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14. ABSTRACT
Medulloblastoma is the most common childhood brain tumor and is a major cause of cancer related mortality in children. Although current treatment strategies reach a 60-80% 5-year survival rate, a proportion of cases remain unresponsive to therapy and lack more effective treatment options. A significant molecular heterogeneity exists within the medulloblastoma subgroups, especially within Group 4 medulloblastoma (G4-MB), challenging the choice and prediction of response to a particular therapy. Our objective is to define the cell-of-origin and candidate drivers of G4-MB, and to develop pre-clinical mouse models that reflect G4-MB tumorigenesis. We have developed a tumor mouse model that allows us to manipulate early iPSC-derived neuroepithelial stem cells, a cell population suggested as the cell-of-origin for but not limited to G4-MB. Using this model we have shown that the G4-MB candidate drivers SRC and ERBB4 induce tumor formation in *TP53*^{WT} and *TP53*^{mut} NESCs. Furthermore we are testing pre-clinical therapies of SRC inhibition in our SRC-ERBB4 tumor-bearing mice and are exploring other genetic G4-MB drivers such as ZMYM3. Our mouse models established here will decipher the yet unknown origin of Group 4 medulloblastoma and inform about basic principles of tumorigenesis in the brain.

15. SUBJECT TERMS
Pediatric Cancer; Medulloblastoma; Neuroepithelial Stem Cells; Tumorigenesis; SRC; ERBB4; ZMYM3

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INTRODUCTION

Group 4 medulloblastoma (G4-MB) is the most common, yet least understood medulloblastoma subgroup: both in terms of driver pathways and cell-of-origin. Despite comprehensive genomic studies, G4-MB cases cannot be explained by known oncogenic drivers. On the molecular level great heterogeneity can be found within the G4-MB subgroup aggravating the understanding of G4-MB biology. G4-MBs show loss of *TP53*-mediated cell cycle checkpoint function, and alterations in chromatin modifiers such as loss-of-function mutations in *ZMYM3* contribute to approximately 30-40% of G4-MB patients. On the proteomic level increased receptor tyrosine kinase (RTK) signaling through activated ERBB4 and SRC has been suggested as a key player in G4-MB biology. However, it is unclear if these alterations represent actual drivers of G4-MB. Mouse models of G4-MB are not available, and the origin of G4-MB is still unclear. Development of targeted G4-MB therapies, which are currently completely lacking, will require identifying both cells-of-origin and drivers of G4-MB tumorigenesis. To test the role of hindbrain stem cells as the G4-MB cell-of-origin and identify key drivers in G4-MB tumorigenesis, we have established a xenograft model of human iPSC-derived neuroepithelial stem cells (NESC), a cell population showing dorsal hindbrain specification. We will use this model to analyze the potential oncogenic function of ERBB4 and SRC, and loss-of-function mutations in *ZMYM3* to form medulloblastoma *in vivo*. Furthermore, we will test potential drug targets of SRC-ERBB4 derived tumors *in vitro* and *in vivo* and analyze the molecular and cellular consequences of aberrant function of ZMYM3 in NESCs. This project will inform about basic principles of development and tumorigenesis in the brain. Expected results from our proposed project will further elucidate the molecular mechanisms in the origin of medulloblastoma and allow us to improve current treatment strategies for medulloblastoma.

KEYWORDS

Pediatric Cancer
Medulloblastoma
Neuroepithelial Stem Cells
Tumorigenesis
SRC
ERBB4
ZMYM3

ACCOMPLISHMENTS

What were the major goals of the project?

Major Goal/Task	Goal	Timeline (Months)	Percentage completed
1	Determine if SRC^{OE}/$ERBB4^{OE}$ signaling in human neuroepithelial stem cells causes G4-MB formation *in vivo*.	4-18	85%
2	Test whether targeted treatment with dasatinib in SRC^{OE}/$ERBB4^{OE}$ $TP53^{WT}$ and $TP53^{MUT}$ NES tumor-bearing mice prolongs survival.	6-20	0%
3	Introduce patient-derived ZMYM3 frameshift mutations P48Lfs*65 and R1111fs*9 into the genome of $TP53^{wt}$ and $TP53^{mut}$ human iPSC-derived NESCs.	4-16	25%
4	Determine if ZMYM3 frameshift mutations P48Lfs*65 and R1111fs*9 in $TP53^{wt}$ and $TP53^{mut}$ NESCs introduces tumor growth *in vivo.*	10-24	0%

What was accomplished under these goals?

Major Goal 1: Determine if SRC^{OE}/$ERBB4^{OE}$ signaling in human neuroepithelial stem cells causes G4-MB formation *in vivo*.

For Major Goal 1 of this proposal, we wanted to test if forced expression of SRC and ERBB4 in neuroepithelial stem cells (NESC) drives tumor formation *in vivo* and will generate tumors with a Group 4 medulloblastoma (G4-MB) signature.

For Subtask 1 under Major Goal 1 we tested if upregulated SRC and ERBB4 signaling in NESCs functions as tumor driver *in vivo*. Therefore, we overexpressed SRC and ERBB4 ($SRC^{OE}ERBB4^{OE}$) in $TP53^{mut}$ or $TP53^{wt}$ human NESCs via lentivirus transduction. If not otherwise stated, for all experiments described in Major Goal 1 $SRC^{OE}ERBB4^{OE}$;$TP53^{mut}$ NESCs and its controls (SRC^{OE};$TP53^{mut}$ NESCs, $ERBB4^{OE}$;$TP53^{mut}$ NESCs, and empty vector $TP53^{mut}$ NESCs or wild-type NSCs) were tested. We then determined tumorigenesis of SRC and ERBB4 overexpression NESCs by injection of 300,000 NES cells into the cerebellum of 6-8-week-old immunocompromised NOD-scid IL2Rgamma (NSG) mice with a group size n=10 for each NESC genotype (Fig. 1A). Mice were monitored for tumor growth with bioluminescence imaging for up to one year after transplantation and euthanized when reaching humane endpoints or at 12 months after injection (Fig. 1B). We found that $SrcA^{OE}$ alone and $SrcA^{OE}$ with $ErbB4^{OE}$ in wild-type NESCs promotes tumor growth *in vivo* and mice reach endpoint around 60 days for $SrcA^{OE}$ alone and around 80 days with combination of $SrcA^{OE}$ and $ErbB4^{OE}$ (Fig. 1C). We see a 10% penetrance with slow tumor formation in $ErbB4^{OE}$ wild-type NESCs with one mouse at endpoint at 190 days, while all other mice in this group did not develop any tumors. In $TP53^{mut}$ NESCs we find slightly more aggressive tumor growth for $SrcA^{OE}$ alone with 40 days of average survival and 60 days

average survival with combination of SrcA^{OE} and ErbB4^{OE} (Fig. 1E). As well for ErbB4^{OE} alone we find a tumor penetrance of 10% (1 mouse) with a survival of 125 days, while all other mice in this group developed no tumors.

Developed tumors were dissected at endpoint. If tumors were visible and of reasonable size (> 2x2x2 mm) tumor tissue was subjected to standard histopathology such as Hematoxylin and Eosin (H&E) staining and immunohistochemistry for Ki67. H&E of coronal sections of the cerebellum show nuclear dense and non-infiltrative areas of tumor tissue with positivity for the cell proliferation marker Ki67 (Fig. 1D).

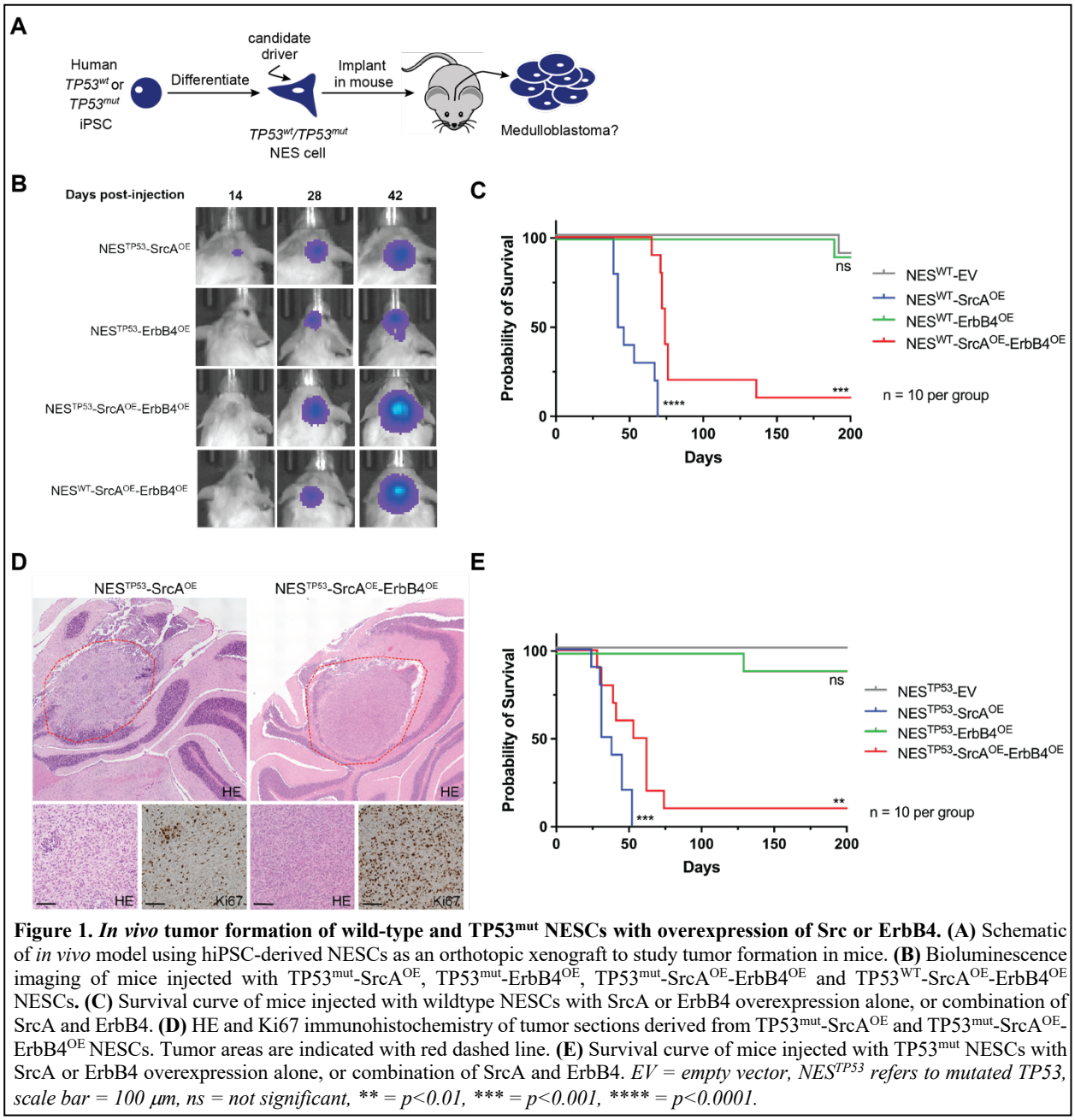

Figure 1. *In vivo* tumor formation of wild-type and TP53^{mut} NESCs with overexpression of Src or ErbB4. (A) Schematic of *in vivo* model using hiPSC-derived NESCs as an orthotopic xenograft to study tumor formation in mice. **(B)** Bioluminescence imaging of mice injected with TP53^{mut}-SrcA^{OE}, TP53^{mut}-ErbB4^{OE}, TP53^{mut}-SrcA^{OE}-ErbB4^{OE} and TP53^{WT}-SrcA^{OE}-ErbB4^{OE} NESCs**. (C)** Survival curve of mice injected with wildtype NESCs with SrcA or ErbB4 overexpression alone, or combination of SrcA and ErbB4. **(D)** HE and Ki67 immunohistochemistry of tumor sections derived from TP53^{mut}-SrcA^{OE} and TP53^{mut}-SrcA^{OE}-ErbB4^{OE} NESCs. Tumor areas are indicated with red dashed line. **(E)** Survival curve of mice injected with TP53^{mut} NESCs with SrcA or ErbB4 overexpression alone, or combination of SrcA and ErbB4. *EV = empty vector, NES^{TP53} refers to mutated TP53, scale bar = 100 µm, ns = not significant, ** = p<0.01, *** = p<0.001, **** = p<0.0001.*

For Subtask 2 and 3 under Major Goal 1 we wanted to molecularly profile obtained tumors from Subtask 1 by DNA methylation and gene expression analysis and compare their profiles with human medulloblastoma samples to define G4-MB specificity. Tumor tissue was harvested from each tumor at endpoint and if visible and of reasonable size (> 2x2x2 mm) and snap frozen. Frozen tissues were subjected to DNA and RNA extraction and further genomic analyses. Tumor DNA was used for whole-genome bisulfite sequencing and DNA methylation clustering was performed using a methylation profiling classifier with a public reference cohort of over 2,800 neuropathological tumors of almost all known entities, including medulloblastoma. We subjected one SrcA^{OE} NES^{TP53mut}, three SrcA^{OE}-ErbB4^{OE} NES^{TP53mut} and three SrcA^{OE} NES^{TP53WT} tumors to DNA methylation profiling. None of the tumors matched with any pediatric neuropathological tumors depicted by a low calibration score in the analysis (Table 1). While this result could be due to low quality of DNA content in our tumor samples and preparations, it is very unlikely. Most likely it shows that our obtained tumors do not resemble medulloblastoma or any other class of pediatric central nervous system tumors by DNA methylation.

Table 1. DNA methylation clustering in SrcA^{OE} and SrcA^{OE}-ErbB4^{OE} TP53^{WT} and TP53^{MUT} NESCs.

Sample	Calibration Score*	Match
NES^{TP53mut}-SrcA^{OE}-1	0.34	no
NES^{TP53mut}-SrcA^{OE}-ErbB4^{OE}-1	0.55	no
NES^{TP53mut}-SrcA^{OE}-ErbB4^{OE}-2	0.6	no
NES^{TP53mut}-SrcA^{OE}-ErbB4^{OE}-3	0.51	no
NES^{TP53WT}-SrcA^{OE}-1	0.45	no
NES^{TP53WT}-SrcA^{OE}-2	0.44	no
NES^{TP53WT}-SrcA^{OE}-3	na	no

* Probability that measure the confidence in the class assignment.
Score >= 0.9 is defined as a match with high confidence. Score < 0.9 is defined as no match.

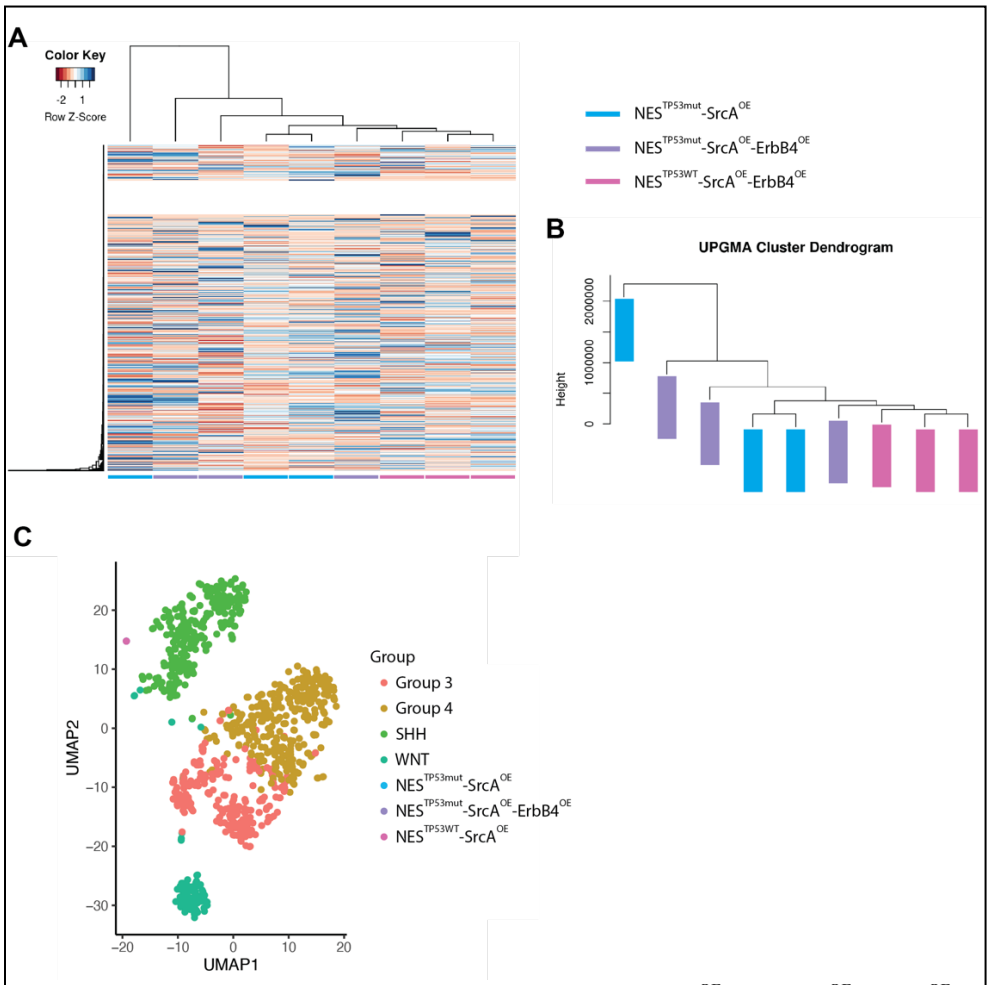

Figure 2. RNA sequencing and gene expression profiling of SrcA^{OE} and SrcA^{OE}-ErbB4^{OE} in TP53^{WT} and TP53^{mut} NESCs. Heat map **(A)** and cluster dendogram **(B)** of hierarchical clustering of NES^{TP53mut}-SrcA^{OE}, NES^{TP53mut}-SrcA^{OE}-ErbB4^{OE} and NES^{TP53WT}-SrcA^{OE}-ErbB4^{OE} tumor gene expression analysis. **(C)** Gene expression profiling of NES^{TP53mut}-SrcA^{OE}, NES^{TP53mut}-SrcA^{OE}-ErbB4^{OE} and NES^{TP53WT}-SrcA^{OE}-ErbB4^{OE} tumors and human medulloblastoma primary tumor samples using UMAP clustering.

For gene expression profiling of the obtained tumors we isolated RNA from three SrcA^{OE} NES^{TP53mut}, three SrcA^{OE}-ErbB4^{OE}

$NES^{TP53mut}$ and three $SrcA^{OE}$ NES^{TP53WT} and ran RNA sequencing and downstream analysis on the obtained data. We performed gene expression clustering of our Src-ErbB4 tumors among them and among medulloblastoma patient samples. We find that the $SrcA^{OE}$ $NES^{TP53mut}$ and $SrcA^{OE}$-$ErbB4^{OE}$ $NES^{TP53mut}$ cluster close together while the $SrcA^{OE}$-$ErbB4^{OE}$ NES^{TP53WT} tumors form their own separate cluster (Fig. 2A and 3B). When clustering our tumor samples with gene expression data of medulloblastoma samples across all subgroups we find that our Src-ErbB4 do not cluster close to any of the subgroups and lie closest to the sonic hedgehog (SSH) medulloblastoma (Fig. 2C). Most surprisingly in this analysis all our Src-ErbB4 tumors cluster very tightly together, which needs to be further investigated to rule out technical issues with the data analysis.

Major Goal 2: Test whether targeted treatment with Dasatinib in SRC^{OE}/$ERBB4^{OE}$ $TP53^{WT}$ and $TP53^{MUT}$ NES tumor-bearing mice prolongs survival.

For Major Goal 2 of this proposal we are planning to test whether targeted treatment strategies against Src pathway activation *in vivo* will prolong survival of SRC^{OE} and SCR^{OE}-$ERBB4^{OE}$ tumor mice. Major Goal 2 is planned to be accomplished in Year 2 (Months 13-24) of this proposal.

Major Goal 3: Determine if mutations in *ZMYM3* drive Group 4 medulloblastoma formation in human neuroepithelial stem cells *in vivo*.

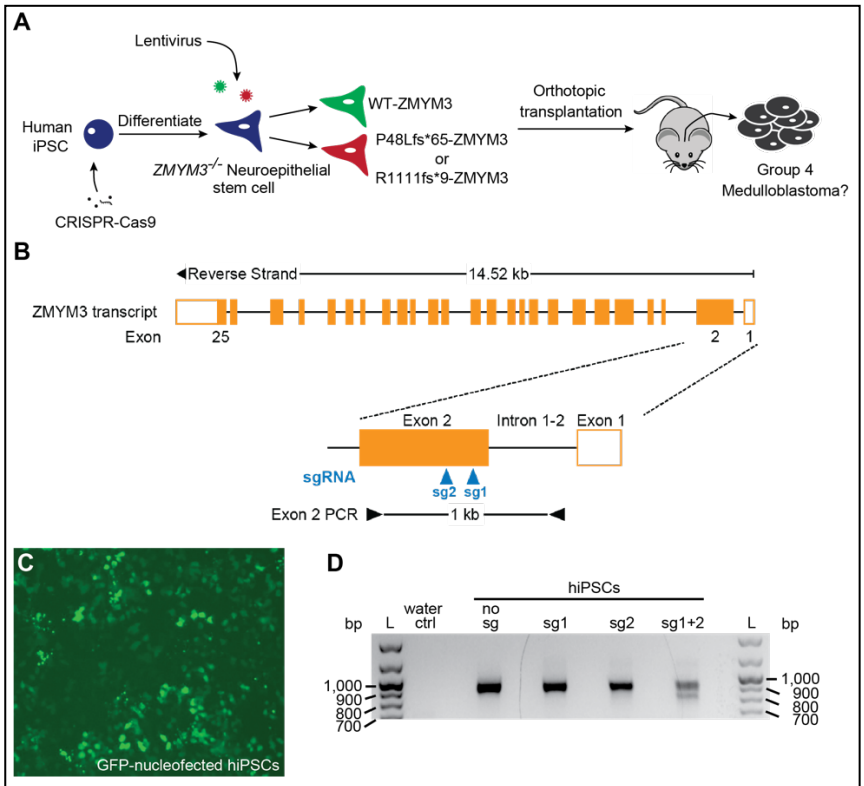

Figure 3. CRISPR-based sgRNA-Cas9 gene-targeted knockout of *ZMYM3* in human iPSCs. (A) Experimental design of introducing patient-derived *ZMYM3* mutations in *ZMYM3*-knockout NESCs to study medulloblastoma formation in mice. **(B)** sgRNA CRISPR design to target exon 2 of *ZMYM3* and introduce deletions and knockout of ZMYM3 in hiPSCs. **(C)** Image of GFP-positive hiPSCs 48 hours after nucleofection with a positive control plasmid driving GFP expression. **(D)** Agarose gel electrophoresis of *ZMYM3* exon 2 PCR-amplified products in hiPSCs nucleofected with sgRNA-Cas9 targeting exon 2 of *ZMYM3*. *sg = sgRNA, bp = base pair, L = ladder.*

For Major Goal 3 of this proposal we wanted to introduce the patient-derived ZMYM3 frameshift mutations P48Lfs*65 and R1111fs*9 into the genome of $TP53^{wt}$ and $TP53^{mut}$ human iPSC-derived NESCs and study their tumorigenic potential *in vivo*.

For Subtask 1 under Major Goal 3 we planned to knockout ZMYM3 in our human iPSCs (TP53 wildtype and TP53 mutated), differentiate them into NESCs and introduce the expression of P48Lfs*65 and R1111fs*9 *ZMYM3* mutations via lentiviral transduction (Fig. 3A). For our knockout experiments we designed sgRNAs targeting the exon 2 locus of ZMYM3 and introduced the sgRNA-Cas9 ribonucleoprotein complex via nucleofection into

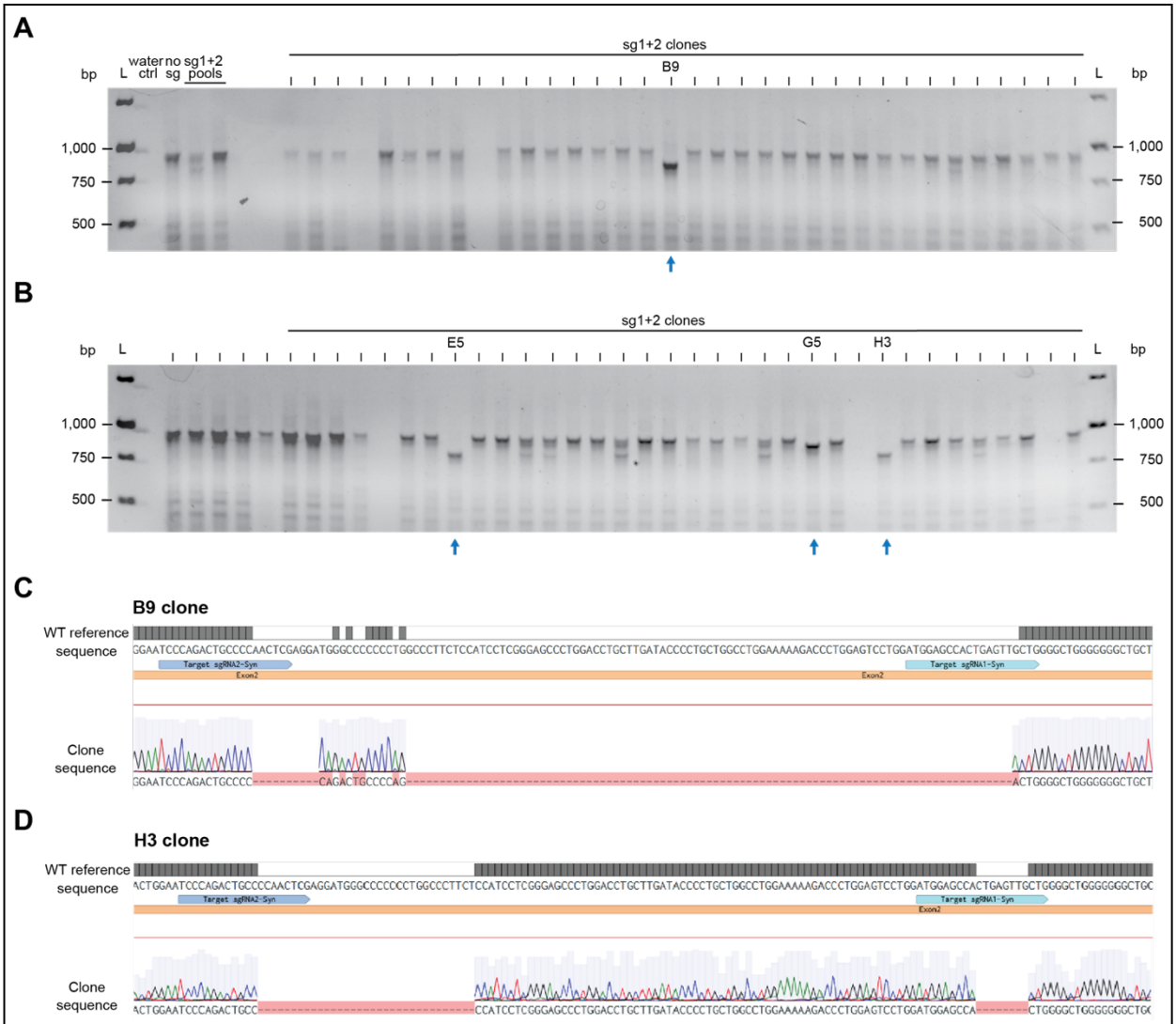

Figure 4. Clonal screening of *ZMYM3* gene-targeting in human iPSCs. (A and B) Agarose gel electrophoresis of *ZMYM3* exon 2 PCR-amplified products in single hiPSCs clones nucleofected with two sgRNA-Cas9 complexes targeting exon 2 of *ZMYM3*. Positive clones are highlighted with a blue arrow. Vertical lines depict one clone and lane on the agarose gel. DNA sequencing of *ZMYM3* exon 2 in clone B9 **(C)** and H3 **(D)**. Sequencing is aligned to the wildtype sequence of *ZMYM3* and location of sgRNAs is depicted in blue arrows along the sequence. Deleted regions are highlighted in red. *sg = sgRNA, bp = base pair, L = ladder, WT = wildtype, Target sgRNA1-Syn = sgRNA1, Target sgRNA2-Syn = sgRNA2.*

hiPSCs. We designed two sgRNAs both targeting exon 2 of ZMYM3 and tested single sgRNA-Cas9 and combination sgRNA-Cas9 nucleofection to introduce larger deletions (Fig. 3B). To analyze the size of our induced deletions we designed a PCR primer pair flanking both sgRNAs in exon 2 and generating a 1 kb PCR product in wildtype ZMYM3. We initially tested the efficiency of our nucleofection using a control GFP plasmid and find most hiPSCs GFP-positive 48 hours after transfection (Fig. 3C). To test the efficiency of our sgRNA-Cas9 we nucleofected cells with single and combination of both sgRNAs, and harvested cells for genomic DNA isolation 7 days later. When running the PCR covering both sgRNAs, from here on referred as ‘exon 2 PCR’, we find only 1 kb products in the single sgRNA conditions, while combination of both sgRNAs also shows a PCR band at around 850 bp indication a local deletion in some of the cells (Fig. 3D).

To generate single-cell clones with a sgRNA-Cas9 induced deletion in ZMYM3 we nucleofected hiPSCs with both our sgRNAs and plated single cells in 96-well plates 48 hours after nucleofection. We grew up single-cell clones and harvested genomic DNA from a total of 74 clones for Exon 2 PCR screening for deletions in exon 2 of ZMYM3. For selection of positive clones we considered the Exon 2 PCR to show no band at 1 kb (wildtype size) but a smaller band visible on the gel. For the 74 screened clones we found 4 positive clones with a PCR band at around 800 bp instead of 1 kb (Fig. 4A and B). Among the 74 screened clones we also found clones with a PCR band below 1 kb and with the wildtype 1kb band. This indicates that the clone came from several cells where some were wildtype and some acquired sgRNA-directed cuts in exon 2. We decided to move forward with the clones showing no wildtype 1 kb band anymore and sequenced the cut regions in exon 2 of ZMYM3 for clone B9 and H3. Clone E5 and G5 unfortunately started to differentiate in culture and therefore were excluded from further analysis and experiments. Clone B9 and H3 showed deleted regions of about 80 bp and 30 bp for the latter clone (Fig. 4C and D).

To understand if the introduced cuts in exon 2 of ZMYM3 will lead to early termination of ZMYM3 transcription and loss of ZMYM3 on the protein level we first performed immunoblot and qRT-PCR analysis in the B9 and H3 clones. On protein level we do not see a knockout or knockdown of *ZMYM3* for both clones compared to wildtype cells (Fig. 5A). For qRT-PCR analysis we designed two primer sets, one primer pair spanning exon 3 to 5 to determine expression levels right after the cut in exon 2, and one primer pair spanning exon 22 to 23 in order to determine expression levels of the 3'end of the full-length *ZMYM3* transcript. We find both regions of *ZMYM3* expressed in both clones indicating that *ZMYM3* mRNA is still being expressed in the cells (Fig. 5B). Furthermore, we also sequenced the expressed transcript of ZMYM3 in the B9 clone by sequencing the reverse transcript generated from mRNA expression. We find that *ZMYM3* in the B9 clone is expressed and still harbors the deletions found in exon 2 through our gene-targeting (Fig. 5C). Despite the introduced frameshift and around 80 bp deletion in exon 2 we find full-length *ZMYM3* mRNA being transcribed in the cells (full sequencing data not shown). This led us to the conclusion that we were not able to generate a gene-edited knockout or significant knockdown of *ZMYM3* and instead might have introduced a truncated version of ZMYM3. To verify our hypothesis, we generated protein transcripts based on our mRNA sequencing results and find that though the deletion in clone B9 introduces an early stop codon at amino acid 53, it also allows for an alternative start site at amino acid 230 that will span protein translation until the C-terminal end of ZMYM3. We therefore conclude that we most likely have generated a N-terminal truncated version of ZMYM3 in our clone that still expresses the functional domains in ZMYM3 and therefore will not be of use for this proposal. In order to reach our goals in this proposal we decided to skip the generation of *ZMYM3* knockout iPSCs and directly overexpress patient-derived *ZMYM3* mutations in our wildtype NESCs via lentiviral infection and conclude with our planned downstream experiments. This will be discussed in detail in the "Changes and Problems" section of this report.

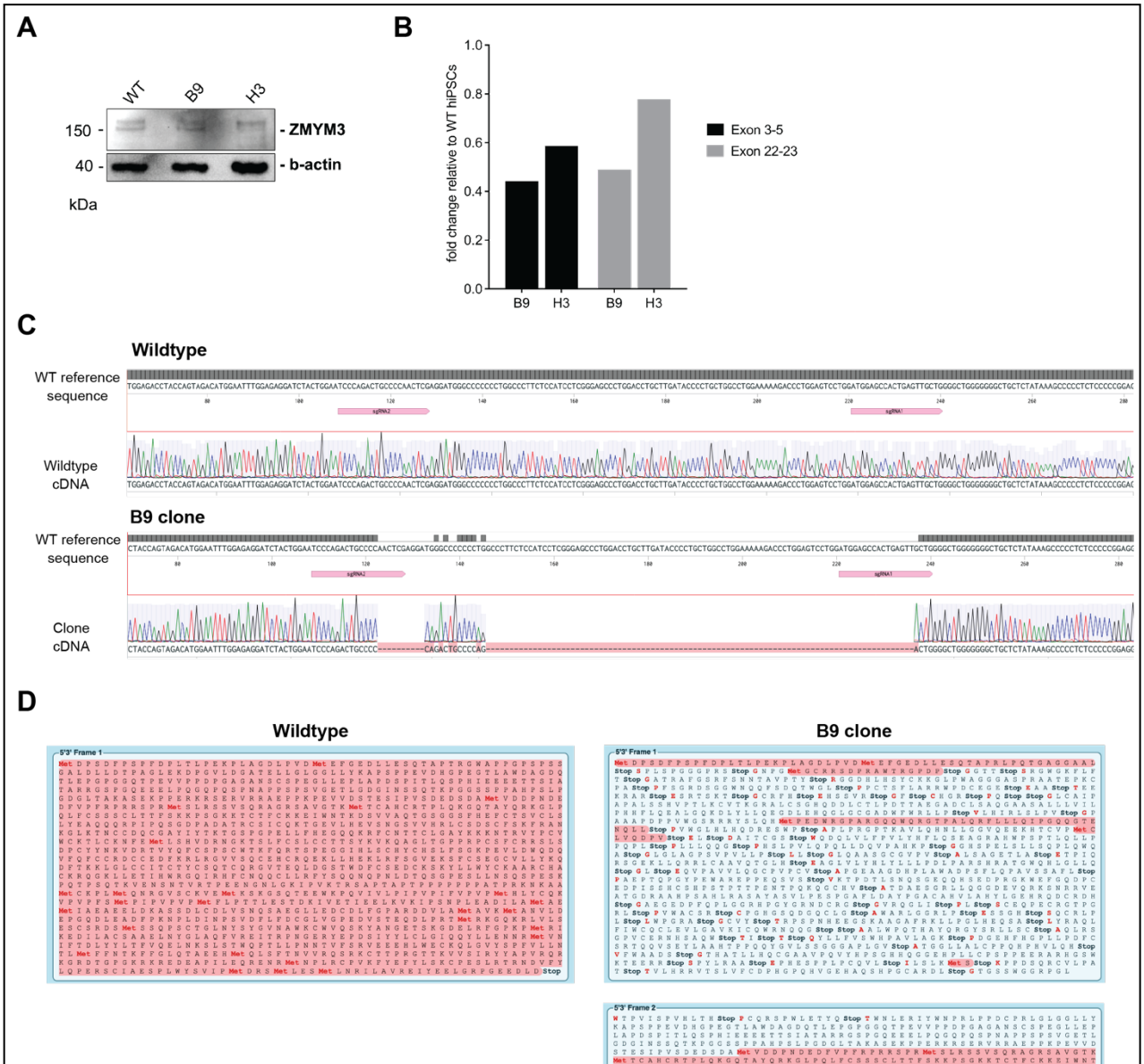

Figure 5. Downstream analysis of *ZMYM3* knockout in B9 and H3 clones. (A) Immunoblotting *ZMYM3* in wildtype and gene-targeted clones B9 and H3. **(B)** Quantitative real-time PCR of *ZMYM3* in iPSC clones B9 and H3. Primers were designed spanning exon 3 to 5 (Exon 3-5) and exon 22 to 23 (Exon 22-23). mRNA expression levels depict fold change to wildtype hiPSCs. **(C)** cDNA sequencing of *ZMYM3* exon 2 in wildtype hiPSCs and B9 clone. Sequencing is aligned to the wildtype sequence of *ZMYM3* and location of sgRNAs is depicted in pink arrows along the sequence. Deleted regions are highlighted in red. **(D)** Protein translation of wildtype *ZMYM3* sequence and B9 *ZMYM3* sequence based on the cDNA sequencing results. Protein translation and predicted protein transcripts were generated using Basic Local Alignment Search Tool (BLAST, website: https://blast.ncbi.nlm.nih.gov/Blast.cgi). Protein start sites are highlighted as 'Met' in red bold font, predicted protein sequence frames are highlighted in red. *WT = wildtype.*

For Subtask 2 under Major Goal 3 we are planning to determine whether *ZMYM3* mutations in NESCs induce a proliferative advantage *in vitro* and regulate DNA repair responses after introducing DNA double-strand breaks in cells. Subtask 2 of Major Goal 3 was planned to be accomplished in Year 1-2 (Months 10-16), but due to the delays in accomplishing Subtask 1 of Major Goal 3 we are planning to carry out Subtask 2 in Year 2 (Months 12-18). The updated plan will be discussed in detail in the "Changes and Problems" section of this report.

Major Goal 4: Determine if ZMYM3 frameshift mutations P48Lfs*65 and R1111fs*9 in $TP53^{wt}$ and $TP53^{mut}$ NESCs introduces tumor growth *in vivo*.

For Major Goal 4 of this proposal we are planning to determine the tumorigenic effect of *ZMYM3* mutations *in vivo* and perform DNA methylation and gene expression profiling analyses of obtained tumors. Major Goal 4 is planned to be accomplished in Year 2 (Months 12-24) of this proposal.

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

As the Principal Investigator of this proposal I have received individual training from both my mentors Dr. William Weiss and Dr. Bjoern Schwer in weekly one-on-one meetings, bi-monthly progress discussion meetings with Dr. Weiss and Dr. Schwer together, and I have presented and discussed this work in our group meetings in both laboratories every 3 months. During all those meetings the progress of the project was discussed in detail and I received mentorship and expertise advice for troubleshooting certain parts of this project. I have also presented and discussed this work with our collaboration partner for this project Dr. Michael D. Taylor and his laboratory and we have discussed the objectives and outcomes of the bioinformatical data analysis. This has increased my knowledge and critical thinking when it comes to data analysis and genomic analyses and will help me to become more skilled in this field.
Due to the COVID19 pandemic in the academic year 2020 and 2021 and Year 1 of this funding period it was not possible for me to attend professional meetings or research conferences to present my work and discuss my work with peers in my research field. This will be a goal for Year 2 of this funding period. Meanwhile to improve my skills in bioinformatics and data analysis of genomic datasets, such as RNA-seq and gene expression arrays, I have participated in a one-week virtual workshop for RNA-seq data analysis hosted by the Bioinformatics Core of the University of California Davis in June 2021. This has allowed me to analyze some of the RNA-seq data we have obtained in this proposal and become more skilled with our sequencing data analysis. I will be able to use this knowledge in Year 2 of our funding period to analyze any RNA-seq datasets we will generate from obtained tumors during this period.

How were the results disseminated to communities of interest?
Nothing to Report.

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

For the next and final reporting period of Year 2 (Months 13-24) of this proposal we will carry out the Major Tasks as stated in our initial SOW for Major Goal/Task 1-4.

Major Goal 1: Determine if SRC^{OE}/$ERBB4^{OE}$ signaling in human neuroepithelial stem cells causes G4-MB formation *in vivo* (85% completed).

For Major Goal 1 of this proposal, we wanted to test if forced expression of SRC and ERBB4 in neuroepithelial stem cells (NESC) drives tumor formation *in vivo* and will generate tumors with a Group 4 medulloblastoma (G4-MB) signature. This goal is almost completed expect for the gene expression profiling analysis of our Src-ErbB4 NESC tumors. We found that our Src^{OE} and SRC^{OE}-$ErbB4^{OE}$ tumors form its own cluster in our gene expression profiling analysis and cluster away from all four medulloblastoma subgroup. We will repeat the analysis with minor modifications to ensure that all our NESC tumors indeed form one tight clusters and are not distinct from each over by gene expression. We plan to accomplish these analyses by month 13-16 of this proposal.

Major Goal 2: Test whether targeted treatment with dasatinib in SRC^{OE}/$ERBB4^{OE}$ $TP53^{WT}$ and $TP53^{MUT}$ NES tumor-bearing mice prolongs survival.

We will test targeted treatment strategies by treating tumor-bearing mice with the novel SRC inhibitor dasatinib. This compound has been suggested to cross the blood-brain barrier in adults and children and is currently tested in several clinical trials for leukemia and two trials for pediatric brain cancer (NCT02015728, NCT02596828). Mice will be randomly enrolled into the placebo and dasatinib treatment arms (n=10 mice per treatment and NESC genotype) 14 days after NESC transplantation. According to the results of Major Goal 1 of this proposal we will enroll SRC^{OE} $TP53^{mut/wt}$ NESC and SRC^{OE}-$ERBB4^{OE}$ $TP53^{mut/wt}$ NESC tumor mice into the treatment study. Treatment arms will include Dasatinib (40 mg/kg BW) and vehicle treatment. Both will be given orally per oral gavage once a day (5 days ON, 2 days OFF). Mice will be treated until moribund and survival will be assessed across both treatment arms. We plan to accomplish the goals in month 13-18 of this proposal.

Major Goal 3: Determine if mutations in *ZMYM3* drive Group 4 medulloblastoma formation in human neuroepithelial stem cells *in vivo*.

For Major Goal 3 of this proposal we wanted to introduce the patient-derived ZMYM3 frameshift mutations P48Lfs*65 and R1111fs*9 into the genome of $TP53^{wt}$ and $TP53^{mut}$ $ZMYM3^{-/-}$ human iPSC-derived NESCs and study their tumorigenic potential *in vivo*.

For Subtask 1 of Major Goal 3 we planned to knock-out endogenous *ZMYM3* in human iPSCs by sgRNA-Cas9 gene-editing. Then differentiate $ZMYM3^{-/-}$ hiPSCs into NESCs and infect cells with lentiviruses overexpressing patient-derived *ZMYM3* mutations P48Ls*65 and R1111fs*9. As reported in the "Accomplishment" section above we were unable to knockout *ZMYM3* in our iPSCs and are making changes to our goals for Major Goal 3 and 4 of this proposal. In order to reach our goals in this proposal we decided to skip the generation of *ZMYM3* knockout iPSCs and directly overexpress patient-derived *ZMYM3* mutations in our wildtype NESCs via lentiviral infection and conclude with our planned downstream experiments. Additionally, instead of introducing the two patient-derived ZMYM3 mutations P48Ls*65 and R1111fs*9 in our cells, we will introduce the *ZMYM3* Q322* mutation that is as well found in Group 4 medulloblastoma. The Q3222* mutation introduces an early stop codon in the *ZMYM3* transcript leading to a loss of the functional domains of ZMYM3. Therefore the cell lines to be generated in this section are NES^{TP3WT}-$ZMYM3^{WT}$, NES^{TP3WT}-$ZMYM3^{Q322*}$, NES^{TP3MUT}-$ZMYM3^{WT}$ and NES^{TP3MUT}-$ZMYM3^{Q322*}$. We plan to accomplish this goal in month 13-15 of this proposal. More details on the changes will be discussed in the "Changes and Problems" section of this report.

For Subtask 2 under Major Goal 3 we will determine whether the Q322* *ZMYM3* mutation induces a proliferative advantage, *ZMYM3*^{Q322*};*TP53*$^{MUT/WT}$ NESCs and its controls will be analyzed with the CyQuant proliferation kit, for cell cycle progression with flow cytometry, and for invasiveness by using the Boyden chamber invasion assay. The role of *ZMYM3*^{WT} and *ZMYM3*^{Q322*} in DNA double-strand break (DSB) repair will be assessed by subjecting *ZMYM3*^{Q322*};*TP53*^{MUT} NESCs and its controls to DNA damage by gamma irradiation (10 Gy) or laser micro-irradiation (405nm). ZMYM3^{WT} and ZMYM3^{Q322*} recruitment to 53BP1 and γH2AX positive DSB sites at 5, 10 and 20 minutes after laser micro-irradiation will be assessed by confocal microscopy. We will also carry out co-immunoprecipitation experiments to determine the interaction of ZMYM3^{WT} and ZMYM3^{Q322*} with histone H2A and the DNA repair factors RAP80, ABRA and BRE after DSB induction. We plan to accomplish the goals in month 15-19 of this proposal.

Major Goal 4: Determine if ZMYM3 Q322* mutation in TP53^{WT} and TP53^{MUT} NESCs introduces tumor growth *in vivo*.

To determine tumorigenic effect of *ZMYM3* Q322* mutation *in vivo*, 300,000 *ZMYM3*^{Q322*} *TP53*$^{WT/MUT}$ NESCs and its controls will be transplanted into the cerebellum of 6-8-week-old immunocompromised NOD-scid IL2Rgamma mice, with a group size of n=10 per NESC genotype. Tumor development will be monitored for up to 9-12 months after transplantation and any tumors that develop will be dissected, and tumor DNA and RNA will be isolated. DNA samples will be subjected to DNA methylation analysis and RNA samples to gene expression analysis for tumor phenotyping and medulloblastoma subgrouping via comparison with patient-derived medulloblastomas, including *ZMYM3*^{mut} medulloblastoma cases. We plan to accomplish the goals in month 115-24 of this proposal.

IMPACT

What was the impact on the development of the principal discipline(s) of the project?
Nothing to Report.

What was the impact on other disciplines?
Nothing to report.

What was the impact on technology transfer?
Nothing to report.

What was the impact on society beyond science and technology?
Nothing to report.

CHANGES/PROBLEMS

Changes in approach and reasons for change

For Specific Aim 2 and Major Task 3 and 4 of this proposal we encountered problems with our approach in Year 1 and will make changes to the experimental design to reach our overall goals during Year 2 of this proposal. As a first step in Specific Aim 2 we wanted to introduce a knockout of *ZMYM3* in the genome of TP53^{wt} and TP53^{mut} human iPSCs via CRISPR sgRNA-Cas9 gene-editing technology.

As described in the "Accomplishment" section of this report we were not able to successfully introduce a knockout of ZMYM3 in our human iPSCs or iPSC-derived NESCs. CRISPR sgRNA efficiency in our NESCs is very low and NESCs cannot be grown as single cell clones. While CRISPR efficiency and selection processes work much better in the human iPSCs, we so far got only partly deleted *ZMYM3* in our iPSCs by the end of Year 1 of this proposal. To avoid further delays and achieve our goals for Specific Aim 2 we will use an alternative way to study *ZMYM3* G4-MB mutations in our NESCs. We will directly overexpress patient-derived *ZMYM3* mutations in our wildtype and P53-mutant NESCs via lentiviral infection and conclude with our planned downstream experiments. We find that endogenous *ZMYM3* in the NESCs is expressed at rather low levels and hypothesize that overexpressing mutant versions of *ZMYM3* in these cells will override the function of the endogenous wildtype *ZMYM3* and allow us to phenotypically study the impact of the *ZMYM3* mutants in medulloblastoma formation. Furthermore, to streamline our studies we will introduce the patient-derived *ZMYM3*^{Q322*} mutation via lentiviral infection in NESCs instead of the planned *ZMYM3*^{P48Lfs*65} and *ZMYM3*^{R1111fs*9} mutations we had planned. Introducing the *ZMYM3*^{Q322*} mutation introduces an early stop codon in the *ZMYM3* transcript and will allow us to study the deletion of the active domains of *ZMYM3* and disruption of the DNA binding domain (Fig. 6A-C). This change in experimental design will have no effects on the hypothesis, experimental approach and expected outcome of our study. For these changes the following cell lines will be generated and used for our approach in Major Task 3: WTC10-*ZMYM3*^{WT}, WTC10-*ZMYM3*^{Q322*}, H1-*ZMYM3*^{WT}, H1-*ZMYM3*^{Q322*}. And the following cell lines will be used for our approach in Major Task 4: H1-*ZMYM3*^{WT} and H1-*ZMYM3*^{Q322*}.

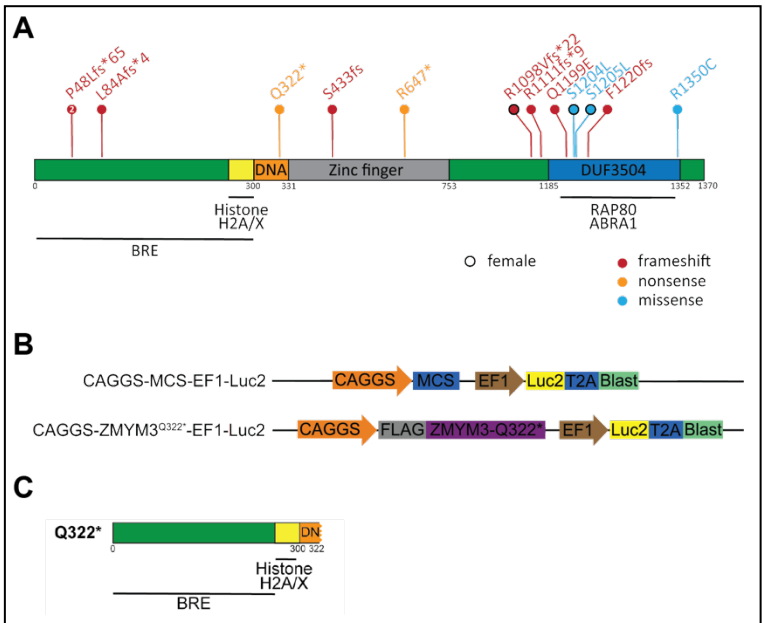

Figure 6. *ZMYM3* mutations and Q322* mutation in G4-MB patients. **(A)** Schematic representation of ZMYM3 and its active domains and mutations found in Group 4 medulloblastoma patients. **(B)** Plasmid constructs for empty vector (CAGGS-MCS-EF1-Luc2) and ZMYM3-Q322* mutation (CAGGS-ZMYM3^{Q322*}-EF1-Luc2) expression in NESCs. **(C)** ZMYM3^{Q322*} mutant transcript leading to an early stop codon in the DNA binding domain and truncated expression of *ZMYM3*.

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

Aim 1 – Determine if aberrant SRC/ERBB4 signaling in human NESCs causes G4-MB formation *in vivo*. For Major Task 2 of Aim 1 we could encounter that either Dasatinib as a SRC inhibitor shows no effect in our SRC-ERBB4 NESCs or has strong toxicity *in vivo* in mice during our treatment study. In the first case we will explore other SRC inhibitors or compounds targeting the EGFR/ERBB2/ERBB4 axis, such as Bosutinib, Tirbanibulin and Epertinib hydrochloride. If we encounter serious toxicity with our Dasatinib treatment regimen in mice we will consult with our veterinarians and animal caretakers on-site to explore ways to improve side effects and toxicity in mice and adjust our dosing and treatment schedule to a more tolerable treatment regimen.

Specific Aim 2 – Determine if mutations in *ZMYM3* drive G4-MB formation in human NESCs. Should we encounter further technical problems, we will work with our colleagues and take advantage of the many resources at UCSF to resolve them. We might find that *ZMYM3* mutations in $TP53^{mut}$ or $TP53^{wt}$ NESCs do not produce tumors at all in our model, or do not produce tumors of the G4-MB subgroup. This would suggest that we are either not targeting the right cell-of-origin for G4-MB or that *ZMYM3* mutations are not a G4-MB driver in the NES cell population. Importantly, such an outcome would not negate the proposed studies: we would nevertheless have produced significant insight into the role of *ZMYM3* mutations as drivers of G4-MB, which addresses an important gap in knowledge.

Changes that had significant impact on expenditures.
Nothing to Report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents.
Nothing to Report.

Significant changes in use or care of human subjects.
Nothing to Report.

Significant changes in use or care of vertebrate animals.
Nothing to Report.

Significant changes in use of biohazards and/or select agents.
Nothing to Report.

PRODUCTS

Publications, conference papers, and presentations

- **Journal publications**
  Nothing to Report.

- **Books or other non-periodical, one-time publications**
  Nothing to Report.

- **Other publications, conference papers, and presentations**
  Nothing to Report.

Website(s) or other Internet site(s)
Nothing to Report.

Technologies or techniques
Nothing to Report.

Inventions, patent applications, and/or licenses
Nothing to Report.

Other Products

- **Data or databases**
  Nothing to Report.

- **Biospecimen collections**
  Nothing to Report.

- **Audio or video products**
  Nothing to Report.

- **Software**
  Nothing to Report.

- **Models**
  Nothing to Report.

- **Educational aids or curricula**
  Nothing to Report.

- **Instruments or equipment**
  Nothing to Report.

- **Research material**
  - Cell lines that were developed in Year 1 of this proposal:
    - $SRC^{OE};TP53^{WT}$ NESCs
    - $ERBB4^{OE};TP53^{WT}$ NESCs
    - SRC^{OE}-$ERBB4^{OE};TP53^{WT}$ NESCs
    - EV-$TP53^{WT}$ NESCs (empty vector)
    - $SRC^{OE};TP53^{mut}$ NESCs
    - $ERBB4^{OE};TP53^{mut}$ NESCs
    - SRC^{OE}-$ERBB4^{OE};TP53^{mut}$ NESCs
    - EV-$TP53^{mut}$ NESCs (empty vector)

- **Clinical interventions**
  Nothing to Report.

- **New business creation**
  Nothing to Report.

- **Other**
  Nothing to Report.

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Christin Schmidt
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	0000-0002-2225-0583
Nearest person month worked:	12
Contribution to the Project:	Designed, performed and analyzed all the current experiments under this project.
Funding Support:	na

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?

Organization Name	Michael D. Taylor Lab Division of Neurosurgery The Hospital for Sick Children
Location of Organization	686 Bay str. 17.9713 Toronto, Canada
Partner's contribution to the project	Bioinformatic analysis of DNA methylation and gene expression of obtained tumors in this project.

SPECIAL REPORTING REQUIREMENTS
Nothing to Report.

APPENDICES
Nothing to Report.