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TITLE: TYK2 as a biomarker and therapeutic target for NF1-associated Malignant Peripheral Nerve Sheath Tumors

PRINCIPAL INVESTIGATOR: Dr. Angela Hirbe

CONTRACTING ORGANIZATION: Washington University, St. Louis, MO

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14. ABSTRACT
Malignant peripheral nerve sheath tumors (MPNST) are aggressive sarcomas that account for approximately 5% of all soft tissue sarcomas. These tumors occur at an increased frequency in patients with the Neurofibromatosis Type 1 (NF1) cancer predisposition syndrome, but also occur sporadically or as a secondary complication of radiation therapy. In the setting of NF1, MPNST arise from malignant transformation of a benign precursor lesion, a plexiform neurofibroma. Initial treatment for MPNST typically involves surgery and radiation with or without chemotherapy. However, despite aggressive therapy, the recurrence rate is high and the vast majority of people with these cancers will die within 5 years of diagnosis. Treatment for metastatic disease is limited to cytotoxic chemotherapy and clinical trials. As such, there is a pressing need to identify novel therapeutic targets. Prior work from our laboratory identified *TYK2* as a gene mutated in a subset of MPNSTs. More recently, we have shown that genetic knockdown of *TYK2* in both human and murine MPNST cell lines results in decreased tumor growth and increased cell death in vitro. Additionally, genetic knockdown of *Tyk2* in murine MPNST cells resulted in decreased tumor burden in subcutaneous tumors and metastatic tumor models. Immunohistochemistry (IHC) for TYK2 was performed on 27 MPNST and 16 plexiform neurofibromas to evaluate TYK2 association with tumor type, overall survival, metastasis and therapeutic response. Additionally, similar to genetic knockdown, pharmacologic inhibition of TYK2 dose-dependently decreased the percent cell confluence and induced apoptosis over time in four MPNST cell-lines, as assessed by IncuCyte proliferation and apoptosis assays. In murine MPNST JW23.3 cells, incubation with TYK2 inhibitors reduced pSTAT3 levels, but not pERK or pS6K. Finally, pharmacologic inhibition of downstream targets STAT3 and Bcl-2 also reduced cell confluence over time, which corresponded with increased cell death, as analyzed by IncuCyte assays. These findings suggest that TYK2 promotes MPNST pathogenesis through STAT3 and inhibition of apoptosis and that TYK2 could serve as a therapeutic target.

15. SUBJECT TERMS
Malignant peripheral nerve sheath tumor, MPNST, tyrosine kinase-2, TYK2, neurofibromatosis-1, NF1, sarcoma, STAT3

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1. INTRODUCTION:

Neurofibromatosis type 1 (NF1) is one of the most common inherited tumor predisposition syndromes, affecting 1:2500 individuals worldwide. Diagnosis is typically made during childhood and is associated with an increased risk of benign and malignant tumors. Approximately 1/3 of patients with NF1 will develop benign nerve sheath tumors called plexiform neurofibromas (PN), and almost half of these tumors will undergo malignant transformation to malignant peripheral nerve sheath tumors (MPNST), a highly aggressive sarcoma. Currently, there are no predictive biological markers of transformation, few therapeutic options for advanced disease, and dismal survival. Once diagnosed with MPNST, patients with localized disease are treated with curative-intent surgery as well as radiation therapy and chemotherapy. Unfortunately, patients with metastatic disease can rarely be cured, and treatment is limited to palliative chemotherapy. Treatment of PN is usually surgical and is generally limited to symptomatic cases given the high morbidity associated with these surgeries. As such, there is a pressing need to identify biomarkers for transformation to MPNST as well as novel therapeutic targets for both PN and MPNST.

2. KEYWORDS:

Malignant peripheral nerve sheath tumor, MPNST, tyrosine kinase-2, TYK2, neurofibromatosis-1 (NF1), sarcoma, STAT3

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: Are TYK2 expression levels associated with overall survival, time to metastasis, and response to therapy in patients with MPNST?
Major Task 2: Does TYK2 expression distinguish atypical neurofibromas from MPNSTs?
Major Task 3: Determine whether STAT-3 medicated activation of Bcl-2 is responsible for TYK-2 mediated protection from cell death.
Major Task 4: Explore the impact of TYK2 downregulation on known signaling nodes to be important in MPNST pathogenesis.
Major Task 5: Determine the impact of TYK2 downregulation on the global expression profile of MPNSTs.
Major Task 6. Does pharmacologic inhibition of TYK2 lead to decreased tumor viability in vitro?
Major Task 7. Does pharmacologic inhibition of TYK2 lead to decreased tumor growth and/or metastasis in vivo in MPNST PDX lines?
Major Task 8. Does pharmacologic inhibition of TYK2 lead to decreased tumor growth and/or metastasis in vivo in mice with an intact immune system?

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.

How were the results disseminated to communities of interest?

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. In this regard, 8-13% of individuals with NF1 will develop these tumors during young adulthood. 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. Leveraging next generation sequencing of NF1-MPNSTs, we recently identified Tyrosine Kinase 2 (TYK2) as a frequently mutated gene, whose function is critical for mouse MPNST survival in vitro and in vivo. Based on these exciting findings, we hypothesize that TYK2 promotes MPNST growth through inhibition of cell death and that TYK2 is a biomarker and therapeutic target for MPNSTs. In this proposal, we will assess the utility of TYK2 expression as a potential prognostic marker for MPNST progression. Additionally, we aim to define the mechanism by which TYK2 promotes MPNST development and growth using engineered primary MPNST cell lines *in vitro* and complementary patient derived xenograft models *in vivo*. Finally, we will assess the utility of therapeutically targeting TYK2. Given the dismal overall survival for patients with MPSNTs and the frequency of these cancers in NF1, there is a pressing need to develop more accurate markers of MPNST development and better targeted therapies for these malignancies. This work has the potential to lead to a biomarker driven therapeutic option and clinical trial for patients in desperate need of an effective therapy.

What was the impact on other disciplines?

The development of new therapeutics and 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

As a result of variability in the short-hairpin system in genetic knockdown, we are now moving to the CRISPR/Cas9 system for Aim 2 as it provides a better genetic knockout and has become a more widely accepted technique than shRNA.

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

With the Covid-19 pandemic, in-person laboratory activities at Washington University were restricted starting in March 2020. While the grant official start date was March 1, 2020, no work started on the grant until July 1, 2020. We were unable to begin work on this grant until July 1, 2020. Covid-19 restrictions limited our lab to 50% capacity between July 1-September 1, 2020, and to 80% capacity between September 1, 2020, to present.

For Aim 1, the commercial antibody against TYK2 (ab39550, Abcam) used in our previous immunohistochemistry (IHC) studies became unavailable during the pandemic. Thus, other antibodies for TYK2 were evaluated and one antibody (ab223733, Abcam) was subsequently optimized for concentration and other IHC conditions before being used on MPNST patient samples.

As a result of variability in the short-hairpin system in genetic knockdown, we are now moving to the CRISPR/Cas9 system for Aim 2 as it provides a better genetic knockout and has become a more widely accepted technique than shRNA. Thus, we proceeded to Aim 3.1 earlier than planned, while switching to CRISPR/Cas9 from shRNA (Aim 2) and due to Covid-19 limitations on in-person staff.

Changes that had a significant impact on expenditures

Due to Covid-19 limitations on in-person staff, work did not begin on this grant until July 1, 2020. Thus, we will request a 6-month no-cost extension at the end of the 3-year grant period.

The new anti-TYK2 antibody for IHC (Aim 1) needs to be used at a higher concentration (1:100 instead of 1:500), which has increased costs for IHC. We are also transitioning from short hairpin RNA (shRNA) to the CRISPR/Cas9 system, which is more expensive, but also provides more consistent genetic knockout. The difference in cost for these experimental changes will be covered by the lab's start-up funding.

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 Aim 1, we obtained subject protocol approval by the Washington University (WU) Institutional Review Board (IRB) on February 28, 2020, and by the Human Research Protection Office (HRPO) in June, 2020, prior to this grant award. 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 3, we obtained protocol approval from IACUC (protocol #20-0117) in April, 2020 and from ACURO (NF190033.e001) in May, 2020. 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.

Planned abstract submission in June, 2021, for the Connective Tissue Oncology Society (CTOS) 2021 Annual Meeting in Vancouver, Canada.

Other publications, conference papers and presentations.

Nothing to report.

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

1. Synapse NF-OSI project space (Synapse Project) for the Hirbe Lab for data related to this grant: https://www.synapse.org/#!Synapse:syn23639889/wiki/607570 2. 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:	6
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:	8
Contribution to Project:	Dr. Borcherding conducted and supervised research activities and analyzed data in this grant project, including IncuCyte assays, IHC, western blotting, qPCR and transfections.
Name:	Sonika Dahiya, M.D.
Project Role:	Professor, Pathology & Immunology
Researcher Identifier (e.g. ORCID ID):	0000-0002-5843-6646
Nearest person month worked:	1
Contribution to Project:	Dr. Dahiya provided pathology expertise for IHC scoring.
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:	Carina Dehner, M.D./Ph.D.
Project Role:	Pathology Resident (Co-Chief)
Researcher Identifier (e.g. ORCID ID):	0000-0001-5214-4813
Nearest person month worked:	1
Contribution to Project:	Dr. Dehner provided pathology expertise for IHC experiments and scoring.
Name:	Kevin He
Project Role:	Undergraduate Student
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	1.5
Contribution to Project:	Mr. He conducted IHC experiments and imaging.
Name:	Neha Amin
Project Role:	Undergraduate Student
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	1
Contribution to Project:	Ms. Amin conducted IncuCyte experiments and analysis.

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?

Dr. Christine Pratilas
John Hopkins University (JHU)
Baltimore, MD
Partner's contribution: Provided MPNST and plexiform neurofibroma specimens for IHC and human MPNST cell-lines.

Dr. Brigitte Widemann
National Cancer Institute (NCI)
Bethesda, MD
Partner's contribution: Provided MPNST and plexiform neurofibroma specimens for IHC.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: N/A

QUAD CHARTS: N/A

9. APPENDICES: See Appendices A-D.

Appendix A: STATEMENT OF WORK

Grant Official Start Date: March 1, 2020
Note: No work started until July 1, 2020, and then only at 50% capacity until September 1st, followed by 80% capacity after September 1, 2020, due to pandemic restrictions. New timeline assumes a no cost extension of 6 months due to the pandemic.

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

Specific Aim 1: We will determine whether TYK2 is a prognostic biomarker for MPNST.	**Timeline (Original)**	**Washington University**	**Percent Complete**	**Timeline (New)**
Major Task 1: Are TYK2 expression levels associated with overall survival, time to metastasis, and response to therapy in patients with MPNST?	Months			Months
Subtask 1: ACURO/HRPO Approval	0	Dr. Hirbe (obtained prior to funding)	100%	0
Subtask 1: Re-review of all 60 MPNST cases with Dr. Dahiya and Chrisinger	0-3	Dr. Hirbe	50%	0-9
Subtask 2: Generation of unstained slides and clinical chart review	3-6	Dr. Hirbe	50%	3-9
Subtask 3: TYK2 staining	6-8	Dr. Hirbe	30%	6-14
Subtask 4: Blinded TYK2 scoring by Dr. Dahiya, Chrisinger	8-10	Dr. Hirbe	30%	10-12
Subtask 5: Statistical analysis	10-12	Dr. Hirbe	On Target	12-14
Milestone(s) Achieved: Determine whether TYK2 expression is correlated with clinical prognostic parameters.	**12**	Dr. Hirbe	**40%**	**14**
Major Task 2: Does TYK2 expression distinguish atypical neurofibromas from MPNSTs?				
Subtask 1: Re-review of all 45 cases of atypical neurofibroma and low grade MPNST cases by Dr. Dahiya and Chrisinger	0-3	Dr. Hirbe	50%	0-9
Subtask 2: Generation of unstained slides and clinical chart review	3-6	Dr. Hirbe	50%	3-9
Subtask 3: TYK2 staining	6-8		30%	6-14
Subtask 4: Blinded TYK2 scoring by Dr. Dahiya, Chrisinger	8-10	Dr. Hirbe	30%	10-14
Subtask 5: Statistical analysis	10-12	Dr. Hirbe	On Target	12-14
Milestone(s) Achieved: Determine whetherTYK2 can be used to distinguish atypical neurofibromas from MPNSTs.	**12**	Dr. Hirbe	**40%**	**14**

<u>Specific Aim 2</u>: We will delineate the mechanism of action of TYK2 in MPNST cells.				
Major Task 3: Determine whether STAT-3 medicated activation of Bcl-2 is responsible for TYK-2 mediated protection from cell death.				
Subtask 1: Generate and test *shSTAT3*, *shBcl2*, and *shLacZ* control lentivirus	0-4	Dr. Hirbe	50%	4-14
Subtask 2: Incucyte cell growth and death assays using lentivirus generated in subtask 1. Cell lines: JW23.3 murine MPNST cells (initially obtained from Dr. Karlyne Reilly, human MPNST 724 cells obtained from Dr. Jonathon Fletcher)	4-8	Dr. Hirbe	20%	14-18
Subtask 3: Incucyte cell growth and death assays using napabucasin (STAT3 inhibitor) and venetoclax (Bcl2 inhibitor) compared to vehicle Cell lines: JW23.3 murine MPNST cells, human MPNST 724 cells	8-12	Dr. Hirbe	50%	10-14
Subtask4: Evaluation of type of cell death Cell lines: JW23.3 murine MPNST cells, human MPNST 724 cells	12-16	Dr. Hirbe	On Target	14-18
Milestone(s) Achieved: Understand the mechanism of TYK2 in MPNST growth and survival.	**16**	Dr. Hirbe	**40%**	**18**
Major Task 4. Explore the impact of TYK2 downregulation on known signaling nodes to be important in MPNST pathogenesis.				
Subtask 1: Western blot analysis to evaluate activation of pathways involved in neurofibromin signaling Cell lines: JW23.3 murine MPNST cells, human MPNST 724 cells	16-20	Dr. Hirbe	50%	6-16
Milestone(s) Achieved: Determine whether TYK2 signaling intersects with neurofibromin signaling.	**20**	Dr. Hirbe	**50%**	**16**
Major Task 5. Determine the impact of TYK2 downregulation on the global expression profile of MPNSTs.				
Subtask 1: RNA extraction *shTYK2* and *shLacZ* control cells Cell lines: JW23.3 murine MPNST cells, human MPNST 724 cells	20-21	Dr. Hirbe	25%	4-21
Subtask 2: RNA sequencing and analysis.	21-24	Dr. Hirbe	20%	6-24
Milestone(s) Achieved: Determine the global expression changes upon knockdown of TYK2.	**24**	Dr. Hirbe	25%	24

<u>Specific Aim 3</u>: Evaluate pharmacologic inhibition of TYK2 with in MPNSTs *in vivo*.				
Major Task 6. Does pharmacologic inhibition of TYK2 lead to decreased tumor viability *in vitro*?				
Subtask 1: Incucyte cell growth and death assays using the TYK2 inhibitors TC JL37, WU12, and WU76. Cell lines: JW23.3 murine MPNST cells, human MPNST 724 cells	16-20	Dr. Hirbe	100%	2-8
Subtask 2: Statistical analysis	20-21	Dr. Hirbe	100%	6-8
Milestone(s) Achieved: Determine IC_{50} for TYK2 inhibitors in MPNST cell lines	**21**	Dr. Hirbe	100%	8
Major Task 7. Does pharmacologic inhibition of TYK2 lead to decreased tumor growth and/or metastasis *in vivo* in MPNST PDX lines?				
Subtask 1: Sciatic nerve injections Patient Derived Xenograft lines: MPNST PDX1, MPNST PDX2, MPNST PDX3, MPNST PDX4, MPNST PDX5 (described in background data, manuscript in preparation)	21-25	Dr. Hirbe	On Target	25-29
Subtask 2: Randomization for drug treatment	22-26	Dr. Hirbe	On Target	26-30
Subtask 3: Monitoring tumor burden and response to treatment	22-32		On Target	26-36
Subtask 4: Statistical analysis and histopatholgical correlations (Drs. Dahiya and Chrisinger)	32-36		On Target	36-40
Milestone(s) Achieved: Determine whether TYK2 inhibition reduces MPNST tumor growth in a genomically heterogenous set of patient derived lines.	**36**	Dr. Hirbe	**On Target**	**40**
Major Task 8. Does pharmacologic inhibition of TYK2 lead to decreased tumor growth and/or metastasis *in vivo* in mice with an intact immune system?				
Subtask 1: Maintenance of *Nf1* heterozygous and wildtype mice	1-36	Dr. Hirbe	On Target	1-42
Subtask 2: Injection of *Nf1* and wildtype mice	21-24	Dr. Hirbe	On Target	27-30
Subtask 2: Monitoring tumor burden and response to treatment	24-33	Dr. Hirbe	On Target	30-39
Subtask 3: Histopathological correlations (Drs. Dahiya and Chrisinger)	33-36	Dr. Hirbe	On Target	39-42
Milestone(s) Achieved: Determine whether TYK2 inhibition reduces MPNST growth in the context of an intact immune system and within the Nf1 heterozyogous context	**36**	Dr. Hirbe	**On Target**	**42**

Appendix B: What was accomplished under these goals?

Major Accomplishments:
Note: While the grant official start date was March 1, 2020, no work started until July 1, 2020 given the shutdown necessitated by the pandemic. Due to pandemic restrictions, our lab was only at 50% capacity for July 1-September 1, 2020, and then at 80% capacity for September 1, 2020, to present.

Specific Aim 1: We will determine whether TYK2 is a prognostic biomarker for MPNST.

Major Task 1: Are TYK2 expression levels associated with overall survival, time to metastasis, and response to therapy in patients with MPNST?

Accomplishments: We obtained ACURO/HRPO approval for these studies and have completed a clinical re-review of all 60 MPNST cases. We also generated unstained slides and H&E stained slides of MPNST tumors from these patients at Washington University and from our collaborators at John Hopkins University (JHU) and the National Cancer Institute (NCI) (**Table 1**).

The commercial antibody against TYK2 (ab39550, Abcam) used in our previous immunohistochemistry (IHC) studies became unavailable during the pandemic. Thus, other antibodies for TYK2 were evaluated and the lead antibody (ab223733, Abcam) was optimized for concentration and other IHC conditions. IHC for TYK2 has been completed on 15 MPNST slides from Washington University patients, and three independent observers have scored these slides in a blinded fashion (**Fig. 1**). Moderate to strong TYK2 staining was observed in 12 of 15 high-grade MPNST samples, with 3 having weak or negative TYK2 staining. In addition, 12 MPNST slides from John Hopkins were also stained for TYK2 and are now in the process of being scored.

Tumor Type	Washington University	John Hopkins University	NCI
MPNST	40	12	5
Atypical/low-grade	15	0	17
Plexiform NF	15	32	0

Table 1. MPNST and atypical neurofibromas obtained for immunohistochemistry staining listed by origin of unstained slides.

Major Task 2: Does TYK2 expression distinguish atypical neurofibromas from MPNSTs?

Accomplishments: Our inventory list quoted in the grant included cases we identified in our pathology clinical database. However, upon requesting cases, a significant number of cases did not have tissue available. As such, we have begun to obtain cases from our collaborators to reach the goals set by our power calculations. Slides were obtained from Johns Hopkins University given that an MTA was already in place. An MTA was put in place with NCI and they are preparing their shipment of slides. **Table 1** is a list of current inventory of cases that have been re-reviewed by a pathologist with expertise in MPNST. In addition, all Washington University and Johns Hopkins cases of plexiform neurofibroma, atypical neurofibroma and low grade MPNST have

been re-reviewed with pathology. We have conducted IHC for TYK2 on 16 plexiform neurofibroma cases from John Hopkins and are now scoring these samples.

<u>Specific Aim 2</u>: We will delineate the mechanism of action of TYK2 in MPNST cells.

Major Task 3: Determine whether STAT-3 mediated activation of Bcl-2 is responsible for TYK-2 mediated protection from cell death.

Accomplishments: We initially generated lentivirus for mouse and human sh*STAT3*, sh*Bcl2* and sh*LacZ* control to transduce MPNST cells to produce stable shRNA lines, and conducted initial knockdown experiments in human MPNST-724 cells (TYK2-deficient and control). However, due to variability in the short-hairpin system in genetic knockdown, we are now moving to the CRISPR/Cas9 system as it provides a better genetic knockout and has become a more widely accepted technique than shRNA. We have transfected, sorted cells by FACS, and are in the process of expanding and validating colonies of two MPNST cell-lines (murine JW23.3 *Nf1/Tp53*-mutant and human JH 2-002) with CRISPR/Cas9 knockout of TYK2 and/or STAT3. We have also added a third cell line JH 2-002 to ensure the translatability of our results.

To further examine the role of STAT3 and Bcl-2 in MPNST cell proliferation and apoptosis, JW23.3 cells were incubated for 72 hours with the STAT3 inhibitor, napabucasin (NP), and Bcl2 inhibitor, venetoclax (ABT-199). Napabucasin and venetoclax both dose-dependently decreased cell confluence over time, which corresponded with increased cell death, as determined by incorporation of the green fluorescent dye YoYo-1 iodide and IncuCyte Zoom (Sartorius) analysis (**Fig. 2**). Thus, pharmacologic inhibition of STAT3 and Bcl2 mimics the anti-proliferative and pro-apoptotic effects that we previously reported with TYK2 genetic deficiency. These initial studies are needed to identify the IC50 for each drug in each cell line. We are currently expanding these studies with the STAT3 and Bcl2 inhibitors to other MPNST cell-lines and in combination with TYK2 inhibitors.

Major Task 4: Explore the impact of TYK2 downregulation on signaling nodes known to be important in MPNST pathogenesis.

Accomplishments: In MPNST, the loss of neurofibromin leads to overactivation of Ras and downstream activation of MEK and mTOR. To investigate the interaction of TYK2 with these signaling pathways, we evaluated the activation of STAT3, ERK, and S6K by western blot (WES system, Protein Simple) in MPNST-724 cells with CRISPR/Cas9 knockout of TYK2 expression (**Fig. 3F-H**). In TYK2-deficient cells, phophorylated-STAT3 (pSTAT3) levels were decreased, but pERK and pS6K were not changed. In MPNST-724 TYK2 KO #5-2 and #6-4 cells, TYK2 mRNA and protein expression was decreased by about 50% vs. control cells (**Fig. 3D-E**). Thus, the moderate decrease in STAT3 activity may be due to incomplete knockdown of TYK2. In addition, we observed reduced cell confluence and elevated apoptosis in TYK2 knockout MPNST-724 cells vs. control, as determined by IncuCyte assays utilizing YoYo-1 fluorescent dye (**Fig. 3A-C**).

We also performed a subset of western blot experiments using TYK2 inhibitor drugs. Similarly, treatment of murine JW23.3 cells with the specific TYK2 inhibitors WU-12 and WU-76 for 48

hours reduced pSTAT3 levels, but did not change pERK or pS6K levels (**Fig. 4**). These results indicate that blocking TYK2 signaling does not significantly inhibit the MEK and mTOR pathways in MPNST, thus suggesting that there may be potential synergism in combination treatments with inhibitors of TYK2 with inhibitors of MEK or mTOR.

Major Task 5: Determine the impact of TYK2 downregulation on the global expression profile of MPNSTs.

Accomplishments: While generating CRISPR/Cas9 knockout cell-lines, we conducted a subset of RNAseq experiments using specific TYK2 inhibitors, WU-12 and WU-76. RNA was isolated from JW23.3 cells treated with vehicle control (DMSO), WU-12 or WU-76 for 48 hours and the impact of TYK2 inhibition on the global expression profile was determined by RNAseq. We have completed QC analysis for these samples and are currently analyzing the RNAseq data in Partek Flow software.

Specific Aim 3: Evaluate pharmacologic inhibition of TYK2 with in MPNSTs *in vivo*.

Major Task 6. Does pharmacologic inhibition of TYK2 lead to decreased tumor viability *in vitro*?

MPNST Cell-line	Drug	IC50
JW23.3	WU-12	27.7 μM
JW23.3	WU-76	18.7 μM
JW23.3	TC-JL-37	27.8 μM
MPNST-724	WU-12	32.1 μM
MPNST-724	WU-76	13.9 μM
MPNST-724	TC-JL-37	*undetermined
JH 2-002	WU-12	26.5 μM
JH 2-002	WU-76	30.8 μM
JH 2-002	TC-JL-37	*undetermined
JH 2-009	WU-12	81.5 μM
JH 2-009	WU-76	80.1 μM
JH 2-009	TC-JL-37	42.8 μM

Table 2. IC50 concentrations for TYK2 inhibitory drugs in MPNST cell-lines. *undetermined value above dose curve upper threshold.

Accomplishments: We proceeded to Aim 3.1 (Major Task 6) earlier than planned, due to pandemic limitations on in-person staff and while switching to the CRISPR/Cas9 system from shRNA in Aim 2. Four MPNST cell-lines, JW23.3, MPNST-724, JH 2-002, and JH 2-009, were incubated for 72 hours with specific TYK2 inhibitors developed at Washington University (WU-12, WU-76), a commercially available TYK2 inhibitor (TC-JL-37), or a control compound (WU-18). WU-12, WU-76 and TC-JL-37 dose-dependently decreased the percent cell confluence, while WU-18 had no effect, in all four MPNST cell-lines, as determined by an IncuCyte cell proliferation assay (**Fig. 5 and 6**). JH 2-009 cells were less responsive to WU-12 and WU-76 than the other cell-lines, while MPNST-724 cells were less responsive to TC-JL-37. Conversely, apoptosis was increased by treatment with WU-12, WU-76 and TC-JL-37 in MPNST cells, as assessed by an IncuCyte cell death assay with YoYo-1 dye (**Fig. 7 and 8**). While TC-JL-37 is a potent TYK2

inhibitor, it can also block JAK1/2/3 signaling. Thus, differences in relative levels of TYK2 and the other JAKs may contribute to varying in responses between cell-lines to TC-JL-37, WU-76 and WU-12. IC50s were calculated for inhibition of cell confluence by the TYK2 inhibitors in each cell-line using GraphPad Prism software (**Table 2**).

Major Task 7. Does pharmacologic inhibition of TYK2 lead to decreased tumor growth and/or metastasis *in vivo* in MPNST PDX lines?

Accomplishments: Patient Derived Xenograft (PDX) lines have been maintained in mice and *in vitro* cultures. Task is on target.

Major Task 8. Does pharmacologic inhibition of TYK2 lead to decreased tumor growth and/or metastasis *in vivo* in mice with an intact immune system?

Accomplishments: *Nf1* heterozygous and wild-type mice have been maintained. Task is on target.

Appendix C: Major Accomplishments Figures

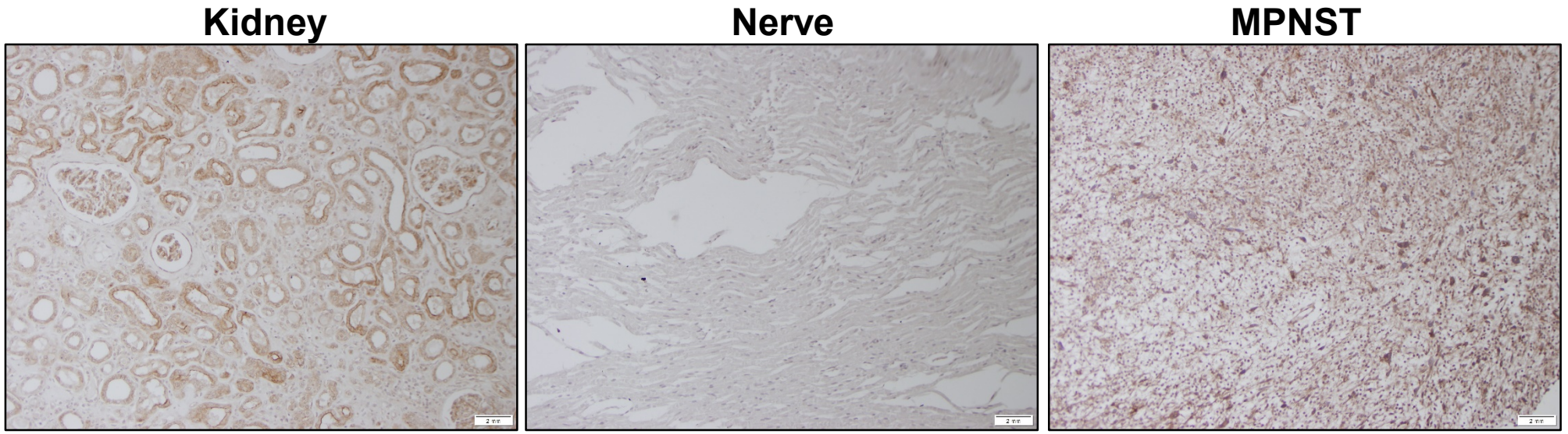

Fig. 1. Tyrosine kinase 2 (TYK2) protein expression by immunohistochemistry (IHC). Representative strong positive TYK2 staining in human kidney and high-grade Malignant Peripheral Nerve Sheath Tumor (MPNST), with negative staining for TYK2 in mouse nerve.

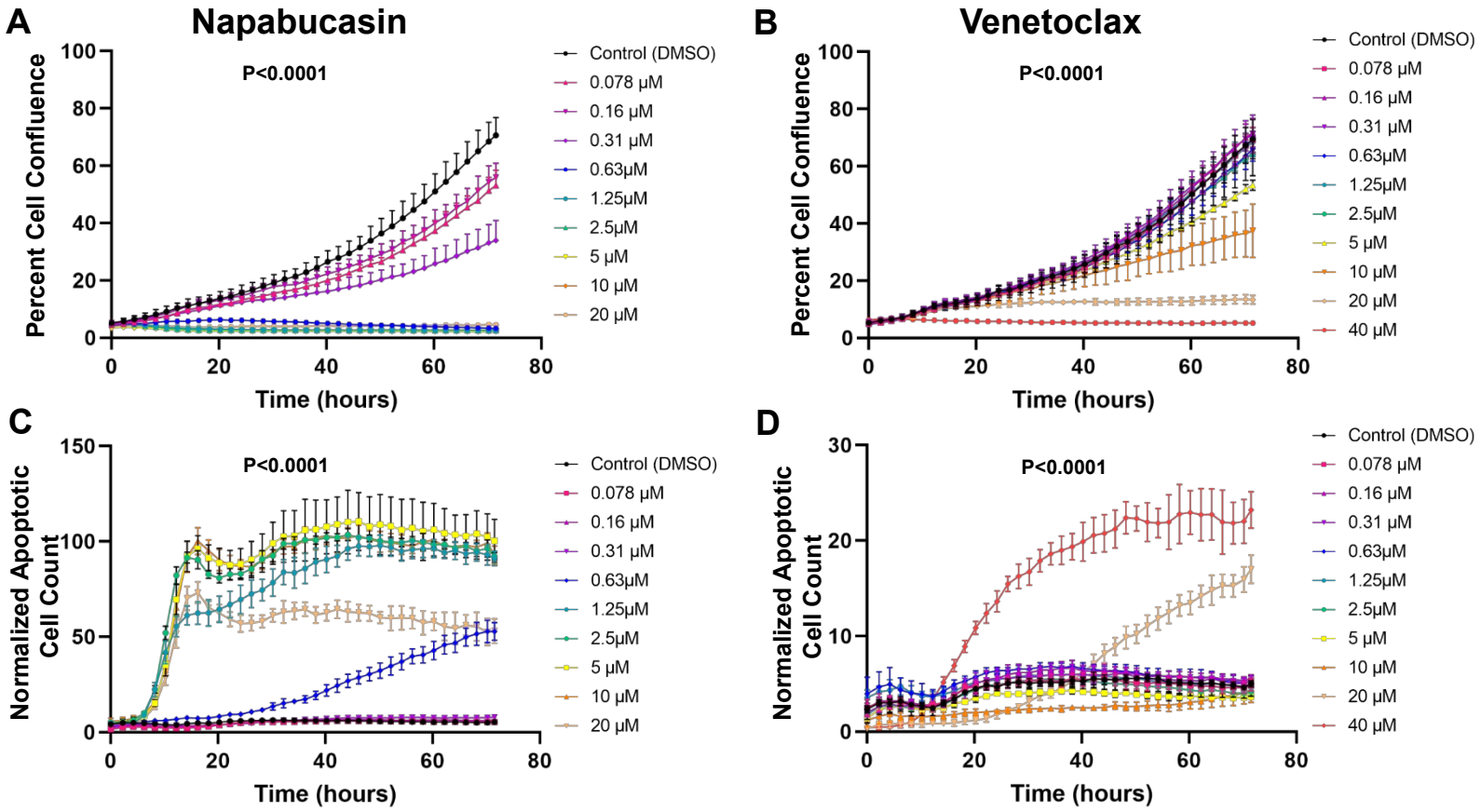

Fig. 2. Pharmacological inhibition of STAT3 or Bcl-2 decreases proliferation and increases apoptosis in MPNST cells. JW23.3 cells were treated with inhibitors of (A, C) STAT3 (napabucasin, NP) and (B, D) Bcl-2 (venetoclax, ABT-199) for 72 hours. Proliferation was determined by an IncuCyte proliferation assay (A, B) and apoptosis by an IncuCyte cell death assay (C, D), as assessed by YoYo-1 iodide green fluorescence.

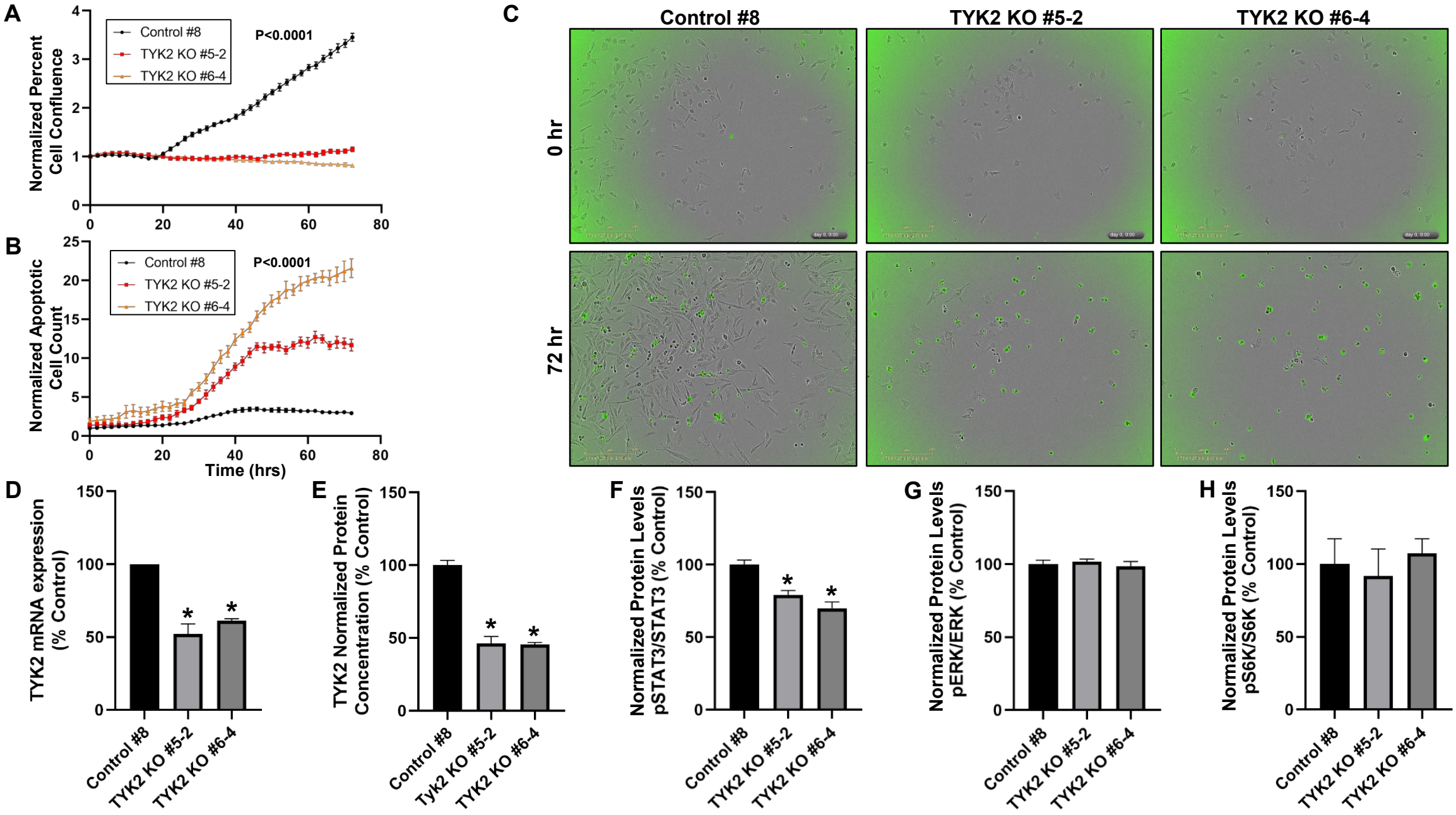

Fig. 3. TYK2-deficient MPNST-724 cells have decreased proliferation and STAT3 activity, but increased apoptosis. An IncuCyte proliferation assay (A, C) reveals decreased cell confluence over time in TYK2 knockdown (KD) MPNST-724 cells. An IncuCyte cell death assay (B, C) depicts increased apoptosis in TYK2 KD MPNST-724 cells, as assessed by YoYo-1 iodide green fluorescence (P<0.0001). (D) TYK2 mRNA expression in TYK2 KD MPNST-724 cells vs. control, as determined by qPCR. (E-H) TYK2 or phosphorylated STAT3, ERK1/2, or S6-kinase (S6K) protein levels in TYK2 KD MPNST-724 cells vs. control, normalized to total protein and analyzed by WES western blotting system (Protein Simple) (P<0.05).

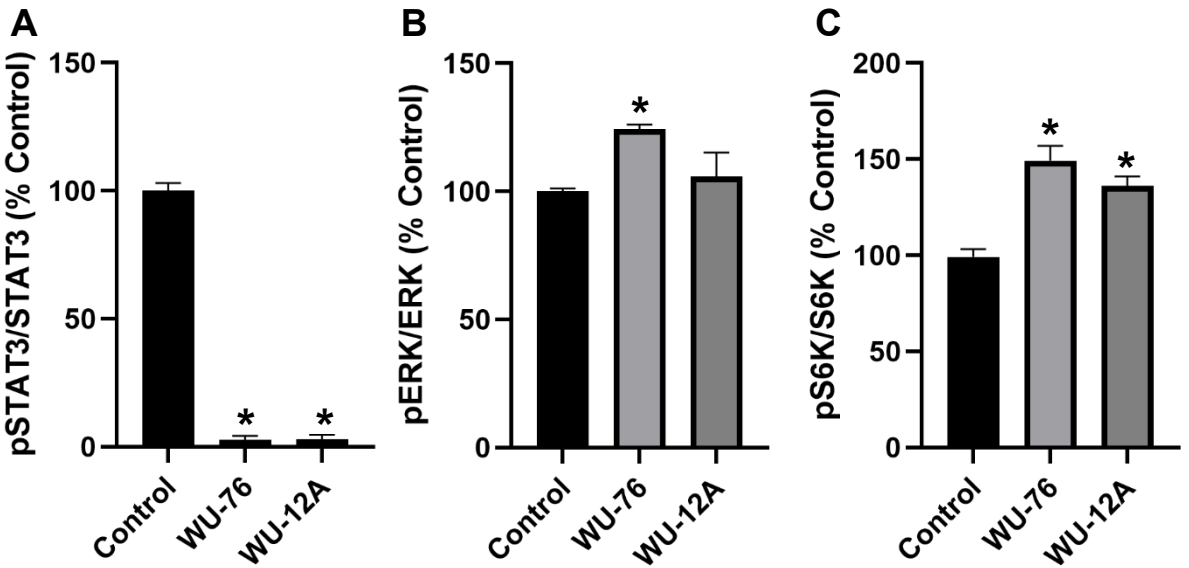

Fig. 4. TYK2 inhibition reduces STAT3 activity, but not activity of ERK1/2 or S6 kinase (S6K). JW23.3 cells were incubated with TYK2 inhibitors, WU-76 and WU-12A, for 48 hours. Lysates were subjected to western blotting using the WES system (ProteinSimple). Phosphorylated proteins were normalized to respective total protein.

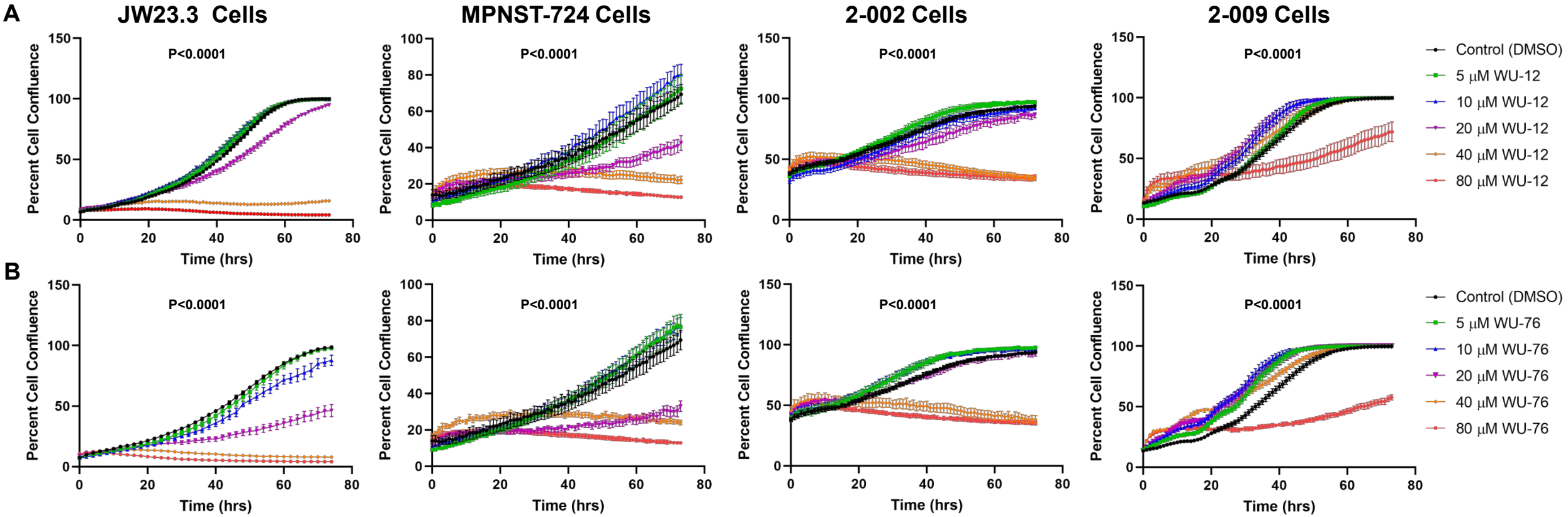

Fig. 5. Inhibition of TYK2 decreases proliferation in four MPNST cell-lines. JW23.3, MPNST-724, 2-002, and 2-009 cells were incubated for 3 days with the specific TYK2 inhibitors (A) WU-12 or (B) WU-76, developed at Washington University. The IncuCyte proliferation assay was used to determine proliferation in cells over time (P<0.0001).

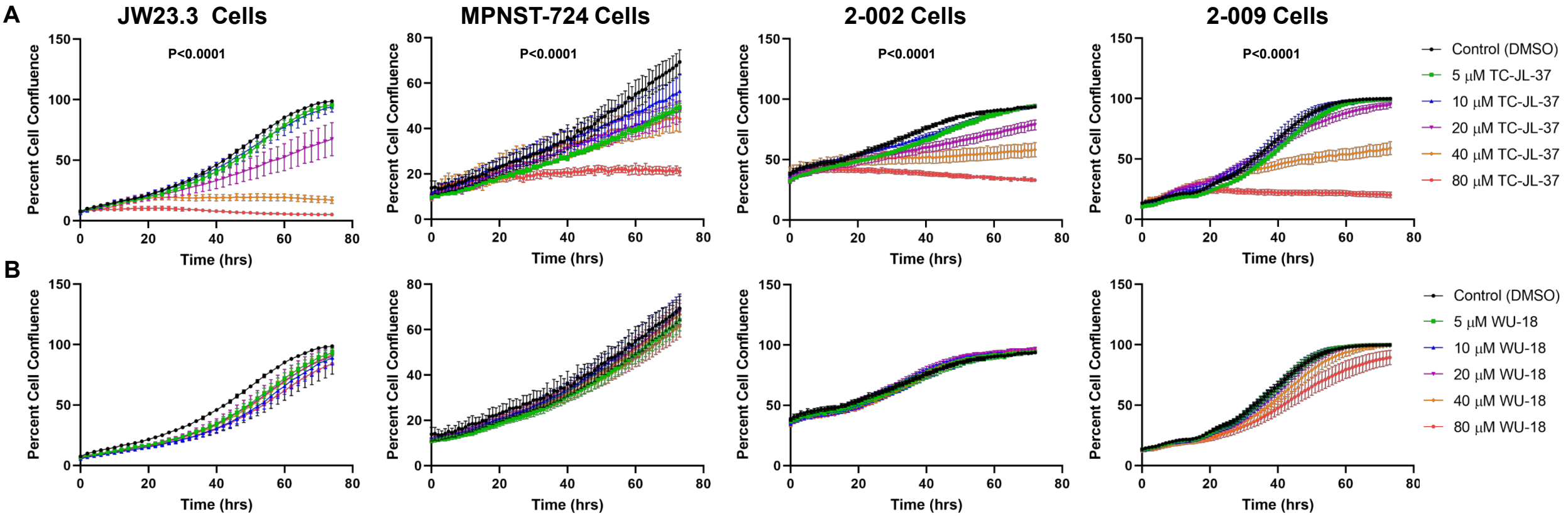

Fig. 6. The TYK2 inhibitor TC-JL-37 reduces cell proliferation in MPNST cells. JW23.3, MPNST-724, 2-002, and 2-009 cells were incubated for 72 hours with (A) TC-JL-37 or (B) WU-18, an inactive control compound. Proliferation was determined by IncuCyte analysis (P<0.0001).

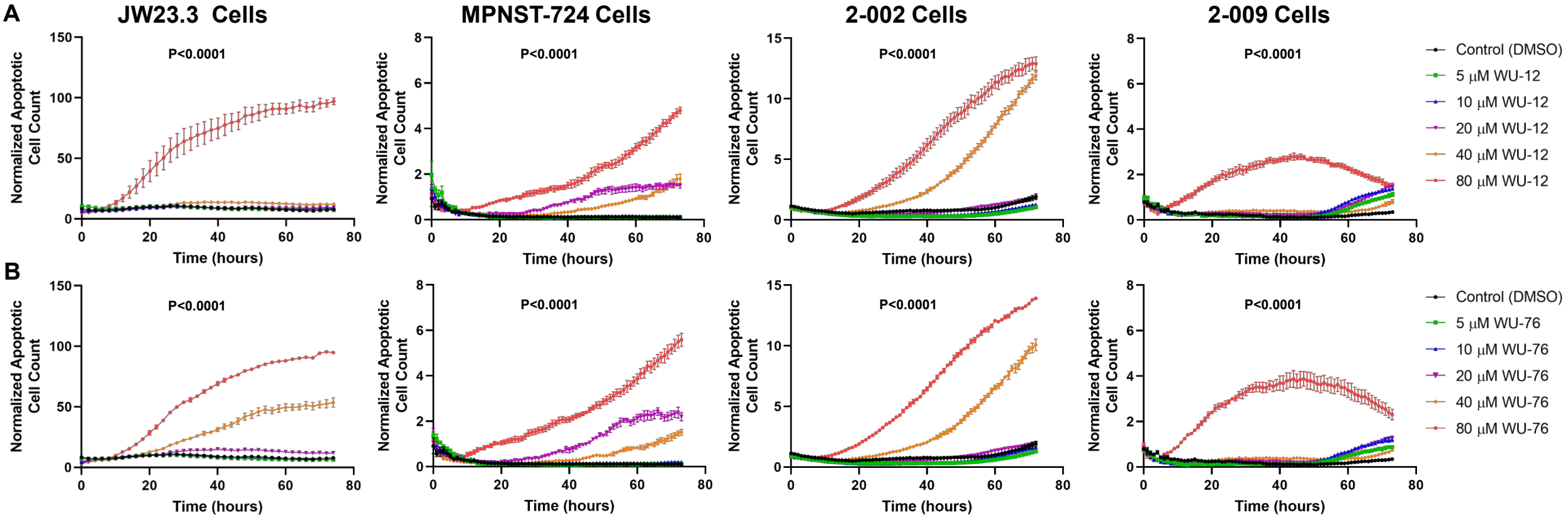

Fig. 7. Inhibition of TYK2 induces apoptosis in four MPNST cell-lines. JW23.3, MPNST-724, 2-002, and 2-009 cells were incubated for 72 hours with the TYK2 inhibitors (A) WU-12 or (B) WU-76. The IncuCyte cell death assay with YoYo-1 fluorescent dye was used to measure apoptosis in cells, with the number of apoptotic green fluorescent cells normalized to percent cell confluence over time (P<0.0001).

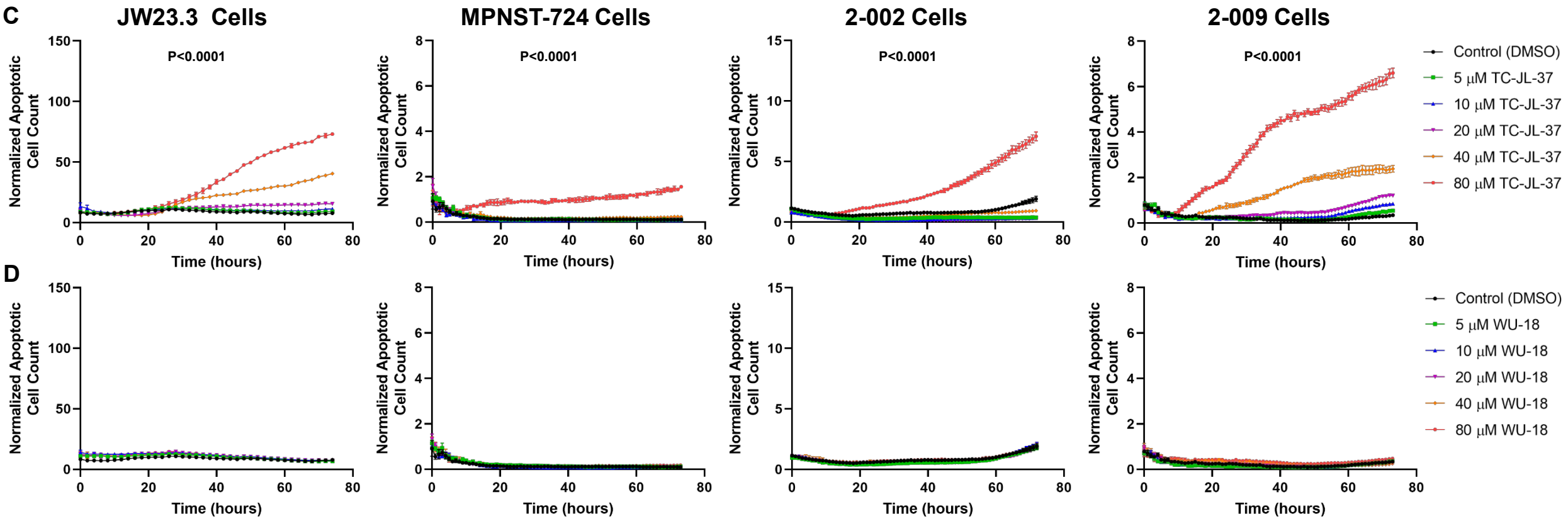

Fig. 8. Inhibition of TYK2 induces apoptosis in four MPNST cell-lines. JW23.3, MPNST-724, 2-002, and 2-009 cells were incubated for 72 hours with (A) the TYK2 inhibitor TC-JL-37 or (B) WU-18, an inactive control compound. The IncuCyte cell death assay with YoYo-1 fluorescent dye was used to measure apoptosis in cells over time. Apoptotic cell count was normalized to percent cell confluence over time (P<0.0001).

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: We will determine whether TYK2 is a prognostic biomarker for MPNST.

Major Task 1: Are TYK2 expression levels associated with overall survival, time to metastasis, and response to therapy in patients with MPNST?

Plans: During the next reporting period (Year 2), we will continue IHC for TYK2 on our remaining MPNST slides, followed by scoring of these slides for TYK2 staining. We will then conduct statistical analysis to determine whether TYK2 levels are associated with overall survival, time to response, and therapy response in MPNST patients.

Major Task 2: Does TYK2 expression distinguish atypical neurofibromas from MPNSTs?

Plans: We plan to complete TYK2 staining and scoring for all of the atypical neurofibromas/low-grade MPNST samples. The diagnostic ability to determine if TYK2 can be used to distinguish atypical neurofibromas from MPNST will be determined with area under the curve (AUC) analyses.

Specific Aim 2: We will delineate the mechanism of action of TYK2 in MPNST cells.

Major Task 3: Determine whether STAT-3 mediated activation of Bcl-2 is responsible for TYK-2 mediated protection from cell death.

Plans: We are in the process of expanding and validating colonies of murine JW23.3 and human 2-002 MPNST cells transfected by CRISPR/Cas9 for knockout of TYK2 and/or STAT3. These genetic knockout cells will then be used for IncuCyte cell growth and death assays. In addition, we will extend IncuCyte proliferation and apoptosis studies with napabucasin, a STAT3 inhibitor, and venetoclax, a Bcl-2 inhibitor, to human MPNST-724 cells. We also plan to evaluate the type of cell death (e.g. apoptosis) involved by TUNEL assay, Western blotting for cleaved caspase-3, and flow cytometry with Annexin-V/propidium iodide staining.

Major Task 4: Explore the impact of TYK2 downregulation on signaling nodes known to be important in MPNST pathogenesis.

Plans: We will continue to explore the interaction of TYK2 signaling (e.g. STAT3) and MPNST known signaling nodes (e.g. the MEK/ERK and mTOR/S6K pathways) by Western blot in TYK2-deficient (CRISPR/Cas9 KO) JW23.3 and 2-002 cells compared to control cells.

Major Task 5: Determine the impact of TYK2 downregulation on the global expression profile of MPNSTs.

Plans: We will continue the analysis of RNAseq data for JW23.3 cells incubated with TYK2 inhibitors (WU-12 or WU-76) for 48 hours. In addition, we will isolate RNA and perform RNAseq

for JW23.3 and 2-002 TYK2 CRISPR KO vs. control cells.

<u>Specific Aim 3</u>: Evaluate pharmacologic inhibition of TYK2 in MPNSTs *in vivo*.

Major Task 6. Does pharmacologic inhibition of TYK2 lead to decreased tumor viability *in vitro*?

Plans: This major task has been completed early during Year 1.

Major Task 7. Does pharmacologic inhibition of TYK2 lead to decreased tumor growth and/or metastasis *in vivo* in MPNST PDX lines?

Plans: Patient Derived Xenograft (PDX) lines will continue to be maintained in mice and *in vitro* cultures. We will plan out the *in vivo* experiments with the goals to begin during the second half of year 2 or year 3.

Major Task 8. Does pharmacologic inhibition of TYK2 lead to decreased tumor growth and/or metastasis *in vivo* in mice with an intact immune system?

Plans: *Nf1* heterozygous and wild-type mice will be maintained over the next year.