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TITLE:

A Cell Line Based Mouse Model of Chronic CDK9 Inhibition to Study Widespread Non-Genetic Transcriptional Elongation Defects (TE^{deff}) in Cancers

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KEYWORDS:

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SUMMARY:

The protocol details an in vitro murine carcinoma model of non-genetic defective transcription elongation. Here, chronic inhibition of CDK9 is used to repress productive elongation of RNA Pol II along pro-inflammatory response genes to mimic and study the clinically overserved TE^{deff} phenomenon, present in about 20% of all cancer types.

ABSTRACT:

We have previously reported that a subset of cancers is defined by global transcriptional deregulations with widespread deficiencies in mRNA transcription elongation (TE)—we call such cancers as TE^{deff}. Notably, TE^{deff} cancers are characterized by spurious transcription and faulty mRNA processing in a large set of genes, such as interferon/JAK/STAT and TNF/NF-κB pathways, leading to their suppression. The TE^{deff} subtype of tumors in renal cell carcinoma and metastatic melanoma patients significantly correlate with poor response and outcome in immunotherapy. Given the importance of investigating TE^{deff} cancers—as it portends a significant roadblock against immunotherapy—the goal of this protocol is to establish an in vitro TE^{deff} mouse model to study these widespread, non-genetic transcriptional abnormalities in cancers and gain new insights, novel uses for existing drugs, or find new strategies against such cancers. We detail the use of chronic flavopiridol mediated CDK9 inhibition to abrogate phosphorylation of serine 2 residue on the C-terminal repeat domain (CTD) of RNA polymerase II (RNA Pol II), suppressing the release of RNA Pol II into productive transcription elongation. Given that TE^{deff} cancers are not classified under any specific somatic mutation, a pharmacological model is advantageous, and best mimics the widespread transcriptional and epigenetic defects observed in them. The use of an optimized sublethal dose of flavopiridol is the only efficacious strategy in creating a generalizable model of non-genetic widespread disruption in transcription elongation and mRNA

processing defects, closely mimicking the clinically observed TE^{deff} characteristics. Therefore, this model of TE^{deff} can be leveraged to dissect, cell-autonomous factors enabling them in resisting immune-mediated cell attack.

INTRODUCTION:

A key rate-limiting step in the expression of nearly all active genes is the transition of RNA polymerase II (RNA Pol II) from promoter-proximal pausing to productive elongation[1,2]. Given that epigenetic dysregulation of transcriptional elongation assists in the progression of multiple human malignancies defined as TE^{deff}, leading to suboptimal signaling in the pro-inflammatory response pathways amounting to a poor response and outcome to immunotherapy[3], the overarching goal of this protocol is to establish a useful in vitro model to study these widespread non-genetic transcriptional abnormalities in cancers. In this light, the use of chronic pharmacological inhibition of CDK9 is an efficacious strategy for creating a generalizable model of non-genetic widespread disruption in transcription elongation and mRNA processing defects. The rationale behind using chronic CDK9 inhibition is that it abrogates phosphorylation of serine 2 residue on the C-terminal repeat domain (CTD) of RNA Pol II, thus repressing the release of RNA Pol II into productive transcription elongation. Also, TE^{deff} cancers, described previously by our group[3], are not classified under any specific somatic mutation. Therefore, a non-genetic (pharmacological) model is advantageous and best mimics the widespread transcriptional and epigenetic defects observed in them. The method herein details the generation and characterization of chronic flavopiridol treatment model of murine cancer cells. This method demonstrably disrupts transcription elongation along genes characterized by longer genomic lengths, with poised promoters and inducible expressions such as TNF/NF-κB and interferon/STAT signaling, profoundly controlled at the level of transcription elongation[3,4,5]. Overall, this optimized murine cell line model of transcriptional elongation defects—the only model to our knowledge to study the newly described TE^{deff} tumors—drives resistance to anti-tumor immune attack, rendering a useful system to exploit and examine the vulnerabilities of non-genetic defects in core transcription machinery in cancers vis-à-vis immune-mediated cell attack.

PROTOCOL:

The Institutional Animal Care and Use Committee and Institutional Biosafety Committee of the Cincinnati Children's Research Foundation approved all animal experimental procedures (IACUC protocol #2017-0061 and IBC protocol #IBC2016-0016), and these experiments were carried out in accordance with standards as described in the NIH Guide to the Care and Use of Laboratory Animals.

1. Chronic inhibition of RNA Pol II by flavopiridol treatment—basic strategy

1.1. Seed B16/F10 mouse melanoma cells in low density (0.2×10^6) in a 10 cm culture plate in their corresponding medium (Dulbecco's Modified Eagle Medium [DMEM], 10% fetal bovine serum [FBS], 1% penicillin and streptomycin [Pen/Strep]) and incubate overnight in a 37 °C, 5% CO_2 humidified incubator.

1.2. Following day, wash the cells with 1x phosphate-buffered saline (PBS) and add a new batch of culture media with a sublethal dose (estimated as 25 nM) of flavopiridol—an inhibitor of the RNA Pol II elongation factor p-TEFb (cyclin T/CDK9)—for one week without further sub-culturing.

1.3. Following a week of flavopiridol treatment, perform confirmatory assays to evaluate the model's ability to recapitulate various attributes of transcriptional elongation defects seen in TE^{deff} cancers.

2. Confirmatory immunoblot assay to assess defective RNA Pol II function and impairment of interferon (IFN) pathway and tumor necrosis factor (TNF) pathway signaling of in the generated mouse TE^{deff} model

2.1. Culture equal number (10^5) of flavopiridol treated B16/F10 mouse melanoma cells and parental B16/F10 mouse melanoma cells in two different sets of 12 well plates (one set for RNA Pol II functional characterization and the other set for cytokine stimulation) at 37 °C in a 5% CO_2 humidified incubator overnight.

2.2. Next day, treat the cells in the cytokine stimulation set with mouse IFN-γ, IFN-α (5 ng/mL), or TNF-α (5 ng/mL) for 45 min at 37 °C.

2.3. Now, extract protein from cells in both cytokine and RNA Pol II functional characterization sets using a radioimmunoprecipitation assay (RIPA) lysis buffer in the following manner:

2.3.1. Wash cells with 1x PBS and lyse it with 50 µL of the lysis buffer per well. Scrape, and then pellet the lysed cells at 4 °C, 21,130 x *g*.

2.3.2. Measure the protein in cell lysate supernatants using a standard colorimetric assay for protein concentration following detergent solubilization (Bradford or a similar assay).

2.4. Load equal amount of measured protein (15 µg) from each sample to run in a 4%−18% sodium dodecyl sulfate (SDS) polyacrylamide gel, and transfer them onto polyvinylidene difluoride (PVDF) membranes.

2.5. Block the PVDF membranes in 5% dry milk in tris-buffered saline−polysorbate 20 (TBST) for 1 h followed by an overnight incubation at 4 °C with primary antibodies (RNA Pol II 1:1000; p-SER2 RNA Pol II 1:1000; p-SER5 RNA Pol II 1:1000; H3K36me3 1:2000; total H3 1:2000; STAT1 1:1000; p-STAT1 1:1000; NFκB 1:1000; p-NFκB 1:1000; β-Actin 1:5000) in 5% bovine serum albumin.

2.6. Following day, wash the PVDF membranes with 1x TBST for 15 min at room temperature (RT), and incubate them with appropriate secondary antibodies (anti-rat [1:5000] for RNA Pol II, p-SER2 RNA Pol II, and p-SER5 RNA Pol II; anti-rabbit (1:5000) for H3K36me3, total H3, STAT1, p-STAT1, NFκB, and p-NFκB) for 50 min at RT. Detect the protein signals using commercially

available horseradish peroxidase (HRP) substrate with enhanced chemiluminescence.

NOTE: β-Actin primary used is HRP-conjugated, therefore it can be developed without a secondary.

3. Confirmatory assay to assess mRNA processing defects in the generated mouse TE^{deff} model

3.1. Seed equal number (0.2 x 10^6) of flavopiridol treated B16/F10 mouse melanoma cells and parental B16/F10 mouse melanoma cells in 6-well plates at 37 °C in a 5% CO_2 humidified incubator overnight.

3.2. Extract total RNA from the cultured cells at 60% confluency using an RNA extraction reagent or kit (**Table of Materials**).

3.3. Deplete rRNA from the total extracted RNA in the following manner:

NOTE: A low-input protocol has been co-opted from a commercially available kit to deplete rRNA

3.3.1. Set one water bath or heat block to 70−75 °C, and another water bath or heat block at 37 °C.

3.3.2. Add the total RNA (100−500 ng in 2 µL of nuclease-free water) with 1 µL of selective rRNA depletion probe and 30 µL of hybridization buffer in a microcentrifuge tube, mix gently by vortexing and incubate them at 70−75 °C for 5 min.

3.3.3. Now, transfer the tubes to a 37 °C water bath/heat block, and allow the sample to cool to 37 °C over a period of 30 min.

3.3.4. Resuspend the selective rRNA depletion probe magnetic beads by vortexing, and aliquot 75 µL of beads in a 1.5 mL RNase-free microcentrifuge tube.

3.3.5. Place the bead suspension on a magnetic separator for 1 min. Allow the beads to settle. Gently aspirate and discard the supernatant. Repeat washing the beads once again by adding 75 µL of nuclease-free water and discarding the supernatant following magnetic separation.

3.3.6. Resuspend the washed beads in 75 µL of hybridization buffer, and aliquot 25 µL of it to another tube and maintain it at 37 °C for later use.

3.3.7. Place the remaining 50 µL beads on a magnetic separator for 1 min and discard the supernatant. Resuspend the beads in 20 µL of hybridization buffer and maintain it at 37 °C for later use.

3.3.8. After the cooling of the RNA/selective rRNA depletion probe mixture to 37 °C for 30 min, briefly centrifuge the tube to collect the sample to the bottom of the tube.

3.3.9. Transfer 33 µL of the RNA/selective rRNA depletion probe mixture to the prepared magnetic beads from step 3.3.7. Mix by low speed vortexing.

3.3.10. Incubate the tube at 37 °C for 15 min. During incubation, gently mix the contents occasionally. Followed by brief centrifugation to collect the sample to the bottom of the tube.

3.3.11. Place the tube on a magnetic separator for 1 min to pellet the rRNA-probe complex. This time do not discard the supernatant. The supernatant contains rRNA-depleted RNA.

3.3.12. Place the tube of 25 µL of beads from step 3.3.6 on a magnetic separator for 1 min. Aspirate and discard the supernatant. Add the supernatant from step 3.3.11 to the new tube of beads. Mix by low speed vortexing.

3.3.13. Incubate the tube at 37 °C for 15 min. During incubation, gently mix the contents occasionally. Briefly centrifuge the tube to collect the sample to the bottom of the tube.

3.3.14. Place the tube on a magnetic separator for 1 min to pellet the rRNA-probe complex. Do not discard the supernatant. Transfer the supernatant (about 53 µL) containing rRNA-depleted RNA to a new tube.

3.3.15. Measure the concentration of the RNA yield by a spectrophotometer.

3.4. Use one half of the rRNA-depleted samples as input to magnetic beads containing oligo (dT)25 to extract polyA$^+$ RNA in the following manner:

NOTE: This protocol of isolating polyA tail messenger RNA using oligo dT sequences bound to the surface of magnetic beads has been co-opted from a commercially available kit (**Table of Materials**).

3.4.1. Resuspend the oligo dT beads in the vial by briefly vortexing for >30 s and transfer 200 µL of oligo dT beads to a tube. Add the same volume (200 µL) of binding buffer, and resuspend.

3.4.2. Place the tube in a magnet for 1 min and discard the supernatant. Now, remove the tube from the magnet and resuspend the washed oligo dT beads in 100 µL of binding buffer.

3.4.3. Adjust the volume of the input rRNA-depleted total RNA sample to 100 µL with 10 mM Tris-HCl pH 7.5. Now, add 100 µL of binding buffer.

3.4.4. Heat to 65 °C for 2 min to disrupt secondary RNA structures. Now, immediately place on ice.

3.4.5. Add the 200 µL of total RNA to the 100 µL washed beads. Mix thoroughly and allow binding by rotating continuously on a rotor for 5 min at RT.

3.5. Measure the purity and concentration of the extracted polyA$^+$ RNA by a spectrophotometer.

NOTE: A 260/280 ratio of 1.90−2.00, and a 260/230 ratio of 2.00−2.20 for all RNA samples are considered acceptable.

3.6. Use the remaining half of the rRNA-depleted samples from section 3.3 as input to protein A columns (provided in the RNA immunoprecipitation [RIP] kit, **Table of Materials**) to immunoprecipitate five-prime capped RNAs using monoclonal 7-methylguanosine antibody in the following manner:

NOTE: This protocol of isolating m7G capped messenger RNA using a commercially available RNA immunoprecipitation kit has been co-opted and further modified.

3.6.1. Wash the protein A magnetic beads obtained from the RIP kit according to manufacturer's protocol to pre-bind the antibody to the beads.

3.6.2. Transfer 3 µg of 7-methylguanosine antibody (rabbit IgG provided in the kit can be used the negative control) to the beads in a microcentrifuge tube suspended in 100 µL wash buffer from the kit.

3.6.3. Incubate with low speed rotation for 30 min at RT. Centrifuge tubes briefly and then place the tubes on a magnetic separator, remove and discard the supernatant.

3.6.4. Remove tubes and add 500 µL of wash buffer from the kit and vortex briefly. Centrifuge the tubes briefly followed by magnetic separation once again, remove and discard the supernatant.

3.6.5. Repeat step 3.6.4 once again.

3.6.6. Add around 120 ng of rRNA depleted (from section 3.3) to the prewashed 7-methylguanosine antibody bound beads. Add 1 µL of RNase inhibitor. Incubate at RT for 1−1.5 h with mild agitation.

3.6.7. Spin down the beads at 300 x *g* for 10 s and remove the supernatant containing uncapped (non-7-methylguanosine) mRNA to a new microcentrifuge tube.

3.6.8. Add 100 µL of wash buffer and wash it two more times similarly. Pool the collected supernatant in the same microcentrifuge tube labelled uncapped (non-7-methylguanosine) mRNA. Store on ice.

3.6.9. Elute the capped (7-methylguanosine) mRNA from the beads with 300 µL of urea lysis

buffer (ULB) containing 7 M urea, 2% SDS, 0.35 M NaCl, 10 mM EDTA and 10 mM Tris, pH 7.5 by heating the beads at 65 °C for 2−3 min.

3.6.10. Mix 300 µL of the eluted samples (capped and uncapped mRNA) with 300 µL of phenol:chloroform:isoamyl alcohol (25:24:1; commercially available) (stored at 4 °C). Mix well by inverting and leave for about 10 min then mix again gently.

3.6.11. Centrifuge at 18,928 x *g* for 2 min and carefully pipette the top layer to fresh tube and discard bottom layer.

3.6.12. Add 300 µL of phenol:chloroform:isoamyl alcohol (25:24:1; stored at 4 °C) to the samples. Mix well by inverting and then centrifuge at 18,928 x *g* for 1 min. Carefully, pipette the top layer to a fresh tube and discard bottom layer.

3.6.13. Add 300 µL of 2-porpanol and 30 µL of 3 M sodium acetate (pH 5.2) to the capped and uncapped RNA. Invert the sample a few times and put it at -20 °C for 20 h.

3.6.14. Now, centrifuge the samples at 18,928 x *g* for 10 min at 4 °C. Carefully remove the supernatant and add 500 µL of 70% ethanol.

3.6.15. Centrifuge again at 18,928 x *g* for 10 min at 4 °C. Carefully discard the supernatant and dry the pellet at RT for less than 5 min. Resuspend the pellet in nuclease-free water.

3.7. Measure the purity and concentration of RNA yield by a spectrophotometer. The 260/280 ratio should be in the range of 1.90−2.00, and the 260/230 ratio in the range of 2.00−2.20 for all RNA samples.

4. Confirmatory assay to assess the response of mouse TE^{deff} model to FasL mediated cell death

4.1. Seed equal number (30,000 cells) of flavopiridol treated B16/F10 mouse melanoma cells and parental B16/F10 mouse melanoma cells in a 96-well culture plate in their corresponding medium (DMEM), and incubate overnight in a 37 °C, 5% CO_2 humidified incubator.

4.2. Treat the cells in a culture hood with different concentrations of h_{his}6FasL (0.1−1000 ng/mL) in the presence of 10 µg/mL anti-His antibody and incubate for 24 h at 37 °C, 5% CO_2 humidified incubator.

4.3. Remove dead cells by washing with 1x PBS buffer. Fix the attached cells in 4% paraformaldehyde for 20 min at RT. Discard the 4% paraformaldehyde (no need to wash), and stain with crystal violet solution (20% methanol, 0.5% crystal violet in 1x PBS) for 30 min.

4.4. Remove excess stain by gently rinsing the plates in tap water. Keep the plates to dry at RT.

4.5. Re-dissolve the crystal violet in 100 µL of 1x nonionic surfactant dissolved in 1x PBS, and measure cell density by measuring the absorbance at 570 nm in a microplate reader.

5. Exploratory assay to assess the response of mouse TE^{deff} model to antigen specific cytotoxic T-cell attack

5.1. Isolation and activation of OT-I CD8+ cytotoxic T-cell attack (CTL)

5.1.1. Purify CD8+ cells from spleens of OT-I TCR Tg RAG-1−/− mice by magnetic cell separation using a mouse CD8 T cell isolation kit as follows:

5.1.1.1. Harvest two spleens from two OT-I TCR Tg RAG-1−/− mice in complete RPMI media.

5.1.1.2. Mash the spleens in a 70 µm filter kept on a 50 mL tube filled with 20 mL of RPMI, using the back of a syringe till only fat is left behind in the filter.

5.1.1.3. Centrifuge the flow through at 220 x *g* for 5 min at 4 °C. Discard the supernatant.

5.1.1.4. Add 1 mL of red blood cell (RBC) lysis buffer to the spleen pellet from the previous centrifugation step and pipette the mixture for 1 min.

5.1.1.5. Neutralize the solution by adding up to 10 mL of RPMI.

5.1.1.6. Centrifuge at 220 x *g* for 5 min at 4 °C. Discard the supernatant and resuspend in 10 mL of RPMI.

5.1.1.7. Take a small aliquot for counting. Centrifuge the remaining at 220 x *g* for 5 min at 4 °C.

5.1.1.8. For every million cells counted, resuspend the pellet in 1 mL of commercially available magnetic separation system buffer (Mojo buffer or a similar buffer).

5.1.1.9. Prepare an antibody cocktail of volume 100 µL for every 1 mL of pelleted cells in step 5.1.1.8. The antibody cocktail includes: biotin anti-CD4, CD105, CD45R/B220, CD11c, CD49b, TER-119, CD19, CD11b, TCR γ/δ, and CD44.

5.1.1.10. Add this cocktail to the 1 mL pelleted cells and keep on ice for 15 min.

5.1.1.11. Add 100 µL of magnetic (streptavidin) beads to every 100 µL of the antibody cocktail

added to the 1 mL resuspended pelleted spleen cells. Keep on ice for 15 min.

5.1.1.12. Add 7 mL of commercially available magnetic separation system buffer. Now, aliquot about 3−4 mL of the mixture to a fresh tube. Mix well and fix it to the magnet for 5 min.

5.1.1.13. Decant the liquid (contains CD8+ cells) to a fresh tube on ice. Now, aliquot the remaining 3−4 mL of mixture from step 5.1.1.12 to the tube and fix it to the magnet for 5 min. Decant the liquid (second batch of CD8+ cells isolated) to the same tube containing the first batch of CD8+ cells kept on ice.

5.1.2. Seed engineered adherent fibroblast APC-(MEC.B7.SigOVA) line to express a specific ovalbumin (OVA)-derived, H-2Kb-restricted peptide epitope OVA257-264 (SIINFEKL), along with the co-stimulatory molecule B7.1, at 75,000 cells per well in 24-well plates, at 37 °C, 5% CO_2 humidified incubator.

NOTE: The adherent fibroblast used is a gift from Dr. Edith Janssen's lab at CCHMC. The line was created originally in Dr. Stephen P. Schoenberger's lab in La Jolla Institute for Allergy and Immunology[6].

5.1.3. After 24 h, wash the monolayer of APC once with Iscove's modified Dulbecco's medium (IMDM) commercially available with HEPES buffer, sodium pyruvate, L-glutamine and high glucose), and add 0.5×10^6 naive OT-I CD8+ cells (from step 5.1.1.13) in 2 mL of IMDM supplemented with 50 mM β-ME, 2 mL EDTA, 4 mM L-glutamine and HEPES and 10% FBS.

5.1.4. After 20 h, gently harvest the non-adherent OT-I cells (by collecting the media in the culture dish with floating OT-I cells and pelleting the cells at 191 x *g* for 2 min; count the viable OT-I cells) and transfer them for co-culture.

5.2. Co-culture of CD8+ cells with B16/F10-OVA cells

5.2.1. Seed OT-I-derived CD8+ cells at a ratio of 1:1 (300,000 cells each) in a co-culture with B16/F10 (lacking the antigen ovalbumin), untreated B16/F10-OVA, and B16/F10-OVA cells pre-treated with flavopiridol (25 nM) for 1 week, in 6-well dishes with complete DMEM media for 20 h at 37 °C in a 5% CO_2 humidified incubator.

5.2.2. After 20 h, remove the OT-I-derived CD8+ cells (by collecting the media in the culture dish with floating OT-I derived CD8+ cells). Wash the adherent B16/F10-OVA cells in 1x PBS.

5.2.3. Trypsinize the three groups of attached B16/F10-OVA cells in 0.05% EDTA containing trypsin for 5 min. Pellet the trypsinized cells at 191 x *g* for 5 min.

5.2.4. Stain the harvested B16/F10-OVA cells by incubating them in cold PBS (containing 0.5% FBS and 0.05% sodium azide) with viability dye and relevant labeled antibodies (fixable viability staining dye e780, AF647-conjugated mouse CD8 and BV421-conjugated mouse CD45).

5.2.5. Analyze the viability of the three groups of B16/F10-OVA cells by flow cytometry.

REPRESENTATIVE RESULTS:

Here, we provide a detailed scheme (**Figure 1**) to establish a TE^{deff} cell model obtained by chronic sub-lethal (**Figure 2**) treatment with flavopiridol at 25 nM. In **Figure 3**, on 3 days of treatment with flavopiridol, B16 OVA cells show partial characteristics of TE^{deff} but after one week of treatment, B16/F10 OVA cells show a profound loss of phosphorylation at serine 2 position on the CTD of RNA Pol II along with a significant decrease in H3K36me3—a histone modification implicated in defining exon boundaries and an inhibitor of run-away cryptic transcriptions. As a consequence, TE^{deff} cell model shows critical mRNA processing defects with manifestly increased ratios of improperly capped and non-poly-adenylated mRNAs (**Figure 4A,B**). Also, specific repression of key inflammatory response pathway genes and FasL mediated cell death pathway are seen in **Figure 5** and **Figure 6**. The imposed resistance to interferon (IFN-α, IFNγ) and TNF-α stimulated phosphorylation of STAT1 and NFκB, and resistance to cell death by the death receptor ligand FasL drastically reduces the cytotoxicity of an immune cell attack against TE^{deff} tumors. These confirmatory techniques are designed to test the extent of influence chronic perturbation of transcription elongation has on a wide array of stimulus-responsive genes, and whether such a perturbation in a given mouse cell line model is adequate enough to prompt an acute dearth of functional mRNA in inflammatory response signaling genes, mimicking the basic essentialities of TE^{deff} cancers clinically. Based on our study of the flavopiridol treatment, the suppression of phosphorylation at the second serine residue (pSER2) of RNA Pol II CTD is critical, as it marks transcription elongation. A sublethal dose for any given mouse carcinoma cell line must achieve a reduction in pSER2 levels in addition to having an insignificant effect on the rate of growth and viability of the cell line. Although we consistently see a reduction in pSER2 and H3K36me3 levels on 25 nM flavopiridol treatments, it does not guarantee a repression of both pSTAT1 and pNFκB levels (on IFN-α, IFNγ and TNF-α stimulations, respectively). Each mouse carcinoma cell line is unique (B16/F10 OVA or CT26 cells cultured in different labs over a period of time may have slightly altered effects) and they may have either JAK1 or CCNT1 partially rescuing the effects of flavopiridol in suppressing the inflammatory response pathway genes. In such cases, the kinetics of pSTAT1 and pNFκB levels may need to be checked at different time points (5−70 min) to understand the temporality of flavopiridol mediated effects and its rescue by either JAK1 or CCNT1. Accordingly, JAK1 and/or CCNT1 may need to be knocked down to establish this model.

Once the flavopiridol model is established and characterized using the aforementioned assays, we provide an exploratory assay to test if the TE^{deff} cell model confers resistance to cytotoxic T-cell (CTL) attack. Based on our optimized protocol, flavopiridol treated B16/F10 cells stably overexpressing the OVA gene (B16 OVA) co-incubated with the activated CD8+ CTLs (specific for the OVA257-264 epitope) having selective toxicity to OVA-expressing cells (a gift from Dr. Stephen P. Schoenberger's lab[6]) were not susceptible to OT-I CTL-mediated tumor lysis. B16/F10 OVA cells (not pretreated with flavopiridol) underwent massive cell death in this system, while B16/F10 parental cells survived, as they do not express OVA antigen (**Figure 7**). It is clear from the outcome of the suggested exploratory assay that chronic flavopiridol-induced TE^{deff} can

bestow a means to escape from anti-tumor immune attack even in vivo. This can be further tested in in vivo tumor models to check the propensity of TE^{deff} models to escape innate and adaptive anti-tumor immune responses. Anti-asialo treatments could be used to regulate the activity of NK cells in vivo in tumor bearing mice. Also, immune checkpoint therapy (anti-CTLA4 and anti-PD1) can be administered to TE^{deff} tumor bearing mice.

In totality, the TE^{deff} confirmatory assays along with the suggested exploratory assay together demonstrate the utility of incorporating this TE^{deff} cell model in a whole host of other tumor-immune testing conditions. This model can help parse out the molecular details resulting from defective transcriptional elongation in tumor cells and their response to immune cell interactions.

FIGURE LEGENDS:

Figure 1: **Schematic representation of the work flow.**

Figure 2: **Cell growth characteristics of B16 OVA cells chronically treated with low-dose flavopiridol: Viability (measured by viability reagent) of control and flavopiridol-treated cells B16 OVA at indicated days post-treatment.** This figure has been modified from Modur et al.[3].

Figure 3: Confirmatory assay to assess RNA Pol II and histone profile: Immunoblots of indicated histone and RNA Pol II marks in B16 OVA cells treated with flavopiridol for 72 h or 1 week. This figure has been modified from Modur et al.[3].

Figure 4: Confirmatory assay to assess severe defects in mRNA processing. Ratios of 5′-uncapped to 5′-capped (**A**) and 3′-non-polyadenylated to 3′-polyadenylated (**B**) mRNA concentrations after rRNA depletion in the indicated cell lines. Error bars represent standard deviation based on three technical replicates.

Figure 5: Confirmatory assay to assess cytokine stimulation profile. Immunoblots of STAT1, pSTAT1, NFκB and p NFκB in control and flavopiridol pre-treated B16 OVA cells stimulated with IFN-α, IFNγ or TNF-α for 30 min at (5 ng/mL). This figure has been modified from Modur et al.[3].

Figure 6: Confirmatory assay to assess resistance to FasL mediated cell death in vitro. Control and flavopiridol pre-treated B16 OVA cells treated with FasL for 24 h readout measured by viability assay. This figure has been modified from Modur et al.[3].

Figure 7: Exploratory assay to assess resistance of TE^{deff} model to antigen-restricted cytotoxic T cell mediated attack in vitro. Left: diagrammatic scheme of the exploratory assay. Right: relative viability of B16/F10-OVA cells co-cultured with activated CD8 + CTLs (1:1 ratio) isolated from the spleens of OT-I mice. *P*: Welch two-sample *t*-test. This figure has been modified from Modur et al.[3].

DISCUSSION:

RNA Pol II elongation control has emerged as a decisive lever for regulating stimulus-responsive gene expression to the benefit of malignant cells[5,7,8]. Overcoming promoter-proximal pausing to elongation and subsequent mRNA production requires the kinase activity of P-TEFb[9,10,11]. Our model utilizes flavopiridol (25 nM), an inhibitor of the essential cyclin-dependent kinase CDK9, to mimic the defects observed during Pol II elongation in TE^{deff} cancers—a previously unknown phenotype in cancers discovered by our group previously[3].

CDK9 kinase activity has long been known to be essential for phosphorylation of serine 2 residues in the CTD of the large subunit of Pol II. Critically, we have succeeded in optimizing flavopiridol treated chronic inhibition of CDK9 (25 nM for 1 week) in B16/F10 OVA such that, in addition to inhibiting CTD phosphorylation, 25 nM flavopiridol treatment for 1 week prevents proper post-transcriptional modifications of mRNA in an unanticipated way and effectively abrogates p-TEFb-dependent productive elongation along long genes such as pro-inflammatory response signaling genes, significantly altering their patterns of expression both at the mRNA and protein levels. To the best of our knowledge, there is no other model described in literature which effectively achieves the same.

This easy to establish, generalizable model of TE^{deff} can therefore be leveraged to dissect, both transcriptional and epigenetic modifications enabling TE^{deff} cancers to adapt to immune-mediated cell attack. Moreover, this murine model retains its TE^{deff}-like reduction of total and phospho- RNA Pol II levels 21 days after flavopiridol release in in vivo growth assay[3] (not mentioned in the protocol here), suggesting the extent of stability of this non-genetic model for further in vivo experimentation. However, care must be taken to optimize the exact sublethal dose of flavopiridol for other murine lines (e.g., about 20 nM flavopiridol treatment for 1 week is the sublethal dose for MC38 murine carcinoma line; not used in this protocol), the impact of variation in cell plating density, culture conditions, and cytokine stimulation conditions may vary for different murine lines. The protocol described here gives a basic framework to minimize the variables known to be critical for the generation of TE^{deff}-like features by chronic CDK9 inhibition. In addition, human carcinoma cell lines, such as T47D and CAL51 have been tested with short-term (3 days) flavopiridol treatments giving rise to similar TE^{deff}-like RNA Pol II profiles, indicating the usefulness of flavopiridol based chronic inhibition of CDK9 mediated transcription elongation in creating even model human lines to study TE^{deff}.

ACKNOWLEDGMENTS:

This work was in part supported by NCI (CA193549) and CCHMC Research Innovation Pilot awards to Kakajan Komurov, and Department of Defense (BC150484) award to Navneet Singh. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Cancer Institute or the Department of Defense. The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

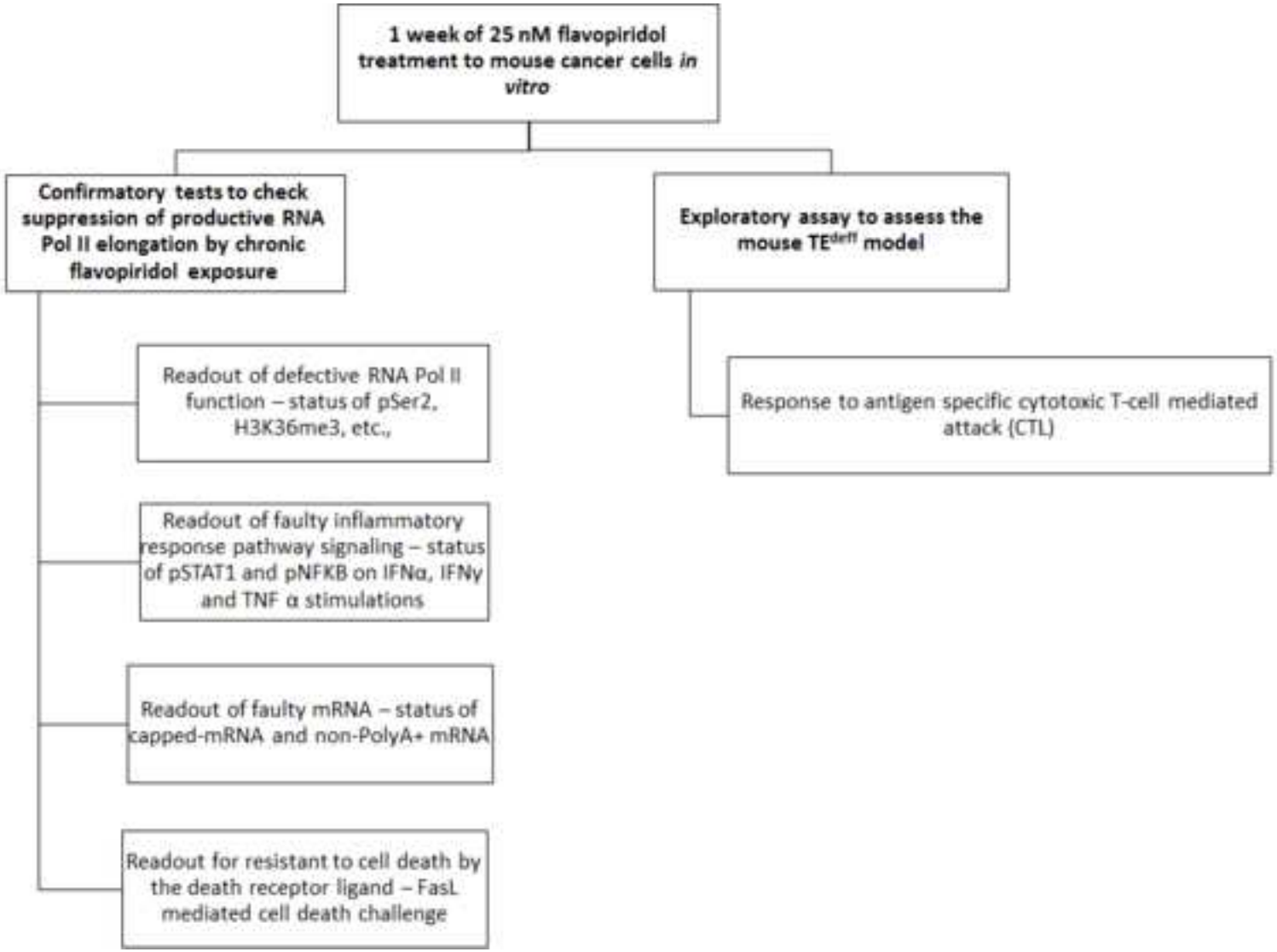


Figure 2

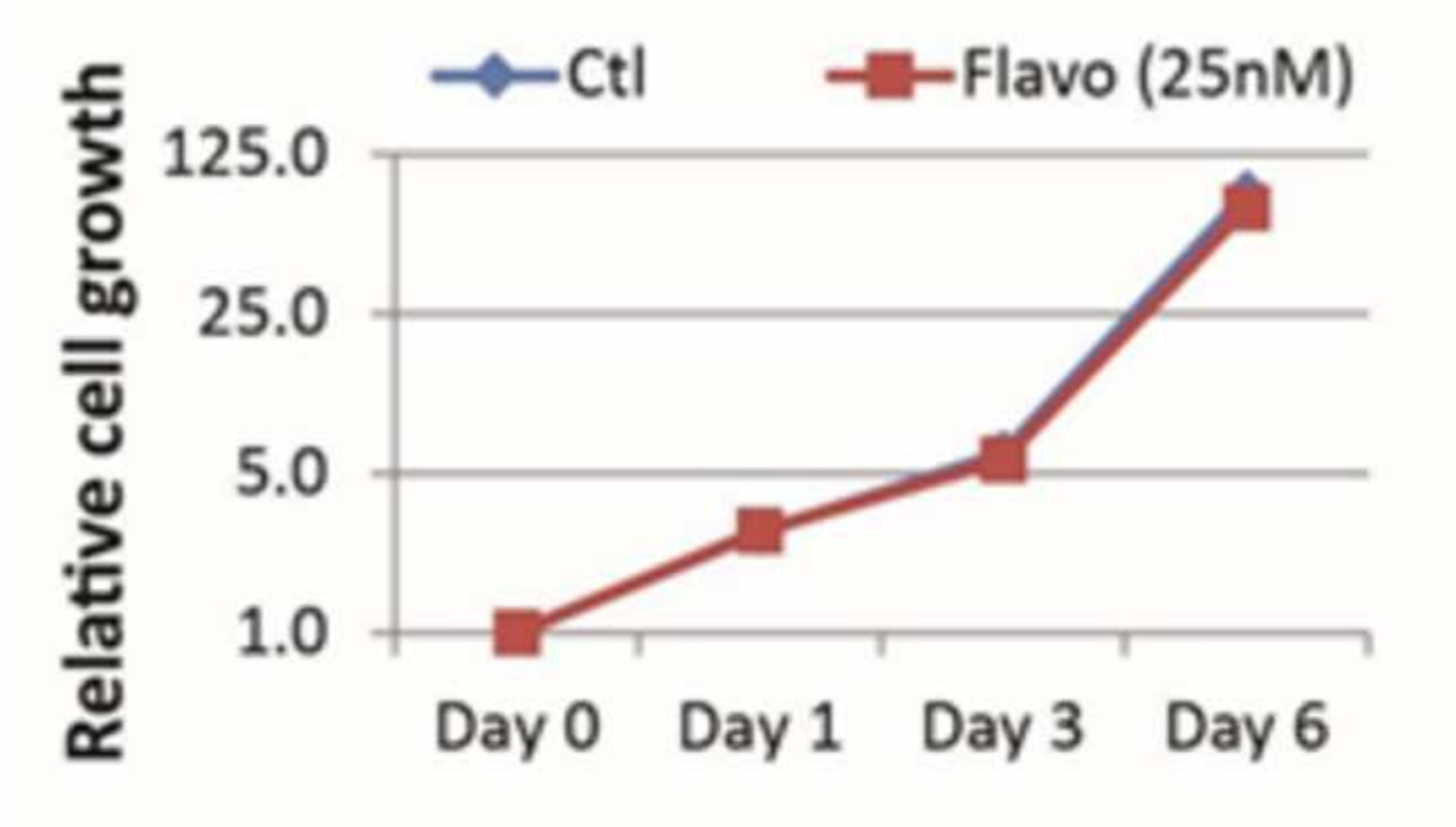


Figure 3

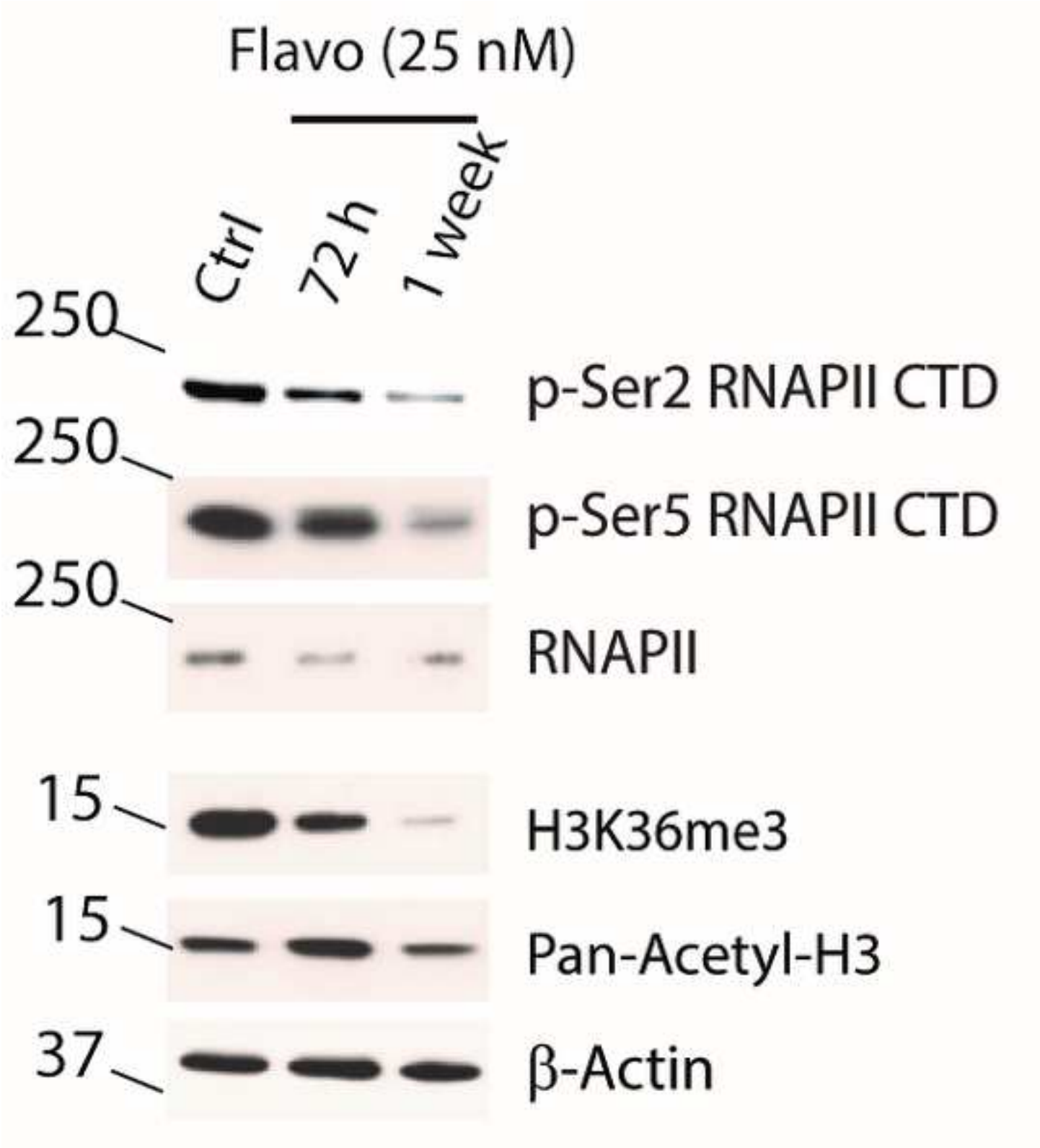


Figure 4

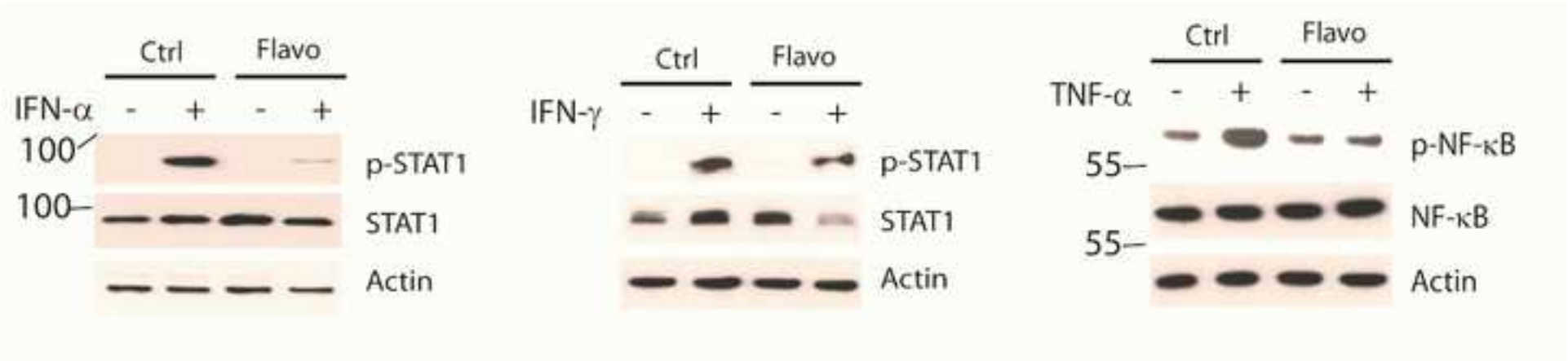


Figure 5

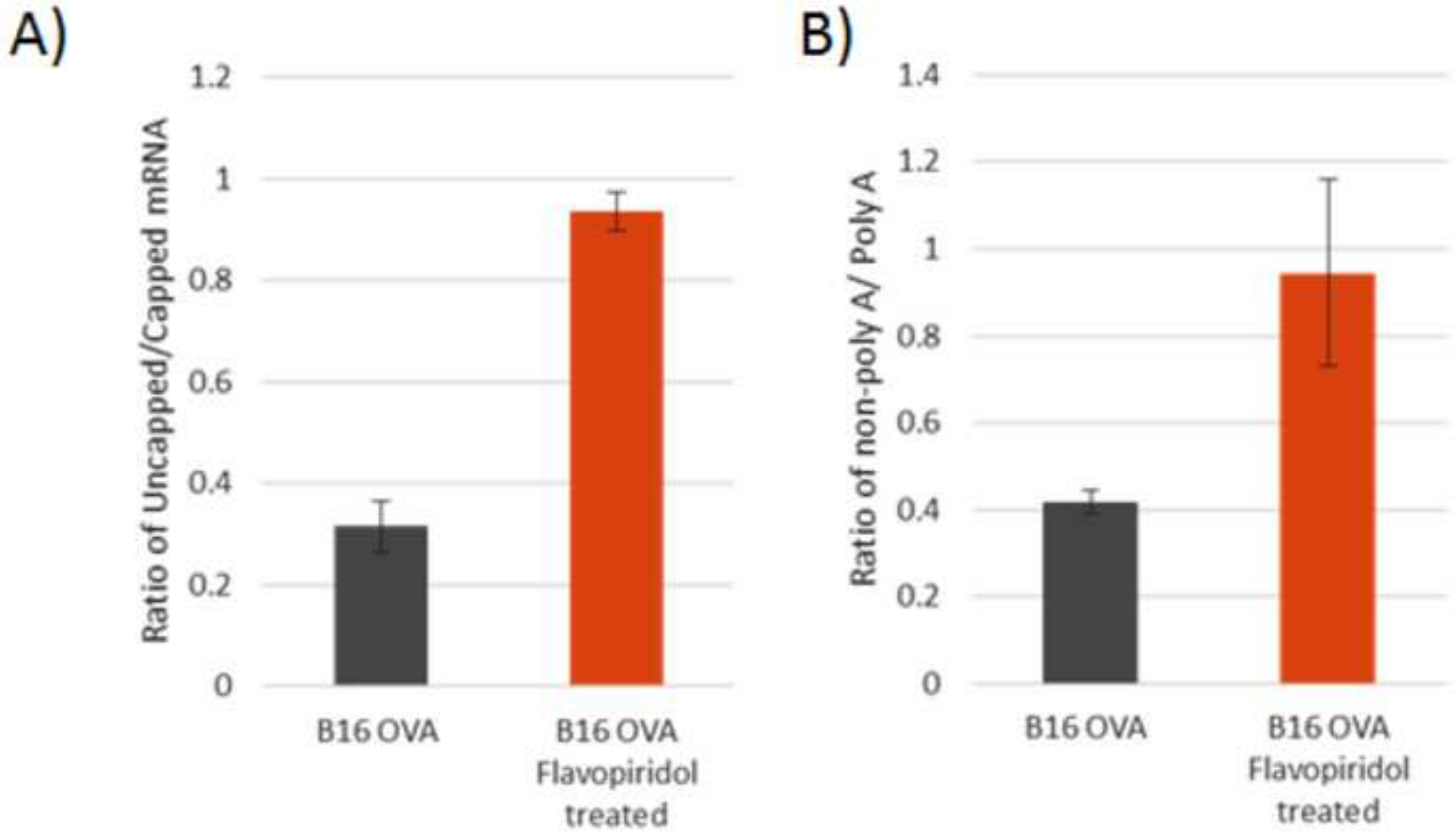


Figure 6

Click here to access/download;Figure;Figure 6.tif

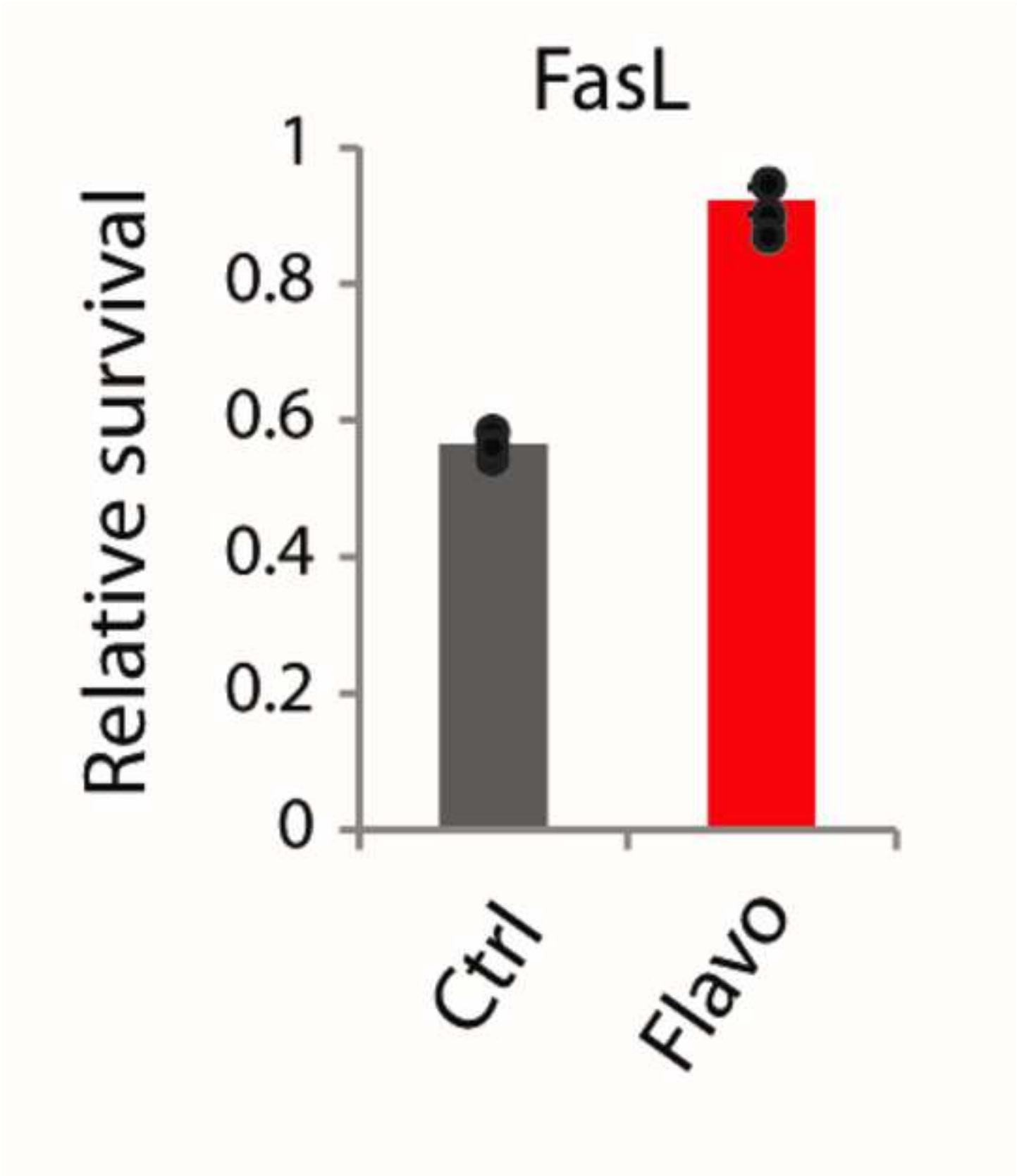
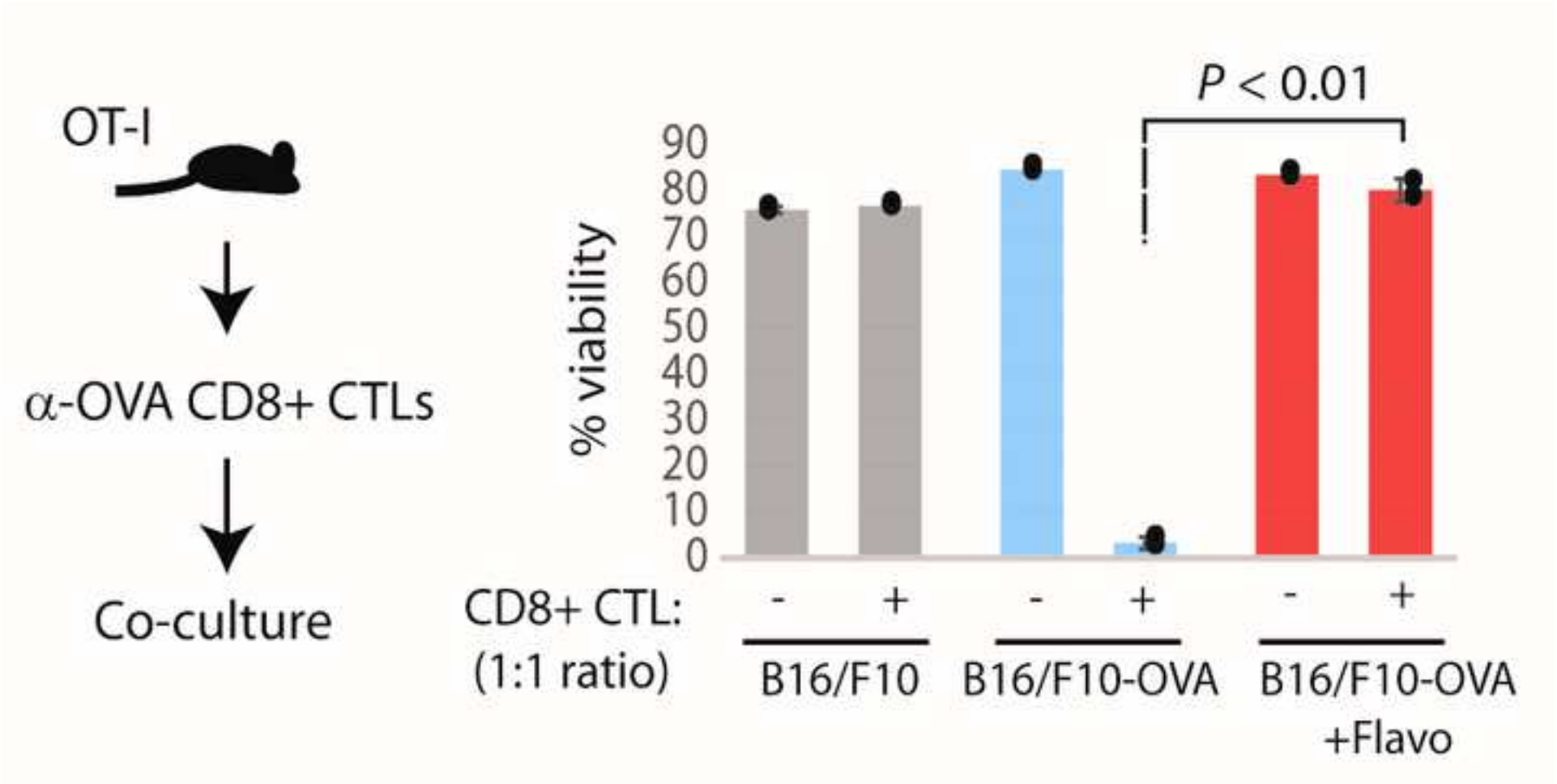


Figure 7



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
hhis6FasL	Cell Signaling	5452	
10X TBS	Bio-Rad	170-6435	
12 well plates	Falcon	353043	
20% methanol	Fisher Chemical	A412-4	
24-well plates	Falcon	351147	
4–18% SDS polyacrylamide gel	Bio-Rad	4561086	
4% Paraformaldehyde	Thermo Fisher Scientific	AAJ19943K2	
5% dry milk	Bio-Rad	170-6404	
7-Methylguanosine antibody	BioVision	6655-30T	
96-well plates	Cellstar	655180	
AF647-conjugated mouse CD8	Biolegend	100727	
antibiotic and antimycotic	Gibco	15240-062	
anti-His antibody	Cell Signaling	2366 P	
Anti-Rabit	Cell Signaling	7074	Dilution 1:5000
Anti-Rat	Cell Signaling	7077S	Dilution 1:5000
Bradford assay Kit	Bio-Rad	5000121	
BSA	ACROS Organics	24040-0100	
BV421-conjugated mouse CD45	Biolegend	109831	
crystal violet	Sigma	C3886-100G	
DMEM	Gibco	11965-092	
Dynabeads Oligo (dT)25	Ambion	61002	
FBS	Gibco	45015	
Fixable Live/Dead staining dye e780	eBioscience	65-0865-14	
Flavopiridol	Selleckchem	S1230	
H3k36me3	Abcam	ab9050	Dilution 1:2000
IFN-α	R&D systems	12100-1	
IFN-γ	R&D systems	485-MI-100	
IMDM	Gibco	12440053	
Immobilon Western Chemiluminescent HRP Substrate	Millipore	WBKLS0500	
MojoSort Mouse CD8 T Cell Isolation Kit	Biolegend	480007	
NF-κB	Cell Signaling	8242s	Dilution 1:1000

PBS	Gibco	14190-144	
p-NF-κB	Cell Signaling	3033s	Dilution 1:1000
p-Ser2-RNAPII	Active Motif	61083	Dilution 1:500
p-Ser5-RNAPII	Active Motif	61085	Dilution 1:1000
p-STAT1	Cell Signaling	7649s	Dilution 1:1000
RiboMinu Eukaryote Kit	Ambion	A10837-08	
RIPA buffer	Santa Cruz Biotechnology	sc-24948	
RNAPII	Active Motif	61667	Dilution 1:1000
STAT1	Cell Signaling	9175s	Dilution 1:1000
TNF-α	R&D systems	410-MT-010	
total H3	Cell Signaling	4499	Dilution 1:2000
Tri reagent	Sigma	T9424	
Triton	Sigma	T8787-50ML	
Tween 20	AA Hoefer	9005-64-5	
β-Actin	Cell Signaling	12620S	Dilution 1:5000
β-ME	G Biosciences	BC98	

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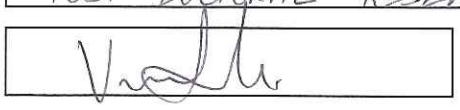
Signature:	
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April 30, 2019.

Dear Xiaoyan Cao, PhD,

I would like to thank you for reviewing our manuscript. The corrections requested have been incorporated and indicated in the tracked changes. I'm happy to re-submit the revised version of our manuscript now titled, "A cell line based mouse model of chronic CDK9 inhibition to study widespread non-genetic transcriptional elongation defects (TE^{deff}) in cancers."

Changes requested and **author's response in bold font**

1) RNA Pol II? Please select one term and use it consistently throughout. Line 81.
Author's Response: RNAPII has been changed to RNA Pol II throughout the manuscript.

2) Please define all abbreviations before use. Line 84.
Author's Response: the full form of DMEM has been added in line 84.

3) Please define abbreviations. Lines 95-96
Author's Response: the full form of IFN and TNF have been added in lines 95-96.

4) At 37 °C? Line 104.
Author's Response: Temperature has been added in line 104.

5) Please convert the unit to x g. Line 110.
Author's Response: Units have been converted to x g in line 110.

6) Please specify the incubation temperature. Please specify the primary antibodies used here including dilution/concentration. Line 120.
Author's Response: Incubation temperature and Primary antibody dilutions have been included in line 120.

7) Please specify the incubation temperature and time. Please specify the secondary antibodies used here including dilution/concentration. What is used to detect protein signals? Please write the sentence in the imperative tense. Note that the highlighted content is not continuous as the readers/viewer will have no idea what the PVDF membranes are and how they are obtained. Please consider highlighting steps 2.3-2.5. Lines 122-124.
Author's Response: Incubation temperature, time, Primary antibody dilutions, and method of protein signal detection have been included in lines 129-133.

8) Please replace RiboMinus with a generic term throughout the manuscript.
Author's Response: rRNA depletion probe has been added in place of RiboMinus throughout the manuscript.

9) Do you mean section 3.3? Line 225.
Author's Response: Section 3.3 is now mentioned appropriately in line 225.

10) From which step? Please specify. Line 252.
Author's Response: This step indicates the addition of 120ng of rRNA depleted RNA from the remaining half of step 3.3. Step 3.6 mentioned this in a general introductory way, but this step (3.6.8) specifically mentions the amount of RNA from the remaining half of step 3.3 to be used.

11) Please spell it out. ULB buffer. Line 257.
Author's Response: It has been added.

12) What is the volume ratio? How is it prepared? Line 262.
Author's Response: The correction has been made. It now reads, Phenol:Chloroform:Isoamyl Alcohol (25:24:1; commercially available) (stored at 4 °C) to the samples. The material has been now listed in the updated Materials List.

13) Please convert it to x g. Line 265.
Author's Response: Has been converted.

14) Chloroform or phenol:chloroform? Line 268. Please convert it to x g. Line 269.
Author's Response: It is in fact, Phenol:Chloroform:Isoamyl Alcohol (25:24:1; commercially available) Has been converted. Lines 268 and 269.

15) Please convert it to x g. Line 274.
Author's Response: Has been converted.

16) 30,000 or 300,000? Line 288.
Author's Response: Has been corrected to 30,000 cells.

17) When are the cells treated? Please specify the temperature. How to fix the attached cells? At what temperature? Please specify the concentration. Lines 292, 295, 301
Author's Response: Corrections have been included.

18) Please convert it to x g. Line 317. Please spell it out. Add buffer to what? Please specify. Line 319.
Author's Response: Corrections have been included.

19) Step 5.1.1.12 does not mention "kept on ice". Please review this step and revise as necessary. Line 345. Please spell it out Line 347. Please specify temperature, CO2%. Line 349.
Author's Response: Corrections have been included.

20) Please number the references in order of appearance. It should be 6 instead of 11. Please update the numbering in the text and in the reference list. Line 353.
Author's Response: The References are renumbered and rearranged as directed.

21) Please spell it out and provide its composition. Line 355. Please describe how. Line 359. 30,000? Line 363. Please specify temperature. Line 365. Please describe how. Line 368. Please

describe how. Line 370. Please replace it with a generic term. Line 373.
Author's Response: Corrections have been included.

22) Please remove commercial language. Line 435.
Author's Response: commercial language has been deleted.

23) Please reference these figures in the results section. Lines 446-458
Author's Response: These figures have now been appropriately cited in the Results section.

Sincerely,
Vishnu Modur