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CONTRACTING ORGANIZATION: University of Washington, St. Louis, MO

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14. ABSTRACT
Malignant peripheral nerve sheath tumors (MPNST) are aggressive sarcomas that account for ~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 secondary to radiation therapy. In the setting of NF1, MPNST arise from malignant transformation of a benign precursor lesion, a plexiform neurofibroma. Despite aggressive therapy, the recurrence rate is high and the vast majority of people with these cancers will die within 5 years of diagnosis. Given limited treatment options, there is a pressing need to identify novel therapeutic targets. Our laboratory previously 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 increased cell death *in vitro* and decreased tumor growth. Here, immunohistochemistry (IHC) shows strong TYK2 staining in 63/112 (56%) MPNST, 13/39 (33%) plexiform neurofibromas and 23/24 (96%) atypical neurofibromas (ANNUBP). Kaplan Meier analysis indicates that TYK2 association with overall survival differs between NF1 and sporadic MPNST patients. In murine MPNST JW23.3 cells, knockout of *TYK2* and *TYK2/STAT3* markedly block proliferation, while *STAT3* knockout only modestly reduces proliferation, as assessed by IncuCyte live cell assays. Similarly, pharmacologic inhibition of TYK2 (WU-12, WU-76, TC-JL-37, and BMS-986165), STAT3 (napabucasin) and Bcl2 (venetoclax) dose-dependently decrease proliferation and induce apoptosis over time in five MPNST cell-lines. In murine JW23.3 and human JH 2-002 cells, incubation with TYK2 inhibitors reduces pSTAT3 levels, while increasing pERK levels, as determined by WES western blot system. Consistent with the TYK2 proliferative effects, RNAseq pathway analysis reveals that TYK2 inhibitory drugs decrease cell cycle, mitotic, and glycolysis pathways. Surprisingly, TYK2 inhibitors stimulate expression of genes in the Ras/MEK/MAP-kinase pathway in MPNST cells, as shown by both RNAseq and a JAK-STAT pathway qPCR array. Combination treatment with inhibitors of TYK2 (WU-12 or BMS-986165) and MEK (mirdametinib) act synergistically to further decrease proliferation and increase apoptosis in MPNST cell-lines. These findings suggest that TYK2 promotes MPNST pathogenesis through stimulation of proliferation, which is partially mediated through STAT3. In addition, the combination of TYK2 and MEK inhibitory drugs serves as a promising therapeutic strategy in MPNST.

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. <u>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.</u>

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, 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 March 1, 2021.

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 moved 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. An amendment to our IACUC/ACCURO animal protocol that added two drugs (TYK2 inhibitor, deucravacitinib, and MEK inhibitor, mirdametinib) was approved in March, 2022.

Significant changes in use of biohazards and/or select agents

Nothing to report.

6. PRODUCTS:

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

Journal publications.

Borcherding DC, He K, Amin NV, Hirbe AC. TYK2 in Cancer Metastases: Genomic and Proteomic Discovery. Cancers. 2021; 13(16):4171. https://doi.org/10.3390/cancers13164171

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

Nothing to report.

Other publications, conference papers and presentations.

D.C. Borcherding, N. Amin, K. He, C. Dehner, P. Ruminski, S. Dahiya, J. Chrisinger, T. Sundby, K. Pollard, B.C. Widemann, C.A. Pratilas, J.F. Shern, and A.C. Hirbe. TYK2 is a Biomarker and Therapeutic Target in Malignant Peripheral Nerve Sheath Tumors. Poster presentation. Connective Tissue Oncology Society (CTOS) 2021 Annual Meeting. Vancouver, Canada (moved online).

- **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**

Provisional patent application in place through the Office of Technology Management at Washington University: **Title: Combined TYK2 inhibition and MEK inhibition**
Reference Number: T-020000

- **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, western blotting and qPCR.

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 for September 1, 2020, to March 1, 2021, due to pandemic restrictions. New timeline assumes a no cost extension of 6 months due to the pandemic (extending to month 42).

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			Month
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	100%	0-9
Subtask 2: Generation of unstained slides and clinical chart review	3-6	Dr. Hirbe	100%	3-9
Subtask 3: TYK2 staining	6-8	Dr. Hirbe	100%	6-18
Subtask 4: Blinded TYK2 scoring by Dr. Dahiya, Chrisinger	8-10	Dr. Hirbe	100%	10-21
Subtask 5: Statistical analysis	10-12	Dr. Hirbe	80%	12-36
Milestone(s) Achieved: Determine whether TYK2 expression is correlated with clinical prognostic parameters.	**12**	Dr. Hirbe	**80%**	**36**
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	100%	0
Subtask 2: Generation of unstained slides and clinical chart review	3-6	Dr. Hirbe	100%	3-9
Subtask 3: TYK2 staining	6-8		100%	6-18
Subtask 4: Blinded TYK2 scoring by Dr. Dahiya, Chrisinger	8-10	Dr. Hirbe	100%	10-21
Subtask 5: Statistical analysis	10-12	Dr. Hirbe	80%	12-36
Milestone(s) Achieved: Determine whetherTYK2 can be used to distinguish atypical neurofibromas from MPNSTs.	**12**	Dr. Hirbe	**80%**	**36**

<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	75%	4-26
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 JH 2-002 cells (obtained from Dr. Christine Pratilas at Johns Hopkins University)	4-8	Dr. Hirbe	50%	16-30
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 JH 2-002 cells	8-12	Dr. Hirbe	100%	10-14
Subtask4: Evaluation of type of cell death Cell lines: JW23.3 murine MPNST cells, human JH 2-002 cells	12-16	Dr. Hirbe	0%	24-36
Milestone(s) Achieved: Understand the mechanism of TYK2 in MPNST growth and survival.	**16**	Dr. Hirbe	**70%**	**36-40**
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 JH 2-002 cells	16-20	Dr. Hirbe	75%	6-36
Milestone(s) Achieved: Determine whether TYK2 signaling intersects with neurofibromin signaling.	**20**	Dr. Hirbe	**75%**	**36**
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 was the initial plan. We have since generated CRISPR knockout to obtain complete knockdown. Cell lines: JW23.3 murine MPNST cells, human JH 2-002 cells	20-21	Dr. Hirbe	75%	4-28
Subtask 2: RNA sequencing and analysis.	21-24	Dr. Hirbe	75%	6-36
Milestone(s) Achieved: Determine the global expression changes upon knockdown of TYK2.	**24**	Dr. Hirbe	75%	**36**

<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, JH 2-002 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, Dehner et al. JCI Insight)	21-25	Dr. Hirbe	On Target	28-30
Subtask 2: Randomization for drug treatment	22-26	Dr. Hirbe	On Target	30-34
Subtask 3: Monitoring tumor burden and response to treatment	22-32		On Target	34-40
Subtask 4: Statistical analysis and histopatholgical correlations (Drs. Dahiya and Chrisinger)	32-36		On Target	40-42
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**	**42**
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	26-30
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 March 1, 2021.

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 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), the National Cancer Institute (NCI), and the University of California at San Francisco (UCSF) (**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 112 MPNST samples from Washington University, JHU, NCI and UCSF patients (**Table 1**). Three independent observers scored these slides in a blinded fashion, and statistical analysis is underway (**Fig. 1**). Moderate to strong TYK2 staining (score ≥ 2) was observed in 56% high-grade MPNST samples, with 44% having weak or negative TYK2 staining (**Table 1**). Sporadic MPNST had a higher rate of strong TYK2 staining than NF1 MPNST samples, at 62% vs. 53% strong TYK2 (**Table 1**). We then compared overall survival for MPNST patients vs. TYK2 levels by Kaplan Meier analysis (**Fig. 1B-D**). For NF1 MPNST samples, weak/negative TYK2 (<2) was associated with poorer overall survival (**Fig. 1C**), with a median overall survival of 28 months vs. 100 months for strong TYK2 (≥2). In contrast, for sporadic MPNST, weak/negative TYK2 (<2) was associated with better overall survival (**Fig. 1D**), with a median overall survival of 94 months vs. 27 months for strong TYK2 (≥2).

Tumor Type	Weak TYK2 (<2)	Strong TYK2 (≥2)
MPNST (all)	49 (44%)	63 (56%)
MPNST: NF1	33 (47%)	37 (53%)
MPNST: Sporadic	16 (38%)	26 (62%)
Plexiform Neurofibroma	26 (67%)	13 (33%)
ANNUBP	1 (4%)	23 (96%)

Table 1. Immunohistochemistry (IHC) for TYK2 protein levels in patient tumor samples. MPNST, plexiform neurofibroma, and ANNUBP samples were stained for TYK2 and the proportion of positive staining was scored on a 0-3 scale.

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 obtained 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 also provided slides. All cases of plexiform neurofibroma, atypical neurofibroma and low grade MPNST have been re-reviewed with pathology. We have conducted IHC for TYK2 on 112 MPNST, 24 Atypical neurofibromatous neoplasm of uncertain biologic potential (ANNUBP), and 39 Plexiform Neurofibroma samples from Washington University, JHU, NCI and UCSF patients (**Fig. 1**). These samples have been scored by three independent observers in a blinded fashion, and we are now completing statistical analyses. Plexiform neurofibromas were largely weak/negative for TYK2 (67%), while most of the ANNUBP tumors (96%) were strongly positive for TYK2 (**Table 1**). This suggests that TYK2 may be a transformative factor from benign precursor tumor to MPNST.

<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 and mouse JW23.3 cells. However, due to variability in the short-hairpin system in genetic knockdown, we moved to the CRISPR/Cas9 system as it provides a better genetic knockout and has become a more widely accepted technique than shRNA.

We have generated colonies for two MPNST cell-lines (murine JW23.3 *Nf1/Tp53*-mutant and human JH 2-002) with CRISPR/Cas9 knockout of *TYK2*, *STAT3*, or double knockout (DKO) of both genes. The JH 2-002 cell line was added to ensure the translatability of our results. Knockout of *TYK2* and/or *STAT3* were verified by next generation sequencing (NGS), by qPCR for mRNA gene expression and/or by WES western blotting system (Protein Simple) for protein levels (**Fig. 2a-b**).

The effect of genetic depletion of *TYK2*, *STAT3* or the combination over time was evaluated by IncuCyte Zoom Live Cell proliferation assays (Sartorius). In JW23.3 cells, *TYK2* knockout (T15-4) dramatically inhibited proliferation to less than 25% of control cells, with DKO of *TYK2* and *STAT3* (TS14-10) having a similar inhibitory effect (**Fig. 2b**). However, *STAT3* knockout (S13-3) only modestly decreased proliferation to about 50-75% of control over time, suggesting that TYK2 proliferative effects may only be partially mediated through STAT3 (**Fig. 2b**). *TYK2* knockout modestly increased the number of apoptotic cells, but *STAT3* knockout or DKO did not affect

apoptosis (**Fig. 2b**). We plan to evaluate proliferation and apoptosis for CRISPR knockout lines (*TYK2*, *STAT3* and DKO) in JH 2-002 cells by IncuCyte Live Cell assays. Additionally, we will evaluate which other STAT proteins may be mediating the downstream effects of TYK2.

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
JW23.3	Napabucasin (NP)	0.338 μM
JW23.3	Venetoclax (ABT-199)	10.3 μM
JW23.3	Mirdametinib (PD0325901)	1.22 μM
MPNST-724	WU-12	32.1 μM
MPNST-724	WU-76	13.9 μM
MPNST-724	TC-JL-37	*undetermined
MPNST-724	Napabucasin (NP)	0.038 μM
MPNST-724	Venetoclax (ABT-199)	1.10 M
MPNST-724	Mirdametinib (PD0325901)	0.279 μM
JH 2-002	WU-12	26.5 μM
JH 2-002	WU-76	30.8 μM
JH 2-002	TC-JL-37	*undetermined
JH 2-002	Napabucasin (NP)	0.297 μM
JH 2-002	Venetoclax (ABT-199)	*undetermined
JH 2-002	Mirdametinib (PD0325901)	0.219 μM
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
JH 2-009	Napabucasin (NP)	0.798 μM
JH 2-009	Venetoclax (ABT-199)	6.52 μM
JH 2-009	Mirdametinib (PD0325901)	0.110 μM
JH 2-031	WU-12	42.4 μM
JH 2-031	WU-76	41.7 μM
JH 2-031	TC-JL-37	63.7 μM
JH 2-031	Napabucasin (NP)	---------
JH 2-031	Venetoclax (ABT-199)	---------
JH 2-031	Mirdametinib (PD0325901)	0.437 μM

Table 2. IC50 concentrations for drugs inhibiting TYK2, STAT3 and Bcl-2 in MPNST cell-lines. *undetermined value above dose curve upper threshold.

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), or 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. 3**). 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. Similarly, the STAT3 and Bcl2 inhibitors decreased proliferation in a panel of other MPNST cell-lines: MPNST-724, JH 2-002, and JH 2-009 (**Table 2**). IC50s were calculated for inhibition of cell confluence by the drugs in each cell-line using GraphPad Prism software (**Table 2**).

Our initial data indicated that genetic depletion of TYK2 resulted in both decreased proliferation and increased apoptosis. However, expanded studies in a larger panel of cell-lines and with TYK2 inhibitory drugs revealed a greater effect on proliferation than on apoptosis. Thus, we are focusing on proliferation and survival currently. We may turn back to evaluating cell death mechanisms, i.e. via Bcl-2 knockout and apoptotic assays at a later time as the true effect of TYK2 is likely multifactorial.

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 cells using TYK2 inhibitor drugs. In JW23.3 and JH 2-002 cells, the TYK2 inhibitors WU-12 and WU-76 (both developed at Washington University) decreased pSTAT3 protein levels at 1, 24 and 48 hours, as evaluated by the WES western blotting system (**Fig. 4A-B**). Interestingly, pERK2 levels were strongly stimulated by 2-3 fold over time by treatment with TYK2 inhibitors (WU-12 and WU-76) in both cell-lines, suggesting that the cells are compensating for TYK2 suppression by upregulating the MEK/ERK pathway. Treatment with the TYK2 inhibitors resulted in moderately increased pS6-kinase levels at 24 and 48 hours in JH 2-002 cells, but not in JW23.3 cells. (**Fig. 4A-B**). We are currently conducting parallel experiments to examine activation of STAT3, ERK, and S6-kinase by incubation with another specific TYK2 inhibitor, deucravacitinib (BMS-986165), which is used clinically in autoimmune conditions, and thus could more easily move into a clinical trial for MPNST.

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. Pathway analysis revealed that inhibition of TYK2 stimulated KRAS signaling, GPCR pathways, and Oxidative Phosphorylation, while decreasing Cell Cycle, Mitotic, and Glycolysis pathways (**Fig. 5B**). Down-regulated genes involved in proliferation and the G1 to S phase transition include *Ccnd2*, *Cdkn1a/c*, *E2f3/4*, *Pole2* and *Mcm7* (**Fig. 6A**). Incubation with TYK2 inhibitors increased expression of genes in the Ras/MEK/MAP-kinase pathway, including *Hras*, *Nras*, *Map3k2/3*, *MAP2k2/4*, *Map2*, and *Jun* (**Fig. 6B**).

In addition, we examined the effect of the TYK2 inhibitor WU-12 on gene expression in human JH 2-002 cells with a qPCR array targeted for JAK-STAT pathway-related genes (**Fig. 5A**). JH 2-002 cells were incubated with WU-12 or vehicle control (DMSO) for 48 hours, and RNA was isolated. A qPCR array was utilized to analyze changes in mRNA for 88 genes of interest (GOIs) related to the JAK-STAT pathway. Gene expression changes revealed a general upregulation of the MEK/MAP-kinase pathway (**Fig. 5A**). We validated these results for select significantly changed genes by qPCR using different primer sets (data not shown). Thus, TYK2 inhibition induced a rapid and sustained increase in pERK1/2 protein levels and MEK/MAP-kinase pathway target gene expression, which we believe is a compensatory mechanism (**Fig. 5C**).

<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*?

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. Five MPNST cell-lines, JW23.3, MPNST-724, JH 2-002, JH 2-009, and JH 2-031, 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 five MPNST cell-lines, as determined by an IncuCyte cell proliferation assay (**Fig. 7C-D and 8**). 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 high doses of WU-12, WU-76 and TC-JL-37 in MPNST cells, as assessed by an IncuCyte cell death assay with YOYO-1 dye (**Fig. 9**). 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. MPNST cell-lines that were more responsive to TYK2 inhibitors, e.g. JH 2-002 and JW23.3, had higher TYK2 gene and protein expression than the less responsive 2-009 cells (**Fig. 7A-B**). IC50s were calculated for inhibition of cell confluence by the TYK2 inhibitors in each cell-line (**Table 2**).

In order to expedite translation to the clinic, we subsequently tested a specific TYK2 inhibitor, deucravacitinib (BMS-986165), and a pan-JAK/TYK2 inhibitor, baricitinib. Both deucravacitinib and baricitinib are used clinically in patients. Deucravacitinib has completed Phase 3 clinical trials and the FDA has accepted the application for approval for treatment of plaque psoriasis. The TYK2 inhibitor, deucravacitinib, dose-dependently reduced JW23.3 cell proliferation with an IC50 value of 6.76 μM (**Fig. 10A**). In contrast, baricitinib decreased cell proliferation with an IC50 of 27.3 μM (**Fig. 10B**). Baricitinib is specific for JAK1/2 as well as TYK2 at higher doses. These results indicate that the inhibition of MPNST cell proliferation is predominantly mediated through TYK2, and not the other JAKs.

Given that inhibition of TYK2 stimulates the MEK/MAP-kinase pathway, we investigated whether adding a MEK inhibitor improved the efficacy of TYK2 inhibitor drugs. Treatment of JW23.3 cells with a MEK inhibitor, mirdametinib, dose-dependently increased the percent inhibition of

cell confluence after 72 hours (**Fig. 11A**). Combination treatment with a TYK2 inhibitor (WU-12) and a MEK inhibitor (mirdametinib) synergistically blocked proliferation in JW23.3 cells (**Fig. 11C-E**). Similarly, the combination of the TYK2 inhibitor, deucravacitinib, and the MEK inhibitor, mirdametinib, synergistically inhibited cell proliferation and increased apoptosis in JW23.3 cells (**Fig. 12**).

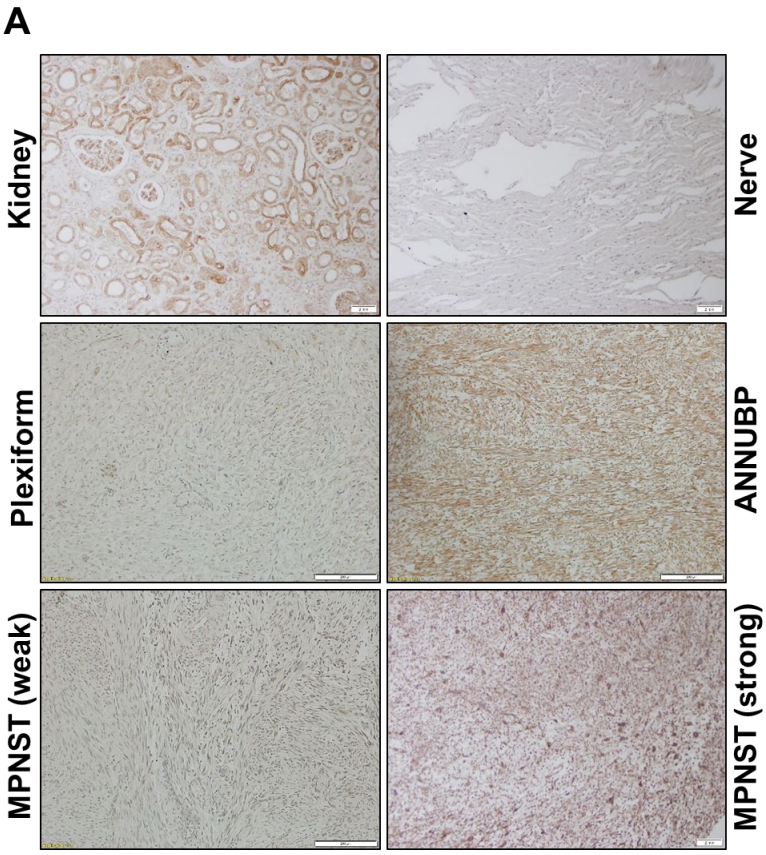
Major Task 7. Does pharmacologic inhibition of TYK2 lead to decreased tumor growth and/or metastasis *in vivo* in MPNST PDX lines?

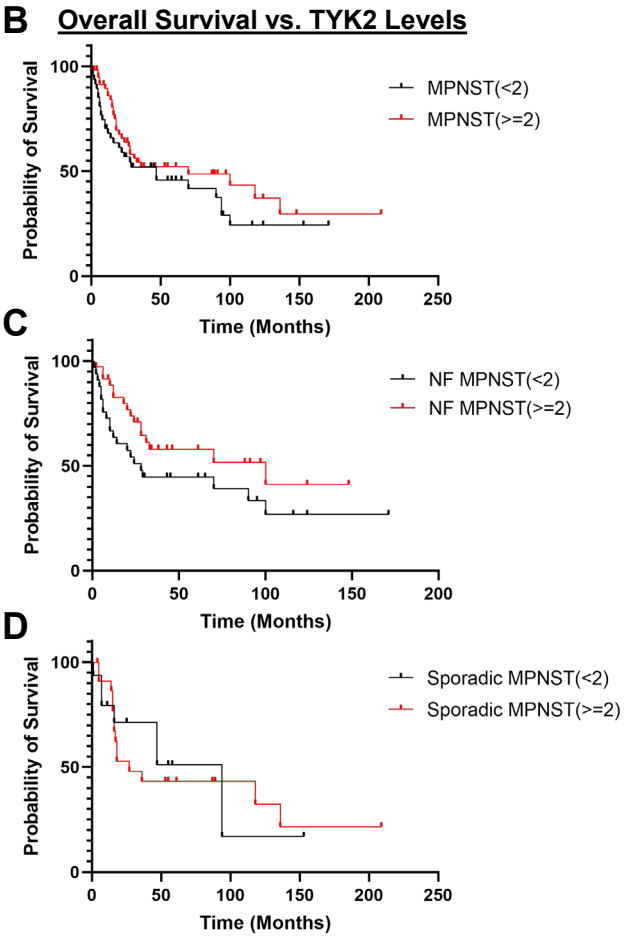
Accomplishments: Patient Derived Xenograft (PDX) lines have been maintained in mice and *in vitro* cultures. Task is on target.

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. In addition, we are now preparing for mouse experiments with JW23.3 (murine *Nf1/Tp53*-mutant) cell xenograft tumors, where mice will be randomized into 4 groups: (1) vehicle control, (2) mirdametinib, (3) deucravacitinib (BMS-986165), and (4) combination of mirdametinib and deucravacitinib. The drugs have been obtained, and we have received approval for the amendment adding these drugs to our IACUC/ACCURO animal protocol.

Appendix C: Major Accomplishments Figures





E

Characteristics	MPNST TYK2 Strong (Proportion>=2)	MPNST TYK2 Weak (Proportion<2)	MPNST	ANNUBP	Plexiform Neurofibromas
Sex—no. (%)					
Male	23	26	49	9	21
Female	40	23	63	11	18
Age Category—no. (%)					
<18 yo	6	7	13	8	16
18-65 yo	55	38	93	12	23
>65 yo	1	3	4	0	0
NF Status—no. (%)					
NF1	37	33	70	18	39
Sporadic	26	16	42	2	0
Average Score	2.57	0.84	1.82	2.73	1.58

Fig. 1. Tyrosine kinase 2 (TYK2) protein expression by immunohistochemistry (IHC). (A) Representative strong positive TYK2 staining in human kidney and negative staining for TYK2 in mouse nerve. Representative TYK2 staining in plexiform neurofibroma, ANNUBP, and high-grade Malignant Peripheral Nerve Sheath Tumor (MPNST). Kaplan Meier plots of overall survival time for patients by TYK2 staining in (A) all MPNST samples, (B) NF1 MPNST samples and (C) sporadic MPNST samples. The proportion of positive TYK2 staining was scored on a 0-3 scale (0.5 increments). (E) Table of patient characteristics.

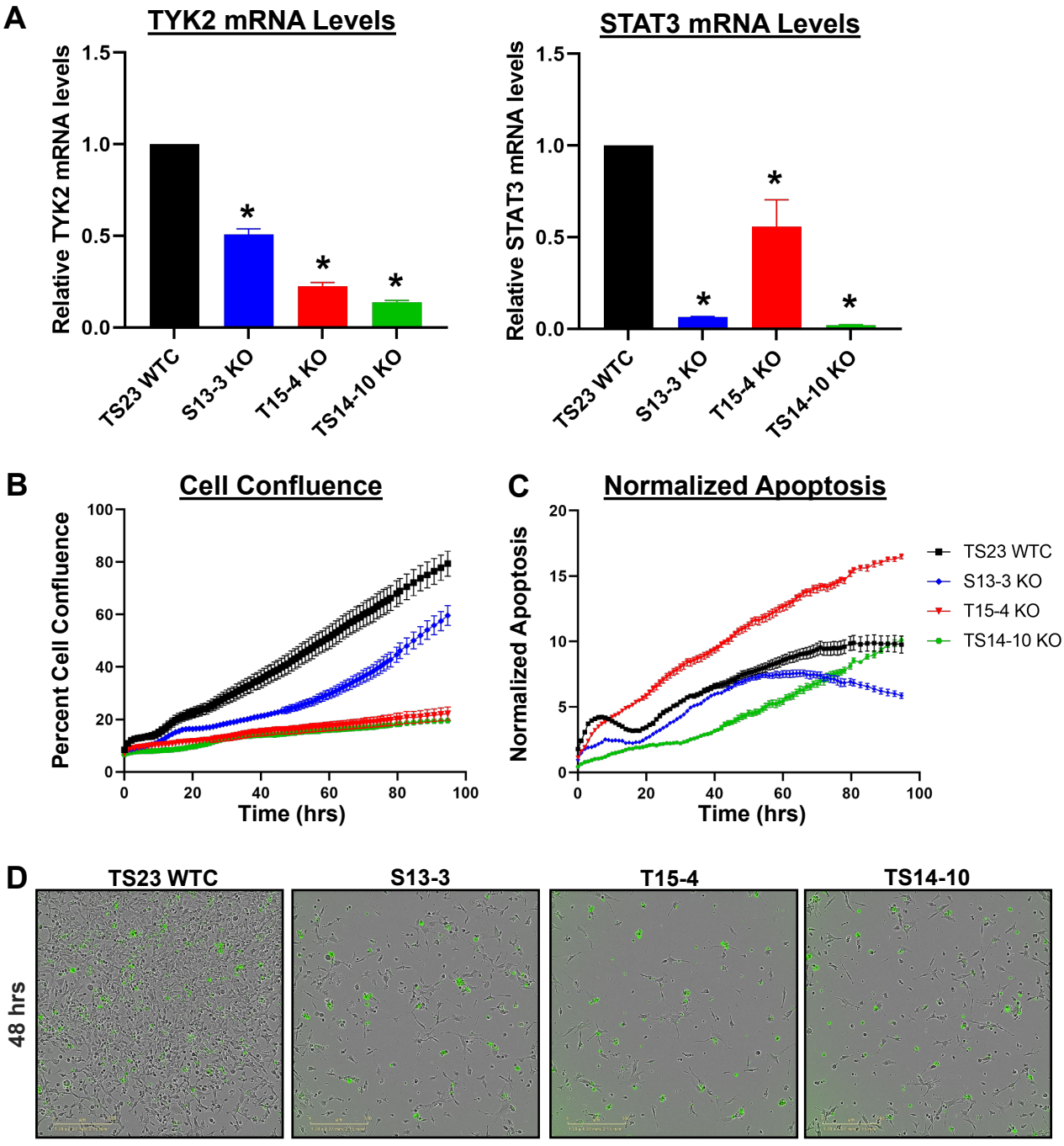


Fig. 2. TYK2 inhibitors reduce proliferation and induce apoptosis in MPNST cells. (A) Relative *TYK2* and *STAT3* mRNA expression in JW23.3 cells for CRISPR/Cas9 knockouts of *TYK2* (T15-4), *STAT3* (S13-3), *TYK2/STAT3* (TS14-10) or wild-type control (TS23 WTC), normalized to housekeeping gene. (B) Percent cell confluence and (C) normalized apoptosis were determined by the IncuCyte live cell imaging assay over 4 days in lines of JW23.3 cells with CRISPR/Cas9 knockout of *TYK2* and/or *STAT3*, in media with 2.5% FBS. Apoptotic cell count (YOYO-1 green fluorescence count), was normalized to percent cell confluence at the same time-point. (D) Representative images of IncuCyte assay at 48 hours, with YOYO-1 green fluorescence as an indicator of apoptosis.

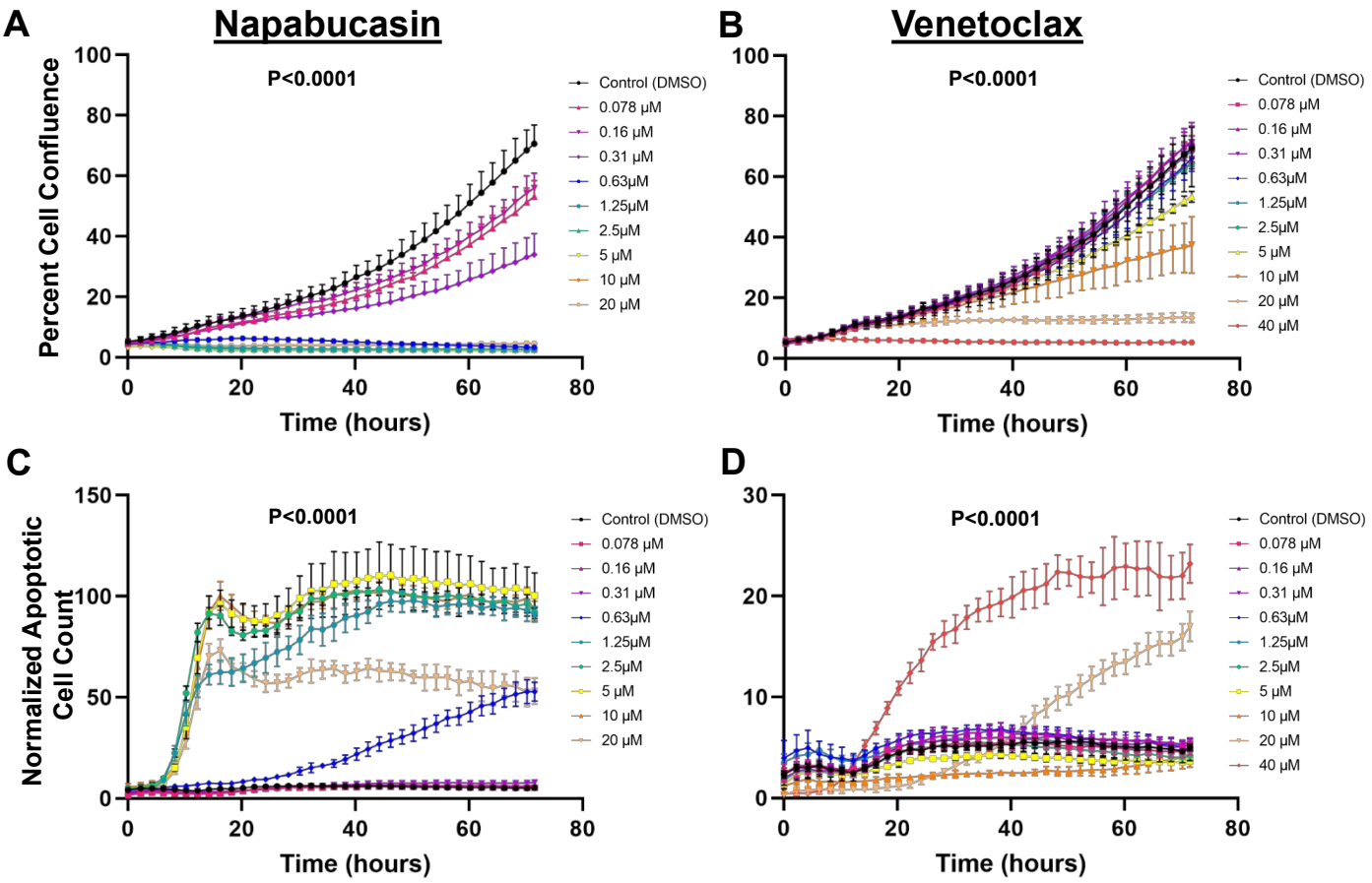


Fig. 3. Pharmacological inhibition of STAT3 or Bcl2 decreases proliferation and increases apoptosis in MPNST cells. JW23.3 cells were treated with inhibitors of (A, C) STAT3 (napabucasin, NP) and (B, D) Bcl2 (venetoclax, ABT-199) for 72 hours. Proliferation was determined by an IncuCyte proliferation assay (A, B) and apoptosis by an IncuCyte cell death assay (C, D), as assessed by YOYO-1 iodide green fluorescence.

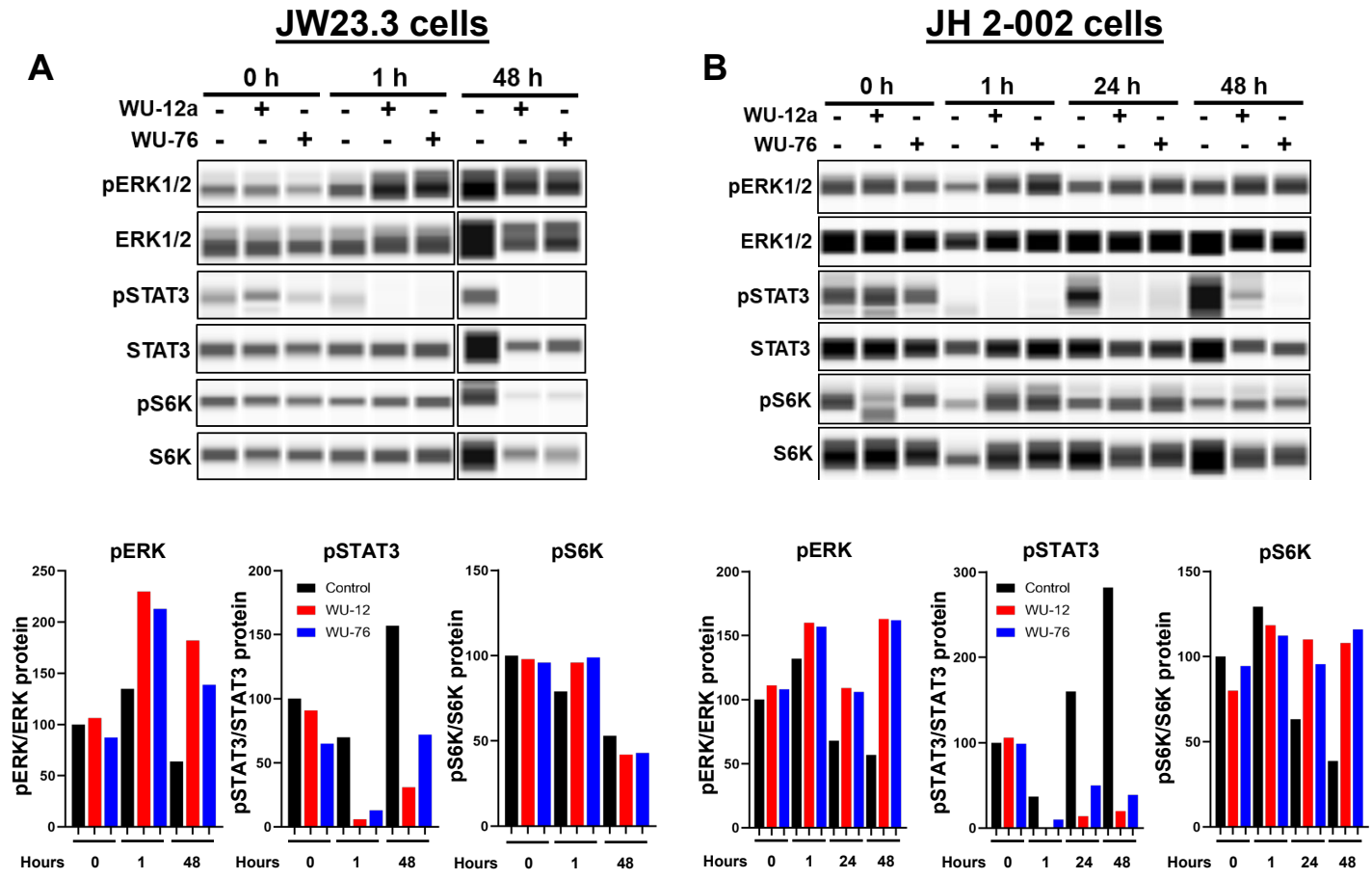
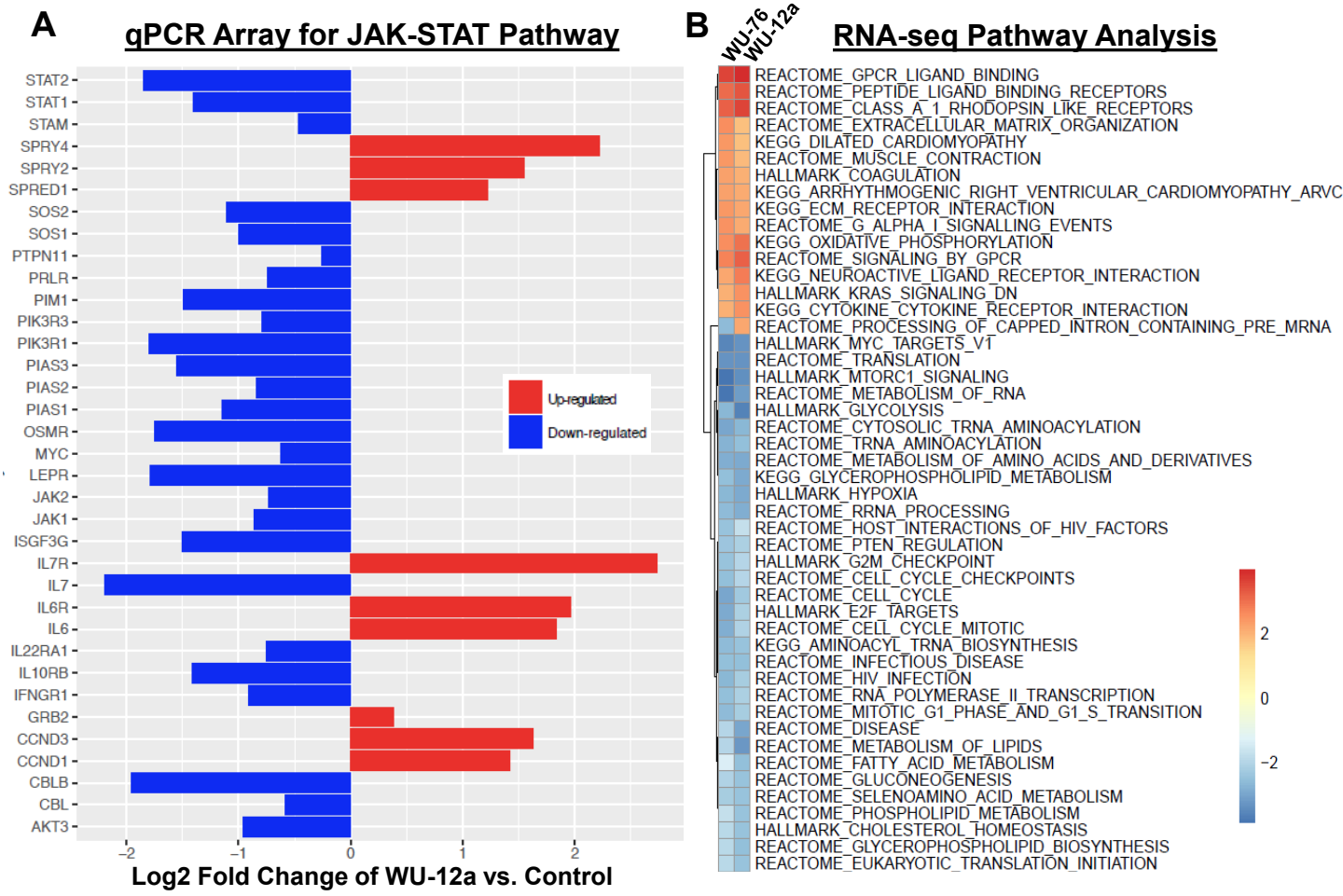


Fig. 4. TYK2 inhibitory drugs decrease activation of STAT3 while increasing activation of ERK1/2 in MPNST cells. (A) JW23.3 cells and (B) JH 2-002 cells were incubated with TYK2 inhibitors (40 μM WU-12 or WU-76) for the indicated times. Phosphorylated and total protein levels for STAT3, ERK1/2, and S6K were analyzed by the WES western system. Bands were analyzed by densitometry, and expressed as phosphorylated normalized to the matching total protein.



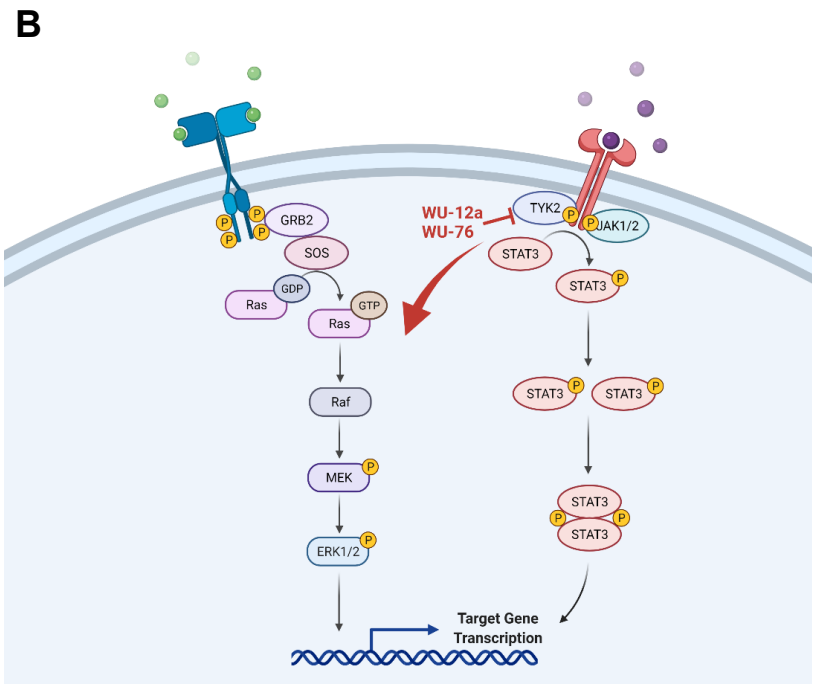


Fig. 5. TYK2 inhibition leads to compensatory stimulation of the MEK/MAP-kinase (MAPK) pathway in MPNST cells. (A) JH 2-002 cells were treated with 40 μM WU-12 for 48 hours, and gene expression was analyzed by qPCR array for JAK/STAT pathway related genes (Takara). (B) JW23.3 cells were treated with 40 μM WU-12 or WU-76 for 48 hours, and global gene expression was determined by RNA-seq pathway analysis. (C) Diagram of TYK2/STAT3 and MEK/MAPK pathways after treatment with TYK2 inhibitors in MPNST cells.

A **RNA-seq pathway analysis: G1 to S cell cycle control**

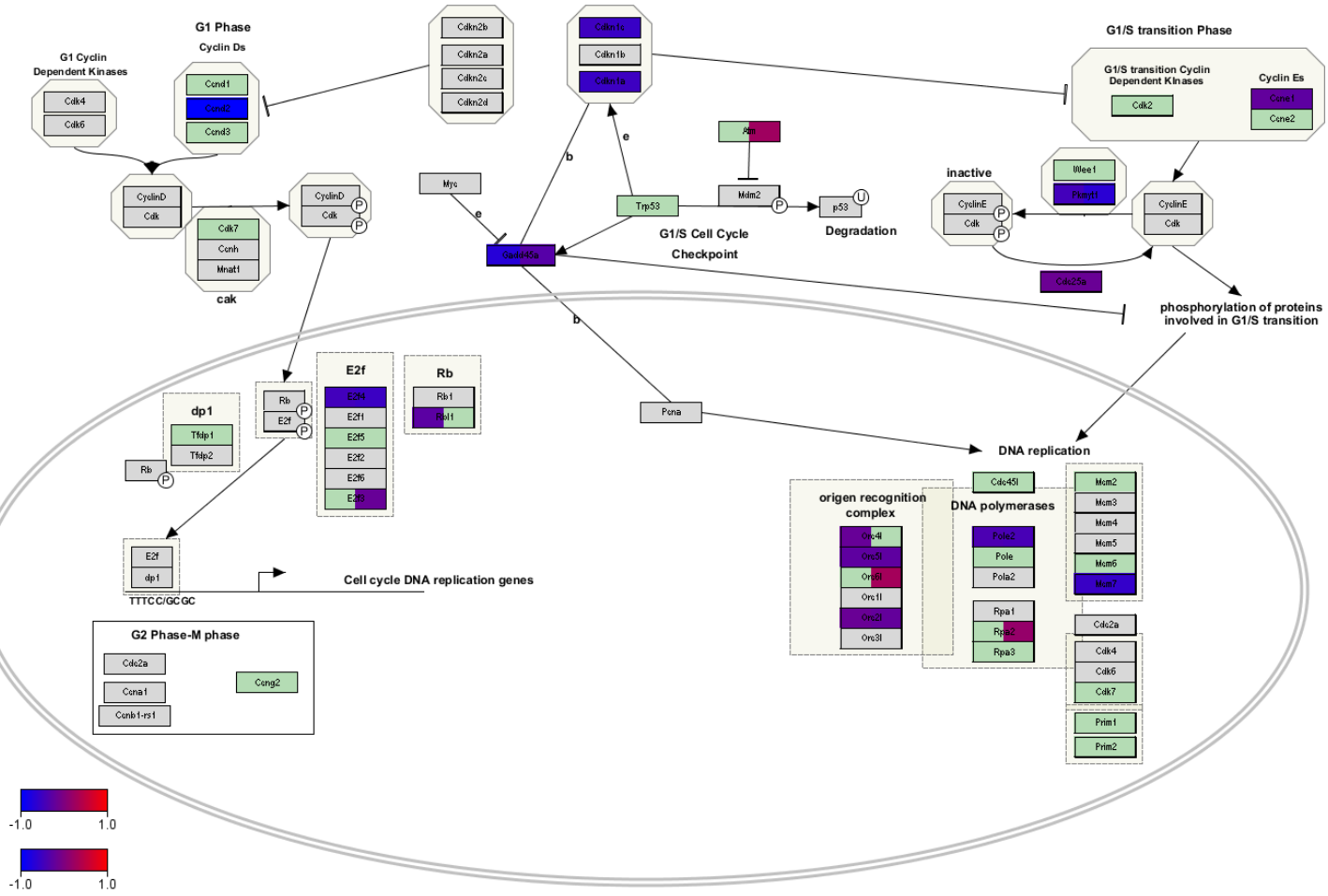


Fig. 6A. TYK2 inhibitors reduce expression of genes involved in proliferation and the G1 to S cycle transition in MPNST cells. JW23.3 cells were treated with 40 μM WU-12 or WU-76 for 48 hours, and global gene expression was determined by RNA-seq pathway analysis.

B **RNA-seq pathway analysis: Ras/MEK/MAPK pathway**

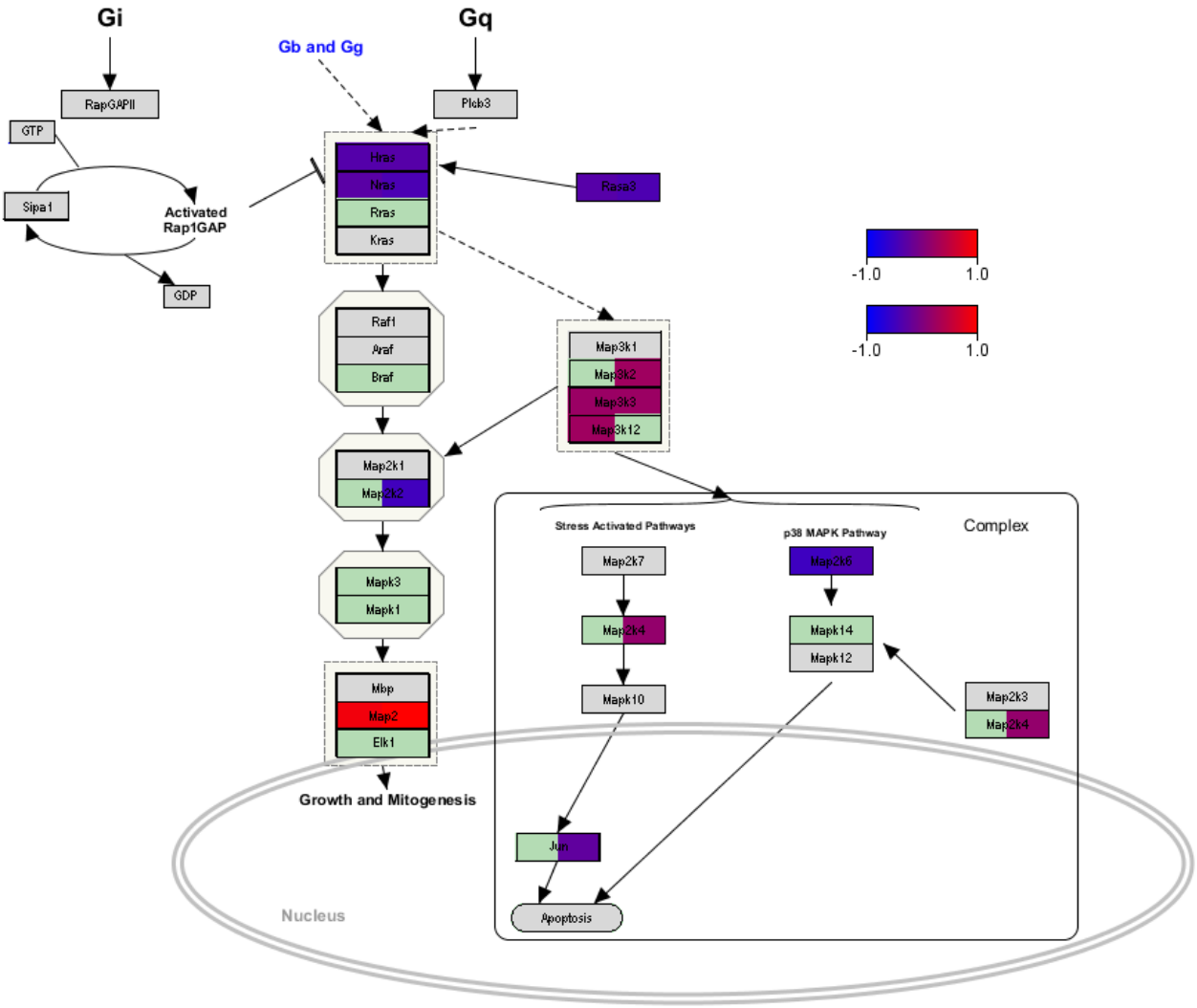


Fig. 6B. TYK2 inhibition leads to compensatory stimulation of the Ras/MEK/MAP-kinase (MAPK) pathway in MPNST cells. JW23.3 cells were treated with 40 μM WU-12 or WU-76 for 48 hours, and global gene expression was determined by RNA-seq pathway analysis.

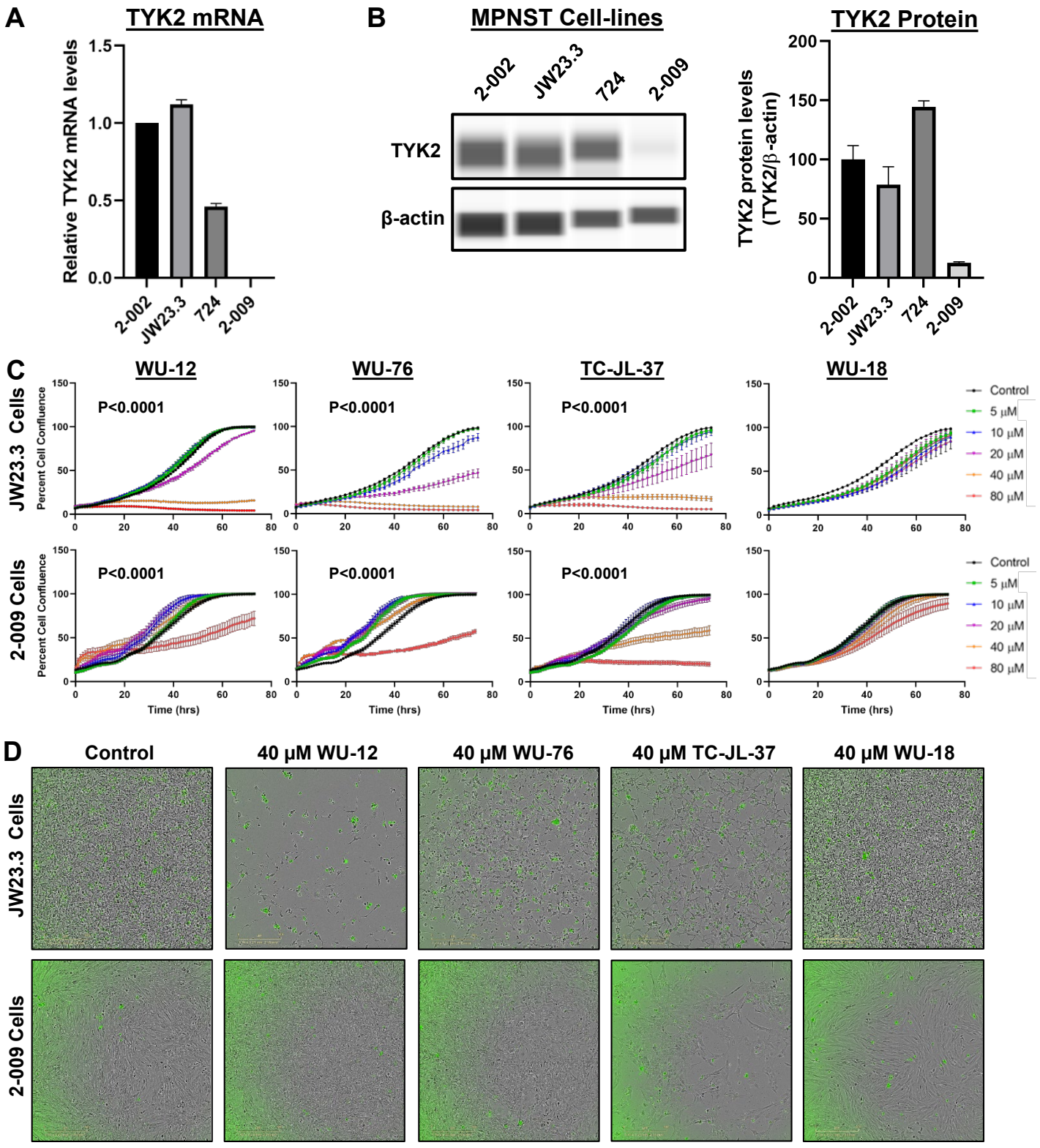


Fig. 7. TYK2 inhibitors reduce proliferation and induce apoptosis in MPNST cells. (A) Relative TYK2 mRNA expression in MPNST cell-lines by qPCR, normalized to housekeeping gene. (B) Relative TYK2 protein levels in MPNST cell-lines by WES. Protein bands analyzed by densitometry, with TYK2 normalized to β-actin. (C) JW23.3 and 2-009 cells were treated with TYK2 inhibitors (WU-12, WU-76, TC-JL-37) or inactive control (WU-18) in media with 5% FBS over 3 days, and cell confluence was determined by the IncuCyte live cell imaging assays. (C) Representative images of IncuCyte assay at 72 hours, with YOYO-1 green fluorescence as an indicator of apoptosis.

Proliferation in MPNST Cell-lines

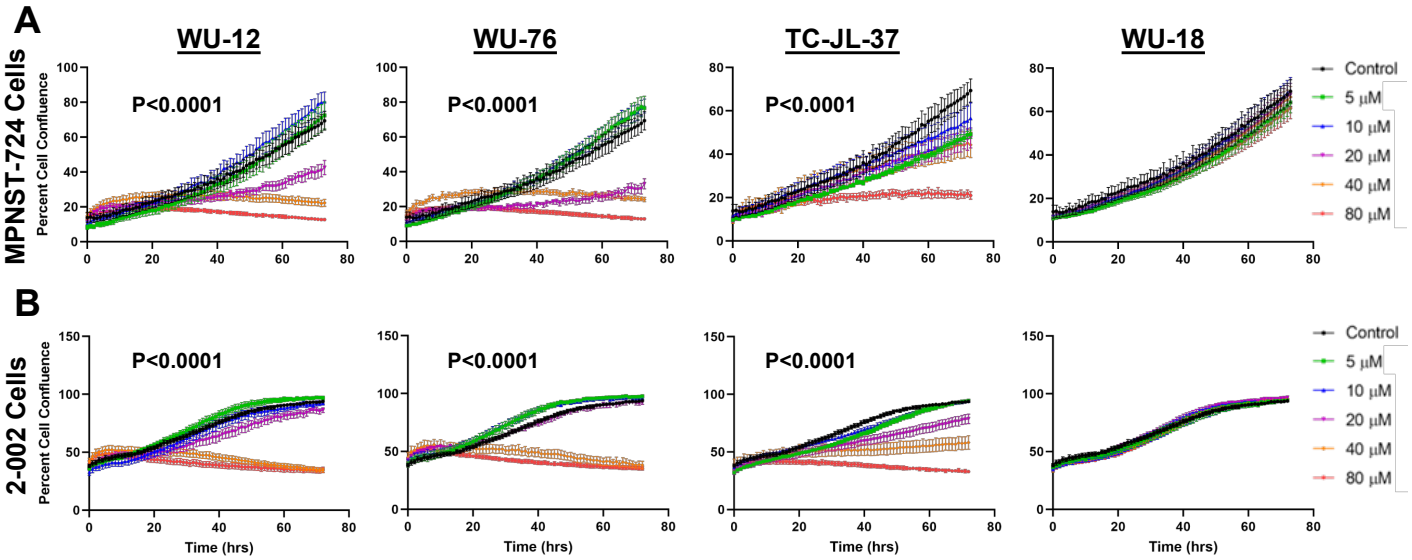


Fig. 8. TYK2 inhibitors reduce proliferation and induce apoptosis in additional MPNST cells. (A) MPNST-724 and JH 2-002 cells were treated with TYK2 inhibitors (WU-12, WU-76, TC-JL-37) or inactive control (WU-18) over 3 days, and cell confluence was determined by the IncuCyte live cell imaging assays. (C) Representative images of IncuCyte assay at 72 hours, with YOYO-1 green fluorescence as an indicator of apoptosis.

Normalized Apoptosis in MPNST Cell-lines

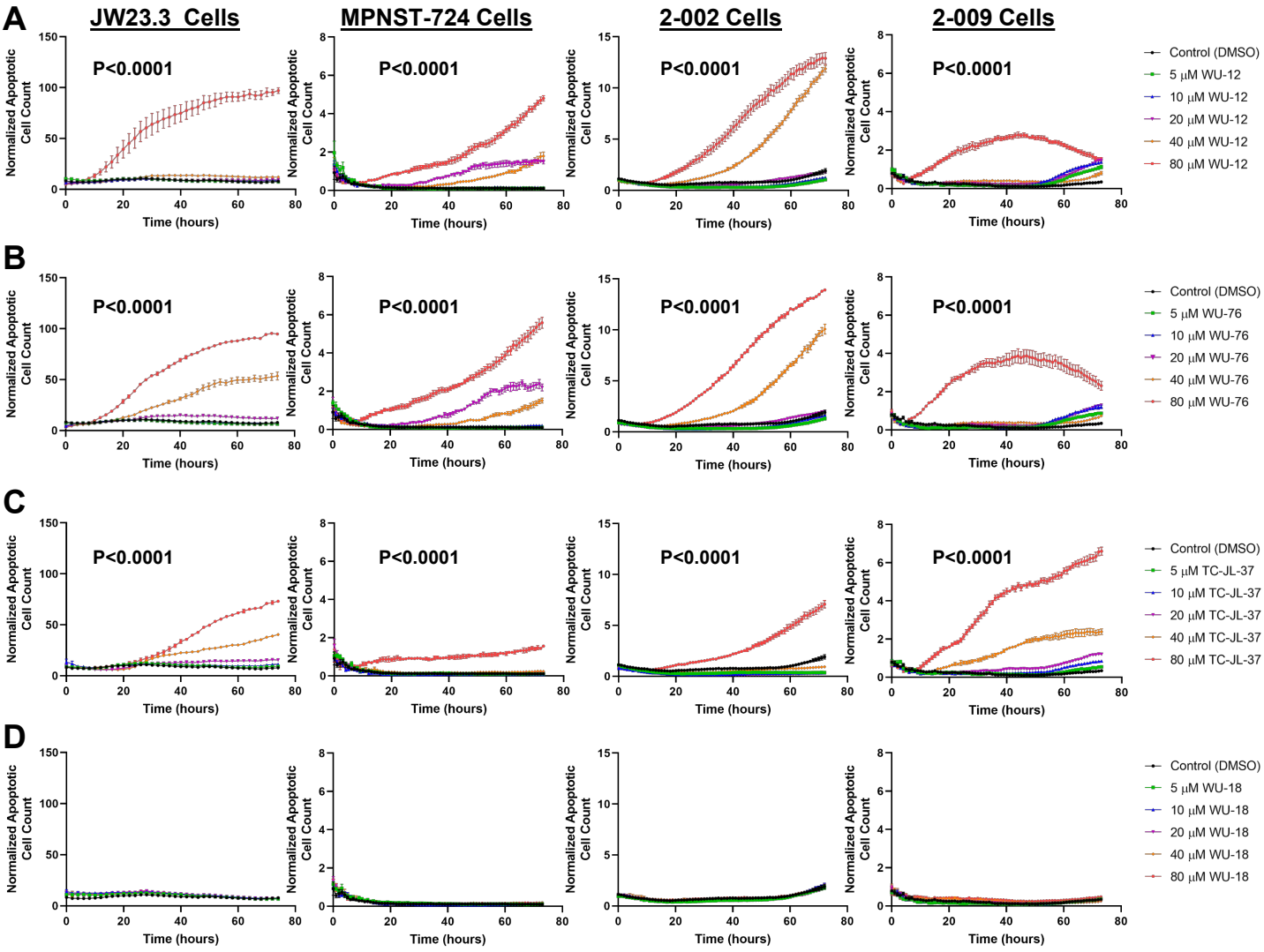


Fig. 9. Inhibition of TYK2 induces apoptosis in four MPNST cell-lines. JW23.3, MPNST-724, 2-002, and 2-009 cells were incubated for 72 hours with (A) the TYK2 inhibitor WU-12, (B) the TYK2 inhibitor WU-76, (C) the TYK2 inhibitor TC-JL-37, or (D) WU-18, an inactive control compound. The IncuCyte cell 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).

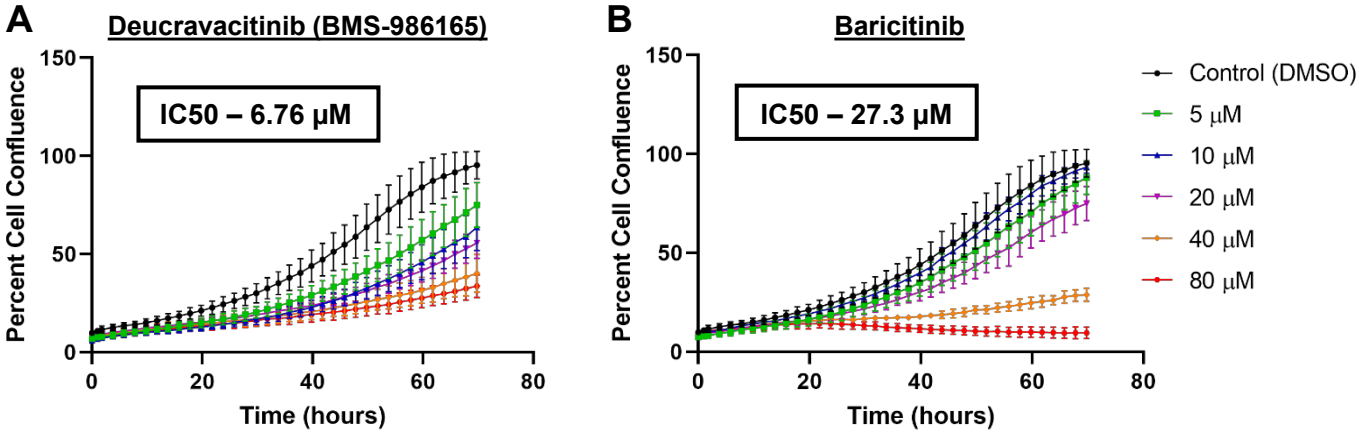


Fig. 10. The specific TYK2 inhibitor, deucravacitinib (BMS-986165), decreases MPNST cell proliferation at lower doses. JW23.3 cells were incubated with (A) the specific TYK2 inhibitor, deucravacitinib (BMS-986165) and (B) the pan-JAK/TYK2 inhibitor, baricitinib, in of IncuCyte live cell assay for 3 days, with YOYO-1 green fluorescence as an indicator of apoptosis. IC50 values were calculated in GraphPad Prism.

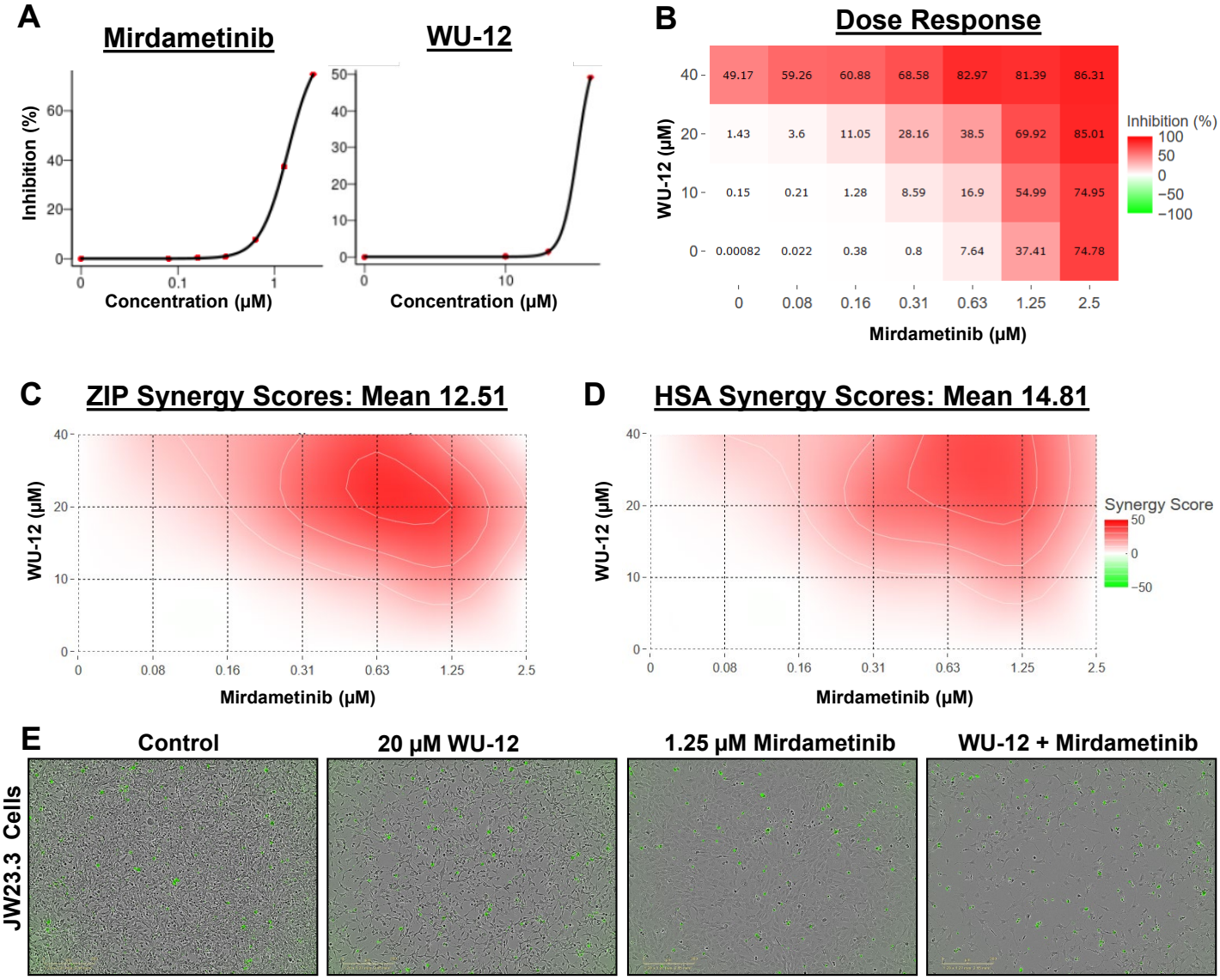


Fig. 11. Inhibitors of TYK2 (WU-12) and MEK (mirdametinib) act synergistically to reduce proliferation in JW23.3 MPNST cells. Cell confluence was analyzed by IncuCyte assay after 72 hour incubation with drugs. Percent inhibition dose response was calculated for each drug alone (A) or in combination (B) by Synergy Finder. Synergy analysis using the ZIP (C) or HSA methods (D). (E) Representative images of JW23.3 cells treated for 48 hours. YOYO-1 green fluorescence indicates apoptotic cells.

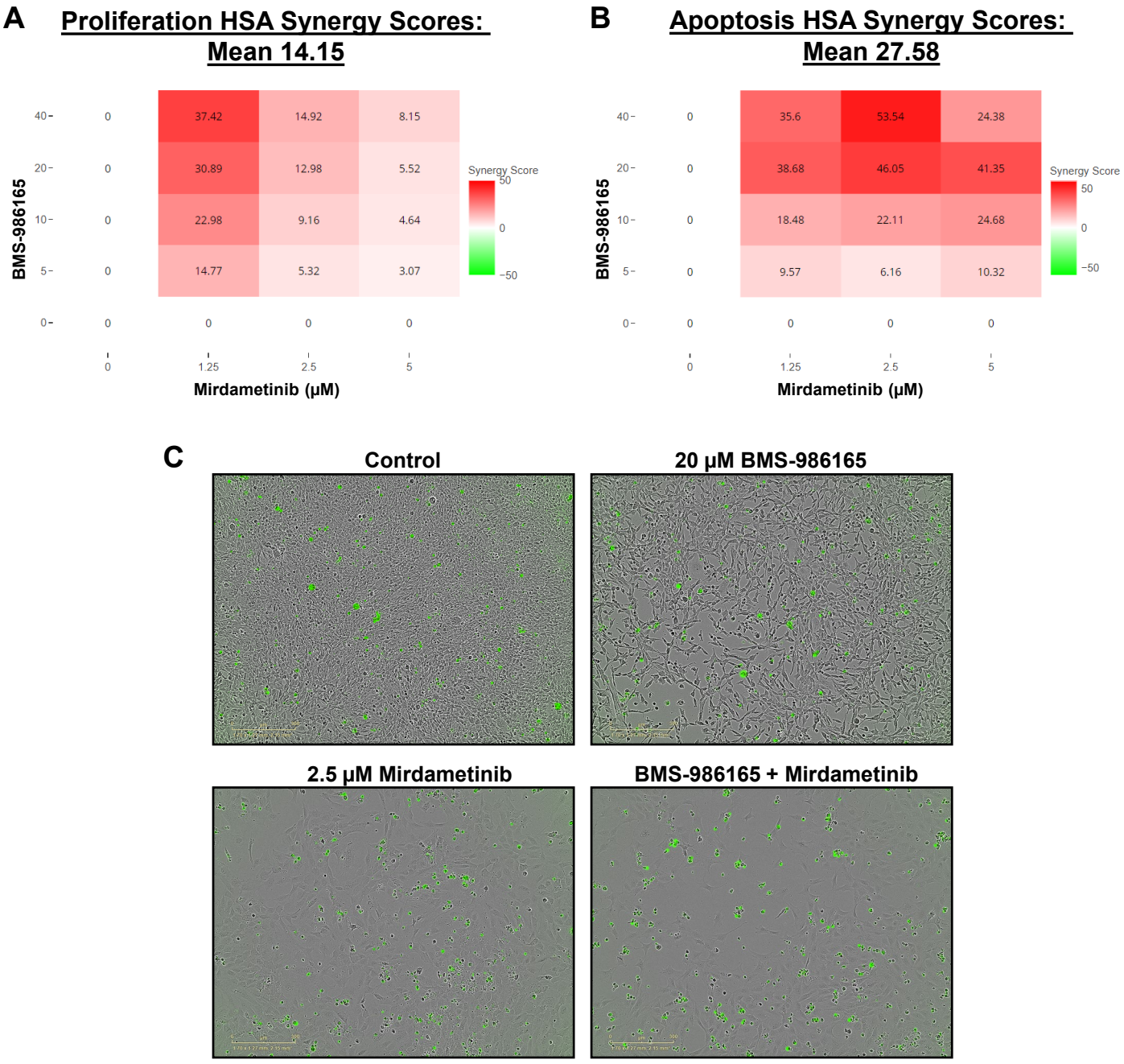


Fig. 12. Inhibitors of TYK2 (Deucravacitinib, BMS-986165) and MEK (mirdametinib) act synergistically to reduce proliferation and increase apoptosis in JW23.3 MPNST cells. Cell confluence and apoptosis was analyzed by the IncuCyte assay after 48 hour incubation with drugs. Synergy was analyzed using Synergy Finder software by the HSA method for (A) inhibition of cell proliferation or (B) apoptosis. (C) Representative images of JW23.3 cells treated for 48 hours. YOYO-1 green fluorescence indicates apoptotic cells.

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

Plans for the next reporting period (Year 3):

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 3), we will continue our statistical analysis of IHC to determine whether TYK2 levels are associated with overall survival, time to response, and therapy response in MPNST patients. If we do not find any significance to TYK2 staining alone, we will determine whether or not we can identify a prognostic signature using a combination of stains including TYK2 as well as H3K27me3 and beta-III-spectrin, two other markers which we believe to be important in MPNST.

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

Plans: We plan to complete statistical analysis of all of the plexiform neurofibromas, atypical neurofibromas, and MPNST samples. We will also evaluate whether TYK2 status is predictive of the progression of plexiform neurofibromas and ANNUBP tumors to MPNST.

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 additional colonies of murine JW23.3 and human 2-002 MPNST cells transfected by CRISPR/Cas9 for knockout of *TYK2* and/or *STAT3*. We will continue IncuCyte cell proliferation and apoptosis assays with the *TYK2*, *STAT3* and *TYK2/STAT3* knockout cells. We will also examine whether STAT1 or STAT5a/b proteins mediate the downstream effects of TYK2. While we plan to focus on proliferative and cell survival effects of TYK2 in MPNST, we may later evaluate cell death mechanisms with apoptosis assays, including Bcl-2 knockout, TUNEL assay, cleaved caspase-3 Western blotting, and flow cytometry for 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 cells deficient for *TYK2*, *STAT3* or both genes (CRISPR/Cas9 KO) in the JW23.3 and 2-002 cell-lines compared to control cells. In addition, we are currently evaluating the effect of the specific TYK2 inhibitor, deucravacitinib (BMS-986165), on the phosphorylation of STAT3, ERK, and S6-kinase in JW23.3 and JH 2-002 cells.

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

Plans: We will complete 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. We have also expanded our studies to include a clinically relevant and specific TYK2 inhibitor, deucravacitinib (BMS-986165), and are currently testing it in our panel of MPNST cell-lines (JW23.3, JH 2-002, JH 2-009, and MPNST-724 cells) to calculate IC50 values.

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 goal to begin during 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 and we plan to start experiments using TYK2 inhibitors with these mice. In addition, we are now preparing for mouse experiments with JW23.3 (murine *Nf1/Tp53*-mutant) cell xenograft tumors. Once tumors develop, mice will be randomized for treatment with (1) vehicle control, (2) mirdametinib, (3) deucravacitinib (BMS-986165), and (4) combination of mirdametinib and deucravacitinib.