P) Protein degradation curves were made after quantification and normalization of the bands from (O).
- Q, R (Q) Western blot of OCT4 during 4 and 8 h treatment with the proteasome inhibitor MG132. (R) graph bars after quantification and normalization of the bands from (Q).

Data information: Data are presented as mean $\pm$ SEM or representative images of $n \geq 3$ independent biological experiments. $^{\$}P < 0.05$, $^{\$\$}P < 0.01$, $^{\$ \$ \$}P < 0.001$ (D0 versus other indicated days). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, n.s = no significant (shScr versus sh1 or sh2). A, B, G, H and I: unpaired Student's t-test; E, N, P and R: ordinary one-way ANOVA.

Source data are available online for this figure.

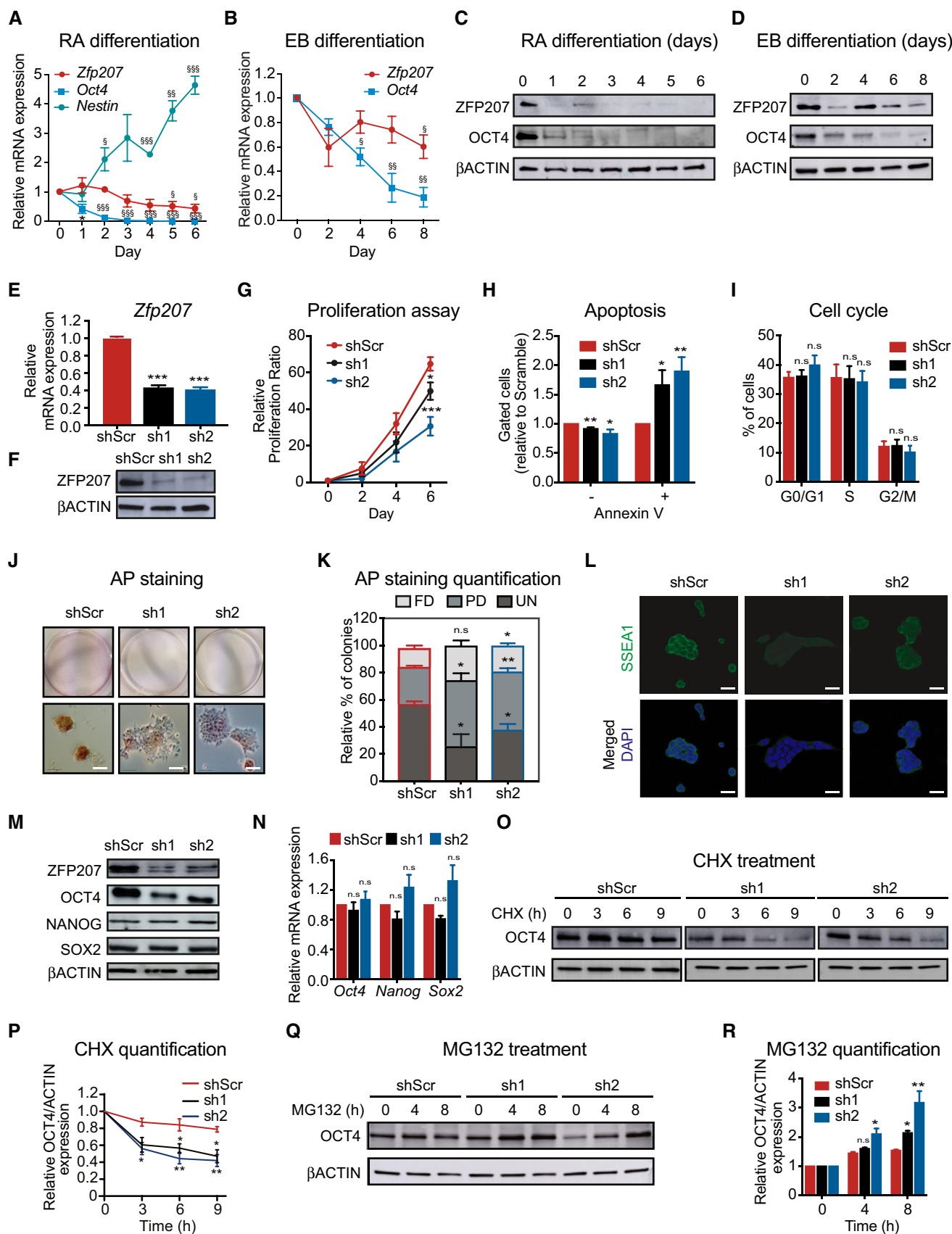


Figure 1.

Zfp207-depleted colonies displayed the typical morphology of differentiating ESCs with flat appearance and undefined colony borders (Fig 1J). Consistently, we detected reduced metabolic activity in ESC after depletion of *Zfp207* determined by alkaline phosphatase (AP) activity assay (Fig 1J). Specifically, downregulation of *Zfp207* resulted in a significant increase in the percentage of partially differentiated colonies, whereas the percentage of undifferentiated colonies was significantly decreased compared to control ESCs (Fig 1K). Such increase in the percentage of partially and finally differentiated colonies in KD1 and KD2 ESCs is a consequence of impaired ESC function as immunofluorescence analysis revealed that silencing of *Zfp207* leads to a decrease of the pluripotency surface marker SSEA1 (Fig 1L).

Since ZFP207 regulates self-renewal and pluripotency in human ESCs, we next analyzed the expression of the core pluripotency factors and observed that *Zfp207* KD1 and KD2 ESCs expressed lower OCT4, but not NANOG and SOX2, compared to control ESCs (Fig 1M). Interestingly, mRNA levels of the three pluripotency factors, including those of *Oct4* (Fig 1N), were unaltered upon silencing of *Zfp207*, indicating that ZFP207 could regulate the expression of OCT4 post-transcriptionally. To test this hypothesis, we treated KD1 and KD2 as well as control ESCs with the protein synthesis inhibitor cycloheximide (CHX). Silencing of *Zfp207* led to a decrease in the half-life of OCT4 (Fig 1O and P), suggesting that ZFP207 promotes the stability of this pluripotency factor. In addition, treatment with the proteasome inhibitor MG132 restored OCT4 protein levels in mouse ESCs depleted of *Zfp207* (Fig 1Q and R), indicating that ZFP207 interferes with the turnover of this pluripotency factor.

To assess whether ZFP207 sustains self-renewal in the naïve ground state, we cultured mouse ESCs in the presence of kinase inhibitors against MAP kinase (MEK) and glycogen synthase kinase 3 β (GSK-3 β ; “2i”) and leukemia inhibitory factor (LIF) (2iL medium) (Martello & Smith, 2014). Although the number of partially differentiated colonies in 2iL-cultured ESCs increased upon silencing of *Zfp207* assessed by a loss of reactivity to AP (Fig EV1A and B), RNA and protein levels of OCT4, NANOG, and SOX2 were unaffected (Fig EV1C and D). Likewise, no changes in SSEA1 staining were observed in 2iL-cultured ESCs upon *Zfp207* knockdown (Fig EV1E), suggesting that ZFP207 is dispensable for the maintenance of pluripotency in the ground state. Therefore, we performed the experiments in mouse ESCs cultured in conventional medium containing serum and LIF, that is, metastable ESCs, where we observed that ZFP207 contributes to stem cell identity by regulating post-transcriptional networks.

ZFP207 is required for proper EB differentiation

We next interrogated the role of ZFP207 during lineage specification by assessing the potential of KD1, KD2, and control mouse ESCs to spontaneously differentiate into EBs recapitulating early mouse embryo development. *Zfp207* KD1 and KD2 ESCs were able to form EBs (Fig 2A). Although the cells remained as solid aggregates, we observed a decrease in the size of both *Zfp207* KD1 and KD2 EBs compared to control EBs (Fig 2B), suggesting intrinsic differences during the differentiation process among the different cell lines. Given that ZFP207 influenced the apoptotic rate of ESCs (Fig 1H), we sought to investigate the mechanisms underlying the reduced size of *Zfp207* KD1 and KD2 compared to control EBs.

Bromodeoxyuridine (BrdU) incorporation revealed that *Zfp207* knockdown cells were associated with a significantly reduced growth rate compared with control EBs after day 4 of differentiation (Fig 2C and Appendix Fig S2A). However, we did not observe differences in apoptosis assessed by Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining performed along the course of differentiation or Annexin V staining of EBs at day 8 (Appendix Fig S2B–D). Altogether these results suggest that, albeit ZFP207 is critical for proper EB proliferation, its expression is not necessary for the viability of the EBs.

As expected, while *Zfp207* expression was gradually downregulated during the course of differentiation in control cells, *Zfp207* mRNA levels remained low in both KD1 and KD2 cells (Fig 2D). The expression of the pluripotent genes *Nanog* and *Sox2* decreased rapidly in all the cell lines, whereas *Oct4* decreased progressively, being its expression higher at the latest stage of the time course differentiation in EBs depleted of *Zfp207* compared to control (Fig 2D). EBs originated from the three shRNA-infected ESCs showed normal levels of the mesodermal (*Msx1* and *Brachyury* or *T*) and endodermal (*Foxa2* and *Sox17*) lineage-specific markers (Fig 2D). However, silencing of *Zfp207* impaired ectodermal specification as shown by decreased expression of *Pax6* and *Sox11* at day 4, and *Nestin* during the course of differentiation in *Zfp207* KD EBs compared to controls (Fig 2D and Appendix Fig S2E).

Silencing of *Zfp207* blocks differentiation of ESCs to neural progenitor cells and subsequently to neurons

To investigate whether ZFP207 plays a role in neurogenesis, KD1, KD2, and control ESCs were subjected to neural differentiation (Fig 3A). Albeit the distinct cell lines did not display major morphological differences at early stages of differentiation (when growing as EBs), KD1 and KD2 underwent a complete block of neuronal differentiation potential at day five (Appendix Fig S3A), and we were not able to characterize *Zfp207* KD1 and KD2 cells at later stages as they died at day 6. However, in control cell lines, NESTIN- and TUJ1-immunopositive neuronal projections appeared at day 8 and were kept in culture until day twelve (Fig 3B). Hence, we sought to also assess the viability and proliferation phenotype of *Zfp207*-depleted mouse ESCs upon neural differentiation. Immunostaining of TUNEL and cleaved caspase 3 revealed that a large proportion of *Zfp207* knockdown cells underwent apoptosis during differentiation (Fig 3C–E, Appendix Fig S3B and C). In addition, *Zfp207* depletion dramatically decreased cell proliferation at day 4 of differentiation as measured by BrdU incorporation (Fig 3F), indicating that ZFP207 play important roles in maintaining neural stem/progenitor cell properties, including their survival and proliferative capacities.

Similar to RA- and EB-mediated differentiation, *Zfp207* expression was downregulated during the course of neurogenesis in control cells, and *Zfp207* mRNA levels remained low in both KD1 and KD2 cells (Fig 3G). We also monitored progression of ESC differentiation toward neural fates by RT-qPCR analysis of neuroectodermal markers. *Zfp207*-depleted cell lines failed to activate the expression of *Pax6*, *Sox11*, and *Nestin* (Fig 3G and Appendix Fig S3D), suggesting a stall at early ectodermal differentiation. No defects were found in the expression of *Tuj1* at the transcriptional level as the upregulation of this neuronal marker occurred at later stages when the KD1 and KD2 cells had died (Fig 3G).

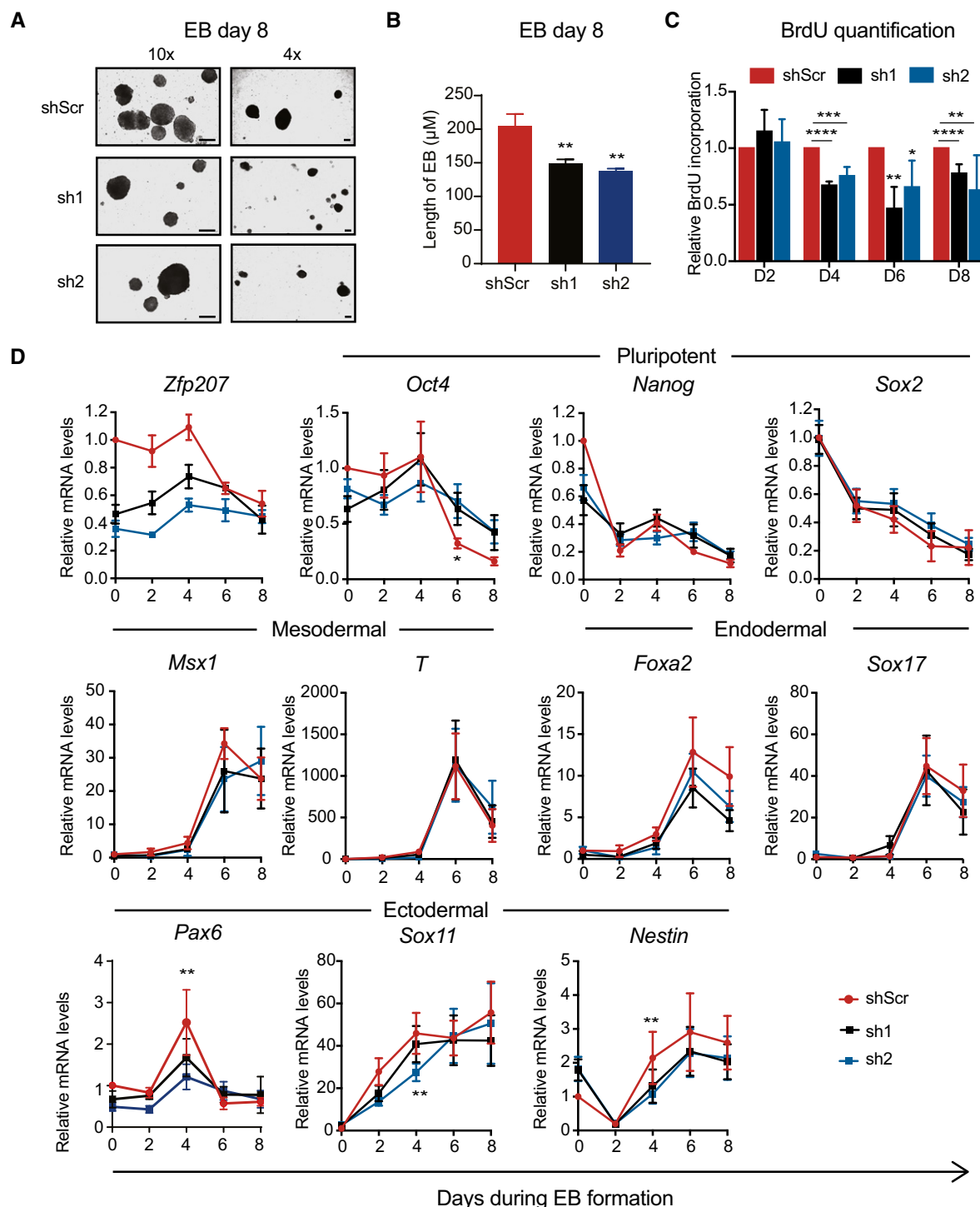


Figure 2. Loss of *Zfp207* results in defective differentiation.

A, B (A) Bright-field images (10 $\times$ (left) and 4 $\times$ (right) magnification) and (B) quantification of embryoid bodies (EB) generated from shScr, sh1, and sh2 at day 8 of differentiation. Scale bars, 200 μM .

C Quantification of BrdU incorporation in shScr, sh1, and sh2 ESCs at the indicated days of EB differentiation. Data are relative to shScr.

D RT-qPCR of *Zfp207*, the pluripotency genes (*Oct4*, *Nanog* and *Sox2*), the mesodermal markers (*Msx1* and *Brachyury* (*T*)), the endodermal markers (*Foxa2*, *Sox17*), and the neural-associated genes (*Pax6*, *Sox11*, and *Nestin*) in shScr, sh1, and sh2 ESCs along the time-course of EB-mediated differentiation. mRNA levels are relative to the expression of shScr at day 0.

Data information: Data are presented as mean $\pm$ SEM or representative images of $n \geq 3$ independent biological experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ (shScr versus sh1 or sh2). B, C: unpaired Student's t -test; D: ratio paired Student's t -test.

We next assessed whether ZFP207 is required for early or terminal differentiation of neurons by derivation of neural progenitor cells (NPCs) from KD1, KD2, and control ESCs (Hanafiah *et al*, 2020) (Fig EV2A). Even though *Zfp207* knockdown mouse ESCs

were successfully differentiated to EBs (day 4), it was immediately apparent that KD1 and KD2 NPCs were smaller and all died after day 6 (Fig EV2B and C). In addition, RT-qPCR analysis indicated that KD1 and KD2 failed to activate the expression of ectodermal

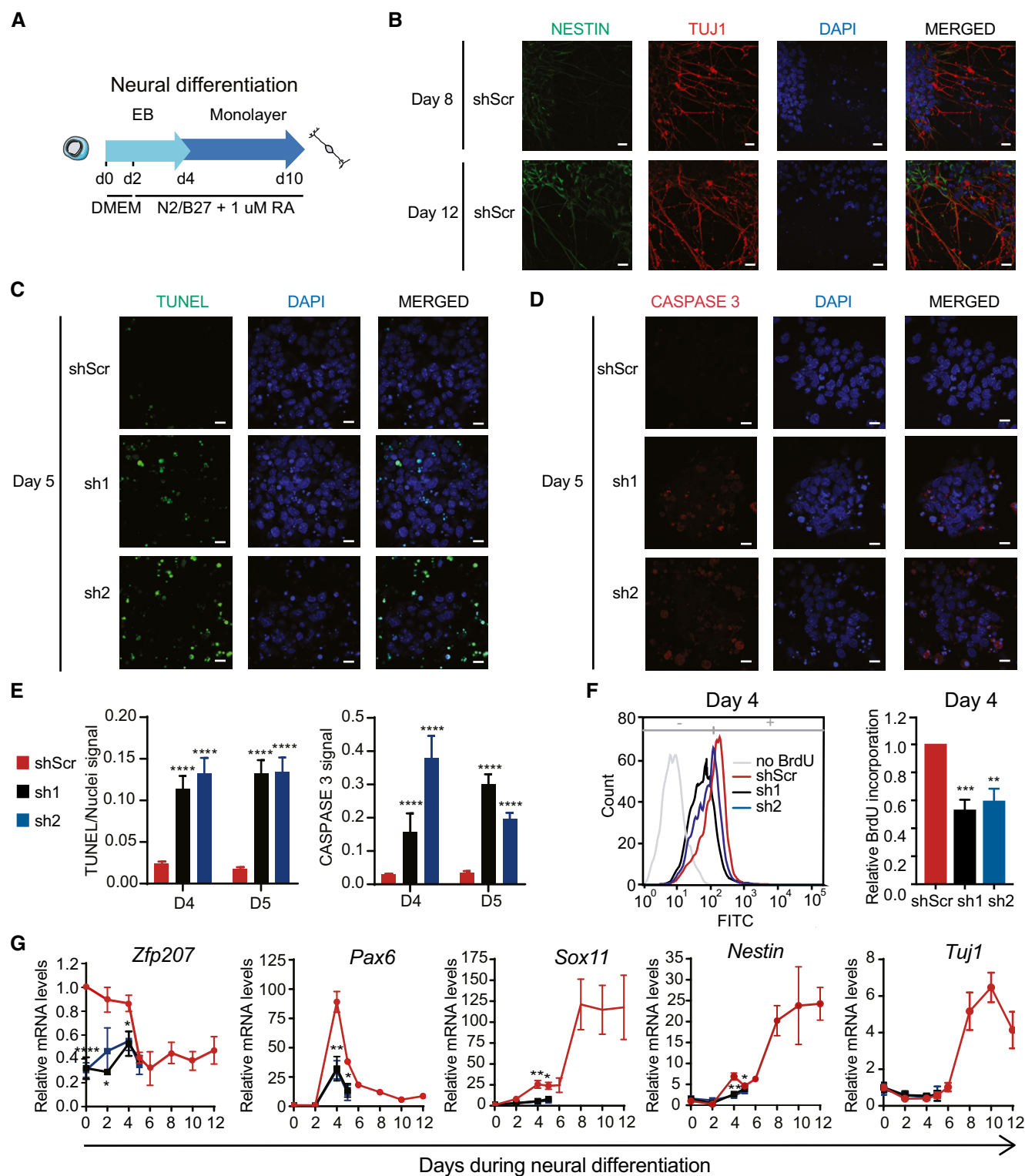


Figure 3.

Figure 3. ZFP207 is essential for neural cell fate specification.

- A Schematic depiction for the neuroectodermal-directed differentiation. DMEM/F12 supplemented with N2B27 and retinoic acid (RA) was added after two days of EB culture.
- B Immunostaining of NESTIN (green) and TUJ1 (red) of neural progenitors generated from shScr on day 8 and 10 of the neuroectodermal differentiation. Nuclei were counterstained with DAPI. Scale bar, 20 μ M.
- C–E (C) TUNEL (green), (D) CASPASE 3 (red) staining and (E) quantification of the signal in shScr, sh1, and sh2 at day 5 of neuroectodermal differentiation. Nuclei were counterstained with DAPI. Scale bar, 20 μ M.
- F Flow cytometric profile and quantification of BrdU incorporation at day 4 of neuroectodermal differentiation.
- G RT-qPCR of *Zfp207* and neural-associated markers in shScr, sh1, and sh2 along the course of neural differentiation. mRNA levels are relative to shScr at day 0.
- Data information: Data are presented as mean $\pm$ SEM or representative images of $n \geq 3$ independent biological experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ (shScr versus sh1 or sh2). E, F, and G: unpaired Student's t-test.

markers, again consistent with a blockade at early ectodermal differentiation upon ZFP207 deficiency (Fig EV2D).

To demonstrate the specificity of the phenotype observed upon silencing of *Zfp207*, we engineered a tet(ON)-ZFP207 cell line in which endogenous *Zfp207* can be depleted by shRNA2 and replaced with a shRNA2-immune exogenous *Zfp207* cDNA responsive to doxycycline (Dox) treatment. As expected, Dox treatment in tet(ON)-ZFP207 KD2 induced the expression of ZFP207 which resulted in an increase of OCT4 protein levels but not *Oct4* mRNA (Fig EV3A and B). In addition, re-expression of ZFP207 rescued the proliferation and apoptotic defect of tet(ON)-ZFP207 KD2 (Fig EV3C and D). Furthermore, upon addition of Dox, tet(ON)-ZFP207 KD2 displayed a characteristic ESC-like morphology, and the number of partially differentiated colonies was increased whereas the percentage of final differentiated colonies was significantly decreased compared to *Zfp207* KD2 ESCs without Dox induction (Fig EV3E and F). Strikingly, re-expression of ZFP207 was not sufficient to return the normal number of undifferentiated colonies (Fig EV3E and F). Nevertheless, the levels of SSEA1 staining of tet(ON)-ZFP207 KD2 upon Dox treatment were comparable to control ESCs (Fig EV3G), confirming that ZFP207 is critical for the maintenance of mouse ESCs.

We next investigated whether the neuroectoderm blockade was specific to the depletion of *Zfp207* by subjecting the tet(ON)-ZFP207 cell line to neural differentiation. Of note, tet(ON)-ZFP207 KD2 cells died at day 4; therefore, it was not possible to characterize the phenotype at later days of differentiation. However, after Dox treatment, tet(ON)-ZFP207 KD2 regained the ability to differentiate being the size of the neurospheres similar to that of the control cells (EV3H). In addition, re-expression of ZFP207 restored the ability to induce the expression of *Pax6*, *Sox11*, and *Nestin*, especially at day 5, where no differences in the expression of the neuroectodermal markers were detected between control and tet(ON)-ZFP207 KD2 induced with Dox (EV3I). However, the blockade phenotype was only partially recovered as rescue cells died at later stages and displayed increase TUNEL staining (EV3J), illustrating a complex regulatory interplay of *Zfp207* isoforms occurring during neural differentiation that will be discussed later.

ZFP207 does not transcriptionally regulate the ectodermal lineage

In order to gain insight on the role of ZFP207 in ESC pluripotency, we analyzed the global transcriptome response to *Zfp207* depletion (Fig 4A). RNA-sequencing (RNA-seq) analysis identified 382 and 522 genes that were downregulated in KD1 and KD2 ESCs, respectively

(fold change > 1.5 ; $P < 0.05$; Fig 4B and Dataset EV1). The differences in the number of downregulated genes between the two KDs could result in the more severe phenotype observed in cells transduced with shRNA2 compared to shRNA1 (Fig 1G). The effect on upregulation was more robust, whereby 1,062 and 1,082 genes were upregulated in KD1 and KD2 ESCs, respectively, compared to control ESCs (Fig 4B). Gene ontology (GO) analysis of biological processes of common downregulated genes revealed generic functions, which included mitotic sister chromatid segregation among other categories (Fig 4C). Strikingly, poly(A)⁺ RNA binding was among the most represented molecular function GO categories (Appendix Fig S4A). According to the reported function of ZFP207 in kinetochore–microtubule attachment (Dai *et al*, 2016), top GO categories for molecular function of common downregulated genes also included microtubule binding (Appendix Fig S4A). We validated these results by performing RT-qPCR analysis of downregulated mitotic sister chromatid segregation genes (e.g., *Cdca8* and *Cep57l1*; Appendix Fig S4B). Upregulated genes were associated with GO biological processes related to RNA splicing and processing, and positive regulation of transcription (Fig 4D). Similar to the common downregulated genes, RNA binding, including poly(A)⁺ and mRNA binding, were among the most represented categories of GO molecular function (Appendix Fig S4C), suggesting a role of ZFP207 in controlling the expression of putative RBPs, primarily involved in RNA splicing and mRNA processing (Appendix Fig S4D).

Depletion of *Zfp207* did not lead to aberrant transcriptional programs that could explain the developmental defects. Hence, there were no differences in the expression of ESC genes upon silencing of *Zfp207* (Fig 4E and F). In addition, we did not find major transcriptional differences in genes associated with the neural lineage that could reflect the stall at early ectodermal differentiation in KD1 and KD2 ESCs (Fig 4G and H). This is in striking contrast to what has been reported in human ESCs where depletion of *ZNF207* impairs neuroectodermal specification by transcriptionally regulating the expression of genes associated with the ectoderm lineage (Fang *et al*, 2018). Noteworthy, in human ESCs, the isoforms A and C of *ZNF207*, retaining the exon 9, are highly abundant compared to mouse ESCs, where the isoforms 1 and 2 of *Zfp207*, retaining the aforementioned exon 9, are highly expressed in differentiated cells (Fig EV4A and B). Hence, in both species, there is an antagonistic switch toward using different isoforms during differentiation, which could partially explain the differences observed between mouse and human ESCs. Noteworthy, *Zfp207* isoform switching occurring during differentiation could also explain why re-expression of one isoform of *Zfp207* in the rescue experiments, that is, isoform 3,

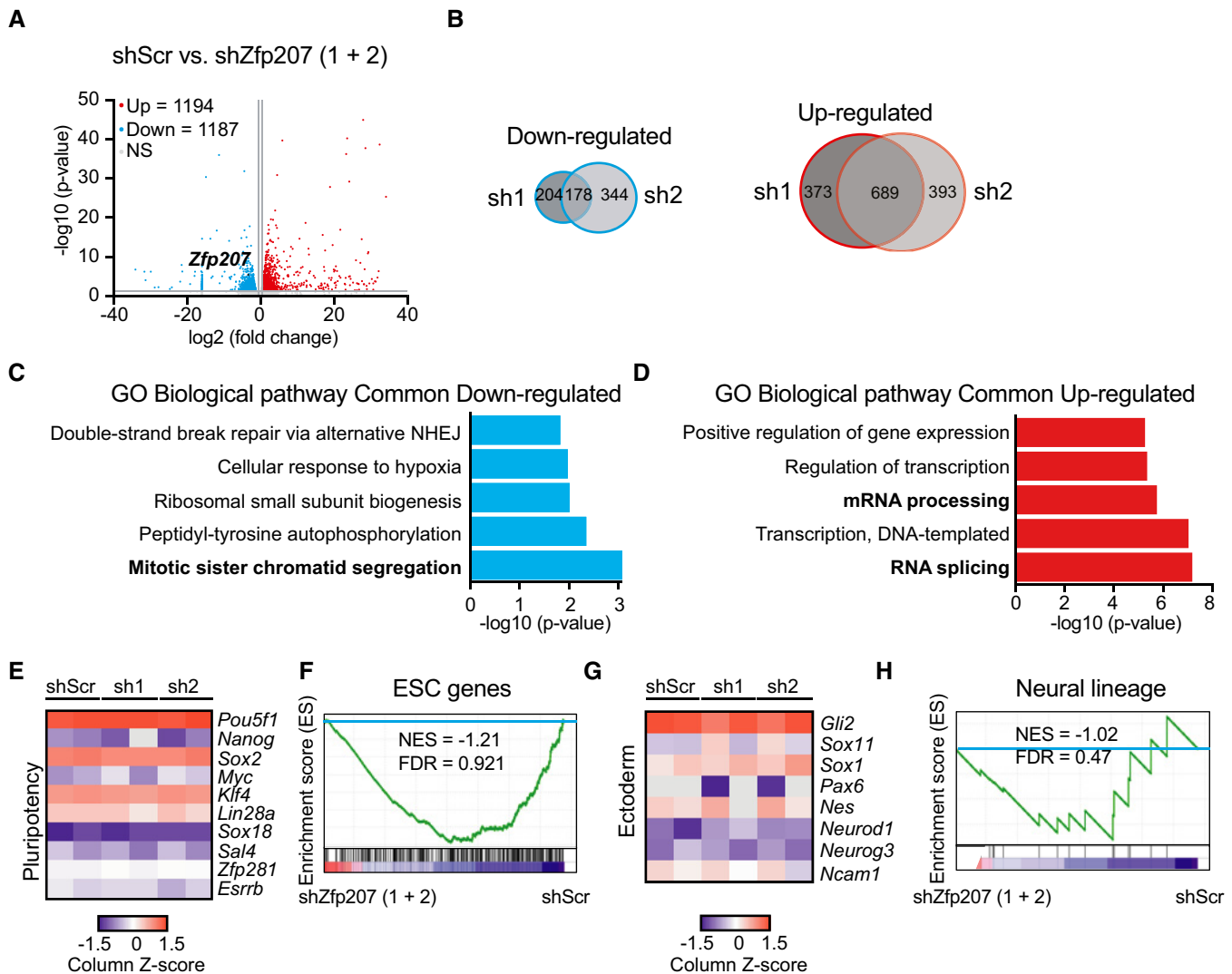


Figure 4. The neural transcriptome is not altered upon depletion of *Zfp207*.

A, B (A) Volcano plots of common differentially expressed genes in shScr and *Zfp207*-depleted (sh1 and sh2) mouse ESCs. Upregulated (Up) and downregulated (Down) genes are indicated in red and blue, respectively ($P < 0.05$; > 1.5 -fold). *Zfp207* is depicted in black. Grey dots indicate non-significant (NS) and < 1.5 -fold differential expressed genes. (B) Venn diagram depicting the overlap of downregulated and upregulated genes between sh1 and sh2 ESCs ($FC > 1.5$ and $P < 0.05$). FDR value was calculated with the Benjamini–Hochberg correction.

C, D Gene ontology (GO) analysis of biological processes associated with common (C) downregulated and (D) upregulated genes in *Zfp207*-depleted ESCs (sh1 and sh2); NHEJ: Nonhomologous end joining.

E–H Heatmap of column z-scores of log2 transformed values of genes related to (E) pluripotency and (G) ectoderm in shScr, sh1, and sh2 ESCs. GSEA plots depicting the expression of (F) ESC-related and (H) ectodermal genes in *Zfp207*-depleted ESCs compared to shScr. High and low expression of genes is represented in red and blue color, respectively. FDR, false discovery rate; NES, normalized enrichment score.

which is highly expressed in mouse ESCs, is not sufficient to fully rescue the phenotype at later stages of differentiation (Fig EV3H and I). As *ZNF207* is a downstream splicing target of *SFRS11* during somatic cell reprogramming (Toh *et al*, 2016), we sought to analyze whether this splicing factor was also modulating mouse *Zfp207* splicing. Similar to what is observed in the human ortholog, depletion of *Sfrs11* increased retention of exon 9, corresponding to the isoforms 1 and 2 of *Zfp207* (Fig EV4C–E). Therefore, *SFRS11* modulates the same splicing event in mouse and human ESCs but with divergent consequences.

ZFP207 regulates alternative splicing in mouse ESCs

AS is a fundamental process that increases proteomic diversity in a species-manner but not organ-specific manner (Barbosa-Morais *et al*, 2012), and it also plays a central role in the regulation of ESC-specific transcriptional programs (Gabut *et al*, 2011). *ZNF207* has been shown to influence pre-mRNA splicing in cancer cells (Wan *et al*, 2015). Consistent with this observation, our GO enrichment analysis of genes upregulated upon *Zfp207* silencing revealed significant enrichment of GO terms related to RNA splicing (Figs 4D and

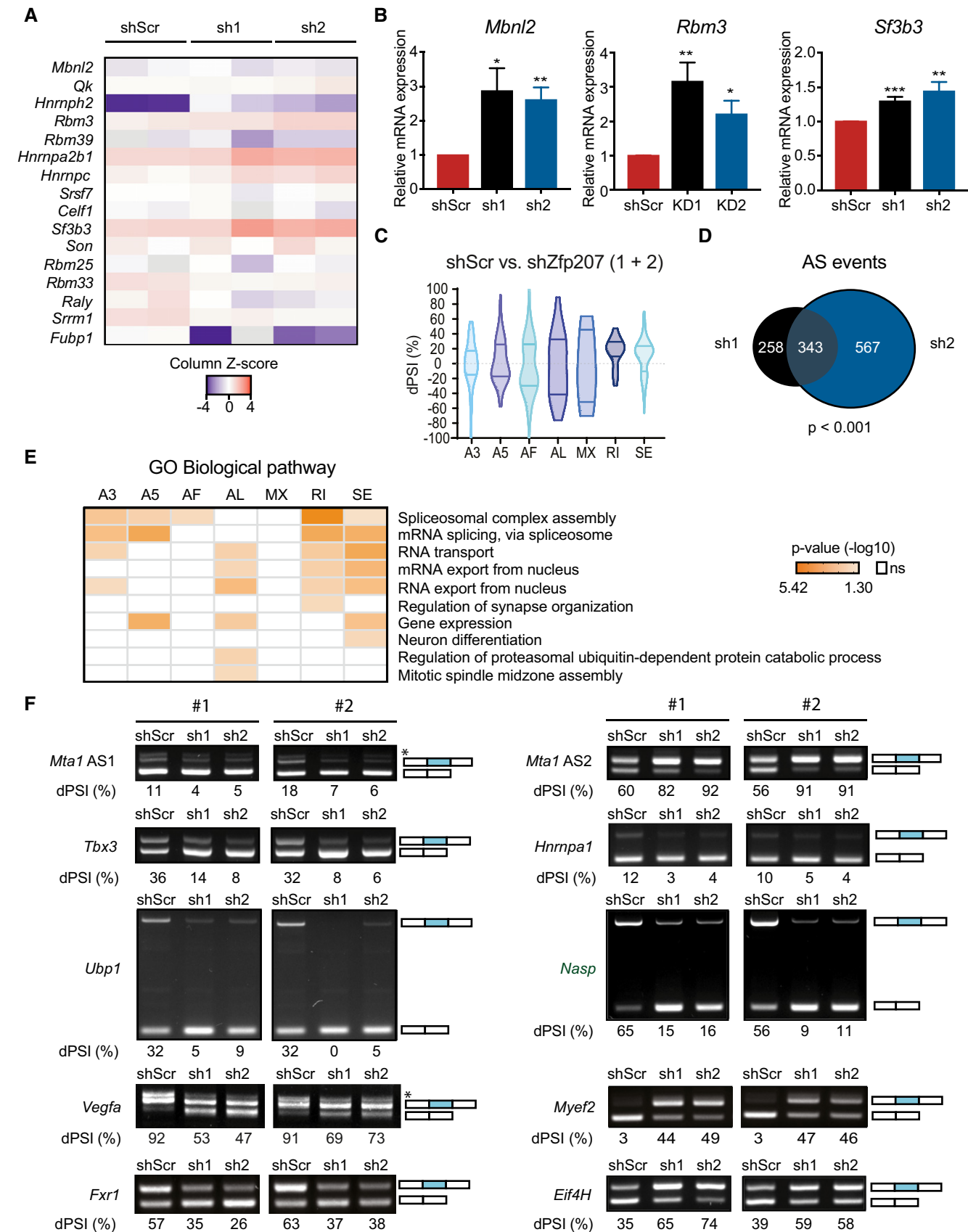


Figure 5.

Figure 5. Silencing of *Zfp207* leads to AS defects.

- A Heatmap of column z-scores of log2 transformed values of genes involved in alternative splicing (AS) in shScr, sh1 and sh2 mouse ESCs.
- B RT-qPCR analysis of *Rbm3*, *Mbnl2*, and *Sf3b3* in shScr, sh1 and sh2 ESCs. mRNA expression is relative to shScr and presented as mean $\pm$ SEM of $n \geq 3$ independent biological experiments; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (shScr versus sh1 or sh2); unpaired Student's *t*-test.
- C Violin plot depicting statistically significant spliced in (PSI) events in each category in sh1 and sh2 related to shScr. Alternative 3' (A3); Alternative 5' (A5); Alternative first exon (AF); Alternative last exon (AL); Mutually exclusive exons (MX); Intron retention (RI); Exon skipping (SE). The dotted line indicates Δ PSI = 0.
- D, E (D) Venn diagram showing the overlap of AS in sh1 and sh2. (E) Gene ontology analysis of common genes undergoing AS in sh1 and sh2 mouse ESCs according to the type of AS event.
- F RT-PCR of *Zfp207*-regulated AS events. For *Mta1*, AS1 (alternative splicing 1) and AS2 (alternative splicing 2). #1 and #2 indicate distinct biological replicates. *Denotes an isoform that was not taken in consideration for the quantification. The structure of each isoform is indicated (not to scale). Alternative exons are blue. The percent spliced in (PSI) was quantified for each condition.

5A), and some randomly selected spliceosomal genes (*Mbnl2*, *Rbm3*, and *Sf3b3*) were further validated by RT-qPCR (Fig 5B).

We therefore sought to investigate whether ZFP207 could control ESC function by a splicing-related mechanism. To this end, reads from RNA-seq data were mapped to exon-splice junction's sites in order to elucidate genome-wide differential AS events (DSEs) (Alamancos *et al*, 2015; Trincado *et al*, 2018), including alternative 5' and 3' splice-site selection (A5 or A3), alternative first (AF) and last (AL) exon selection, exons that are spliced in a mutually exclusive manner (MX) or that are skipped (SE), and changes in intron retention (RI) (Fig EV5A). Comparisons of AS isoform levels were performed in KD1 and KD2 ESCs versus control shRNA ESCs, and differences in AS isoforms were calculated as the change in percent spliced in (dPSI) (Fig 5C). Alternative first exon selection and exon skipping appeared to be the most predominant splice events (Fig EV5B). Our analysis identified 601 and 910 splicing events in KD1 and KD2 compared to control corresponding to 419 and 609 genes, respectively (false discovery rate [FDR] < 0.05 ; Fig 5D and Dataset EV2). Alterations in DSEs upon knockdown of *Zfp207* were not due to changes in transcription as gene expression levels were not correlated to dPSI (Fig EV5C). The overlapped AS genes were significantly enriched in functional categories associated with RNA splicing, RNA transport and export from the nucleus, and neurogenesis, among others (Figs 5E and EV5D), suggesting that splicing switches occurring upon silencing of *Zfp207* are relevant to the severe blocking defect towards the neural lineage observed in KD1 and KD2 ESCs. DSEs were validated by RT-PCR amplifying AS exons that differ in size and band intensity were assessed in order to estimate the exon-inclusion ratios. Specifically, we validated randomly selected ZFP207-mediated DSEs in control, KD1, and KD2 ESCs (Fig 5F), including the chromatin regulatory cofactor *Mta1* (AS1 and AS2); the AS factors *Tbx3* and *Hnrnpa1*; the transcription factor *Ubp1*; the histone chaperone *Nasp*; the neuronal factors *Vegfa*, *Myef2* and *Fxr1*; and the translation initiation factor *eIF4H*. Furthermore, we also interrogated the aforementioned DSEs in neural differentiation, and found that the majority of the splicing switches validated in KD1 and KD2 ESCs were also prevalent in differentiated cells (Fig EV5E). Collectively, these data indicate that depletion of *Zfp207* elicits aberrant AS patterns that resemble the differentiated cell-like pattern, and consequently influences mouse ESC identity.

ZFP207 is a newly identified RBP

Because ZFP207 influenced AS and it has been identified as a mRNA binder using a quantitative proteomics approach (Baltz *et al*, 2012;

Castello *et al*, 2012), we assessed the innate binding propensity of ZFP207 in mouse ESCs by employing a modified *in vitro* RNA immunoprecipitation (RIP) protocol (see methods section). We identified 1,400 transcriptome-wide binding events for ZFP207 in our assay (Dataset EV3). In total, 942 of these binding sites resided within coding regions of protein-coding genes (Fig 6A). Since ZFP207 is located in the nucleus, we also found binding to intronic sequences (178/1,400). In addition, we detected binding to untranslated regions (UTRs) (267/1,400) and exons of noncoding transcripts (17/1,400). It is possible that there are more ZFP207-binding events to nascent RNAs, which might have been missed since most intronic sequences are depleted by this method. The 1,400 ZFP207 binding events were distributed over 1,171 genes. Based on a fold-change weighted by the relative expression levels (referred to as binding score), we identified the strongest binding sites within the *Ubp1*, *Marf1*, *Sae1*, *Erp29*, and *Nsd1* transcripts (Fig 6B). Surprisingly, three of the top five binding sites (*Marf1*, *Sae1*, and *Nsd1*) were found over exon-exon junctions (Appendix Fig S5A). Such binding events are difficult to envision *in vivo*, unless ZFP207 is part of the exon-exon junction complex and connects exonic ends directly.

Overlapping of the *in vitro* RIP-seq and AS data sets revealed 13.7% of DSEs that were directly bound by ZFP207 (Fig 6C), including *Mta1*, *Ubp1*, *Vegfa*, and *Fxr1* with AS switches occurring upon silencing of *Zfp207* that were validated (Fig 5F). However, we cannot exclude that dysregulated expression of genes encoding spliceosomal factors upon depletion of *Zfp207* was a major contributor to ESC-differential AS events. *In vivo* ZFP207 binding at randomly selected transcripts, such as the pluripotency factors *Klf4* and *Esrrb*, the RBPs *Igf2bp1* and *Fxr1*, which play a critical role in neurogenesis (Ravanidis *et al*, 2018), and the highly enriched *Sae1* transcript were validated by *in vivo* RIP followed by RT-qPCR, thus validating our *in vitro* approach (Appendix Fig S5B and C).

In order to classify the bound genes into functional groups, we performed a pathway enrichment analysis for biological processes, and we found that the 1,171 genes fell into 693 significantly enriched pathways over the whole murine transcriptome (FDR ≤ 0.05) (Fig 6D). Interestingly, GO analysis revealed DNA repair, chromosome organization, and chromosome segregation among the most represented categories, suggesting that ZFP207 can also regulate the aforementioned pathways not just by interacting with Bub3 (Jiang *et al*, 2014; Toledo *et al*, 2014) but also by directly binding to target transcripts and influencing RNA fate. Noteworthy, GO terms that were affected upon *Zfp207* silencing such as mRNA processing, splicing, positive regulation of neuron differentiation, and neuron projection development were highly enriched. This

suggests that in addition to regulating a pluripotent AS signature, ZFP207 controls the ESC state through post-transcriptional gene regulatory mechanisms. Indeed, ZFP207-bound transcripts fell into two major ESC gene signatures, namely, the Core and Myc modules,

which are highly active in the pluripotent state, whereas the Polycomb repressive (PRC) module, operating in differentiation, was underrepresented (Appendix Fig S5D) (Kim *et al*, 2010), which is in agreement with the observation that ZFP207 regulates pluripotency

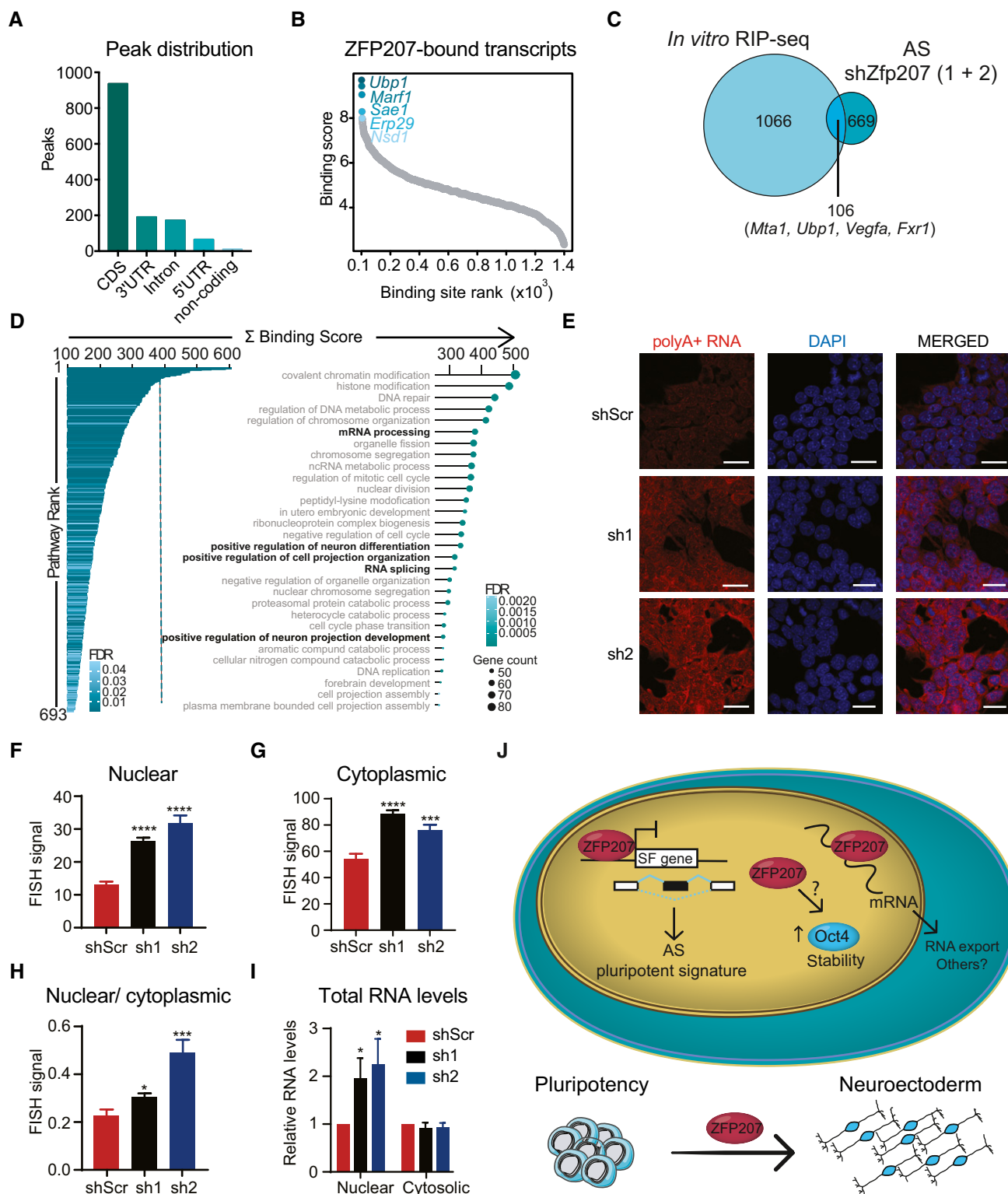


Figure 6.

Figure 6. ZFP207 is a novel RBP.

- A–D (A) Distribution of ZFP207-binding sites across common transcript features. (B) Scatterplot depicting ranked distribution of ZFP207 binding sites. The top 5 strongest binding sites are highlighted. (C) Venn diagram of *in vitro* RIP-seq and AS upon *Zfp207* knockdown. (D) Bar- and dot plots show significantly enriched GO terms for biological processes for ZFP207 bound genes. Top 30 GO terms are shown.
- E–I (E) FISH of poly(A)⁺ RNA distribution in shScr, sh1 and sh2 stained with 5'Cys3-oligo dT(50). Nuclei were counterstained with DAPI. Scale bar, 20 μ M. Quantification of (F) nuclear, (G) cytoplasmic, and (H) of nuclear/cytoplasmic poly(A)⁺ FISH signals. (I) Relative nuclear and cytosolic total RNA levels in sh1 and sh2 compared to shScr. Data are presented as mean $\pm$ SEM of $n \geq 3$ independent biological experiments. * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$ (shScr versus sh1 or sh2); unpaired Student's t-test.
- J ZFP207 modulates mouse ESCs pluripotency and neuroectodermal differentiation by integrating co- and post-transcriptional mechanisms.

by transcriptional-independent programs. The fact that ZFP207-bound transcripts were enriched in the Myc module, which is active in various cancers and predicts cancer outcome, may reveal additional post-transcriptional regulatory functions of ZFP207 not just in pluripotency but also in human diseases. Depiction of the binding score of the ZFP207-bound transcripts showed a similar binding activity pattern within the Core, Myc, and PRC modules (Appendix Fig S5E).

Because RBPs play important roles not just in mRNA splicing but also in other aspects of the post-transcriptional mRNA fate such as nuclear export, localization, or nuclear decay (Ye & Blelloch, 2014), we analyzed the distribution of bulk poly(A)⁺ RNAs by fluorescence in situ hybridization (FISH) using an oligo(dT) probe in scramble control, KD1 and KD2 ESCs. mRNA was accumulated in nuclear poly(A)⁺ foci upon silencing of *Zfp207* (Fig 6E and F), suggesting that ZFP207 might play a role in nuclear export. Although cytoplasmic poly(A)⁺ RNA FISH signals were also brighter in *Zfp207*-depleted cells compared to that in control cells (Fig 6E and G), the intensity of cytoplasmic poly(A)⁺ RNA pixel, and the nuclear to cytoplasmic ratios were less profound in *Zfp207* knockdown compared to control ESCs (Fig 6G and H). In addition, cellular fractionation showed a clear accumulation of nuclear RNA upon silencing of *Zfp207*, whereas no differences were detected in the amount of RNA in the cytoplasmic fractions of the distinct cell lines (Fig 6I). Although it remains possible that some mRNA will get exported from the nucleus, these results indicate that at least some particular mRNAs are nuclear retained and largely aggregate into poly(A)⁺ foci in *Zfp207* KD1 and KD2 ESCs.

In summary, we show that ZFP207 plays an important role in maintaining mouse ESC self-renewal and neuroectodermal specification by controlling co- and post-transcriptional networks. We propose a model whereby ZFP207 regulates the expression of splicing factors to promote *bona fide* splicing of a specific set of transcripts that alter cellular fate. Additionally, ZFP207 acts as an RBP to facilitate mRNA nuclear export. Hence, the versatility in regulatory capacities of ZFP207 is important for normal expression of pluripotency and neural commitment genes in order to ensure mouse ESC identity (Fig 6J).

Discussion

Previous investigations have focused the attention on the properties of ZNF207 as a transcription factor and its function in transcriptional control (Fang *et al*, 2018). An important aspect of our findings is the observation that ZFP207 regulates mouse ESC maintenance through co- and post-transcriptional mechanisms similarly to other

members of the ZNF/ZFP family that can act as regulators of RNA metabolism (Hall, 2005; Razin *et al*, 2012). On the contrary to human ESCs, mouse ESCs have a naïve pluripotency with unbiased differentiation capacity, and they have proven to be an invaluable model to study development. Hence, although the human ortholog ZNF207 has been described as a critical regulator for self-renewal and pluripotency elsewhere (Fang *et al*, 2018), we found divergent functional characteristics between both studies. These dissimilarities may reflect two distinct pluripotent states that represent mouse and human ESCs: naïve and primed, respectively (Ginis *et al*, 2004). In addition, these differences could arise from opposite AS-shifting patterns of *Zfp207* and *ZNF207* that occur during cellular differentiation of mouse and human ESCs, respectively.

ZFP207 is essential for cellular viability in near-haploid cells (Blomen *et al*, 2015). In line with this, we were unable to fully disrupt the *Zfp207* gene in mouse ESCs using CRISPR/Cas9 technology. Hence, we anticipate that ablation of ZFP207 is incompatible with ESC maintenance. Moreover, our results show that depletion of *Zfp207* in ESCs resulted in cell proliferation defects by triggering apoptosis, which further shifts the self-renewal phenotype toward differentiation. Similar to human ESCs, we also found that ZFP207 controlled the expression of the pluripotency factor OCT4. However, such regulation did not occur transcriptionally as *Oct4* mRNA levels were not affected upon silencing of *Zfp207*. OCT4 is targeted for ubiquitination and degradation, and this post-translational modification plays a critical role in OCT4 expression in ESCs (Xu *et al*, 2009). Our data suggest that ZFP207 would increase OCT4 protein stability by preventing degradation of OCT4 through the proteasome. Consistently, Jiang *et al* (2014) reported that ZFP207 functions as a chaperone that binds and stabilizes the spindle assembly checkpoint protein Bub3 by protecting Bub3 from degradation by the proteasome. It remains to be elucidated the mechanism by which ZFP207 protects OCT4 from proteasomal degradation as none of the core pluripotency factors were identified in mass spectrometric analysis of mouse ESCs using ZFP207 as bait (Jiang *et al*, 2014). However, such study identified the E3 ubiquitin ligase UBR5 as a ZFP207-interacting partner, suggesting that ZFP207 could exert a secondary role in controlling proteasome-mediated degradation. Strikingly, such mechanism would only operate in mouse ESCs cultured in serum medium, displaying a metastable pluripotency state, as ZFP207 does not play a critical role in the maintenance of pluripotency when ESCs are cultured in 2iL conditions where it does not modulate the expression of OCT4. This finding supports the model in which depleting a single component of the pluripotency network (such as Brd4) is not sufficient to induce perturbations in ground state pluripotency (Finley *et al*, 2018).

We assessed the function of ZFP207 during *in vitro* differentiation to the three germ layers, recapitulating the early events in embryogenesis, by performing EB-directed differentiation. Although EB formation and growth were defective after silencing of *Zfp207*, we could conclude from these experiments that endodermal and mesoderm specification was normal whereas the neuroectodermal lineage was highly compromised. Indeed, upon neural induction, EBs depleted of *Zfp207* failed to efficiently upregulate neural markers, and neuronal differentiation appeared to stall at an early stage. In agreement with this observation, *Zfp207* knockdown ESCs were not able to generate NPCs. In contrast to human ESCs, RNA-seq analysis revealed that ZFP207 did not drive the transcription of neuronal gene expression programs and could not explain the blockade of neuroectodermal differentiation upon *Zfp207* silencing. However, ZFP207 controlled the transcription of multiple splicing factors, which potentially could lead to aberrant AS patterns with critical functions in pluripotency. For instance, we validated the upregulated expression of *Mbnl2*, *Rbm3*, and *Sf3b3*. Accordingly, *Mbnl2* and *Rbm3* have been shown to promote differentiated-cell-specific AS patterns (Han et al, 2013; Xia et al, 2018; Yan et al, 2019). To our knowledge, no specific function of the splicing factor SF3B3 in ESCs has been described. Global profiling of the AS landscape revealed alterations of a broad number of genes. Although such splicing defects could be a consequence of an indirect effect, through dysregulation of spliceosome gene expression, we cannot exclude the possibility that ZFP207 also regulates AS in a direct manner, through interaction with spliceosome factors (Jiang et al, 2014). Hence, it has been shown that ZFP207 interacts with the splicing components U2AF65 and SF3a3, and transcriptomic analysis revealed splicing defects after depletion of ZFP207 in cancer cells, although such splicing events were not thoroughly characterized (Wan et al, 2015). Our results expand on these prior observations reporting that ZFP207 is a major splicing regulator that plays a critical role in modulating post-transcriptional networks that influence cell fate.

Functional categories of these AS transcripts included RNA metabolism and neuron differentiation. Interestingly, mitotic spindle midzone assembly was also among the top identified GO categories, suggesting that ZNF207 might not only promote spindle assembly by undergoing coacervation and hence, concentrating microtubules and tubulin through ZNF207 droplets (Jiang et al, 2015), but also by an RNA-mediated mechanism. Randomly chosen DSE have been experimentally validated in this study. Most of them (eight out of ten) displayed a switching to a differentiated cell-like AS pattern upon *Zfp207* knockdown, indicating that ZFP207 regulates an AS signature that controls pluripotency. In addition, several of the stem cell switches that we identified, such as *Nasp* or *Tbx3*, have been shown to be involved in cancer (Aleksiev et al, 2011; Krstic et al, 2020).

By using a modified *in vitro* RIP protocol, we characterized ZFP207 as a novel RBP. ZFP207 has an annotated C2H2-ZNFs through which RNA binding is facilitated (Brannan et al, 2016), and two independent interactome capture studies have retrieved ZFP207 in the mRNA-bound proteome (Baltz et al, 2012; Castello et al, 2012). As the extent of intronic binding events that ZFP207 could potentially have inside the nucleus can be orders of magnitude higher than the ones we identified with the *in vitro* RIP, it would therefore be tempting to speculate that ZFP207 regulates AS by directly binding to specific transcripts. However, it is also plausible that the RNA-binding activity of ZFP207 provides an additional

function as we observed an accumulation of poly(A)⁺ foci in KD1 and KD2 ESCs. Interestingly, it has been shown that the poly(A)⁺ RNA foci that were accumulated upon depletion of *Zfp207* are primarily formed by exosome target mRNAs (Fan et al, 2018). Thus, it is possible that ZFP207 functions in the turnover of poly(A)⁺ RNA in general, and in the export of pluripotency and neuroectodermal mRNAs in ESCs. It would be interesting to determine in future studies whether the distinct *Zfp207* isoforms target a distinct set of RNAs. If that would be the case, the ESC-specific switch in the exon 9 could drive divergent RNA-metabolism-based programs required for ESC self-renewal and pluripotency.

Albeit the function of most ZNFs/ZFPs is still poorly understood, a group of proteins of this large family has been associated with the functionality of the nervous system (Al-Naama et al, 2020). Hence, the Zic family of ZNFs is involved in the differentiation of neuronal progenitors in the medial forebrain and the cerebellum, whereas the Ikaros family has a similar function in the striatal medium spiny neurons (Inoue et al, 2008; Alsio et al, 2013). Similarly, ZNF367 has been shown to modulate neuroblast proliferation and neuroectodermal differentiation, suggesting a role in M phase exit or in the spindle checkpoint control prior to anaphase (Naef et al, 2018). Many of these ZNFs/ZFPs exert their function through RNA binding. For instance, midlife crisis (Mdlc) is required for the maintenance of neuronal differentiation in *Drosophila*, a mechanism that is dependent on the CCCH zinc-finger domain and involves splicing (Carney et al, 2013). Another example is Unkempt, which modulates RNA trafficking to control early morphology of murine neurons (Murn et al, 2015). Here, we illustrate an additional ZNF/ZFP with a critical RNA-mediated function in neuroectoderm specification, suggesting that post-transcriptional regulation might be a common feature shared by a subgroup of ZNFs/ZFPs.

This study contributes to a general and better understanding of the coordinated control of gene expression mediated by ZNFs/ZFPs during neuronal differentiation. In addition, it sheds light on the function of ZFP207 and on our understanding of how co- and post-transcriptional programs ensure gene expression to maintain the ESC state. Given that ZFP207 binds both DNA and RNA, the relative contributions between transcriptional and post-transcriptional networks cannot be discriminated. Hence, we cannot exclude the possibility that ZFP207 may also regulate self-renewal transcriptionally, that is, by regulating the expression of splicing factors. Thus, ZFP207 provides an extra layer of control to fine-tune the balance between self-renewal and neuronal lineage commitment in mouse ESCs.

Materials and Methods

Constructs

cDNA of *Zfp207* isoform 3 from mouse ESCs was generated (Rev-erAid[®] First Strand cDNA Synthesis Kit) and cloned into pGEM[®]-T easy vector (Promega), and ultimately subcloned into the p3XFLAG-CMV-8 (Sigma-Aldrich) vector using *HindIII* and *BglII* as restriction enzymes. To generate the rescue construct, p3XFLAG-CMV-8-*Zfp207* was used to mutate the region of *Zfp207* targeted by shRNA2 by using the Quikchange Lightning multisite-directed mutagenesis kit (Agilent). DNA sequencing confirmed the single nucleotide mutations. The mutated *Zfp207* was then amplified with specific

primers harboring restrictions' sites extensions for *Esp3I* and *XhoI* and subcloned in the Lenti-iCas9 neo vector (Addgene 85400), which was digested with the same restriction enzymes, resulting in the loss of the iCas9 region. The resulting construct was thereafter referred to as tet(ON)-ZFP207. All primers used for cloning purposes are described in the Appendix Table S1.

Antibodies

The following commercially available antibodies were used at the indicated concentrations for western blot: Anti-ZFP207 (Santa Cruz Biotechnology, sc-271943, 1:500), Anti- β -ACTIN (Sigma-Aldrich, A5441, 1:2,500), Anti-OCT3/4 (Santa Cruz Biotechnology, sc8628, 1:2,500), Anti-Nanog (Santa Cruz Biotechnology, sc-374001, 1:1,000), Anti-Sox2 (Santa Cruz Biotechnology, sc-398254, 1:1,000), Anti-SFRS11 antibody (Abcam, ab196801, 1:2,000), Goat Anti-Rabbit IgG H&L (HRP) (Abcam, ab6721, 1:5,000), Goat Anti-Mouse IgG H&L (HRP) (Abcam, 1:1,000, ab6789), Rabbit Anti-Goat IgG H&L (HRP) (Abcam, 1:5,000, ab6741), and Rabbit Anti-goat IgG (HRP) (Abcam, ab6771, 1:5,000), Anti-Flag (Sigma, F3165, 1:1,000). In all western blots, β -ACTIN was used as a loading control (Sigma, A5441, 1:2,500). For IF staining, we used Anti-SSEA1 (Invitrogen, MA5-17042, 1:250), Anti-Nestin (Abcam, ab81462, 1:50), Anti-Tuj1 (Abcam, ab18207, 1:200), Anti-Caspase 3 Antibody, active (cleaved) form (MERK, AB3623, 1:100), Goat anti-mouse IgG AF488 (Invitrogen, A11029, 1:1,000), Goat anti-rabbit IgG AF568 (Invitrogen, A11011, 1:1,000), Anti-Mouse IgG HRP (Abcam, ab6789, 1:1,000), and Donkey Anti-Goat IgG AF594 (Invitrogen, A11058, 1:250).

Cell culture

CCE murine ESCs were cultured on 0.1% gelatin-coated tissue culture plates under feeder free culture conditions at 37°C with 5% CO₂ in a humidified incubator. The media composition consists of Dulbecco's modified Eagle's medium (DMEM) high glucose, 15% fetal bovine serum (FBS; Gibco), 1% MEM non-essential amino acids (Sigma-Aldrich), 0.1 mM of 2- β mercaptoethanol, 1% L-glutamine (Hyclone) and 1% penicillin/streptomycin (Gibco) and Leukemia inhibitory factor (LIF; R&D systems). ESCs in the 2iL medium were grown in a 1:1 mix of DMEM/F12 (Gibco) and Neurobasal medium (Gibco) with 1 $\times$ N2-Supplement (Gibco, Cat. 17502048), 1 $\times$ B27 minus insulin (Gibco, Cat. A1895601), 0.05% BSA (Gibco), Leukemia inhibitory factor (LIF; R&D systems), 2 mM Glutamine (Gibco), 1% penicillin/streptomycin (Gibco), 1 μ M PD03259010 (Mek Inhibitor; MedChem-Express), 3 μ M CHIR99021 (GSK3b Inhibitor; Stem cell technology), 1.4 $\times$ 10⁻⁴ M Monothioglycerol (Sigma) at a density of 1 $\times$ 10⁵ per well. The media was replaced every 48 h.

Lentiviruses production and generation of *Zfp207* KD mouse ESCs

Lentiviral particles were generated by transfecting HEK-293T with the following lentiviral plasmids: (i) pLKO.1-Puro containing shRNA1 and shRNA2 against *Zfp207* (Appendix Table S1); (ii) pLKO.1-Puro containing shRNA1, shRNA2 and shRNA3 against *Sfrs11* (Appendix Table S1); and (iii) tet(ON)-ZFP207 with the packaging vector pCMV-dR8.2 and the helper plasmid pCMV-VSV-G using jet-PEI (Polyplus) as per the manufacturer's instructions.

Lentiviral supernatants were collected after 48 h of incubation and concentrated using Amicon Ultra-15 Centrifugal Filter Units (Merck). Early passage mouse ESCs were transduced with the lentiviral particles in mouse ESC media supplemented with polybrene (8 μ g/ml) for 24 h. After 36 h, infected cells were treated with puromycin (2 μ g/ml) for additional 6 days.

Generation of ZFP207-inducible ESCs

Mouse ESCs were transduced with tet(ON)-ZFP207 with concentrated viral particles as indicated above. At 48 h upon transduction, cells were treated with Neomycin (250 μ g/ml) for 6 days to obtain a stable cell line. The knockdown of endogenous *Zfp207* was done using pLKO.1 shRNA2 as described above. Infected cells were selected by addition of puromycin (2 μ g/ml) into the culture media and simultaneously induced with doxycycline (250 ng/ml) during the entire experimental period.

Generation of CRISPR/Cas9 knockout ESCs

sgRNAs were designed using the E-CRISP online tool (<http://www.e-crisp.org/E-CRISP/aboutpage.html>) (Appendix Table S1) (Ran et al, 2013). All sgRNA-Cas9 plasmids were obtained by ligation (T7 DNA ligase, Fermentas) of annealed complementary oligonucleotides of the 20-nucleotides target sequences with the pSpCas9 (BB)-2A-Puro (PX459) vector (Addgene plasmid #62988) digested with BbsI (BpII) (Thermo Scientific). One day before transfection, mouse ESCs were seeded in a 12-well plate at a density of 80,000 cells/well. A day after that, cells were transfected using Lipofectamine (Invitrogen) with 0.8 μ g of Cas9 expression vector containing the corresponding sgRNAs. After 24 h, transfected cells were diluted and treated with puromycin to obtain isogenic cell clones. Isogenic cell clones were picked and expanded for 10–15 days to identify indels (insertion and deletion). KO were screened with PCR, Sanger sequencing and evaluated by western blot analysis.

EB and RA differentiation assays

EBs were obtained by growing mouse ESCs in low-attachment dishes in the presence of complete medium (DMEM high glucose, 15% FBS (Gibco), 1% MEM nonessential amino acids (Sigma-Aldrich), 0.1 mM of 2- β mercaptoethanol, 1% L-glutamine (Hyclone), and 1% penicillin/streptomycin (Gibco) without LIF at a density of 8.8 $\times$ 10⁴ cells/cm². Medium was replenished every 48 h. EBs were harvested for extraction of total RNA, whole cell extract, and nuclear protein extraction at the indicated time points. RA differentiation was performed plating mouse ESCs onto tissue culture dishes pre-coated with 0.1% gelatin at a density of 2.1 $\times$ 10³ cells/cm² in complete media (without LIF) supplemented with 5 μ M RA (Sigma-Aldrich) for 6 days. Differentiation media containing RA was replenished every 48 h.

Neural differentiation assay

Differentiation toward the neuroectodermal lineage was performed as previously described (Aguilo et al, 2017). Briefly, 5 $\times$ 10⁴ cells/ml were plated in low-attachment dishes in the presence of complete medium without LIF. Following EB formation for 2 days, EBs were

cultured in media containing DMEM/F-12, 0.1 mM of 2-mercaptoethanol, N2 supplement (100×) (Gibco, Cat. 17502048), B27 supplement (50×) (Gibco, cat. 17504044), and penicillin/streptomycin (Gibco) supplemented with 1 μ M of RA (Sigma-Aldrich) for 4 days to allow neuroectodermal differentiation. On day 4, EBs were transferred onto tissue culture dishes and precoated with 0.1% gelatin, and neuroectodermal differentiation media was replenished every 48 h. Differentiated cells were harvested for total RNA, nuclear protein extraction, and immunostaining analysis at the indicated time points. Cell cultures were maintained at 37°C with 5% CO₂ in a humidified incubator.

Generation of neural progenitor cells

Generation of NPCs was based on EB formation as described by Hanafiah *et al* (2020). Briefly, 1×10^5 cells were seeded per plate in low-attachment dishes in the presence of a complete medium without LIF for EB formation. After 4 days, the culture media was supplemented with 5 μ M of RA (Sigma-Aldrich) for 4 days. Cells were harvested for total RNA at different time points, and media was replenished every 48 h. Cell cultures were maintained at 37°C with 5% CO₂ in a humidified incubator.

Cellular proliferation, cell cycle, and apoptosis assays

Cellular proliferation, cell cycle, and apoptosis assays were carried out using a Muse Cell Analyzer (Millipore, Sigma-Aldrich) following the manufacturer's recommendations.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

TUNEL assay (in situ Cell Death Detection Kit, TMR red; Roche, Mannheim, Germany) for EBs and NE differentiation was performed as per manufacturer indication with slight modifications. Briefly, EBs were collected in tubes at days 4, 6, and 8 and fixed with 4% paraformaldehyde for 30 min and permeabilized with a solution containing 0.5% Triton-100 and 0.1% Tween-20 in 1× PBS for 1 h at RT. Thereafter, EBs were incubated in 50 μ l of TUNEL staining mix solution in a humidified chamber for 1 h at 37°C in darkness. Following incubation, EBs were washed with PBS and stained with DAPI (4',6-diamidino-2-phenylindole; Invitrogen) for 5 min at RT, washed again and mounted on slides. Whole mount EBs were analyzed using the Zeiss 710 Confocal Microscope. For NE, the same protocol was followed except that for day 5, cells were grown and fixed on cover slips. Images were acquired using a Nikon microscope (ZEN lite software) and analyzed using ImageJ. For quantification of TUNEL staining, images containing at least 20–30 nuclei were only considered. For EB, images were taken simultaneously in different focal planes. Then, the total fluorescence signal was measured using a threshold setting. Thereafter, data are represented as the ratio of TUNEL to nuclei signal. All the images were exposed with the same magnitude of gain.

BrdU incorporation assay

BrdU incorporation assay was conducted to monitor DNA replication of EBs at days 2, 4, 6, and 8 of differentiation using a FITC BrdU

Flow Kit following the manufacturer's protocol with some modifications. Briefly, the cells were incubated without or with 10 μ M of BrdU (Acros Organics) for 3 h in the differentiation media. After incubation, EBs were harvested by washing with 1× PBS and fixed with cold 70% ethanol at –20°C for 4 h. After washing twice with 1× PBS, EBs were denatured in 4 M of HCl for 20 min at RT. Thereafter, EBs were washed with phosphate/citrate acid buffer (pH 7.4) followed by filtering to remove any large clump. Then the cells were resuspended and washed in antibody stain solution (0.15 Triton X and 1% BSA in 1× PBS) twice, and resuspended in 100 μ l of antibody staining solution. The cells were stained with 5 μ l of anti-BrdU-FITC antibody (eBiosciences 11-5071-42) for 30 min at RT. BrdU incorporation data were collected in a ZE5 flow cytometer and analyzed using FlowJo Software.

Alkaline phosphatase staining

Alkaline phosphatase staining was measured using the Alkaline Phosphatase Detection kit (Stemgent) according to standard protocols from the manufacturer. Brightfield images were obtained using a Zeiss microscope and analyzed by ZEN lite software.

Immunocytochemistry analysis

Cells were fixed in 4% formaldehyde (Sigma-Aldrich) and permeabilized with 0.25% Triton X-100 (Sigma-Aldrich). Following permeabilization, cells were washed twice with PBS and blocked with 10% goat serum (Invitrogen) and bovine serum albumin (Hyclone) at RT for 1 h. Cells were then stained with primary antibodies overnight at 4°C at corresponding dilution as previously indicated, and secondary staining was performed using corresponding secondary antibodies. DAPI was used for DNA nuclear staining. Images were acquired using a Nikon microscope (ZEN lite software) and analyzed using ImageJ. The total fluorescence signal was measured using a threshold setting.

CHX treatment and proteasome inhibition

Mouse ESCs were seeded at a density of 1×10^5 cells/ml in 6-well plate and after 24 h; cells were treated with cycloheximide (CHX) at a final concentration of 50 μ g/ml for 0, 3, 6, and 9 h, respectively. For proteasome inhibition, cells were incubated with MG-132 at a final concentration of 10 μ g/ml for 0, 4, and 8 h, respectively. Cells were harvested for whole cell extracts at indicated time points and subjected to immunoblotting. Blots were quantified using Image Lab software.

RT-qPCR

The extraction of total RNA was performed using the RNeasy Plus Mini Kit (Qiagen). One microgram of total RNA was used for complementary DNA synthesis using RevertAid First-Strand cDNA Synthesis kit (Invitrogen) as per the manufacturer's instructions. RT-qPCR was performed in triplicate using the PowerUp SYBR Green Mix (Thermo Fisher Scientific) and the primers listed in Appendix Table S1 on a QuantStudio Real-time PCR instrument (Applied Biosystems). β -Actin levels were used to normalize input amounts. After each round of amplification, melting curve analysis

was carried out in order to confirm the PCR product specificity. Ct values obtained for each gene were analyzed using the $\Delta\Delta C_t$ method from at least three biological replicates.

RNA-Seq and differential gene expression analysis

RNA-seq library preparation was carried out at Novogene facilities (<https://en.novogene.com/>) and sequenced using Illumina HiSeq 2500 platform (Illumina) as 150-bp pair-ended reads. FASTQ reads were aligned to the ENSEMBL mouse transcriptome using BWA-MEM. Then, the calculation of normalized reads per transcript was retrieved by Express (Roberts & Pachter, 2013). Thereafter, DEG was obtained by using a test under the assumption of a negative binomial distribution where the variance is linked to the mean via a locally regressed smooth function of the mean (Anders & Huber, 2010) and *P*-values were adjusted by estimation of the false discovery rate for multiple hypothesis. We only considered the genes with TPM > 0 in at least 2 of the samples. FDR value was calculated with the Benjamini–Hochberg correction.

AS and data analysis

SUPPA2 was used as a pipeline for AS analysis (Patro *et al.*, 2017). Briefly, FASTQ reads from RNA-seq experiment were aligned and pseudo quantified to mouse genome (mm10) using Salmon (Trinacado *et al.*, 2018). Splicing events in the mouse genome were obtained using a specific SUPPA2 script from a mouse GTF file. Thereafter, percent splicing inclusion (PSI) values for each event were obtained, and the differential PSI values for each condition were calculated along with a *P*-value for each event.

RT-PCR

Total RNA was extracted using the RNeasy Mini Kit (Qiagen). Two micrograms of total RNA were reverse transcribed using the RevertAid First Strand cDNA Synthesis kit (Invitrogen). RT-PCR was performed using the DreamTaq Green Master mix (Thermo Fisher Scientific). The specific primers used for AS are listed in Appendix Table S1. The ratios between splice variants were determined by densitometry using ImageJ software. The percentage of inclusion was calculated by dividing the area of the inclusion band by the combined area of both inclusion and exclusion bands and multiplied by 100.

Gene Ontology (GO) analysis

Gene ontology (GO) analysis was performed using the web tool The Database for Annotation, Visualization and Integrated Discovery (DAVID) (<http://david.abcc.ncifcrf.gov/>).

Heat maps

Heat maps were made using Z-score, which was calculated as following: $Z = (X - X_{av}) / X_{sd}$, where *X* is the log₂-transformed expression level for a given gene in a specific sample, *X_{av}* is the mean of log₂-transformed expression values for that gene in all samples, and *X_{sd}* is the standard deviation of log₂-transformed expression values for that gene across all samples.

In vitro RNA immunoprecipitation (RIP) and data analysis

cDNA of *Zfp207* isoform 3 from mouse ESCs was generated (RevertAid® First Strand cDNA Synthesis Kit) and cloned into a HaloTag backbone as a ZFP207-Halo fusion (primers are described in Appendix Table S1). *Zfp207*-Halo plasmid was linearized, *in vitro* transcribed (MegaScript T7 Transcription kit, Thermo Fisher #AM1333) and *in vitro* translated (Wheat Germ *in vitro* translation kit, Promega #L4380). The *in vitro*-translated ZFP207-Halo fusion product was incubated with total RNA from mouse ESCs. Prior to pull-down mouse ESCs, total RNA was chemically fragmented at 95°C for 4 min in 100 mM of Tris-HCl and 10 mM of MgCl₂ resulting in a population of ~50 nt long RNA fragments that can be bound by ZFP207 and cloned subsequently using a small RNA library preparation protocol. After this binding reaction, the RBP–RNA complex was purified and the bound RNA eluted for subsequent Illumina library preparation (NEXTFLEX small RNA library preparation kit v3, PerkinElmer #NOVA-5132-06) and paired-end (40 × 43 cycles) sequenced on an Illumina NextSeq 500. Sequencing reads were trimmed, adapters and ribosomal RNA reads removed and aligned against the reference genome GRCm38.p6 retrieved from GENCODE (<https://www.gencodegenes.org/mouse/>) using HISAT2 v2.1.0 with default parameters. Only uniquely mapping reads were further considered. PCR duplicate reads were removed. The libraries of the two replicates, HaloTag and input were read count normalized. The input RNA size was 50 nt in length. Under this consideration, we expect that fragments bound by ZFP207 will result in an enrichment of alignable reads with respect to HaloTag and Input control libraries. This peak enrichment is an indication of binding events and binding sites. Binding events (peaks) were called by using MACS2 v2.2.6 (<https://github.com/macs3-project/MACS/wiki/Advanced:-Call-peaks-using-MACS2-subcommands>). Genomic regions enriched over Halo and Input signals are kept and further filtered at a FDR ≤ 0.01, a fold change (FC) enrichment FC ≥ 3 (Halo) and FC ≥ 2 (Input) and RPM ≥ 2 in both replicates. Only consensus peaks between the two replicates were considered further. Genomic features (R Genomic Features package (v1.36.4) and R Genomic Ranges package (v1.36.1)) were used to annotate the peaks, and pathway enrichment was conducted using R ClusterProfiler (v3.12.0) to compute the significance of the enrichments and org.Mm.db (v3.8.2) to retrieve Entrez IDs required by ClusterProfiler using the entire murine transcriptome as a background model. Each peak has a reported Binding Score computed as the base 2 logarithm of the product between the log₂RPM and the mean enrichment over the two negative controls (HaloTag and Input). For GO, each term was ranked by the sum of all binding scores of all peaks present in this pathway. Circle diameter represents the number of genes bound by ZFP207 in that specific pathway.

In vivo RNA immunoprecipitation (RIP)

Mouse ESCs were transfected with 15 µg of pCMV-3XFLAG ZFP207 or empty pCMV-3XFLAG using Lipofectamine LTX reagent with Plus reagent (Invitrogen). After 48 h, cells were harvested and lysed with 1× NP lysis buffer (50 mM of Tris-HCl pH 7.5, 100 mM of NaCl, 0.5% (v/v) NP40, 2 mM of EDTA, 1× Protease inhibitor cocktail (Thermo), and 1× diluted Ribolock RNase inhibitor (Thermo)) with gentle rotation for 30 min at 4°C. The supernatant was separated

after centrifugation at 12,000 g for 15 min at 4°C. The lysate was precleared with protein A agarose magnetic beads (Pierce) for 30 min at RT with gentle rotation. Thereafter, the lysate was incubated with FLAG antibodies in a ratio of 1 µg of FLAG antibodies to 2 mg of lysate for 1 h at RT with gentle rotation, and subsequently immunoprecipitated with protein A beads for 3 h at 4°C. Beads were washed three times with IP wash buffer (50 mM of Tris-HCl pH 7.5, 300 mM of NaCl, 0.05% (v/v) NP40), three times with high salt wash buffer (50 mM of HEPES-KOH pH 7.5, 500 mM of NaCl) and twice with PNK buffer (50 mM of Tris-HCl pH 7.5, 50 mM of NaCl, 10 mM of MgCl₂). All washes were carried out at 4°C with gentle rotation for 5 min. One-tenth of the samples were analyzed by SDS-PAGE in order to check IP efficiency. For RT-qPCR analysis, proteins were removed by proteinase K digestion, RNA was extracted using Trizol, and RNA was reverse-transcribed with the SuperScript VILO cDNA synthesis kit (Thermo). RT-qPCR analysis of the cDNA was performed with specific primers as indicated in Appendix Table S1.

Subcellular RNA fractionation

Two million cells were harvested and washed twice with PBS. After centrifugation for 10 min at 500 g, the pellets were resuspended in 2 packed volumes of cytoplasmic lysis buffer (10 mM of Tris-HCl pH 7.5, 0.15% NP-40, 150 mM of NaCl supplemented with proteinase and RNase inhibitors) and mixed well by pipetting up and down 15 times. The mix was then incubated on ice for 10 min and 1.5 times volumes of chilled sucrose buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 24% sucrose) were added to the lysate. The lysate was then centrifuged at 4°C, 9,500 g for 3 min, and the supernatant was collected as cytoplasmic fraction. The nuclear pellet was resuspended in 2 packed volumes of cytoplasmic lysis buffer without NP-40 and mixed with 1.5 times volumes of chilled sucrose buffer followed by centrifugation at 9,500 g 5 min at 4°C. The washed nuclear fraction was resuspended with chilled glycerol buffer (20 mM of Tris-HCl pH 7.9, 75 mM of NaCl, 0.5 mM EDTA, and 50% glycerol) followed by addition of equal volume of cold nuclei lysis buffer (10 mM of HEPES pH 7.6, 1 mM of DTT, 7.5 mM of MgCl₂, 0.2 mM of EDTA, 0.3 M of NaCl, 1 M of Urea, 1% NP-40 supplemented with proteinase and RNase inhibitor). The mixture was vortexed twice for 5 s each and incubated on ice for 2 min followed by centrifugation at high speed for 5 min. The supernatant was collected as a nuclear fraction. Total RNA from cytosolic and nuclear fraction was extracted using the RNeasy Mini Kit (Qiagen).

RNA FISH

Cells were grown on microscope coverslips placed in tissue culture dishes. After washing twice with PBS, cells were fixed in 4% paraformaldehyde (Sigma-Aldrich) and permeabilized with 70% Ethanol for 2 h at 4°C. Following permeabilization, cells were washed twice with 25% of RNA wash buffer (25% formamide, 2× SSC) for 5 min at RT. Cells were stained with hybridization mix (10% dextran sulfate, 25% formamide, 2 × SSC, 1 mg/ml *E. coli* tRNA, 0.02% BSA, 10 mM vanadyl-ribonucleoside complex) and 2 µM 5'Cys3-oligo dT(50) probe for 24 h at 30°C. The following day, coverslips were washed with 25% of RNA wash buffer for

60 min at 30°C and incubated with 20 ng/ml DAPI (RNase-free) in 25% RNA wash 30 min at 30°C. The coverslips were then rinsed with 2 × SSC buffer followed by RNA equilibration buffer (10 mM of Tris-HCl pH 7.5, 2 × SSC and 0.4% glucose). Thereafter, coverslips were mounted using an anti-bleach buffer (10 mM trolox, 37 ng/µl glucose oxidase, catalase in RNA equilibration buffer). Images were acquired using a Nikon microscope (ZEN lite software) and analyzed using ImageJ. For quantifying nuclear poly(A)⁺ RNA signals (N), images containing at least 30 nuclei were only considered. DAPI staining was masked and overlaid on top of the 5'Cys3-oligo d(T) signal, after that intensity of pixel was measured using analyze particles. Then, the total FISH signal was measured as fluorescence using a threshold setting. And the cytosolic signal was measured as subtracting the nuclear signal from the total signal. All the images were exposed to the same magnitude of gain.

Statistical analysis

Data are shown as mean ± SEM. GraphPad Prism version 8.0.0 for Windows was used to perform the statistical analysis (GraphPad Software, La Jolla California USA, www.graphpad.com). The significance was determined using the student's *t*-test, the ordinary one-way ANOVA, two-way ANOVA and ratio paired *t*-test where indicated including correction for multiple comparison. Probability values of **P* < 0.05, ***P* < 0.01, ****P* < 0.001 *****P* < 0.0001 were considered as statistically significant.

Data availability

All next-generation sequencing data can be publicly accessed in ArrayExpress webserver (E-MTAB-10108, <http://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-10108/> and E-MTAB-10113, <http://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-10113/>).

Expanded View for this article is available online.

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Author contributions

FA conceived and designed the study. SM, DPB, PG, DM-S, and CM-G performed experiments, IA and CK performed *in vitro* RIP analysis, A-CR, DZ, and D-FL performed bioinformatics analysis. FA wrote the manuscript. All authors reviewed and edited the manuscript.

Disclosure statement and competing interests

The authors declare that they have no conflict of interest.

References

- Aguilo F, Zakirova Z, Nolan K, Wagner R, Sharma R, Hogan M, Wei C, Sun Y, Walsh MJ, Kelley K *et al* (2017) THAP1: role in mouse embryonic stem cell survival and differentiation. *Stem Cell Rep* 9: 92–107
- Aguilo F, Zhang F, Sancho A, Fidalgo M, Di Cecilia S, Vashisht A, Lee D-F, Chen C-H, Rengasamy M, Andino B *et al* (2015) Coordination of m(6)A mRNA methylation and gene transcription by ZFP217 regulates pluripotency and reprogramming. *Cell Stem Cell* 17: 689–704
- Alamancos GP, Pages A, Trincado JL, Bellora N, Eyraes E (2015) Leveraging transcript quantification for fast computation of alternative splicing profiles. *RNA* 21: 1521–1531
- Alekseev OM, Richardson RT, Tsuruta JK, O'Rand MG (2011) Depletion of the histone chaperone tNASP inhibits proliferation and induces apoptosis in prostate cancer PC-3 cells. *Reprod Biol Endocrinol* 9: 50
- Al-Naama N, Mackeh R, Kino T (2020) C2H2-type zinc finger proteins in brain development, neurodevelopmental, and other neuropsychiatric disorders: systematic literature-based analysis. *Front Neurol* 11: 32
- Alsio JM, Tarchini B, Cayouette M, Livesey FJ (2013) Ikaros promotes early-born neuronal fates in the cerebral cortex. *Proc Natl Acad Sci USA* 110: E716–725
- Anders S, Huber W (2010) Differential expression analysis for sequence count data. *Genome Biol* 11: R106
- Baltz AG, Munschauer M, Schwanhäusser B, Vasile A, Murakawa Y, Schueler M, Youngs N, Penfold-Brown D, Drew K, Milek M *et al* (2012) The mRNA-bound proteome and its global occupancy profile on protein-coding transcripts. *Mol Cell* 46: 674–690
- Barbosa-Morais NL, Irimia M, Pan Q, Xiong HY, Gueroussov S, Lee LJ, Slobodeniuc V, Kutter C, Çolak R *et al* (2012) The evolutionary landscape of alternative splicing in vertebrate species. *Science* 338: 1587–1593
- Blomen VA, Májek P, Jae LT, Bigenzahn JW, Nieuwenhuis J, Staring J, Sacco R, van Diemen FR, Olk N, Stukalov A *et al* (2015) Gene essentiality and synthetic lethality in haploid human cells. *Science* 350: 1092–1096
- Bradley A, Evans M, Kaufman MH, Robertson E (1984) Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines. *Nature* 309: 255–256
- Brannan KW, Jin W, Huelga SC, Banks CAS, Gilmore JM, Florens L, Washburn MP, Van Nostrand EL, Pratt GA, Schwinck MK *et al* (2016) SONAR discovers RNA-binding proteins from analysis of large-scale protein-protein interactomes. *Mol Cell* 64: 282–293
- Carney TD, Struck AJ, Doe CQ (2013) midlife crisis encodes a conserved zinc-finger protein required to maintain neuronal differentiation in *Drosophila*. *Development* 140: 4155–4164
- Cassandri M, Smirnov A, Novelli F, Pitolli C, Agostini M, Malewicz M, Melino G, Raschella G (2017) Zinc-finger proteins in health and disease. *Cell Death Discov* 3: 17071
- Castello A, Fischer B, Eichelbaum K, Horos R, Beckmann BM, Strein C, Davey NE, Humphreys DT, Preiss T, Steinmetz LM *et al* (2012) Insights into RNA biology from an atlas of mammalian mRNA-binding proteins. *Cell* 149: 1393–1406
- Dai XX, Xiong H, Zhang M, Sun S, Xiong B (2016) Zfp207 is a Bub3 binding protein regulating meiotic chromosome alignment in mouse oocytes. *Oncotarget* 7: 30155–30165
- Fan J, Kuai B, Wang K, Wang L, Wang Y, Wu X, Chi B, Li G, Cheng H (2018) mRNAs are sorted for export or degradation before passing through nuclear speckles. *Nucleic Acids Res* 46: 8404–8416
- Fang F, Xia N, Angulo B, Carey J, Cady Z, Durruthy-Durruthy J, Bennett T, Sebastiano V, Reijo Pera RA (2018) A distinct isoform of ZNF207 controls self-renewal and pluripotency of human embryonic stem cells. *Nat Commun* 9: 4384
- Finley LWS, Vardhana SA, Carey BW, Alonso-Curbelo D, Koche R, Chen Y, Wen D, King B, Radler MR, Rafii S *et al* (2018) Pluripotency transcription factors and Tet1/2 maintain Brd4-independent stem cell identity. *Nat Cell Biol* 20: 565–574
- Gabut M, Samavarchi-Tehrani P, Wang X, Slobodeniuc V, O'Hanlon D, Sung H-K, Alvarez M, Talukder S, Pan Q, Mazzoni EO *et al* (2011) An alternative splicing switch regulates embryonic stem cell pluripotency and reprogramming. *Cell* 147: 132–146
- Ginis I, Luo Y, Miura T, Thies S, Brandenberger R, Gerecht-Nir S, Amit M, Hoke A, Carpenter MK, Itskovitz-Eldor J *et al* (2004) Differences between human and mouse embryonic stem cells. *Dev Biol* 269: 360–380
- Hall TM (2005) Multiple modes of RNA recognition by zinc finger proteins. *Curr Opin Struct Biol* 15: 367–373
- Han H, Irimia M, Ross PJ, Sung H-K, Alipanahi B, David L, Golipour A, Gabut M, Michael IP, Nachman EN *et al* (2013) MBNL proteins repress ES-cell-specific alternative splicing and reprogramming. *Nature* 498: 241–245
- Hanafiah A, Geng Z, Wang Q, Gao Z (2020) differentiation and characterization of neural progenitors and neurons from mouse embryonic stem cells. *J vis Exp* 159: e61446
- Inoue T, Ogawa M, Mikoshiba K, Aruga J (2008) Zic deficiency in the cortical marginal zone and meninges results in cortical lamination defects resembling those in type II lissencephaly. *J Neurosci* 28: 4712–4725
- Jiang H, He X, Wang S, Jia J, Wan Y, Wang Y, Zeng R, Yates 3rd J, Zhu X, Zheng Y (2014) A microtubule-associated zinc finger protein, BuGZ, regulates mitotic chromosome alignment by ensuring Bub3 stability and kinetochore targeting. *Dev Cell* 28: 268–281
- Jiang H, Wang S, Huang Y, He X, Cui H, Zhu X, Zheng Y (2015) Phase transition of spindle-associated protein regulate spindle apparatus assembly. *Cell* 163: 108–122
- Kim J, Woo AJ, Chu J, Snow JW, Fujiwara Y, Kim CG, Cantor AB, Orkin SH (2010) A Myc network accounts for similarities between embryonic stem and cancer cell transcription programs. *Cell* 143: 313–324
- Krstic M, Hassan HM, Kolendowski B, Hague MN, Anborgh PH, Postenka CO, Torchia J, Chambers AF, Tuck AB (2020) Isoform-specific promotion of breast cancer tumorigenicity by TBX3 involves induction of angiogenesis. *Lab Invest* 100: 400–413
- Lee DF, Walsh MJ, Aguilo F (2016) ZNF217/ZFP217 Meets Chromatin and RNA. *Trends Biochem Sci* 41: 986–988
- Martello G, Smith A (2014) The nature of embryonic stem cells. *Annu Rev Cell Dev Biol* 30: 647–675
- Murn J, Zarnack K, Yang YJ, Durak O, Murphy EA, Cheloufi S, Gonzalez DM, Teplova M, Turk T, Zuber J *et al* (2015) Control of a neuronal morphology program by an RNA-binding zinc finger protein, unkempt. *Genes Dev* 29: 501–512
- Naef V, Monticelli S, Corsinovi D, Mazzetto MT, Cellerino A, Ori M (2018) The age-regulated zinc finger factor ZNF367 is a new modulator of neuroblast proliferation during embryonic neurogenesis. *Sci Rep* 8: 11836
- Nichols J, Smith A (2009) Naive and primed pluripotent states. *Cell Stem Cell* 4: 487–492

- Patro R, Duggal G, Love MI, Irizarry RA, Kingsford C (2017) Salmon provides fast and bias-aware quantification of transcript expression. *Nat Methods* 14: 417–419
- Ran FA, Hsu PD, Wright J, Agarwala V, Scott DA, Zhang F (2013) Genome engineering using the CRISPR-Cas9 system. *Nat Protoc* 8: 2281–2308
- Ravanidis S, Kattan FG, Doxakis E (2018) Unraveling the pathways to neuronal homeostasis and disease: mechanistic insights into the role of RNA-binding proteins and associated factors. *Int J Mol Sci* 19: 2280
- Razin SV, Borunova VV, Maksimenko OG, Kantidze OL (2012) Cys2His2 zinc finger protein family: classification, functions, and major members. *Biochemistry* 77: 217–226
- Roberts A, Pachter L (2013) Streaming fragment assignment for real-time analysis of sequencing experiments. *Nat Methods* 10: 71–73
- Toh C-XD, Chan J-W, Chong Z-S, Wang HF, Guo HC, Satapathy S, Ma D, Goh GYL, Khattar E, Yang L *et al* (2016) RNAi reveals phase-specific global regulators of human somatic cell reprogramming. *Cell Rep* 15: 2597–2607
- Toledo CM, Herman JA, Olsen JB, Ding Y, Corrin P, Girard EJ, Olson JM, Emili A, DeLuca JG, Paddison PJ (2014) BuGZ is required for Bub3 stability, Bub1 kinetochore function, and chromosome alignment. *Dev Cell* 28: 282–294
- Trincado JL, Entizne JC, Hysenaj G, Singh B, Skalic M, Elliott DJ, Eyraes E (2018) SUPPA2: fast, accurate, and uncertainty-aware differential splicing analysis across multiple conditions. *Genome Biol* 19: 40
- Wan Y, Zheng X, Chen H, Guo Y, Jiang H, He X, Zhu X, Zheng Y (2015) Splicing function of mitotic regulators links R-loop-mediated DNA damage to tumor cell killing. *J Cell Biol* 209: 235–246
- Xia W, Su L, Jiao J (2018) Cold-induced protein RBM3 orchestrates neurogenesis via modulating Yap mRNA stability in cold stress. *J Cell Biol* 217: 3464–3479
- Xu H, Wang W, Li C, Yu H, Yang A, Wang B, Jin Y (2009) WWP2 promotes degradation of transcription factor OCT4 in human embryonic stem cells. *Cell Res* 19: 561–573
- Yan J, Goerne T, Zelmer A, Guzman R, Kapfhammer JP, Wellmann S, Zhu X (2019) The RNA-binding protein RBM3 promotes neural stem cell (NSC) proliferation under hypoxia. *Front Cell Dev Biol* 7: 288
- Ye J, Belloch R (2014) Regulation of pluripotency by RNA binding proteins. *Cell Stem Cell* 15: 271–280



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RESEARCH ARTICLE

Patient-derived iPSCs link elevated mitochondrial respiratory complex I function to osteosarcoma in Rothmund-Thomson syndrome

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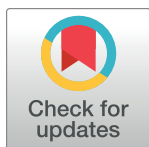
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Abstract

Rothmund-Thomson syndrome (RTS) is an autosomal recessive genetic disorder characterized by poikiloderma, small stature, skeletal anomalies, sparse brows/lashes, cataracts, and predisposition to cancer. Type 2 RTS patients with biallelic *RECQL4* pathogenic variants have multiple skeletal anomalies and a significantly increased incidence of osteosarcoma. Here, we generated RTS patient-derived induced pluripotent stem cells (iPSCs) to dissect the pathological signaling leading to RTS patient-associated osteosarcoma. RTS iPSC-derived osteoblasts showed defective osteogenic differentiation and gain of *in vitro* tumorigenic ability. Transcriptome analysis of RTS osteoblasts validated decreased bone morphogenesis while revealing aberrantly upregulated mitochondrial respiratory complex I gene expression. RTS osteoblast metabolic assays demonstrated elevated mitochondrial



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respiratory complex I function, increased oxidative phosphorylation (OXPHOS), and increased ATP production. Inhibition of mitochondrial respiratory complex I activity by IACS-010759 selectively suppressed cellular respiration and cell proliferation of RTS osteoblasts. Furthermore, systems analysis of IACS-010759-induced changes in RTS osteoblasts revealed that chemical inhibition of mitochondrial respiratory complex I impaired cell proliferation, induced senescence, and decreased MAPK signaling and cell cycle associated genes, but increased H19 and ribosomal protein genes. In summary, our study suggests that mitochondrial respiratory complex I is a potential therapeutic target for RTS-associated osteosarcoma and provides future insights for clinical treatment strategies.

Author summary

Rothmund-Thomson syndrome (RTS) is an autosomal recessive genetic disease characterized by an array of clinical phenotypes affecting multiple tissues. Type 2 RTS is caused by pathogenic variants in the *RECQL4* gene encoding the RECQL4 DNA helicase. Type 2 RTS patients are prone to developing multiple primary osteosarcomas and have limited chemotherapy options due to organ toxicities or lifetime limits on active agents such as anthracyclines. There is currently no available RTS model that recapitulates the bone malignancy phenotype in this disease, severely limiting the ability to explore new treatment avenues which are greatly needed for these patients. To overcome this problem, we established a Type 2 RTS disease model using a human induced pluripotent stem cell platform. We then applied an unbiased approach to explore novel molecular mechanisms involved in *RECQL4* mutation-induced osteosarcoma to explore therapeutic interventions. Our findings indicate that mitochondrial respiratory complex I is an “Achilles’ heel” of RTS osteosarcoma and that cancers harboring *RECQL4* mutations/deletions, in general, may be vulnerable to mitochondrial respiratory complex I inhibition.

Introduction

Rothmund-Thomson syndrome (RTS) is a rare autosomal recessive disorder first described by Dr. Auguste Rothmund in 1868 in his patients who had the characteristic poikilodermatous skin rash and bilateral juvenile cataracts. In 1921 Dr. Sydney Thomson reported a similar condition but in association with radial ray defects [1–3]. The clinical phenotype of RTS was subsequently further delineated to include other features including growth retardation, hair, nail, and skeletal abnormalities, and a predisposition to cancer, specifically osteosarcoma [1–3]. Pathogenic variants in the *ANAPC1* and *RECQL4* genes have been identified in Type 1 (OMIM #618625) and Type 2 (OMIM #268400) RTS patients, respectively [4,5]. *RECQL4* pathogenic variants associated with RTS include nonsense, frameshift, splicing, and intronic deletions, all of which impair RECQL4 cellular function. In contrast to Type 1 RTS patients, who have no elevation in risk of osteosarcoma, Type 2 RTS patients with biallelic *RECQL4* pathogenic variants have a significantly elevated lifetime risk of osteosarcoma. While the clinicopathological features of RTS-associated osteosarcomas are similar to osteosarcomas arising in the general population [6], they occur at an earlier age, and RTS patients are at risk of developing multiple primary tumors.

One hallmark feature of cancer cells is their ability to metabolically reprogram mitochondrial metabolism to support continued growth [7]. The mitochondria are central cellular

organelles responsible for metabolizing carbohydrates, lipids, and amino acids and for generating over 90% of cellular ATP and metabolic intermediates such as FADH₂, NADH, and NADPH for the electron transport chain (ETC) and oxidative phosphorylation (OXPHOS). Cancer cells may facilitate growth by reprogramming their energy metabolism to favor several pathways, including acquisition of aerobic glycolysis (Warburg effect), increase in fatty acid oxidation, enhancement of glutamine and amino acid metabolism, and activation of OXPHOS [8,9]. Although the majority of cancer cells preferentially employ aerobic glycolysis to meet anabolic demands, some cancer cells rely uniquely on OXPHOS to support cell proliferation and survival [10]. In addition to promoting tumor proliferation, OXPHOS also drives tumor migration, metastasis, and immortalization [11,12]. These findings indicate the potential of therapeutically targeting OXPHOS-dependent tumors via their mitochondrial functions.

Although immortalized cancer cell lines, primary patient samples and cultured cells, and small organism models (e.g., fruit fly, zebrafish, and mouse) have made innumerable contributions to our understanding of the molecular mechanisms of cancer initiation, progression, metastasis, and recurrence, the complexity of the cancer genome and differences among species frequently limit clinical translation of many preclinical findings. Patient-derived induced pluripotent stem cells (iPSCs) provide unparalleled advantages as a model system, allowing investigators to study a cell continuously from the moment it differentiates from a multipotent progenitor into a differentiated cell type of interest [13,14]. Cancer disease modeling using patient-derived iPSCs has been employed successfully to study malignancies driven by a defined genetic background [15,16], including osteosarcoma in Li-Fraumeni syndrome (LFS) patients [17–20], leukemia in myelodysplastic syndrome (MDS) and Noonan syndrome patients [21,22], and colorectal cancers in familial adenomatous polyposis (FAP) patients [23]. These findings illustrate the feasibility of using patient-derived iPSCs as a platform to elucidate the pathological mechanisms involved in tumor initiation and development.

In this study, we established RTS iPSCs to analyze the transcriptomic alterations in RTS osteoblasts and to determine the pathological signature involved in RTS-associated osteosarcoma. We then applied these findings through multiple *in vitro* assays to identify a treatment strategy with promising activity against *RECQL4*-mutated osteoblasts. Our study has the potential to offer a paradigm shift in the treatment of osteosarcoma in RTS patients and *RECQL4*-mutated osteosarcoma. Suppression of mitochondrial respiratory complex I by OXPHOS inhibitor may offer effective salvage therapies, which currently do not exist for RTS patients as well as for recurrent sporadic osteosarcoma patients with somatic *RECQL4* mutations.

Results

Generation and characterization of iPSCs from RTS patients and unaffected parents

To gain insight into RTS-associated bone malignancies, we employed a patient-derived iPSC disease platform constructed from a cohort of four individuals from two RTS families, each with one affected RTS proband and one unaffected parent. Both RTS probands (labeled as RTS-A and RTS-B) had biallelic *RECQL4* pathogenic variants (Fig 1A), while parents (labeled as Family control A (FC-A) and Family control B (FC-B)) each had a *RECQL4* variant on one allele. Both RTS probands presented with classic Type 2 RTS features, including poikiloderma, radial ray defects, growth retardation, multiple skeletal and dental abnormalities (see Fig 1B for photos and Table 1 for clinical features), and both developed osteosarcomas. The *RECQL4* pathogenic variants in RTS patients were confirmed by Sanger sequencing (Fig 1C). Immunoblotting indicated the loss of full-length *RECQL4* protein in both RTS-A and RTS-B cells, suggesting impairment of *RECQL4* function in these RTS patients (Fig 1D).

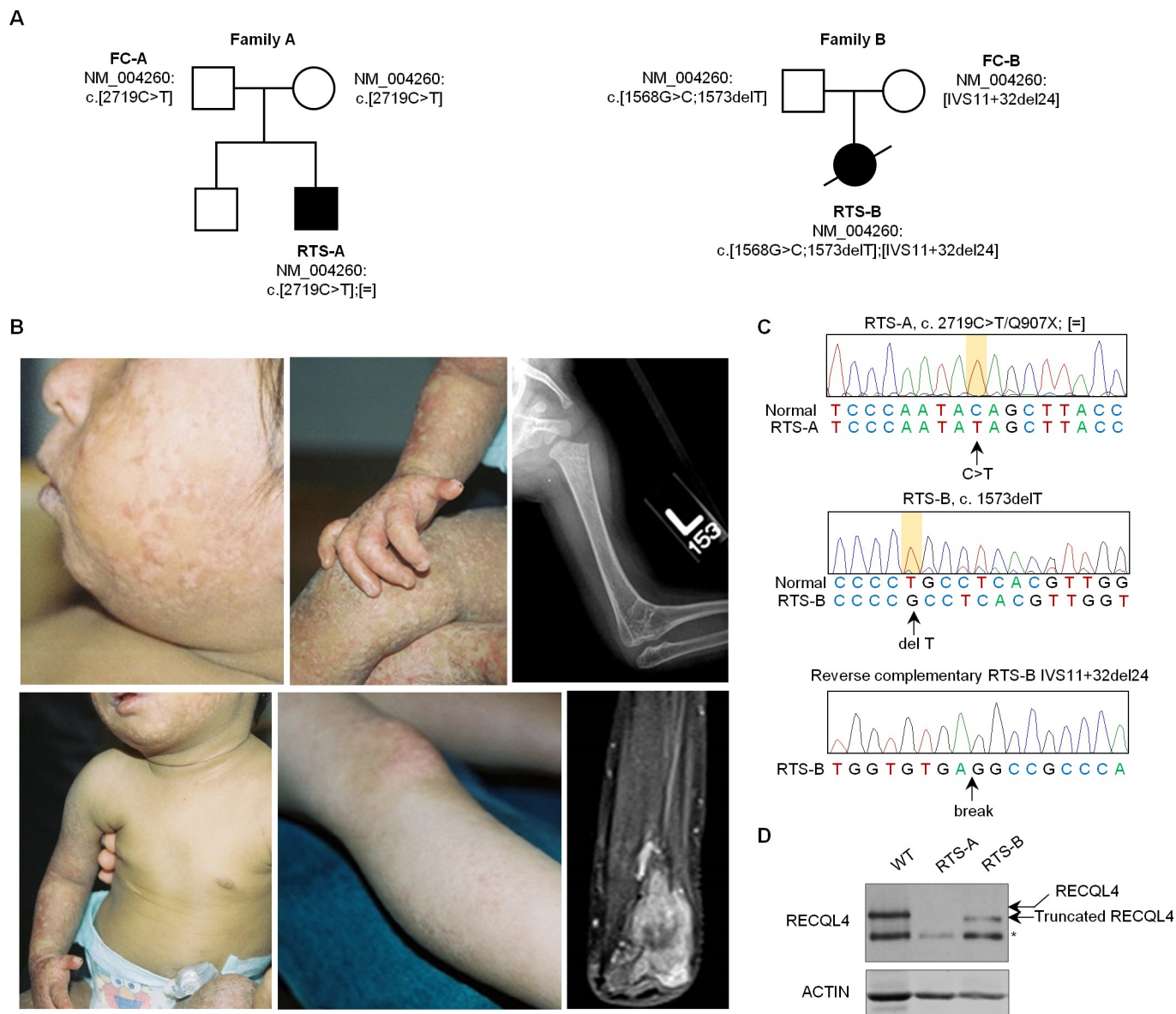


Fig 1. Clinical characterization of Type 2 Rothmund-Thomson Syndrome patients in this study. (A) Pedigrees of the two families with RTS Type 2 and *RECQL4* pathogenic variants reported in this study. (B) Photos show the classic poikiloderma on the face, ears, and arms of individual RTS-A at age 2 years (left panels, top and bottom). Note the sparing of the trunk and abdomen as well as sparse scalp hair and absence of eyebrows and presence of gastrostomy feeding tube. Middle panels illustrate variability in the severity of poikiloderma on the lower extremities in RTS-A (upper) and RTS-B (lower). Note hypoplastic thumb and dystrophic nails in RTS-A. Both individuals developed osteosarcoma. The right top panel shows an x-ray of the skeletal defect (radiohumeral synostosis, age 2 years) in RTS-A, and the bottom panel shows an MRI image of osteosarcoma that developed in the right proximal radius of RTS-A at age 10 years. This T1 fat-saturated, coronal, post-contrast view demonstrates diffuse solid enhancement of the tumor with some central areas of non-enhancement. (C) Sanger sequencing verifies biallelic *RECQL4* (c. 2719C>T/Q907X) pathogenic variant in RTS-A fibroblasts (upper panel) and *RECQL4* (1568G>C;1573delT and VS11+32del24) in RTS-B fibroblasts (middle and lower panels). (D) Immunoblotting indicates the loss of RECQL4 protein in RTS-A fibroblasts and the truncated RECQL4 protein in RTS-B fibroblasts. *, non-specific band.

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Fibroblasts from RTS individuals and their parents were transduced with four Yamanaka factors (NANOG, OCT4, SOX2, and MYC) [24,25] using a Sendai virus (SeV)-based cell reprogramming method. iPSC clones were successfully isolated and expanded from fibroblasts, leading to generation of iPSC clones from two affected RTS probands (RTS-A and RTS-B) and two parents (FC-A and FC-B). The iPSCs exhibited hESC clonal morphology and

Table 1. Clinical characteristics of RTS Type 2 individuals with RECQL4 mutations.

	RTS-A	RTS-B
Gender	M	F
Skin	Poikiloderma, hyperkeratosis	Poikiloderma
Hair	Sparse scalp hair, absent eyebrows, eyelashes	Sparse scalp hair, absent eyebrows, eyelashes
Eyes	Astigmatism, epiphoria	Astigmatism, epicanthal folds
ENT	Recurrent OM, mild hearing loss, mild nasal and choanal stenosis	Normal
Teeth	Dental crowding	Dental caries, erosion, enamel defects
Nails	Dystrophic nails	Normal
Skeletal	Bilateral thumb and digit hypoplasia, left radiohumeral synostosis, metaphyseal trabeculation defects; osteopenia	Bilateral thumb and digit hypoplasia, left radius hypoplasia, limited elbow extension
Growth and Development	Small stature, speech and motor delay	Small stature, delayed milestones
GI	History of vomiting diarrhea, malabsorption, feeding tube	History of severe vomiting as infant
Cancer	Osteosarcoma, diagnosed at 10 years	Osteosarcoma, diagnosed at 11 years

Abbreviations: M-male; F-female; ENT-Ear, Nose Throat; OM-otitis media; GI-Gastrointestinal

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ubiquitously expressed the pluripotency transcription factors NANOG and OCT4 as well as human embryonic stem cell (hESC) surface antigens TRA-1-81 and SSEA4 (Fig 2A). Quantitative reverse transcription PCR (qRT-PCR) confirmed comparable expression of NANOG, OCT4, and SOX2 mRNA transcripts in iPSC lines compared to hESC H1 cells (Fig 2B). Teratoma formation assay was performed to test the ability of these cell lines to differentiate into all three germ layers *in vivo*. H&E examination of teratomas revealed successful differentiation of all generated lines into ectodermal, endodermal, and mesodermal lineages (S1A Fig), confirming their pluripotency. We further verified the loss of exogenous SeV-OCT4, SOX2, KLF4, and MYC transgenes by genomic PCR detection and confirmed absence of detectable SeV transgenes in these iPSCs (S1B Fig), indicating that these RTS and FC iPSCs are generated with zero genetic footprint. To further investigate if additional mutation burden was created during reprogramming, we applied whole exome sequencing (WES) to compare RTS fibroblasts and iPSCs. In comparison with RTS-B fibroblasts, 6 and 146 additional nonsynonymous somatic variants were detected in RTS-B2 and RTS-B3, respectively (S1C Fig and S1 Table). The number of somatic variants resembled the level of mutations found in reprogrammed iPSCs as previously reported [26]. No mutations related to known tumor suppressor genes and oncogenes (e.g., *p53*, *RB1*, and *RAS*) were detected in RTS iPSCs. Taken together, these iPSC characterizations suggested the reliability of the RTS iPSC platform to model and study RTS pathology.

RTS iPSC-derived osteoblasts demonstrate osteogenic differentiation defects and premalignant phenotypes

We differentiated the newly generated iPSC lines to mesenchymal stem cells (MSCs) using an optimized MSC differentiation protocol [27]. During the 8-week MSC differentiation period, RTS and FC iPSCs exhibited morphological changes under brightfield microscopy from compact iPSCs to elongated and swirling cellular patterns characteristic of MSCs (Fig 2C). Immunostaining of these differentiated cells demonstrated presence of typical MSC surface markers CD44, CD73, and CD105 (Fig 2C), confirming successful differentiation. These MSCs were further differentiated into osteoblasts, a potential cell-of-origin for osteosarcoma, by a defined osteoblast differentiation method [17]. We further characterized cell proliferation of RTS and FC samples at different stages (iPSCs, MSCs, and osteoblasts) and found that RTS iPSCs and

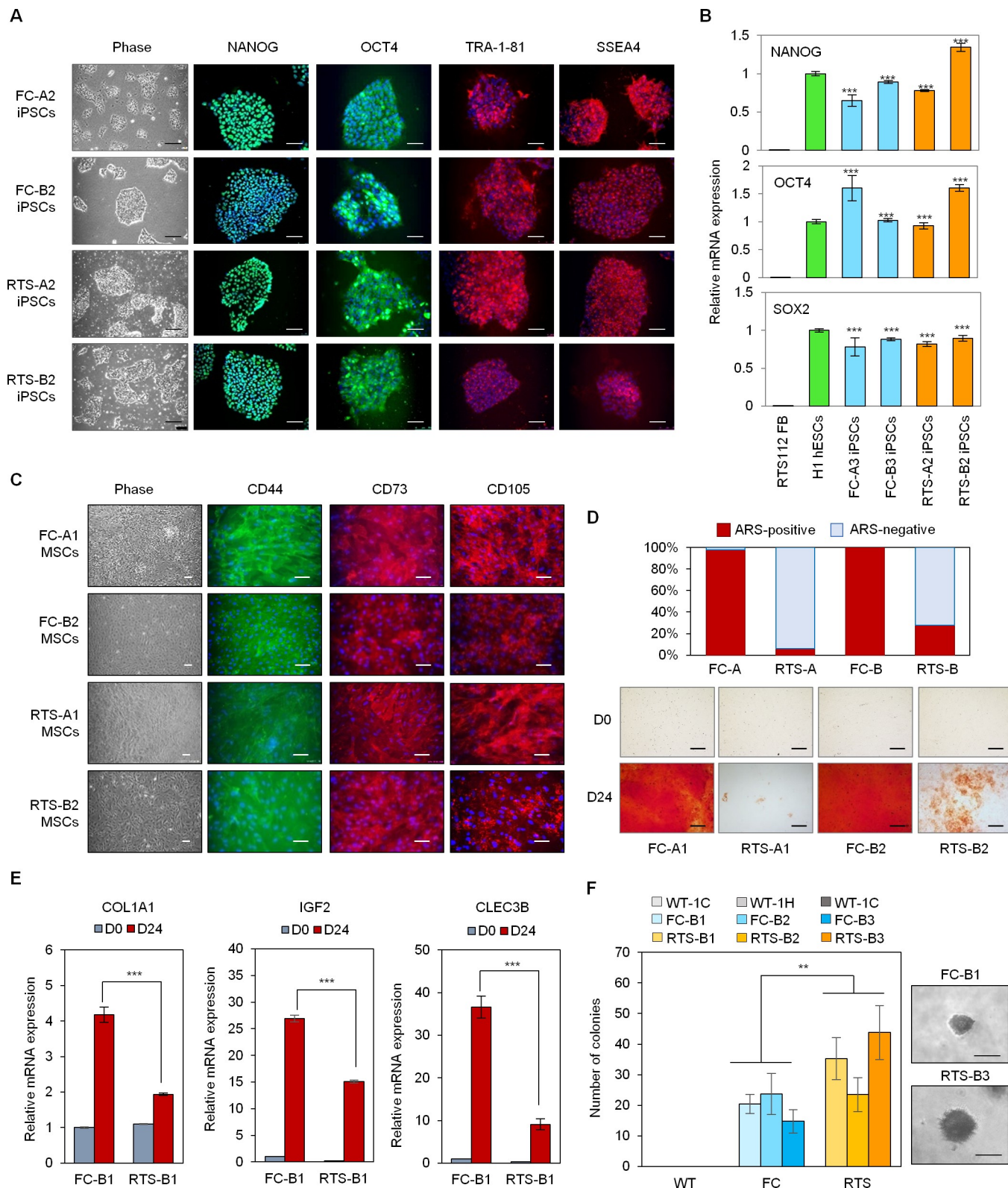


Fig 2. Generation of RTS iPSCs and iPSC-derived osteoblasts. (A) RTS and FC iPSCs exhibit hESC morphology and express pluripotency transcription factors NANOG and OCT4 as well as hESC surface antigens TRA-1-81 and SSEA44 ubiquitously. Scale bar, 100 μ m. (B) qRT-PCR assay for pluripotency genes NANOG, OCT4, and SOX2 in RTS and FC iPSCs. Error bars indicate SEM of triplicates. $n = 3$ biological replicates. (C) Immunostaining demonstrates that

iPSC-derived MSCs exhibit swirling morphology (phase contrast image) and express MSC surface markers CD44, CD73, and CD105. Scale bar, 100 μ m. (D) ARS staining reveals attenuated mineral deposition ability in RTS osteoblasts. Scale bar, 100 μ m. (E) qRT-PCR assay for osteoblast lineage genes *COL1A1*, *IGF2*, and *CLEC3B* in RTS and FC osteoblasts. Error bars indicate SEM of triplicates. $n = 3$ biological replicates. (F) AIG assay for *in vitro* tumorigenicity demonstrates that RTS osteoblasts present an increased clonal growth ability in soft agar in comparison with FC and WT osteoblasts. Positive colonies after 1.5-month growth of differentiated osteoblasts in osteoblast differentiation medium are those larger than 50 μ m (scale bar, 50 μ m). $n = 3$ biological replicates.

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MSCs have decreased cell proliferation ability compared to FC counterparts, indicating the essential role of RECQL4 in maintaining cell growth (S2A Fig, upper and middle panels). Importantly, there were no significant growth differences between RTS and FC osteoblasts (S2A Fig, lower panel). Consistent with the role of RECQL4 in regulating the cell cycle [28], cell cycle profile analyses revealed that RTS iPSCs have an increased G2/M cell population, whereas RTS MSCs and osteoblasts do not show differences in their G2/M cell population (S2B Fig). These results imply potential pro-oncogenic transformation (e.g., enhanced cell growth ability) in RTS osteoblasts. Wound healing assays demonstrated no difference in cell migration abilities between RTS and FC osteoblasts (S2C Fig), which may reflect the clinical observation that RTS patient osteosarcomas do not have a prevalence of metastases [6]. Furthermore, Alizarin Red S (ARS) staining for mineral deposition by functional osteoblasts was decreased in RTS compared to FC samples (Fig 2D), suggesting impaired osteogenic differentiation in RTS osteoblasts. Consistently, in comparison with FC osteoblasts, RTS osteoblasts showed lower expression of the osteoblastic genes *COL1A1*, *IGF2*, and *CLEC3B* during osteogenesis (Fig 2E). Anchorage-independent growth (AIG) assay showed that RTS osteoblasts demonstrate an increased clonal growth ability in soft agar in comparison with FC and wild-type (WT) osteoblasts (lacking biallelic *RECQL4* pathogenic variants) (Fig 2F), supportive of an *in vitro* tumorigenic ability of RTS osteoblasts. Remarkably, FC osteoblasts could maintain a certain clonal growth ability in soft agar, implying a potential tumor suppressor role of RECQL4 at the tumor initiation stage. Taken together, these findings demonstrate that aspects of the Type 2 RTS phenotype, in particular abnormal skeletal development and premalignant ability, can be recapitulated within the RTS iPSC disease platform.

Increased mitochondrial respiratory gene transcripts in RTS osteoblasts

To interrogate the molecular dysregulation leading to osteosarcomagenesis in RTS patients, RTS and FC iPSC-derived cells were collected for transcriptome analysis by RNA-seq at three different osteogenic differentiation stages: MSC stage (day 0; D0); pre-osteoblast stage (day 15; D15); and mature osteoblast stage (day 24; D24). We applied gene set enrichment analysis (GSEA) to identify gene ontology biological processes (GO_BPs) enriched in RTS or FC cells at these differentiation stages. GO_BPs for many mitochondrial energy production functions (e.g., ATP synthesis coupled electron transport, mitochondrial respiratory chain complex assembly, mitochondrial electron transport NADH to ubiquinone, NADH dehydrogenase complex assembly, OXPHOS, etc.) were enriched in RTS osteoblasts compared to FC osteoblasts (Fig 3A and 3B). Interestingly, enrichment of these pathways was only observed in osteoblast but not MSC lineages, indicating that the alteration of mitochondrial energy production occurs during, but not prior to, osteogenic differentiation. In contrast, GO_BP analyses revealed significantly depleted genes in skeletal development pathways (e.g., bone morphogenesis, bone development, cell matrix adhesion, extracellular matrix assembly, etc.) in the RTS pre-osteoblasts and osteoblasts compared to FC counterparts (Fig 3A and 3B), supportive of the small stature and skeletal abnormalities seen in RTS patients (Fig 1B and Table 1) and the impaired osteogenic differentiation observed in RTS osteoblasts (Fig 2D).

Consistently, KEGG pathway analyses highlighted increased mitochondrial ATP production pathways, including OXPHOS and tricarboxylic acid (TCA) cycle pathway genes, but

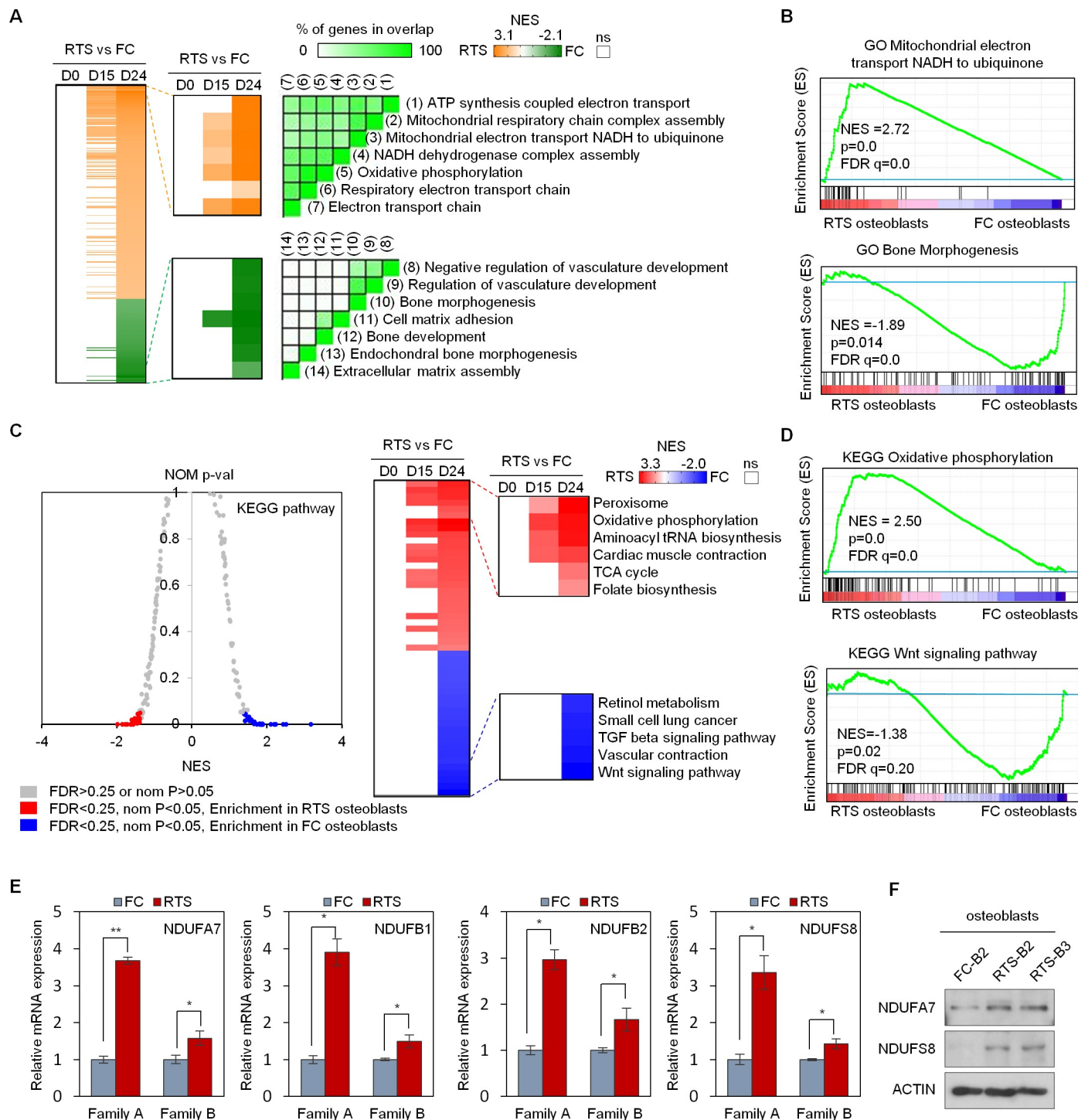


Fig 3. Transcriptional analysis reveals increased OXPHOS in RTS osteoblasts. (A) GSEA analyses identify gene ontology biological processes (GO_BP) from a collection of 7525 genes sets (left) enriched in RTS or FC osteoblasts. GO_BP gene sets enriched (orange, corresponding to a positive normalized enrichment score (NES)) or depleted (green, corresponding to a negative NES) in the transcriptome of RTS compared to FC osteoblasts are shown. Enriched gene sets are selected based on statistical significance (normalized p-value < 0.05 and FDR q-value < 0.25). Middle, heatmap of significantly altered GO_BPs in RTS MSCs (D0), pre-osteoblasts (D15), and osteoblasts (D24) compared to FC counterparts. Right, GSEA leading edge analysis demonstrates the overlap between gene sets enriched in RTS and FC osteoblasts. (B) GSEA GO_BP results for mitochondrial electron transfer NADH to ubiquinone and bone morphogenesis. (C) KEGG pathway analyses identify enriched pathways in RTS and FC osteoblasts. Left, 183 total pathways are included in KEGG pathway analysis. Pathways enriched in the transcriptome of FC osteoblasts have a negative normalized enrichment score (NES) (blue), while pathways with a positive NES (shown in red) are enriched in the transcriptome of RTS osteoblasts. Enriched pathways are selected based on statistical significance (normalized p-value < 0.05 and FDR q-value < 0.25). Right, heatmap showing

significantly altered pathways in RTS MSCs (D0), pre-osteoblasts (D15), and osteoblasts (D24) compared with FC counterparts. (D) Representative KEGG pathways differentially regulated in RTS osteoblasts, including OXPHOS and Wnt signaling pathway. (E) Individual genes (*NDUFA7*, *NDUFB1*, *NDUFB2*, and *NDUFS8*) that compose complex I of the electron transport chain are significantly upregulated in RTS osteoblasts. *n* = 3 biological replicates. (F) Immunoblotting indicates that *NDUFA7* and *NDUFS8* are upregulated in RTS osteoblasts compared to FC osteoblasts.

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decreased osteogenic Wnt signaling genes in RTS compared to FC osteoblasts (Fig 3C and 3D). These findings were further supported by Reactome biologic pathways and process analyses showing an increase in transcription of genes related to respiratory electron transport and respiratory electron transport ATP synthesis but decreased transcription of genes related to extracellular matrix organization in RTS compared to FC osteoblasts (S3A Fig). We validated the upregulation of select OXPHOS genes in RTS osteoblasts using qRT-PCR and confirmed upregulation of *NDUFA7*, *NDUFB1*, *NDUFB2*, and *NDUFS8*, which are involved in both the accessory and catalytic subunits of mitochondrial respiratory complex I [29], in RTS compared to FC osteoblasts (Fig 3E). The upregulation of *NDUFA7* and *NDUFS8* was further confirmed by immunoblotting (Fig 3F). These findings are consistent with transcriptome analyses of osteosarcoma cell lines and specimens demonstrating increased mitochondrial ATP production pathways (e.g., OXPHOS, respiratory electron transport chain, TCA cycle, and mitochondrial respiratory complex assembly) in sporadic osteosarcoma cell lines compared with normal bone tissues and osteoblasts (S3B Fig), suggesting that elevated expression of mitochondrial ATP production genes contributes to osteosarcomagenesis.

Increased mitochondrial respiratory complex I activity in RTS osteoblasts facilitates elevated OXPHOS

To explore the biochemical activity of the electron transport chain in RTS and FC osteoblasts, we systematically analyzed the enzymatic activities of the major mitochondrial respiratory protein complexes including complex I, complex I and III, complex II, complex II and III, and citrate synthase enzyme activity. We discovered that RTS osteoblasts had significantly increased mitochondrial respiratory complex I activity (Fig 4A), but no difference in complex II, III, IV, or citrate synthase enzyme activities (S4A–S4E Fig). These mitochondrial enzymatic activity assays were consistent with transcriptome results showing increased mRNA expression of mitochondrial respiratory complex I genes *NDUFA7*, *NDUFB1*, *NDUFB2*, and *NDUFS8* (Fig 3E). These findings lead us to conclude that RTS osteoblasts are characterized by increased mitochondrial respiratory complex I gene transcription and activity.

Mitochondrial respiratory complex I is of particular importance as it is the first step in the electron transport chain, establishing the electrochemical gradient necessary for ATP production [30]. Next, we explored the cellular metabolism of RTS osteoblasts using Seahorse metabolic flux assays. Our results demonstrated an increased oxygen consumption rate (OCR) in RTS osteoblasts compared to FC osteoblasts (Fig 4B). The basal respiration rate of RTS osteoblasts, as measured by ATP production, was also significantly increased compared to FC osteoblasts (Fig 4C). RTS osteoblasts also had significantly increased proton leak under normal culture conditions (Fig 4D), indicating an increased ability to create the proton gradient across the mitochondrial inner matrix for ATP synthase activity. Indeed, RTS osteoblasts showed increased ATP-linked (i.e., mitochondrial) respiration and maximal respiration (Fig 4E and 4F). RTS osteoblasts also retained significantly more spare respiratory capacity (Fig 4G). In contrast, RTS and parental osteoblasts had similar non-mitochondrial respiration rates (Fig 4H), suggesting that mitochondria-associated OXPHOS is the cause of increased oxygen consumption.

We next compared extracellular acidification rates (ECAR) to assay for glycolysis in RTS osteoblasts. In agreement with their elevated OXPHOS, RTS osteoblasts shift away from

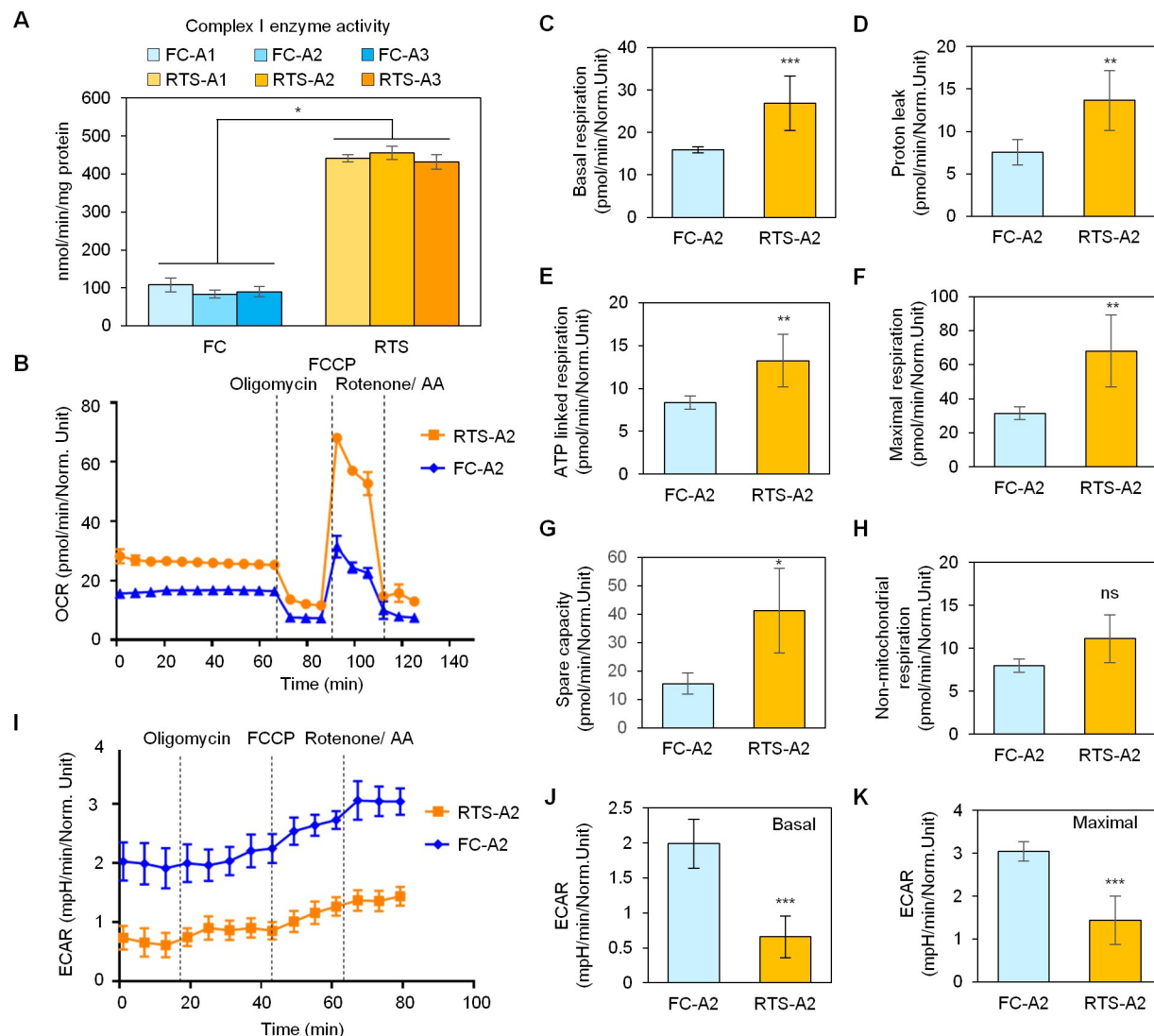


Fig 4. Metabolic differences between RTS and FC osteoblasts demonstrate the role of OXPHOS in increasing ATP production in RTS osteoblasts. (A) Enzyme activity of complex I is increased in RTS osteoblasts. $n = 3$ biological replicates. (B) Seahorse assays indicate increased oxygen consumption rate, a measure of OXPHOS, in RTS osteoblasts. $n = 3$ biological replicates. (C-H) Individual calculations of basal respiration (C), proton leak (D), ATP-linked respiration (E), maximal respiration (F), and spare capacity (G) in RTS osteoblasts as measured by seahorse assay, all of which are significantly upregulated. Non-mitochondrial respiration (H), which is not attributed to OXPHOS, is comparable between RTS and parental osteoblasts. $n = 3$ biological replicates. (I) Seahorse assays show decreased extracellular acidification rate, a measure of glycolysis, in RTS osteoblasts. $n = 3$ biological replicates. (J-K) Significantly decreased basal extracellular acidification rate (J) and maximal acidification rate (K) in RTS osteoblasts indicate RTS osteoblast reliance on OXPHOS for ATP production. $n = 3$ biological replicates.

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glycolysis toward OXPHOS as a means of ATP production (Fig 4I) and demonstrated a significant decrease in both basal and maximal ECAR (Fig 4J and 4K), indicating that RTS osteoblasts have little reliance on glycolytic metabolism. Also, a decrease of total ATP levels was found in RTS osteoblasts (S4F Fig). These data demonstrate that, compared to FC osteoblasts, RTS osteoblasts upregulate OXPHOS and mitochondrial respiratory complex I-mediated ATP production at the expense of decreased glycolysis. Taken together, our systems-based analyses demonstrating an increase in mitochondrial respiratory transcripts and activities lead us to hypothesize that elevated complex I enzymatic activity in RTS osteoblasts may play a critical role in RTS-driven osteosarcomagenesis.

IACS-010759 selectively inhibits OXPHOS and cell viability of RTS osteoblasts

OXPHOS inhibitor IACS-010759 binds to and inhibits mitochondrial respiratory complex I of the electron transport chain, thereby selectively inhibiting growth and survival of cells dependent on complex I function [31,32]. A preclinical study of IACS-010759 demonstrated robust suppression of proliferation and induction of cell death in tumor cells reliant on OXPHOS. This agent is being evaluated in phase I clinical trials against acute myeloid leukemia (AML) and solid tumors [31]. Based on our results highlighting that RTS osteoblasts meet their increased energy demands via hyperactivated mitochondrial respiratory complex I function, we investigated whether RTS premalignant osteoblasts are selectively vulnerable to inhibition of mitochondrial respiratory complex I function. We first examined the effect of IACS-010759 on ATP production via OXPHOS in RTS osteoblasts. IACS-010759 treatment resulted in decreased OXPHOS (Fig 5A), with significantly reduced ATP-linked respiration, maximal respiration, and spare respiratory capacity in these cells (Fig 5B). We then assayed osteoblast growth following IACS-010759 inhibition. Upon IACS-010759 treatment, RTS osteoblasts, but not FC osteoblasts, altered their cell morphology from a spindle shape into an irregular shape (Figs 5C and S5A), indicating cellular effects on RTS osteoblasts. Consistently, cell proliferation assays demonstrated selectively inhibited RTS osteoblast growth following IACS-010759 treatment but only limited effects on FC osteoblast proliferation (Fig 5D), suggesting that inhibition of mitochondrial respiratory complex I activity selectively suppresses RTS cell survival. In contrast, Rotenone, another known mitochondrial respiratory complex I inhibitor, not only presented less efficacy in inhibiting RTS osteoblasts compared to FC counterparts but also showed a certain inhibitory effect on FC osteoblast growth (S5B Fig). These findings suggest that IACS-010759 is a better choice for clinical application.

We next investigated the cellular effects of IACS-010759-mediated RTS osteoblast growth inhibition. As shown in S5C Fig, RTS osteoblasts exhibited an increased reactive oxygen species (ROS) production compared to FC osteoblasts, but IACS-010759 did not change ROS levels in both RTS and FC osteoblasts. Caspase 3/7 activity assays indicated that IACS-010759 leads to decreased cell apoptosis in both RTS and FC osteoblasts. (S5D Fig). Interestingly, IACS-010759 selectively induced cellular senescence of RTS osteoblasts (Fig 5E) and altered the cell cycle profile of RTS osteoblasts by increasing G0/G1 and decreasing S and G2/M cell populations (S5E Fig). These findings suggest that induction of senescence, but not ROS production and apoptosis, is one of the pharmacological mechanisms involved in IACS-010759-induced RTS osteoblast growth inhibition. Taken together, these results demonstrate that inhibition of mitochondrial respiratory complex I abrogates OXPHOS and induces cell senescence in RTS osteoblasts and underscore the possibility of targeting RTS osteosarcomas using IACS-010759.

To shed light on the molecular effects of IACS-010759 in halting RTS osteoblast viability, we next examined the transcriptional changes in RTS osteoblasts upon IACS-010759 treatment. A scatter plot comparing gene expression between IACS-010759 and DMSO treated RTS osteoblasts indicated that IACS-010759 treatment upregulates long non-coding RNA H19 and numerous ribosome proteins (e.g., RSP27, RPL11, RPL32, RPL35A, RPL11, etc.) and downregulates cell cycle processes (e.g., EPGN, EREG, GPSM2, etc.) and the MAP kinase pathway (STK39, HGF, GHR, etc.) in these cells (Fig 5F). Enrichr-based GO_BP analysis of differentially expressed genes (>1.5 fold changes) between DMSO and IACS-010759-treated RTS osteoblasts demonstrated an increase in protein targeting, T cell mediated cytotoxicity, and protein translation pathway transcripts and a decrease in cell cycle process and MAP kinase (MAPK) pathway transcripts in IACS-010759-treated RTS osteoblasts (Figs 5G

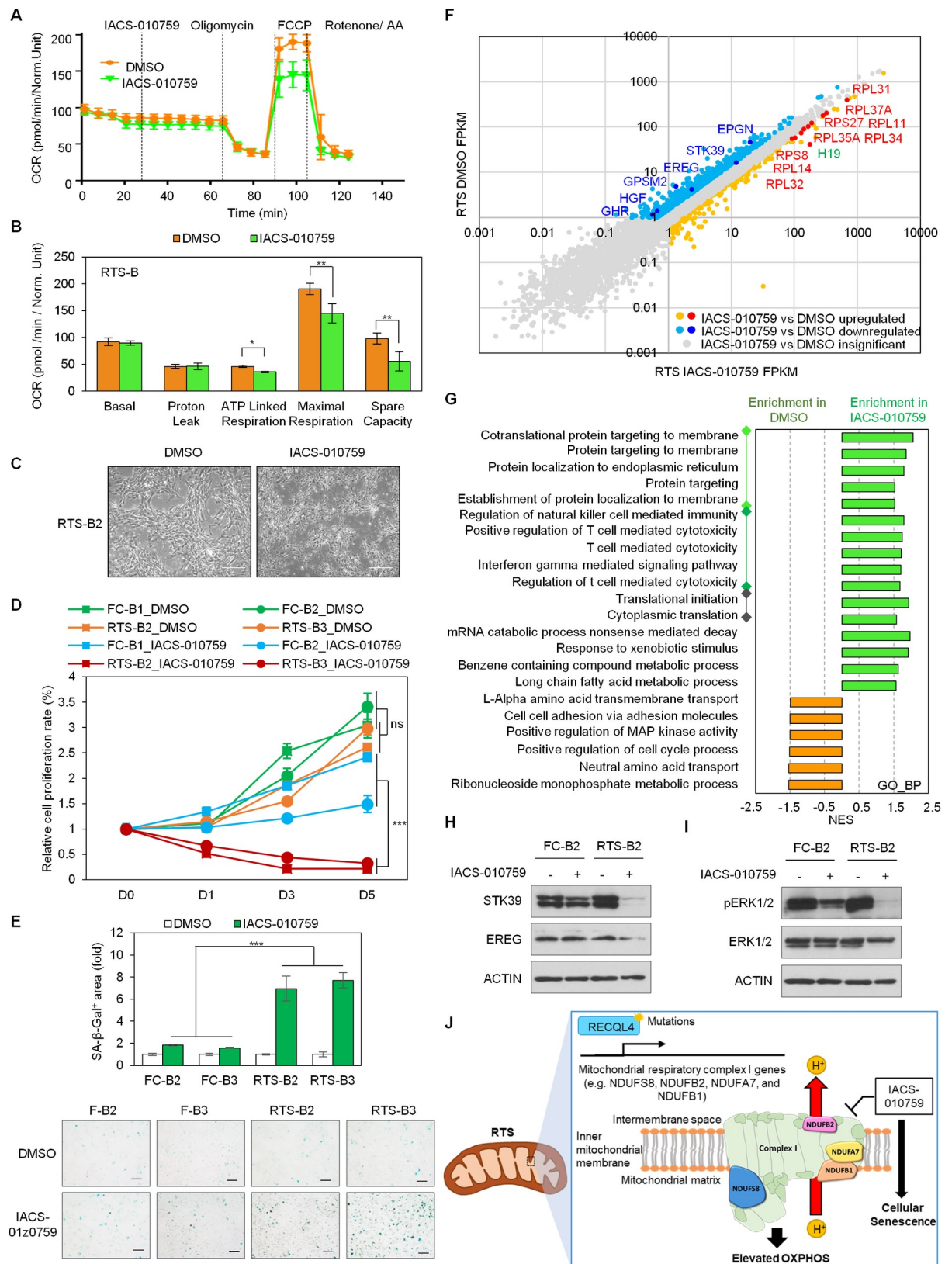


Fig 5. Inhibition of mitochondrial respiratory complex I specifically impairs ATP respiration, reliance on OXPHOS, and survival in RTS osteoblasts. (A-B) RTS osteoblasts treated by 100 nM of IACS-010759 show reduced ATP respiration, maximal respiration, and spare

capacity. Differences in OCR with seahorse assay are most apparent after the addition of FCCP, which measures the maximum potential of ATP production through the electron transport chain. $n = 3$ biological replicates. (C) IACS-010759 alters RTS osteoblast cell morphology from a spindle shape to an irregular shape. Scale bar, 100 μm . (D) IACS-010759 selectively inhibits cell proliferation of RTS osteoblasts. Cell proliferation assays after treatment with 100 nM of IACS-010759 show growth inhibition of RTS osteoblasts but little effect on FC osteoblasts. $n = 4$ biological replicates. (E) IACS-010759 induces the cellular senescence of RTS osteoblasts. The SA- β -Gal $^{+}$ areas are measured using ImageJ. $n = 3$ biological replicates. (F) Scatter plot indicates a marked difference of transcripts between DMSO and 100 nM of IACS-010759-treated RTS osteoblasts. H19 and numerous ribosomal protein genes are upregulated but MAPK pathway and cell cycle-associated genes are downregulated upon IACS-010759 treatment. (G) GO_BP analysis indicates dysregulated pathways in RTS osteoblasts upon IACS-010759 treatment. IACS-010759 activates protein targeting, T cell-mediated toxicity, and protein translation related biological processes but inhibited MAPK and cell cycle processes in RTS osteoblasts. (H) Immunoblotting indicates a decrease MAPK pathway related STK9 and EREG in RTS osteoblasts upon IACS-010759 treatment. (I) IACS-010759 impairs ERK1/2 activity in RTS osteoblasts. (J) Model for the elevated mitochondrial respiratory complex I function in RTS osteoblasts. In RTS patients, impaired RECQL4 function leads to an increase of mitochondrial respiratory complex I gene expression and function, and thereby induces premalignant phenotypes. Pharmacological inhibition of mitochondrial respiratory complex I function by IACS-010759 can be applied to prevent and/or treat RTS patient associated osteosarcomas.

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and S5F). Downregulation of cell cycle regulator EREG and MAPK signaling molecule STK39 (Fig 5H) and ERK1/2 activity (Fig 5I) in IACS-010759-treated RTS osteoblasts were validated by immunoblotting. KEGG pathway analysis of significantly upregulated genes in IACS-010759-treated RTS osteoblasts further validated the upregulation of the ribosome pathway (S5G Fig). In summary, our systematic transcriptome analyses revealed multiple potential pharmacologic mechanisms for IACS-010759-mediated cell growth inhibition in RTS osteoblasts.

Discussion

In this study, we established an RTS iPSC disease model to investigate altered pathological mechanisms involved in RTS patient-associated osteosarcoma. By differentiating RTS iPSCs to osteoblasts and characterizing them along this pathway of differentiation, we were able to establish specific osteoblastic defects induced by the *RECQL4* genotypes that correlate with the RTS Type 2 phenotype. The advantage of the iPSC platform lies in its ability to establish highly flexible pluripotent human cell lines from an established unique genetic alteration and proven background. In this case, our approach allowed us to dissect the impact of *RECQL4* pathogenic variants on RTS-associated pathological consequences such as defective osteogenesis and osteosarcomagenesis. The unique self-renewal/pluripotency and differentiation abilities of iPSCs distinguish them from all existing model systems. In addition to MSCs and osteoblasts, RTS iPSCs are capable of being differentiated into other germ layers. With appropriate differentiation protocols, these models can be used to study other clinical phenotypes of RTS, such as cataracts, poikiloderma, and abnormal dentition, that affect other tissues and organ systems.

We examined the dysregulated pathways in RTS iPSC-derived osteoblasts using multiple gene set categories including GO_BP, KEGG pathway and Reactome. All tools for systems analyses pointed to the same alteration of increased energy metabolism and upregulated OXPHOS in RTS osteoblasts, indicating that *RECQL4* pathogenic variants result in elevated OXPHOS in RTS osteoblasts. Accumulating studies of energy metabolism in leukemias, lymphomas, pancreatic ductal adenocarcinoma, melanoma, and endometrial carcinoma reveal the essential role of OXPHOS in contributing to tumor formation and progression [10]. Our transcriptomic analyses also identified increased expression of genes involved in OXPHOS, particularly mitochondrial respiratory complex I, in RTS osteoblasts. Consistently, bioenergetics in RTS osteoblasts revealed the preference of RTS osteoblasts for utilizing OXPHOS but not aerobic glycolysis to meet anabolic demands. Since total ATP production is decreased in RTS cells (S4F Fig), an increase of OXPHOS by upregulating mitochondrial respiratory complex I gene expression to enhance ATP production would provide an essential advantage to overcome

ATP deficiency due to *RECQL4* pathogenic variants. Increased production of NAD⁺ through mitochondrial respiratory complex I has been shown to immortalize tumor cells at the tumor initiation stages [11]. We speculate that elevated mitochondrial respiratory complex I genes and OXPHOS provide dual RTS survival benefits, including energy for a higher level of growth as well as increased NAD⁺ to drive RTS osteoblast immortalization toward malignancy.

As a DNA helicase, *RECQL4* not only plays an important role in modulating DNA replication and repair but also preserves mitochondrial DNA integrity. *RECQL4* was suggested to interact with mitochondrial polymerase (PolyA/B2) and play a role in the proofreading activities of PolyA/B2 [33]. In agreement with elevated ROS in RTS osteoblasts (S5C Fig), *RECQL4* homozygous deficient fibroblasts from RTS patients exhibited increased oxidative stress and a markedly decreased repair capacity for DNA damage [34]. Mutant *RECQL4* particularly translocates from nucleus to mitochondria and then interacts with mitochondrial replication helicase PEO1, which leads to elevated mtDNA synthesis and mitochondrial dysfunction [35]. Our study added a new finding to link mitochondrial dysfunction to RTS pathology and uncovered the upregulated mitochondrial respiratory complex I gene expression and activities in RTS osteoblasts. Together, these findings shed light on additional *RECQL4* functions in mitochondria and the involvement of mitochondria dysregulation in RTS etiology.

Our thorough transcriptional and biochemical studies conclude that elevated mitochondrial respiratory complex I function in RTS osteoblasts is a potential therapeutic target to treat RTS osteosarcoma. Indeed, the preclinical compound IACS-010759, a mitochondrial respiratory complex I inhibitor specific for the ND1 subunit that blocks the intermembrane transport of H⁺ [31], selectively impairs cell viability of RTS osteoblasts. Our RTS osteoblast transcriptome analyses demonstrated that IACS-010759 treatment not only impairs MAPK signaling and cell cycle gene expression, but also upregulates H19 and ribosomal gene expression. The effect of the RTS genotype on the MAPK pathway has been previously shown in RTS fibroblasts, which demonstrated an increased cellular lifespan and proliferation rate due to MAPK activation [36]. H19 has also been shown to function as a tumor suppressor to promote osteogenesis and inhibit osteosarcomagenesis in LFS osteoblasts and osteosarcoma [17], implying that IACS-010759 may shift cells from a tumorigenic and undifferentiated profile towards normal osteoblast differentiation. Furthermore, IACS-010759 upregulates the expression of ribosomal protein genes such as *RSP27*, *RPL11*, *RPL32*, *RPL35A*, and *RPL11*, mutations which are found in osteosarcoma-prone Diamond-Blackfan anemia (DBA) patients [37–39]. Homeostasis of ribosomal biogenesis may be an essential process to maintain survival of RTS osteoblasts.

In summary, we established an RTS iPSC disease platform to dissect the pathological mechanisms involved in RTS-associated osteosarcoma (Fig 5J). Transcriptome analysis revealed upregulation of genes involved in OXPHOS, especially mitochondrial respiratory complex I, in RTS osteoblasts. The upregulation of oxidative metabolism in RTS is a druggable pathway, and our preclinical studies using IACS-010759 suggest favorable, clinically relevant changes in RTS osteoblasts treated with this drug. Overall, our study suggests strong clinical potential for therapies targeting mitochondrial respiratory complex I in RTS patients with osteosarcoma.

Materials and methods

Ethics statement

The protocol entitled "Molecular Basis of Clinical Spectrum of Rothmund-Thomson Syndrome" (IRB# H-9106) was approved by the Institutional Review Board of Baylor College of Medicine and Affiliated Hospitals. Written formal consent for participation in this IRB-approved protocol was obtained by Dr. Lisa L. Wang (P.I.) from the legally authorized representative of each subject at the time of enrollment.

Cell culture

RTS and family control (FC) fibroblasts were cultured and maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (vol/vol) fetal bovine serum (FBS) (Opti-Gold performance enhanced F0900-050, GenDEPOT, Barker, TX), L-glutamine, non-essential amino acids, β -mercaptoethanol, and penicillin/streptomycin. iPSC-derived MSCs were maintained in gelatin-coated plates and cultured in DMEM supplemented with 10% FBS, L-glutamine, and penicillin/streptomycin. iPSC-derived osteoblasts were cultured in osteogenic differentiation medium (α -MEM supplemented with 10% FBS, 0.1 μ M dexamethasone, 10 mM β -glycerol phosphate, and 200 μ M ascorbic acid) [17,40].

Somatic cell reprogramming RTS and FC fibroblasts

RTS individuals and their parents provided informed consent to participate in an institutional review board-approved study at Baylor College of Medicine (Houston, TX). Sequence analysis of the *RECQL4* gene was performed by Baylor Genetics laboratories (Houston, TX) and confirmed the pathogenic variants in subjects used in this study. Fibroblasts were isolated from skin biopsies from two RTS patients and two first-degree relatives and reprogrammed to iPSCs by transducing Sendai viruses (SeV) expressing the four reprogramming factors OCT4, SOX2, KLF4, and MYC (CytoTune-iPS 2.0 Sendai Reprogramming Kit, Thermo Fisher Scientific, A16517) according to the manufacturer's protocol. The reprogrammed cells were maintained in hESC media (KnockOut DMEM/F12 (Thermo Fisher Scientific, 12660012) containing 20% (vol/vol) KnockOut Serum Replacement (Thermo Fisher Scientific), L-glutamine, non-essential amino acids, β -mercaptoethanol, penicillin/streptomycin, and bFGF). At 3–4 weeks post-induction, individual clones with hESC/iPSC morphology and positive TRA-1-81 staining were selected, passaged on irradiated mouse embryonic fibroblasts (MEFs), and maintained for 10–15 passages. Clones were examined for loss of SeV by genomic DNA PCR using specific primers targeting SeV (5'-GGATCACTAGGTGATATCGAGC-3' and 5'-ATGCACCGCTACGACGTGAGCGC-3'), SOX2 (5'-ATGCACCGCTACGACGTGAGCGC-3' and 5'-AATGTATCGAAGGTGCTCAA-3'), and KLF4 (5'-TTCCTGCATGCCAGAGGAGCCC-3' and 5'-AATGTATCGAAGGTGCTCAA-3').

Teratoma formation assay

Teratoma formation assay was performed as described previously [41]. H&E staining for histology was performed by Histowiz (Brooklyn, NY).

In vitro differentiation of iPSCs to MSCs

In vitro differentiation of iPSCs from RTS individuals and unaffected family members to MSCs was performed by a defined optimized MSC differentiation protocol described previously [27]. Briefly, 80% confluent iPSCs were cultured in StemMACS iPSC-Brew XF medium (Miltenyi Biotec, 130-104-368) supplemented with 10 μ M TGF β inhibitor SB-431542 (MilliporeSigma, S4317) in Matrigel-coated plates at 37°C and 7.5% CO₂ and passaged at 80–90% confluence by Accutase cell detachment solution (StemCell Technology, 07922). After 25 days in culture, cells at the edge of differentiated cell clusters became spindle-shaped. These differentiated cells were trypsinized into single cells and maintained in gelatin-coated plates using modified human ESC-MSC medium (KnockOut DMEM/F-12 supplemented with 10% KnockOut Serum Replacement, L-glutamine, non-essential amino acids, β -mercaptoethanol, 20 ng/ml bFGF2, 10 ng/ml EGF, 10 μ M SB-431542, and penicillin/streptomycin). The medium was changed daily. When cells reached 80–90% confluence, cells were passaged at a 1 to 3 ratio

for three weeks. The MSCs were characterized by staining for MSC surface markers CD44, CD73, and CD105.

Osteogenic differentiation and ARS staining

Osteogenic differentiation was carried out based on previously described protocols [17,18,40]. MSCs were seeded at a density of 3×10^5 cells/per well in a 6-well dish and cultured in osteoblast differentiation medium (α MEM supplemented with 10mM β glycerol phosphate, 2% penicillin/streptomycin, 200 μ M ascorbic acid, and 10% FBS). The medium was changed every three days. Differentiated osteoblasts were fixed in ice-cold 70% ethanol at room temperature for 2 h, washed with tap water, and stained by ARS solution (0.02g/ml alizarin red S, pH 4.1–4.3) for 30 min. Stained osteoblasts were imaged under a Leica DMI8 microscope.

qRT-PCR

qRT-PCR was performed as described previously [41]. PCR primers used to detect gene expression included *NANOG* (5'-TTTGTGGGCCTGAAGAAACT-3' and 5'-AGGGC TGTCCTGAATAAGCAG-3'), *OCT4* (5'-AACCTGGAGTTTGTGCCAGGGTTT-3' and 5'-TGAACCTCACCTTCCCTCCAACCA-3'), *SOX2* (5'-AGAAGAGGAGAGAGAAAGAA AGGGAGAGA-3' and 5'-GAGAGAGGCAAACTGGAATCAGGATCAAA-3'), *COL1A1* (5'-GAGGGCCAAGACGAAGACATC-3' and 5'-CAGATCACGTCATCGCACAAAC-3'), *IGF2* (5'-GTGGCATCGTTGAGGAGTG-3' and 5'-CACGTCCCTCTCGGACTTG-3'), *CLEC3B* (5'-CCCAGACGAAGACCTTCCAC-3' and 5'-CGCAGGTACTCATACAGGG C-3'), *NDUFA7* (5'-TGCAGCTACGCTACCAGGA-3' and 5'-GAGCTTGTGGCTAGGAC CC-3'), *NDUFB1* (5'-GTCCCTATGGGATTTGTCATTGG-3' and 5'-CAGTTAGCCGTT CATCACTCTT-3'), *NDUFB2* (5'-GGAGGCCGCCTTTTCAGAA-3' and 5'-GGAAGGATC AGGATACGGAAAGT-3'), *NDUFS8* (5'-CCATCAACTACCCGTTTCGAGA-3' and 5'-CCG CAGTAGATGCACTTGG-3'), and internal control *GAPDH* (5'-CCACTCCTCCACCTTT GAC-3' and 5'-ACCCTGTTGCTGTAGCCA-3').

In vitro AIG assay

In vitro AIG assay was described previously [17]. Briefly, RTS, FC, and WT iPSC-derived MSCs were differentiated into osteoblasts. 1×10^4 differentiating osteoblasts (D7) were resuspended in osteoblast differentiation medium with 0.4%–0.5% LMP agarose. The cell suspensions were then plated in 12-well plates containing solidified 0.8% agarose in osteoblast differentiation medium. Osteoblasts were maintained in osteoblast differentiation medium for 1.5 months with medium changes every 3 days. Colonies (considered to have a diameter $\geq 50 \mu\text{m}$) were counted under a Leica DMI8 microscope.

Immunofluorescence staining and immunoblotting

Immunofluorescence staining and immunoblotting was described previously [41–43]. Antibodies used in this study included *NANOG* (R&D Systems, AF1997), *OCT4* (Santa Cruz, SC-9081), TRA-1-81-Alexa Fluor 555-conjugated (R&D Systems, FAB3195A), SSEA4-PE conjugated (R&D Systems, FA1435P-025), CD44 (BD PharMingen, 559942), CD73 (BD PharMingen, 550257), CD105 (eBioscience, 12-1057), RECQL4 (MilliporeSigma, SAB1410002), *NDUFA7* (ABclonal Science, A8441), *NDUFS8* (ABclonal Science, A13034), *STK39* (ABclonal Science, A2275), *EREG* (ABclonal Science, A16372), *ERK1/2* (Cell Signaling, 4695), phospho-*ERK1/2* (Cell Signaling, 9101), and *ACTIN* (MilliporeSigma, A2228) antibodies.

RNA-seq, GSEA, and enrichment map analysis

iPSC-derived MSCs and osteoblasts were lysed and RNA samples were prepared by Trizol RNA isolation reagent (Thermo Fisher Scientific, 15596026). RNA-sequencing (RNA-seq) was performed by BGI Genomics (Cambridge, MA) and UTHealth Cancer Genomics Center (Houston, TX). All RNA-seq data analyses were performed using Galaxy Community Hub to calculate FPKM (Fragments Per Kilobase of transcript per Million) as described previously [17]. Differential gene expression was analyzed by DESeq2 [44]. Gene Set Enrichment Analysis (GSEA) was employed to analyze differentially regulated Gene Ontology biological processes (GO_BPs), Kyoto Encyclopedia of Gene and Genomes (KEGG) pathways, Reactome, oncogenic signatures, and transcription factor targets (TFTs) [45]. Differences in gene sets were considered significant if p-value <0.05 and FDR q-value <0.25. Enrichment map analysis was performed to analyze enriched GO_BPs in osteosarcoma tissues using a published osteosarcoma microarray dataset (GSE36001) [46].

Cell proliferation assay

RTS and FC osteoblasts were seeded in flat bottom 96-well plates (Corning, 3598) at a density of 3,000 cells per well and cultured in 200 μ l osteoblast differentiation medium with or without IACS-010759 (dissolved in DMSO) for 3 days. Cell viability upon IACS-010759 treatment was examined by PrestoBlue cell viability reagent (Thermo Fisher Scientific, A13262) following the manufacturer's instructions. The quantitative measure of viability was read by absorbance at 570 nm.

Sequencing data processing and variant calling

WES of RTS fibroblasts and RTS-B2 and RTS-B3 iPSCs was performed using Novogene exome hybridization protocol followed by barcoding and sequencing of paired-end 150-bp reads on an Illumina NovaSeq 6000 instrument (Illumina Inc., San Diego, CA). Trim Galore! (Version 0.6.6) was then applied for adapter and quality trimming to the FastQ files (DOI: <https://doi.org/10.14806/ej.17.1.200>). Sequencing reads mapping to the human genome (GRCh38/hg38), initial variant calling and quality filtration were performed using the best practices in BWA-GATK4 (v4.2.2.0) pipeline with the tumor-only mode on [47,48]. Single nucleotide variants were detected using MuTect2 [49], while indels were not interrogated given the high rates of false-positive calls. Additional filters were applied to eliminate false-positive somatic variant calls: 1) read depth lower than 25th percentile of the sequencing coverage [50], 2) read depth higher than 75th percentile of the sequencing coverage [51], 3) present in dbSNP [52]. Functional annotation of the remaining somatic variants was carried out using ANNOVAR (v0.4.0.0) [53].

Cell cycle analysis

G0/G1, G2, and S-phase cells were stained with PI, examined by the BD flow cytometer, and analyzed by FlowJo as previously described [54].

Reactive oxygen species assay

Intracellular oxidation of osteoblasts was assayed by measuring the conversion of DCFH to DCF using the Reactive Oxygen Species (ROS) Fluorometric Assay Kit (Elabscience, E-BC-K138-F) according to the manufacturer's instructions. In short, DCFH-DA was added to osteoblasts and incubated for 1 h, then fluorescence of DCF was measured at 525nm using TECAN Infinite M Plex multimode microplate reader.

Senescence staining

Senescent cells were measured using the Senescence β -Galactosidase Staining Kit (Cell Signaling Technology, #9860). Osteoblasts, either treated with 100 nM IACS-010759 or DMSO for 3 days, were washed, fixed, then stained with β -Galactosidase overnight at 37°C. The population of β -Galactosidase positive cells was calculated by ImageJ.

Apoptotic activity assay

Apoptosis was measured via Caspase 3/7 activity using the SensoLyte Homogeneous AMC Caspase-3/7 Assay Kit (Anaspec, AS-71118). The assay was carried out according to the manufacturer's instructions. FC and RTS osteoblasts were cultured in a 6-well plate and then treated with 100 nM IACS-010759 for 3 days. Cell lysates were incubated with Caspase 3/7's fluorogenic substrate Ac- Asp-Glu-Val-Asp (DEVD) -AMC for 4 h in the dark. Fluorescence was measured at Ex/Em = 354nm/442nm using TECAN Infinite M Plex multimode microplate reader.

ATP detection assay

ATP levels of RTS and FC Day 24 osteoblasts were measured using the ATPlite Luminescence Assay System (Perkin Elmer, 6016943) and following the manufacturer's instructions. Briefly, cell lysis was incubated with ATPlite solution containing luciferase and D-Luciferin firefly. The ATP concentration is proportional to the emitted luminescence. The samples were incubated at room temperature in the dark for 10 minutes. Luminescence was measured by TECAN Infinite M Plex multimode microplate reader. Results were compared to the supplied ATP standard.

Electron transport chain enzymatic assays

Electron transport chain (ETC) enzymatic activity and citrate synthase (CS) activity assays were performed as previously described [55,56]. The total protein content of osteoblast lysates was quantified by Bradford protein assay (Bio-Rad, 5000006) and used for normalization.

Seahorse assay

Seahorse assays (Agilent) were carried out according to the manufacturer's protocol with modifications as described below. 30,000 osteoblasts were seeded into each well of a Seahorse cell culture plate, with at least 4 wells per assay left blank as controls. Osteoblasts were cultured in 200 μ l osteoblast differentiation medium at 37°C for 3 days. A Seahorse assay plate was prepared by incubating in water overnight and replacing with calibration medium for 4 h prior to performing the assay. Each well in the Seahorse assay plate was filled with 180 μ l of assay medium supplemented with 10 mM pyruvate, 2 mM glutamine, 25 mM glucose, and 4 mg/ml BSA. The tissue culture plate was then incubated at 37°C with 0% CO₂ and 0% humidity. Assay chemicals were dissolved in the assay medium without BSA.

Assay measurements were made at baseline and every 6 min over 18 min (3 cycles each entailing a 3-min mix and 3-min measurement). Next, IACS 010759 was injected from port A over 30 min and measurements were made at time 0 and at 6-min intervals over 30 min (5 cycles each of 3-min mix and 3-min measurement). Lastly, 15 μ M Oligomycin, 30 μ M FCCP, and 5 μ M Rotenone/Antimycin A were injected for 18 min from port B. Immediately following each assay, osteoblasts were fixed with 4% paraformaldehyde and stained with DAPI (1:5000 in DPBS). Cell numbers were counted using a Biotek Lionheart automatic microscope with default settings for TPP 96 well TC flat bottom plates. Nuclei were counted by

thresholding for a minimum size of 5 μm and a maximum size of 50 μm including edge objects. The rates of change of dissolved oxygen (OCAR) and pH (ECAR) were calculated using the Wave software, normalized to total cell number.

Statistical analysis

Statistical analysis was performed in Prism 8.0 with a two-tailed Student's t-test. Results are expressed as the mean $\pm$ SEM. Difference between two groups was compared by two-tailed unpaired Student's t-test or ANOVA in Prism 8.0. ns, non-significant; *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$.

Supporting information

S1 Fig. Characterization of RTS and FC iPSCs. (A) *In vivo* teratoma formation assay demonstrates three germ-layer differentiation abilities of RTS and FC iPSCs. Scale bar, 200 μm . (B) PCR detection of SeV genome and transgenes indicates that RTS and FC iPSCs are footprint-free. (C) Somatic variant analysis of RTS-B2 and RTS-B3 iPSCs suggests that limited somatic mutations are generated during cellular reprogramming.

(TIF)

S2 Fig. Characterization of RTS and FC iPSCs, MSCs, and osteoblasts. (A) Relative cell proliferation of RTS and FC iPSCs (upper panel), MSCs (middle panel), and osteoblasts (lower panel). $n = 3$ biological replicates. (B) Cell cycle profiles of RTS and FC iPSCs, MSCs, and osteoblasts are performed using PI staining by flow cytometry. (Upper) Cell cycle profiles of RTS iPSCs, MSCs, and osteoblasts. (Lower) Bar histogram shows the percentage of cells in the sub-G1, G0/G1, S, and G2/M phases. $n = 3$ biological replicates. (C) Wound healing assay determines the cell migration ability of RTS and FC osteoblasts. Wound healing assays were performed at 72 hours in RTS and FC osteoblasts. (Left) Representative phase-contrast microscope images show the area covered by RTS and FC osteoblasts at 72 hours after wounding. (Right) The cell migration rate is determined by the rate of cells moving towards the scratched area upon time measured. $n = 3$ biological replicates.

(TIF)

S3 Fig. Transcriptional analysis of RTS osteoblasts and osteosarcoma. (A) Reactome analysis demonstrates enrichment of multiple canonical pathways in RTS compared to FC MSCs (D0), pre-osteoblasts (D15), and osteoblasts (D24). (B) Left, GO_BPs enriched in osteosarcoma by EnrichmentMap analysis. Network visualization of gene sets enriched in osteosarcoma compared with normal bone tissues and osteoblasts (p value < 0.05 , FDR q value < 0.1) indicates that GO_BPs involved in DNA repair, chromatin regulation, cell cycle, and mitochondrial ATP production and repair are enriched in osteosarcoma. The number of enriched genes in each GO is displayed as node size, with closer distance between nodes representing increased overlap between genes in ontologies. Enriched gene sets in osteosarcoma are displayed in red and enriched gene sets in bone tissues and osteoblasts in blue. Right, representative enriched gene sets (ATP synthesis coupled electron transport and respiration electron transport chain) involved in mitochondrial ATP production are shown.

(TIF)

S4 Fig. Metabolic differences between RTS and FC osteoblasts. (A-E) Enzyme activity of complexes I and III (A), complex II (B), complexes II and III (C), complex IV (D), and citrate synthase (E) show no significant difference between RTS and FC osteoblasts, leaving complex I as the only component of the electron transport chain with upregulated activity. $n = 3$ biological replicates. (F) The decrease of total ATP levels is observed in RTS osteoblasts compared to

FC osteoblasts. n = 4 biological replicates.
(TIF)

S5 Fig. Biological effects and transcriptome alterations in IACS-010759-treated RTS osteoblasts. (A) IACS-010759 alters RTS osteoblast cell morphology from a spindle shape to an irregular shape. Scale bar, 100 μ m. (B) Cell proliferation assay indicates that Rotenone impairs cell proliferation of RTS and FC osteoblasts. n = 4 biological replicates. (C) ROS activity assay demonstrates that IACS-010759 does not induce ROS production in both RTS and FC osteoblasts. n = 3 biological replicates. (D) Cell apoptosis assay indicates a decrease of Caspase 3/7 activities upon IACS-010759 treatment in both RTS and FC osteoblasts. n = 3 biological replicates. (E) Cell cycle profiles of RTS and FC osteoblasts are performed upon IACS-010759 treatment. (Upper) Cell cycle profiles of IACS-010759-treated RTS and FC osteoblasts. (Lower) Bar histogram shows the percentage of cells in the sub-G1, G0/G1, S, and G2/M phases. n = 3 biological replicates. (F) Representative GO_BPs influenced by IACS-010759, including protein targeting to membrane, translational initiation, positive regulation of MAPK activity, and positive regulation of cell cycle process. (G) KEGG pathway analysis confirms upregulation of ribosome function in IACS-010759-treated RTS osteoblasts.
(TIF)

S1 Table. The mutation analysis of RTS-B2 and RTS-B3 iPSCs.
(XLSX)

Acknowledgments

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References

1. Lu L, Jin W, Liu H, Wang LL. RECQ DNA helicases and osteosarcoma. *Adv Exp Med Biol.* 2014; 804:129–45. https://doi.org/10.1007/978-3-319-04843-7_7 PMID: 24924172.
2. Lu L, Jin W, Wang LL. RECQ DNA Helicases and Osteosarcoma. *Adv Exp Med Biol.* 2020; 1258:37–54. https://doi.org/10.1007/978-3-030-43085-6_3 PMID: 32767233.
3. Wang LL, Plon SE. Rothmund-Thomson Syndrome. In: Adam MP, Ardinger HH, Pagon RA, Wallace SE, Bean LJH, Stephens K, et al., editors. *GeneReviews*(R). Seattle (WA)1993.
4. Wang LL, Gannavarapu A, Kozinetz CA, Levy ML, Lewis RA, Chintagumpala MM, et al. Association between osteosarcoma and deleterious mutations in the RECQL4 gene in Rothmund-Thomson syndrome. *J Natl Cancer Inst.* 2003; 95(9):669–74. <https://doi.org/10.1093/jnci/95.9.669> PMID: 12734318.
5. Ajeawung NF, Nguyen TTM, Lu L, Kucharski TJ, Rousseau J, Molidperee S, et al. Mutations in ANAPC1, Encoding a Scaffold Subunit of the Anaphase-Promoting Complex, Cause Rothmund-Thomson Syndrome Type 1. *Am J Hum Genet.* 2019; 105(3):625–30. <https://doi.org/10.1016/j.ajhg.2019.06.011> PMID: 31303264; PubMed Central PMCID: PMC6731352.
6. Hicks MJ, Roth JR, Kozinetz CA, Wang LL. Clinicopathologic features of osteosarcoma in patients with Rothmund-Thomson syndrome. *J Clin Oncol.* 2007; 25(4):370–5. <https://doi.org/10.1200/JCO.2006.08.4558> PMID: 17264332.
7. Phan LM, Yeung SC, Lee MH. Cancer metabolic reprogramming: importance, main features, and potentials for precise targeted anti-cancer therapies. *Cancer Biol Med.* 2014; 11(1):1–19. <https://doi.org/10.7497/j.issn.2095-3941.2014.01.001> PMID: 24738035; PubMed Central PMCID: PMC3969803.
8. Koppenol WH, Bounds PL, Dang CV. Otto Warburg's contributions to current concepts of cancer metabolism. *Nat Rev Cancer.* 2011; 11(5):325–37. <https://doi.org/10.1038/nrc3038> PMID: 21508971.
9. Pavlova NN, Thompson CB. The Emerging Hallmarks of Cancer Metabolism. *Cell Metab.* 2016; 23(1):27–47. <https://doi.org/10.1016/j.cmet.2015.12.006> PMID: 26771115; PubMed Central PMCID: PMC4715268.
10. Ashton TM, McKenna WG, Kunz-Schughart LA, Higgins GS. Oxidative Phosphorylation as an Emerging Target in Cancer Therapy. *Clin Cancer Res.* 2018; 24(11):2482–90. <https://doi.org/10.1158/1078-0432.CCR-17-3070> PMID: 29420223.
11. Bonnay F, Veloso A, Steinmann V, Kocher T, Abdusselamoglu MD, Bajaj S, et al. Oxidative Metabolism Drives Immortalization of Neural Stem Cells during Tumorigenesis. *Cell.* 2020; 182(6):1490–507 e19. <https://doi.org/10.1016/j.cell.2020.07.039> PMID: 32916131.
12. Denisenko TV, Gorbunova AS, Zhivotovsky B. Mitochondrial Involvement in Migration, Invasion and Metastasis. *Front Cell Dev Biol.* 2019; 7:355. <https://doi.org/10.3389/fcell.2019.00355> PMID: 31921862; PubMed Central PMCID: PMC6932960.
13. Gingold J, Zhou R, Lemischka IR, Lee DF. Modeling Cancer with Pluripotent Stem Cells. *Trends Cancer.* 2016; 2(9):485–94. <https://doi.org/10.1016/j.trecan.2016.07.007> PMID: 27722205; PubMed Central PMCID: PMC5050918.
14. Papapetrou EP. Patient-derived induced pluripotent stem cells in cancer research and precision oncology. *Nat Med.* 2016; 22(12):1392–401. <https://doi.org/10.1038/nm.4238> PMID: 27923030; PubMed Central PMCID: PMC5233709.
15. Liu M, Tu J, Gingold JA, Kong CSL, Lee DF. Cancer in a dish: progress using stem cells as a platform for cancer research. *Am J Cancer Res.* 2018; 8(6):944–54. PMID: 30034933; PubMed Central PMCID: PMC6048395.
16. Zhu D, Kong CSL, Gingold JA, Zhao R, Lee DF. Induced Pluripotent Stem Cells and Induced Pluripotent Cancer Cells in Cancer Disease Modeling. *Adv Exp Med Biol.* 2018; 1119:169–83. https://doi.org/10.1007/5584_2018_257 PMID: 30069853.
17. Lee DF, Su J, Kim HS, Chang B, Papatsenko D, Zhao R, et al. Modeling familial cancer with induced pluripotent stem cells. *Cell.* 2015; 161(2):240–54. <https://doi.org/10.1016/j.cell.2015.02.045> PMID: 25860607; PubMed Central PMCID: PMC4397979.
18. Kim H, Yoo S, Zhou R, Xu A, Bernitz JM, Yuan Y, et al. Oncogenic role of SFRP2 in p53-mutant osteosarcoma development via autocrine and paracrine mechanism. *Proc Natl Acad Sci U S A.* 2018; 115(47):E11128–E37. <https://doi.org/10.1073/pnas.1814044115> PMID: 30385632; PubMed Central PMCID: PMC6255152.

19. Zhou R, Xu A, Gingold J, Strong LC, Zhao R, Lee DF. Li-Fraumeni Syndrome Disease Model: A Platform to Develop Precision Cancer Therapy Targeting Oncogenic p53. *Trends Pharmacol Sci.* 2017; 38(10):908–27. <https://doi.org/10.1016/j.tips.2017.07.004> PMID: 28818333; PubMed Central PMCID: PMC5752137.
20. Pang LK, Pena M, Zhao R, Lee DF. Modeling of osteosarcoma with induced pluripotent stem cells. *Stem Cell Res.* 2020; 49:102006. <https://doi.org/10.1016/j.scr.2020.102006> PMID: 33022533.
21. Kotini AG, Chang CJ, Boussaad I, Delrow JJ, Dolezal EK, Nagulapally AB, et al. Functional analysis of a chromosomal deletion associated with myelodysplastic syndromes using isogenic human induced pluripotent stem cells. *Nat Biotechnol.* 2015; 33(6):646–55. <https://doi.org/10.1038/nbt.3178> PMID: 25798938; PubMed Central PMCID: PMC4464949.
22. Mulero-Navarro S, Sevilla A, Roman AC, Lee DF, D'Souza SL, Pardo S, et al. Myeloid Dysregulation in a Human Induced Pluripotent Stem Cell Model of PTPN11-Associated Juvenile Myelomonocytic Leukemia. *Cell Rep.* 2015; 13(3):504–15. <https://doi.org/10.1016/j.celrep.2015.09.019> PMID: 26456833; PubMed Central PMCID: PMC4618050.
23. Crespo M, Vilar E, Tsai SY, Chang K, Amin S, Srinivasan T, et al. Colonic organoids derived from human induced pluripotent stem cells for modeling colorectal cancer and drug testing. *Nat Med.* 2017; 23(7):878–84. <https://doi.org/10.1038/nm.4355> PMID: 28628110; PubMed Central PMCID: PMC6055224.
24. Fusaki N, Ban H, Nishiyama A, Saeki K, Hasegawa M. Efficient induction of transgene-free human pluripotent stem cells using a vector based on Sendai virus, an RNA virus that does not integrate into the host genome. *Proc Jpn Acad Ser B Phys Biol Sci.* 2009; 85(8):348–62. <https://doi.org/10.2183/pjab.85.348> PMID: 19838014; PubMed Central PMCID: PMC3621571.
25. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell.* 2006; 126(4):663–76. <https://doi.org/10.1016/j.cell.2006.07.024> PMID: 16904174.
26. Rouhani FJ, Nik-Zainal S, Wuster A, Li Y, Conte N, Koike-Yusa H, et al. Mutational History of a Human Cell Lineage from Somatic to Induced Pluripotent Stem Cells. *PLoS Genet.* 2016; 12(4):e1005932. <https://doi.org/10.1371/journal.pgen.1005932> PMID: 27054363; PubMed Central PMCID: PMC4824386.
27. Zhao Q, Gregory CA, Lee RH, Reger RL, Qin L, Hai B, et al. MSCs derived from iPSCs with a modified protocol are tumor-tropic but have much less potential to promote tumors than bone marrow MSCs. *Proc Natl Acad Sci U S A.* 2015; 112(2):530–5. <https://doi.org/10.1073/pnas.1423008112> PMID: 25548183; PubMed Central PMCID: PMC4299223.
28. Fang H, Niu K, Mo D, Zhu Y, Tan Q, Wei D, et al. RecQL4-Aurora B kinase axis is essential for cellular proliferation, cell cycle progression, and mitotic integrity. *Oncogenesis.* 2018; 7(9):68. <https://doi.org/10.1038/s41389-018-0080-4> PMID: 30206236; PubMed Central PMCID: PMC6134139.
29. Formosa LE, Dibley MG, Stroud DA, Ryan MT. Building a complex complex: Assembly of mitochondrial respiratory chain complex I. *Semin Cell Dev Biol.* 2018; 76:154–62. <https://doi.org/10.1016/j.semcdb.2017.08.011> PMID: 28797839.
30. Brandt U. Energy converting NADH:quinone oxidoreductase (complex I). *Annu Rev Biochem.* 2006; 75:69–92. <https://doi.org/10.1146/annurev.biochem.75.103004.142539> PMID: 16756485.
31. Molina JR, Sun Y, Protopopova M, Gera S, Bandi M, Bristow C, et al. An inhibitor of oxidative phosphorylation exploits cancer vulnerability. *Nat Med.* 2018; 24(7):1036–46. <https://doi.org/10.1038/s41591-018-0052-4> PMID: 29892070.
32. Vangapandu HV, Alston B, Morse J, Ayres ML, Wierda WG, Keating MJ, et al. Biological and metabolic effects of IACS-010759, an OxPhos inhibitor, on chronic lymphocytic leukemia cells. *Oncotarget.* 2018; 9(38):24980–91. <https://doi.org/10.18632/oncotarget.25166> PMID: 29861847; PubMed Central PMCID: PMC5982765.
33. Gupta S, De S, Srivastava V, Hussain M, Kumari J, Muniyappa K, et al. RECQL4 and p53 potentiate the activity of polymerase gamma and maintain the integrity of the human mitochondrial genome. *Carcinogenesis.* 2014; 35(1):34–45. <https://doi.org/10.1093/carcin/bgt315> PMID: 24067899.
34. Chi Z, Nie L, Peng Z, Yang Q, Yang K, Tao J, et al. RecQL4 cytoplasmic localization: implications in mitochondrial DNA oxidative damage repair. *Int J Biochem Cell Biol.* 2012; 44(11):1942–51. <https://doi.org/10.1016/j.biocel.2012.07.016> PMID: 22824301; PubMed Central PMCID: PMC3461334.
35. Wang JT, Xu X, Alontaga AY, Chen Y, Liu Y. Impaired p32 regulation caused by the lymphoma-prone RECQ4 mutation drives mitochondrial dysfunction. *Cell Rep.* 2014; 7(3):848–58. <https://doi.org/10.1016/j.celrep.2014.03.037> PMID: 24746816; PubMed Central PMCID: PMC4029353.
36. Davis T, Tivey HS, Brook AJ, Grimstead JW, Rokicki MJ, Kipling D. Activation of p38 MAP kinase and stress signalling in fibroblasts from the progeroid Rothmund-Thomson syndrome. *Age (Dordr).* 2013; 35

- (5):1767–83. <https://doi.org/10.1007/s11357-012-9476-9> PMID: 23001818; PubMed Central PMCID: PMC3776094.
37. Lin YH, Jewell BE, Gingold J, Lu L, Zhao R, Wang LL, et al. Osteosarcoma: Molecular Pathogenesis and iPSC Modeling. *Trends Mol Med*. 2017; 23(8):737–55. <https://doi.org/10.1016/j.molmed.2017.06.004> PMID: 28735817; PubMed Central PMCID: PMC5558609.
38. Choesmel V, Fribourg S, Aguisa-Toure AH, Pinaud N, Legrand P, Gazda HT, et al. Mutation of ribosomal protein RPS24 in Diamond-Blackfan anemia results in a ribosome biogenesis disorder. *Hum Mol Genet*. 2008; 17(9):1253–63. <https://doi.org/10.1093/hmg/ddn015> PMID: 18230666.
39. Chae H, Park J, Lee S, Kim M, Kim Y, Lee JW, et al. Ribosomal protein mutations in Korean patients with Diamond-Blackfan anemia. *Exp Mol Med*. 2014; 46:e88. <https://doi.org/10.1038/emm.2013.159> PMID: 24675553; PubMed Central PMCID: PMC3972785.
40. Zhou R, Xu A, Tu J, Liu M, Gingold JA, Zhao R, et al. Modeling Osteosarcoma Using Li-Fraumeni Syndrome Patient-derived Induced Pluripotent Stem Cells. *J Vis Exp*. 2018;(136). <https://doi.org/10.3791/57664> PMID: 29985349; PubMed Central PMCID: PMC6101728.
41. Jewell BE, Liu M, Lu L, Zhou R, Tu J, Zhu D, et al. Generation of an induced pluripotent stem cell line from an individual with a heterozygous RECQL4 mutation. *Stem Cell Res*. 2018; 33:36–40. <https://doi.org/10.1016/j.scr.2018.10.003> PMID: 30312871; PubMed Central PMCID: PMC6317900.
42. Lee DF, Su J, Ang YS, Carvajal-Vergara X, Mulero-Navarro S, Pereira CF, et al. Regulation of embryonic and induced pluripotency by aurora kinase-p53 signaling. *Cell Stem Cell*. 2012; 11(2):179–94. <https://doi.org/10.1016/j.stem.2012.05.020> PMID: 22862944.
43. Lee DF, Kuo HP, Chen CT, Hsu JM, Chou CK, Wei Y, et al. IKK beta suppression of TSC1 links inflammation and tumor angiogenesis via the mTOR pathway. *Cell*. 2007; 130(3):440–55. <https://doi.org/10.1016/j.cell.2007.05.058> PMID: 17693255.
44. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol*. 2014; 15(12):550. <https://doi.org/10.1186/s13059-014-0550-8> PMID: 25516281; PubMed Central PMCID: PMC4302049.
45. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci U S A*. 2005; 102(43):15545–50. <https://doi.org/10.1073/pnas.0506580102> PMID: 16199517; PubMed Central PMCID: PMC1239896.
46. Merico D, Isserlin R, Stueker O, Emili A, Bader GD. Enrichment map: a network-based method for gene-set enrichment visualization and interpretation. *PLoS One*. 2010; 5(11):e13984. <https://doi.org/10.1371/journal.pone.0013984> PMID: 21085593; PubMed Central PMCID: PMC2981572.
47. DePristo MA, Banks E, Poplin R, Garimella KV, Maguire JR, Hartl C, et al. A framework for variation discovery and genotyping using next-generation DNA sequencing data. *Nat Genet*. 2011; 43(5):491–8. <https://doi.org/10.1038/ng.806> PMID: 21478889.
48. Doi A, Park IH, Wen B, Murakami P, Aryee MJ, Irizarry R, et al. Differential methylation of tissue- and cancer-specific CpG island shores distinguishes human induced pluripotent stem cells, embryonic stem cells and fibroblasts. *Nat Genet*. 2009; 41(12):1350–3. <https://doi.org/10.1038/ng.471> PMID: 19881528.
49. Cibulskis K, Lawrence MS, Carter SL, Sivachenko A, Jaffe D, Sougnez C, et al. Sensitive detection of somatic point mutations in impure and heterogeneous cancer samples. *Nat Biotechnol*. 2013; 31(3):213–9. <https://doi.org/10.1038/nbt.2514> PMID: 23396013.
50. Sukhai MA, Misyura M, Thomas M, Garg S, Zhang T, Stickle N, et al. Somatic Tumor Variant Filtration Strategies to Optimize Tumor-Only Molecular Profiling Using Targeted Next-Generation Sequencing Panels. *J Mol Diagn*. 2019; 21(2):261–73. <https://doi.org/10.1016/j.jmoldx.2018.09.008> PMID: 30576869.
51. Kwon EM, Connelly JP, Hansen NF, Donovan FX, Winkler T, Davis BW, et al. iPSCs and fibroblast subclones from the same fibroblast population contain comparable levels of sequence variations. *Proc Natl Acad Sci U S A*. 2017; 114(8):1964–9. <https://doi.org/10.1073/pnas.1616035114> PMID: 28167771; PubMed Central PMCID: PMC5338363.
52. Sherry ST, Ward MH, Kholodov M, Baker J, Phan L, Smigielski EM, et al. dbSNP: the NCBI database of genetic variation. *Nucleic Acids Res*. 2001; 29(1):308–11. <https://doi.org/10.1093/nar/29.1.308> PMID: 11125122; PubMed Central PMCID: PMC29783.
53. Wang K, Li M, Hakonarson H. ANNOVAR: functional annotation of genetic variants from high-throughput sequencing data. *Nucleic Acids Res*. 2010; 38(16):e164. <https://doi.org/10.1093/nar/gkq603> PMID: 20601685.
54. Darzynkiewicz Z, Juan G, Bedner E. Determining cell cycle stages by flow cytometry. *Curr Protoc Cell Biol*. 2001;Chapter 8:Unit 8.4. <https://doi.org/10.1002/0471143030.cb0804s01> PMID: 18228389.

55. Chen CJ, Sgritta M, Mays J, Zhou H, Lucero R, Park J, et al. Therapeutic inhibition of mTORC2 rescues the behavioral and neurophysiological abnormalities associated with Pten-deficiency. *Nat Med.* 2019; 25(11):1684–90. <https://doi.org/10.1038/s41591-019-0608-y> PMID: 31636454; PubMed Central PMCID: PMC7082835.
56. Ma Y, Bai RK, Trieu R, Wong LJ. Mitochondrial dysfunction in human breast cancer cells and their transmitochondrial cybrids. *Biochim Biophys Acta.* 2010; 1797(1):29–37. <https://doi.org/10.1016/j.bbabo.2009.07.008> PMID: 19647716; PubMed Central PMCID: PMC2787670.



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

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-suppressive 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 μ M ascorbic acid, and 0.1 μ M 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: AATTGtagaaaATGCGGCCCGCCGACCAGAATCATGCAAGT GCGTAAGATAGTCGCGGGTCGGCGGCCCGCATctgtctgggG; S1m_Reverse: AATTCcccagcagATGCGGCCCGCCGACCCGC GACTATCTTACGCACTTGCATGATTCTGGTCGGCGGCC GCATttttacC), 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 ($\geq 50 \mu\text{m}$) 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 MgCl₂, 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 MgCl₂, 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 $\pm$ 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 ≥ 1 and fold change ≥ 5) and 81 downregulated genes (FPKM ≥ 1 and fold change ≥ 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

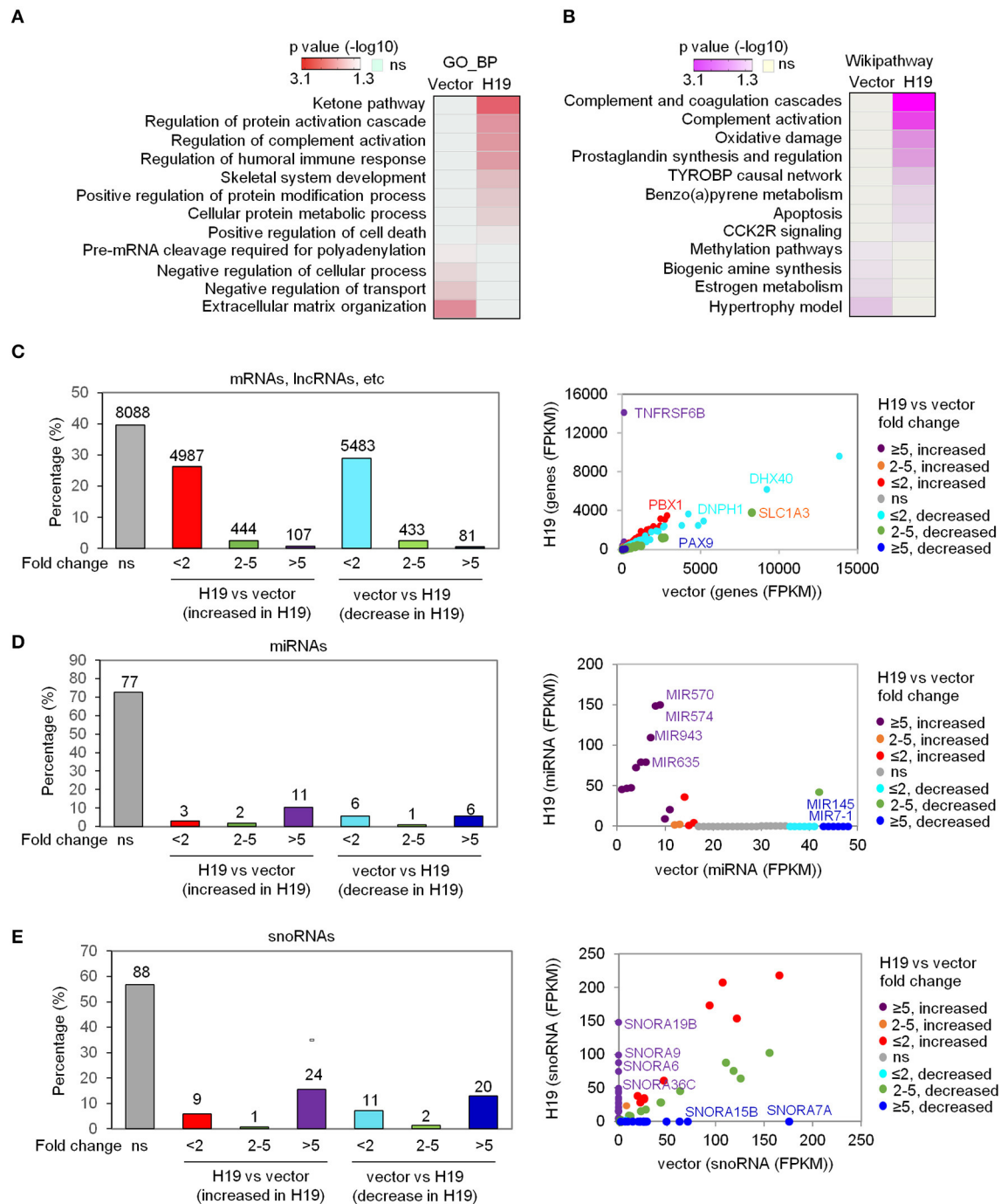


FIGURE 1 | H19 significantly affects miRNA and snoRNA expression. **(A,B)** Heatmaps show the GO_BP **(A)** and Wikipathway **(B)** categories enriched in either H19-expressing LFS osteoblasts (right column) or vector control LFS osteoblasts (left column) compared with each other based on mRNA expression 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.

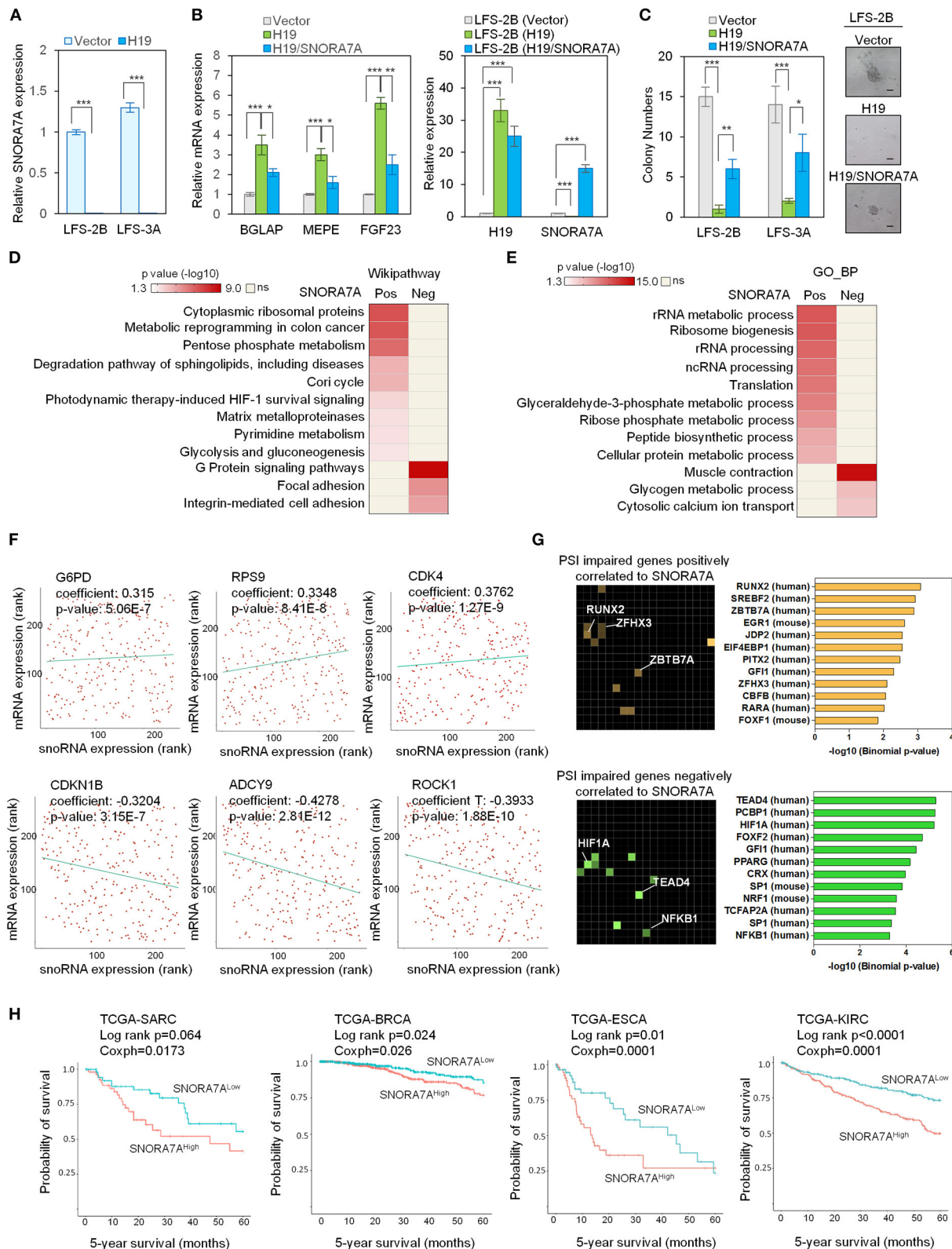


FIGURE 2 | Systems analyses of the oncogenic role of SNORA7A from TCGA datasets. **(A)** Ectopic expression of H19 downregulates SNORA7A expression in LFS osteoblasts. qRT-PCR data are represented as mean $\pm$ SEM; $n = 3$ biological replicates; statistical significance is determined using two-tailed 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 $\pm$ 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 50 μ m diameter. Data are represented as mean $\pm$ 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 PWMs 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 ZFH3 (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.

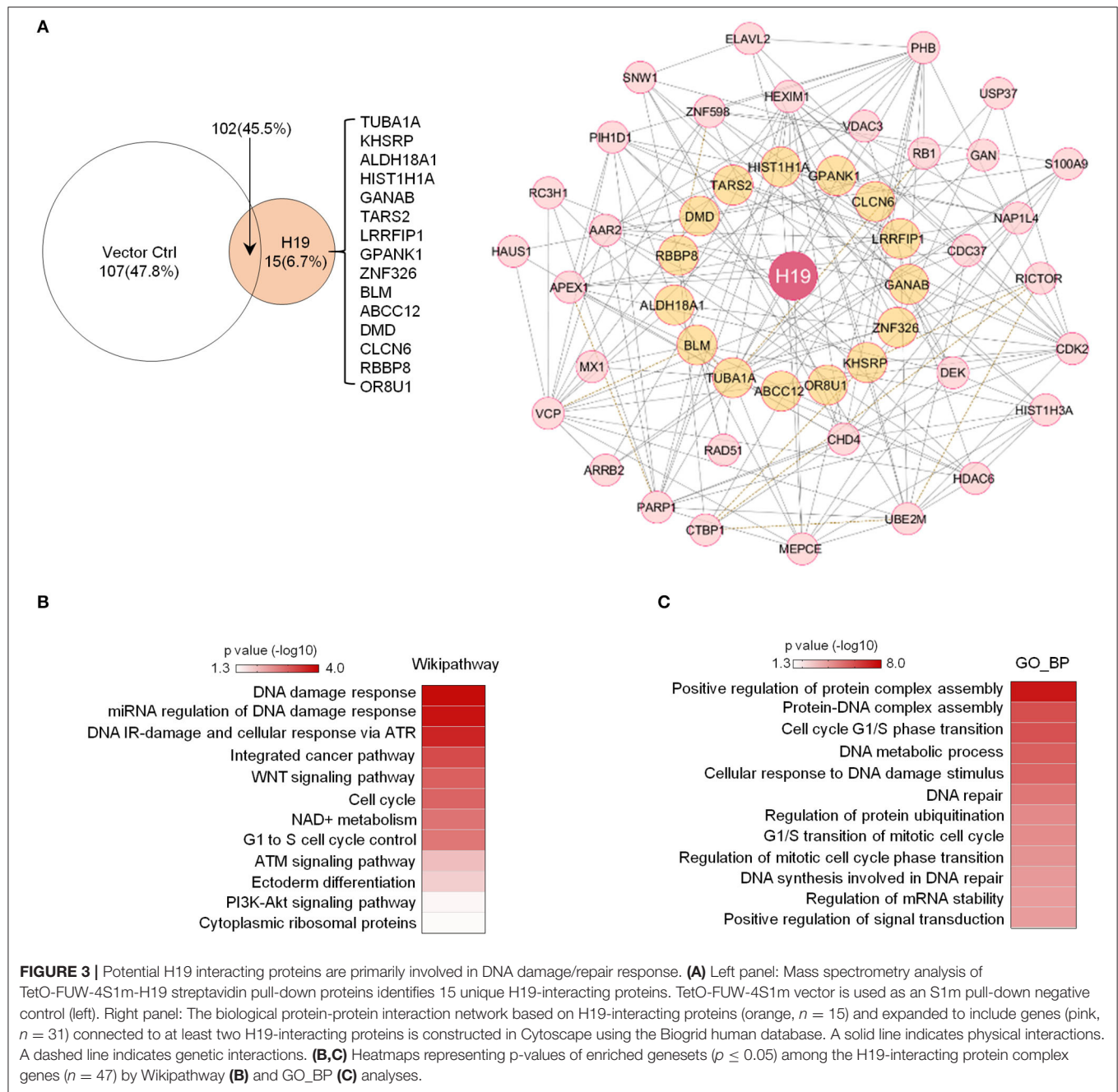
We next analyzed the expression of SNORA7A in multiple cancers using TCGA datasets and correlated its expression with patient survival data. The five-year overall survival analyses showed that high SNORA7A levels were significantly associated with poorer survival in multiple cancer types including sarcoma (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 $\times$ 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



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) (Gabori 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. SNORA7A 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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In memory of our mentor and friend IL (1953-2017), who inspired us with his lifelong passion to understand stem cell biology.

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>

REFERENCES

- Amelio, I., Mancini, M., Petrova, V., Cairns, R. A., Vikhrev, P., Nicolai, S., et al. (2018). p53 mutants cooperate with HIF-1 in transcriptional regulation of extracellular matrix components to promote tumor progression. *Proc. Natl. Acad. Sci. U.S.A.* 115, E10869–E10878. doi: 10.1073/pnas.1808314115
- Bachellerie, J. P., Cavaillie, J., and Huttenhofer, A. (2002). The expanding snoRNA world. *Biochimie* 84, 775–790. doi: 10.1016/S0300-9084(02)01402-5
- Carvajal-Vergara, X., Sevilla, A., D'Souza, S. L., Ang, Y. S., Schaniel, C., Lee, D. F., et al. (2010). Patient-specific induced pluripotent stem-cell-derived models of LEOPARD syndrome. *Nature* 465, 808–812. doi: 10.1038/nature09005
- Chen, E. Y., Tan, C. M., Kou, Y., Duan, Q., Wang, Z., Meirelles, G. V., et al. (2013). Enrichr: interactive and collaborative HTML5 gene list enrichment analysis tool. *BMC Bioinform.* 14:128. doi: 10.1186/1471-2105-14-128
- Chen, P. L., Liu, F., Cai, S., Lin, X., Li, A., Chen, Y., et al. (2005). Inactivation of CtIP leads to early embryonic lethality mediated by G1 restraint and to tumorigenesis by haploid insufficiency. *Mol. Cell Biol.* 25, 3535–3542. doi: 10.1128/MCB.25.9.3535-3542.2005
- Cheng, T., Xu, M., Qin, B., Wu, J., Tu, Y., Kang, L., et al. (2019). lncRNA H19 contributes to oxidative damage repair in the early age-related cataract by regulating miR-29a/TDG axis. *J. Cell Mol. Med.* 23, 6131–6139. doi: 10.1111/jcmm.14489
- Concetti, J., and Wilson, C. L. (2018). NFKB1 and cancer: friend or foe? *Cells* 7:9. doi: 10.3390/cells7090133
- David, C. J., Chen, M., Assanah, M., Canoll, P., and Manley, J. L. (2010). HnRNP proteins controlled by c-Myc deregulate pyruvate kinase mRNA splicing in cancer. *Nature* 463, 364–368. doi: 10.1038/nature08697

- Falaleeva, M., Pages, A., Matuszek, Z., Hidmi, S., Agranat-Tamir, L., Korotkov, K., et al. (2016). Dual function of C/D box small nucleolar RNAs in rRNA modification and alternative pre-mRNA splicing. *Proc. Natl. Acad. Sci. U.S.A.* 113, E1625–1634. doi: 10.1073/pnas.1519292113
- Gabory, A., Ripoche, M. A., Le Digarcher, A., Watrin, F., Ziyat, A., Forne, T., et al. (2009). H19 acts as a trans regulator of the imprinted gene network controlling growth in mice. *Development* 136, 3413–3421. doi: 10.1242/dev.036061
- Gazy, I., Zeevi, D. A., Renbaum, P., Zeligson, S., Eini, L., Bashari, D., et al. (2015). TODRA, a lncRNA at the RAD51 locus, is oppositely regulated to RAD51, and enhances RAD51-dependent DSB (double strand break) repair. *PLoS ONE* 10:e0134120. doi: 10.1371/journal.pone.0134120
- Gingold, J., Zhou, R., Lemischka, I. R., and Lee, D. F. (2016). Modeling cancer with pluripotent stem cells. *Trends Cancer* 2, 485–494. doi: 10.1016/j.trecan.2016.07.007
- Giovarelli, M., Bucci, G., Ramos, A., Bordo, D., Wilusz, C. J., Chen, C. Y., et al. (2014). H19 long noncoding RNA controls the mRNA decay promoting function of KSRP. *Proc. Natl. Acad. Sci. U.S.A.* 111, E5023–5028. doi: 10.1073/pnas.1415098111
- Gong, J., Li, Y., Liu, C. J., Xiang, Y., Li, C., Ye, Y., et al. (2017). A pan-cancer analysis of the expression and clinical relevance of small nucleolar RNAs in human cancer. *Cell Rep.* 21, 1968–1981. doi: 10.1016/j.celrep.2017.10.070
- Gravel, S., Chapman, J. R., Magill, C., and Jackson, S. P. (2008). DNA helicases Sgs1 and BLM promote DNA double-strand break resection. *Genes Dev.* 22, 2767–2772. doi: 10.1101/gad.503108
- Hao, Y., Crenshaw, T., Moulton, T., Newcomb, E., and Tycko, B. (1993). Tumour-suppressor activity of H19 RNA. *Nature* 365, 764–767. doi: 10.1038/365764a0
- Hsu, T. Y., Simon, L. M., Neill, N. J., Marcotte, R., Sayad, A., Bland, C. S., et al. (2015). The spliceosome is a therapeutic vulnerability in MYC-driven cancer. *Nature* 525, 384–388. doi: 10.1038/nature14985
- Hu, Q., Zhang, B., Chen, R., Fu, C., Fu, A. J., et al. (2019). ZFH3 is indispensable for ERbeta to inhibit cell proliferation via MYC downregulation in prostate cancer cells. *Oncogenesis* 8:28. doi: 10.1038/s41389-019-0138-y
- Hu, W. L., Jin, L., Xu, A., Wang, Y. F., Thorne, R. F., Zhang, X. D., et al. (2018). GUARDIN is a p53-responsive long non-coding RNA that is essential for genomic stability. *Nat. Cell Biol.* 20, 492–502. doi: 10.1038/s41556-018-0066-7
- Itzhaki, I., Maizels, L., Huber, I., Zwi-Dantsis, L., Caspi, O., Winterstern, A., et al. (2011). Modelling the long QT syndrome with induced pluripotent stem cells. *Nature* 471, 225–229. doi: 10.1038/nature09747
- Kallen, A. N., Zhou, X. B., Xu, J., Qiao, C., Ma, J., Yan, L., et al. (2013). The imprinted H19 lncRNA antagonizes let-7 microRNAs. *Mol. Cell* 52, 101–112. doi: 10.1016/j.molcel.2013.08.027
- Keniry, A., Oxley, D., Monnier, P., Kyba, M., Dandolo, L., Smits, G., et al. (2012). The H19 lincRNA is a developmental reservoir of miR-675 that suppresses growth and Igf1r. *Nat. Cell Biol.* 14, 659–665. doi: 10.1038/ncb2521
- Kim, H., Yoo, S., Zhou, R., Xu, A., Bernitz, J. M., Yuan, Y., et al. (2018). Oncogenic role of SFRP2 in p53-mutant osteosarcoma development via autocrine and paracrine mechanism. *Proc. Natl. Acad. Sci. U.S.A.* 115, E11128–E11137. doi: 10.1073/pnas.1814044115
- Koh, C. M., Bezzi, M., Low, D. H., Ang, W. X., Teo, S. X., Gay, F. P., et al. (2015). MYC regulates the core pre-mRNA splicing machinery as an essential step in lymphomagenesis. *Nature* 523, 96–100. doi: 10.1038/nature14351
- Komori, T. (2019). Regulation of proliferation, differentiation and functions of osteoblasts by Runx2. *Int. J. Mol. Sci.* 20:7. doi: 10.3390/ijms20071694
- Koukourakis, M. I., Giatromanolaki, A., Sivridis, E., Simopoulos, C., Turley, H., Talks, K., et al. (2002). Hypoxia-inducible factor (HIF1A and HIF2A), angiogenesis, and chemoradiotherapy outcome of squamous cell head-and-neck cancer. *Int. J. Radiat. Oncol. Biol. Phys.* 53, 1192–1202. doi: 10.1016/s0360-3016(02)02848-1
- Kuleshov, M. V., Jones, M. R., Rouillard, A. D., Fernandez, N. F., Duan, Q., Wang, Z., et al. (2016). Enrichr: a comprehensive gene set enrichment analysis web server 2016 update. *Nucleic Acids Res.* 44, W90–97. doi: 10.1093/nar/gkw377
- Lee, D. F., Kuo, H. P., Chen, C. T., Hsu, J. M., Chou, C. K., Wei, Y., et al. (2007). IKK beta suppression of TSC1 links inflammation and tumor angiogenesis via the mTOR pathway. *Cell* 130, 440–455. doi: 10.1016/j.cell.2007.05.058
- Lee, D. F., Kuo, H. P., Liu, M., Chou, C. K., Xia, W., Du, Y., et al. (2009a). KEAP1 E3 ligase-mediated downregulation of NF-kappaB signaling by targeting IKKbeta. *Mol. Cell* 36, 131–140. doi: 10.1016/j.molcel.2009.07.025
- Lee, D. F., Su, J., Ang, Y. S., Carvajal-Vergara, X., Mulero-Navarro, S., Pereira, C. F., et al. (2012a). Regulation of embryonic and induced pluripotency by aurora kinase-p53 signaling. *Cell Stem Cell* 11, 179–194. doi: 10.1016/j.stem.2012.05.020
- Lee, D. F., Su, J., Kim, H. S., Chang, B., Papatsenko, D., Zhao, R., et al. (2015). Modeling familial cancer with induced pluripotent stem cells. *Cell* 161, 240–254. doi: 10.1016/j.cell.2015.02.045
- Lee, D. F., Su, J., Sevilla, A., Gingold, J., Schaniel, C., and Lemischka, I. R. (2012b). Combining competition assays with genetic complementation strategies to dissect mouse embryonic stem cell self-renewal and pluripotency. *Nat. Protoc.* 7, 729–748. doi: 10.1038/nprot.2012.018
- Lee, G., Papapetrou, E. P., Kim, H., Chambers, S. M., Tomishima, M. J., Fasano, C. A., et al. (2009b). Modelling pathogenesis and treatment of familial dysautonomia using patient-specific iPSCs. *Nature* 461, 402–406. doi: 10.1038/nature08320
- Leppik, K., and Stoecklin, G. (2014). An optimized streptavidin-binding RNA aptamer for purification of ribonucleoprotein complexes identifies novel ARE-binding proteins. *Nucleic Acids Res.* 42:e13. doi: 10.1093/nar/gkt956
- Li, A., Mallik, S., Luo, H., Jia, P., Lee, D. F., and Zhao, Z. (2020). H19, a long non-coding, RNA, mediates transcription factors and target genes through interference of MicroRNAs in pan-cancer. *Mol. Ther. Nucleic Acids* 21, 180–191. doi: 10.1016/j.omtn.2020.05.028
- Lian, Q., Lye, E., Suan Yeo, K., Khia Way Tan, E., Salto-Tellez, M., Liu, T. M., et al. (2007). Derivation of clinically compliant MSCs from CD105+, CD24- differentiated human ESCs. *Stem Cells* 25, 425–436. doi: 10.1634/stemcells.2006-0420
- Liang, J., Wen, J., Huang, Z., Chen, X. P., Zhang, B. X., and Chu, L. (2019). Small nucleolar RNAs: insight into their function in cancer. *Front. Oncol.* 9:587. doi: 10.3389/fonc.2019.00587
- Liang, W. C., Fu, W. M., Wong, C. W., Wang, Y., Wang, W. M., Hu, G. X., et al. (2015). The lncRNA H19 promotes epithelial to mesenchymal transition by functioning as miRNA sponges in colorectal cancer. *Oncotarget* 6, 22513–22525. doi: 10.18632/oncotarget.4154
- Lin, K. C., Park, H. W., and Guan, K. L. (2017a). Regulation of the hippo pathway transcription factor TEAD. *Trends Biochem. Sci.* 42, 862–872. doi: 10.1016/j.tibs.2017.09.003
- Lin, Y. H., Jewell, B. E., Gingold, J., Lu, L., Zhao, R., Wang, L. L., et al. (2017b). Osteosarcoma: molecular pathogenesis and iPSC modeling. *Trends Mol. Med.* 23, 737–755. doi: 10.1016/j.molmed.2017.06.004
- Liu, M., Tu, J., Gingold, J. A., Kong, C. S. L., and Lee, D. F. (2018). Cancer in a dish: progress using stem cells as a platform for cancer research. *Am. J. Cancer Res.* 8, 944–954.
- Liu, X. S., Haines, J. E., Mehanna, E. K., Genet, M. D., Ben-Sahra, I., Asara, J. M., et al. (2014). ZBTB7A acts as a tumor suppressor through the transcriptional repression of glycolysis. *Genes Dev.* 28, 1917–1928. doi: 10.1101/gad.245910.114
- Luo, M., Li, Z., Wang, W., Zeng, Y., Liu, Z., and Qiu, J. (2013). Long non-coding RNA H19 increases bladder cancer metastasis by associating with EZH2 and inhibiting E-cadherin expression. *Cancer Lett.* 333, 213–221. doi: 10.1016/j.canlet.2013.01.033
- Lustig-Yariv, O., Schulze, E., Komitowski, D., Erdmann, V., Schneider, T., de Groot, N., et al. (1997). The expression of the imprinted genes H19 and IGF-2 in choriocarcinoma cell lines. Is H19 a tumor suppressor gene? *Oncogene* 15, 169–177. doi: 10.1038/sj.onc.1201175
- Ma, H., Yuan, L., Li, W., Xu, K., and Yang, L. (2018). The lncRNA H19/miR-193a-3p axis modifies the radio-resistance and chemotherapeutic tolerance of hepatocellular carcinoma cells by targeting PSEN1. *J. Cell Biochem.* 119, 8325–8335. doi: 10.1002/jcb.26883
- Marcel, V., Ghayad, S. E., Belin, S., Therizols, G., Morel, A. P., Solano-Gonzalez, E., et al. (2013). p53 acts as a safeguard of translational control by regulating fibrillarin and rRNA methylation in cancer. *Cancer Cell* 24, 318–330. doi: 10.1016/j.ccr.2013.08.013
- Mei, Y., Liao, J., Shen, J., Yu, L., Liu, B., Liu, L., et al. (2012). Small nucleolar RNA 42 acts as an oncogene in lung tumorigenesis. *Oncogene* 31, 2794–2804. doi: 10.1038/onc.2011.449
- Mulero-Navarro, S., Sevilla, A., Roman, A. C., Lee, D. F., D'Souza, S. L., Pardo, S., et al. (2015). Myeloid dysregulation in a human induced pluripotent stem cell

- model of PTPN11-associated juvenile myelomonocytic leukemia. *Cell Rep.* 13, 504–515. doi: 10.1016/j.celrep.2015.09.019
- Sanchez Calle, A., Kawamura, Y., Yamamoto, Y., Takeshita, F., and Ochiya, T. (2018). Emerging roles of long non-coding RNA in cancer. *Cancer Sci.* 109, 2093–2100. doi: 10.1111/cas.13642
- Shi, Z., He, F., Chen, M., Hua, L., Wang, W., Jiao, S., et al. (2017). DNA-binding mechanism of the Hippo pathway transcription factor TEAD4. *Oncogene* 36, 4362–4369. doi: 10.1038/onc.2017.24
- Ulitsky, I., and Bartel, D. P. (2013). lincRNAs: genomics, evolution, and mechanisms. *Cell* 154, 26–46. doi: 10.1016/j.cell.2013.06.020
- Wan, G., Hu, X., Liu, Y., Han, C., Sood, A. K., Calin, G. A., et al. (2013). A novel non-coding RNA lncRNA-JADE connects DNA damage signalling to histone H4 acetylation. *EMBO J.* 32, 2833–2847. doi: 10.1038/emboj.2013.221
- Wu, L., Chang, L., Wang, H., Ma, W., Peng, Q., and Yuan, Y. (2018). Clinical significance of C/D box small nucleolar RNA U76 as an oncogene and a prognostic biomarker in hepatocellular carcinoma. *Clin. Res. Hepatol. Gastr.* 42, 82–91. doi: 10.1016/j.clinre.2017.04.018
- Wu, M., Deng, L., Zhu, G., and Li, Y. P. (2010). G Protein and its signaling pathway in bone development and disease. *Front. Biosci.* 15:957–985. doi: 10.2741/3656
- Yagi, T., Ito, D., Okada, Y., Akamatsu, W., Nihei, Y., Yoshizaki, T., et al. (2011). Modeling familial Alzheimer's disease with induced pluripotent stem cells. *Hum. Mol. Genet.* 20, 4530–4539. doi: 10.1093/hmg/ddr394
- Yoshimizu, T., Miroglio, A., Ripoché, M. A., Gabory, A., Vernucci, M., Riccio, A., et al. (2008). The H19 locus acts *in vivo* as a tumor suppressor. *Proc. Natl. Acad. Sci. U.S.A.* 105, 12417–12422. doi: 10.1073/pnas.0801540105
- You, Z., and Bailis, J. M. (2010). DNA damage and decisions: CtIP coordinates DNA repair and cell cycle checkpoints. *Trends Cell Biol.* 20, 402–409. doi: 10.1016/j.tcb.2010.04.002
- Zhang, Y., Xu, C., Gu, D., Wu, M., Yan, B., Xu, Z., et al. (2017). H/ACA box small nucleolar RNA 7A promotes the self-renewal of human umbilical cord mesenchymal stem cells. *Stem Cells* 35, 222–235. doi: 10.1002/stem.2490
- Zheng, Z. G., Xu, H., Suo, S. S., Xu, X. L., Ni, M. W., Gu, L. H., et al. (2016). The essential role of H19 contributing to cisplatin resistance by regulating glutathione metabolism in high-grade serous ovarian cancer. *Sci. Rep.* 6:26093. doi: 10.1038/srep26093
- Zhou, R., Xu, A., Gingold, J., Strong, L. C., Zhao, R., and Lee, D. F. (2017). Li-fraumeni syndrome disease model: a platform to develop precision cancer therapy targeting oncogenic p53. *Trends Pharmacol. Sci.* 38, 908–927. doi: 10.1016/j.tips.2017.07.004
- Zhou, R., Xu, A., Tu, J., Liu, M., Gingold, J. A., Zhao, R., et al. (2018). Modeling osteosarcoma using li-fraumeni syndrome patient-derived induced pluripotent stem cells. *J. Vis. Exp.* 13:57664. doi: 10.3791/57664
- Zhu, D., Kong, C. S. L., Gingold, J. A., Zhao, R., and Lee, D. F. (2018). Induced pluripotent stem cells and induced pluripotent cancer cells in cancer disease modeling. *Adv. Exp. Med. Biol.* 1119, 169–183. doi: 10.1007/5584_2018_257
- Zhu, Q. N., Wang, G., Guo, Y., Peng, Y., Zhang, R., Deng, J. L., et al. (2017). LncRNA H19 is a major mediator of doxorubicin chemoresistance in breast cancer cells through a cullin4A-MDR1 pathway. *Oncotarget* 8, 91990–92003. doi: 10.18632/oncotarget.21121

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.

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