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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Neurofibromatosis type 1 (NF1) is a common cancer predisposition syndrome, affecting 1 in 3,000 individuals worldwide. While most of the cancers are benign neoplasms (neurofibromas, low-grade gliomas), 10-13% of patients will develop an aggressive sarcoma, termed a malignant peripheral nerve sheath tumor (MPNST). Composed of high-grade neoplastic Schwann cells, MPNST most often arise from a benign precursor lesion, such as plexiform neurofibroma (PN). Unfortunately, even with aggressive multi-modality therapy, these cancers recur in >50% of individuals, and most patients die within five years of diagnosis. For this reason, there is an unmet need for better therapeutic modalities. To this end, our group has defined the molecular heterogeneity of MPNST using sequencing of patient-derived xenografts (PDX) models. We have demonstrated that MPNST exhibit a high degree of aneuploidy, while their benign PN counterparts are usually diploid. Additionally, we showed that MPNST uniformly demonstrate gains involving the long arm of chromosome 8 (Chr8q), where several cancer-related genes are located. These genes are highly expressed in primary MPNST, and are maintained in their corresponding PDX. Pathway analyses suggest that Chr8q gain leads to increased expression of genes involved in diverse processes including connective tissue development, stem cell maintenance, and ribosomal RNA processing offering potential mechanisms for Chr8q cancer promotion. Moreover, leveraging The Cancer Genome Atlas (TCGA) database, we found that Chr8q gain was associated with reduced overall survival (OS) in patients with soft tissue sarcomas, which may explain the poor prognosis of MPNST. **Based on these observations, we hypothesize that Chr8q gain is a critical driver of MPNST progression, and functions by increasing the expression of a set of cancer genes responsible for the tumor growth and progression.**

15. SUBJECT TERMS
Malignant peripheral nerve sheath tumor, MPNST, neurofibromatosis-1, NF1, sarcoma,

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1. INTRODUCTION:

Neurofibromatosis type 1 (NF1) is an autosomal dominant tumor predisposition syndrome with a prevalence of 1:2500-3000 worldwide. MPNST occur in individuals with NF1 at a significantly elevated estimated lifetime frequency of 8-13%. In the setting of NF1, MPNST arise from malignant transformation of a benign precursor lesion, a plexiform neurofibroma (PN). Despite surgical resection, radiation, and chemotherapy, MPNST recur in almost 50% of individuals, and most people die within five years of diagnosis. A better understanding of these aggressive cancers is imperative to improve new therapeutic approaches. We previously analyzed the CNV profiles of patient MPNST, corresponding PDX and a set of PN. We found a high degree of aneuploidy in NF1-MPNST, whereas the PN samples harbored diploid genomes. Notably, 80% of the cases showed a Chr8q gain, but this event was not observed in any of the examined PN. Additionally, we previously showed that Chr8q gain is associated with poor overall survival for soft tissue sarcomas. Comparing our bulk RNA sequencing data, we also observed many differentially expressed genes in tumors that had Chr8gain compared to those that did not. We hypothesize that Chr8q gain induces genome-wide perturbations, leading to an altered transcriptome and proteome that promotes cancer progression. By characterizing the effects of Chr8q gain in MPNST alongside the protein and signaling changes, we can identify protein biomarkers that mediate the global effects of Chr8q gain in MPNST.

2. KEYWORDS:

Malignant peripheral nerve sheath tumor, MPNST, neurofibromatosis-1 (NF1), sarcoma, aneuploidy, chromosome 8

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Please see **Appendix A** for detailed major goals and target dates in the SOW:
Major Task 1: Define which genes on Chr8q are necessary for MPNST progression
Major Task 2: Determine whether expression of one or more of these genes is sufficient to drive MPNST formation
Major Task 3: Globally characterize the transcripts and proteins that are altered in MPNST through multi-regional tumor sampling
Major Task 4. Employ network-based modeling to identify mediators of the Chr8q phenotype
Major Task 5. Does the degree of Chr8 gain correlate with OS or other clinical factors?

What was accomplished under these goals?

Please see **Appendix B** and **Appendix C** for major accomplishments.

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

Nothing to Report.

N/A

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

Please see **Appendix D** on project plans.

4. IMPACT:

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

The most common malignancy affecting adults with neurofibromatosis type 1 (NF1) is the malignant peripheral nerve sheath tumor (MPNST), a highly aggressive sarcoma that commonly develops from benign plexiform neurofibromas. MPNST arises approximately in 8-13% of patients with NF1. Currently there are few therapeutic options, and the vast majority of people with these cancers will die within 5 years of diagnosis. Moreover, there is a pressing need to identify accurate biological markers of plexiform neurofibroma malignant transformation. While NF1 loss is necessary for MPNST development, it is not sufficient for malignant transformation. This conclusion is supported by numerous genetically-engineered mouse studies, demonstrating that conditional Nf1 gene inactivation in Schwann cell precursors results in benign plexiform neurofibroma formation only.In both mouse and human MPNST, alterations in the TP53, CDKN2A, and EED/SUZ12 genes have been reported as cooperating secondary genetic alterations that promote MPNST development. However, none of these mutations are universal to MPNST. In this regard, loss of CDKN2A occurs in approximately 70% of cases; EED/SUZ12 mutations occur in 60-70% of cases, and TP53 mutations occur in only 25% of cases. Importantly, the role of copy number alterations and their impact on cellular signaling that gives rise to MPNST pathogenesis has been understudied. Current treatment options for NF1-MPNST are limited, with surgery for localized disease and radiation to reduce the risk of local recurrence. One of the major barriers to the improving outcomes for these patients is the lack of preclinical models that accurately represent the genetic heterogeneity of these cancers. To address this, we generated and fully characterized eight NF1-MPNST PDX from eight corresponding biopsy-proven NF1-MPNST,representing the largest characterized set of NF1-MPNST PDX reported to date. Whole exome and bulk RNA sequencing showed TP53 mutations in 12.5% (1/8 pairs) and SUZ12 mutations in 62.5% (5/8 pairs), with the other 25% (2/8 pairs) harboring mutations in other cancer related pathways, such as DNA repair genes. Collectively these models more accurately reflect the heterogeneity seen in human MPNST. In addition, we analyzed the CNV profiles of patient MPNST, corresponding PDX and a set of PN. We found a high degree of aneuploidy in NF1-MPNST, whereas the PN samples harbored diploid genomes. We next examined the clonal structure of each PDX generated with single cell data using the inferCNV method and found that all PDX exhibited Chr8q gain in all or a portion of the tumor cells, showing that Chr8q gain was the most prevalent and dominant CNV, present in >90% of cells. To identify potential candidates residing on Chr8, we searched an internal database of cancer related genes as well as the Database of Curated Mutations We next interrogated the expression levels of genes on Chr8q using our bulk RNAseq data. The six genes with the highest expression included *RECQL4, SOX17, HEY1, C-MYC, RAD21,* and *UBR5*. ***Given the high levels of expression of these genes, it is likely that one or more of these candidates promotes progression of MPNST. This work has the potential to identify which of these genes are drivers and potential targets for MPNST therapy.***

What was the impact on other disciplines?

The identification of biomarkers in our studies have the potential to be extended to other types of sarcomas to potentially develop more effective therapies.

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

We are in the process of selecting a replacement MPNST cell line for the JH-2-009 cells due to STR profiling results. We have conducted the parallel experiments in the two other listed MPNST cell-lines, JH-2-002 and MPNST-724, including generation of the CRISPR/Cas9 knockout lines and IncuCyte live cell experiments. MYC and RAD21 do not appear to have a functional significance in MPNST. As such, we are not further evaluating these genes. We are performing a CRISPR screen to evaluate the importance of all Chr8 genes.

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

For the studies in Aims 2-3, we obtained subject protocol approval by the Washington University (WU) Institutional Review Board (IRB) and by the Human Research Protection Office (HRPO) prior to this grant award beginning. Annual renewals occur each year. No changes have been made since protocol approval.

Significant changes in use or care of vertebrate animals

For the mouse studies in Aim 1, we obtained protocol approval from IACUC (protocol 22-041) on 3/15/22 and from ACURO. No changes have been made since approval.

Significant changes in use of biohazards and/or select agents

Nothing to report.

6. PRODUCTS:

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

Journal publications.

Nothing to report.

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

Nothing to report.

Other publications, conference papers and presentations.

Poster Presentation

Acar, S., Bhatia, H., Borcherding, D., Jones, P., Lyu, Y., Zhang, X., He, K., Hirbe, A.C.. (2023, June 25). *UBR5, a chromosome 8 gene, regulates proliferation and cell survival in MPNST* [Poster presented]. Children's Tumor Foundation NF Conference 2023. Scottsdale AZ, USA.

Acar, S., Bhatia, H., Borcherding, D., Jones, P., Lyu, Y., Zhang, X., He, K., Hirbe, A.C.. (2023, October 12). *UBR5 is a critical regulator of cell survival in MPNST* [Poster presented]. The Midwest Society for Pediatric Research. Chicago, IL, USA.

Oral Presentation

Acar, S., Bhatia, H., Borcherding, D., Jones, P., Lyu, Y., Zhang, X., He, K., Hirbe, A.C.. (2022, November 03). *UBR5, a chromosome 8 gene, promotes tumor growth of malignant peripheral nerve sheath tumors (MPNST)* [Oral Presentation]. Neurofibromatosis Young Investigators' Forum. Houston, TX, USA

Acar, S., Bhatia, H., Borcherding, D., Jones, P., Lyu, Y., Zhang, X., He, K., Hirbe, A.C.. (2023, February 28). *UBR5 regulates tumor growth of malignant peripheral nerve sheath tumors (MPNST)* [Oral Presentation]. Rare Disease Day at Washington University St.Louis School of Medicine. St. Louis, MO, USA.

Acar, S., Bhatia, H., Borcherding, D., Jones, P., Lyu, Y., Zhang, X., He, K., Hirbe, A.C.. (2023, April 29). *UBR5, a chromosome 8 gene, promotes tumor growth of malignant peripheral nerve sheath tumors (MPNST)* [Oral Presentation]. Pediatric Academic Societies (PAS) Meeting. Washington , DC, USA.

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

1. Hirbe lab website at Washington University: https://hirbelab.wustl.edu/

- **Technologies or techniques**

Nothing to report.

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

Nothing to report.

- **Other Products**

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: Angela Hirbe, M.D./Ph.D.
Project Role: PI
Researcher Identifier (e.g. ORCID ID): 0000-0003-1719-0771
Nearest person month worked: 1.2
Contribution to Project: Dr. Hirbe coordinated and supervised all the research activities of this grant project.

Name: Dana Borcherding, Ph.D.
Project Role: Senior Scientist
Researcher Identifier (e.g. ORCID ID): 0000-0002-8849-2139
Nearest person month worked: 3
Contribution to Project: Dr. Borcherding conducted and supervised research activities and analyzed data in this grant project, including IncuCyte assays and transfections.

Name: Simge Acar, MD
Project Role: Visiting Researcher
Researcher Identifier (e.g. ORCID ID): 0000-0002-9757-5059
Nearest person month worked: 3
Contribution to Project: Dr. Acar conducted research activities and analyzed daha in this grant project including Incucyte assays, collection of patient samples and cell culture.

Name: John Chrisinger, M.D.
Project Role: Assistant Professor, Pathology & Immunology
Researcher Identifier (e.g. ORCID ID): 0000-0002-7138-0923
Nearest person month worked: 1
Contribution to Project: Dr. Chrisinger provided pathology expertise for IHC scoring.

Name: Himanshi Bhatia
Project Role: Postdoctoral Fellow
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 1
Contribution to Project: Dr. Bhatia conducted and supervised research activities and analyzed data in this grant project, including IncuCyte assays, qPCR, transfections and mouse experiments.

Name: Kuangying Yang
Project Role: Undergraduate Student
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 3
Contribution to Project: CRISPR screen to identify other important Chr8 genes

Name: xxxx
Project Role: Undergraduate Student
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked:
Contribution to Project:

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?

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: N/A

QUAD CHARTS: N/A

9. APPENDICES: See Appendices A-D.

STATEMENT OF WORK
PROPOSED START DATE: September 1, 2022

Washington University
660 S. Euclid Ave, St. Louis, MO 63110
PI: Angela C Hirbe, MD, PhD

Specific Aim 1: Define the genes on Chr8q that are essential for MPNST progression.	**Timeline (Original)**	**Washington University**	**Percent Complete**	**Timeline (New)**
Major Task 1: *Define which genes on Chr8q are necessary for MPNST progression.*	Months			
Subtask 1: ACURO/HRPO Approval	0	Dr. Hirbe (obtained prior to funding)	100%	0
Subtask 1: CRISPR/Cas9 KO of *C-MYC* in MPNST 724, JH2-002, and JH2-009 (2 lines each)	0-3	Dr. Hirbe	100%	0-3
Subtask 2: CRISPR/Cas9 KO of *RAD21* in MPNST 724, JH2-002, and JH2-009 (2 lines each); addition of KO of *RAD21* to 2 clones with *C-MYC* KD to generate the triple KO lines.	3-6	Dr. Hirbe	50%	3-18
Subtask 3: CRISPR/Cas9 KO of *UBR5* in MPNST 724, JH2-002, and JH2-009 (2 lines each); addition of KO of *UBR5* to 2 clones with *C-MYC/RAD21* KD to generate the triple KO lines.	6-9	Dr. Hirbe	50%	6-18
Subtask 4: Incucyte Live Cell Analysis system to assess the effects of KD of each gene as well as the triple KO on (1) cell proliferation (2) cell death and (3) invasion and migration.	9-12	Dr. Hirbe	50%	12-18
Subtask 5: Screen other Chr8 genes if necessary; utilization of trisomy 8 *NF1 -/-* hiPSC if necessary (these are currently being generated)	12-24	Dr. Hirbe and Genome Engineering and iPSC core	50%	6-18
Subtask 6: Organization of data on Synapse	35-36	Dr. Allaway	On Target	35-26
Milestone(s) Understand which genes on chr8 are necessary for MPNST progression.	**12-24**		On Target	18-24
Major Task 2: *Determine whether expression of one or more of these genes is* ***sufficient*** *to drive MPNST formation.*				
Subtask 1: Lentiviral transductions to express *UBR5* in *NF1* -/- hiPSC	9-10	Dr. Hirbe	On Target	15-18
Subtask 2: *UBR5*, in *NF1* -/- hiPSC SCP differentiation	10-11	Dr. Hirbe	On Target	18-21
Subtask 3: Colony formation assays *in vitro* using the Incucyte Live system comparing the	11-13	Dr. Hirbe	On Target	18-21

3 overexpression lines to control *NF1 -/-* hiPSC SCP				
Subtask 4: 10^6 Chr8 gene-hiPSC-SCPs will be surgically implanted into the sciatic nerves of NRG mice and followed for up to 12 months for tumor formation; trisomy 8 *NF1 -/-* SCPs if necessary	13-25	Dr. Hirbe	On Target	15-27
Subtask 5: Breeding GFAP-Cre *Nf1* $^{flox/null}$ mice	0-36	Dr. Hirbe	On Target	12-36
Subtask 6: Sciatic nerve lentiviral injections to express *Ubr5* in GFAP-Cre *N; f1* $^{flox/null}$ mice and followed for up to 12 months for tumor formation	13-25		On Target	15-27
Subtask 7: Testing of other genes or utilization of trisomy 8 *NF1 -/-* hiPSC differentiated to SCPs if necessary	25-36	Dr. Hirbe	On Target	25-36
Subtask 8: Organization of data on Synapse	35-36	Dr. Allaway	On Target	35-36
Milestone(s) Achieved: Understand which Chr8 genes are sufficient for MPNST progression.	**25-36**		On Target	25-36
<u>Specific Aim 2</u>: We will define the dysregulated signaling pathways in cells with Chr8q gain.				
Major Task 3: *Globally characterize the transcripts and proteins that are altered in MPNST through multi-regional tumor sampling*.				
Subtask 1: Collect multiregional samples from 3 MPNST	0-18	Dr. Hirbe	50%	0-18
Subtask 2: WGS and copy number analysis	18-24	Dr. Hirbe	On Target	18-24
Subtask 3: Proteomics	18-24	Dr. Gosline	On Target	18-24
Subtask4: Single cell RNAseq	18-24	Dr. Shern	On Target	18-24
Subtask 5: Organization of raw data on synapse	24	Dr. Allaway	On Target	24
Milestone(s) Achieved: Performed the first multiregional genomic/proteomic analysis on MPNST.	**16**		On Target	16
Major Task 4. *Employ network-based modeling to identify mediators of the Chr8q phenotype*.				
Subtask 1: Identify transcriptional regulators and differentially expressed proteins and map them to published protein-protein interaction data.	24-36	Dr. Gosline	On Target	24-26
Milestone(s) Achieved: Understand how Chr8gain alters protein expression and signaling.	**20**		On Target	20
<u>Specific Aim 3</u>: Determine if the degree of Chr8 gain observed in FISH or cell-free				

DNA correlates with OS or development of resistance.				
Major Task 5. Does the degree of Chr8 gain correlate with OS or other clinical factors?				
Subtask 1: Pull and review 60 MPNST cases between Wash U, JHU, and NCI	0-12	Dr. Hirbe/Dr. Chrisinger	50%	0-18
Subtask 2: Have FFPE slides cut	12-18	Dr. Hirbe	On Target	18-24
Subtask 3: Perform FISH	18-21	Dr. Hirbe	On Target	24-27
Subtask 4: Score FISH	21-24	Dr. Hirbe	On Target	27-30
Subtask 5: Correlate FISH score with clinical parameters	24-30	Dr. Hirbe	On Target	30-34
Subtask 6: Organization of data on Synapse	30-32	Dr. Allaway	On Target	34-36
Subtask 7: Manuscript preparation	32-36	Dr. Hirbe	On Target	32-36
Milestone(s) Achieved: Determine if Chr8 is a prognostic biomarker	**36**		On Target	36

Appendix B: What was accomplished under these goals?

Major Accomplishments:

<u>Specific Aim 1</u>: Define the genes on Chr8q that are essential for MPNST progression.

Major Task 1: *Define which genes on Chr8q are necessary for MPNST progression.*

Accomplishments: We first evaluated the expression of two candidate chromosome 8 (Chr8) cancer-related genes, *UBR5* and *Rad21*, in MPNST and benign precursor PN tumors. RNAseq analysis showed elevated *UBR5* and *Rad21* expression in MPNST compared to PN tumors (**Fig. 1A**). Our previously established MPNST PDX lines were then examined for RNA expression of the Chr8 genes, *UBR5* and *Rad21.* In 13 matching pairs of MPNST parental tumors and PDX samples, *UBR5* and *Rad21* gene expression was similar in PDX tumors and their corresponding MPNST parental tumors (**Fig. 1B**), indicating that *UBR5* and *Rad21* overexpression is generally preserved in the PDX lines. Additionally, real-time qPCR revealed high levels of *UBR5* and *Rad21* expression in two human MPNST cell lines, JH-2-002 and MPNST724, compared to Schwann cells (**Fig. 1C**). Protein levels of UBR5 and Rad21 were also increased in the MPNST cell lines, JH-2-002 and MPNST724, vs. the ipNF95.6 PN cell line, as determined by an automated WES western blotting system (**Fig. 1D**).

To define the gene or genes essential for the selective advantage conferred by Chr8q gain, we performed CRISPR/Cas9-mediated knockout (KO) in two MPNST cell lines, MPNST 724 and JH-2-002. We generated single KO of each gene, a triple KO, and control line for MPNST 724 and JH-2-002 (**Fig. 2A-C**). We are in the process of finding a replacement cell line instead of JH-2-009 cells, due to STR profiling results. These combinations are listed in **Table 1**. Two clones for each genetic combination were generated in each cell line for a total of 20 lines. We subsequently performed Incucyte Live Cell Analysis system to assess the effects of KO of each gene on (1) cell proliferation, (2) cell death, and (3) migration.

MPNST 724 cells	**JH-2-002 cells**
Control	Control
C-MYC KO	*C-MYC* KO
UBR5 KO	*UBR5* KO
RAD21 KO	*RAD21* KO
C-MYC/UBR5/RAD21 KO	*C-MYC/UBR5/RAD21* KO

Table 1. Knockout (KO) of chromosome 8 genes in MPNST cell lines.

To explore the function of *UBR5* and *Rad21* genes *in vitro*, we first investigated the effects of CRISPR/Cas9-mediated knockdown of the *UBR5* gene in MPNST cells. In JH-2-002 and MPNST 724 cells, we confirmed via next generation sequencing (NGS) and qPCR that *UBR5* gene expression is decreased in heterozygous (+/-) CRISPR clones compared to wild-type (+/+) control clones (**Fig. 3A-B and E**). Partial knockout of *UBR5* in the heterozygous (+/-) UBR5 clones significantly decreased cell proliferation in both MPNST724 and JH-2-002 cell lines (**Fig. 3C-D and F-G**).

Given that CRISPR/Cas9-mediated *UBR5* heterozygous knockdown decreased cell proliferation, we also utilized shRNA-mediated knockdown of *UBR5* and *Rad21* genes individually in MPNST cell lines. Knockdown of *UBR5* gene expression by multiple targeted shRNA was confirmed by qPCR in two NF1-MPNST cell-lines, human JH-2-002 cells and mouse JW23.3 cells (**Fig. 4A and D**). Consistent with the CRISPR/Cas9 experiments, reduced *UBR5* expression in shRNA cells was associated with decreased cell proliferation in JH-2-002 and JW23.3 cells in IncuCyte live cell proliferation assays (**Fig. 4B and E**). Representative pictures of decreased cell proliferation in JH-2-002 and JW23.3 upon *UBR5* knockdown are shown in **Fig. 4C-F**. Similarly, shRNA knockdown reduced *UBR5* mRNA levels in a sporadic human MPNST cell line, MPNST 724, and proliferation was lower in *UBR5* shRNA cells than control cells (**Fig. 5A-C**).

Knockdown of the *Rad21* gene by shRNA decreased *Rad21* mRNA expression to less than 50% of control, as determined by qPCR (**Fig. 6A and C**). However, shRNA-mediated loss of *Rad21* did not significantly change cell proliferation in JH-2-002 and MPNST724 cell lines (**Fig 6B-D**). In order to obtain a better knockout, we generated Rad21 CRISPR-Cas9 heterzygous (+/-) and wild-type (+/+) controls in MPNST724 cells (**Fig. 7A-B**). Similar to the shRNA knockdown, heterozygous (+/-) Rad21 CRISPR/Cas9 clones did not reduce proliferation of MPNST 724 cells (**Fig. 7C**). In addition, partial knockout of *c-Myc* by CRISPR/Cas9 did not affect proliferation of MPNST724 cells (**data not shown**).

In IncuCyte cell death assays, apoptosis was significantly increased by 3-10 fold for CRISPR heterozygous *UBR5* (+/-) clones in both MPNST724 and JH-2-002 cell lines (**Fig. 8**). Apoptotic cells were visualized by YOYO-1 green fluorescent dye, as shown in representative pictures (**Fig. 8B and D**). Similarly, *UBR5* shRNA knockdown significantly stimulated apoptosis in JH-2002, JW23.3 and MPNST724 cell lines (**Fig. 9 and Fig. 10**). However, *Rad21* shRNA knockdown in MPNST724 and JH-2002 cells was not associated with significantly increased cell death (**Fig. 11**).

The effect of *UBR5* on MPNST cell migration was explored in IncuCyte scratch wound migration assays. For both MPNST724 and JH-2-002 cells, heterozygous *UBR5* (+/-) clones had slower wound healing than wild-type (+/+) controls, as determined by lower relative wound density in IncuCyte migration assays (**Fig. 12**). Thus, depletion of *UBR5* expression decreases MPNST cell migration. In contrast, migration of heterozygous *Rad21* (+/-) MPNST cells was not significantly different than that of wild-type controls (**Fig. 13**).

Major Task 2: *Determine whether expression of one or more of these genes is* ***sufficient*** *to drive MPNST formation.*

Accomplishments: We have generated and maintained *NF1* -/- hiPSC. Next, lentiviral transductions will be performed to express each *UBR5* in the *NF1* -/- hiPSC. The task is on target. We also have maintained Chr8gain- *NF1* -/- hiPSC generated by reprogramming trisomy 8 fibroblasts to hiPSC and then using CRISPR to knockdown *NF1.* This will allow us to determine if the gain of Chr8 in conjunction with *NF1* loss is sufficient for tumor formation.

<u>Specific Aim 2</u>: We will define the dysregulated signaling pathways in cells with Chr8q gain.

Major Task 3: *Globally characterize the transcripts and proteins that are altered in MPNST through multi-regional tumor sampling*.

Accomplishments: We are in the process of collecting samples for multi-regional tumor sampling. We have collected samples from two cases to date and are in the process of performing FISH. After we receive the results, samples will be sent the PNNL for proteomics analysis. The task is on target.

Major Task 4: *Employ network-based modeling to identify mediators of the Chr8q phenotype*.

Accomplishments: After we obtain multi-regional samples from three resection cases, and have performed the multi-omic experiments, we will employ our modeling to explain the link between Chr8q gain and the observed changes in gene and protein expression. The task is on target.

<u>Specific Aim 3</u>: Determine if the degree of Chr8 gain observed in FISH or cell-free DNA correlates with OS or development of resistance.

Major Task 5: *Does the degree of Chr8 gain correlate with OS or other clinical factors?*

Accomplishments: We have pulled 30 cases to review and do FISH analysis so far. We are planning to pull 30 more cases and continue with FISH. The task is on target.

Appendix C: Major Accomplishments Figures

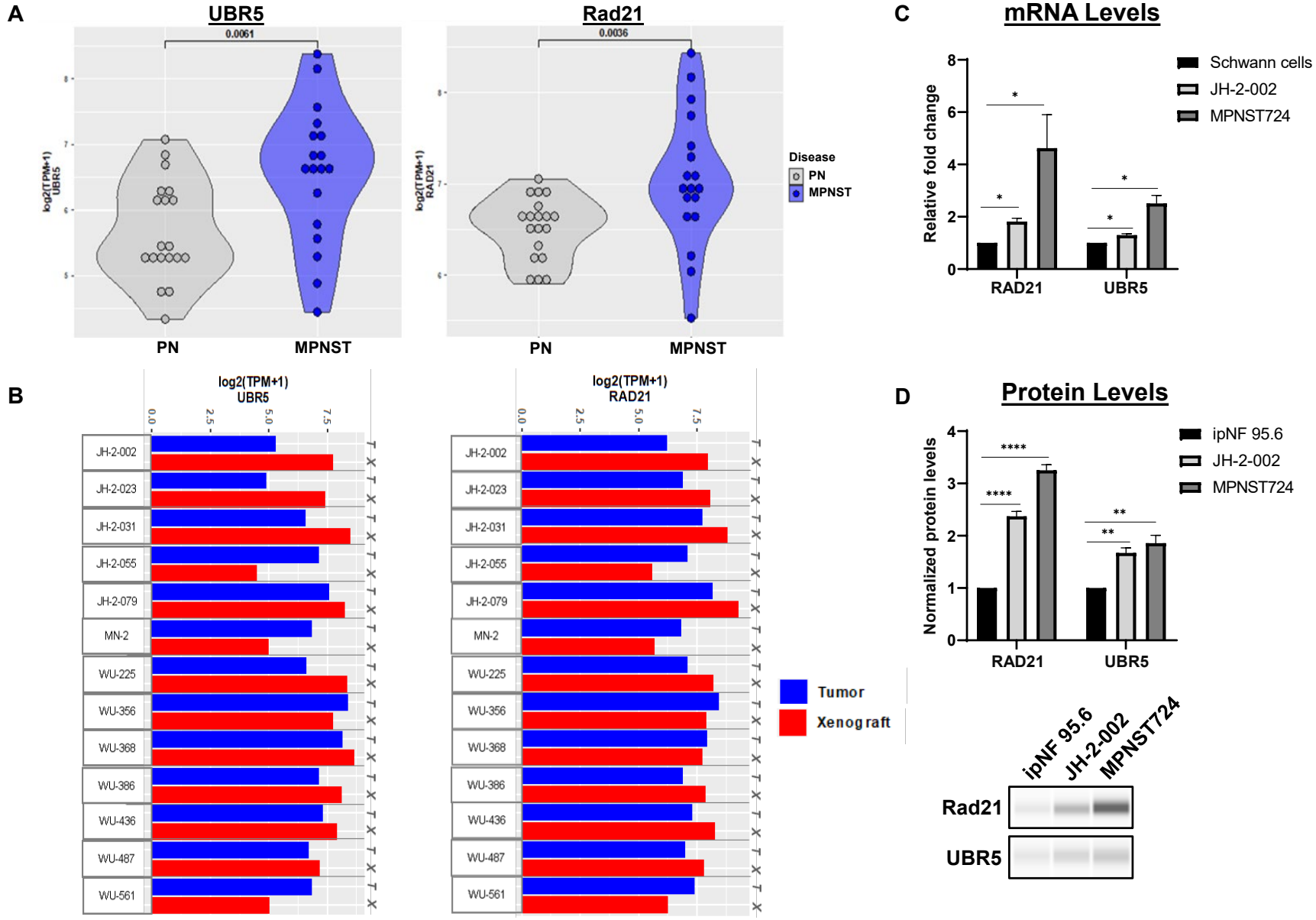


Figure 1. Expression of UBR5 and Rad21 is elevated in parental tumors, PDX, and MPNST cell lines compared with plexiform neurofibromas (PN). A) Violin plots depicting the expression of UBR5 and Rad21 in PN v/s MPNST. B) Expression levels of UBR5 and Rad21 in each PDX-tumor pair. C) Real-time qPCR quantification of UBR5 and Rad21 mRNAs in Schwann, JH-2-002, and MPNST724 cells normalized to GAPDH. D) Automated WES protein expression analysis measuring UBR5 and Rad21 expression in MPNST724 and JH-2-002 cells v/s PN cells, ipNF 95.6. Expression was normalized to total protein.

A

	Single cell KO clones	Total reads	sp7_wt	Indel
MPNST724 cells	UBR5 wild-type	3049	3032 (99.4%)	0 (+/+)
	UBR5 clone#1	1684	806 (47.9%)	-24 (+/-)
	Rad21 wild-type	3111	3092 (99.4%)	0 (+/+)
	Rad21 clone#1	2804	1319 (47.0%)	1 (+/-)
	Rad21 clone#2	2892	1442 (49.9%)	-2 (+/-)
	Rad21 clone#3	2881	1482 (51.4%)	-2 (+/-)
	Myc clone#1	1656	790 (47.7%)	-3 (+/-)
	Myc clone#2	1628	639 (39.3%)	-1 (+/-)
	Myc wild-type	1602	1598 (99.8%)	0 (+/+)
JH-2-002 cells	UBR5 clone#1	5845	1549 (26.5%)	-6 (+/-)
	UBR5 clone#2	6142	2363 (38.5%)	-7 (+/-)
	UBR5 wild-type	5164	5127 (99.3%)	0 (+/+)
	Rad21 clone#1	5588	2890 (51.7%)	1 (+/-)
	Rad21 clone#2	5578	1520 (27.2%)	4 (+/-)
	Rad21 wild-type	5400	5332 (98.7%)	0 (+/+)

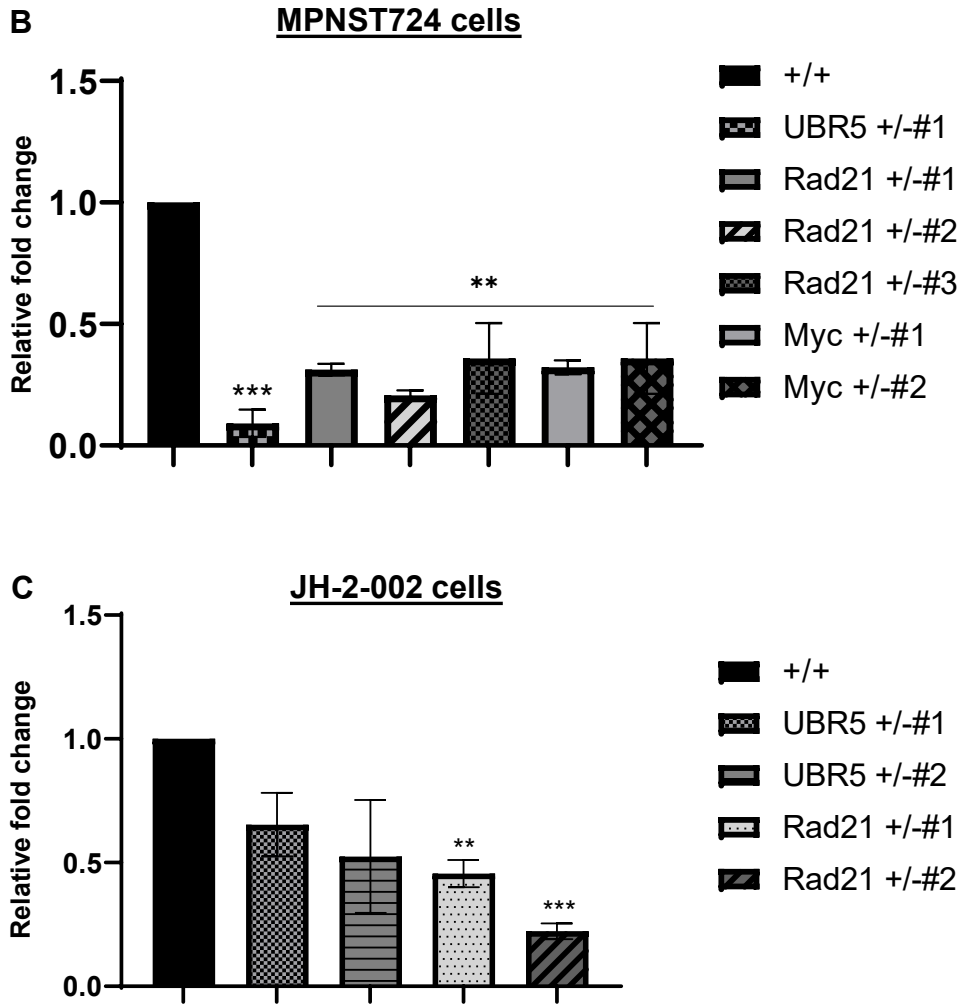


Figure 2. Generation of knockouts (KO) of *UBR5*, *Myc*, and *Rad21* genes by the CRISPR/Cas9 system. A) Details of Insertions/deletions (Indel) of single gene wild-type controls and heterozygous clones, as determined by NGS, in in MPNST724 and JH-2-002 cells. Real-time qPCR quantification of UBR5 mRNA expression in B) MPNST724 and C) JH-2-002 cells for wild-type and heterozygous clones, normalized to GAPDH mRNA levels.

A

	Single cell KO clones	Total reads	sp7_wt	Indel
724	UBR5 wild-type	3049	3032 (99.4%)	0 (+/+)
724	UBR5 clone#1	1684	806 (47.9%)	-24 (+/-)
JH-2-002	UBR5 clone#1	5845	1549 (26.5%)	-6 (+/-)
JH-2-002	UBR5 wild-type	5164	5127 (99.3%)	0 (+/+)

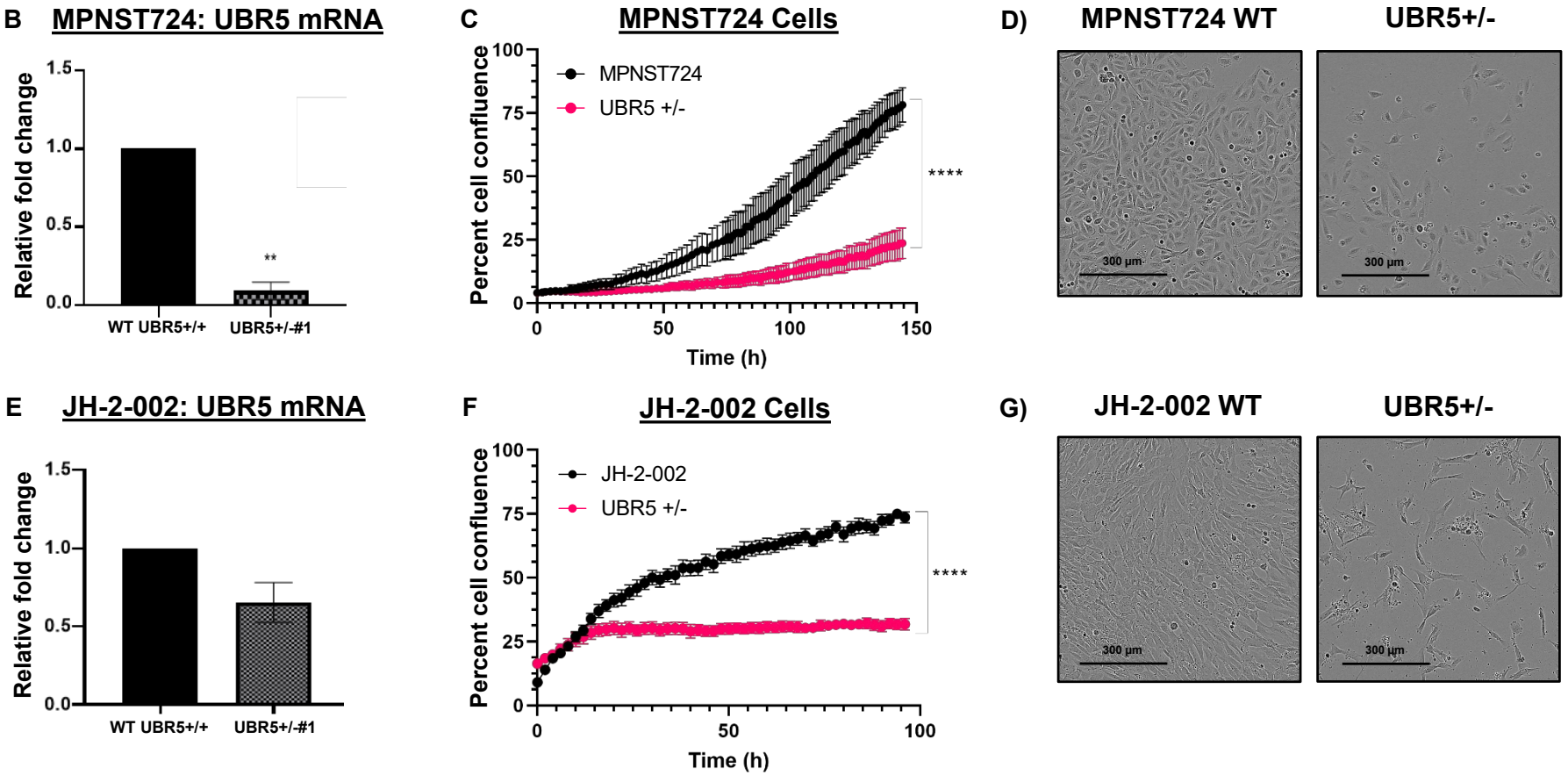


Figure 3. CRISPR/Cas9-mediated knockdown of UBR5 in MPNST724 and JH-2-002 cells leads to reduced cell proliferation. A) Indel details of single cell wild-type and heterozygous clones of UBR5 in MPNST724 and JH-2-002 cells. Real-time PCR quantification of UBR5 mRNA expression in B) MPNST724 and E) JH-2-002 wild-type and heterozygous clones normalized to GAPDH mRNA levels. Incucyte cell proliferation assays measuring confluence over time in C) MPNST724 or F) JH-2-002 wild-type and UBR5 heterozygous clones. Representative images of IncuCyte cell proliferation assays with wild-type or UBR5 heterozygous clones of D) MPNST724 or G) JH-2-002 cells.

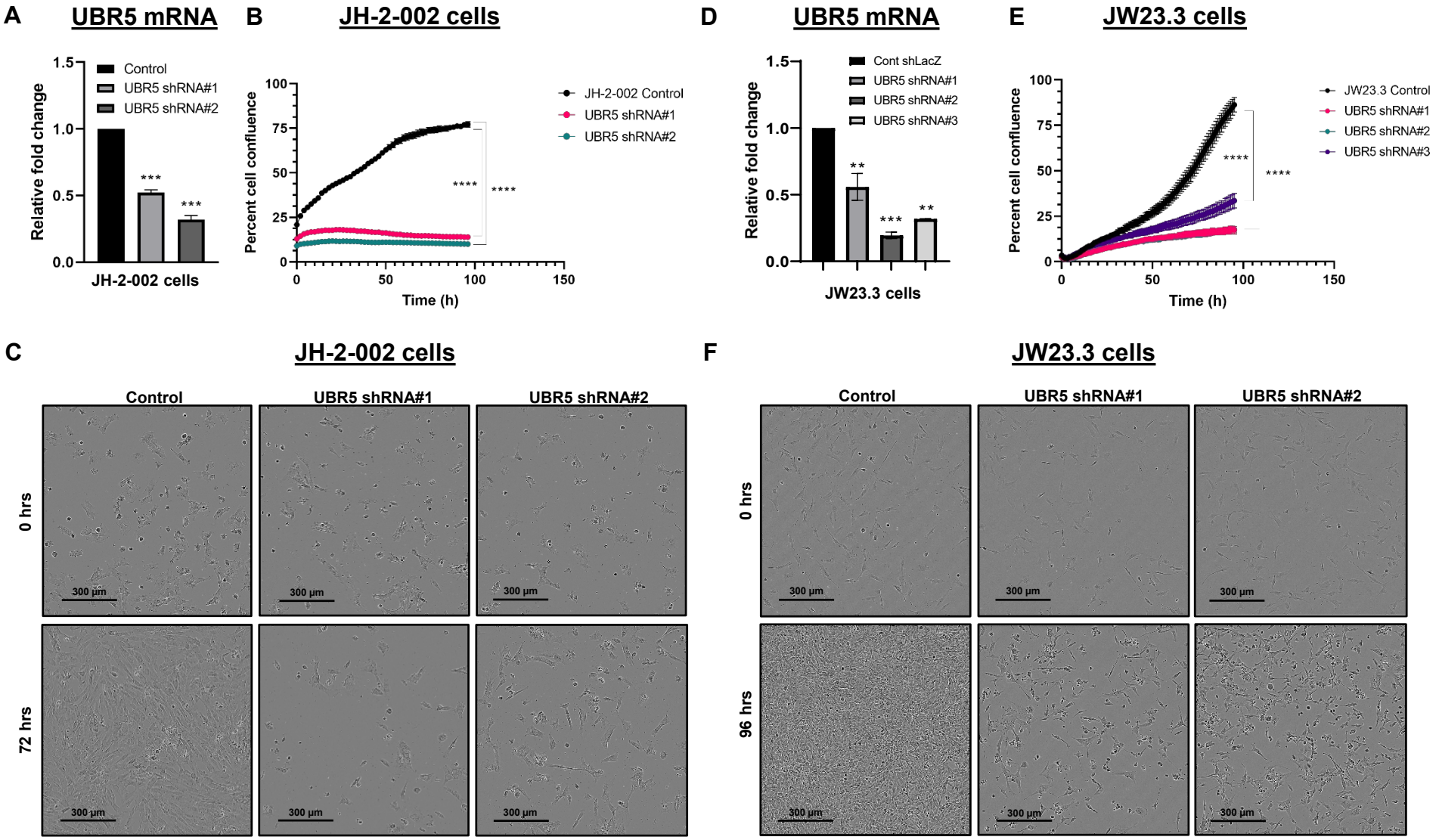


Figure 4. shRNA-mediated loss of *UBR5* in JH-2-002 and JW23.3 MPNST cells leads to reduced cell proliferation. Real-time qPCR quantification of *UBR5* mRNA levels in shRNA-infected JH-2-002 (A) and JW23.3 (D) cells, normalized to *GAPDH*. Incucyte cell proliferation assays measuring confluence over time in *UBR5* shRNA-infected JH-2-002 (B-C) and JW23.3 murine MPNST cells (E-F).

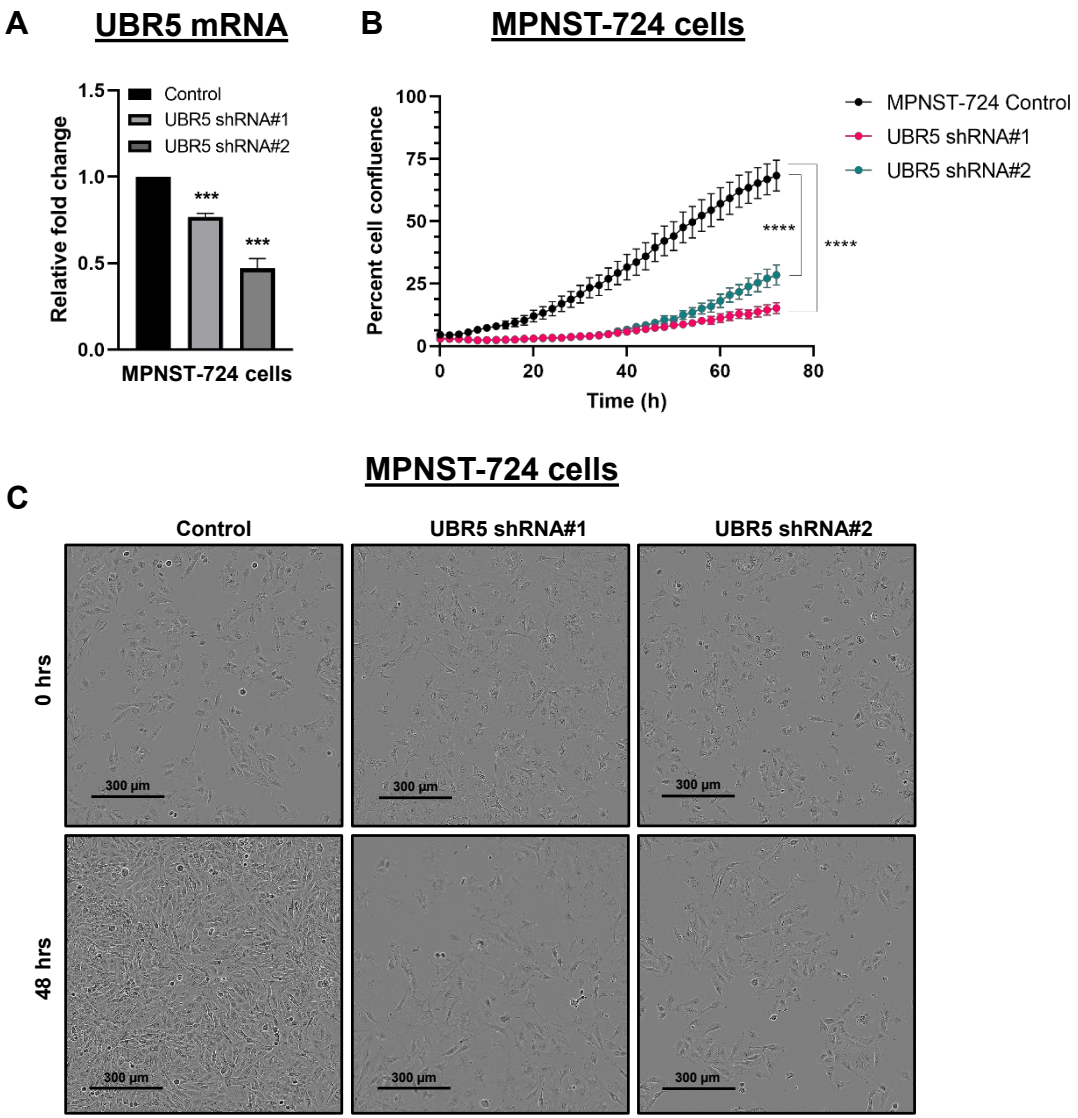


Figure 5. shRNA-mediated loss of UBR5 in human MPNST-724 cells leads to reduced cell proliferation. (A) Real-time PCR quantification of UBR5 mRNA levels in shRNA-infected MPNST-724 cells normalized to GAPDH. (B) Incucyte cell proliferation assay measuring confluence over time in UBR5 shRNA-infected MPNST-724 cells. (C) Representative images of an IncuCyte proliferation assay with MPNST-724 UBR5 shRNA and control cells at the indicated times.

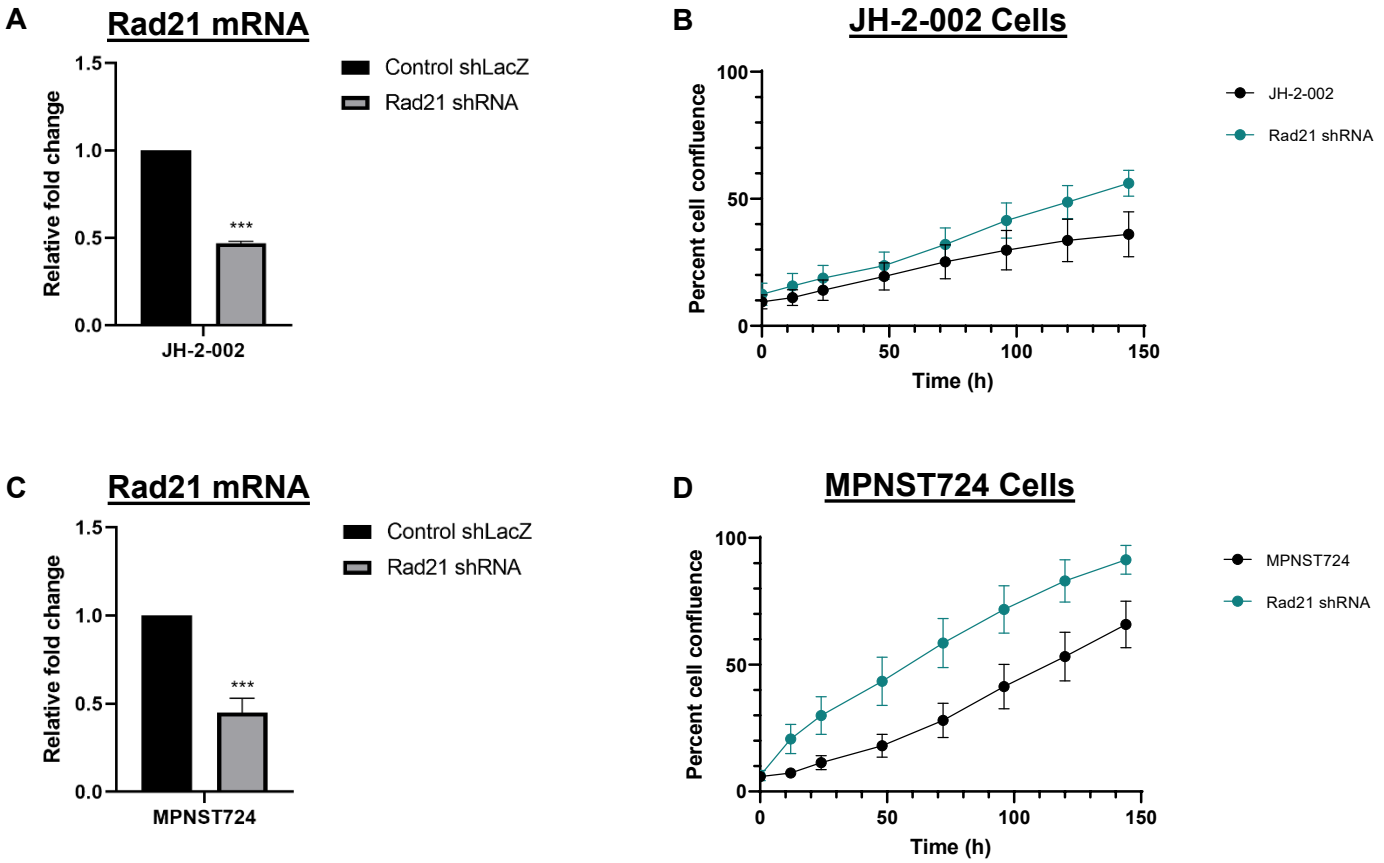
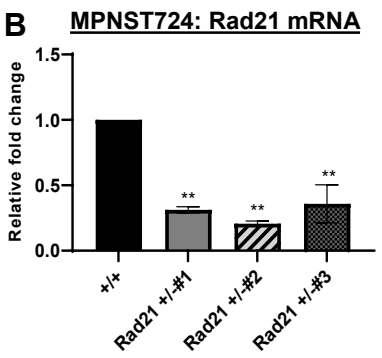


Figure 6. shRNA-mediated loss of Rad21 in JH-2-002 and MPNST724 cells showed no statistically significant difference in cell proliferation. Real-time PCR quantification of Rad21 gene expression in JH-2-002 (A) and MPNST724 (C) cells infected with shRNA for Rad21 or LacZ control, normalized to GAPDH. IncuCyte cell proliferation assays analyzed percent cell confluence over time in JH-2-002 (B) or MPNST724 cells (D) infected with shRNA LacZ control or Rad21 knockdown.

A

	Single cell KO clones	Total reads	sp7_wt	Indel
MPNST724	Rad21 wild-type	3111	3092 (99.4%)	0 (+/+)
	Rad21 clone#1	2804	1319 (47.0%)	1 (+/-)
	Rad21 clone#2	2892	1442 (49.9%)	-2 (+/-)
	Rad21 clone#3	2881	1482 (51.4%)	-2 (+/-)



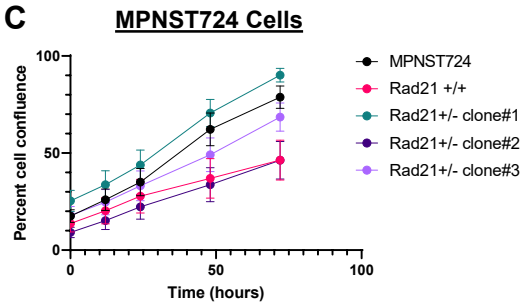


Figure 7. CRISPR/Cas9-mediated knockdown of Rad21 in MPNST724 has no effect on cell proliferation. A) Indel details of single cell wild-type and heterozygous CRISPR/Cas9 clones for Rad21 knockout in MPNST724 cells. B) Real-time qPCR quantification of Rad21 gene expression in MPNST724 heterozygous clones and controls, normalized to GAPDH. C) IncuCyte cell proliferation assays measuring percent cell confluence over time in MPNST724 clones.

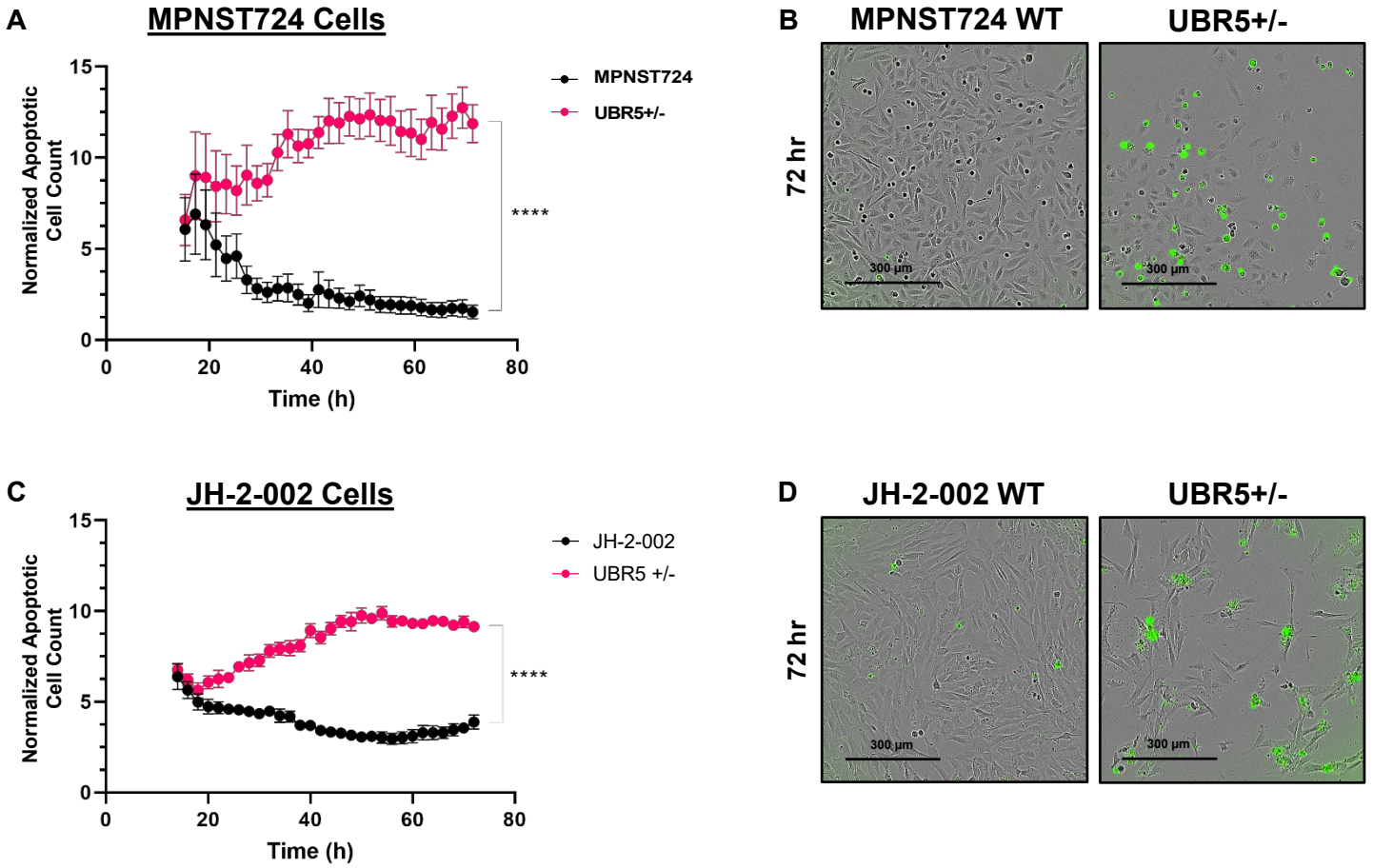


Figure 8. CRISPR/Cas9-mediated knockdown of UBR5 increases apoptosis in MPNST cells. Incucyte apoptosis assays measuring cell death over time in A) MPNST724 and C) JH-2-002 wild-type and heterozygous clones, respectively. Representative photomicrographs of B) MPNST724 or D) JH-2-002 cells stained with YOYO-1 green fluorescent apoptotic dye after 48 hours or 72 hours, respectively.

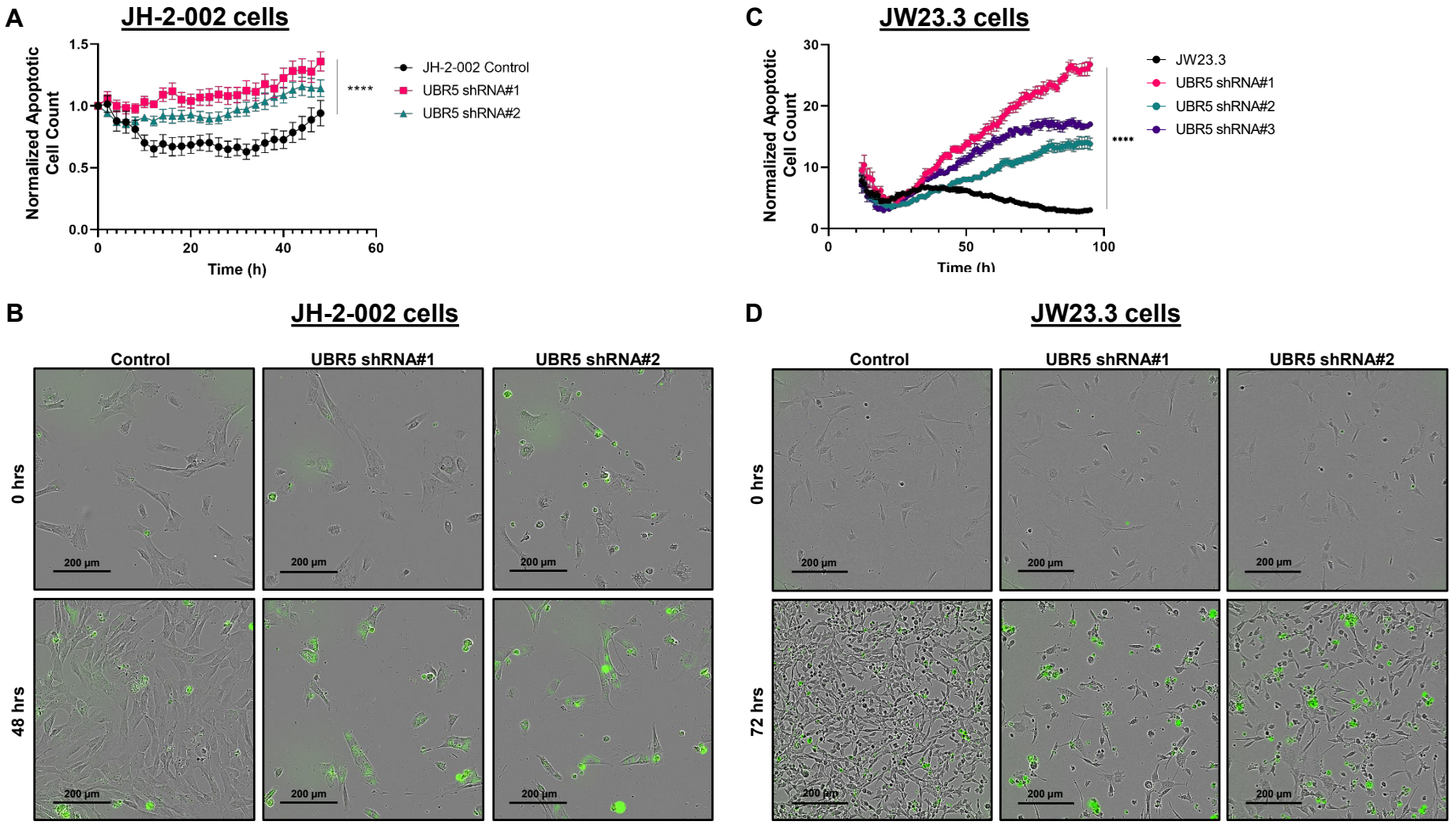


Figure 9. Knockdown of UBR5 induces apoptosis in human and mouse MPNST cells. (A-B) Human JH-2-002 and (C-D) mouse JW23.3 were infected with UBR5 shRNA or control. Apoptosis over time was determined using Incucyte cell death assays, and normalized apoptotic cell count is shown for (A) JH-2-002 and (C) JW23.3 cells. Representative images of IncuCyte apoptosis assays at the indicated times for (B) JH-2-002 and (D) JW23.3 cells, with YOYO-1 green fluorescence dye as an indicator of apoptotic cells.

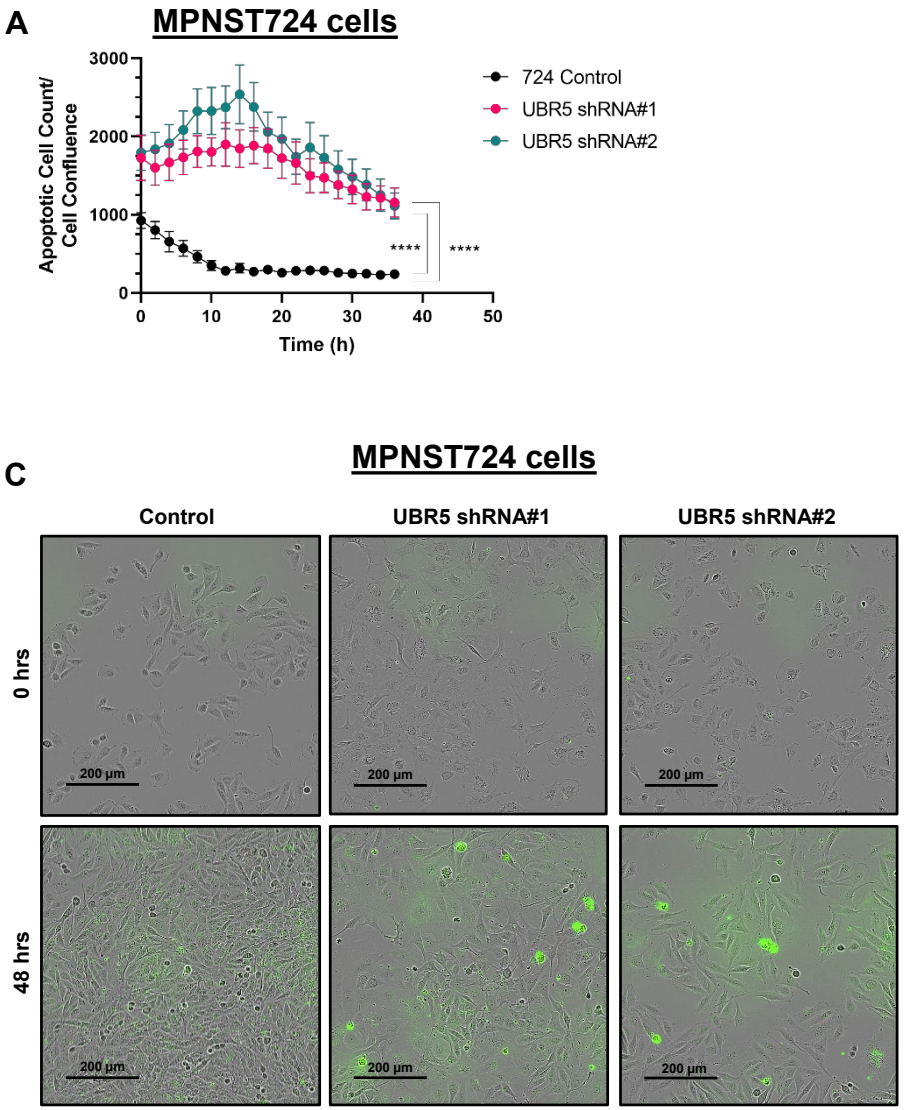


Figure 10. Knockdown of UBR5 induces apoptosis in sporadic MPNST cell line. MPNST724 cell line was infected with UBR5 shRNA or control. Apoptosis over time was determined using Incucyte cell death assays, and apoptotic cell count is shown in (A) an (B). Representative images of IncuCyte apoptosis assays at the indicated times with YOYO-1 green fluorescence dye as an indicator of apoptotic cells (C)

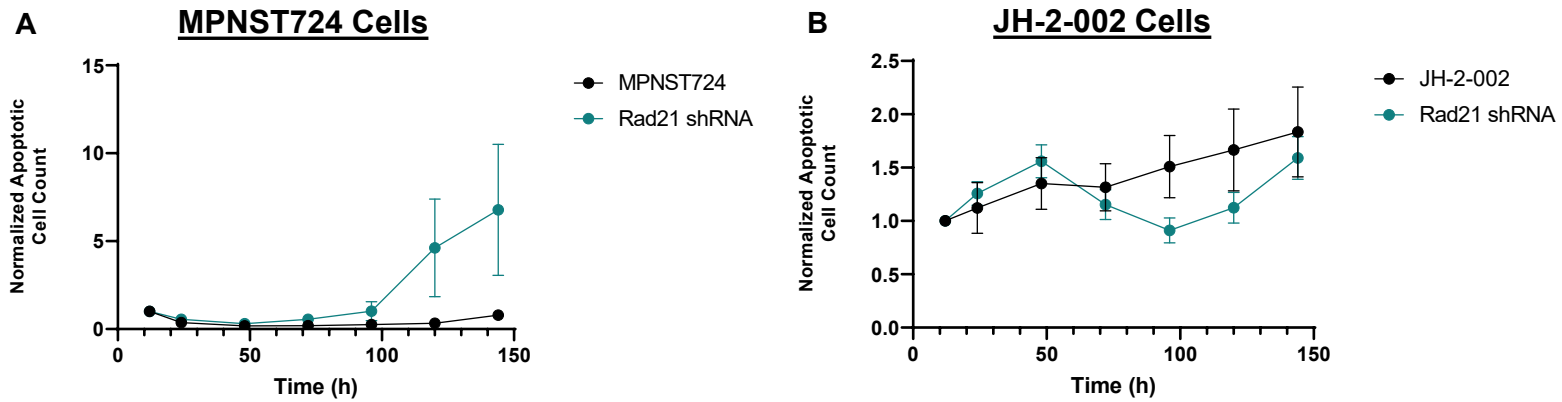


Figure 11. shRNA-mediated loss Rad21 in MPNST724 and JH-2-002 showed no statistically significant difference in cell death.

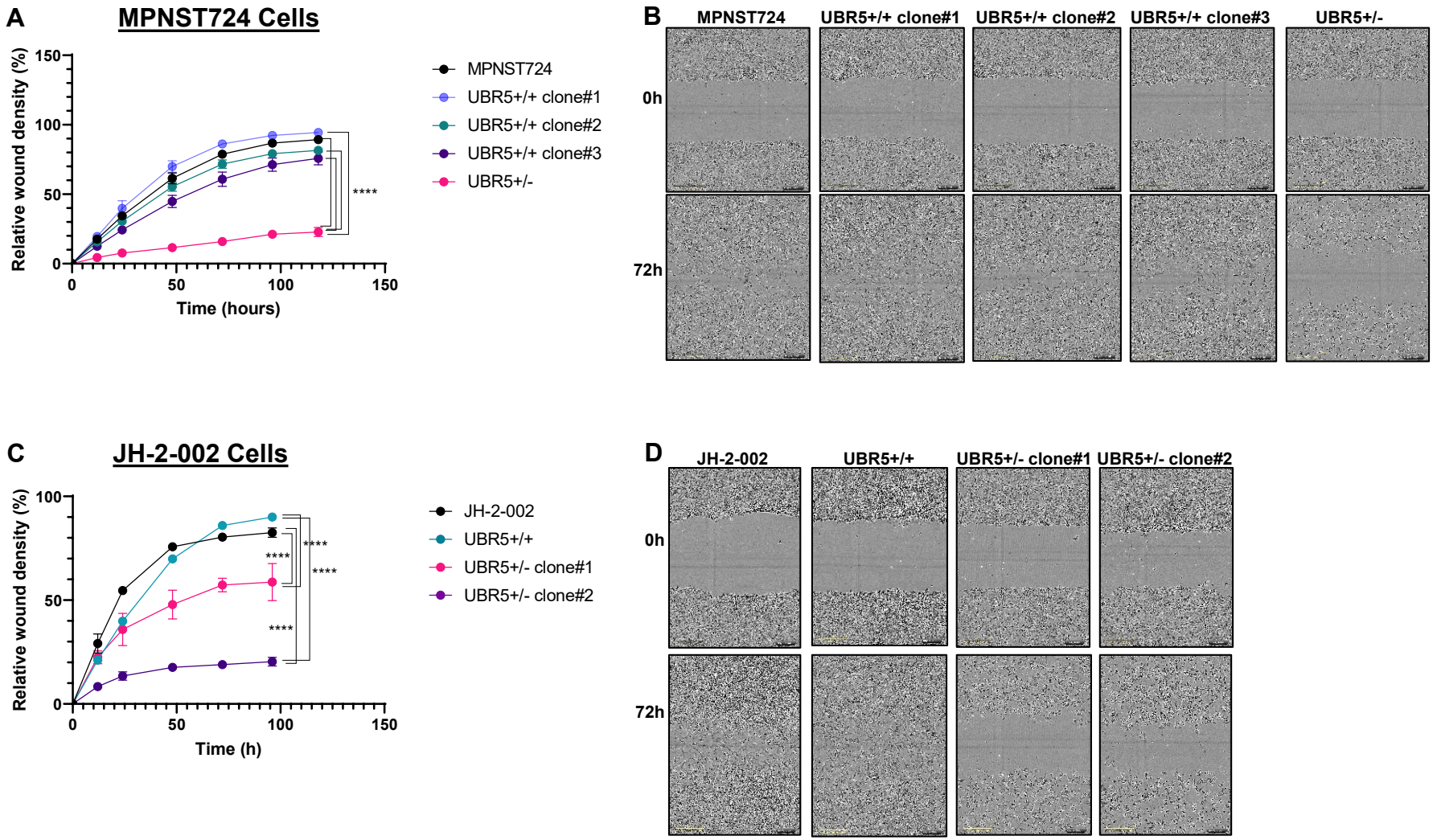


Figure 12. UBR5 knockdown decreases migration in MPNST cells. IncuCyte scratch wound assay for cell migration over time in (A) MPNST724 and (C) JH-2-002 clones with CRISPR/Cas9-mediated knockdown of UBR5 or control. Representative images of scratch wound assay in (B) MPNST724 and (D) JH-2-002 cells.

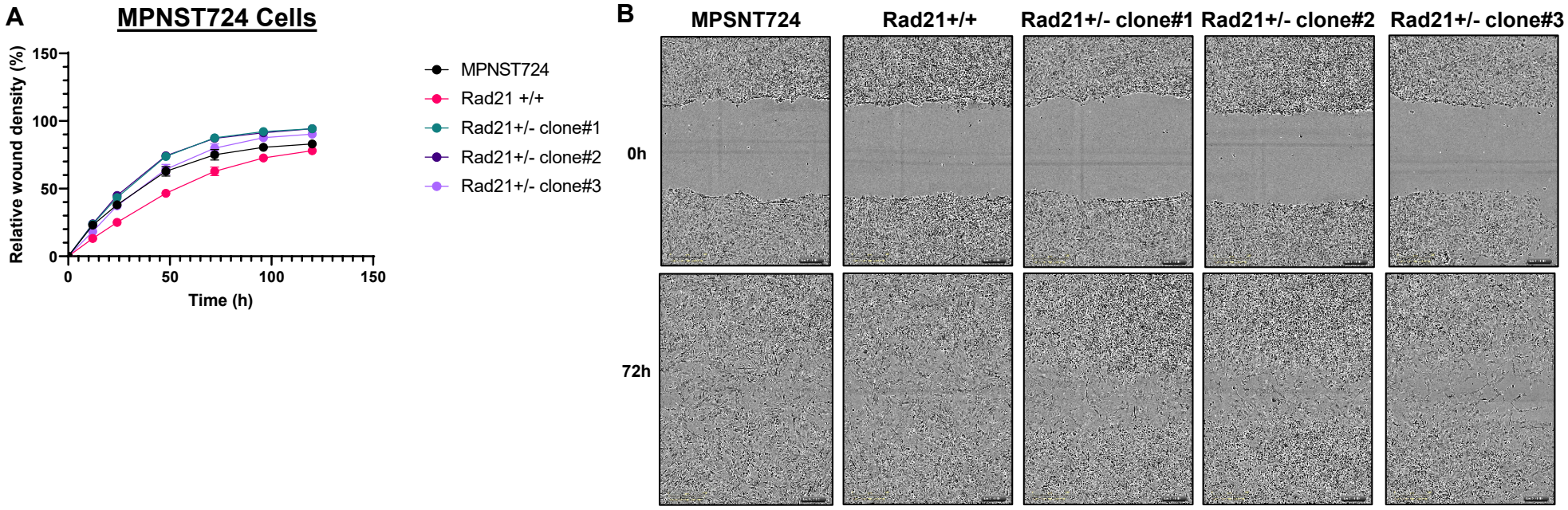


Figure 13. CRISPR/Cas9-mediated knockdown of Rad21 in MPNST724 has no effect on cell migration. A) Incucyte cell migration scratch wound assay measuring migration over time in MPNST724 Rad21 (+/-) clones vs. wild-type control. (B) Representative pictures of the migration assay.

Appendix D: What do you plan to do during the next reporting period to accomplish the goals? Plans for the next reporting period (Year 2):

Specific Aim 1: Define the genes on Chr8q that are essential for MPNST progression.

Major Task 1: *Define which genes on Chr8q are necessary for MPNST progression.*

Plans: During the next reporting period (Year 2) we will continue to explore the role of *UBR5* in MPNST pathogenesis. *Rad21* and *MYC* do not seem to have a leading role in tumor progression in MPNST, therefore we will not going to further evaluate those genes. Additionally, we are performing a CRISPR screen to determine if there are other genes of importance on Chr8.

Major Task 2: *Determine whether expression of one or more of these genes is* ***sufficient*** *to drive MPNST formation.*

Plans: We are in the process of lentiviral transduction to express *UBR5* in *NF1*-/- hiPSCs. Later, we will differentiate them to schwann cell precursors (SCP) and perform Incucyte Live cell analysis system to compare the overexpression cell lines. After differentiating them to SCP, we will also surgically implant 10^6 Chr8 gene-hiPSC-SCPs to sciatic nerves of NRG mice. Those mice will be followed for 12 months for tumor growth. We have also injected Chr8gain *NF1*-/- SCP into the sciatic nerves of mice and are 6 months into the follow up period. We will continue to follow these mice.

Specific Aim 2: We will define the dysregulated signaling pathways in cells with Chr8q gain.

Major Task 3: *Globally characterize the transcripts and proteins that are altered in MPNST through multi-regional tumor sampling.*

Plans: We have collected multi-regional samples from 2 MPNST resections so far and we plan to collect 1 more in the following months. After that, we will proceed with WGS, copy number analysis of the samples and proteomics analysis.

Major Task 4: *Employ network-based modeling to identify mediators of the Chr8q phenotype.*

Plans: After transcriptomic and proteomic analysis of the samples, this task will be completed during the third year.

<u>Specific Aim 3</u>: Determine if the degree of Chr8 gain observed in FISH or cell-free DNA correlates with OS or development of resistance.

Major Task 5: *Does the degree of Chr8 gain correlate with OS or other clinical factors?*

Plans: We are in the process of collecting MPNST cases between Wash U, JHU and NCI. We will have FFPE slides cut and perform FISH when we reach 60 samples in the following period.