ATR IS A NOVEL THERAPEUTIC TARGET FOR MEDULLOBLASTOMA
IDENTIFIED BY ITS ROLE IN CEREBELLAR DEVELOPMENT

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A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment for the degree of Doctor of Philosophy in the Department of Cell Biology and Physiology (Program in Cellular and Molecular Physiology) in the School of Medicine.

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ABSTRACT

Patrick Y. Lang: ATR is a novel therapeutic target for medulloblastoma identified by its role in cerebellar development
(Under the direction of Timothy R. Gershon)

Microcephaly and brain tumors can both arise as a consequence of dysregulated expansion of neural progenitor cells. Inadequate progenitor proliferation, premature cell cycle exit, and inappropriate cell death can all result in neurodevelopmental disorders characterized by microcephaly. In contrast, brain tumors may form in response to overactive progenitor proliferation, failed cell cycle exit, and/or escape from cell death. This dissertation focuses on the role of the DNA damage response protein ATR (Ataxia-telangiectasia and Rad3-related) in the survival of cerebellar granule neuron progenitors (CGNPs) and CGNP-derived medulloblastoma, a cerebellar tumor that is the most common brain cancer of childhood. Chapter 1 provides background information on medulloblastoma, cerebellar development, CGNP biology, the ATR-mutated microcephalic disorder Seckel syndrome, ATR’s mechanism of action, and the apoptotic pathway. Chapter 2 is written in the form of a review article on how insights from primary microcephalic disorders in general can inform the development of novel brain cancer therapeutics. Chapter 3 is a published article that explores the function of ATR in CGNPs during neonatal cerebellar development. Specifically, we show that Atr deletion in CGNPs leads to widespread, p53-mediated, BAX/BAK-dependent apoptosis in the early postnatal period due to the accumulation of severe chromosomal abnormalities. This chapter also provides initial data suggesting the therapeutic value of targeting ATR using a novel nanoparticle formulation of the small molecule ATR inhibitor VE-822, pVE-822, for treating medulloblastoma. Chapter 4 presents ongoing work in the
form of two research-in-brief articles in progress. The first section further investigates the possibility of treating CGNP-derived medulloblastoma with pVE-822, showing that pVE-822 has an anti-tumor effect in mice with established, spontaneous medulloblastoma. In the second section, data are presented demonstrating that in vivo inhibition of the mitotically-limited, microtubule-associated motor protein KIF11/EG5 potently arrests CGNPs in mitosis without producing DNA damage or apoptosis. Finally, Chapter 5 provides a discussion on how, if at all, ATR performs a special and unique function in CGNPs compared to other brain cells and whether ATR inhibition for the treatment of medulloblastoma should be further pursued.
To my uncle Yong Lang and my grandmother Xi Hua Li:
Gone but not forgotten.
ACKNOWLEDGMENTS

I would like to thank the UNC MD/PhD Program for their unfailing support over the course of my medical and graduate training. In particular, I thank Dr. Eugene P. Orringer: a charismatic friend and mentor who will always be remembered. Drs. David Siderovski and Kimryn Rathmell, who have since left UNC, were always incredibly supportive during their tenures as Director of Basic Research and Director of Clinical Research, respectively. I would further like to acknowledge the ongoing mentorship of the current Director of Basic Research, Dr. Mohanish Deshmukh, and the current Director of Clinical Research, Dr. Toni Darville. Additionally, Alison Regan and Carol Herion provide so much day-to-day background support such that my training would truly not be possible without them.

I thank the members of my graduate dissertation committee, Drs. Mohanish Deshmukh, Tim Gershon, Ian Davis, Ben Major, and Carol Otey, for their sage advice and patience over the years. My mentor and supervisor Dr. Gershon has been a paragon both inside and outside of the lab for the kind of man that I would one day like to become. My gratitude towards Dr. Gershon for his patronage these past four years is truly immense.

For Chapter 2, we thank the members of the Gershon lab for their assistance in reviewing and providing feedback on this manuscript.

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on the RNA-Seq data; Matthew Soloway (UNC Lineberger Comprehensive Cancer Center) for uploading RNA-Seq and microarray data to GEO; Jing Gao (UNC Eshelman School of Pharmacy) for technical assistance with formulating pVE-822; Eric Brown (University of Pennsylvania, Pennsylvania, PA, USA) for $Atr^{loxP/loxP}$ mice; David Rowitch (UCSF, San Francisco, CA, USA) and Robert Wechsler-Reya (Sanford-Burnham Medical Research Institute, La Jolla, CA, USA) for $Math1-Cre$ mice; and Eva Anton (UNC Neuroscience Center) for $hGFAP-Cre$ mice.

For Chapter 4, we thank the UNC Center for Gastrointestinal Biology and Disease Histology Core for processing tissue sections and staining for hematoxylin and eosin, the UNC Translational Pathology Laboratory for help in staining and digitizing brain sections and quantifying staining, and the UNC Flow Cytometry Core Facility for FACS assistance. We would like to thank Shi Yan (UNC Lineberger Comprehensive Cancer Center Genomics Core) for help in determining tumor cell RNA quality and Michael Vernon (UNC Functional Genomics Core) for providing valuable assistance in performing the microarray analysis. We thank Jing Gao (UNC Eshelman School of Pharmacy) for technical assistance with formulating pVE-822. We are grateful to Eric Brown (University of Pennsylvania, Pennsylvania, PA) for the $Atr^{loxP/loxP}$ mice, David Rowitch (UCSF, San Francisco, CA) and Robert Wechsler-Reya (Sanford-Burnham Medical Research Institute, La Jolla, CA) for the $Math1-Cre$ mice, and Eva Anton (UNC Neuroscience Center) for the $hGFAP-Cre$ mice.

Finally, I would like to acknowledge the vital support, camaraderie, and care provided by my friends and family during the course of my graduate training. I have been incredibly fortunate to meet so many people from all over the world as a part of my professional development, and they have all influenced my life in profound ways. Most of all, my father, Adam Lang, has been a steadfast friend and teacher, and I hope that my completion of this dissertation will make him proud.
PREFACE

I began my research career as a junior in high school, where I studied the etiology of Parkinson’s disease with Dr. Paul Carvey at Rush Medical University in Chicago, IL. My high school, the IL Mathematics and Science Academy, encouraged lab-based research and it was the influence of my grandfather, a neurosurgeon in Harbin, China, that caused me to seek out Dr. Carvey. After high school, I continued researching Parkinson’s for four years with Dr. Mark Stacy at Duke University. Incredibly, it took me six years to realize that I was neither interested in Parkinson’s disease nor in neuroscience. Just goes to show how much of what one does depends on to what one has been exposed. This thought prevailed in my mind throughout my undergraduate years, compelling me to gain exposure in several areas, including medical specialties like Radiology, Neurosurgery, Psychiatry, and Geriatrics, as well as basic science fields like Genetics, Developmental Biology, and Cancer Biology. I had passing interest in most of these topics, electing to try something else after one semester in each case, but the study of Developmental and Cancer Biology with Dr. Robert Wechsler-Reya held my captivation. It was ultimately my research with Dr. Wechsler-Reya that led me to join the lab of Dr. Timothy Gershon at UNC, where I continued my research on the interrelated processes of normal development and tumor formation. Before joining Dr. Gershon’s lab, I rotated with Dr. Mark Zylka, whose work is primarily on brain development, and with Dr. Channing Der, whose work is primarily on cancer pathogenesis and treatment. These rotations helped me realize that I was interested in the intersection of the development and cancer fields. As such, my graduate training with Dr. Gershon has been profoundly rewarding and has set me up for a career researching development and cancer.
TABLE OF CONTENTS

List of Tables ........................................................................................................... xv
List of Figures ......................................................................................................... xvi
List of Abbreviations and Symbols ........................................................................ xviii

Chapter 1: Introduction ........................................................................................... 1
  Overview ................................................................................................................ 1
  Normal cerebellar development and medulloblastoma ..................................... 2
    Medulloblastoma background ........................................................................ 2
  Normal cerebellar development .................................................................... 7
  Cerebellar granule neuron progenitors ..................................................... 10
  The Sonic hedgehog pathway ....................................................................... 13
  Seckel syndrome and ATR ............................................................................... 18
    The history of Seckel syndrome .............................................................. 18
    The genetics of Seckel syndrome ............................................................ 20
    Animal models of Seckel syndrome ....................................................... 23
    ATR kinase and the DNA damage response ........................................ 26
    ATM kinase and the consequences of replication fork collapse ........ 32
  Apoptosis ......................................................................................................... 34
  Tables ............................................................................................................... 39
  Figures ............................................................................................................. 41
  References ....................................................................................................... 43

Chapter 2: Familial microcephalic disorders suggest new approaches to brain tumor treatment ................................................................. 79
  Overview ......................................................................................................... 79
Introduction ................................................................................................... 80

*KIF11* is mutated in primary microcephaly and upregulated in glioma .... 81

*ASPM* is mutated in primary microcephaly and upregulated in glioma and medulloblastoma ............................................. 84

*CDK6* is mutated in primary microcephaly and upregulated in glioma and medulloblastoma ............................................. 87

*ATR* is mutated in Seckel syndrome and plays an important role in medulloblastoma tumorigenesis ............................................. 90

Discussion ...................................................................................................... 94

Competing Interests .................................................................................... 103

Author Contributions .................................................................................. 103

Funding ........................................................................................................ 103

Tables ........................................................................................................... 104

Figures ......................................................................................................... 107

References .................................................................................................... 109

Chapter 3: ATR maintains chromosomal integrity during postnatal cerebellar neurogenesis and is required for medulloblastoma formation ................................................................................. 129

Overview ...................................................................................................... 129

Introduction ................................................................................................. 130

Results ......................................................................................................... 131

*Atr* deletion induces CGNP apoptosis and cerebellar hypoplasia .... 131

Deletion of *Bax* and *Bak* prevents cell death in *Atr*-mutant CGNPs ................................................................................. 133

*Atr*- and *p53*-double-mutant CGNPs undergo caspase-independent cell death ............................................................................. 134

Accelerated cell cycle exit in *Atr;Bax;Bak^{M-cre}* CGNPs .................... 135

Cell cycle checkpoint failure in ATR-deficient CGNPs

with DNA damage ....................................................................................... 136
ATR protects CGNP chromosome integrity ......................................... 138

Transcriptomic adaptations to ATR deficiency are predominantly p53-driven ................................................................. 139

ATR is required for medulloblastoma tumorigenesis .................. 142

ATR inhibitor administered in vivo induces DNA damage specifically in CGNPs ................................................................. 143

Discussion .................................................................................................... 144

Materials and Methods ............................................................................... 147

Mice ..................................................................................................... 147

Immunostaining of cerebellar sections .............................................. 148

Quantification of immunostaining ..................................................... 148

CGNP isolation ................................................................................... 148

Flow cytometry ................................................................................... 149

Cytogenetic analysis ........................................................................... 149

Mutation analysis ............................................................................... 150

Western blot ........................................................................................ 150

Spectral karyotyping .......................................................................... 150

RNA-Seq and differential expression analysis .................................. 150

Pathway analysis ................................................................................ 151

pVE-822 in vivo administration ......................................................... 151

Competing Interests .................................................................................... 152

Author Contributions .................................................................................. 152

Funding ........................................................................................................ 152

Data Availability .......................................................................................... 152

Supplementary Materials and Methods ..................................................... 153

Measurement of cerebellar cellularity ............................................... 153

Survival analysis ................................................................................ 153
BrdU injection ................................................................. 153
Microarray validation ....................................................... 153
Mutation analysis ................................................................. 154
Western blot analysis ........................................................... 154
pVE-822 formulation .......................................................... 154
Antibodies for IHC/IF ............................................................ 156

Tables ........................................................................... 157
Figures ............................................................................ 160
References ........................................................................ 182

Chapter 4: Ongoing work .................................................. 189

ATR inhibition by pVE-822 for the treatment of medulloblastoma .......... 189

Introduction ..................................................................... 189

Results ........................................................................... 192

ATR inhibition by pVE-822 induces DNA damage 
and tumor regression in mice with medulloblastoma .... 192

Acute ATR inhibition by pVE-822 does not result 
in cell cycle changes in medulloblastoma tumor cells .... 194

No transcriptional changes are seen in Atr-deleted 
medulloblastoma mice ..................................................... 195

VE-822 has a combined antiproliferative effect with 
Vismodegib or Etoposide on CGNPs ............................... 196

Discussion ...................................................................... 198

Materials and Methods .................................................... 204

Mice ............................................................................ 204
Genotyping ........................................................................ 204
pVE-822 formulation and in vivo administration ..................... 206
Immunostaining brain sections ......................................... 206
Quantification of immunostaining ...................................... 206
The role of ATR in the developing cerebellum ............................................. 249

The proliferative nature of CGNPs could explain
  cerebellar dependence on ATR ............................................................. 249

ATR may function in a unique manner in CGNPs ..................................... 253

ATR inhibition as treatment for medulloblastoma .................................... 264

ATR inhibitors with good in vivo activity and
  their uses in cancer treatment .............................................................. 265

ATR inhibition for the treatment of TP53-
  mutated medulloblastoma ................................................................. 274

References .................................................................................................... 281

Endnotes ........................................................................................................ 297
LIST OF TABLES

Table 1.1 - Many secreted factors can positively or negatively regulate SHH-mediated CGNP proliferation .............................................. 39

Table 1.2 - Seckel syndrome is a genetically heterogeneous disease defined by possible mutations in several genes ......................... 40

Table 2.1 - Several KIF11/EG5 inhibitors are in active clinical trials for a diverse range of non-primary brain cancers ......................... 104

Table 2.2 - Three CDK6 inhibitors are in active clinical trials for CNS cancers .... 105

Table 2.3 - Two ATR inhibitors are in active clinical trials, but not for primary brain malignancies ..................................................... 105

Table 2.4 - Genes mutated in microcephalic disorders code for products that may be attractive targets for brain cancer therapies .... 106

Table 3.1 - Pathway enrichment analysis on significantly differentially expressed genes between CGNPs from \( P3 Atr;Bax;Bak^{M-cre} \) and \( Bax;Bak^{M-cre} \) cerebella ................................................................. 157

Table 3.S1 - Differential gene expression of \( P3 Atr;Bax;Bak^{M-cre} \) vs. \( Bax;Bak^{M-cre} \) : Partial list .......................................................... 158

Table 3.S2 - Differential gene expression of \( P3 Atr;p53^{M-cre} \) vs. \( p53^{M-cre} \) : Full list ............................................................................. 159

Table 3.S3 - Pathway enrichment analysis on significantly differentially expressed genes between CGNPs from \( P3 Atr;p53^{M-cre} \) and \( p53^{M-cre} \) cerebella ...................................................... 159

Table 4.S1 - In CGNPs, VE-822 combines with Vismodegib or Etoposide to produce an enhanced anti-proliferative effect, but combines with Etoposide, SB-743921, or XRT to decrease DNA damage and apoptosis ......................................................... 222

Table 4.S2 - Mitotic arrest of CGNPs increases over time following \textit{in vivo} administration of SB-743921, accompanied by increased p4EBP1 ............ 222
LIST OF FIGURES

Figure 1.1 - The cerebellum and how it develops ............................................................. 41

Figure 2.1 - Microcephalic disorders are characterized by disruptions in mitotic regulators, which could be targeted for brain cancer therapy ...... 107

Figure 3.1 - Atr deletion in CGNPs induces postnatal DNA damage, p53 activation, apoptosis, and cerebellar hypoplasia ..................... 160

Figure 3.2 - Co-deletion of Bax and Bak, but not p53, blocks cell death in ATR-deficient CGNPs .......................................................... 162

Figure 3.3 - Increased DNA damage and inappropriate mitotic entry in Atr-deleted CGNPs ................................................................. 164

Figure 3.4 - Atr deletion compromises the chromosome integrity of proliferating CGNPs ................................................................. 166

Figure 3.5 - The p53 pathway controls the CGNP transcriptomic response to Atr deletion ................................................................. 167

Figure 3.6 - Atr deletion blocks medulloblastoma tumorigenesis, and acute, in vivo ATR inhibition induces DNA damage and apoptosis in the postnatal cerebellum .................................................. 169

Figure 3.7 - ATR is required to maintain G2/M checkpoint integrity and prevent apoptosis induced by proliferation-associated DNA damage ...... 171

Figure 3.S1 - Normal proliferation in Atr-deleted CGNPs during early postnatal development despite later reduced cerebellar cellularity and hypoplasia ................................................................. 173

Figure 3.S2 - Deletion of Bax and Bak alone fails to rescue the ATR cerebellar phenotype ................................................................. 175

Figure 3.S3 - p53 deletion in ATR-deficient CGNPs is associated with DNA damage accumulation but not change in proliferation .......... 176

Figure 3.S4 - Atr deletion increases the fraction of CGNPs with DNA damage, especially in M-phase .................................................. 178

Figure 3.S5 - Atr deletion in CGNPs is not associated with a specific mutational pattern ................................................................. 180
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.S6</td>
<td>In vitro characterization of pVE-822</td>
<td>181</td>
</tr>
<tr>
<td>4.1</td>
<td>ATR inhibition by pVE-822 induces medulloblastoma tumor shrinkage</td>
<td>223</td>
</tr>
<tr>
<td>4.2</td>
<td>SB-743921 induces transient cell cycle arrest in prometaphase in CGNPs</td>
<td>225</td>
</tr>
<tr>
<td>4.S1</td>
<td>ATR inhibition by pVE-822 produces no survival benefit in mice with medulloblastoma</td>
<td>227</td>
</tr>
<tr>
<td>4.S2</td>
<td>pVE-822 treatment does not alter tumor cell cycle dynamics in mice with medulloblastoma</td>
<td>229</td>
</tr>
<tr>
<td>4.S3</td>
<td>VE-822 combined with Vismodegib or Etoposide enhances reduction of CGNP proliferation</td>
<td>231</td>
</tr>
<tr>
<td>4.S4</td>
<td>SB-743921 does not induce DNA damage or apoptosis in CGNPs</td>
<td>233</td>
</tr>
<tr>
<td>Abbreviation</td>
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<tr>
<td>°C</td>
<td>Degree(s) Celsius or Centigrade</td>
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</tr>
<tr>
<td>%</td>
<td>Percent</td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>Knockout (as part of gene)</td>
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<tr>
<td>μg</td>
<td>Microgram</td>
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</tr>
<tr>
<td>μm</td>
<td>Micrometer</td>
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<td>Number</td>
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<td>γH2A.X</td>
<td>Phosphorylated histone H2A, member X</td>
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<td>+</td>
<td>Positive or wild-type (as part of gene)</td>
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<td>A549</td>
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<td>AIF</td>
<td>Apoptosis-inducing factor</td>
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<td>ALL</td>
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<tr>
<td>AKT</td>
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<td>AML</td>
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<td>ATG5 or Atg5</td>
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<td>ATM or Atm</td>
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<td>Atoh1</td>
<td>Atonal homolog 1</td>
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ATR or Atr: Ataxia-telangiectasia and Rad3-related

Atr;Bak\textsuperscript{M-cre}: Math1-Cre;Atr\textsuperscript{loxP/loxP};Bak\textsuperscript{-/-}

Atr;Bax\textsuperscript{M-cre}: Math1-Cre;Atr\textsuperscript{loxP/loxP};Bax\textsuperscript{loxP/loxP}

Atr;Bax;Bak\textsuperscript{M-cre}: Math1-Cre;Atr\textsuperscript{loxP/loxP};Bax\textsuperscript{loxP/loxP};Bak\textsuperscript{-/-}

Atr\textsuperscript{E-cre}: Emx1-Cre;Atr\textsuperscript{loxP/loxP}

Atr\textsuperscript{G-cre}: hGFAP-Cre;Atr\textsuperscript{loxP/loxP}

ATRIP: ATR-interacting protein

Atr\textsuperscript{M-cre}: Math1-Cre;Atr\textsuperscript{loxP/loxP}

Atr\textsuperscript{N-cre}: Nestin-Cre;Atr\textsuperscript{loxP/loxP}

Atr;\textsuperscript{p53}M-cre: Math1-Cre;Atr\textsuperscript{loxP/loxP};p53\textsuperscript{loxP/loxP}

A.V.K.: Alexander V. Kabanov

AXIN2: Axis inhibition protein 2

B: Bursa of Fabricius

BAD: BCL2-associated death promoter

BAK or Bak: BCL2 antagonist/killer

BAK1 or Bak1: BCL2 antagonist/killer 1

BAX or Bax: BCL2-associated X

Bax;Bak\textsuperscript{M-cre}: Math1-Cre;Bax\textsuperscript{loxP/loxP};Bak\textsuperscript{-/-}

BBC3: BCL2-binding component 3

BCAS2: Breast carcinoma-amplified sequence 2

BCL2: B-cell lymphoma 2

BCL2L1: BCL2-like 1

BCL2L11: BCL2-like 11

BD: Becton, Dickinson, and Company

bFGF: Basic fibroblast growth factor

bHLH: Basic helix-loop-helix

BID: BH3-interacting domain death agonist
<table>
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<th>Full Form</th>
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<tr>
<td>BIM</td>
<td>BCL2-interacting mediator of cell death</td>
</tr>
<tr>
<td>BLM</td>
<td>Bloom syndrome RecQ-like helicase</td>
</tr>
<tr>
<td>BM</td>
<td>Biocare Medical</td>
</tr>
<tr>
<td>BMP2</td>
<td>Bone morphogenic protein 2</td>
</tr>
<tr>
<td>BMP4</td>
<td>Bone morphogenic protein 4</td>
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<td>BMP7</td>
<td>Bone morphogenic protein 7</td>
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<td>BRCA1</td>
<td>Breast cancer 1</td>
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<td>Breast cancer 2</td>
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<tr>
<td>BRCT</td>
<td>BRCA1 C-terminus</td>
</tr>
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<td>Bromo-deoxy-uridine</td>
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<td>c-ABL</td>
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<tr>
<td>CAD</td>
<td>Caspase-activated DNase</td>
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<td>Cyclic adenosine monophosphate</td>
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<td>CREB-binding protein</td>
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<td>cC7</td>
<td>Cleaved Caspase-7</td>
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<tr>
<td>CCD</td>
<td>Charge-coupled device</td>
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<td>CCG</td>
<td>Children’s Cancer Group</td>
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<td>CCNU</td>
<td>1-(2-chloroethyl)-3-cyclohexyl-1-nitrosurea</td>
</tr>
<tr>
<td>CD2</td>
<td>Cyclin D2</td>
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<td>Cluster of differentiation 95</td>
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<td>CDK5</td>
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INK4A  Inhibitor of CDK4 A (p16)
INK4B  Inhibitor of CDK4 B (p15)
INK4C  Inhibitor of CDK4 C (p18)
INK4D  Inhibitor of CDK4 D (p19)
IP     Intraperitoneal
IR     Ionizing radiation
IV     Intravenous
Jag1   Jagged 1
J.S.P.  Joel S. Parker
k      Thousand
KCl    Potassium chloride
KEGG   Kyoto encyclopedia of genes and genomes
kg     Kilogram(s)
Kif7   Kinesin family member 7
KIF11 or Kif11  Kinesin family member 11
KIP1   Kinesin-like protein 1 (p27)
KIP2   Kinesin-like protein 2 (p57)
KO     Knockout
KRAS   Kirsten rat sarcoma viral oncogene homolog
KSP    Kinesin spindle protein
L      Liter(s)
L1CAM  L1 cell adhesion molecule
LC     Loading capacity
LE     Loading efficiency
LoVo   Human colorectal adenocarcinoma cells
loxP   Locus of X(cross)-over in P1
M      Mitotic phase

xxvii
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mM Millimolar
mm Millimeter
MMS Methyl methanesulfonate
MN Minnesota
MNAT1 Ménage à trois homolog 1
MO Missouri
MOPDII Microcephalic osteodysplastic primordial dwarfism type II
MRE11 Meiotic recombination 11 homology
MRI Magnetic resonance imaging
MRN MRE11, RAD50, and NBS1
mRNA Messenger RNA
M-SmoM2 Math1-Cre;SmoM2
M.S.-P. Marina Sokolsky-Papkov
mTOR Mammalian target of Rapamycin
MutL Mutator L
MutS Mutator S
MYC or Myc V-Myc avian myelocytomatosis viral oncogene homolog
MYCN or MycN MYC neuroblastoma-derived
MYOD1 Myogenic differentiation 1
n Number
NBN Nibrin (p95)
NBS Nijmegen breakage syndrome
NBS1 Nijmegen breakage syndrome 1 (p95)
NC North Carolina
NCI National Cancer Institute
Neg. Negative
NES or Nes Nestin

xxix
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xxx
p4EBP1  Phosphorylated 4EBP1
p53    Tumor protein p53
p-53BP1 Phosphorylated 53BP1
p53<sup>M-cre</sup>  Math1-Cre; p53<sup>loxP/loxP</sup>
PA     Pennsylvania
PACAP  Pituitary adenylate cyclase-activating polypeptide
PARP   Poly(ADP-ribose)polymerase
p-ATM  Phosphorylated ATM
Pax6   Paired box 6
PC1    First principal component
PC2    Second principal component
PC3    Human prostate adenocarcinoma cells
PCA    Principal component analysis
PCL    Purkinje cell layer
PCNA   Proliferating cell nuclear antigen
PCR    Polymerase chain reaction
PCTN   Pericentrin
PDI    Polydisperity index
PDS    Papain dissociation system
PEG    Poly-ethylene glycol
PGP    P-glycoprotein
pH3    Phosphorylated histone H3
PI     Isoelectric point
PI3K   Phosphoinositide 3-kinase
PIAS3  Protein inhibitor of activated STAT3
PIN1   Peptidylpropyl cis/trans isomerase, NIMA-interacting 1
PKA    Protein kinase A
PlCL  Plasma cell leukemia
PLK1  Polo-like kinase 1
PLL  Prolymphocytic leukemia
PMAIP1  Phorbol-12-myristate-13-acetate-induced protein 1
PMC  PubMed Central
p-MLKL  Phosphorylated MLKL
PNET  Primitive neuroectodermal tumor
POG  Personalized onco-genomics
Pos.  Positive
POT1  Protection of telomeres 1
POx  Poly(Methyl-Butyl-Methyl) oxazoline
PP2A  Protein phosphatase 2A
PP5  Protein phosphatase 5
p-p53  Phosphorylated p53
PPM1D  Protein phosphatase 1D
PRPF19  Pre-mRNA processing factor 19
PSN-1  Human pancreatic ductal adenocarcinoma cells
Ptc  Patched
PTCH1  Patched 1
PTCH2  Patched 2
PTEN  Phosphatase and tensin homolog
PUMA  p53-upregulated modulator of apoptosis
pVE  pVE-822
pVE-822  Polymeric micelle-encapsulated VE-822
P.Y.L.  Patrick Yunlong Lang
Q12x3  Once every 12 hr, 3 times
QD  Once daily

xxxii
QDx5 Once daily, 5 times
RB or Rb Retinoblastoma
RBBP8 Retinoblastoma-binding protein 8
RBFOX3 RNA-binding protein, FOX1 homolog 3
RHNO1 9-1-1-interacting nuclear orphan 1
RIPK1 Receptor-interacting Ser/Thr kinase 1
RIPK3 Receptor-interacting Ser/Thr kinase 3
RMA Robust multi-array average
RNA Ribonucleic acid
RNA-Seq RNA sequencing
ROCK1 Rho-associated, coiled-coil-containing protein kinase 1
RPA Replication protein A
RPM Revolutions per minute
RT Read-through
RT-PCR Reverse transcription PCR
S Synthesis phase
s Second(s)
SAS6 Spindle assembly abnormal protein 6 homolog
SASS6 SAS6 centriolar assembly protein
SB SB-743921
SCAN SRE-ZBP, CTfin51, AW-1, and Number 18 cDNA
SCLC Small cell lung cancer
SDF-1α Stromal cell-derived factor 1 alpha
SEM or s.e.m. Standard error of the mean
Ser Serine
SHH Sonic hedgehog
SIAH2 Seven in absentia homolog 1
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>siRNA</td>
<td>Small or short interfering RNA</td>
</tr>
<tr>
<td>SIRT1</td>
<td>Silent mating-type information regulation 2 homolog 1</td>
</tr>
<tr>
<td>SK-BR-3</td>
<td>Human breast adenocarcinoma cells</td>
</tr>
<tr>
<td>SKY</td>
<td>Spectral karyotyping</td>
</tr>
<tr>
<td>SMAC</td>
<td>Second mitochondria-derived activator of caspase</td>
</tr>
<tr>
<td>SMAD</td>
<td>Mothers against decapentaplegic</td>
</tr>
<tr>
<td>SMARCAL1</td>
<td>SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily A-like 1</td>
</tr>
<tr>
<td>SMC1A</td>
<td>Structural maintenance of chromosomes 1A</td>
</tr>
<tr>
<td>SMC3</td>
<td>Structural maintenance of chromosomes 3</td>
</tr>
<tr>
<td>SMO or Smo</td>
<td>Smoothened</td>
</tr>
<tr>
<td>SmoA1</td>
<td>Smoothened mutant A1</td>
</tr>
<tr>
<td>SmoM2</td>
<td>Smoothened mutant M2</td>
</tr>
<tr>
<td>SmoM2(^{G-cre})</td>
<td>hGFAP-Cre;SmoM2(^{loxP/loxP})</td>
</tr>
<tr>
<td>SmoM2(^{M-cre})</td>
<td>Math1-Cre;SmoM2(^{loxP/loxP})</td>
</tr>
<tr>
<td>SmoM2(^{G-cre})</td>
<td>hGFAP-Cre;SmoM2</td>
</tr>
<tr>
<td>SN</td>
<td>SN-38</td>
</tr>
<tr>
<td>SNF</td>
<td>Sucrose-non-fermentable</td>
</tr>
<tr>
<td>SNIP1</td>
<td>SMAD nuclear-interacting protein 1</td>
</tr>
<tr>
<td>S. pombe</td>
<td>Schizosaccharomyces pombe</td>
</tr>
<tr>
<td>SPSS</td>
<td>Statistical package for the social sciences</td>
</tr>
<tr>
<td>SRC</td>
<td>Rous sarcoma</td>
</tr>
<tr>
<td>SRE</td>
<td>Serum response factor</td>
</tr>
<tr>
<td>SS</td>
<td>Seckel syndrome</td>
</tr>
<tr>
<td>SSB</td>
<td>Single-stranded DNA break</td>
</tr>
<tr>
<td>SSC</td>
<td>Saline-sodium citrate</td>
</tr>
<tr>
<td>ssDNA</td>
<td>Single-stranded DNA</td>
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</tbody>
</table>
STAT3  Signal transducer and activator of transcription 3
SUFU   Suppressor of fused
SUMO   Small, ubiquitin-like modifier
SUMO1  Small, ubiquitin-like modifier 1
SVZ    Subventricular zone
SWI    Switch
T      Thymus (cell) or Thymidine (nucleic acid sequence)
t     Translocation
Taq    Thermus aquaticus
tBID   Truncated BID
TCF3   Transcription factor 3
TFS    ThermoFisher Scientific
Thr    Threonine
TIM    Timeless circadian clock
TIPIN  Timeless-interacting protein
TNF-α  Tumor necrosis factor alpha
TNFR   Tumor necrosis factor receptor
TNFR1  Tumor necrosis factor receptor 1
TOPBP1 DNA topoisomerase 2-binding protein 1
TP53   Tumor protein p53
TP53BP1 p53-binding protein 1
TRADD  TNFR1-associated Death domain protein
TRAIP  TNFR-associated factor-interacting protein
TREX1  Three-prime repair exonuclease 1
T.R.G.  Timothy Robin Gershon
TRO    Trophinin
TUJ1   Neuron-specific class III beta-tubulin

xxxv
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase dUTP Nick-End labeling</td>
</tr>
<tr>
<td>U2OS</td>
<td>Human osteosarcoma cells</td>
</tr>
<tr>
<td>UCSF</td>
<td>University of California at San Francisco</td>
</tr>
<tr>
<td>UK</td>
<td>United Kingdom</td>
</tr>
<tr>
<td>Ulk3</td>
<td>Unc51-like kinase 3</td>
</tr>
<tr>
<td>UNC</td>
<td>University of North Carolina at Chapel Hill</td>
</tr>
<tr>
<td>Unc51</td>
<td>Uncoordinated 51</td>
</tr>
<tr>
<td>UPF1</td>
<td>Up-frameshift suppressor 1 homolog</td>
</tr>
<tr>
<td>US</td>
<td>United States</td>
</tr>
<tr>
<td>USA</td>
<td>United States of America</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>V</td>
<td>Volt(s)</td>
</tr>
<tr>
<td>VCP</td>
<td>Vincristine, CCNU (Lomustine), and Prednisone</td>
</tr>
<tr>
<td>V(D)J</td>
<td>Variable, diversity, and joining</td>
</tr>
<tr>
<td>VE</td>
<td>VE-822</td>
</tr>
<tr>
<td>Veh</td>
<td>Vehicle</td>
</tr>
<tr>
<td>Vis</td>
<td>Vismodegib</td>
</tr>
<tr>
<td>VTN</td>
<td>Vitronectin</td>
</tr>
<tr>
<td>VZ</td>
<td>Ventricular zone</td>
</tr>
<tr>
<td>WDR62 or Wdr62</td>
<td>WD repeat domain 62</td>
</tr>
<tr>
<td>WIP1</td>
<td>Wild-type p53-induced phosphatase 1</td>
</tr>
<tr>
<td>WNT or Wnt</td>
<td>Wingless-related integration site</td>
</tr>
<tr>
<td>WRN</td>
<td>Werner syndrome RecQ-like helicase</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
</tr>
<tr>
<td>WWOX</td>
<td>WW domain-containing oxidoreductase</td>
</tr>
<tr>
<td>XIAP</td>
<td>X-linked IAP</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>XPA</td>
<td>Xeroderma pigmentosum group A</td>
</tr>
<tr>
<td>XPD</td>
<td>Xeroderma pigmentosum group D</td>
</tr>
<tr>
<td>XRCC1</td>
<td>X-ray cross-complementing group 1</td>
</tr>
<tr>
<td>XRCC3</td>
<td>X-ray cross-complementing group 3</td>
</tr>
<tr>
<td>XRT</td>
<td>X-ray therapy</td>
</tr>
<tr>
<td>ZBP</td>
<td>Zona pellucida B protein</td>
</tr>
<tr>
<td>ZIC or Zic</td>
<td>Zinc finger protein</td>
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<tr>
<td>Zic1</td>
<td>Zinc finger protein 1</td>
</tr>
<tr>
<td>Zic2</td>
<td>Zinc finger protein 2</td>
</tr>
<tr>
<td>Zic3</td>
<td>Zinc finger protein 3</td>
</tr>
<tr>
<td>ZSCAN21 or Zscan21</td>
<td>Zinc finger and SCAN domain-containing 21</td>
</tr>
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CHAPTER 1: INTRODUCTION

Overview

Cerebellar hypoplasia and medulloblastoma are severe childhood neurological disorders that both result from failed regulation of progenitor proliferation ([Wechsler-Reya and Scott, 1999; Garel et al., 2011; Chizhikov et al., 2007]). Hypoplasia of the cerebellum is seen in Seckel syndrome, which is caused by mutation of the gene coding for the DNA damage response protein ATR (Ataxia-telangiectasia and Rad3-related) ([O'Driscoll et al., 2003]). Deficiency of ATR throughout the brain specifically disrupts the growth of cerebellar progenitors, leading to cerebellar hypoplasia ([Lee et al., 2012; Lang et al., 2016]), although the mechanism for this pathogenesis was not well understood until recently ([Lang et al., 2016]). Cerebellar progenitors are also uniquely vulnerable to transformation ([Yang et al., 2008; Dey et al., 2012; Mao et al., 2006]), giving rise to medulloblastoma, a tumor of cerebellar origin that is the most common pediatric brain cancer ([CBTRUS, 2010; Rutka, 1997; McNeil et al., 2002; Smoll and Drummond, 2012]). Seckel syndrome and medulloblastoma both involve genomic instability from accumulated DNA damage ([Fernandez et al., 2012; Kalay et al., 2011; Kool et al., 2014]), highlighting the potential for DNA damage to disrupt cerebellar growth. Importantly, medulloblastoma remains profoundly sensitive to DNA damage, as radiation and chemotherapy are highly effective against this tumor ([Bartlett et al., 2013; Packer et al., 1991]). These non-specific therapies, however, are associated with significant morbidity in young patients, and not all patients are responsive ([Schroeder and Gururangan, 2014; Miralbell et al., 1997; Ris et al., 2001]). One of the goals of my dissertation was to determine the precise function of ATR in cerebellar
granule neuron progenitors (CGNPs) and elucidate how ATR disruption leads to cerebellar hypoplasia, providing knowledge of Seckel syndrome pathogenesis. I further predicted that medulloblastoma cells, like cerebellar progenitors, depend on ATR for growth and that ATR would therefore be an effective therapeutic target in medulloblastoma that could produce improved efficacy with reduced toxicity. To appreciate the scope of these aims and the results that have followed requires a foundational understanding of medulloblastoma, normal cerebellar development, Seckel syndrome, and ATR function.

Medulloblastoma and normal cerebellar development

Medulloblastoma background

Medulloblastoma (MB) is a malignant pediatric brain tumor of the cerebellum (Rorke, 1983; Parsons et al., 2011). It was initially described as a glioma – a tumor derived from cells of the glial lineages – until Bailey and Cushing provided evidence in 1925 for its non-glial cell origins (Bailey and Cushing, 1925). They observed that MB predominantly presented in the cerebellum and frequently extended into the fourth ventricle, causing blockage of cerebrospinal fluid (CSF) flow, resulting in hydrocephalus. In addition, most cases of MB were found to occur in pediatric patients – that is, in individuals younger than 20.

Since Bailey and Cushing's seminal study, MB has been alternately designated as an infratentorial primitive neuroectodermal tumor (PNET) (Hart and Earle, 1973; Rorke, 1983) and as a tumor distinct from PNETs (Kleihues et al., 1993; McManamy et al., 2007; Rubinstein, 1985). The controversy as to this cancer’s classification was a result of limited knowledge regarding its cell of origin. Whereas PNETs derive from pluripotent neural crest cells, MBs can arise from mutation in more committed progenitor cells (Wechsler-Reya and Scott, 1999; Yang et al., 2008; Molenaar and
Regardless of its designation, MB is an invasive tumor that represents a serious disease burden. In 2010, the cost of care for MB in the United States (US) was estimated at $40 million (NCI, 2010). Between 2004 and 2007, MB comprised 1% of primary brain and central nervous system (CNS) tumors among all age groups and nearly 20% of newly diagnosed cases of CNS tumors in the pediatric population (CBTRUS, 2010; Crawford et al., 2007). Approximately 13% of children between 0 - 14-years-old and 3.9% of teens between 15 - 19 were living with MB during those years. Generally, this cancer is seen more in boys than in girls (2:1), although girls tend to have a better prognosis (Bloom et al., 1969). As a rare disease, MB has a prevalence of less than 200,000 affected individuals and an incidence of approximately 2 per 100,000 people, or 350 new cases each year in the US (CBTRUS, 2010). Despite this low prevalence, diagnosis of MB has been facilitated by decades of research.

Clinically, MB presents as a cerebellar mass – frequently localized initially to the surface of the cerebellum – that impinges upon the fourth ventricle (Bailey and Cushing, 1925). Hydrocephalus causes the majority of outwardly manifest symptoms in patients, including headache, lethargy, vomiting, and gait imbalance (MacDonald et al., 2003). Diagnosis is confirmed by magnetic resonance imaging (MRI), computerized tomography (CT) scan, and histology on tumor biopsy (Chang et al., 1969; Tomlinson et al., 1992a,b). Histological examination of human tumor samples have revealed at least three subtypes of MB: classic, desmoplastic/nodular, and large-cell anaplastic (Gilbertson and Ellison, 2008). Classic MB cells tend to be small, round-to-oval, and contain little cytoplasm (Kleihues et al., 1993; Katsetos et al., 1988). Most cells appear undifferentiated on the basis of lineage marker analysis, although some may express markers of neurons and glia. In addition, approximately 40% of classic MB samples are characterized by Homer-Wright rosettes – circular or spherical groupings of tumor cells organized around a central
cavity of neurofibrils. Classic MB comprises the majority (approximately 50%) of diagnosed MB cases and is associated with a medium outcome and prognosis.

Desmoplastic, or nodular, MB is distinguished by regions of densely packed, undifferentiated cells with extensive reticulin fibers surrounding islands of more differentiated cells (Katsetos et al., 1988; Leary et al., 2011). This subtype represents approximately 25% of all MB cases and is associated with a better prognosis than the classic subtype. In contrast, the large-cell anaplastic variant is thought to correlate with low survival and the worst overall prognosis of all three MB subtypes (Eberhart et al., 2002a; Leonard et al., 2001; von Hoff et al., 2010). These tumors are noted for their invasiveness, which is a consequence of highly proliferative cells. Compared to classic MB cells, those in the large-cell anaplastic subtype tend to be 2-3 times larger, dedifferentiated (reversion of cells to less specialized form), and in densely packed clusters. While histology has allowed for the categorization of MB into distinct variants, correlating these subtypes with genetic and molecular mechanisms is not always straightforward.

Risk for MB is associated with a number of genetic factors. Losses in chromosomes 6q, 16q, 10q, and, most frequently, 17p have been seen in many tumor samples (Rasheed and Bigner, 1991). Notably, the human p53 (TP53, tumor protein p53) tumor suppressor gene lies within chromosome 17p (chromosome 11 in mouse) and the PTEN (Phosphate and tensin homolog) tumor suppressor gene lies within 10q; however, complete loss of these genes have been absent in nearly all cases of MB. On the other hand, mutations in known genes, such as MYC (V-Myc avian myelocytomatosis viral oncogene homolog), MYCN (MYC neuroblastoma-derived), TP53, Sonic hedgehog (SHH) pathway members (PTCH1/2 (Patched 1/2), SMO (Smootherned), and SUFU (Suppressor of fused)), and WNT (Wingless-related integration site) pathway members (CTNNB1 (Catenin-beta 1), GSK3B (Glycogen synthase kinase 3 beta), AXIN2 (Axis inhibition protein 2), and APC(Adenomatous
polyposis coli), are found in many MBs (Kool et al., 2012; Northcott et al., 2012; Taylor et al., 2012). Indeed, the expression pattern of certain genes is perhaps linked to histological MB subtypes and therefore associated with disease prognosis. For instance, loss of wild-type $Ptc$ in conjunction with $MycN$ overexpression in CGNPs is able to induce the formation of tumors in mice that resemble the desmoplastic subtype of MB (Kessler et al., 2009). Constitutive Smo activation (SmoM2) in CGNPs in mice results in classic-like medulloblastoma (Mao et al., 2006). As another example, disruption of $p53$ acting together with $Myc$ overexpression in cerebellar stem cells may be causative in large-cell anaplastic MB (Pei et al., 2012).

Despite this wide array of chromosomal aberrations and genetic mutations, few risk factors are actually inherited. However, certain heritable conditions have been implicated as increasing the risk for acquiring MB (Taylor et al., 2000). Predisposition for MB as a consequence of these other diseases is thought to be a result of mutations in similar pathways. For instance, patients with inherited Gorlin syndrome (also known as basal cell nevus syndrome or nevoid basal cell carcinoma syndrome) have on average a 3 - 5% higher chance for acquiring MB than people in the general population (Evans et al., 1991b,a). Gorlin usually develops as a result of mutations in $PTCH1$, which encodes a SHH pathway antagonist (Bale et al., 1998). Furthermore, patients with heritable Turcot, Li-Fraumeni, and Rubinstein-Taybi syndromes have also shown increased incidence of MB (Taylor et al., 2000; Bourdeaut et al., 2014; Skousen et al., 1996). In both Turcot (a type of adenoma) and some cases of MB, the gene for $APC$, which is a part of the WNT signaling pathway and has been implicated in MB tumorigenesis, is mutated (Eberhart et al., 2000; Gibson et al., 2010; Huang et al., 2000; Zurawel et al., 1998). Studies on conditions such as Gorlin and Turcot have contributed to a greater understanding of the genetic background and molecular pathways underlying MB.

At present, 5-year mortality can be as high as 30% among MB patients receiving
treatment (Ray et al., 2004). While a combination of surgery, radiation, and chemotherapy is commonly used in MB, side effects frequently include severe developmental defects, cognitive impairment, and early strokes (Packer and Vezina, 2008). Prognosis tends to be worse in very young patients, who also stand a greater chance of suffering adverse side effects as a result of treatment (Raffel, 2004). Children under 3-years-old are generally not given radiation so as to minimize the chances of developing intellectual deficits (Bouffet, 2010; Copeland et al., 1999; Jakacki et al., 2004; Kellie, 1999; Walter et al., 1999). However, among older patients, radiation is the tool of choice for preventing the metastasis of MB cells through the CSF (Packer et al., 1999; Sirachainan et al., 2011). Current chemotherapeutic agents tend to be general, non-specific compounds like Cisplatin, Vincristine, Etoposide, and Cyclophosphamide (Packer and Vezina, 2008). Popular treatment regimens for children include eight-in-one: Carmustine, Cisplatin, Cyclophosphamide, Cytarabine, Hydroxyurea, Procarbazine, Prednisone, and Vincristine (Gentet et al., 1995). VCP, developed by the Children’s Cancer Group (CCG), involves Vincristine, Lomustine (CCNU), and Prednisone (Mulhern et al., 1998). A Pediatric Oncology Group (POG) therapy plan entails Vincristine, Cisplatin, Cyclophosphamide, and Etoposide (Packer et al., 2006). Targeted molecular approaches, such as the SHH pathway inhibitors Cyclopamine and Vismodegib, have only recently come under consideration (Bar and Stearns, 2008; Robinson et al., 2015). The difficulty in developing MB-specific drugs comes in part from an incomplete understanding of the disease subtypes, but it is also a result of the lack of strong in vitro models for rapid testing of therapeutics. Therefore, a better understanding of MB onset and progression will lead to advances in treating this cancer.
Normal cerebellar development

Classically, the study of cancer has equated with the study of normal developmental processes and the mechanisms underlying their aberration (Weinberg, 2013; Hanahan and Weinberg, 2011, 2000). For medulloblastoma, this has meant first and foremost a firm understanding of normal cerebellar development (Bihannic and Ayrault, 2016; Marshall et al., 2014; Roussel and Hatten, 2011; Wechsler-Reya and Scott, 2001). The cerebellum is a prominent hindbrain structure tucked beneath the occipital lobe of the cerebellar cortex and posterior to both the fourth ventricle and, by extension, the pons of the brainstem (Glickstein et al., 2009). Anatomically, the cerebellum appears highly folded due to a crumpled outer layer of cortex overlaying an inner region of white matter. Top-to-bottom subdivisions (anterior, posterior, and flocculonodular lobes) emphasize anatomical distinctions whereas medial-to-lateral subdivisions (vermis, spinocerebellum, cerebrocerebellum) relate to functional differences (Manto, 2008) (Fig. 1.1A).

The vermis is the medial-most region of the cerebellum and is thought to influence speech, language, social behavior, and fear (Riva and Giorgi, 2000b,a). Most MBs in the pediatric population arise within this specific area of the cerebellum, and as such, MB patients often experience speech deficits and altered behavior early in disease progression (Koeller and Rushing, 2003). Lateral to the vermis is the spinocerebellum, or paleocerebellum, which plays a role in fine-tuning motor control by responding to input from the spinal cord (Perciavalle et al., 1995). Lesions to this region result in the onset of ataxias, or neurodegenerative disorders characterized by balance difficulty and uncoordinated movements (Duenas et al., 2006). The cerebrocerebellum, or neocerebellum, is the lateral-most division of the cerebellum. Function of this area is not well understood, but is perhaps related to motor planning and information processing (Ishikawa et al., 2016; Timmann and Daum, 2007).

Finally, the flocculonodular lobe at the base of the cerebellum participates in balance...
and spatial orientation (Schmahmann, 1991). Disturbance to this region is found in patients with gait imbalance. As MB progresses, tumor expansion can damage all of these cerebellar areas, leading to the problems described.

At the cellular level, the adult cerebellum can be further subdivided into three distinct layers: the molecular cell layer (MCL), the Purkinje cell layer (PCL), and the granular cell layer (GCL) (Carletti and Rossi, 2008). The MCL is the outermost sheet of the cerebellar cortex and is dense with dendritic projections extending from the PCL and with axons from neurons in the granular layer (Chu et al., 2012; Sotelo, 2015). Scattered throughout these projections are inhibitory neurons in the form of star-shaped stellate cells and basket cells with axons that form cone-like structures. Beneath the MCL is a region enriched for the cell bodies of Purkinje neurons (Kapfhamer, 2004; Zhang et al., 2010). These easily-recognizable cells have an intricate dendritic meshwork and some of the longest axons of any neuron in the brain. Their dendrites receive excitatory signals (glutamate) from the underlying GCL, which is populated by small granule neurons that constitute nearly half of all neurons in the CNS. In contrast, inhibitory messages like GABA (γ-aminobutyric acid) for Purkinje cells originate from the MCL. Glutamate and GABA signals combine to modulate the strength of the inhibitory message outputted by Purkinje axons to neurons within the cerebellar white matter. Those white matter neurons reside in the deep cerebellar nuclei, which also receive excitatory signals from the cerebral cortex and the brainstem. The deep cerebellar nuclei integrate inhibitory and excitatory signals to regulate motor control.

Establishment of the cerebellar landscape begins with closing of the neural tube during early development (Goldowitz and Hamre, 1998). Specifically, the flat, oval fetal cerebellum arises as a protrusion of the neural tube’s alar plate within the metencephalon region (Hatten and Heintz, 1995). Unlike other brain structures, the developing cerebellum initially contains two germinal zones: the ventricular zone
CVZ) and the rhombic lip, which gives rise to the external granular layer (EGL) (Voogd and Glickstein, 1998) (Fig. 1.1B, top). In mice, neurons of the deep nuclei and Purkinje cells begin migrating away from the VZ between embryonic day (E) 10 and E12.5 (Butts et al., 2014). Purkinje cells form a temporary plate-like structure just caudal and rostral to the rhombic lip. Around the same time, the precursors to granule neurons (CGNs), CGNPs, begin migrating tangentially, in a caudorostral stream, away from the rhombic lip (E13 in mice) and over the dorsal surface of the cerebellar anlage to populate the EGL (Martinez et al., 2013). CGNP migration concludes around E18.5 in mice, after which these cells enter a period of rapid proliferation in response to SHH signaling from underlying Purkinje cells. Starting around postnatal day (P) 3, CGNPs form a T-shaped process as they begin migrating radially inward past the PCL to populate the internal granular layer (IGL), which becomes the GCL in adults (Fig. 1.1B, bottom).

CGNP migration is mediated by the radial fibers of Bergmann glia (astrocytes that are also known as Golgi epithelial cells) and signaling molecules like NMDA (N-methyl-D-aspartic acid) and neuregulin (Meissirel et al., 2011; Rieff et al., 1999; Xu et al., 2013). En route to the IGL, CGNPs cease proliferation and commence terminal differentiation into mature CGNs (Behesti and Marino, 2009; Gazit et al., 2004; Mellor et al., 1998). By P21, CGNPs have completed migration to the IGL, the EGL has disappeared, and the cerebellum has assumed its mature, complex structure with deep fissures and large folia. However, mutations in the SHH pathway can cause CGNPs to remain in a proliferative state in the EGL, potentially leading to the formation of MB (Fernandez et al., 2009; Kim et al., 2003; Schuller et al., 2008; Wechsler-Reya and Scott, 1999; Dey et al., 2012; Mao et al., 2006).
Cerebellar granule neuron progenitors

CGNPs are the precursors of CGNs – the most abundant type of neuron in the adult mammalian brain (Altman and Bayer, 1997). Microscopically, CGNPs appear small and round, resembling the neoplastic cells seen in tumors of some patients with MB (Gilbertson and Ellison, 2008; Wechsler-Reya and Scott, 2001). The striking similarity between CGNPs and cells in MB has led to efforts to better characterize CGNPs at the molecular level. As a part of this interest in these cells, the transcription factor Math1 (Mammalian atonal homolog 1; Atoh1, Atonal homolog 1) was identified as being expressed exclusively by CGNPs within the cerebellum (Helms and Johnson, 1998). In mice, Math1 expression is weak in mid-postnatal development (P7) and mostly absent beyond P20. This suggests that Math1 is only expressed in CGNPs and not in their differentiated daughter CGNs.

Math1 is the mammalian homolog to the atonal gene in Drosophila, which codes for a basic helix-loop-helix (bHLH) transcription factor (Akazawa et al., 1995). In Drosophila, atonal is critical for proper development of photoreceptors and proprioception – loss or mutation results in uncoordinated flies lacking photoreceptors (Jarman et al., 1993, 1995). Similarly, other eukaryotic organisms like mice and yeast also possess bHLHs, which control a diverse array of developmental processes, including cell differentiation and proliferation (Massari and Murre, 2000). Math1 and its homologs are activated through dimerization with other bHLHs like TCF3 (Transcription factor 3) (Bertrand et al., 2002; Johnson et al., 1992; Hu et al., 1992; Murre et al., 1989). Once dimerized, Math1 regulates transcription of its target genes by docking at enhancers with the E-box motif (CANNTG hexonucleotide sequence) (Chien et al., 1996; Klisch et al., 2011). In CGNPs, Math1 is implicated in both cellular proliferation and, paradoxically, differentiation, and its expression is necessary for normal CGNP development (Ben-Arie et al., 1997; Helms et al., 2001; Gazit et al., 2004).
Math1 expression can first be detected by E13 in mice (Akazawa et al., 1995). Although the mechanism leading to this early activation is not well understood, research has suggested that once activated, Math1 promotes its own expression through positive auto-regulation (Helms et al., 2000). Increased Math1 activity correlates with upregulation of proteins in the Notch signaling pathway (Gazit et al., 2004) (Fig. 1.1C). This includes the Notch receptor (Notch2 in CGNPs) and its corresponding ligands, DLL1 (Delta-like 1) and JAG1 (Jagged 1). The binding of ligand to receptor causes cleavage of the cytosolic portion of Notch, which then translocates to the nucleus to act as a transcription factor (Artavanis-Tsakonas et al., 1999). Within the nucleus, Notch2 activates transcription of Hes1 (Hairy and enhancer of split 1) and Hes5, which code for negative regulators of Math1 (Gazit et al., 2004; Zine and de Ribaupierre, 2002; Lanford et al., 2000; Solecki et al., 2001). Thus, Math1 seems to participate in both positive and negative auto-regulation. This careful balance between Math1 activation and repression is likely responsible for delineating the brief developmental interval when Math1 is active in CGNPs.

The importance of balancing Math1 expression is due to its implicated role in both CGNP differentiation and proliferation. One study found that normal Math1 expression was required for the proper differentiation of CGNPs into CGNs (Gazit et al., 2004). However, constitutive or overexpression of Math1 has also been shown to prevent CGNP differentiation (Helms et al., 2001). Math1 hyperactivity led to the expression of some genes involved in CGNP differentiation, but ultimately prevented actual morphological differentiation. These results suggest that the normally brief window of Math1 activity is required for the expression of CGNP differentiation genes; however, Math1 must then be silenced to permit differentiation to occur. Silencing is likely maintained by Notch signaling as these cells differentiate and migrate to the IGL – indeed, Notch pathway activity is detected within the EGL and IGL in early postnatal development (Solecki et al., 2001; Irvin et al., 2004; Kusumî
et al., 2001; Fan et al., 2004).

In terms of the proliferative role of Math1, murine Math1 appears to bind to an E-box-rich enhancer on Gli2 (Glioma-associated oncogene family zinc finger 2), which encodes a SHH pathway transcription factor that activates genes involved in cell proliferation (Flora et al., 2009; Stanton and Peng, 2010). Furthermore, deletion of Math1 was found to prevent tumor development in the SmoM2 mouse model of MB. On its own, Math1 deletion in otherwise normal mice leads to the establishment of a diminished rhombic lip and a subsequently reduced EGL (Ben-Arie et al., 1997, 2000). Within this shrunken rhombic lip resides migratory Math1-independent cells that will populate various areas of the cerebellum (Jensen et al., 2004). However, their fate and function are not fully understood. In the context of cancer, MATH1 is found to be highly expressed in MB tumor samples, especially in those correlated with poor patient prognosis (Salsano et al., 2004). Taken together, these results strongly suggest that MB tumorigenesis likely involves some interaction between MATH1 and the SHH pathway in CGNPs.

Apart from Math1, expression of the genes Pax6 (Paired box 6) (Engelkamp et al., 1999; Yamasaki et al., 2001), Zscan21 (Zipro1; Zinc finger and SCAN domain-containing 21) (Yang et al., 1996), Zic1/2/3 (Zinc finger protein 1/2/3) (Yokota et al., 1996), En2 (Engrailed 2) (Liu and Joyner, 2001), and NeuroD1/2 (Neuronal differentiation 1/2) (Miyata et al., 1999; Olson et al., 2001) is also characteristic of cells of the granule lineage. Some of these markers can be helpful as Math1-adjunctive identifiers of CGNPs, while others are used to delineate the difference between CGNPs and mature CGNs. PAX6 is most recognized for its role in eye development – a role that demonstrates remarkable interspecies conservation (Callaerts et al., 1997). In the developing cerebellum, the Pax6 gene product is thought to mediate CGNP cytoskeletal organization and migration (Yamasaki et al., 2001). Zscan21, according to one study, encodes a zinc-finger transcription factor
that can induce increased postnatal CGNP proliferation in mice (Yang et al., 1999). Yet, in that same study, it was determined that mitotic activity and cerebellar development were not apparently altered in mice with loss of Zscan21.

Like Zscan21, Zic encodes several zinc-finger transcription factors (Aruga et al., 1996). In one study, expression of Zic members was detected not only in CGNPs, but in mature CGNs as well (Yokota et al., 1996). Furthermore, ZIC proteins were found in 26 of 29 MB samples, but not in any cases among 70 other tumors, suggesting a role for ZIC in genesis of MB through cells of the granule lineage. However, other studies have also shown that ZIC proteins are only detected in mature CGNs, thereby putting into question its usefulness as a marker of CGNPs (Aruga et al., 1998, 2002).

The homeobox protein EN2 is thought to control a diverse set of developmental functions, including CGNP migration and proliferation and cerebellar pattern formation (Cheng et al., 2010). NEUROD proteins, on the other hand, are bHLHs that are important for CGNP survival and differentiation (Miyata et al., 1999; Olson et al., 2001). In the end though, while markers like PAX6, ZSCAN21, ZIC, EN2, and NEUROD are useful for distinguishing granule neurons from other cerebellar cells, their roles in MB tumorigenesis have not been extensively studied. Furthermore, most of these genes (Pax6, Zic1/2/3, Zscan21, and En2) are active both in CGNPs and terminally differentiated CGNs. In contrast, NeuroD and, by many indications, Zic, are only expressed in granule neurons, and not at all in CGNPs. Thus, Math1 remains an important protein for distinguishing granule neurons from other cerebellar cells, but also for separating CGNPs from their mature daughters.

The Sonic hedgehog pathway

The importance of Math1 is emphasized by its role in medulloblastomas with SHH pathway mutation (Flora et al., 2009; Salsano et al., 2004). SHH was originally identified as a morphogen in the classic Heidelberg screens of Nusslein-Volhard and
Wieschaus (Nusslein-Volhard and Wieschaus, 1980). Since then, SHH has also been recognized as a potent mitogen (Fuccillo et al., 2006). A morphogen is a signaling molecule that controls the pattern of tissue formation and organogenesis during organismal development. Mitogens are secreted proteins that trigger activation of mitotic pathways upon binding cognate receptors on their target cells. This dual role of SHH underscores its pivotal role in development and its continued, post-developmental relevance as a tumorigenic factor.

At the molecular level, SHH acts by binding a transmembrane receptor called PTC (PTCH1/2) (Fuccillo et al., 2006). SHH binding to PTC is thought to release inhibition of the G protein-coupled receptor SMO (Vaillant and Monard, 2009) (Fig. 1.1D). Once freed, SMO liberates GLI2/3 in a still poorly understood mechanism in mammals. Unstimulated cells are thought to have GLI2/3 bound to a destruction complex, which subsequently marks GLI for degradation by the proteasome (Wang and Zoghbi, 2001). Proteasomal cleavage of GLI3 results in the formation of a small peptide fragment, which enters the nucleus to act as a transcriptional co-repressor of GLI2/3 target genes. The gli destruction complex has most extensively been studied in Drosophila and consists of at least three proteins: fu (fused) (Therond et al., 1996), sufu (suppressor of fused) (Methot and Basler, 2000), and the kinesin-related protein cos2 (costal-2) (Zadorozny et al., 2015). The mammalian homologs – ULK3 (Unc51-like kinase 3), SUFU, and KIF7 (Kinesin family member 7), respectively – have been shown to perform similar functions. Fu/ULK3 is a serine/threonine kinase that potentially modifies GLI2/3 for nuclear entry in the absence of SUFU (Maloverjan and Piirsoo, 2012). Kinesins like Cos2/KIF7 regulate intracellular mobility through interaction with microtubules. Thus, KIF7 has been thought to play a role in coordinating translocation of the GLI destruction complex and to allow its interaction with SMO (Cheung et al., 2009; Endoh-Yamagami et al., 2009; Liem et al., 2009). Untethered SMO could act to dissociate SUFU from the destruction
complex, thereby permitting Fu/ULK3 to activate GLI2/3 for nuclear localization (Kim et al., 2011). Within the nucleus, GLI2/3 initiates transcription of genes such as the SHH pathway antagonist P{ch1}/2 and the transcription factor Gli1. In turn, GLI1 activates expression of the cell cycle proteins Cyclin D and Cyclin E and of the proto-oncogene MycN (Katoh and Katoh, 2009).

Cyclins are the actual effectors of cell proliferation in the SHH signal cascade. On the other hand, promotion of P{ch1}/2 transcription is thought to provide a negative feedback signal for this pathway. Mouse models show that complete loss of Ptc is embryonically lethal (death by 10 days into gestation), while loss of one wild-type Ptc allele can sometimes cause MB formation by 4-6 months of age (Goodrich et al., 1997; Wetmore et al., 2000). These Ptc-mutant-heterozygous mice also display an increased incidence of polydactyly, acromegaly, and rhabdomyosarcoma (Hahn et al., 1999; Goodrich et al., 1997). With the proto-oncogene MycN, one study found that cultured CGNPs treated with SHH showed increased MycN expression (Kenney et al., 2003). When its protein product NMYC was inhibited, the SHH-induced CGNP proliferative response was attenuated. In mouse models of MB, animals heterozygous for Ptc mutation only develop tumors 15 - 25% of the time (Pietsch et al., 1997; Vorechovsky et al., 1997). However, these Ptc heterozygotes developed tumors resembling desmoplastic MB 100% of the time when MycN was forcibly overexpressed (Kessler et al., 2009). Indeed, MYCN is amplified in a subset of MB tumors in human patients (Eberhart et al., 2002b; Aldosari et al., 2002; Pomeroy et al., 2002). While altered MYCN expression in human MBs has been described in all four molecular subgroups, it is most perturbed in the MBs with SHH pathway hyperactivation (Roussel and Robinson, 2013). Mutations in SMO represent the second-most common genetic alteration, after PTCH1 mutation, in patients with SHH subgroup MB and these mutations tend to occur more frequently in adult MB (Kool et al., 2014). When CGNPs in mice are engineered to express a mutant,
constitutively active form of Smo, SmoM2, MB-like tumors develop 100% of the time in early postnatal life (Schuller et al., 2008). Expression of an alternate mutant form of Smo, SmoA1, causes mice to develop tumors later in life but only about 50% of mice get tumors and the age of phenotypic tumor onset can be highly variable (Hallahan et al., 2004).

Apart from the main components of the SHH pathway, other molecules have also demonstrated roles in mediating SHH signaling (Table 1.1). The complexity of cerebellar development begets an understanding of how these additional factors may also influence SHH-mediated CGNP proliferation. PKA (Protein kinase A) is thought to phosphorylate GLI3 and thus cause its degradation by the proteasome (Tempe et al., 2006). SHH signaling was found to inhibit PKA activity in CGNPs, and, in turn, sustained PKA activation blocked CGNP proliferation in vitro despite stimulation by SHH (Barzi et al., 2010; Nicot et al., 2002). In the cerebellum, PKA may be activated through signaling by PACAP (pituitary adenylate cyclase activating polypeptide) – a molecule released by Purkinje cells (Vaudry et al., 1998). Secreted PACAP is received in the outer and inner EGL by CGNPs, which express PACAP receptors during early development (Basille et al., 1993, 1995; Favit et al., 1995).

Another negative regulator of CGNP proliferation, and an inducer of their differentiation, is the extracellular matrix (ECM) glycoprotein VTN (Vitronectin), which can be found from the inner EGL through the IGL (Wechsler-Reya, 2001; Hashimoto et al., 2016). Although the method by which VTN acts on CGNPs is not well understood, one possibility is that it binds to receptors on CGNPs to activate PKA. Research along this line has also shown that PKA achieves transcriptional control by stimulating CREB (cAMP (cyclic adenosine monophosphate) response element-binding protein), which is a transcription factor found in CGNPs (Delghandi et al., 2005; Pons et al., 2001). Thus, the emerging picture is that as CGNPs migrate inward from the outer EGL, VTN and PACAP activate PKA, which phosphorylates
GLI3 and CREB. This marks GLI3 and CREB for degradation and heralds cellular exit from proliferation and the commencement of differentiation.

Several potential positive regulators of CGNP proliferation also exist along the outer EGL. The ECM protein laminin has been shown to increase the proliferative ability of CGNP cultured with SHH \cite{Pons2001}. Additionally, HSPGs (heparan sulfate proteoglycans) and the chemokine SDF-1α (stromal cell-derived factor-1α; CXCL12, Chemokine C-X-C motif ligand 12) have also demonstrated the ability to act synergistically with SHH signaling \cite{Rubin2002, Klein2001}. On their own (without SHH signal), these molecules can only stimulate modest CGNP proliferation. In contrast, growth factors such as EGF (epidermal growth factor), IGF1 (insulin-like growth factor 1), and bFGF (basic fibroblast growth factor) can induce CGNP proliferation without SHH \cite{Gao1991}. Interestingly, when these factors are provided in addition to SHH, EGF and IGF1 cause a mild suppression of CGNP proliferation, and bFGF, likely acting through CREB, significantly reduces the SHH-mediated proliferative response \cite{Wechsler-Reya1999}. Yet, another study found that IGF1 and IGF2 signaling increased CGNP proliferation both synergistically with SHH and independent of SHH \cite{Fernandez2010}.

The significance of these pro- and anti-proliferative factors is realized when viewed in light of the complexity of cerebellar development. In the cerebellum, Purkinje cells secrete SHH \cite{Wallace1999}. CGNPs initially proliferate in response to this signal while in the EGL during early development. Maturation of CGNPs into CGNs requires cessation of proliferation, migration to the IGL, and terminal differentiation. Paradoxically, CGNPs achieve this maturation as they travel from the EGL past the PCL – the very source of SHH signaling. While CGNPs could somehow convert a proliferative SHH signal into a differentiative one, it appears that SHH is actually unnecessary for CGNP maturation \cite{Lewis2004}. A
possibility is that extracellular SHH is maximal in the EGL through secretion from the tips of Purkinje axons, rather than being maximal in the ECM of the PCL. Switching from proliferation to differentiation could also involve reduced contact with proliferative signals in the outer EGL such as laminin as increased contact with differentiative signals in the inner EGL such as VTN, as CGNPs migrate inwards. Thus, normal CGNP development conceivably requires a delicate interplay between the factors promoting SHH-mediated proliferation and those signaling for differentiation through blocking SHH activity. The timing of these interactions is also likely to be of the utmost importance, with dysregulation of signaling events potentially leading to the formation of cancer.

**Seckel syndrome and ATR**

**The history of Seckel syndrome**

In 1960, the German-American pediatrician Helmut Paul George Seckel described thirteen cases of what he called bird-headed dwarfism from researching the literature, with two cases being from personal interaction (Seckel, 1960). Seckel borrowed the term bird-headed dwarf from the German physician Rudolf Ludwig Carl Virchow, who first observed isolated instances of the condition in the late-19th century (Virchow, 1892). Affected patients displayed intrauterine growth restriction, microcephaly, mental disability, and characteristic facies including a sloping/receding forehead and chin with a protruding nose. Among the documented cases, several occurred in siblings with normal, consanguineous parents at equal frequency between both sexes, suggesting autosomal recessive inheritance. This condition was thereafter called Seckel syndrome (SS), and since its initial report, only approximately one hundred other cases have been chronicled.

Before the age of genetic testing, SS was diagnosed solely based on clinical
findings. Seckel himself delineated what he considered the most important features in diagnosing SS as opposed to other types of primordial dwarfism, intrauterine growth restriction, or microcephaly. That is, characteristic craniofacial deformities must be present in addition to microcephaly and mental disability. Indeed, throughout the years, various authors have questioned earlier historical accounts of SS, putting into doubt the true number of cases of Seckel that have ever been diagnosed (Thompson and Pembrey, 1985; Majewski and Goecke, 1982). As it stands, SS is an extremely rare condition with unknown incidence and prevalence.

Soon after Seckel’s initial report on his eponymous syndrome, John Black described two sisters with bird-headed dwarfism and microcephaly consistent with SS (Black, 1961). Over the next five years, four more patients were purported to have SS (Aarons, 1964; De La Cruz, 1963; Szalay, 1964). Then in 1967, pairs of siblings in three separate families were reported as having SS by two groups (McKusick et al., 1967; Harper et al., 1967). Only five more descriptions of SS surfaced globally through the course of the next ten years, emphasizing the rarity of this condition (Nunez et al., 1971; Sauk et al., 1973; Anoussakis et al., 1974; Cervenka et al., 1979; Lambotte et al., 1976). Interestingly, a meta-analysis in 1982 reputed some of the SS diagnoses made by several authors, including Seckel himself, stating that only seventeen cases had been reported to date that fit the physiological criteria of SS (Majewski and Goecke, 1982). A decade later, a separate group expressed support for these conclusions (Sugio et al., 1993), and also included one additional case that they had recently identified as well as two cases identified by Butler and colleagues in 1987, who proposed to expand the SS criteria since they observed chromosome breakage in the cells of two patients, one of whom also had pancytopenia (Butler et al., 1987). Similarly, a patient with SS who later in life developed acute myeloid leukemia (AML) and was treated with chemotherapy that produced severe hematological toxicity further raised the question of whether SS patients
reproducibly harbor some hematological abnormality (Hayani et al., 1994).

The first neuroimaging on SS patients was performed in 1997 on three siblings from a consanguineous family (Shanske et al., 1997). On CT scan, the authors noted agenesis of the corpus callosum, pachygyria, and cortical cysts. More than fifty additional cases of SS were reported over the next two decades, including one instance accompanied by extensive congenital heart anomalies and one patient with a hypoplastic corpus callosum (Abou-Zahr et al., 1999; Can et al., 2010; Mokrani-Benhelli et al., 2013; Borglm et al., 2001; Goodship et al., 2000). Genetic testing that was performed in one of those studies on five affected patients in two unrelated, consanguineous families suggested chromosome 3q22.1-q24 as the SS locus (Goodship et al., 2000). Another study on four SS patients from a consanguineous family identified instead chromosome 18p11.31-q11.2 as the putative SS locus (Borglm et al., 2001). With this greater ease of genetic mapping and growing interest in SS, it increasingly appeared to be a genetically heterogeneous condition, which was supported by findings in eleven SS patients spanning six consanguineous families who all lacked mutations in these two loci (Faivre et al., 2002).

The genetics of Seckel syndrome

Two candidates were proposed in 2003 as the gene mutated in SS (Table 1.2). In one study, among eighteen SS patients across thirteen families, individuals in five families demonstrated mutation in chromosome 14q23, which contains the MNAT1 (Ménage à trois homolog 1) gene that is involved in cell cycle and transcriptional regulation (Kilinc et al., 2003; Tassan et al., 1995). Later, the authors amended their findings in light of faulty data, and MNAT1 is no longer considered a SS gene nor 14q23 a SS locus – formerly referred to as causative of SS type 3. In another study that same year, Goodship and colleagues, who identified the 3q22.1-q24 SS locus,
performed further studies on their five patients and found that the *ATR* gene, which codes for a DNA damage response protein, specifically was mutated in that locus, so that SS type 1 is now considered to be defined by *ATR* mutation (*O’Driscoll et al.*, 2003). Over the next decade, seven more genes would be identified as mutated in different subtypes of SS.

SS type 4 was described in 2007 in three individuals who harbored mutations in *PCTN* (Pericentrin; chromosome 21q22.3), which codes for a protein that ensures proper mitotic spindle formation (*Griffith et al.*, 2008; *Doxsey et al.*, 1994). Alternately, SS type 4 has been proposed to be due to mutations in *CENPJ* (Centromere protein J; chromosome 13q12.12-q12.13) – responsible for centriole duplication and centrosome maintenance – which were found in five affected individuals from a consanguineous family (*Al-Dosari et al.*, 2010; *McIntyre et al.*, 2012). The authors of the latter study suggested that the purported SS patients with PCTN mutation would more accurately be described as having microcephalic osteodysplastic primordial dwarfism type II (MOPDII) rather than SS since their presentation was more MOPDII-like than SS-like. Shortly thereafter, in rapid succession, SS types 2, 5, and 6 were defined in 2011. Using the SS patients in which chromosome 18p11.31-q11.2 anomalies had been discovered, Qvist and colleagues determined the *RBBP8* (Retinoblastoma-binding protein 8) gene, which codes for an endonuclease associated with ATM- (Ataxia-telangiectasia mutated) and ATR-mediated DNA repair, to be mutated in all affected individuals (*Qvist et al.*, 2011; *Huertas and Jackson*, 2009; *Sartori et al.*, 2007). Three years later, this same mutation was found in an unrelated SS patient, and SS associated with this mutation is now categorized as SS type 2 (*Shaheen et al.*, 2014). SS types 5 and 6 are defined by mutations in genes that code for the interacting/cooperative centrosomal proteins *CEP152* (Centrosomal protein 152) and *CEP63*, respectively (*Blachon et al.*, 2008; *Smith et al.*, 2009; *Brown et al.*, 2013). Four subjects from three
consanguineous families and one unrelated subject were diagnosed with SS and found to have mutations in *CEP152* (chromosome 15q21.1-q21.2) (Kalay et al., 2011). Independently that same year, *CEP63* (chromosome 3q22.2) was seen to be mutated in three female cousins from consanguineous families (Sir et al., 2011).

Although SS type 3 has been dropped from the SS spectrum, a gene near *MNAT1*, *NIN* (Ninein; chromosome 14q22.1), is considered to be mutated in SS type 7. Two sisters born from distantly-related parents were diagnosed with a SS-like condition based on pre- and postnatal growth impairment, microcephaly, mental disability, and facies such as prominent nose, and found to have mutations in *NIN*, which codes for a centrosomal protein (Dauber et al., 2012; Ou et al., 2002). SS type 8 was categorized based on observed mutations in *DNA2* (DNA replication helicase/nuclease 2; chromosome 10q21.3), which codes for a nuclear and mitochondrial DNA replication and repair enzyme, seen in two members of a consanguineous family with distinct features of SS (Shaheen et al., 2014; Zheng et al., 2008). Most recently, whole-exome sequencing has determined a mutation in three unrelated SS patients of the *TRAIP* (Tumor necrosis factor receptor-associated factor interacting protein; chromosome 3p21.31) gene whose protein product negatively regulates innate immune signaling and has a putative role in resolving DNA replication forks (Harley et al., 2016).

Apart from the nine categorized subtypes of SS, mutations in other genes have also been described in SS patients. In 2012, three patients were found to have mutations in the *ATRIP* (ATR-interacting protein) gene (Ogi et al., 2012). ATRIP forms a complex with ATR and is required for its recruitment and activation (Cortez et al., 2001), so given that *ATR* mutation causes SS type 1, it was reasonable that *ATRIP* mutation should also produce a form of SS. A final gene thought to be mutated in a type of SS is *CDK5RAP2* (CDK (Cyclin-dependent kinase) 5 regulatory subunit-associated protein 2; chromosome 9q33.2), whose protein regulates mitotic spindle orientation and spindle checkpoint activation (Yigit et al., 2015; Lizarraga

22
Two sibling pairs from two consanguineous families with characteristics of SS were sequenced as having splice-site mutations in CDK5RAP2 that produced a truncated, unstable protein associated with spindle disorganization and mitotic defects. Altogether, this wide array of genetic alterations reported in patients with SS underscores the genotypic heterogeneity and helps explain some of the phenotypic diversity of this condition. That is, beyond the intrauterine growth restriction, microcephaly, mental disability, and characteristic facies that typify SS, patients can suffer from a multitude of comorbidities, as described in the case reports above.

**Animal models of Seckel syndrome**

In 2009, the first animal model of SS, specifically SS type 1, was introduced (Murga et al., 2009). To create this model, the authors replaced exons 8, 9, and 10 of the mouse Atr coding sequence with the human counterparts and altered exon 9 with the splicing mutation previously observed in Seckel patients that causes skipping of that exon (O'Driscoll et al., 2003). Exons 8 and 10 were also introduced into mice because the authors reasoned that although ATR exons are conserved between man and mouse, the introns are divergent and represent important regions for splicing reactions. Mice with mutant, humanized Atr expressed very low, but life-sustaining, levels of the protein, as in human patients. This last consideration was important since prior research had shown that complete loss of ATR is embryonic lethal in mice by E8.5, although maintenance of one allele results in decreased ATR levels, but enough to sustain life (de Klein et al., 2000). Atr mutation in mice modeled human SS by recapitulating several important phenotypic findings: intrauterine growth restriction, postnatal dwarfism, microcephaly, craniofacial dysmorphia including a receding forehead and prominent nose, and MRI findings of agenesis of the corpus callosum and cortical cysts. While ATR protein levels were globally very low in mutant neonatal mice, tissues such as the testes and lungs partially regained
normal ATR levels later in postnatal life, suggesting clonal selection for isolated cells with normally-spliced rather than mutant Atr and highlighting the critical importance of ATR. Although the lifespan and long-term medical consequences of SS patients has not been well-documented, SS type 1-like Atr-mutant mice typically died by 20 weeks of life, with none surviving past 35 weeks, due to multiple organ failure. That is, in later postnatal development, these mice appeared progeric, with canities, kyphosis, osteoporosis, bone marrow fat accumulation, and pancytopenia. Molecular analysis revealed that the SS-like phenotype from Atr mutation was due to widespread DNA damage, despite attempted repair by ATM and DNA-PKc (DNA-dependent protein kinase, catalytic subunit), that led to decreased proliferation through G2/M arrest and p53-mediated, caspase-dependent apoptosis during embryonic development and focally to the brain compared to other organs in neonatal development. Molecular and phenotypic events were exacerbated in Atr/p53-double-mutants, who interestingly never developed tumors though despite very high levels of widespread DNA damage.

Animal models for SS types 4 and 7 were then simultaneously revealed in November 2012. A hypomorphic mutation in Cenpj, as found in human SS type 4 patients, was developed in mice that led to greatly reduced levels of the normal protein product due to alternative splicing of a critical exon (McIntyre et al., 2012). Mutant animals experienced intrauterine growth restriction and showed signs of postnatal growth impairment, microcephaly, abnormal bone ossification, and characteristic facies such as a prominent nose, receding chin, and high forehead. Cellular studies found that neuropathology was due to increased DNA damage that led to caspase-dependent apoptosis, but not attenuated proliferation, in neural stem and progenitor cells of the embryonic brain. DNA damage, in turn, was shown to be a result of dysregulated mitotic processes, normally controlled by CENPJ, that led to supernumerary centrosomes, chromosome damage, and aneuploidy.
Although *NIN* mutation is considered the putative defining characteristic of SS type 7 (Zheng et al., 2016), the original authors who identified the only known *NIN* mutations believed they had discovered a novel locus for an unreported type of primordial dwarfism rather than SS specifically (Dauber et al., 2012). The affected siblings with *NIN* mutations had many hallmarks of SS, but the authors felt that some physical deformities did not match up well. Nevertheless, the authors acknowledged the recognized phenotypic heterogeneity of SS and conceded that the patients could have a new form of SS. Primary fibroblasts isolated from the patients showed normal levels of *NIN* that localized to interphase, but not mitotic, centrosomes. Cell proliferation and mitotic progression were curiously normal in these cells. Morpholino knockdown of *ninein* in zebrafish led to embryonic neurodevelopmental disturbances, including prominently at the rhombic lip, and the postnatal microcephaly and craniofacial deformities reminiscent of SS. Recently, a different group mutated *nin* in Drosophila, but found that decreased *nin* expression had no impact on embryonic or neural development, suggesting different dependencies on NIN between vertebrates and flies (Zheng et al., 2016).

A final animal model for SS involves mutation of *CEP63*, as seen in SS type 6. Mice were engineered to express gene-trapped alleles of *Cep63*, significantly reducing *Cep63* expression and producing mice with postnatal growth impairment and microcephaly, albeit with normal lifespans (Marjanovic et al., 2015). Looking specifically at neural stem and progenitor cells in *Cep63*-mutant E14.5 mice, the authors found normal or increased levels of mitosis and weak levels of DNA damage but strong induction of p53-mediated, caspase-dependent apoptosis. Co-deletion of *p53* rescued microcephaly but co-deletion of *Atm* or *Chk2* failed to rescue pathology, indicating that the cellular response to CEP63 deficiency is independent of the double strand break repair pathway. Increased cell death from CEP63 disruption was seen to be a result of failed centriole duplication, abnormal centrosome
formation and numbers, and mitotic spindle defects that led to spindle assembly checkpoint activation, which can induce apoptosis. *Cep63*-mutant neural stem and progenitor cells were also seen with aneuploidy in G1-phase, suggesting escape of mitotic arrest, which could precipitate apoptosis later in the cell cycle. These four animal models of SS thus suggest that microcephaly in this condition occurs due to apoptosis of neural stem and progenitor cells.

**ATR kinase and the DNA damage response**

ATR was one of the first genes linked to SS and its protein function has been extensively studied. The importance of ATR in SS is emphasized by observations of disrupted ATR pathway signaling not just in SS type 1 but in more than half of all of the recognized subtypes of SS, including type 2 (*RBBP8* mutation), type 5 (*CEP152* mutation), type 6 (*CEP63* mutation), type 8 (*DNA2* mutation), and an unclassified subtype defined by *ATRIP* mutation. In a way, disruption of ATR pathway signaling has come to define SS. Therefore, understanding of SS pathogenesis has been heavily informed by studies of ATR function in different cells and the consequences of its absence.

ATR was first described in yeast, as Esr1 (Essential gene required for DNA repair and meiotic recombination 1) or Mec1p (Mitosis entry checkpoint protein 1) ([Kato and Ogawa, 1994](#)). Complete absence of the Atr homolog was lethal and mutant yeast with hypomorphic *Atr* experienced increased sensitivity to ultraviolet (UV) light and the DNA replication fork stalling agent methyl methanesulfonate (MMS). Esr1 was seen to be induced during mitosis and analysis of its structure revealed similarities to the DNA repair enzyme Rad3 (XPD, Xeroderma pigmentosum group D, in mammals), the regulator of chromosome segregation Cut1 (ESPL1, Extra spindle pole bodies-like 1 in mammals), and the PI3K (phosphoinositide 3-kinase) family. Shortly after the characterization of Esr1/Mec1p in yeast, the mammalian homolog
ATR (originally named FRP1, Fluorescence recovery after photobleaching-related protein 1) was discovered by cDNA cloning and gene mapping in human Jurkat T-cells (Cimprich et al., 1996). The ATR gene was mapped to chromosome 3q22-q24 and structural analysis showed its similarity to yeast Mec1p and Rad3. Since these initial reports, an enormous body of literature has minutely dissected the ATR pathway, revealing that ATR is broadly possibly involved in numerous processes through interactions with hundreds of putative targets (Matsuoka et al., 2007). The exact function of ATR may differ by cell type and depend on the cellular situation, but suggested or confirmed processes include cell cycle control, signal transduction, cell proliferation and differentiation, immune function, protein metabolism and modification, mRNA transcription, and DNA replication, recombination, and repair.

Perhaps the most well-studied function of ATR is in responding to DNA damage and inducing cell cycle arrest to allow for repair (Nam and Cortez, 2011). In dividing cells, DNA damage can occur as a result of endogenous processes such as oxidation (Willis et al., 2013), transcription-replication collision (Deshpande and Newlon, 1996; Takeuchi et al., 2003; Ivessa et al., 2003), transcription-associated recombination and mutation (Paques and Haber, 1999; Prado and Aguilera, 2005), and R-loop formation (Santamaria et al., 1998, 2000; Viguera et al., 1996). Exogenous sources like UV light, X-ray irradiation, and chemical mutagens can also produce DNA damage (Ciccia and Elledge, 2010). The presence of DNA damage induced by such sources promotes stalling of the replication fork in S-phase (Mirkin and Mirkin, 2007). Similarly, fork stalling can occur as a result of depletion of materials requisite for replication such as nucleotides and RPA (replication protein A), which must coat single-stranded DNA (ssDNA) at open replication forks (Poli et al., 2012; Toledo et al., 2013).

DNA replication is a highly regulated event requisite for distribution of genetic material and cell division. During the first interphase, areas of the genome are
marked as replication origins by the origin recognition complex (ORC), a hetero-hexameric scaffold composed of ORC1 - ORC6 (Dutta and Bell, 1997). The ORC binds to specific DNA sequences, including AT-rich sequences or dinucleotide repeats in general (Paixao et al., 2004; Altman and Fanning, 2004; Wang et al., 2004), asymmetrical purine-pyrimidine regions (Wang et al., 2004), and parts of the DNA that attach to the nuclear matrix (Schaarschmidt et al., 2004). The minichromosome maintenance (MCM) complex, a hexameric structure composed of MCM2 - MCM7, is then loaded onto the ORC along with CDC6 (Cell division cycle 6) and CDT1 (Chromatin licensing and DNA replication factor 1), generating the pre-replication complex (pre-RC) (Wyrick et al., 2001). Activation of the pre-RC, which is required for binding of further components needed to initiate replication, is achieved by phosphorylation by CDC7 and CDK2-Cyclin A/CDK2-Cyclin E (Woo and Poon, 2003).

In mammalian cells, not all pre-RCs will initiate replication and it is an active area of research as to what dictates which pre-RCs will fire (Edwards et al., 2002; Hyrien et al., 2003), but may be related to the influence of certain DNA elements outside the pre-RC (Aladjem et al., 1995), chromatin accessibility (Pasero et al., 2002; Pappas et al., 2004), and transcriptional activity (Nieduszynski et al., 2005; Haase et al., 1994). However, the function of the excess, non-firing pre-RCs seems to be in maintaining genomic integrity and that they will activate to ensure complete DNA replication when the replication fork stalls at other regions initiated by primary pre-RCs (Woodward et al., 2006; Ge et al., 2007; Ibarra et al., 2008).

In S-phase, binding of additional factors to the pre-RC, namely CDC45 and the GINS complex (Go-ichi-ni-san; composed of GINS1, GINS2, GINS3, and GINS4), establishes the nascent replisome, or active area of DNA replication (Takayama et al., 2003; Kamada et al., 2007). A fully functional replication fork requires further recruitment of proteins like helicase (MCM), topoisomerase, DNA primase, DNA polymerase, DNA ligase, fork stability/protection factors, and RPA (Masai et al.,
Establishment of a functional replication fork allows DNA replication to proceed, which can be hindered by factors that stall the replication fork, as detailed above. Replication fork stalling promotes the uncoupling of the MCM helicase and DNA polymerase, which provides a signal for the binding of ATRIP to the RPA that coats strands of unwound DNA in active forks (Byun et al., 2005; Zou and Elledge, 2003). ATRIP recognition of RPA is enhanced by, but does not require, interaction with CINP (CDK2-interacting protein), which forms a complex with CEP152 (Kalay et al., 2011; Lovejoy et al., 2009). ATR exists in a functional dimer with ATRIP, so recruitment of ATRIP to RPA recruits ATR as well (Cortez et al., 2001).

Independently, RPA also attracts the RAD17 protein, which localizes the 9-1-1 (RAD9-RAD1-HUS1) complex (Zou et al., 2002). The 9-1-1 complex has been found to recruit TOPBP1 (DNA topoisomerase 2-binding protein 1) and RHNO1 (9-1-1-interacting nuclear orphan 1), which are essential activators of ATR (Delacroix et al., 2007; Cotta-Ramusino et al., 2011). Claspin is another component of the stalled replication fork recognition complex that is recruited independently of ATR/ATRIP and RAD17/9-1-1 and is suggested to act as a scaffold for localization of CHK1 (Chekpoint kinase 1) and mediate the interaction between ATR and CHK1 through TOPBP1 (Kumagai and Dunphy, 2000; Liu et al., 2006b). Claspin may be tethered to RPA through the action of TIPIN (Timeless-interacting protein), which also facilitates TIM (Timeless circadian clock)-mediated modification of ATR to allow for CHK1 activation (Kondratov and Antoch, 2007; Kemp et al., 2010). Ultimately, a major effect of this complicated network is CHK1 activation by ATR through phosphorylation on Ser-317 and Ser-345 (Liu et al., 2000).

CHK1 activity is mostly limited to S- and G2-phases to reflect the maximal periods of possible ATR activity (Lukas et al., 2001). In support of the hypothesis that replication forks regularly stall from endogenous sources in proliferative cells, activated CHK1 has been found in cells not exposed to exogenous insults (Kaneko et al., 2010).
CHK1 is a serine/threonine kinase that was originally described in *S. pombe* as a controller of progression through the G2/M checkpoint ([Walworth et al., 1993](#)). Since its original discovery, several roles have been elucidated for CHK1, and other controllers of its activation have also been found. In S-phase, CHK1 phosphorylates CDC25A, targeting it for proteasomal degradation ([Sorensen et al., 2003](#); [Mailand et al., 2000](#); [Shimuta et al., 2002](#)). CDC25A removes inhibitory phosphate groups from CDK2, which promotes the activity of the CDK2-Cyclin E and CDK2-Cyclin A complexes that are important for G1-S transition, S-phase progression, and S-G2 transition ([Shen and Huang, 2012](#)). Thus, CHK1 activation in S-phase acts to slow S-phase progression and delay transition into G2-phase. Delay is also induced by CHK1 phosphorylation and inhibition of CDC7, preventing activation of the pre-RC ([Heffernan et al., 2007](#)). Evidence suggests that CHK1 can additionally remove CDC45 from replisomes, further halting S-phase progression ([Liu et al., 2006a](#)). In essence, these delays in S-phase progression are a consequence of reduced replication origin firing as a means of reducing the amount of possible stalled forks. The role of CHK1 in regulating G2/M transition is mediated by phosphorylation and inactivation of CDC25C, which, when active, removes inhibitory phosphates from CDK1 (CDC2), an important controller of mitotic entry ([Furnari et al., 1999](#); [Blasina et al., 1999](#); [Peng et al., 1997](#)). CHK1 also phosphorylates and activates WEE1, which further inactivates CDK1 and prevents G2/M transition ([Lee et al., 2001](#)). Similar to CDC25C, another member of that phosphatase family, CDC25B, can also activate CDK1. CHK1 apparently associates with interphase centrosomes to inactivate CDC25B and thereby prevent inappropriate mitotic entry ([Kramer et al., 2004](#)). Finally, arrest at G2/M can be achieved by ATR targeting of CEP63 to block assembly of the mitotic spindle ([Smith et al., 2009](#); [Marjanovic et al., 2015](#)). Just as continued DNA replication and cell cycle progression in the presence of stalled replication forks
can be toxic, so too can continued transcription potentially exacerbate fork stalling. In response, CHK1 phosphorylates histone H3 to repress transcription (Shimada et al., 2008).

The primary goal of halting the cell cycle through ATR activation of CHK1 is to allow for the resolution of stalled replication forks, which often requires DNA repair. ATR is capable of phosphorylating and activating several substrates involved in homologous recombination (HR), including BRCA1 (Breast cancer 1) (Tibbetts et al., 2000), WRN (Werner syndrome RecQ-like helicase) (Pichierri et al., 2003), and BLM (Bloom syndrome RecQ-like helicase) (Davies et al., 2004; Li et al., 2004). WRN is believed to interact with the nuclease DNA2 in processing DNA damage and promoting replication fork restart (Thangavel et al., 2015). Similarly, CHK1 can activate the HR proteins RAD51 (Syljuasen et al., 2005; Sorensen et al., 2005) and BRCA2 (Bahassi et al., 2008). Besides HR, ATR can also promote DNA repair through FANCD2 (Fanconi anemia complementation group D2)-mediated crosslink repair (Andreassen et al., 2004) and XPA (Xeroderma pigmentosum group A)-mediated nucleotide excision repair (Wu et al., 2007). However, it still unclear how ATR dictates whether DNA repair is requisite at a stalled fork and how it then decides which type of repair to utilize, but this choice could relate to cell cycle phase and the availability of homologous chromosomes. Should DNA damage be extensive, ATR and CHK1 are both capable of directly activating p53, or indirectly through inactivation of the p53 ubiquitin ligase MDM2 (Mouse double minute 2 homolog), which can either further halt the cell cycle, especially at the G1/S checkpoint, or initiate the apoptotic program (Tibbetts et al., 1999; Nghiem et al., 2002). Once DNA damage has been repaired or the source of fork stalling otherwise resolved, ATR phosphorylates MCM2, a component of the replication helicase, which allows for the docking of PLK1 (Polo-like kinase 1) (Trenz et al., 2008; Cortez et al., 2004; Yoo et al., 2004). The addition of PLK1 to the replication fork promotes fork restart, at least in
part through inhibition of CHK1 and the re-recruitment of CDC45. In this way, ATR self-regulates its signaling to allow for cell cycle arrest and DNA repair in the presence of stalled replication forks and then fork restart upon resolution of stalling.

**ATM kinase and the consequences of replication fork collapse**

As the name ATR suggests, it is morphologically and functionally related to another PI3K-like protein, ATM. Although originally thought to be involved in relatively separate and independent DNA damage response processes, namely ssDNA DNA break (SSB) repair at stalled replication forks for ATR and dsDNA break (DSB) repair for ATM, the prevailing belief now is that ATR and ATM have many of the same substrates and can also promote the activation of each other (Cimprich and Cortez, 2008; Smith et al., 2010). At sites of DNA damage at stalled replication forks, ATR is able to phosphorylate H2A.X (Histone H2A, member X) on Ser-139, generating γH2A.X, which is thought to recruit ATM (Ward and Chen, 2001). Once recruited, ATM can be activated by ATR through phosphorylation of Ser-1981 (Stiff et al., 2006). Activated ATM can mediate repair of DSBs, but has also been shown to localize and promote the activity of TOPBP1, thereby activating ATR in kind (Yoo et al., 2007, 2009; Wang et al., 2011). ATM may further enhance ATR pathway signaling by stabilizing the 9-1-1 complex through RAD9 phosphorylation (Shin et al., 2012). Downstream of ATR/ATM activation, among the hundreds of putative ATR targets, many are shared by ATM, although the functional significance of most of these interactions is still not well understood (Matsuoka et al., 2007).

An understanding of ATM function is important in light of the consequences of replication fork collapse. As discussed, stalled replication forks trigger ATR/CHK1-mediated cell cycle arrest and attempted DNA repair. However, extensive DNA damage can overwhelm the repair machinery, prolonging replication fork stalling, which can also be caused by absence of ATR. Prolonged fork stalling leads to
replication fork collapse, or the formation of DSBs, through an as yet poorly understood mechanism (Sirbu et al., 2011; Hanada et al., 2007). One possible method of fork collapse is the digestion of ssDNA by endonucleases into DSBs (Hanada et al., 2007; Lopes et al., 2006, 2001; Sogo et al., 2002). Regardless of how exactly stalled forks collapse, the consequence is the generation of DSBs, considered the most toxic form of DNA damage, which can also form in response to ionizing radiation (IR), radiomimetic chemicals, and oxygen free radicals (Brown and Baltimore, 2000; Ragland et al., 2013; Saleh-Gohari et al., 2005). DSBs rapidly recruit the MRN complex consisting of the endo- and exonuclease MRE11 (Meiotic recombination 11 homology), the DNA binding element RAD50, and the recruiter of ATM NBS1 (Nijmegen breakage syndrome 1) (Shiloh, 2003; Shiloh and Ziv, 2013; Shiloh, 2006). MDC1 (Mediator of DNA damage checkpoint 1) is recruited next to a DSB site if the site contains γH2A.X, which is phosphorylated by ATR, ATM, and DNA-PKc (Lukas et al., 2004; Mukherjee et al., 2006; Reitsema et al., 2005). The last member recruited to DSBs is 53BP1 (p53-binding protein 1), an activator of p53 (Wang et al., 2004). The presence of MDC1 is thought to stabilize the long-term DNA association of the MRN and 53BP1. ATM is recruited by the MRN and enhances the activity of NBS1, MDC1, and 53BP1, which in turn promote its recruitment and activation through a cyclical process. While ATM can be activated from replication fork collapse, or the conversion of SSBs to DSBs, so too has it been shown that DSBs, such as formed directly from IR, can be converted to SSBs via nuclease digestion of a single strand of DNA in order to recruit ATR (Jazayeri et al., 2006; Myers and Cortez, 2006; Adams et al., 2006; Cuadrado et al., 2006).

The canonical target of activated ATM is the serine/threonine kinase CHK2, which is closely related to CHK1 and acts on many of the same downstream effectors (Smith et al., 2010; Shiloh, 2006, 2003). Together, ATM and CHK2, like ATR and CHK1, increase the activity of the important tumor suppressor p53 both directly and
through inactivation of MDM2, which allows p53 to halt the cell cycle and/or induce apoptosis (Meek, 2004; Stommel and Wahl, 2004; Kurz and Lees-Miller, 2004). CHEK2 can also promote cell cycle arrest via similar mechanisms used by ATR/CHK1 such as activation of G1/S and intra-S checkpoints through phosphorylation of CDC25A and activation of the G2/M checkpoint through phosphorylation of CDC25C (Falck et al., 2001; Thanasoula et al., 2012). Arrest of the cell cycle facilitates repair of the causative DNA damage, which can be accomplished by several molecules. CHK2 directly activates the HR protein BRCA1 and indirectly activates the related HR protein BRCA2 and the base excision repair factor XRCC1 (X-ray cross-complementing group 1) through stabilization of the FOXM1 (Forkhead box M1) transcription factor (Lee et al., 2000; Tan et al., 2007). DNA repair by HR requires exposure of template DNA, which is facilitated by MRE11, BRCA1, and the nuclease CtIP (C-terminal-binding protein-interacting protein; RBBP8), which is activated indirectly by CHK2 through CDK2 (Sartori et al., 2007).

**Apoptosis**

When genotoxicity overwhelms the capabilities of the DNA repair machinery, or should any component of that machinery be faulty due to mutation, then programmed apoptotic cell death can be initiated. As discussed, the DNA damage response proteins ATR and ATM can both directly and indirectly activate the master regulator of apoptosis, p53. How precisely p53 decides whether DNA repair, temporary cell cycle arrest, cell cycle exit/senescence, or apoptosis should occur is an area of active investigation. Presumably, widespread and prolonged presence of DNA damage allows for differential activation of p53 to promote the apoptotic program (Elmore, 2007; Fridman and Lowe, 2003). Once convinced to induce apoptosis, an important function of p53 is to transcriptionally and mechanically activate
pro-apoptotic members of the BCL2 (B-cell lymphoma 2) family such as BAX (BCL2-associated X) (Miyashita et al., 1994), BAK/BAK1 (BCL2 antagonist/killer 1) (Perfettini et al., 2004; Leu et al., 2004), BAD (BCL2-associated death promoter) (Yang et al., 1995), BID (BH3-interacting domain death agonist) (Sax et al., 2002), BIM/BCL2L11 (BCL2-interacting mediator of cell death/BCL2-like 11) (O'Connor et al., 1998), PUMA/BBC3 (p53-upregulated modulator of apoptosis/BCL2-binding component 3) (Nakano and Vousden, 2001), and NOXA/PMAIP1 (Phorbol-12-myristate-13-acetate-induced protein 1) (Oda et al., 2000). High levels of pro-apoptotic mediators overcomes the anti-apoptotic effects of other BCL2 members like BCL2 (Vaux et al., 1988; Tsujimoto, 1989), BCL-xL/BCL2L1 (BCL2-like protein 1) (Minn et al., 1997), and MCL1 (Myeloid cell leukemia 1) (Perciavalle et al., 2012). In the absence of a cell death signal, anti-apoptotic BCL2 proteins reside in the mitochondria and prevent the association of the pro-apoptotic factors BAX and BAK as well as directly alter the mitochondrial membrane to stop release of molecules such as Cytochrome C and AIF (Apoptosis-inducing factor) (Liu et al., 1996; Kluck et al., 1997; Yang et al., 1997). Pro-apoptotic BCL2 proteins can promote cell death either through direct activation of BAX and BAK, the key mediators of mitochondrial outer membrane permeabilization, or bind and inactivate anti-apoptotic BCL2 proteins. For instance, BIM and BID are capable of inducing activating oligomerization of BAX and BAK (Letai et al., 2002), whereas BIM, BID, BAD, PUMA, and NOXA suppress either certain or all members of the pro-apoptotic BCL2 family (Chen et al., 2005; Willis et al., 2007, 2005).

Regulation of pro-apoptotic proteins like PUMA and NOXA is predominantly controlled at the transcriptional level by p53 (Michalak et al., 2008). BIM activity may be controlled both at the transcriptional level, though not necessarily directly by p53, or through post-translational modification and altered protein localization (Herold et al., 2013; Clybouw et al., 2012; Gogada et al., 2013; Gillings et al., 2009).
Finally, BAX (Gross et al., 1998; Wolter et al., 1997), BAK (Cartron et al., 2014), BAD (Kitada et al., 1998), and BID (Billen et al., 2008) are regulated through changes to their structure that adjust their function or location to enhance apoptotic signaling (Kutuk and Letai, 2008; Elkholi et al., 2011). Through activation of BAX and BAK and inhibition of anti-apoptotic factors, BAX and BAK are able to localize in activated forms to the mitochondrial outer membrane where their pore-forming ability releases Cytochrome C from the mitochondria (Jurgensmeier et al., 1998; Eskes et al., 1998). Cytosolic Cytochrome C can then associate with APAF1 (Apoptosis peptidase-activating factor 1) to form the apoptosome (Tsujimoto, 1998; Adrain and Martin, 2001; Chinnaiyan, 1999). p53 acts as a transcriptional activator of APAF1 (Robles et al., 2001; Portin et al., 2001), although BCL2 and BCL-xL, potentially through intermediaries, may prevent APAF1 association with Cytochrome C until their activity is attenuated by the pro-apoptotic factors (Hu et al., 1998; Pan et al., 1998; Inohara et al., 1998; Chau et al., 2000). A fully functional apoptosome also includes binding of pro-Caspase-9 to APAF1, an interaction that is again denied by BCL2 and BCL-xL in the absence of pro-apoptotic factors (Hill et al., 2004; Bratton and Salvesen, 2010). Pro-Caspase-9 clustering on the apoptosome leads to its activation into the initiator Caspase-9, which is capable of cleaving and activating executioner Caspases-3, -6, and -7 responsible for continuing apoptosis (Wurstle et al., 2012). However, as another layer of protection against inappropriate apoptosis, which would be highly detrimental to a tissue, executioner caspases are inactivated by inhibitor of apoptosis proteins (IAPs) like XIAP (X-linked IAP) (Deveraux et al., 1997; Suzuki et al., 2001; Shiozaki et al., 2003). In addition to Cytochrome C and AIF, permeabilization of the mitochondrial outer membrane also releases the IAP inhibitor SMAC/DIABLO (Second mitochondria-derived activator of caspase, Direct inhibitor of apoptosis-binding protein with low PI) (Wu et al., 2000; Chai et al., 2000; Du et al., 2000). Thus, through BAX/BAK activity, the apoptosome
forms to activate executioner caspases that are freed from inhibition.

Caspase-9 is able to directly cleave and activate Caspases-3 and -7, and Caspase-3 in turn is able to activate Caspases-6 and -7 (Logue and Martin, 2008). Caspase-3 is considered the primary executioner caspase as Caspases-6 and -7 perform many redundant functions albeit with differing specificities (Slee et al., 2001; Walsh et al., 2008). These executioner caspases cleave many substrates that are either directly vital to cell survival or activate molecules that then directly destroy key cellular components. For instance, α-fodrin maintains cytoskeletal integrity and is cleaved by the executioner caspases (Janicke et al., 1998). Another group targeted for degradation is the cytokeratins, which are also important for providing mechanical support to cells (Caulin et al., 1997; MacFarlane et al., 2000). Gelsolin is a center for actin polymerization, creating a permissive environment for intracellular signal transduction (Sun et al., 1999). When this molecule is cleaved by Caspase-3, its fragments degrade integral actin filaments in the cell, thereby preventing intracellular transport and signal transduction and compromising the cytoskeletal architecture (Kothakota et al., 1997). Nuclear destruction proceeds in part through cleavage, especially by Caspases-3 and -6, of NUMA (Nuclear mitotic apparatus protein), a molecule that both organizes the mitotic spindle and enforces the organized structure of the nuclear matrix (Hirata et al., 1998). In addition, CAD (Caspase-activated DNase) is released from its inhibitor partner ICAD (Inhibitor of CAD), allowing it to migrate to the nucleus and actively degrade genomic DNA (Sakahira et al., 1998). Possibly as a mechanism to prevent attempted cellular repair of degraded DNA during apoptosis, Caspases-3 and -7 also inactivate the nuclear repair enzyme and transcriptional activator of DNA repair genes PARP (Poly[ADP-ribose]polymerase) (Kaufmann et al., 1993; Boulares et al., 1999).

One consequence of complete intracellular breakdown is that the phospholipid membrane component phosphatidylserine will flip from the inside to the outside of
the cell (Bratton et al., 1997). This conformational change facilitates recognition by phagocytes through a non-inflammatory mechanism, enabling the uptake of dying cells (Fadok et al., 2001). Grossly, the changes that occur to a cell as a result of apoptosis can be appreciated as a sequence of defined events (Hacker, 2000). Early on, an apoptotic cell shrinks in size, making the organelles more compact, and its chromatin becomes highly condensed in what is known as pyknosis. Shrinking of the cell is likely a result of caspase-mediated interference with the normal cytoskeleton architecture, however the exact mechanism of pyknosis is still being explored, but thought to involve the action of caspase-activated DNases like CAD (Tone et al., 2007; Topiwala et al., 2013). As apoptosis proceeds, membrane blebbing occurs in response to novel actin-myosin attachments between the membrane and cytoskeleton that are promoted by the caspase-activated kinase ROCK1 (Rho-associated, coiled-coil-containing protein kinase 1), which also contributes to the contractile activity of these newly formed filaments (Coleman et al., 2001). This event is closely followed by further nuclear destruction in what is termed karyorrhexis, or widespread DNA fragmentation likely resulting from activation of DNases and nucleases. Just as the DNA fragments during apoptosis, so too does the entire cell into small phospholipid-enclosed entities known as apoptotic bodies. Cellular fragmentation, along with phosphatidylserine exposure, allows rapid uptake of destroyed cells by macrophages and parenchymal cells without the release of intracellular materials, circumventing the necessity for an inflammatory response (Savill and Fadok, 2000; Kurosaka et al., 2003). Apoptosis is complete upon the engulfment of all cellular components.
### TABLES

#### Table 1.1: Many secreted factors can positively or negatively regulate SHH-mediated CGNP proliferation.

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Effect on CGNP proliferation</th>
<th>Location</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laminin</td>
<td>↑</td>
<td>oEGL†</td>
<td>Pons 2001</td>
</tr>
<tr>
<td>HSPG</td>
<td>↑</td>
<td>oEGL</td>
<td>Rubin 2002</td>
</tr>
<tr>
<td>SDF-1α</td>
<td>↑</td>
<td>oEGL</td>
<td>Klein 2001</td>
</tr>
<tr>
<td>BMP7</td>
<td>↑</td>
<td>oEGL</td>
<td>Krizhanovsky 2006</td>
</tr>
<tr>
<td>BMP2/4</td>
<td>↓</td>
<td>iEGL</td>
<td>Zhao 2008</td>
</tr>
<tr>
<td>EGF§</td>
<td>↓</td>
<td>-</td>
<td>Gao 1991, Wechsler-Reya 1999</td>
</tr>
<tr>
<td>IGF1§</td>
<td>↓ or ↑</td>
<td>-</td>
<td>Fernandez 2010, Gao 1991, Wechsler-Reya 1999</td>
</tr>
<tr>
<td>bFGF§</td>
<td>↓</td>
<td>-</td>
<td>Gao 1991, Wechsler-Reya 1999</td>
</tr>
</tbody>
</table>

*The effect on proliferation in the presence of SHH.
†oEGL: outer external granular layer, iEGL: inner external granular layer.
‡PACAP receptors only found on CGNPs in oEGL
§Molecules not naturally found in significant concentrations in the developing cerebellum.
Table 1.2: Seckel syndrome is a genetically heterogeneous disease defined by possible mutations in several genes.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Location</th>
<th>Function</th>
<th>Subtype</th>
<th>Animal model?</th>
<th>References</th>
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<tr>
<td>ATR</td>
<td>Chr. 3q23</td>
<td>DNA damage response, replication fork repair, mitotic entry</td>
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<td>Yes</td>
<td>O’Driscoll 2003, Murga 2009, Lang 2016</td>
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<tr>
<td>RBBP8</td>
<td>Chr. 18q11.2</td>
<td>ATM/ATR-mediated DNA repair</td>
<td>2</td>
<td>No</td>
<td>Sartori 2007, Huertas 2009, Qvist 2011</td>
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<tr>
<td>MNAT1</td>
<td>Chr. 14q23</td>
<td>Cell cycle/transcription regulation</td>
<td>3*</td>
<td>No</td>
<td>Tassan 1995, Kilinc 2003</td>
</tr>
<tr>
<td>PCTN</td>
<td>Chr. 21q22.3</td>
<td>Mitotic spindle assembly</td>
<td>4‡</td>
<td>No</td>
<td>Doxsey 1994, Griffith 2008</td>
</tr>
<tr>
<td>CENPJ</td>
<td>Chr. 13q12.12-q12.13</td>
<td>Centrosome maintenance</td>
<td>4</td>
<td>Yes</td>
<td>Al-Dosari 2010, McIntyre 2012</td>
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<tr>
<td>NIN</td>
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<td>Anchor interphase microtubules to centrosome</td>
<td>7</td>
<td>Yes</td>
<td>Ou 2002, Dauber 2012, Zheng 2016</td>
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<tr>
<td>DNA2</td>
<td>Chr. 10q21.3</td>
<td>DNA replication and repair</td>
<td>8</td>
<td>No</td>
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<td>TRAIP</td>
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<td>Immune signaling</td>
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<tr>
<td>ATRIP</td>
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<td>DNA damage response</td>
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<tr>
<td>CDK5RAP2</td>
<td>Chr. 9q33.2</td>
<td>Mitotic spindle orientation</td>
<td>-</td>
<td>No</td>
<td>Lizarraga 2010, Yigit 2015</td>
</tr>
</tbody>
</table>

Locations are cytogenetic bands on human chromosomes.

Subtypes as listed by Online Mendelian Inheritance in Man. Blanks indicate genetic mutations not currently associated with a recognized subtype.

Mutations in the region containing MNAT1 were originally described as causative of SS type 3, but the authors later amended their findings and retracted the claim.

PCTN mutation was originally described as causative of SS type 4 but later retracted, as reported subjects more likely had MOPDII.
Figure 1.1: The cerebellum and how it develops. (A) The developed, adult cerebellum shown in mid-sagittal (left) and inferior axial (right) views. Adapted from (Netter, 2011).
(B) CGNPs and their precursors migrate tangentially away from the rhombic lip, a transitory dorsal hindbrain germinal zone, in mid-embryonic development to populate the EGL (top). CGNPs then proliferate in the EGL in early postnatal development, gradually migrating radially inwards past the MCL and PCL to populate the IGL (bottom). Ages refer to mouse development. (C) The Math1 transcription factor is activated in mid-embryonic development, as CGNPs and their precursors begin migrating away from the rhombic lip. Math1 both directly increases its own expression and increases expression of its repressors in the Notch pathway, which eventually silence Math1 and allow for CGNP differentiation. (D) In the absence of SHH, the PTC transmembrane receptor inhibits SMO, and GLI2/3 is marked for destruction by a complex consisting of Fu/ULK3, SUFU, and Cos2/KIF7 (top). The degraded GLI3 fragment is a transcriptional repressor. SHH binding releases SMO from PTC, liberating GLI2/3, which activates transcription of targets like \textit{Ptch1/2}, \textit{Gli1}, and \textit{MycN} (bottom). D: dorsal, V: ventral, A: anterior, P: posterior, R: rostral, C: caudal, EGL: external granular layer, MCL: molecular cell layer, PCL: Purkinje cell layer, IGL: internal granular layer.
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CHAPTER 2: FAMILIAL MICROCEPHALIC DISORDERS SUGGEST NEW APPROACHES TO BRAIN TUMOR TREATMENT

Overview

Current brain tumor treatments that rely on inducing DNA damage through radiation and chemotherapy are limited in efficacy and produce excessive side effects. Many types of brain tumors, including glioblastoma and other high grade gliomas, frequently develop resistance to radiation and chemotherapy. While medulloblastoma, the most common malignant brain tumor in children, is often cured by conventional therapy, survivors suffer treatment-induced cognitive impairment, hearing loss, early strokes, and growth failure. New therapies for primary brain tumors are thus urgently needed.

Brain tumors arise from mutations in diverse stem and progenitor cells that ultimately originate from the neuroectoderm. Familial microcephaly also develops in response to genetic mutations in these cell types, but with the opposite result: limited brain growth due to hypoproliferation, premature senescence, and/or apoptosis. Some brain tumors retain many similarities to their neuroectoderm-derived stem or progenitor cell of origin. These tumors may therefore be susceptible to disruptions in genes that are mutated in microcephaly. The potential for the products of microcephaly genes to be therapeutic targets in brain tumors is highlighted by recent literature on KIF11/EG5, ASPM, CDK6, and ATR. Treatments that disrupt these targets may open new avenues for brain tumor therapy.
Introduction

Strict control of neural stem and progenitor cell proliferation prevents both developmental malformation resulting from hypo-proliferation and tumor formation resulting from hyper-proliferation. Microcephaly is a neurodevelopmental disorder defined by reduced head size due to impaired brain development, or microencephaly. A broad and diverse host of causes have been identified for microcephaly, ranging from genetic conditions to fetal trauma to maternal exposures. In general, the common link between these sources is that pathogenesis involves insufficient proliferation of neural stem or progenitor cells, premature neuronal differentiation or senescence, and/or inappropriate cell death (Jamuar and Walsh, 2015). Familial microcephaly and congenital microcephalic disorders thus identify genes that are vital in proper brain development. Specifically, these hereditary conditions reveal how certain gene products critically regulate processes necessary for the normal expansion and survival of neural stem and progenitor cells.

Improved understanding of the basic biology of neural stem and progenitor cells is important for the study of brain tumors. Whereas microcephaly is a consequence of failed stem/progenitor expansion, brain tumors can form in response to mutations in these cells that lead to over-expansion. The most common primary, malignant tumors of the brain are glioblastoma in adults and medulloblastoma in children. The cell of origin for glioblastoma is still under investigation but candidates include Nestin\(^+\) neural stem cells, multipotent progenitors of the subventricular zone (SVZ), or lineage-restricted progenitors that give rise to astrocytes or oligodendrocytes (Alcantara Llaguno et al., 2009; Holland et al., 2000; Gibson et al., 2010; Lindberg et al., 2009). For medulloblastoma, the cell of origin is either a neural stem cell of the cerebellar ventricular zone (CVZ) or a progenitor cell of the cerebellum or rhombic lip, depending on the molecular subgroup (Gibson et al., 2010; Gilbertson and Ellison, 2008; Northcott et al., 2012). With both glioblastoma and medulloblastoma,
either a majority or a distinct population of tumor cells retain genetic and molecular characteristics of the stem/progenitor cell of origin (Alderton, 2011; Baryawno et al., 2010; Dirks, 2003; Modrek et al., 2014; Schuller et al., 2003; Zong et al., 2012).

Therefore, proteins that regulate growth and survival in neural stem/progenitor cells should retain their function in brain tumors. Familial microcephaly and congenital microcephalic disorders identify these proteins, which may be attractive targets for brain tumor treatment.

In this review, we focus on four genes that are each mutated in a different form of hereditary microcephaly and either over-expressed or uniquely important in a primary brain tumor: KIF11, ASPM, CDK6, and ATR. Interestingly, the products of these genes all exert some form of control over mitotic entry or progression, underscoring the particular importance of mitosis for normal brain development and tumor formation. For each of the four candidates, preclinical studies have demonstrated efficacy in targeting the gene products for attenuating tumorigenesis in glioblastoma and/or medulloblastoma. These examples ultimately highlight the importance of studying normal brain development for understanding the pathogenesis and treatment of brain tumors.

**KIF11 is mutated in primary microcephaly and upregulated in glioma**

A recent report identified KIF11 (Kines family member 11) mutations as responsible for a form of primary, autosomal dominant microcephaly (Ostergaard et al., 2012). Examination of ten families found diverse KIF11 mutations that were associated with microcephaly, chorioretinopathy, and/or lymphedema. All of the mutations were predicted to alter the KIF11 protein product, EG5 (KSP, Kinesin spindle protein), which is a bipolar molecular motor protein of the kinesin family. KIF11 expression begins as early in mammalian development as the blastula stage and EG5 is required for initial cleavage events in embryogenesis (Castillo and
As a result, Kif11-null mice perish prior to gastrulation (Chauviere et al., 2008).

Beyond embryogenesis, EG5 interacts crucially with microtubules of the mitotic spindle during mitosis (Kapoor et al., 2000; Sawin and Mitchison, 1995; Valentine et al., 2006). Chromatid separation in mitosis requires formation of a bipolar spindle apparatus with two centrosomes located on opposite sides of the nucleus (Fig. 2.1A). The heterotetrameric shape and plus-end-directed motor activity of EG5 allow it to crosslink antiparallel microtubules and push apart the duplicated centrosomes in prophase (Kashina et al., 1997; Sawin et al., 1992). Continued EG5 activity into prometaphase completes establishment of a bipolar mitotic spindle that is capable of pulling apart sister chromatids (Blangy et al., 1995; Kashina et al., 1996). Indeed, early studies of EG5 disruption with the small molecule inhibitor Monastrol resulted in monoasters with radial spokes of microtubules surrounded by a band of duplicated, unsegregated chromosomes (Kapoor et al., 2000; Mayer et al., 1999).

Formation of this aberrant structure in turn leads to activation of the spindle assembly checkpoint via MAD2 (Mitotic arrest-deficient 2) and subsequent mitotic arrest (Kapoor et al., 2000; Chin and Herbst, 2006). Prolonged mitotic arrest triggers the cell death program through the intrinsic apoptotic pathway involving activation of Caspase-3 (Valensin et al., 2009). The exact mechanism, however, for how mitotic arrest actually leads to apoptosis is an area of active investigation. One theory proposes that prolonged mitotic arrest produces cellular stress, which induces activation of Caspases-9 and -7 and the caspase-activated DNase (Orth et al., 2012). This nuclease damages the DNA of arrested cells, triggering full engagement of the p53-dependent apoptotic pathway.

Although it is still unclear how exactly KIF11 mutation leads to microcephaly, it follows that based on the known mechanism of EG5 and the consequences of its inhibition in other cells, that reduced EG5 activity could lead to reduced
proliferation and increased apoptosis in mitotically active neural stem and progenitor cells. In the developing zebra fish nervous system, eg5 inhibition was found to attenuate the rate of proliferation and aggravate the rate of cell death in neural stem and progenitor cells (Johnson et al., 2014). KIF11 mutations that reduce rather than completely abrogate EG5 activity could reasonably produce microcephaly through an identical mechanism.

Importantly, while EG5 is essential to the microtubules of mitosis and its inhibition can lead to apoptosis, EG5 function is limited to mitosis (Kapoor et al., 2000; Sawin and Mitchison, 1995; Mayer et al., 1999). That is, EG5 does not seem important in non-mitotic microtubules, which are required for diverse cellular processes, including axonal transport in the central nervous system (Conde and Caceres, 2009). Thus, KIF11/EG5 inhibitors may disrupt the mitotic spindle without producing the neurotoxicity of microtubule-directed agents such as vinca alkaloids (Vinblastine and Vincristine) and taxanes (Paclitaxel and Docetaxel) (Grisold et al., 2012; Marcus et al., 2005). Overexpression of KIF11 has been identified in bladder and pancreatic cancers and its expression was correlated with higher clinical grades and stages (Ding et al., 2011; Liu et al., 2010). A 2015 study found KIF11 upregulation in human glioblastoma (Venere et al., 2015). KIF11/EG5 inhibition by the small molecule inhibitor Ispinesib reduced the proliferation of neural stem-like glioblastoma cells in vitro and in mouse xenograft models by preventing mitotic progression. Ispenisib treatment increased the survival of mice with glioblastoma from 24 to 36 days.

KIF11/EG5 inhibitors have not yet been tested for primary brain cancers in clinical trials in the United States. However, several small molecule EG5 inhibitors are being tested in patients with various other cancers, including Ispinesib (SB-715992), Litronesib (LY-2523355), Filanesib (ARRY-520), SB-743921, 4SC-205, AZD-4877, MK-0731, and ARQ-621 (Table 2.1). For all of these agents, patients
experienced minimal neurotoxicity, as predicted – neutropenia and leukopenia were the most common side effects since mitotic inhibition targets all rapidly dividing cells (Knight and Parrish, 2008). Unfortunately, EG5 inhibition as monotherapy has mostly had limited benefit in the cancers in which it has been tested due to the short half-life of many of the inhibitors, which prevented targeting relatively slower cycling neoplastic cells in the tested cancers (Huszar et al., 2009). ARRY-520 and 4SC-205 have shown longer half-life, but in general, EG5 inhibitors are being tested in combination with other chemotherapeutics (Khoury et al., 2012). Regardless of these findings in other cancers, EG5 inhibition could potentially still hold great promise even as monotherapy for glioblastoma since the cancer cells of glioblastoma are highly proliferative, which should allow even short half-life inhibitors to exert a potent effect. Moreover, the recent reports on EG5 inhibition in preclinical models of glioblastoma (Valensin et al., 2009; Venere et al., 2015) are encouraging and recommend further exploration of inhibiting EG5 for the treatment of high grade gliomas.

**ASPM is mutated in primary microcephaly and upregulated in glioma and medulloblastoma**

Like EG5, ASPM (Abnormal spindle microtubule assembly) has a role in orienting and maintaining the mitotic spindle, and it is especially important in neural stem/progenitor cells (Kouprina et al., 2005; Zhong et al., 2005). ASPM is a microtubule minus-end-associated protein that is found at the mitotic spindle poles from prophase through telophase (Kouprina et al., 2005; Higgins et al., 2010). In *vitro* studies in non-neural human cells demonstrated that ASPM is required for cell division and that cytokinesis fails in its absence (Higgins et al., 2010). In *Drosophila* neuroblasts, the ASPM homolog asp is required for proper completion of mitosis and its disruption leads to mitotic arrest at metaphase (do Carmo Avides and Glover, 84)}
In contrast, loss of ASPM in mammalian neural stem and progenitor cells does not prevent mitotic progression (Fish et al., 2006; Pulvers et al., 2010; Williams et al., 2015). Rather, ASPM is thought to control symmetric divisions in epithelial stem cells of the neuroectoderm and its acute inhibition by RNA-i led to increased asymmetric divisions that depleted the stem cell population (Fish et al., 2006). A conflicting report by the same group found no such changes to the proportion of symmetric versus asymmetric neuroepithelial divisions when Aspm was mutated, but the authors suggested that this discrepancy could be due to partially preserved ASPM function with the mutation and/or a difference between acute inhibition and gradual acclimation (Pulvers et al., 2010). In the more lineaged-committed cerebellar granule neuron progenitor (CGNP), ASPM mediates cell cleavage plane orientation (Williams et al., 2015). Conditional Aspm deletion in CGNPs led to a relative increase in the proportion of divisions oriented transverse to the pial surface in the external granule layer (EGL) of the early postnatal mouse cerebellum, consistent with inappropriately increased progenitor cell cycle exit and differentiation (Fig. 2.1B).

Mutations in ASPM are the most common cause of familial microcephaly (Bond et al., 2002; Roberts et al., 2002). Nearly all of the identified mutations lead to ASPM truncation (Bond et al., 2002; Roberts et al., 2002), and although the mutations affect all tissues, it is still unclear why ASPM is particularly important to neural stem/progenitor cells compared to other proliferating cells (Kouprina et al., 2005; Luers et al., 2002). Indeed, Aspm-null mice develop microcephaly and hypogonadism, but other tissues seem relatively unaffected (Pulvers et al., 2010; Fujimori et al., 2014). Research into the molecular function of ASPM indicates that microcephaly associated with ASPM mutation is due at least in part to impaired self-renewal of neural stem and progenitor cells (Fish et al., 2006; Pulvers et al., 2010; Williams et al., 2015). In addition, apoptosis from ASPM loss may be important in generating
microcephaly through cerebellar hypoplasia (Williams et al., 2015), but cell death does not appear to occur in ASPM-inhibited neuroepithelial cells (Pulvers et al., 2010). Altogether, these studies emphasize the disproportionate importance of ASPM in the developing brain compared to other tissues and demonstrate how ASPM disruption decreases proliferation and increases cell death in neural stem/progenitor cells.

In contrast to microcephaly, ASPM expression is increased in malignant gliomas (Horvath et al., 2006; Hagemann et al., 2008; Bikeye et al., 2010). In an early study using 120 patient samples of glioblastoma, an unbiased screen identified ASPM as being significantly overexpressed (Horvath et al., 2006). A separate study using RT-PCR and immunohistochemistry on tumor samples from 15 patients with low-grade (II) astrocytoma and 15 patients with glioblastoma showed that ASPM mRNA and protein were low in the astrocytomas but much higher in glioblastomas (Hagemann et al., 2008). Similar results were obtained from 175 patient glioma samples, representing 8 grade II, 75 grade III, and 92 grade IV gliomas – ASPM mRNA and protein levels increased with tumor grade and were also higher in recurrent tumors (Bikeye et al., 2010). siRNA knockdown of ASPM in glioblastoma cell lines reduced proliferation, increased cell death, and sensitized cells to DNA damaging agents like X-rays and Cisplatin (Bikeye et al., 2010; Horvath et al., 2006; Kato et al., 2011). ASPM is also upregulated in medulloblastoma (Vulcanti-Freitas et al., 2011). In a primary mouse model of medulloblastoma, conditional Aspm deletion was found to increase DNA damage in tumors and attenuate overall tumorigenesis (Williams et al., 2015). Further research in in vivo systems is needed to illuminate the exact mechanism by which ASPM disruption reduces tumor formation or destroys tumors. Nonetheless, these studies highlight the shared dependence of neural stem/progenitor and brain tumor cells on ASPM. Despite these promising findings, there are currently no ASPM inhibitors in clinical trials in the
**CDK6 is mutated in primary microcephaly and upregulated in glioma and medulloblastoma**

Data collected on eight generations of a large, consanguineous family in which 10 individuals had apparent primary, autosomal recessive microcephaly found recurrent mutations in *CDK6* (Cyclin-dependent kinase 6) ([Hussain et al., 2013](#)). CDK6 protein levels were not reduced in the microcephalic patients, however its function and behavior were altered. In control human fibroblasts, CDK6 associated with centrosomes in mitosis – this association was absent in *CDK6* mutant fibroblasts, which displayed abnormal mitotic spindle alignment and supernumerary centrosomes that persisted into interphase. Mutant patient fibroblasts had abnormal cell polarity and reduced cell motility *in vitro* ([Fig. 2.1C](#)). While these observations have yet to be confirmed directly in neural stem or progenitor cells, the authors did detect CDK6 by immunofluorescence in neuroepithelial and neural progenitor cells of the embryonic mouse brain. They hypothesized that microcephaly was a consequence of increased neural stem/ progenitor apoptosis and decreased cell proliferation due to reduced RB (Retinoblastoma) phosphorylation, which were seen in the mutant fibroblasts.

The most well-studied role for CDK6 is in control of the cell cycle. CDK6 interacts with Cyclin D in G\(_1\)-phase to phosphorylate RB ([Meyerson and Harlow, 1994](#)). This phosphorylation allows cell cycle progression from G\(_1\)- to S-phase ([Sherr et al., 2016](#); [Tigan et al., 2016](#)). Cyclin D binding is required for CDK6 activity, and the levels of this cyclin are controlled by growth factors and mitogens ([Sherr and Roberts, 1999](#)). In turn, CDK6 levels are thought to be regulated by p21 (CIP1, CDK-interacting protein 1), p27 (KIP1, Kinesin-like protein 1), and p57 (KIP2), which may control CDK6 assembly and nuclear translocation ([Blain et al., 1997](#); [Cheng et al., 1998](#));
Besides Cyclin D, CDK6 activity is also regulated by its inhibitors p15 (INK4B, Inhibitor of CDK4 B), p16 (INK4A), p18 (INK4C), and p19 (INK4D) (Ruas and Peters, 1998).

Tight control of CDK6’s cell cycle activity is imperative as it is expressed at some point in almost all tissues. Nevertheless, CDK6 has a homolog in mammals, CDK4, that is able to carry out many of the same functions (Bates et al., 1994; Lucas et al., 1995). Indeed, Cdk6-null mice are viable and mostly develop normally, but suffer from anemia and defective T-cell maturation (Hu et al., 2009; Malumbres et al., 2004). Cdk4-null mice are also viable and instead experience pancreatic and pituitary hypoplasia (Rane et al., 1999; Tsutsui et al., 1999). In contrast, loss of both Cdk4 and Cdk6 results in embryonic lethality, underscoring redundant roles for CDK4 and CDK6 (Malumbres et al., 2004). The distinct phenotypes of the two null mice, however, suggest some non-overlapping functions for the kinases. Apart from a role in hematopoiesis, CDK6 may regulate the apoptotic program (Nagasawa et al., 2001). Cultured mouse embryonic fibroblasts overexpressing Cdk6 upregulated Tp53 and subsequent exposure to ultraviolet (UV) irradiation caused rapid, widespread, BAX (BCL2 (B-cell lymphoma 2)-associated X)-mediated apoptosis.

In the central nervous system (CNS), Cdk6 expression is high in cortical progenitor and striatal stem cells and decreases once the cells cease proliferating and differentiate (Ferguson et al., 2000). Cdk6 disruption prolonged G1-phase in these cells and inhibited entry into S-phase, inhibiting their proliferation. G1-phase lengthening has been shown in mouse neuroepithelial cells to induce premature differentiation, explaining the decreased proliferation associated with Cdk6 disruption (Calegari and Huttner, 2003). Juvenile mice lacking Cdk6 similarly had precursor cells in the SVZ and subgranular zone of the dentate gyrus that experienced prolonged G1-phase, premature cell cycle exit, and attenuated proliferation (Beukelaers et al., 2011). A final putative role for CDK6 in the CNS is
suggested by an experiment where astrocytes from neonatal mice that were induced to overexpress Cdk6 in vitro appeared more bi-polar and fibroblast-like than normal, multi-polar astrocytes (Ericson et al., 2003).

CDK6 upregulation is found in several types of tumors, including malignant glioma and medulloblastoma (Costello et al., 1997; Mendrzyk et al., 2005; Lam et al., 2000; Tadesse et al., 2015). In gliomas, CDK6 expression increases with higher tumor grade and is correlated with a worse prognosis (Li et al., 2012; Chen et al., 2013). siRNA knockdown of CDK6 in cultured glioblastoma cells reduced proliferation and increased apoptosis following treatment with Temozolomide (Chen et al., 2013). Two miRNAs, miRNA-495 and miRNA-340, have been identified that suppress CDK6 activity (Li et al., 2012, 2015). They are both downregulated in human glioblastoma and their expression was found to arrest tumor cells in G1, leading to less proliferation. SUMO1 (Small, ubiquitin-like modifier 1) has also recently been identified to interact with CDK6 in glioblastoma by preventing its degradation in order to permit tumor cell hyper-proliferation (Bellail et al., 2014). As in glioblastoma, CDK6 inhibition in medulloblastoma cell lines was found to induce G1 arrest due to RB hypo-phosphorylation, attenuating cell proliferation (Whiteway et al., 2013). Moreover, CDK6 inhibition significantly limited the survival of medulloblastoma cells exposed to radiation. The role of CDK6 in mitotic progression, as suggested by microcephaly, has yet to be investigated in these cancers.

A multitude of CDK6 inhibitors, though not all specific for CDK6, have been tested in clinical trials over the past twenty years for a variety of cancers (Asghar et al., 2015). Few, however, have been tried in primary brain cancers (Table 2.2). The CDK4/6 inhibitor Palbociclib (PD-0332991) has the most ongoing clinical trials for mostly breast cancers, but also several for primary or metastatic brain tumors. Ribociclib (LEE-011), another CDK4/6 inhibitor, has active Phase I/II clinical trials for gliomas. Finally, Abemaciclib (LY-2835219), also a CDK4/6 inhibitor, has one
active Phase I trial for children with diffuse intrinsic pontine glioma. Early results with these agents as monotherapy in non-brain cancers have shown good clinical outcomes, with neutropenia and some gastrointestinal toxicity as the most common limiting side effects. However, anti-tumor effects have generally been modest and not all cancers are responsive. Further research will reveal the feasibility of targeting CDK6 in humans to treat primary brain tumors.

**ATR is mutated in Seckel syndrome and plays an important role in medulloblastoma tumorigenesis**

ATR (Ataxia-telangiectasia and Rad3-related) is a serine/threonine kinase that activates substrates involved in DNA repair, cell cycle arrest, and apoptosis (Abraham, 2001). Specifically, ATR responds to the presence of single-stranded DNA (ssDNA), which forms as a normal part of DNA replication and pathologically as a result of DNA damage (Cimprich and Cortez, 2008). In vitro studies demonstrate that in undamaged dividing cells, ATR checks genomic integrity at S-phase and slows cell cycle progression to prevent replication stress from causing DNA damage (Shiloh, 2001). Conversely, ssDNA resultant from DNA damage can cause ATR activation in all phases of the cell cycle (Shiloh, 2003; Germann et al., 2014; Gray et al., 2013). The presence of DNA damage prompts ATR to act in two possible ways – cell cycle arrest and DNA repair if the damage is minimal, or apoptosis if the damage is extensive (Smith et al., 2010). Yet, whether DNA damage from ATR deficiency occurs in specific areas of the genome or is randomly distributed is not well understood. Moreover, although ATR can promote apoptosis, loss of ATR is actually associated with increased cell death due to the greater role of ATR in mitigating DNA damage and preventing cell death (Yang et al., 2003). There is some debate as to how cell death proceeds from ATR deficiency. Cleaved Caspase-3 (cC3) activation in Atr-deleted cells indicates that cell death occurs via apoptosis (Myers et al., 2003;
In contrast to typical apoptosis after genotoxic stress, however, ATR-deficient cell death has been suggested to be p53-independent (Heffernan et al., 2009; Lee et al., 2012). This is unexpected given the pivotal role of p53 in bridging DNA damage with apoptosis (Offer et al., 2002; Roos and Kaina, 2006; Wang, 2001).

The role of ATR in proliferating cells is especially important due to its ability to relieve physiologic replicative stress, which would otherwise cause DNA damage (Paulsen and Cimprich, 2007). Complete loss of ATR is embryonic lethal in mice, as widespread chromosome fragmentation precipitates caspase-mediated apoptosis (Brown and Baltimore, 2000). In the brain, ATR is essential to cerebellar growth, where proliferation of CGNPs continues longer than almost all other neural cell types (Carletti and Rossi, 2008). Conditional deletion of Atr in the embryonic mouse CNS was found to almost exclusively affect two areas of the developing brain – the ganglionic eminence, which produces cortical, striatal, and olfactory cells, and the nascent cerebellum (Lee et al., 2012). Neural stem cells of the embryonic brain required ATR for normal proliferation and expansion, and its loss resulted in microcephaly, with especially prominent cerebellar hypoplasia, and early postnatal lethality. In the absence of ATR, neural stem cell populations of the embryonic ganglionic eminence and nascent cerebellum experienced extensive DNA damage that led to either apoptosis or impaired proliferation, respectively. Interestingly, inactivation of p53 neither significantly rescued nor exacerbated the ATR phenotype, despite partial attenuation of apoptosis in the forebrain, leading the authors to conclude that neuropathology from ATR loss is not p53-dependent.

The function of ATR appears to be slightly different in more lineage-committed neural progenitors at later stages of development. Our group has found that Atr deletion in CGNPs causes chromosomal damage and widespread apoptosis but no defect in proliferation at the early postnatal stage, when CGNP proliferation is
maximal (Lang et al., 2016). Cerebellar hypoplasia was partially rescued by co-deletion of either Bax and Bak or p53. However, in the Atr/Bax/Bak-deleted mice, pathology persisted due to continued activation of p53 by ATM in CGNPs, leading to p21-mediated cell cycle arrest and premature cell cycle exit. CGNPs in Atr/p53-deleted mice experienced caspase-independent cell death that likely induced cerebellar hypoplasia. Furthermore, ATR loss was found to increase the fraction of CGNPs in M-phase, suggesting an important role for ATR in mediating mitotic entry in CGNPs. This checkpoint role is supported by the finding that Atr deletion significantly increased the fraction of CGNPs with a spectrum of chromosomal abnormalities (Fig. 2.1D). Finally, sequencing and pathway analyses demonstrated the critical importance of p53 in mediating the response to ATR loss in CGNPs.

While ATR may have slightly different functions in neural stem cells compared to neural progenitors, the impact of its loss in these cell types clearly demonstrates its importance in brain development. This importance is highlighted by the congenital disorder Seckel syndrome, which is caused by hypomorphic mutations in ATR and characterized by intrauterine growth restriction, cryptorchidism, distinctive facies, pancytopenia, and microcephaly (Goodship et al., 2000; O'Driscoll et al., 2003). Research into the pathogenesis of Seckel syndrome, and especially the effect of ATR deletion on neural stem/progenitor cells, as discussed above, has implications for developing novel brain cancer therapeutics. The unique requirement of cerebellar progenitors for ATR (Lee et al., 2012; Lang et al., 2016) suggests that ATR may be an effective therapeutic target for the cerebellar cancer medulloblastoma (Amakye et al., 2013). Activating mutations in the SHH signaling pathway are responsible for 25% of human medulloblastoma (Wechsler-Reya and Scott, 2001), and CGNPs are thought to be the cells of origin for this molecular subset (Schuller et al., 2008; Wechsler-Reya and Scott, 1999; Fernandez et al., 2009; Kim et al., 2003; Dey et al., 2012; Mao et al., 2006). Indeed, genetically engineered mice with neuronal
hyper-activation of the Sonic hedgehog (SHH) pathway reproducibly develop cerebellar tumors that resemble human medulloblastoma (Pietsch et al., 1997; Dey et al., 2012; Mao et al., 2006). As with other types of cancer cells, medulloblastoma cells suffer from genetic instability owing to rapid rounds of proliferation (Blow and Gillespie, 2008; Negrini et al., 2010; Kool et al., 2014). Radiation and chemotherapy, the current standard of care for medulloblastoma, take advantage of this genomic instability by further damaging the DNA of neoplastic cells – enough to push them towards cell death (Roos and Kaina, 2006; Herr and Debatin, 2001). However, radiation can be extremely toxic to the normal brain, and chemotherapy damages healthy, endogenous dividing cells such as found in the GI tract and bone marrow (Barnett et al., 2009). These effects can be particularly devastating in young patients.

We have found that Atr deletion in two different mouse models of medulloblastoma either significantly reduced tumorigenesis or completely abrogated tumor formation (Lang et al., 2016). Our group is currently testing pharmacological ATR inhibition in mice with established medulloblastoma using the novel ATR inhibitor pVE-822, in which the small molecule ATR inhibitor VE-822 is packaged in polymeric micelle nanoparticles. Preliminary results show that pVE-822 delivered by intraperitoneal injection effectively crosses the blood-brain barrier and induces DNA damage and apoptosis in neonatal wild-type mice. In medulloblastoma-bearing mice, pVE-822 treatment increased tumor DNA damage and reduced tumor size. Ongoing research aims to determine whether pVE-822 produces a survival benefit in mice with medulloblastoma and how pVE-822 may be combined with other therapies to improve treatment outcomes. At this time, the ATR inhibitor VX-970 (VE-822) is in Phase I clinical trials with radiation therapy for secondary brain cancer (metastases from non-small cell lung cancer (NSCLC)). VX-970 is also being actively tested in Phase I and II trials for other non-brain malignancies (Table 2.3). In addition, the ATR inhibitor AZD-6738 is currently in Phase I trials for solid tumors
refractory to other therapies.

A particular benefit of ATR inhibition for the treatment of brain cancers is that in the adult brain, ATR seems to be dispensable for the survival and function of post-mitotic neurons (Lang et al., 2016; Kemp and Sancar, 2016; Ruzankina et al., 2007). It has been shown in mice, however, that ATR is still required for normal proliferation of remaining neural stem cell populations, such as in the hippocampus, in the adult mouse brain (Onksen et al., 2011). Of course, with medulloblastoma, since most patients are young with still-developing brains, ATR inhibitors may produce greater toxicity than in adults. Yet, our findings in neonatal and juvenile medulloblastoma-bearing mice suggest fairly minimal CNS toxicity. In gliomas, although ATR upregulation has yet to be reported, the deleterious effect of ATR disruption in the neural stem cells from which gliomas may derive supports investigating ATR inhibition in these tumors. Here especially, the apparent CNS tolerability for ATR loss intimates the possible improved therapeutic benefit of ATR inhibition over traditional chemotherapy.

Discussion

The summary presented here highlights how insights from developmental disorders can inform advances in cancer therapeutics. Using the brain as an example, we make the case that molecules downregulated in microcephaly may be attractive targets for brain tumor treatment, where they may be upregulated, and since the two conditions lie on opposite ends of the same spectrum of proliferation regulation. Of the four discussed proteins, three – KIF11/EG5, ASPM, and CDK6 – have been shown to play a role in orienting the mitotic spindle in neural stem and progenitor cells (Sawin and Mitchison, 1995; Kapoor et al., 2000; Kouprina et al., 2005; Zhong et al., 2005; Hussain et al., 2013). Indeed, the primary role of EG5 and ASPM seems to be in spindle maintenance (Valentine et al., 2006; Sawin et al., 1992;
Higgins et al., 2010), while CDK6 is better recognized for controlling the G\textsubscript{1}-S checkpoint and has only recently been implicated in spindle assembly in neural stem/progenitor cells (Hussain et al., 2013; Sherr et al., 2016; Tigan et al., 2016). Like CDK6, ATR has been most extensively studied for its non-mitotic functions, such as restarting stalled replication forks and responding to DNA damage (Abraham, 2001; Cimprich and Cortez, 2008; Shiloh, 2003). Our group has shown that, as with EG5, ASPM, and CDK6, ATR also has an important function related to mitosis in neural stem/progenitors in that it seems to control mitotic entry in CGNPs (Lang et al., 2016).

That four genes mutated in microcephalic disorders code for proteins involved with mitosis is not necessarily surprising since primary microcephaly has been described as essentially a mitotic disorder (Faheem et al., 2015). Rather, the examples we have presented emphasize the similar molecular dependencies between neural stem/progenitor cells and brain cancer cells and suggest that the disproportionate importance of mitosis in neural stem/progenitor cells should provide incentive to more intensively study mitotic inhibition as targeted treatment for brain cancers. With high-grade gliomas, evidence suggests that populations of neural stem or progenitor-like cells maintain tumor growth, and these cells are resistant to radiation and alkylating chemotherapy (Venere et al., 2013, 2011). Yet, the similarity of these tumor cells to neural stem/progenitor cells implies that they too may rely on mitotically active microcephaly proteins, and a variety of experiments have certainly shown this to be the case (Venere et al., 2015; Bikeye et al., 2010; Hagemann et al., 2008; Horvath et al., 2006; Chen et al., 2013; Li et al., 2012, 2015). Thus, inhibition of microcephaly proteins like EG5, ASPM, and CDK6 may not only efficiently target malignant glioma cells, but also produce reduced neurotoxicity due to the relative absence of neural stem/progenitor cells in the developed brain outside of tumors.

Likewise, in medulloblastoma, tumor cells often bear resemblance to cerebellar
stem/progenitor cells (Wang and Wechsler-Reya, 2014). The mitotic spindle proteins ASPM and CDK6 are upregulated in medulloblastoma and their disruption has been shown to impair tumor growth (Williams et al., 2015; Vulcani-Freitas et al., 2011; Mendrzyk et al., 2005; Whiteway et al., 2013). Although ATR has not been shown to be upregulated in human medulloblastoma, it clearly plays a pivotal role in CGNP survival and is required for tumorigenesis in the SHH subgroup of medulloblastoma (Lang et al., 2016). Current standard-of-care for children with medulloblastoma involves surgical resection of the primary mass accompanied by radiation and chemotherapy, all of which can have substantial deleterious effects on the still-developing CNS (Roos and Kaina, 2006; Herr and Debatin, 2001; Barnett et al., 2009). Here again, targeted therapy against microcephaly proteins may produce substantial therapeutic benefit with relatively lower toxicity, as has been suggested by studies of ATR inhibition (Lang et al., 2016; Kemp and Sancar, 2016).

A concern might be that while the four discussed proteins, and mitotic regulators in general, may be viable targets for monotherapy, inhibitors of such molecules may not synergize well with other chemotherapeutics since blocking mitosis would halt cell cycle progression and most existing chemotherapeutic agents disrupt cycling cells (Roberts et al., 2012; Dean et al., 2012). In the cases of ASPM and ATR inhibition, neural stem/progenitor cells continue to cycle, at least initially, and their disrupted expansion was due to processes other than solely cell cycle arrest (Fish et al., 2006; Pulvers et al., 2010; Williams et al., 2015; Lang et al., 2016). Another consideration for inhibiting some of these targets is that proteins like EG5, CDK6, and ATR function in many tissues and organs. Systemic administration of inhibitors against these molecules may produce non-neural toxicities, which must be studied, and raises the possibility of need for direct tumoral delivery.

Apart from KIF11, ASPM, CDK6, and ATR, several other genes that are mutated in microcephalic disorders may be viable candidates for cancer therapy (Table 2.4).
**NBS1** (*NBN, Nibrin; p95*) is mutated in Nijmegen breakage syndrome (NBS), which is characterized by growth impairment, immunodeficiency, radiation sensitivity, microcephaly, and, paradoxically, predisposition to malignancies like lymphoma, rhabdomyosarcoma, glioma, and medulloblastoma (*Digweed and Sperling, 2004*). The protein coded by this gene senses DNA double-strand breaks (DSBs) and mediates S-phase arrest and DNA repair through ATM (Ataxia-telangiectasia mutated) and BRCA1 (Breast cancer 1) (*Branzei and Foiani, 2008; Lim et al., 2000; Zhu et al., 2000*). L1CAM (L1 cell adhesion molecule) regulates NBS1 expression and activity and L1CAM expression is associated with a worse prognosis in glioma (*Izumoto et al., 1996; Suzuki et al., 2005*). Disruption of L1CAM and NBS1 were found to increase the radiation sensitivity of stem-like cells in glioblastoma (*Cheng et al., 2011*). However, considering the importance of NBS1 in DSB repair, NBS1 inhibition may have limited therapeutic use, especially considering that its mutation actually increases the frequency of brain tumors.

Mutations in *IGF1R* (Insulin-like growth factor 1 receptor) have been identified in patients with microcephaly and general pre- and post-natal growth impairment (*Abuzzahab et al., 2003; Juanes et al., 2015; Walenkamp et al., 2006*). IGF1R is a receptor tyrosine kinase that is broadly expressed in almost all tissues and mediates cell proliferation through IGF1 signaling, ensuring adequate growth of many organs, including the brain (*Klammt et al., 2008*). As in humans, mice carrying mutations in *Igf1r* experience growth failure and microcephaly, although complete loss of IGF1R is embryonic lethal due to respiratory failure (*Baker et al., 1993; Liu et al., 1993*). In the CNS, IGF1R is indispensable for the expansion of neural stem and progenitor cells, including CGNPs, during development (*Ziegler et al., 2015; de Pablo and de la Rosa, 1995*). It continues to be important in mature neurons, ensuring their survival and promoting synaptogenesis (*Joseph D’Ercole and Ye, 2008*). Some research has suggested that signaling through IGF1R may contribute to the genesis of
medulloblastoma and increased IGF1R expression has been found in a set of human medulloblastoma samples (Del Valle et al., 2002). A recent report has described increased IGF1 presence in the CSF of patients with metastatic medulloblastoma, and the authors proposed targeting IGF1R as a novel treatment for advanced medulloblastoma (Svalina et al., 2016). Many IGF1R inhibitors exist, but thus far, they have had mixed success in clinical trials as anti-tumor therapies, with the broad biological role of IGF1R possibly limiting the therapeutic index (Chen and Sharon, 2013).

Aicardi-Goutieres syndrome is a congenital disorder that develops in response to hyper-activation of the immune system that primarily leads to postnatal microcephaly and widespread skin lesions (Aicardi and Goutieres, 1984; Bonnemann and Meinecke, 1992; Lanzi et al., 2003). Genetic studies have identified several genes that may contribute to this condition, including TREX1 (Three prime repair exonuclease 1), which codes for a 3’ exonuclease involved in DNA proofreading and repair (Crow et al., 2006a, b; Rice et al., 2009, 2012, 2014). Decreased TREX1 function allows for the intracellular accumulation of DNA fragments that would normally be degraded, essentially mimicking an effect of viral infection, thereby stimulating a prolonged immune response (Ishii and Akira, 2005; Lehtinen et al., 2008; Yang et al., 2007). Inappropriate activation of the immune response is thought to be neurotoxic, either directly or indirectly, and thus cause microcephaly (Peschke et al., 2016). Since TREX1 dysfunction increases immune-mediated neurotoxicity, one group has suggested that TREX1-mutated lymphocytes could impede cancer growth (Pulliero et al., 2012). The authors cultured neuroblastoma, a cancer of the peripheral nervous system, cells in the presence of TREX1-mutated T-cells and saw that tumor cell growth was significantly inhibited. This study illustrates how insights from microcephalic disorders can even inform development of novel cancer immunotherapies.
Another possible target for cancer therapy is WDR62 (WD repeat domain 62), which is mutated in primary, autosomal recessive microcephaly (Bastaki et al., 2016; Bhat et al., 2011; Kousar et al., 2011). Patients with WDR62 mutations have microcephaly, pachygyria, polymicrogyria, gyral thickening, microlissencephaly, and cortical dysplasia (Bilguvar et al., 2010; Murdock et al., 2011). Interestingly, WDR62 has a similar function to ASPM, so microcephaly may proceed from dysregulation of analogous mechanisms. In Drosophila, the WDR62 ortholog cg7337 was described as a microtubule-associated protein that stabilizes interphase microtubules in neuroblasts, regulates their mitotic entry, and ensures proper mitotic centrosome positioning and spindle orientation (Ramdas Nair et al., 2016). Similarly, in mammalian cells, WDR62 has been found to co-localize with ASPM to mitotic spindle poles in proliferating neural precursor cells (Nicholas et al., 2010; Yu et al., 2010). Wdr62 deletion in mice leads to mitotic arrest in neural stem and progenitor cells as a consequence of spindle instability and activation of the spindle assembly checkpoint, which ultimately results in cell death (Chen et al., 2014). It has also been shown that deficiency of WDR62 in cortical progenitors can induce premature differentiation (Xu et al., 2014). Thus far, one study has found a link between WDR62 and cancer (Zhang et al., 2013). WDR62 protein levels were increased in 6 ovarian cancer cell lines and ovarian tumor samples from 75 patients, with greater expression in more advanced cancers. Similar to CDK6 depletion, overexpression of WDR62 was associated in tumor samples with supernumerary centrosomes, suggesting that CDK6 and WDR62 may have opposing roles in mitotic spindle organization (Hussain et al., 2013). More research is needed to determine if WDR62 is also upregulated in other cancers, but nonetheless, its similar role to ASPM in neural stem/progenitor cells includes it as a possible target for brain cancer therapy.

One recent report has implicated SASS6 (SAS6 centriolar assembly protein) mutations as being responsible for a form of primary, autosomal recessive
microcephaly (Khan et al., 2014). Three members of a consanguineous Pakistani family were affected with microcephaly and found to have missense mutations in SASS6. The protein coded by this gene, SAS6 (Spindle assembly abnormal protein 6 homolog), regulates centrosome cohesion, assembly, and duplication (Rodrigues-Martins et al., 2007). Mutation in CEP135 (Centrosomal protein 135) has also been linked to human microcephaly, and its protein product is believed to directly interact with SAS6 to regulate centrosome activity (Hussain et al., 2012; Lin et al., 2013). To date, only one report has suggested SASS6 over-expression in human cancers (Shinmura et al., 2015). The authors found SASS6 mRNA and protein to be increased in a panel of colorectal cancer samples, and SASS6 overexpression in colorectal cancer cells was seen to precipitate mitotic chromosomal abnormalities. Probing of the cancer genome atlas also revealed slightly increased SAS6 levels in bladder urothelial carcinoma, breast invasive carcinoma, head and neck squamous cell carcinoma, kidney renal clear cell carcinoma, lung adenocarcinoma, lung squamous cell carcinoma, prostate carcinoma, and uterine corpus endometrial carcinoma. Despite the paucity of research on SAS6, that it is a centrosome-associated protein disrupted in microcephaly like EG5, ASPM, CDK6, and WDR62 hints at its importance in neural stem/progenitor cells, which could make it a viable target for brain tumor treatment.

CENPE (Centromere protein E) is a centromere kinetochore-associated protein that accumulates in G2-phase and has activity in M-phase (Gudimchuk et al., 2013; Kim et al., 2010; Yen et al., 1991). The microtubule-dependent motor activity of CENPE allows it to align chromosomes in prometaphase (Cai et al., 2009; Kim et al., 2008). Depletion of CENPE results in chromosomal instability and mitotic arrest (Putkey et al., 2002; Yao et al., 2000). Mutation of the CENPE gene has been described in two siblings with microcephalic primordial dwarfism (Mirzaa et al., 2014). Although CENPE expression was not reduced in the patients cells, its activity
was altered, as the protein failed to accumulate in mitosis. Lymphoid cells from
these patients were found to display mitotic spindle abnormalities (monopolar and
multipolar), altered chromosome segregation, and mitotic arrest. While CENPE
function is disrupted in microcephaly, its expression has been found to be increased
in triple-negative breast cancers (Kung et al., 2014). siRNA knockdown and small
molecule inhibition of CENPE were found to inhibit proliferation of triple-negative
breast cancer cell lines and reduce tumor growth in a breast cancer xenograft model.
Similarly, CENPE inhibition has been shown to reduce the viability of pancreatic
carcinoma cell lines (Henderson et al., 2009). A high-throughput screen identified a
novel CENPE inhibitor that induced mitotic arrest and apoptosis in these cells.
Furthermore, CENPE inhibition had a strong anti-tumor effect on colon
adenocarcinomas xenografted onto mice – treatment increased mitotic arrest that
led to apoptosis (Wood et al., 2010). Another broad screen described sensitivity to
CENPE inhibition in multiple types of cancer cell lines (Ohashi et al., 2015), and
indeed, targeting CENPE for cancer treatment has gained attention (Wacker and
Kapoor, 2010). Several CENPE inhibitors are being explored in preclinical trials,
and one, GSK-923295, has completed Phase I clinical trials for refractory cancers.
The similar molecular function of CENPE to EG5 supports research into its
inhibition in treating malignant gliomas.

Cornelia de Lange syndrome is a congenital disorder that presents with a
spectrum of abnormalities, including growth failure, hypertrichosis, hearing
impairment, cleft palate, visual defects, cardiac abnormalities, seizures, and
microcephaly (Ireland et al., 1993; Jackson et al., 1993). Underlying etiology involves
mutation of the NIPBL (Nipped-B-like) gene in most cases, although mutations in
SMC1A (Structural maintenance of chromosomes 1A), SMC3, and HDAC8 (Histone
deaacetylase 8) have also been reported (Deardorff et al., 2007; Krantz et al., 2004;
Musio et al., 2006; Tonkin et al., 2004). All four genes code for cohesin proteins that
hold together sister chromatids after replication until their separation in mitosis, and disruption of at least NIPBL leads to mitotic arrest (Peters et al., 2008; Watrin et al., 2006). Studies in the developing CNS of zebrafish have shown that loss of the nipblb paralog promoted neural stem and progenitor cell apoptosis with concomitant reduced activity in the Wnt (Wingless-related integration site) signaling pathway (Pistocchi et al., 2013). These findings suggest that pathogenesis of the microcephalic component of Cornelia de Lange syndrome may be a result of NIPBL disruption leading to prolonged mitotic arrest that triggers apoptosis in neural stem/progenitor cells. On the other hand, upregulation of NIPBL has been described in tumor samples from patients with NSCLC, where its expression was correlated with worse pathologic features (Xu et al., 2015). NIPBL was also upregulated in NSCLC cell lines and its disruption promoted apoptosis, as with neural stem cells, and increased sensitivity to Paclitaxel and Gemcitabine. An oncogenic or therapeutic role in brain cancer has yet to be described.

A final candidate for novel brain cancer therapy is MECP2 (Methyl CpG-binding protein 2), a transcriptional regulator with a prominent role in the brain (Shahbazian et al., 2002b). Mutations in MECP2 are responsible for the X-linked neurodevelopmental disorder Rett syndrome, which presents around one-year of age as autism-like behaviors, motor deficits, GI disturbances, and sometimes microcephaly (Shahbazian and Zoghbi, 2002; Shahbazian et al., 2002a; Jacob et al., 2009). The pathogenesis of Rett syndrome is still not entirely clear, as the functions of MECP2 have yet to be fully elucidated (Guy et al., 2011). Nevertheless, the timing of symptoms in Rett syndrome suggests that MECP2 predominantly functions in neurons rather than neural stem or progenitor cells, and MECP2 has been shown to be important in synaptic function and neurotransmission (Armstrong, 2001; Armstrong et al., 1995; Armstrong, 2005). A recent study in zebrafish, however, found mecp2 to be important in promoting neural stem cell differentiation, and its
depletion increased the proportion of nestin+ stem cells in embryonic development (Gao et al., 2015). With regards to tumorigenesis, MECP2 silencing in prostate cancer cell lines was found to both decrease proliferation and increase apoptosis (Yaqinuddin et al., 2008). A separate study demonstrated human prostate cancer cell proliferation was dramatically attenuated by MECP2 disruption and that ectopic MECP2 expression actually promoted prostate cancer proliferation (Bernard et al., 2006). These opposing effects of MECP2 in neural stem cells versus prostate cancer cells raise concerns as to its viability as a cancer therapeutic, especially for stem/progenitor-derived brain cancers. Even as treatment for non-brain malignancies, inhibiting MECP2 may produce severely limiting neurotoxicity, which is supported by the observation that Rett syndrome is a postnatal condition and therefore MECP2 must be vitally important in post-mitotic neurons.

**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**

P.Y.L and T.R.G. conceived, wrote, and edited the manuscript.

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TABLES

Table 2.1: Several KIF11/EG5 inhibitors are in active clinical trials for a diverse range of non-primary brain cancers.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Cancer</th>
<th>Combination</th>
<th>Phase</th>
<th>Study Start</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ispinesib (SB-715992)</td>
<td>Recurrent head &amp; neck cancer</td>
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<td>II</td>
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<td>Metastatic prostate cancer</td>
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<td>II</td>
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<td>Metastatic malignant melanoma</td>
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<td>II</td>
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<td>II</td>
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<td>II</td>
<td>2004</td>
</tr>
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<td>Relapsed ovarian cancer</td>
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<td></td>
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<td>Solid tumors</td>
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<td>II</td>
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<td>Ovarian, NSCLC, prostate, colorectal, gastroesophageal, or head &amp; neck cancer</td>
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<td>2010</td>
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<td>Acute leukemia</td>
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<td>2011</td>
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<td>(Peg)filgrastim</td>
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<td>Filgrastim</td>
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<td>2015</td>
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<td>Advanced solid or hematologic cancers</td>
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Information from clinicaltrials.gov.

* Indicates current active phase or most recently reported completed phase.
Table 2.2: Three CDK6 inhibitors are in active clinical trials for CNS cancers.

<table>
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<th>Compound</th>
<th>Cancer</th>
<th>Combination</th>
<th>Phase</th>
<th>Study Start</th>
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<td>Palbociclib (PD-0332991)</td>
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<td>II</td>
<td>2009</td>
</tr>
<tr>
<td></td>
<td>Recurrent glioblastoma</td>
<td>None</td>
<td>II (T)</td>
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<td></td>
<td>Recurrent, refractory, or progressive CNS tumors</td>
<td>None</td>
<td>I (S)</td>
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<tr>
<td></td>
<td>Recurrent oligodendroglioma or oligoastrocytoma</td>
<td>None</td>
<td>II</td>
<td>2015</td>
</tr>
<tr>
<td></td>
<td>Triple-negative breast cancer w/ brain metastasis</td>
<td>Trastuzumab</td>
<td>II</td>
<td>2016</td>
</tr>
<tr>
<td></td>
<td>Metastatic malignant neoplasm to brain</td>
<td>None</td>
<td>II</td>
<td>2016</td>
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<tr>
<td>Ribociclib (LEE-011)</td>
<td>High grade gliomas after radiation</td>
<td>None</td>
<td>I/II</td>
<td>2015</td>
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<td></td>
<td>Recurrent glioblastoma or anaplastic glioma</td>
<td>None</td>
<td>I</td>
<td>2015</td>
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<tr>
<td></td>
<td>Preoperative glioma and meningioma</td>
<td>None</td>
<td>0</td>
<td>2016</td>
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<tr>
<td>Abemaciclib (LY-2835219)</td>
<td>Diffuse intrinsic pontine glioma</td>
<td>None</td>
<td>I</td>
<td>2015</td>
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Information from clinicaltrials.gov.
* Indicates current active phase or most recently reported completed phase. S: suspended, T: terminated.

Table 2.3: Two ATR inhibitors are in active clinical trials, but not for primary brain malignancies.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Cancer</th>
<th>Combination</th>
<th>Phase</th>
<th>Study Start</th>
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</thead>
<tbody>
<tr>
<td>VX-970 (VE-822)</td>
<td>Advanced solid tumors</td>
<td>None</td>
<td>I</td>
<td>2014</td>
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<td></td>
<td>Advanced/metastatic solid tumors</td>
<td>Irinotecan</td>
<td>I</td>
<td>2015</td>
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<tr>
<td></td>
<td>Head &amp; neck squamous cell carcinoma</td>
<td>Radiation &amp; Cisplatin</td>
<td>I</td>
<td>2015</td>
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<td></td>
<td>Brain metastases from NSCLC</td>
<td>Radiation</td>
<td>I</td>
<td>2015</td>
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<td></td>
<td>Recurrent ovarian, primary peritoneal, or fallopian tube cancer</td>
<td>Gemcitabine &amp;/or Carboplatin</td>
<td>II</td>
<td>2015</td>
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<tr>
<td></td>
<td>SCLC</td>
<td>Topotecan</td>
<td>I/II</td>
<td>2015</td>
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<td></td>
<td>Metastatic urothelial cancer</td>
<td>Gemcitabine &amp; Cisplatin</td>
<td>II</td>
<td>2015</td>
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<tr>
<td></td>
<td>Refractory solid tumors</td>
<td>Veliparib &amp; Cisplatin</td>
<td>I</td>
<td>2016</td>
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<td></td>
<td>Relapsed/refractory CLL, PLL, or B-cell lymphoma</td>
<td>None</td>
<td>I</td>
<td>2013</td>
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<td>AZD-6738</td>
<td>Advanced solid tumors</td>
<td>Radiation, Carboplatin, Olaparib, or MEDI-4736</td>
<td>I</td>
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<td>Refractory, metastatic cancer</td>
<td>Paclitaxel</td>
<td>I</td>
<td>2015</td>
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Information from clinicaltrials.gov.
* Indicates current active phase or most recently reported completed phase.
<table>
<thead>
<tr>
<th>Gene of interest</th>
<th>Protein function</th>
<th>Associated microcephalic disorder</th>
<th>Suggested roles in cancer</th>
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<td><strong>KIF11</strong></td>
<td>Mitotic spindle organization through microtubule association</td>
<td>Decreased function in primary, autosomal dominant microcephaly</td>
<td>Increased expression in bladder transitional carcinoma, pancreatic adenocarcinoma, and glioblastoma: disruption reduces proliferation</td>
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<td><strong>ASPM</strong></td>
<td>Mitotic spindle organization through microtubule association</td>
<td>Decreased function in primary, autosomal recessive microcephaly</td>
<td>Increased expression in glioblastoma and medulloblastoma: disruption reduces tumorigenesis</td>
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<td><strong>CDK6</strong></td>
<td>G₁-S checkpoint regulation and possibly mitotic spindle organization</td>
<td>Decreased function in primary, autosomal recessive microcephaly</td>
<td>Increased expression in many cancers, including glioblastoma and medulloblastoma: disruption reduces proliferation</td>
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<td><strong>ATR</strong></td>
<td>DNA damage response and possibly M-phase checkpoint regulation</td>
<td>Decreased function in Seckel syndrome</td>
<td>Disruption reduces medulloblastoma tumorigenesis</td>
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<td><strong>NBS1</strong></td>
<td>DNA damage response</td>
<td>Decreased function in Nijmegen breakage syndrome</td>
<td>Disruption increases radiation sensitivity in glioblastoma cells</td>
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<td><strong>IGF1R</strong></td>
<td>Mutli-tissue cell proliferation and survival; CNS synaptogenesis</td>
<td>Decreased function in primary, autosomal recessive microcephaly</td>
<td>Increased expression in medulloblastoma, especially metastatic</td>
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<td><strong>TREX1</strong></td>
<td>DNA proofreading and repair</td>
<td>Decreased function in Aicardi-Goutieres syndrome</td>
<td>Disruption in T-cells increases immune-mediated killing of neuroblastoma cells</td>
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<td><strong>WDR62</strong></td>
<td>Mitotic spindle organization through microtubule association</td>
<td>Decreased function in primary, autosomal recessive microcephaly</td>
<td>Increased expression in ovarian cancer</td>
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<td><strong>SASS6</strong></td>
<td>Centrosome cohesion, assembly, and duplication</td>
<td>Decreased function in primary, autosomal recessive microcephaly</td>
<td>Increased expression in colorectal cancer</td>
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<td><strong>CENPE</strong></td>
<td>Mitotic chromosome alignment</td>
<td>Decreased function in microcephalic primordial dwarfism</td>
<td>Disruption reduces viability of triple-negative breast cancer, pancreatic carcinoma, and colon adenocarcinoma cells</td>
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<td><strong>NIPBL</strong></td>
<td>Sister chromatid cohesion</td>
<td>Decreased function in Cornelia de Lange syndrome</td>
<td>Increased expression in non-small cell lung cancer: disruption induces apoptosis</td>
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<td><strong>MECP2</strong></td>
<td>Transcriptional repression and neurotransmission</td>
<td>Decreased function in Rett syndrome</td>
<td>Disruption reduces proliferation in prostate cancer cells</td>
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Figure 2.1: Microcephalic disorders are characterized by disruptions in mitotic regulators, which could be targeted for brain cancer therapy. (A) EG5/KIF11 is a heterotetrameric, plus-end-directed microtubule motor protein that pushes apart the duplicated centrosomes to create a bipolar mitotic spindle in prophase and prometaphase, which allows for separation of sister chromatids (left). In the absence of EG5/KIF11, the centrosomes fail to separate, resulting in the formation of a monoaster, which triggers mitotic arrest that can lead to apoptosis (right). (B) ASPM is a microtubule minus-end-associated protein found at mitotic spindle poles that has been implicated in both mediating symmetric cell divisions and orienting the cell division plane in neural stem and progenitor cells (left). The absence of ASPM creates more asymmetric divisions and altered division planes, both of which can promote differentiation over continued proliferation (right). (C) One report has found that CDK6 associates with mitotic centrosomes, possibly mediating centrosome duplication, spindle assembly, and cellular motility (left). The CDK6 mutation associated with microcephalic patients did not reduce CDK6 protein levels but rather altered its function and behavior such that it no longer associated with centrosomes, which led to supernumerary centrosomes, inappropriate mitotic spindle alignment, abnormal cellular polarity, and reduced cell motility (right). (D) In CGNPs, endogenous, replication-associated DNA damage activates the DNA damage response protein ATR, which recognizes single-stranded DNA coated by RPA at stalled replication forks, simplified here. Activated ATR can promote G$_2$/M arrest by barring mitotic entry through preventing CDK1/CDC2 activation and by targeting CEP63 to block assembly of the mitotic spindle (left). Absence of ATR both limits DNA repair and permits mitotic entry in CGNPs. Progression through mitosis exacerbates DNA damage in the form of severe chromosomal aberrations. Overwhelming DNA damage causes ATM to activate p53, which primarily induces apoptosis in postnatal CGNPs.
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involved in malignant progression of gliomas and constitutes an attractive

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Tuysuz, B., Caglayan, A. O., Gokben, S., Kaymakcalan, H., Barak, T., Bakircioglu,
M., Yasuno, K., Ho, W., Sanders, S., Zhu, Y., Yilmaz, S., Dincer, A., Johnson, M. H.,
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Kumandas, S., Topcu, M., Ozmen, M., Sestan, N., Lifton, R. P., State, M. W., and

Blain, S. W., Montalvo, E., and Massague, J. (1997). Differential interaction of the
cyclin-dependent kinase (cdk) inhibitor p27kip1 with cyclin a-cdk2 and cyclin

Phosphorylation by p34cdc2 regulates spindle association of human eg5, a


CHAPTER 3: ATR MAINTAINS CHROMOSOMAL INTEGRITY DURING POSTNATAL CEREBELLAR NEUROGENESIS AND IS REQUIRED FOR MEDULLOBLASTOMA FORMATION

Overview

Microcephaly and medulloblastoma may both result from mutations that compromise genomic stability. We report that ATR, which is mutated in the microcephalic disorder Seckel syndrome, sustains cerebellar growth by maintaining chromosomal integrity during postnatal neurogenesis. Atr deletion in cerebellar granule neuron progenitors (CGNPs) induced proliferation-associated DNA damage, p53 activation, apoptosis and cerebellar hypoplasia in mice. Co-deletions of either p53 or Bax and Bak prevented apoptosis in Atr-deleted CGNPs, but failed to fully rescue cerebellar growth. ATR-deficient CGNPs had impaired cell cycle checkpoint function and continued to proliferate, accumulating chromosomal abnormalities. RNA-Seq demonstrated that the transcriptional response to ATR-deficient proliferation was highly p53-dependent and markedly attenuated by p53 co-deletion. Acute ATR inhibition in vivo by nanoparticle-formulated VE-822 reproduced the developmental disruptions seen with Atr deletion. Genetic deletion of Atr blocked tumorigenesis in medulloblastoma-prone SmoM2 mice. Our data show that p53-driven apoptosis and cell cycle arrest – and, in the absence of p53, non-apoptotic cell death – redundantly limit growth in ATR-deficient progenitors. These mechanisms may be exploited for treatment of CGNP-derived medulloblastoma using ATR inhibition.
Introduction

Appropriate brain development requires genomic fidelity. Diverse mutations that increase DNA damage can restrict brain growth, causing microcephaly (Lee and McKinnon, 2007; McMahon et al., 2014; Orii et al., 2006; Rosin et al., 2015; Williams et al., 2015). Combined with p53 deletion, mutations in DNA repair genes may produce unrestricted growth in medulloblastoma (Frappart et al., 2009; Lee and McKinnon, 2002; Tong et al., 2003), a malignant tumor of neural progenitors. The serine/threonine kinase ATR (Ataxia Telangiectasia and Rad3-related protein) mitigates proliferation-associated DNA damage (Brown and Baltimore, 2000) and has been implicated in microcephaly in Seckel syndrome (O’Driscoll et al., 2003). In mice, conditional deletion of Atr throughout the CNS during embryonic development similarly induces microcephaly, but particularly affects the cerebellum and ganglionic eminence (Lee et al., 2012). The precise mechanisms that cause DNA damage to impair growth specifically in the brain are unknown. We investigated the processes that restrict growth when Atr is deleted in the cerebellum in order to shed light on the pathogenesis of microcephaly associated with DNA damage and to determine whether the reliance of neural progenitors on ATR persists in progenitor-derived medulloblastoma.

Cerebellar growth depends on the postnatal proliferation of cerebellar granule neuron progenitors (CGNPs) in the external granular layer (EGL) along the outside of the cerebellum, which peaks between postnatal day (P) 5 and 7 (Hatten and Heintz, 1995). Although Atr deletion in Nestin (NES)+ progenitors blocks cerebellar development prior to postnatal neurogenesis (Lee et al., 2012), the specific cellular processes restricting the growth of ATR-deficient progenitors have not been discerned. In vitro, ATR reduces DNA damage during proliferation by stabilizing stalled replication forks and by arresting the cell cycle to allow for DNA repair (Cimprich and Cortez, 2008). Replication fork collapse following ATR disruption
produces DNA double-strand breaks (DSBs), which are considered the most toxic form of DNA damage (Couch et al., 2013). ATR also maintains S- and G2-phase checkpoints by phosphorylating CHK1 (CHEK1) in response to DNA damage (Smith et al., 2010). Determining how these functions combine in vivo to sustain neural progenitors is essential to understanding both the pathogenesis of Seckel syndrome and the requirement for ATR in brain development.

We found that ATR-deficient CGNPs continued to proliferate despite accumulating DNA damage, which induced population-wide, p53-dependent apoptosis. Blocking apoptosis in ATR-deficient CGNPs through co-deletion of the key apoptotic mediators Bax and Bak (Bak1) or co-deletion of p53 (Tp53) did not fully rescue the resulting cerebellar hypoplasia. Rather, premature cell cycle exit in Atr//Bax/Bak mutants or non-apoptotic cell death in Atr/p53 mutants redundantly limited CGNP population growth. Atr-deleted CGNPs also demonstrated diverse chromosomal abnormalities that were intensified by restriction of apoptosis. CGNPs are the cells of origin for Sonic hedgehog (SHH) subgroup medulloblastomas; we found that Atr deletion blocked the tumorigenic effect of constitutive SHH activation in transgenic SmoM2 mice. These investigations define a crucial role for ATR in maintaining genomic integrity during brain development and suggest that ATR dependence might be exploited for medulloblastoma therapy.

Results

Atr deletion induces CGNP apoptosis and cerebellar hypoplasia

To analyze ATR function in CGNPs, we generated mice with conditional Atr deletion (Brown and Baltimore, 2003) in the MATH1 lineage. Math1 (Atoh1) is expressed by CGNPs in a rostrocaudal progression beginning at the anterior margins of the cerebellar cortex at embryonic day (E) 12.5 (Hatten and Roussel, 2013).
We crossed the Math1-Cre and Atr\textsuperscript{loxP/loxP} mouse lines to generate Math1-Cre; Atr\textsuperscript{loxP/loxP} (Atr\textsuperscript{M-cre}) mice.

Atr\textsuperscript{M-cre} mice were viable and fertile, but by P12 displayed tremor and ataxia, suggesting impaired postnatal neurogenesis. These animals were born with hindbrains of normal appearance, with the EGL spread over the primordial cerebellum. By P3, however, the EGL in Atr\textsuperscript{M-cre} mice was noticeably thinner and less foliated than in Math1-Cre littermate controls with intact Atr (Math1-Cre; Atr\textsuperscript{+/+} or Math1-Cre; Atr\textsuperscript{loxP/+}). Cerebellar hypoplasia in Atr\textsuperscript{M-cre} mice became progressively more apparent with age (Fig. 3.1A).

We noted sparing of the posterior regions of the cerebellum in Atr\textsuperscript{M-cre} mice (Fig. 3.1A, middle row). To determine if this sparing was the result of incomplete recombination in these regions or was due to reduced dependence on ATR, we used hGFAP-Cre to delete Atr throughout the CGNP population. hGFAP-Cre mice express Cre recombinase in neural stem cells, targeting all cells of the cerebellum except the Purkinje cells (Andrae et al., 2001). In the resulting hGFAP-Cre; Atr\textsuperscript{loxP/loxP} (Atr\textsuperscript{G-cre}) mice, we found hypoplasia across the entire EGL. The EGL was thinned by P0 and completely lost by P7. These mice showed more severe tremor and died by P18 (Fig. 3.1A, bottom row; Fig. 3.S1A,B).

To understand the cause of cerebellar hypoplasia in Atr mutants, we analyzed patterns of proliferation, DNA damage and apoptosis in Atr\textsuperscript{M-cre} and Atr\textsuperscript{G-cre} mice by immunohistochemistry (IHC). Although prior studies showed that deletion of Atr in Nes-expressing progenitors blocks the prenatal proliferation of CGNP precursors (Lee et al., 2012), we observed no reduction in the postnatal proliferation of CGNPs in P3 Atr\textsuperscript{M-cre} or P0 Atr\textsuperscript{G-cre} cerebella, as demonstrated by the fraction of EGL cells expressing the mitosis marker phosphorylated histone H3 (pH3) (Fig. 3.1B,C, Fig. 3.S1C) or the cycling cell marker proliferating cell nuclear antigen (PCNA) (Fig. 3.S3D). However, we found significantly increased DNA damage as demonstrated by
phosphorylated histone H2A.X (γH2A.X), increased activation of the cellular DNA damage response as shown by phosphorylated ATM (p-ATM) and p53 (p-p53), and increased apoptosis as shown by the levels of cleaved Caspase-3 (cC3) (Fig. 3.1B,C, Fig. 3.S1C,D). Atr-deleted CGNPs therefore proliferate robustly, but accrue extensive DNA damage and undergo apoptosis.

**Deletion of Bax and Bak prevents cell death in Atr-mutant CGNPs**

Extensive apoptosis complicated further analysis of ATR-deficient CGNPs. Prior studies showed that deletion of the apoptosis gatekeeper Bax is sufficient to stabilize CGNPs with radiation-induced or proliferation-associated DNA damage (Chong et al., 2000; Garcia et al., 2013; Williams et al., 2015). We found, however, that apoptosis of Atr-deleted CGNPs and cerebellar hypoplasia were not prevented by Bax co-deletion in Math1-Cre;Atr<sup>loxP/loxP</sup>;Bax<sup>loxP/loxP</sup> (Atr;<sup>M-cre</sup>Bax) animals (Fig. 3.2A, Fig. 3.S2A,B). Co-deletion of Atr and the Bax homolog Bak also failed to stabilize the CGNP population in Math1-Cre;Atr<sup>loxP/loxP</sup>;Bak<sup>−/−</sup> (Atr;<sup>M-cre</sup>Bak) mice (Fig. 3.S2A,B). However, combining genetic deletion of both Bax and Bak in ATR-deficient mice (Math1-Cre;Atr<sup>loxP/loxP</sup>;Bax<sup>loxP/loxP</sup>;Bak<sup>−/−</sup>, or Atr;Bax;Bak<sup>M-cre</sup>) effectively prevented CGNP apoptosis, partially rescuing cerebellar growth (Fig. 3.2A,B, Fig. 3.S2A,C). TUNEL staining did not detect non-apoptotic cell death in Atr;Bax;Bak<sup>M-cre</sup> cerebella that might have been missed by cC3 studies (Fig. 3.2B,D). These data show that Atr deletion in CGNPs, in contrast to other pro-apoptotic stimuli such as radiation, activated both BAX- and BAK-driven apoptosis.

We compared markers of proliferation, DNA damage and the DNA damage response in CGNPs of P3 Atr;<sup>M-cre</sup>Bax, Atr;Bax;Bak<sup>M-cre</sup>, and Atr-intact Math1-Cre;Bax<sup>loxP/loxP</sup>;Bak<sup>−/−</sup> (Bax;Bak<sup>M-cre</sup>) controls by IHC. The fractions of pH3<sup>+</sup> and PCNA<sup>+</sup> CGNPs were similar in Atr-mutant and Atr-intact genotypes (Fig. 3.2D).
CGNPs in Atr;Bax<sup>M-cre</sup> and Atr;Bax;Bak<sup>M-cre</sup> mice showed similar levels of γH2A.X. However, p-ATM, p-p53, and the cell cycle inhibitor p21 (CDKN1A) were all markedly increased in Atr;Bax;Bak<sup>M-cre</sup> CGNPs (Fig. 3.2B,D, Fig. 3.S2B,C). Apoptosis-disabled, ATR-deficient CGNPs were thus able to activate p53 without undergoing cell death.

**Atr- and p53-double-mutant CGNPs undergo caspase-independent cell death**

To determine the functional significance of p53 activation in CGNPs of Atr<sup>M-cre</sup> mice, we examined the phenotype of Math1-Cre;Atr<sup>loxP/loxP</sup>;p53<sup>loxP/loxP</sup> (Atr;p53<sup>M-cre</sup>) mice. p53 deletion prevents CGNP apoptosis after radiation-induced DNA damage (Herzog et al., 1998). p53 deletion did not completely rescue cerebellar hypoplasia in Atr mutants (Fig. 3.2A, Fig. 3.S3A), despite effective suppression of the p53 axis, as demonstrated by the absence of p21 and p53 activation (Fig. 3.2C,D). The fraction of pH3<sup>+</sup> and PCNA<sup>+</sup> CGNPs in Atr;p53<sup>M-cre</sup> mice did not differ from Atr- intact controls at P3 (Fig. 3.2D, Fig. 3.S3B,D). Atr;p53<sup>M-cre</sup> CGNPs accumulated significantly more DNA damage than Atr<sup>M-cre</sup> or Atr;Bax;Bak<sup>M-cre</sup> CGNPs (Fig. 3.2D). This increased level of DNA damage did not trigger activation of Caspase-3 (Fig. 3.2D). However, TUNEL staining demonstrated that cell death had occurred without caspase activation (Fig. 3.2C,D).

Atr;p53<sup>M-cre</sup> CGNPs showed abnormal morphologies that were different from the pyknotic nuclei found in the Atr<sup>M-cre</sup> EGL. These features included giant, multinucleated cells and cells with micronuclei (Fig. 3.S3D), consistent with published descriptions of p53-deficient cancer cells undergoing necrosis after DNA damage (Vakifahmetoglu et al., 2008). To examine whether Atr;p53<sup>M-cre</sup> CGNPs were dying through regulated necrosis, we compared the phosphorylation of MLKL, an essential step in the necroptosis pathway (Cai et al., 2014). We found scattered
CGNPs showing phosphorylated MLKL (p-MLKL) in Math1-Cre, p53<sup>M-cre</sup>, and Atr;p53<sup>M-cre</sup> mice, but no increase in the Atr;p53<sup>M-cre</sup> genotype (Fig. 3.S3F). Thus, ATR/p53-deficient CGNPs undergo cell death without evidence of activating the apoptotic or necroptotic pathways.

**Accelerated cell cycle exit in Atr;Bax;Bak<sup>M-cre</sup> CGNPs**

To account for the incomplete rescue of cerebellar growth in Atr;Bax;Bak<sup>M-cre</sup> and Atr;p53<sup>M-cre</sup> mice despite the prevention of CGNP apoptosis, we investigated CGNP proliferation over time. We compared the mitotic rate of Atr<sup>M-cre</sup>, Atr;Bax;Bak<sup>M-cre</sup>, and Atr;p53<sup>M-cre</sup> CGNPs in the EGL with Atr-intact controls at P3, P5 and P7. We first determined that EGL mitotic rates did not vary significantly between the Atr-intact Math1-Cre, Bax;Bak<sup>M-cre</sup>, and p53<sup>M-cre</sup> control genotypes, allowing us to pool controls (Fig. 3.S3G). The mitotic rates of Atr<sup>M-cre</sup> CGNPs did not differ from controls with any statistical significance at P3, P5 or P7: both groups showed a small decrease in proliferation by P7 (Fig. 3.2E). By contrast, the proliferation rate of Atr;Bax;Bak<sup>M-cre</sup> CGNPs decreased markedly from P3 to P5 and from P5 to P7, and was significantly lower than in controls at both P5 and P7. These data show that non-proliferating cells were increased in the P5 and P7 EGL of Atr;Bax;Bak<sup>M-cre</sup> mice but not in Atr<sup>M-cre</sup> mice with intact apoptosis. This increased cell cycle exit at P5 and P7 was p53-dependent, as the mitotic rate of apoptosis-disabled Atr;p53<sup>M-cre</sup> CGNPs was significantly higher than the Atr;Bax;Bak<sup>M-cre</sup> CGNP mitotic rate at both P5 and P7.

We noted clusters of cells in P20 Atr;Bax;Bak<sup>M-cre</sup> cerebella in the typically depopulated region where the EGL had been. These cells expressed the neuronal marker NEUN (RBFOX3) (Fig. 3.2F) and were PCNA<sup>-</sup> (data not shown), identifying them as differentiated neurons. The location of these neurons suggests that they derive from CGNPs, and similar ectopic neurons were not seen in Atr<sup>M-cre</sup>,
Atr;\textit{p53}\textsuperscript{M-cre}, or Atr-intact control cerebella. These observations provide evidence that premature, p53/p21-driven CGNP cell cycle exit after P3 limits the rescue effect of disabled apoptosis in Atr;Bax;Bak\textsuperscript{M-cre} cerebella.

**Cell cycle checkpoint failure in ATR-deficient CGNPs with DNA damage**

ATR coordinates the cellular response to replication stress by activating cell cycle checkpoints (Abraham, 2001). Our studies of cerebellar sections indicated that Atr-deleted CGNPs continue to proliferate after developing detectable DNA damage. To assess checkpoint function in Atr-mutants, we used fluorescence-activated cell sorting (FACS) to analyze freshly isolated CGNPs from the cerebella of P3 mice with and without Atr deletion, including A\textit{tr}\textsuperscript{M-cre}, Atr;Bax;Bak\textsuperscript{M-cre}, and Atr;\textit{p53}\textsuperscript{M-cre} knockout genotypes as well as Math1-Cre, Bax;Bak\textsuperscript{M-cre}, and \textit{p53}\textsuperscript{M-cre} controls. All cells were stained for DNA content (FxCycle Violet), \(\gamma\)H2A.X, and pH3.

Atr-deleted genotypes showed a strongly \(\gamma\)H2A.X\(^+\) subpopulation that was not seen in controls (Fig. 3.S4A). To generate Atr-intact CGNPs with comparable DNA damage, we subjected P3 wild-type (WT) mice to X-ray radiation (2Gy) and then isolated and analyzed CGNPs 2hr. later. These WT, irradiated CGNPs showed \(\gamma\)H2A.X staining that was comparable in intensity to the \(\gamma\)H2A.X\(^+\) CGNPs in Atr-deleted mice (Fig. 3.S4A). DNA content staining effectively identified cells at G\textsubscript{1}-, S-, and G\textsubscript{2}/M-phases (Fig. 3.S4A), and pH3 staining distinguished G\textsubscript{2} from M-phase (Fig. 3.S4B). We found no statistically significant differences in cell cycle distribution between non-irradiated control genotypes (Fig. 3.S4C), and these controls were pooled for further analysis.

Without \(\gamma\)H2A.X selection, comparison of P3 CGNP cell cycle distribution between controls and Atr-deleted genotypes showed a significant difference only in the Atr;Bax;Bak\textsuperscript{M-cre} CGNPs. These cells showed enrichment of G\textsubscript{2} at the expense of G\textsubscript{1}-phase (Fig. 3.3A), consistent with either delayed progression or G\textsubscript{2} arrest.
However, comparing the γH2A.X+ subsets from Atr-deleted genotypes and irradiated, WT mice, we noted marked differences in M-phase. All three Atr-deleted genotypes demonstrated CGNPs that were both γH2A.X+ and pH3+ (Fig. 3.3B). By contrast, pH3+ CGNPs were almost undetectable in the irradiated, WT mice (Fig. 3.3B).

FACS results were confirmed by dual immunofluorescence (IF) for pH3 and γH2A.X or phospho-53BP1 (p-53BP1; 53BP1 is also known as TP53BP1), another marker of DNA damage (Fig. 3.3C). First, in WT, non-irradiated controls, pH3+ cells were always negative for γH2A.X and p-53BP1. Second, in irradiated, WT mice, CGNPs that were γH2A.X+ or p-53BP1+ were never pH3+. Finally, in all Atr-deleted genotypes, γH2A.X+/pH3+ and p-53BP1+/pH3+ cells were common (Fig. 3.3C). The γH2A.X+/pH3+ CGNPs in Atr-deleted genotypes either entered M-phase with DNA damage or persisted in M-phase after acquiring DNA damage. These data do not conclusively show whether γH2A.X+, Atr-deleted CGNPs are able to complete mitosis. However, Atr deletion clearly permitted cells with DNA damage to be mitotic—a condition that is not seen in Atr-intact CGNPs.

Additionally, in each of the Atr-deleted genotypes, we found that γH2A.X+ CGNPs were distributed across all phases of the cell cycle, with relative enrichment in S-, G2-, and M-phases, compared with γH2A.X+ CGNPs from irradiated, WT mice (Fig. 3.3D). Consistent with these data, IF studies showed colocalization of γH2A.X with markers of G1- (Cyclin D1), S- (BrdU), and G2- (Cyclin B1) phases (Fig. 3.3D) in AtrM-cre cerebella. G1 depletion in Atr-deleted, γH2A.X+ CGNPs could indicate either loss of H2A.X phosphorylation in G1 cells through DNA repair, or slowed return of γH2A.X+ cells to G1. The abundance of γH2A.X+ cells in each phase of the cell cycle, however, suggests that CGNPs with Atr deletion and DNA damage can progress through the cell cycle and, in the case of Atr;Bax;BakM-cre, have not all permanently arrested by P3. Altogether, at this single time point, most ATR-deficient CGNPs did not show evidence of impaired cell cycle function. However, Atr
deletion increased the fraction of CGNPs with DNA damage and altered the barriers that normally prevent them from undergoing mitosis.

FACS data confirmed the increased cell death seen in Atr<sup>M-cre</sup> and Atr;<i>p53</i><sup>M-cre</sup> CGNPs, which both showed increased sub-G<sub>1</sub> fractions. We did not detect an increase in sub-G<sub>1</sub> γH2A.X<sup>+</sup> CGNPs in Atr;<i>Bax;Bak</i><sup>M-cre</sup> cerebella (Fig. 3.3E). These increases in sub-G<sub>1</sub> fractions are consistent with our observations of apoptotic cell death in the Atr<sup>M-cre</sup> genotype and non-apoptotic cell death in the Atr;<i>p53</i><sup>M-cre</sup> genotype.

**ATR protects CGNP chromosome integrity**

Our finding of impaired checkpoint function suggested that genomic fidelity might be impaired in Atr-deleted CGNPs. In vitro studies have shown incomplete DNA replication and chromosomal fragmentation in Atr-mutant cells (Brown and Baltimore, 2000). To determine if chromosomal damage accumulates in ATR-deficient cells in vivo and to quantify the damage, we karyotyped Atr-deleted CGNPs freshly harvested from P3 cerebella. We dissociated CGNPs from Atr<sup>M-cre</sup>, Atr;<i>Bax;Bak</i><sup>M-cre</sup>, and Atr;<i>p53</i><sup>M-cre</sup> mice and Math1-Cre, Bax;<i>Bak</i><sup>M-cre</sup>, and <i>p53</i><sup>M-cre</sup> controls. Dissociated cells were treated ex vivo with Colcemid, and metaphase spreads were analyzed by conventional karyotyping and spectral karyotyping (SKY). We noted frequent chromosome breaks in all Atr-mutant genotypes and a spectrum of abnormalities, including dicentric chromosomes, end-to-end fusions, deletions, translocations, and whole chromosome gains or losses (Fig. 3.4A). In addition, Atr;<i>p53</i><sup>M-cre</sup> CGNP chromosomes showed segregation defects (premature sister chromatid separation). These abnormalities were not detected in controls (Atr<sup>M-cre</sup> 35%, P<0.01; Atr;<i>Bax;Bak</i><sup>M-cre</sup> 48%, P<0.01; Atr;<i>p53</i><sup>M-cre</sup> 49%, P<0.01; Student’s t-test).

For statistical analysis, we divided metaphases with varying degrees of
chromosomal damage into four bins: 1-10 chromosome breaks/cell; >10 chromosome breaks/cell and <50% chromosomes fragmented (Mild); >10 chromosome breaks/cell, >50% chromosomes fragmented, and some preserved chromosome morphology (Moderate); and >10 chromosome breaks/cell, >50% chromosomes fragmented, and complete loss of chromosome morphology (Severe) (Fig. 3.4B). In all Atr-deleted genotypes, the frequencies of cells with all degrees of chromosomal damage were increased, but the increases in AtrM-cre cells with Moderate and Severe damage were not statistically significant. Moderate and Severe damage, however, were particularly enriched in the apoptosis-incompetent Atr;Bax;BakM-cre and Atr;p53M-cre genotypes (Fig. 3.4C). These data show that CGNPs require ATR to maintain chromosomal integrity during proliferation, and that apoptosis limits the accumulation of genetic abnormalities by removing damaged cells from the population. Karyotype analyses also reaffirm that CGNPs with damaged DNA progress to M-phase in Atr-mutants.

**Transcriptomic adaptations to ATR deficiency are predominantly p53-driven**

We used RNA-Seq to determine the transcriptomic alterations induced by proliferation-associated DNA damage in the absence of Atr. Despite a wide body of literature on the cellular consequences of ATR loss, the impact on the transcriptome had not previously been reported. The prolonged survival of Atr-deleted CGNPs in Atr;Bax;BakM-cre and Atr;p53M-cre mice allowed us to analyze mRNA abundance without losing cells with DNA damage from the population through cell death. We isolated and purified total RNA for RNA-Seq analysis from the cerebella of apoptosis-incompetent, ATR-deficient P3 mice (Atr;Bax;BakM-cre and Atr;p53M-cre) and corresponding age-matched controls (Bax;BakM-cre and p53M-cre). The abundance of mRNAs may be altered by changes in either production or degradation. ATR has been implicated in RNA processing (Chandris et al., 2010), and Atr deletion may
alter both gene transcription and mRNA stability. Our data thus identify transcriptomic, rather than transcriptional changes.

We noted that Atr deletion evoked a richer, more complex pattern of transcriptomic changes in the Bax/Bak-co-deleted background compared with the p53-co-deleted background. Principal component analysis (PCA) demonstrated that the second component of variation explained 16% of variance in global gene expression, and separated Atr;Bax;Bak\textsuperscript{M-cre} from Bax;Bak\textsuperscript{M-cre} (P=4.41x10\textsuperscript{-7}, Hotelling’s t-square test) but not Atr;p53\textsuperscript{M-cre} from p53\textsuperscript{M-cre} (P=0.20, Hotelling’s t-square test) (Fig. 3.5A). This difference in separation on PCA indicates that p53-deleted CGNPs are more restricted in their transcriptomic response to ATR deficiency. Consistent with reduced transcriptional responsiveness with Atr/p53-co-deletion, the number of genes differentially expressed in Atr;Bax;Bak\textsuperscript{M-cre} cerebella compared with controls was significantly greater than in Atr;p53\textsuperscript{M-cre} cerebella compared with controls (P=2.8x10\textsuperscript{-52}, Fisher’s exact test). Using a false discovery rate (FDR) of <0.01 and considering only genes meeting a fold-change (FC) criterion of |log\textsubscript{2}(knockout/control)| >1.5, we identified 339 genes differentially expressed by ATR-deficient cells in the Bax/Bak-deleted background, as compared with 56 genes differentially expressed by ATR-deficient cells in the p53-deleted background, with 9 genes represented in both sets (Fig. 3.5B, Tables 3.S1 and 3.S2). This overlap demonstrates a high degree of correspondence between the gene sets (P=2.0x10\textsuperscript{-8}, hypergeometric test). However, P-values for differentially expressed genes were consistently smaller in the p53-deleted comparison, as visualized in plots of FC versus significance (Fig. 3.5C), indicating an overall smaller transcriptomic effect of Atr deletion in the absence of p53. Expression microarray analysis comparing mRNA from Atr;Bax;Bak\textsuperscript{M-cre} versus Bax;Bak\textsuperscript{M-cre} cerebella defined a gene set that corresponded well with the differential gene set defined by RNA-Seq (Fig. 3.5A). By contrast, microarray analysis was unable to identify any
differentially expressed genes in the $Atr;p53^{M-cre}$ versus $p53^{M-cre}$ comparison, consistent with an overall low signal-to-noise ratio in the absence of $p53$.

Pathway analysis of the 339 genes differentially expressed in $Atr;Bax;Bak^{M-cre}$ CGNPs demonstrated a strong correspondence between these genes and the molecular signature of p53-dependent transcription. Activation of p53 can induce both apoptosis and cell cycle arrest (Zuckerman et al., 2009), and we noted that regulators of both processes were enriched in the $Atr;Bax;Bak^{M-cre}$ gene set (Table 3.1). By contrast, we did not identify any pattern in $Atr;p53^{M-cre}$ CGNPs that could be attributed to a specific transcriptional regulator (Table 3.3) and, consistent with the lack of increased MLKL activation, we found no elevation of necroptosis-associated genes such as RIP kinases.

We used IF to determine the protein expression patterns of Trophinin (TRO), which was among the 9 genes identified as differentially expressed in both $Atr$-deleted genotypes, and for which we were able to obtain effective antibodies. Examining $Bax;Bak^{M-cre}$ and $p53^{M-cre}$ control brains, we found that TRO was expressed in all differentiated neurons of the brain, but absent in progenitor regions (Fig. 3.5D). In $Atr$-deleted $Atr;Bax;Bak^{M-cre}$ and $Atr;p53^{M-cre}$ cerebella, however, TRO was detected within the CGNP layer (EGL), indicating upregulation of the protein as well as the RNA.

From the larger set of 124 genes that were upregulated in the $Atr$-mutant cerebella of both $p53$-deleted and $Bax/Bak$-deleted backgrounds with FDR<0.01, irrespective of FC, we selected $Eif4ebp1$ for further analysis. EIF4EBP1 is known to regulate protein translation downstream of mTOR (Fingar et al., 2002), a PIKK kinase homologous to ATR (Lovejoy and Cortez, 2009). $Atr$-deleted CGNPs demonstrated marked upregulation of EIF4EBP1 protein. In control cerebella, EIF4EBP1 was limited to a subset of CGNPs at the outer margin of the EGL, whereas in $Atr$-deleted cerebella EIF4EBP1 was detected throughout the EGL (Fig.
Our *in situ* IF data, along with our microarray results, thereby validate our RNA-Seq findings.

We further examined our RNA-Seq data to determine whether *Atr* deletion produced recurrent point mutations or other changes in RNA sequence. We did not detect a significant change in mismatch rate associated with *Atr* deletion ([Fig. 3.S5B](#)). Fusion transcript detection software suggested a small number of fusion products, only one of which, with unknown biological function, emerged with statistical significance ([Fig. 3.S5C](#)). Together with our karyotype analysis, these data suggest that, although *Atr* deletion significantly altered chromosome structure, it did not substantially increase the rate of point mutations or recurrent fusion transcripts.

**ATR is required for medulloblastoma tumorigenesis**

Prior studies have shown that deletion of diverse DNA repair pathway genes, combined with *p53* deletion, induces medulloblastoma in mice ([Frappart et al., 2007], [2009]; [Holcomb et al., 2006]; [Lee and McKinnon, 2002]). To determine if *Atr* mutation predisposes mice to medulloblastoma, we followed the viability and neurologic function of 12 *Atr;p53* M-cre and 12 *Atr;Bax;Bak* M-cre mice for more than 300 days ([supplementary Materials and Methods](#)). These mice showed no progressive neurological changes or other evidence of tumorigenesis, and their cerebella remained hypoplastic (data not shown). The absence of tumors despite impaired genomic stability and *p53* deletion suggests that medulloblastoma tumorigenesis, like postnatal neurogenesis, might require ATR function.

We directly tested this suggested requirement for ATR in medulloblastoma tumorigenesis by deleting *Atr* in tumor-prone *hGFAP-Cre;SmoM2* (*SmoM2* G-cre) mice ([Schuller et al., 2008]). CGNPs in *SmoM2* G-cre mice with intact *Atr* gave rise to medulloblastoma with 100% frequency by P7, causing the mice to die from tumor
progression by P20 (Fig. 3.6A). By contrast, Atr deletion in SmoM2;Atr\textsuperscript{G-cre} animals completely blocked tumor formation, as seen and quantified by Hematoxylin and Eosin (H&E) staining and by IF for PCNA, which marks proliferating tumor cells (Fig. 3.6A). The inability of Atr-deleted CGNPs in SmoM2;Atr\textsuperscript{G-cre} mice to give rise to tumors suggests that ATR inhibition might effectively restrict medulloblastoma growth.

**ATR inhibitor administered in vivo induces DNA damage specifically in CGNPs**

To determine whether acute disruption of ATR function during postnatal neurogenesis in WT mice would recapitulate conditional Atr deletion, we developed a novel formulation of the small molecule ATR inhibitor VE-822 (pVE-822) (Charrier et al., 2011; Fokas et al., 2012). We first demonstrated that VE-822 induces DNA damage and apoptosis in isolated CGNPs in vitro in a time- and dose-dependent manner (Fig. 3.6A). To enhance delivery of VE-822 across the blood-brain barrier, we encapsulated VE-822 in poly(2-oxazoline) micelles, generating pVE-822 (Fig. 3.6B,C). We found that pVE-822, administered by intraperitoneal (IP) injection, induced γH2A.X and cC3 in the EGL, without affecting the surrounding differentiated neurons in the cerebellum or cortical neurons (Fig. 3.6B,C). As with Atr deletion, acute ATR inhibition by pVE-822 promoted upregulation of EIF4EBP1 in CGNPs, demonstrating that EIF4EBP1 is an effective biomarker of ATR disruption (Fig. 3.6D). These results show that pVE-822 effectively crosses the blood-brain barrier in bioactive concentrations after IP administration, and that acute, global loss of ATR function specifically disrupts CGNPs. The absence of neuronal toxicity associated with pVE-822 administration is consistent with recent literature demonstrating that ATR inhibition does not damage non-proliferating, differentiated neurons (Kemp and Sancar, 2016).
Discussion

Our data show that ATR is required to mitigate endogenous DNA damage during postnatal CGNP proliferation in order to maintain genomic stability. A prior study that deleted Atr using Nes-Cre, which is expressed by E9.5 with an effect by E10.5, found that embryonic CGNP precursors exit the cell cycle prematurely, blocking cerebellar development before postnatal CGNP proliferation (Lee et al., 2012). By contrast, in Atr\textsuperscript{G-cre} and Atr\textsuperscript{M-cre} mice, where Cre expression does not occur until E11.5 and E12.5 (Andrae et al., 2001; Machold and Fishell, 2005), respectively, CGNPs proliferated normally in the postnatal period and the effect of Atr deletion was not observed until P0 and P3, respectively. Postnatal proliferation in Atr-deleted CGNPs produced widespread DNA damage that activated ATM and p53 and induced apoptosis, resulting in cerebellar hypoplasia.

Blocking apoptosis by co-deletion of Bax and Bak or p53 did not fully rescue cerebellar hypoplasia. Cerebellar growth failure persisted despite attenuation of apoptosis due to inappropriate cell cycle exit and differentiation in Atr\textsuperscript{;Bax;Bak\textsuperscript{M-cre}} CGNPs or caspase-independent cell death in Atr\textsuperscript{;p53\textsuperscript{M-cre}} CGNPs. Karyotype analysis showed that Atr-deleted CGNPs developed extensive chromosomal abnormalities, and RNA-Seq analysis revealed the p53 pathway to be the predominant driver of the transcriptomic response to ATR-deficient proliferation.

Atr deletion in medulloblastoma-prone mice (SmoM2;Atr\textsuperscript{G-cre}) completely abrogated tumor formation, suggesting a therapeutic potential for ATR inhibition as a novel treatment for medulloblastoma. The anti-tumor effect of targeting ATR might be magnified in our model, as early deletion of Atr by hGFAP-Cre depletes the pool of CGNPs from which tumors may originate. However, CGNPs were present in P0 Atr\textsuperscript{G-cre} mice (Fig. 3.1A), and SmoM2 expression consistently failed to induce tumor growth from these cells in SmoM2;Atr\textsuperscript{G-cre} animals. Future work will assess the therapeutic potential of the ATR inhibitor pVE-822 in mice with established
medulloblastoma. Indeed, we have already shown here that acute, in vivo ATR inhibition by pVE-822 in WT mice reproduces the effects of conditional Atr deletion. Taken together, these results define a crucial role for ATR in maintaining genomic integrity in rapidly proliferating neural progenitors and in medulloblastoma cells.

The finding that 5-9% of Atr-deleted CGNPs were cC3⁺ demonstrates a strong induction of apoptosis. Unlike CGNP death after radiation, the death of these cells is not expected to be a synchronous process. Moreover, dying cells are cC3⁺ for only a limited time because they are rapidly cleared by phagocytosis and cC3 is known to have a short half-life (Elliott and Ravichandran, 2010; Walsh et al., 2011). Detecting 5-9% of cells expressing cC3 thus indicates a high rate of cell death.

Apoptosis in Atr-deleted CGNPs, in contrast to irradiated Atr-intact CGNPs (Chong et al., 2000; Williams et al., 2015), was not prevented by co-deletion of Bax, but was prevented by co-deletion of both Bax and Bak. In other cell types, BAK-driven apoptosis can be activated by genotoxic stress during mitosis (Chu et al., 2012; Flores et al., 2012). Atr-deleted CGNPs with DNA damage were markedly more prone to be mitotic than irradiated CGNPs in Atr-intact mice. The mitosis of Atr-deleted CGNPs with DNA damage might activate BAK-driven apoptosis that is not activated by radiation in WT mice.

The dependence of CGNPs on ATR to prevent DNA damage suggests that these cells are particularly prone to endogenous replication stress. ATR is activated in response to stalled DNA replication forks (Nam and Cortez, 2011), which can be caused by exogenous (Harley et al., 2016; Harper et al., 2010) or endogenous (Willis et al., 2013) factors. We have previously shown that normal CGNP proliferation produces endogenous DNA damage, detectable as small γH2A.X⁺ foci (Williams et al., 2015). We now show that ATR mitigates endogenous, proliferation-associated DNA damage in CGNPs in order to maintain replication fidelity.

To account for the phenotype of Atr deletion we propose a model in which: (1)
rapid proliferation promotes replication fork stalling from endogenous sources (Mirkin and Mirkin, 2007); (2) absence of ATR prolongs and collapses forks with consequent formation of DSBs (Couch et al., 2013); (3) ATM is activated and recruited to sites of DSBs (Shiloh, 2003); and (4) p-ATM activates p53, which ultimately induces apoptosis (Shiloh and Ziv, 2013) through BAX and BAK, and cell cycle arrest through p21 (Fig. 3.7). Because DNA damage and apoptosis occur throughout the entire CGNP population, cerebellar growth is effectively halted.

Apoptosis functions to cull CGNPs that acquire chromosomal abnormalities; thus, disabling apoptosis in ATR-deficient mice increases chromosomal fragmentation. p21 upregulation in Atr;Bax;Bak<sup>M-cre</sup> CGNPs demonstrates that p53-induced cell cycle exit acts as an additional barrier to the propagation of genomic abnormalities in apoptosis-incompetent, Atr-deleted CGNPs. Genomic stability in CGNPs may be particularly important for preventing their rapid postnatal proliferation from degenerating into tumorigenesis. Medulloblastoma, the most common malignant pediatric brain tumor, arises from hindbrain neural progenitors including CGNPs (Yang et al., 2008). Chromosome damage has been observed in medulloblastoma, frequently in association with p53 mutation (Rausch et al., 2012). Importantly, despite severe chromosomal abnormalities in Atr;Bax;Bak<sup>M-cre</sup> and Atr;p53<sup>M-cre</sup> mice, we never observed tumor formation. Indeed, we found that medulloblastoma tumorigenesis, like cerebellar development, requires ATR.

Since the physiological requirement for ATR in neural progenitors is recapitulated in the pathological growth of medulloblastoma, ATR may be a promising target for novel anti-tumor therapy. pVE-822 effectively crosses the blood-brain barrier in bioactive concentrations and induces DNA damage in proliferating cells. By damaging DNA in proliferating cells, pVE-822 might have a similar toxicity to conventional chemotherapeutic agents such as etoposide. An advantage of ATR inhibition over standard chemotherapy, however, is suggested by
our data showing that \textit{Atr} deletion kills cells even in the absence of p53. Whether the activity of pVE-822 against proliferating, non-tumor cells will limit its therapeutic potential remains to be tested.

\textbf{Materials and Methods}

\textbf{Mice}

We generated \(Atr^{M-\text{cre}}\) and \(Atr^{G-\text{cre}}\) mice by crossing \(Atr^{\text{loxP/loxP}}\) (Brown and Baltimore, 2003) mice with the \textit{Math1-Cre} (Jackson Labs, stock 011104) (Matei et al., 2005) and \(hGFAP-Cre\) (Jackson Labs, stock 012886) (Zhuo et al., 2001) mouse lines, respectively. \(Bax^{\text{loxP/loxP} ; Bak^{-/-}}\) mice (Takeuchi et al., 2005) were obtained from Jackson Labs (stock 006329). To generate mice with co-deletion of \(Atr/Bax/Bak\), \(Atr/Bax\), \(Atr/Bak\), and \(Bax/Bak\), we crossed \(Atr^{M-\text{cre}}\) and \(Bax^{\text{loxP/loxP} ; Bak^{-/-}}\) animals, intercrossed the progeny, and selected mice of \(Atr;Bax;Bak^{M-\text{cre}}\), \(Atr;Bax^{M-\text{cre}}\), \(Atr;Bak^{M-\text{cre}}\), and \(Bax;Bak^{M-\text{cre}}\) genotypes. We generated \(Atr;p53^{M-\text{cre}}\) mice by crossing \(Atr^{M-\text{cre}}\) with \(p53^{\text{loxP/loxP}}\) mice (Jonkers et al., 2001) provided by the NCI (strain 01XC2). Medulloblastoma-prone animals with and without \(Atr\) deletion were born from the cross between \(hGFAP-Cre; Atr^{\text{loxP/+}}\) and \(SmoM2^{\text{loxP/loxP} ; Atr^{\text{loxP/loxP}}}\), in which tumorigenesis is induced by deletion of a loxP-flanked Stop cassette between the \textit{Smo} promoter and coding region (Schuller et al., 2008). All mice were of species \textit{Mus musculus} and crossed into the C57BL/6 background through at least five generations. We used equal numbers of male and female mice, as we did not observe any differences based on sex. Numbers (n) in figures indicate biological replicates, which were determined so as to measure a 25\% difference in means with power=80\% and \(\alpha=0.05\). Animal use was in keeping with the policies of the University of North Carolina at Chapel Hill Institutional Animal Use and Care Committee. P3 mice of the indicated genotypes received 2 Gy
whole-body X-ray irradiation and were sacrificed 2 hr later.

**Immunostaining of cerebellar sections**

Mouse brains were processed and immunostained as previously described (Gershon et al., 2013). Primary antibodies are listed in the supplementary Materials and Methods. Secondary antibodies of the indicated fluorophores were used at 1:2000 for IF. Cell death was detected by TUNEL assay [ThermoFisher Scientific (TFS) C10617] (Galluzzi et al., 2009). DAPI and Hematoxylin were used as nuclear counterstains. BrdU analysis is described in the supplementary Materials and Methods.

**Quantification of immunostaining**

Stained slides were digitally imaged and positively-stained cells were counted using Aperio Software (Aperio Technologies) for chromogen-stained slides or Tissue Studio (Definiens) for fluorescence, as previously described (Williams et al., 2015). The entire EGL region in each section was manually annotated and used for quantifications, which were normalized to the total number of nucleated cells in the designated region. The measurement of cerebellar cellularity is described in the supplementary Materials and Methods.

**CGNP isolation**

CGNPs from P3 Atr-deleted mice and controls were isolated as previously described (Lee et al., 2009). Briefly, we separated cerebella from the rest of the brain, removed the meninges, and dissociated the tissue in 20 units/ml papain (Worthington Biochemical Corporation PDS) at 37°C for 15 min. CGNPs were then purified from the dissociated cerebellar tissue by successive rounds of centrifugation, discontinuous density gradient, and mesh filtering.
Flow cytometry

Flow cytometry on ATR-deficient CGNPs was performed by first fixing and permeabilizing (TFS GAS-004) isolated CGNPs suspended in HBSS containing 33 mM glucose, as previously described (Stahl et al., 2008). CGNPs were then stained successively for DNA damage with e660-conjugated anti-γH2A.X Ser139 (eBioscience 50-9865) at 1:10, for M-phase with 488-conjugated anti-pH3 (Cell Signaling Technology 9708) at 1:25, and for DNA content with FxCycle Violet (TFS F-10347) at 1:50. Technical controls included no stain, single-stained and fluorescence-minus-one samples. FACS was performed on an LSR Fortessa (BD Biosciences). For all experiments, 10,000-50,000 cells were counted. Analysis of FACS data was performed using FlowJo v10.0.8 (FlowJo).

Cytogenetic analysis

Cytogenetic analysis was performed on Atr-deleted CGNP metaphase spreads. Freshly isolated CGNPs from P3 animals were treated for 30 min with 100 nM Colcemid (TFS 15210-040) in Neurobasal-A Medium (TFS 10888-022) supplemented with 1x GlutaMAX-I (TFS 35050), 1x penicillin-streptomycin and 25 mM KCl at 37°C to block cell cycle progression in M-phase. CGNPs were then resuspended in 75 mM KCl, incubated at 37°C for 5 min, and fixed in methanol:acetic acid (3:1). The fixed cell suspension was dropped onto slides, stained in 0.08 µg/ml DAPI in 2x SSC for 3 min, and mounted in antifade solution (Vector Laboratories H-1200). The stained slides were scanned using a Zeiss Axioplan 2i epifluorescence microscope equipped with a megapixel CCD camera (CV-M4+CL, JAI) controlled by Isis 5.2 imaging software (Metasystems International). Chromatid breaks were counted as single-break events, tri-radials and quadri-radials as two-break events each, and other complex chromatid exchanges were converted into the minimum number of breaks required for their theoretical reconstruction. Chromosome aberrations
(fragments, rings, dicentric/tricentric and large marker chromosomes) were recorded and the breaks required for these rearrangements were not added to the frequency of chromatid breaks.

**Mutation analysis**

The frequency of mismatches or of transcript fusions associated with *Atr* deletion were determined as described in the supplementary Materials and Methods.

**Western blot**

P3 WT CGNPs subject to VE-822 or vehicle were analyzed for γH2A.X and cC3 (with β-actin loading control) by western blot as described in the supplementary Materials and Methods.

**Spectral karyotyping**

Metaphase spreads from the fixed cell suspensions were hybridized with SKY painting probes according to the manufacturer’s protocol (Applied Spectral Imaging). SKY images were acquired with an SD300 Spectracube (Applied Spectral Imaging) mounted on a Nikon Eclipse E800 microscope using a custom-designed optical filter (SKY-1) (Chroma Technology). For each sample, a minimum of 20, but usually 50, metaphases were captured and fully karyotyped. The breakpoints on the SKY-painted chromosomes were determined by comparison with the corresponding DAPI karyotype and chromosomal abnormalities were described according to (ISCN, 2013).

**RNA-Seq and differential expression analysis**

For RNA-Seq analysis, total RNA was purified using the RNeasy Mini Kit (Qiagen 74104) from freshly dissected whole P3 cerebella. RNA quality and quantity were assessed by spectrophotometry and capillary gel electrophoresis. We generated
stranded mRNA libraries using an Illumina TruSeq Stranded mRNA Library Prep Kit (Illumina RS-122-2101). 60 ng/μl poly(A)-selected RNA from each sample was run in two lanes of a HiSeq 2000 sequencing instrument (Illumina) for 100 cycles of multiplexed paired-end reads.

We performed read pseudo-alignment and quantification using Kallisto v0.42.4 ([Bray et al., 2016] with 200 bootstraps/paired-end read, aligning against the mouse mm9 genomic assembly, GRCm38 transcriptome definition. Downstream analysis was performed using the edgeR v3.12.0 software package ([Robinson et al., 2010] in R-Studio v0.99.491 with R v2.11.1 ([Team, 2014]). To assess differential expression, we used a general linear model in edgeR comparing Atr-deleted with control, setting the minimum counts per million at 2 and requiring greater than half the samples to pass the count threshold for a given transcript. Microarray validation is detailed in the supplementary Materials and Methods.

**Pathway analysis**

Biological pathway enrichment was determined by comparing transcriptomic data with Kyoto Encyclopedia of Genes and Genomes v77.1 ([Kanehisa and Goto, 2000]), WikiPathways ([Kutmon et al., 2016]) and Gene Ontology v1.2 ([Ashburner et al., 2000]) databases using EnrichNet v1.1 ([Glaab et al., 2012]), Panther v10.0 ([Mi et al., 2013]), and WebGestalt ([Wang et al., 2013]). Only genes above the differential expression significance threshold of log₂(FC)>1.5 and FDR<0.01 were used in pathway analysis.

**pVE-822 in vivo administration**

For in vivo studies, WT mice were subject to IP injection with 60 mg/kg pVE-822 (see supplementary Materials and Methods for formulation) or an equal volume of vehicle every 12 hr for 3 total injections from P3-5. 12 hr following the final injection, animals were sacrificed and brains were prepared for immunostaining.
Competing Interests

The authors declare no competing or financial interests.

Author contributions

P.Y.L. and T.R.G. conceived and designed the experiments and wrote the manuscript. G.J.N. and C.S. obtained and analyzed karyotype and SKY data. M.S.-P. and A.V.K. conceived the nanoparticle formulation of VE-822 in collaboration with P.Y.L. and T.R.G., and prepared and analyzed pVE-822 with the assistance of D.H. J.S.P. performed mutational analysis and consulted on transcriptomic studies. All other experiments were performed by P.Y.L. and analyzed by P.Y.L. and T.R.G.

Funding

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Data availability

RNA-Seq and microarray data are available at Gene Expression Omnibus under accession number GSE85394.
Supplementary Materials and Methods

Measurement of cerebellar cellularity

Cerebellar cellularity was defined as the IGL density (cells/mm\(^2\)) as measured on H&E-stained, sagittal brain sections. We measured statistical significance using the 2-sided independent samples t-test assuming unequal variances in SPSS Statistics 23 (IBM, Armonk, NY).

Survival analysis

Atr-deleted and control mice were followed for event-free survival. Animals were sacrificed either at the censure age of 300 days-old or at observation of severe weight loss, marked difficulty ambulating, and/or significantly diminished physical activity.

BrdU injection

To label cells in S-phase, 200 mg/kg BrdU (BD Biosciences 550891) dissolved in HBSS was administered by IP injection to mice of the indicated genotypes 2 hr before harvest.

Microarray validation

Total RNA was purified using the RNeasy Mini Kit (Qiagen 74104, Hilden, Germany) from freshly-dissected whole P3 cerebella. RNA quality and quantity were assessed by spectrophotometry and capillary gel electrophoresis. 500 ng of RNA from each sample was run on an Affymetrix GeneChip Mouse Transcriptome Assay 1.0 (Affymetrix, Santa Clara, CA). Differential gene expression was analyzed in Partek Genomics Suite (Partek Inc., Chesterfield, MO) using RMA normalization and significance thresholds of FDR<0.01 and |Log\(_2\)(FC)|>1.5. Data available at GEO: GSE85394.
Mutation analysis

Mismatch error rate was calculated from the set of all mutations/mismatches after MapSplice v2.2.0 (Wang et al., 2010) alignment to the mouse mm9 genomic assembly. Mismatches were captured using the bcftools -A" option to keep all possible alternate alleles at variant sites. Neighboring nucleotides of each mismatch were collected using a custom software. The frequency of all 96 possible trinucleotide mutations were calculated and plotted as a histogram. We measured statistical significance using the 2-sided independent samples t-test assuming unequal variances in SPSS Statistics 23 (IBM, Armonk, NY).

Transcript fusion products were detected using FusionCatcher v0.99.5a (Nicorici et al., 2014), employing Bowtie v1.1.2 (Langmead et al., 2009) for alignment to mouse genome build mm9 and STAR v2.5.1b (Dobin et al., 2013) for mapping reads to find the fusion junction. Statistical significance was measured by Barnard’s exact test in R Studio.

Western blot analysis

CGNPs isolated from P3 WT mice were cultured with 0.5 g/mL sonic hedgehog (R&D Systems, Minneapolis, MN) and DMSO or VE-822 (MedKoo 406258, Chapel Hill, NC) dissolved in DMSO at 0.1, 1, or 5 M. Cells were harvested after 2, 6, or 24 hr and lysates were probed for γH2A.X Ser139 at 1:50 (CST 9718, Batch 10), cC3 at 1:100 (Biocare Medical CP229C, Batch 060115), and β-actin at 1:5000 (CST 3700, Batch 3) by Western blot.

pVE-822 formulation

The amphiphilic triblock copolymer poly(Methyl-Butyl-Methyl) oxazoline (POx), P(MeOx_{37}-b-BuOx_{21}-b-MeOx_{36}), was purchased from Rainer Jordan (Technische Universität, Dresden, Germany). VE-822-loaded polyoxazoline micelles (pVE-822)
were prepared as previously described (Luxenhofer et al., 2010), with slight modifications. Briefly, VE-822 was dissolved in acetone and mixed with POx solution in acetone (final drug:polymer of 4 mg:5 mg). Following complete removal of acetone, the resulting thin film was rehydrated with saline at 60°C. Samples were allowed to cool to room temperature and then centrifuged at 10k RPM for 3 min to remove any remaining residual solids. Only the transparent supernatant solution was used for subsequent experiments. Micelle hydrodynamic diameter and polydispersity index (PDI) were determined by dynamic light scattering (DLS) using a Malvern Nanosizer (Malvern Instruments Ltd., Worcestershire, UK). VE-822 concentration was determined by HPLC. Final VE-822 concentration was 26 mg/mL and non-loaded POx micelles were used as Vehicle control.
### Antibodies for IHC/IF

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Dilution</th>
<th>Source</th>
<th>Batch</th>
</tr>
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<tr>
<td>BrdU</td>
<td>1:200</td>
<td>CST 5292</td>
<td>3</td>
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<tr>
<td>cC3</td>
<td>1:50</td>
<td>BM CP229C</td>
<td>060115</td>
</tr>
<tr>
<td>Cyclin b1</td>
<td>1:100</td>
<td>CST 4138</td>
<td>2</td>
</tr>
<tr>
<td>Cyclin d1</td>
<td>1:500</td>
<td>CST 2978</td>
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<tr>
<td>Eif4ebp1</td>
<td>1:3000</td>
<td>CST 9644</td>
<td>10</td>
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<td>NeuN</td>
<td>1:1000</td>
<td>EMD M MAB377</td>
<td>A60</td>
</tr>
<tr>
<td>p-Atm Ser1981</td>
<td>1:5000</td>
<td>EMD M 05740</td>
<td>2276333</td>
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<td>p-MLKL Ser345</td>
<td>1:300</td>
<td>Abcam ab196436</td>
<td>GR238752-10</td>
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<tr>
<td>p-p53 Ser15</td>
<td>1:50</td>
<td>CST 9284</td>
<td>15</td>
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<td>p21</td>
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<td>5</td>
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<tr>
<td>PCNA</td>
<td>1:2000</td>
<td>CST 2586</td>
<td>10</td>
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<td>pH3 Ser10</td>
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<td>CST 9706</td>
<td>9</td>
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<td>Trophinin</td>
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<td>GR259420-1</td>
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<td>γH2A.X Ser139</td>
<td>1:2000</td>
<td>CST 9718</td>
<td>10</td>
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</table>

*a CST = Cell Signaling Technology, Danvers, MA, USA.
Antibody validation information: [http://www.cellsignal.com/common/content/content.jsp?id=ourApproach-validation-principles](http://www.cellsignal.com/common/content/content.jsp?id=ourApproach-validation-principles)

*b Abcam, Cambridge, UK.
Antibody validation information: [http://www.abcam.com/content/validating-our-products](http://www.abcam.com/content/validating-our-products)

c EMD M = EMD Millipore, Billerica, MA, USA.

d BM = Biocare Medical, Concord, CA, USA.
Antibody validated on brain sections from WT mice at P0, P3, P7, P12, and P20 (n = 10 for each) and WT mice exposed to 10 Gy XRT at P0, P3, P7, P12, and P20 (n = 10 for each).
Table 3.1: Pathway enrichment analysis on significantly differentially expressed genes between CGNPs from P3 Atr;Bax;Bak<sup>cre</sup> and Bax;Bak<sup>cre</sup> cerebellum.

<table>
<thead>
<tr>
<th>Database</th>
<th>Pathway</th>
<th>Fold enriched</th>
<th>FDR</th>
<th>Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kyoto Encyclopedia of Genes and Genomes</td>
<td>p53 signaling pathway&lt;sup&gt;2&lt;/sup&gt;</td>
<td>27</td>
<td>5.9E-8</td>
<td>↑Cdkn1a, ↑Fas, ↑Mdm2, ↑Pmaip1, ↑Sesn2, ↑Trp73, ↓Cdk1, ↓Mdm4</td>
</tr>
<tr>
<td>Cell cycle</td>
<td></td>
<td>9</td>
<td>2.0E-3</td>
<td>↑Cdkn1a, ↑Mdm2, ↓Anapc1, ↓Cdk1, ↓Mcm7</td>
</tr>
<tr>
<td>Pathways in cancer</td>
<td></td>
<td>7</td>
<td>1.4E-3</td>
<td>↑Cdkn1a, ↑Fas, ↑Fgfr3, ↑Itga6, ↑Mdm2, ↑Sos2, ↓Apc</td>
</tr>
<tr>
<td>MAPK signaling pathway</td>
<td></td>
<td>9</td>
<td>1.1E-5</td>
<td>↑Cacnb4, ↑Fas, ↑Fgfr3, ↑Mapkapk3, ↑Pla2g2f, ↑Sos2, ↓Hspa8</td>
</tr>
<tr>
<td>Wiki Pathways&lt;sup&gt;1&lt;/sup&gt;</td>
<td>Apoptosis</td>
<td>13</td>
<td>6.0E-4</td>
<td>↑Fas, ↑Mdm2, ↑Pmaip1, ↑Tnfrsf10b, ↑Trp73</td>
</tr>
<tr>
<td>miRNA regulation of DNA damage response</td>
<td></td>
<td>24</td>
<td>6.0E-7</td>
<td>↑Cdkn1a, ↑Fas, ↑Mdm2, ↑Pmaip1, ↑Tnfrsf10b, ↓Cdk1, ↓Mcm7</td>
</tr>
<tr>
<td>G&lt;sub&gt;1&lt;/sub&gt; to S cell cycle control</td>
<td></td>
<td>14</td>
<td>1.4E-3</td>
<td>↑Cdkn1a, ↑Mdm2, ↓Cdk1, ↓Mcm7</td>
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<tr>
<td>Gene Ontology Biological Process&lt;sup&gt;4&lt;/sup&gt;</td>
<td>p53 signal transduction</td>
<td>15</td>
<td>1.7E-4</td>
<td>↑Cdkn1a, ↑Mdm2, ↑Pmaip1, ↑Sesn2, ↑Trp53inp1, ↓Mdm4</td>
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<tr>
<td>Cell cycle</td>
<td></td>
<td>4</td>
<td>4.4E-4</td>
<td>↑Cdkn1a, ↑Mdm2, ↑Trp53inp1, ↓Anapc1, ↓Cdk1, ↓Mcm7</td>
</tr>
<tr>
<td>Nervous system development</td>
<td></td>
<td>3</td>
<td>3.8E-5</td>
<td>↑ApoE, ↑Fas, ↑Fgfr3, ↑Gfap, ↑Myh10, ↓Apc, ↓Otx1, ↓Smarca1</td>
</tr>
</tbody>
</table>

Differentially expressed genes defined as |log₂(FC)| > 1.5 and FDR < 0.01. Subset by consensus between RNA-Seq and microarray. Arrows indicate whether an individual gene within that pathway is upregulated or downregulated.  
Accessed via WebGestalt.  
Accessed via Panther.  
Also as determined by KEGG accessed via EnrichNet.
158


Table 3.S2: Differential gene expression of P3 Atr; p53<sup>M-cre</sup> vs. p53<sup>M-cre</sup>: Full list.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Log&lt;sub&gt;2&lt;/sub&gt;(FC)</th>
<th>FDR&lt;sup&gt;′&lt;/sup&gt;</th>
<th>Gene</th>
<th>Log&lt;sub&gt;2&lt;/sub&gt;(FC)</th>
<th>FDR&lt;sup&gt;′&lt;/sup&gt;</th>
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<tr>
<td>Hist1h2oc</td>
<td>2.36</td>
<td>1.08E-04</td>
<td>Ank2</td>
<td>1.54</td>
<td>1.81E-04</td>
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<tr>
<td>Tiam1</td>
<td>-10.00</td>
<td>1.90E-51</td>
<td>Ptgsd</td>
<td>-2.62</td>
<td>2.04E-04</td>
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<tr>
<td>Glice</td>
<td>-10.53</td>
<td>2.36E-45</td>
<td>Cyb5r4</td>
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<td>2.67E-04</td>
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<td>Suv420h1</td>
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<td>1.73E-34</td>
<td>Inpp4e</td>
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<td>Fgf21</td>
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<td>Gm12896</td>
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<td>4.97E-04</td>
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<tr>
<td>Cd209c</td>
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<td>1.09E-18</td>
<td>Rps13</td>
<td>2.74</td>
<td>5.66E-04</td>
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<tr>
<td>Rps3a1</td>
<td>2.72</td>
<td>2.84E-13</td>
<td>Nfib</td>
<td>-1.95</td>
<td>5.76E-04</td>
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<tr>
<td>Hist1h1c</td>
<td>1.58</td>
<td>1.57E-12</td>
<td>Arpp19</td>
<td>-1.97</td>
<td>6.07E-04</td>
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<tr>
<td>Olfr1372-ps1</td>
<td>2.16</td>
<td>5.23E-12</td>
<td>Zfp335</td>
<td>3.99</td>
<td>9.48E-04</td>
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<tr>
<td>Mbp</td>
<td>-1.89</td>
<td>1.06E-09</td>
<td>Scnn1</td>
<td>1.54</td>
<td>2.15E-03</td>
</tr>
<tr>
<td>Trim66</td>
<td>1.55</td>
<td>1.06E-09</td>
<td>Rps2-ps10</td>
<td>3.13</td>
<td>2.69E-03</td>
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<tr>
<td>Xpo1</td>
<td>11.80</td>
<td>1.08E-08</td>
<td>Senp7</td>
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<td>2.69E-03</td>
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<tr>
<td>Mobp</td>
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<td>3.56E-08</td>
<td>Abl2</td>
<td>-1.52</td>
<td>2.87E-03</td>
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<td>Abi1</td>
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<td>Myh10</td>
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<td>3.32E-03</td>
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<tr>
<td>Pcdh9</td>
<td>10.62</td>
<td>2.06E-06</td>
<td>Gm10925</td>
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<td>3.69E-03</td>
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<tr>
<td>Brp1f</td>
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<td>Pkia</td>
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<tr>
<td>Gemin8</td>
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<td>2.55E-06</td>
<td>Reln</td>
<td>1.63</td>
<td>5.22E-03</td>
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<tr>
<td>Mr1</td>
<td>1.71</td>
<td>9.69E-06</td>
<td>Slc3a4</td>
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<td>Rab7</td>
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<td>1.37E-05</td>
<td>Nav1</td>
<td>1.75</td>
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<tr>
<td>ATP6</td>
<td>1.85</td>
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<td>Stxbp5</td>
<td>-1.66</td>
<td>6.28E-03</td>
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<tr>
<td>Atp6v0a1</td>
<td>7.17</td>
<td>4.70E-05</td>
<td>Csur1</td>
<td>2.45</td>
<td>6.88E-03</td>
</tr>
<tr>
<td>Arhgef2</td>
<td>2.83</td>
<td>6.13E-05</td>
<td>Psd2</td>
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<td>7.67E-03</td>
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<td>Tro</td>
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<td>6.37E-05</td>
<td>Nop56</td>
<td>1.84</td>
<td>7.88E-03</td>
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<tr>
<td>Cflap53</td>
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<td>6.50E-05</td>
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<td>4932433A13Rik</td>
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<td>6.57E-05</td>
<td>Usps46</td>
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<tr>
<td>Atrc3</td>
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<td>Dek</td>
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<tr>
<td>Synj1</td>
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<td>1.26E-04</td>
<td>Aars</td>
<td>1.70</td>
<td>9.99E-03</td>
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</tbody>
</table>

Differentially expressed genes defined as |log<sub>2</sub>(FC)| > 1.5 and FDR < 0.01, from RNA-Seq. Genes ordered by FDR.

Table 3.S3: Pathway enrichment analysis on significantly differentially expressed genes between CGPNPs from P3 Atr; p53<sup>M-cre</sup> and p53<sup>M-cre</sup> cerebellum.

<table>
<thead>
<tr>
<th>Database</th>
<th>Pathway</th>
<th>Fold enriched</th>
<th>FDR</th>
<th>Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene</td>
<td>Hindbrain development</td>
<td>25</td>
<td>3.0E-5</td>
<td>↑Aars, ↑Abl1, ↑Myh10, ↓Nfib, ↓Pax6</td>
</tr>
<tr>
<td>Ontology Process</td>
<td>Neuron differentiation</td>
<td>7</td>
<td>9.2E-6</td>
<td>↑Atrc3, ↑Ahi1, ↑Myh10, ↑Reln, ↓Abl2, ↓Mbp, ↓Nfib, ↓Pax6, ↓Stxbp5, ↓Tiam1, ↓Vhl</td>
</tr>
<tr>
<td>Biological Process</td>
<td>Axonogenesis</td>
<td>12</td>
<td>3.0E-5</td>
<td>↑Myh10, ↑Reln, ↓Mbp, ↓Nfib, ↓Pax6, ↓Stxbp5, ↓Tiam1</td>
</tr>
</tbody>
</table>

Differentially expressed genes defined as |log<sub>2</sub>(FC)| > 1.5 and FDR < 0.01, by RNA-Seq. Arrows indicate whether an individual gene within that pathway is upregulated or downregulated. Accessed via Webgestalt.
Figure 3.1: *Atr* deletion in CGNPs induces postnatal DNA damage, p53 activation, apoptosis and cerebellar hypoplasia. (A) H&E-stained sagittal brain sections from mice of the indicated genotypes and ages.
(B) Sagittal cerebellar sections from P3 mice of the indicated genotypes stained for pH3, γH2A.X and cC3. (C) Quantification of replicate cerebella of the indicated genotypes, stained as in B and Fig. 3.S1. Average is shown with s.e.m. P-values by two-sided independent samples t-test assuming unequal variances. All stainings were repeated on at least three biological replicates (n). Scale bars: 800 μm in A; 400 μm (main) and 200 μm (inset) for pH3, 50 μm for cC3 (inset) in B.
Figure 3.2: Co-deletion of Bax and Bak, but not p53, blocks cell death in ATR-deficient CGNPs. (A) H&E-stained, sagittal brain sections from P12 mice of the indicated genotypes, showing partial rescue of cerebellar development in Atr;Bax;Bak^{M-cre} and Atr;p53^{M-cre} mice. (B) Sagittal cerebellar sections from P3 mice of the indicated genotypes stained for p-p53, TUNEL, and p21.
(C) Sagittal cerebellar sections from P3 mice of the indicated genotypes stained for γH2A.X, TUNEL, and p21. (D) Quantification of replicate cerebella of the indicated genotypes, stained as in B,C, Fig. 3.S2 and Fig. 3.S3. Bax;Bak<sup>M-cre</sup> and p53<sup>M-cre</sup> controls were not significantly different from the Math1-Cre controls presented in Fig. 3.1. (E) pH3 quantification in the external granular layer (EGL) of mice of the indicated genotypes at P3, P5, and P7. (F) NEUN staining on P20 sagittal cerebellar sections from mice of the indicated genotypes. Average with s.e.m. P-values by two-sided independent samples t-test assuming unequal variances. All stainings were repeated on at least three biological replicates (n). Scale bars: 800 μm in A; 400 μm (main) and 50 μm (inset) for p-p53, 200 μm for TUNEL (inset) in B; 400 μm (main) and 200 μm (inset) in C; 200 μm (main) and 50 μm (inset) in F.
Figure 3.3: Increased DNA damage and inappropriate mitotic entry in Atr-deleted CGNPs. (A) FACS analysis without subsetting for γH2A.X on CGNPs from P3 mice of the indicated genotypes. M-phase was determined by pH3 labeling. P-values by chi-square test. (B) Quantification of γH2A.X+/pH3+ CGNPs from P3 mice of the indicated genotypes. P-values by two-sided independent samples t-test assuming unequal variances.
(C) Double labeling for $\gamma$H2A.X/pH3 and p-53BP1/pH3 on sagittal cerebellar sections from P3 mice of the indicated genotypes. Arrowheads indicate double positives. Scale bars: 100 $\mu$m (main) and 50 $\mu$m (inset). (D) FACS analysis with subsetting for $\gamma$H2A.X$^+$ CGNPs from P3 mice of the indicated genotypes. P-values by chi-square test. (E) Quantification of 'sub-G$_1$' DNA content detected by FACS in CGNPs from mice of the indicated genotypes. P-values by two-sided independent samples t-test assuming unequal variances. Average with s.e.m. All stainings were repeated on at least three biological replicates (n).
Figure 3.4: Atr deletion compromises the chromosome integrity of proliferating CGNPs. (A) Metaphase chromosome spread and SKY analysis on $Atr;Bax;Bak^{M\text{-cre}}$ and $Atr;p53^{M\text{-cre}}$ CGNPs. (B) Severity of chromosome damage in CGNPs with >10 chromosome breaks/cell, grouped into Mild, Moderate, and Severe. (C) The distribution of CGNPs with chromosomal abnormalities by genotype. Average with s.e.m. P-values by two-sided independent samples t-test assuming unequal variances. n, number of biological replicates, with 35-50 metaphases counted per replicate.
Figure 3.5: The p53 pathway controls the CGNP transcriptomic response to Atr deletion. (A) PCA on gene expression of CGNPs from P3 mice of the indicated genotypes, as determined by RNA-Seq. (B) Differential gene expression analysis of RNA-Seq data using the indicated criteria comparing mice of the indicated genotypes. (C) Volcano plots of \( \log_2(FC) \) versus -\( \log_{10}(P\text{-value}) \) for Atr;Bax;Bak\(^{M\text{-cre}}\) compared with Bax;Bak\(^{M\text{-cre}}\) and for Atr;p53\(^{M\text{-cre}}\) compared with p53\(^{M\text{-cre}}\), with color threshold (teal dots) set at \( \log_2(FC)>1.5 \) and FDR<0.01 (lines).
(D) IF for TRO and EIF4EBP1 on sagittal cerebellar sections from P3 mice of the indicated genotypes. Arrows indicate TRO$^+$ cells. Scale bar: 100 $\mu$m. IGL, internal granular layer. All stains were repeated on at least three biological replicates (n).
Figure 3.6: Atr deletion blocks medulloblastoma tumorigenesis, and acute, in vivo ATR inhibition induces DNA damage and apoptosis in the postnatal cerebellum. (A) Sagittal H&E- and PCNA-stained brain sections at P7 and P20 show the absence of tumor formation in SmoM2;Atr\textsuperscript{G-cre} mice. Bar charts compare total area and percentage PCNA\textsuperscript{+} cells in the P20 posterior fossa by genotype.
(B-D) Sagittal sections of whole brains, cerebella and cortices from P5 WT mice injected with pVE-822 or vehicle, stained for (B) γH2A.X, (C) cC3 or (D) EIF4EBP1. Arrows indicate EIF4EBP1⁺ cells. Average with s.e.m. P-values by two-sided independent samples t-test assuming unequal variances. All stainings were repeated on at least three biological replicates (n). Scale bars: 2 mm in A; 2 mm (brain), 500 μm (cerebellum) and 100 μm (EGL and cortex) in B-D.
Figure 3.7: ATR is required to maintain G₂/M checkpoint integrity and prevent apoptosis induced by proliferation-associated DNA damage. (A) CGNPs proliferate in the postnatal cerebellum, generating DNA damage (lightning bolt) from endogenous processes, which stalls replication forks. ATR is recruited by replication protein A (RPA), which binds single-stranded DNA at stalled replication forks. Activated ATR initiates checkpoint arrest, promoting DNA repair with minimal engagement of the p53 pathway.
(B) ATR loss prolongs replication fork stalling across the CGNP genome, which degenerates into widespread DSBs that are sensed by ATM. Resultant p53 activation triggers apoptosis through BAX/BAK and Caspase-3 (C-3) due to overwhelming DNA damage, with some concurrent activation of p21 and cell cycle arrest. G2/M checkpoint failure from ATR absence allows CGNPs with DNA damage to enter mitosis, incurring chromosomal abnormalities; however, apoptosis removes cells with aberrant chromosomes. (C) Co-deletion of Bax and Bak in ATR-deficient CGNPs prevents apoptosis. Instead, cells with DNA damage and p53 activation are driven to p21-mediated cell cycle exit, attenuating CGNP proliferation over time. However, cells that escape arrest then inappropriately enter mitosis and experience chromosome damage, which accumulates due to apoptotic failure. (D) In Atr;p53M-cre CGNPs, DSBs from collapsed replication forks accumulate and propagate due to the inability to undergo both ATR-mediated G2/M arrest and p53-mediated apoptosis and cell cycle arrest. Excessive DNA damage triggers an as yet unidentified form of non-apoptotic cell death.
Supplemental Figure 3.1: Normal proliferation in Atr-deleted CGNPs during early postnatal development despite later reduced cerebellar cellularity and hypoplasia. (A) Conditional Atr deletion in CGNPs (AtrM-cre) and neural stem cells (AtrG-cre) leads to cerebellar hypoplasia by P12, as measured by IGL cellular density (cells/mm²). Average with s.e.m. P-values by two-sided independent samples t-test assuming unequal variances. (B) Atr deletion in CGNPs (AtrM-cre) has no effect on mouse survival, as seen on Kaplan-Meier curve, although earlier and more broad neural deletion of Atr (AtrG-cre) significantly reduces survival. P-values by log-rank test. (C) P0 AtrG-cre cerebella show preserved mitotic rate (pH3), increased DNA damage (γH2A.X), increased phosphorylation of ATM (p-ATM) and p53 (p-p53), and cleavage of Caspase-3 (cC3). Scale bars: 400 μM and 200 μM (inset) on pH3, and 50 μM (inset) on p-ATM.
(D) P3 Atr$^{M-cre}$ cerebella have increased activation of ATM and p53. Scale bars: 400 μM and 200 μM (inset). IGL = internal granular layer. All stains were repeated on at least three biologic replicates (n).
Supplemental Figure 3.2: Deletion of Bax or Bak alone fails to rescue the ATR cerebellar phenotype. (A) Co-deletion of Bax and Bak (Atr;Bax;Bak<sup>M-cre</sup>), but not Bax alone (Atr;Bax<sup>M-cre</sup>), partially rescues the Atr-deleted cerebellar phenotype at P0 and P3 when compared to Bax;Bak<sup>M-cre</sup> control, as seen on representative H&E-stained, sagittal sections. Scale bar = 800 μM. (B) P3 Atr;Bax<sup>M-cre</sup> and Atr;Bak<sup>M-cre</sup> cerebella have equivalently elevated levels of γH2A.X, p-p53, cC3, TUNEL, and p21, with no difference in pH3 compared to control (Fig. 2D). Scale bars = 400 μM and 200 μM (inset) on pH3, and 50 μM (inset) on p-p53. (C) Atr;Bax;Bak<sup>M-cre</sup> cerebella have increased levels of γH2A.X, but equivalent levels of pH3 and cC3 compared to Bax;Bak<sup>M-cre</sup> controls. Scale bars: 400 μM and 200 μM (inset) on pH3, and 50 μM (inset) on cC3. All stains were repeated on at least three biologic replicates.
Supplemental Figure 3.3: p53 deletion in ATR-deficient CGNPs is associated with DNA damage accumulation but no change in proliferation. (A) Co-deletion of Atr and p53 (Atr; p53M-cre) partially rescues the ATR-deficient (AtrM-cre) cerebellar phenotype at P3 when compared to p53M-cre control, as seen on representative H&E-stained, sagittal sections. Scale bar: 800 µM.
(B) P3 $\text{Atr; } p53^{\text{M-cre}}$ cerebella have increased activation of DNA damage response as seen by p-ATM, but no change in proliferation as assessed by pH3 and PCNA when compared to $p53^{\text{M-cre}}$ control. Scale bars: 400 $\mu$M and 200 $\mu$M (inset). (C) IF for PCNA in P3 $\text{Atr}$-deleted and ATR-intact cerebella shows no difference in proliferation induced by $\text{Atr}$ deletion in CGNPs, reflecting the results of pH3 staining and quantification. Scale bars: 400 $\mu$M and 200 $\mu$M (inset). (D) Quantification of replicate cerebella of the indicated genotypes, stained for PCNA as in B and C. (E) Pyknotic nuclei (red arrow heads), characteristic of apoptotic cells, are found in the EGL of P3 $\text{Atr}^{\text{M-cre}}$ mice (middle panel), as seen on representative H&E-stained, sagittal sections. In contrast, the EGL of P3 $\text{Atr;p53}^{\text{M-cre}}$ mice (right panel) contain multi-nucleated giant cells (yellow arrow) and cells with micronuclei (yellow arrow head), typical of necrotic cells. Scale bar: 50 $\mu$M. (F) Similar proportions of cells positive for the necroptosis marker p-MLKL can be found in the EGL of P3 $\text{Math1-Cre}, \text{Atr}^{\text{M-cre}}$, and $\text{Atr;p53}^{\text{M-cre}}$ mice. Scale bars: 200 $\mu$M and 100 $\mu$M (inset). (G) Quantification of pH3 staining in control cerebella of the indicated genotypes shows no differences in mitotic rate between controls at P3, P5, and P7. Average with s.e.m. P-values by two-sided independent samples t-test assuming unequal variances. All stains were repeated on at least 3 biologic replicates (n).
Supplemental Figure 3.4: *Atr* deletion increases the fraction of CGNPs with DNA damage, especially in M-phase. (A) Live CGNPs from P3 cerebella of the indicated genotypes can be found in all phases of the cell cycle by staining for DNA content with FxCycle Violet. γH2AX+ cells are found in *Atr*-deleted genotypes and WT +XRT, but not in controls. P-values by two-sided independent samples t-test assuming unequal variances.
(B) More γH2A.X+ CGNPs in G2/M from Atr-deleted cerebella are pH3+ compared to γH2A.X+ CGNPs in G2/M from WT +XRT cerebella or all CGNPs in G2/M from control cerebella. (C) No difference in cell cycle distribution is noted between the indicated ATR-intact control genotypes. P-values by chi square test. Average with s.e.m. (D) IF labeling of P3 AtrM-cre cerebellar sections shows co-localization of γH2A.X with markers of G1- (Cyclin d1+), S- (BrdU+), and G2-phases (Cyclin b1+/pH3+) of the cell cycle. Scale bars: 100 μM and 50 μM (inset). All stains were repeated on at least three biologic replicates (n).
Supplemental Figure 3.5: Atr deletion in CGNPs is not associated with a specific mutational pattern. (A) Microarray validates 76% of the genes found by RNA-Seq to be significantly differentially expressed between P3 Atr;Bax;Bak<sup>M-cre</sup> and Bax;Bak<sup>M-cre</sup>. (B) Mismatch rate quantification shows no increase in mutation in Atr;Bax;Bak<sup>M-cre</sup> and Atr;p53<sup>M-cre</sup> samples versus Atr-intact controls. P-values by two-sided independent samples t-test assuming unequal variances. (C) Transcript fusion analysis on Atr;Bax;Bak<sup>M-cre</sup>, Atr;p53<sup>M-cre</sup>, and Atr-intact controls shows that Atr deletion is correlated with an increase in certain fusion products. However, the sole fusion product that is significantly increased, Gm6976-Gm29096, is only detected in Atr;Bax;Bak<sup>M-cre</sup> samples and is only representative of a read-through error fusing two predicted genes of unknown function on chromosome 10. P-value by Barnards exact test. Average with s.e.m. RT = read-through. n = number of biologic replicates.
Supplemental Figure 3.6: In vitro characterization of pVE-822. (A) CGNPs freshly dissociated from P3 WT mice were cultured in the presence of Sonic Hedgehog and the ATR inhibitor VE-822 or DMSO at the indicated concentrations and then collected for Western blot analysis after 2, 6, or 24 hr. γH2A.X and cC3 levels increase in time- and dose-dependent manners. Experiment replicated twice. (B) Micelle size and size distribution, as determined by DLS. (C) VE-822 and POx concentrations and pVE-822 loading efficiency (LE) and loading capacity (LC).
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ATR inhibition by pVE-822 for the treatment of medulloblastoma

Introduction

Medulloblastoma is a cerebellar tumor that is the most common brain cancer of childhood (McNeil et al., 2002; Smoll and Drummond, 2012). Current standard of care involves some combination of surgical resection of the primary tumor, cranio-spinal radiation to destroy metastatic cells, and/or chemotherapy for any residual cancer (Packer and Vezina, 2008; Packer et al., 1999, 2006, 1991). However, these methods can produce substantial toxicity as they also damage normal, untransformed, proliferating cells, which is of special concern in developing children. Notable side effects associated with medulloblastoma treatment include growth impairment, mental disability, hearing loss, and early onset strokes (Packer and Vezina, 2008; Bartlett et al., 2013). Moreover, not all patients respond to existing therapies, and indeed, brain cancer is the second leading cause of death by disease in children in the United States (CBTRUS, 2010). As a result, more effective, targeted therapies that can reduce side effects and treat a greater fraction of patients are required.

Medulloblastoma generally responds favorably to radiation and chemotherapeutics like Cisplatin, which produce an anti-tumor effect by inducing DNA damage, highlighting the sensitivity of this cancer to genotoxicity (Bar and Stearns, 2008; Bloom et al., 1969; Gentet et al., 1995; Klesse and Bowers, 2010). ATR is a serine/threonine kinase that regulates the DNA damage response and its canonical role is in mediating the resolution of stalled replication forks in S-phase.
that form as a result of genomic stress from either endogenous or exogenous sources (Cimprich and Cortez, 2008; Shiloh, 2001). Seckel syndrome is a congenital developmental disorder that can be caused by hypomorphic ATR mutations and is characterized by intrauterine growth restriction, postnatal growth impairment, microcephaly, mental disability, craniofacial deformities, and sometimes hematological abnormalities (O’Driscoll et al., 2003; Seckel, 1960). Microcephaly from ATR disruption has been shown in mice to arise due to failed expansion of neural stem cells specifically, and surprisingly only, in the ganglionic eminence and the nascent cerebellum (Lee et al., 2012), suggesting tissue-specific dependence on ATR in the developing brain. Deleting Atr slightly later in embryonic development mostly affected the cerebellum during postnatal development, again highlighting the special cerebellar requirement for ATR (Lang et al., 2016).

Indeed, our recent research provides evidence that ATR is required to maintain genomic integrity in cerebellar granule neuron progenitors (CGNPs) of the neonatal cerebellum and that ATR disruption results in widespread CGNP apoptosis that produces cerebellar hypoplasia (Lang et al., 2016). CGNPs are one of the most highly proliferative cells in the postnatal brain, with peak proliferation stimulated by the potent mitogen Sonic hedgehog (SHH) in the external granular layer (EGL) of the cerebellum between postnatal day (P) 3 and P7 in mice, generating the largest population of neurons in the adult brain, the cerebellar granule neurons (CGNs) (Hatten and Heintz, 1995; Wechsler-Reya and Scott, 1999, 2001). Molecular characterization of human medulloblastoma has divided the cancer into four subgroups, one of which is characterized by hyperactivation of the SHH pathway (Gibson et al., 2010; Gilbertson and Ellison, 2008; Northcott et al., 2012; Taylor et al., 2012). This SHH subgroup of medulloblastoma is thought to arise from mutations in CGNPs, and mice with only activating mutations in the SHH pathway in CGNPs develop medulloblastoma (Gilbertson and Ellison, 2008; Schuller et al.,
Importantly, tumor cells in SHH medulloblastoma retain many molecular and genomic similarities to CGNPs, suggesting that programs critical to CGNP survival may also be important in medulloblastoma tumorigenesis (Gilbertson and Ellison, 2008; Wechsler-Reya and Scott, 2001). Thus, given the critical dependence of CGNPs on ATR, targeting ATR may represent a viable and novel therapeutic for medulloblastoma.

For preclinical testing of pharmacological ATR inhibition as a treatment for medulloblastoma, we have previously developed a novel nanoparticle formulation of the small molecule ATR inhibitor VE-822, called pVE-822, for efficient in vivo delivery (Lang et al., 2016). We have shown in neonatal wild-type (WT) mice that intraperitoneal (IP)-injected pVE-822 is able to cross the blood-brain barrier in bioactive concentrations and inhibit ATR to induce DNA damage and apoptosis specifically in CGNPs without obvious effect to the rest of the brain. In one model of medulloblastoma-prone mice, Atr deletion completely abrogated tumor formation, again while apparently sparing the rest of the brain. However, the possibility remained that the anti-tumor effect of Atr deletion could be attributed to early depletion of the CGNP pool from which tumor cells arise. Nevertheless, the complete absence of tumors in medulloblastoma-prone mice with Atr deletion and the specific cytotoxic effect of acute ATR inhibition by pVE-822 on CGNPs encourage further investigation of ATR disruption to treat medulloblastoma.

Here, we show that pVE-822 administration after the point of tumor formation in two mouse models of medulloblastoma induces tumor regression by specifically increasing intra-tumoral DNA damage. Increased DNA damage, however, was not associated with activation of the p53 pathway or caspase-mediated apoptosis. Tumor regression caused by ATR inhibition could also not be explained by altered cell cycle dynamics, as no difference was observed compared to Vehicle-treated mice with medulloblastoma. Similarly, tumor cells from medulloblastoma mice treated with
pVE-822 had high levels of >4N DNA content, but this type of aneuploidy was also detected in cells from Vehicle-treated animals. Survival analysis showed no benefit to treating medulloblastoma mice with pVE-822. Moreover, medulloblastoma mice with Atr deletion did not survive any longer following a course of radiation therapy than control medulloblastoma mice with intact Atr. Curiously, microarray analysis of mice with Atr-deleted medulloblastoma showed no differentially expressed genes compared to Atr-intact control mice with medulloblastoma. In vitro studies in primary CGNPs failed to reveal any compounds that synergize with VE-822 to increase DNA damage or cell death. While pVE-822 and ATR disruption in general clearly have an anti-tumor effect in medulloblastoma, further studies are required to determine the therapeutic value of inhibiting ATR to treat medulloblastoma.

**Results**

**ATR inhibition by pVE-822 induces DNA damage and tumor regression in mice with medulloblastoma**

To investigate the effect of ATR inhibition in a preclinical model of medulloblastoma, we treated mice with established, spontaneous SmoM2 medulloblastoma, which have a Cre-inducible, constitutively active form of the Smoothened (SMO) receptor that drives SHH pathway activity (Schuller et al., 2008), with a nano-formulation of the small molecule ATR inhibitor VE-822, pVE-822, by IP injection, as previously described (Lang et al., 2016). Murine medulloblastoma is slightly different in the SmoM2 model of tumorigenesis depending on whether SHH pathway hyperactivation, and consequently tumor formation, is driven by the hGFAP (Human glial fibrillary acidic protein) promoter (G-SmoM2), expressed in neural stem cells, or by the Math1 (Mammalian atonal homolog 1) promoter (M-SmoM2), expressed in CGNPs. We have seen that tumor progression is more rapid and aggressive in the G-SmoM2 model and these tumors are more resistant to the effects.
of therapeutic radiation. With *G-SmoM2* mice, tumor formation is apparent by P12 due to symptoms such as ataxia, lethargy, growth impairment, reduced grooming, and posterior skull deformity (*Schuller et al.*, 2008). These mice typically perish by P20, with median survival until P18. With *M-SmoM2* mice, tumor formation is apparent by P16 and mice can survive until P40, with median survival until P20 (*Crowther et al.*, 2016). We analyzed the effects of pVE-822 treatment in both of these medulloblastoma models to discern any differences. In order to determine the pharmacodynamics of pVE-822 treatment in mice with medulloblastoma, we injected tumor-bearing mice once per day for five consecutive days starting either at P8 or P12 and sacrificed animals 12 hr. following the fifth injection.

Treatment beginning at P12 had a stronger anti-tumor effect in both models of medulloblastoma, as seen on histology of brain sections (*Fig. 4.1A*). In order to ascertain the anti-tumor effect of pVE-822, we compared the density of the posterior fossa between drug- and vehicle-treated mice, measured on mid-sagittal brain sections as the number of nuclei per mm$^2$ in the posterior fossa (*Fig. 4.1B*). Using this method, we detected a significant anti-tumor effect of pVE-822 treatment in *G-SmoM2* mice, but not in *M-SmoM2* mice. Separation of the components of this density measurement fails to detect an anti-tumor effect in both models of medulloblastoma (*Fig. 4.S1A,B*). That is, there was no significant difference in the number of nuclei in the posterior fossa or the size, in mm$^2$, of the posterior fossa between pVE-822- and Vehicle-treated mice.

In order to further understand the tumorigenic effects of ATR inhibition by pVE-822 treatment in mice with *SmoM2* medulloblastoma, we looked at changes in cell proliferation in the posterior fossa as measured by PCNA (Proliferating cell nuclear antigen), mitosis as measured by pH3 (phosphorylated Histone H3), DNA damage as measured by γH2A.X (phosphorylated Histone H2A, member X), cell cycle arrest and apoptosis as measured by p-p53 (phosphorylated p53), and cell
death as measured by cC3 (cleaved Caspase-3) (Figs 4.1C, D, 4.S1C-E). On quantification, we saw that cell proliferation in tumors was significantly reduced by pVE-822 treatment in both models of medulloblastoma and that there was a concurrent increase in DNA damage (Fig. 4.1E). Surprisingly, we did not detect changes in mitosis, activation of cell cycle arrest, or apoptosis from pVE-822 treatment despite decreased proliferation, increased DNA damage, and smaller tumors. As we have shown previously (Lang et al., 2016), pVE-822 treatment does not illicit a strong response in the rest of the brain, measured as the percentage of cortical cells staining positive for PCNA, pH3, γH2A.X, p-p53, or cC3 (Fig. 4.S1F). Nevertheless, there was no apparent survival benefit associated with pVE-822 treatment compared to Vehicle in G-SmoM2 and M-SmoM2 mice given 60 mg/kg of drug or vehicle once per day for five days per week starting at P12 (Fig. 4.S1G, H).

**Acute ATR inhibition by pVE-822 does not result in cell cycle changes in medulloblastoma tumor cells**

Despite lack of activation of the potent cell cycle inhibitor p53 (Agarwal et al., 1995; Shaw, 1996), we hypothesized that altered cell cycle dynamics could account for the reduced proliferation and tumor regression seen in medulloblastoma mice treated with pVE-822. We interrogated acute changes induced by ATR inhibition to the cell cycle of medulloblastoma cells by injecting P16 G-SmoM2 and M-SmoM2 mice with 60 mg/kg pVE-822 or Vehicle and then sacrificing animals 6 hr. later to harvest tumor cells for fluorescence-activated cell sorting (FACS). By staining for DNA content with FxCycle Violet, we could separate cells in G$_1$-phase versus S-phase versus G$_2$/M-phase. Additional staining with pH3 allowed us to separate G$_2$- and M-phases (Fig. 4.S2A). In our initial studies with a small cohort of animals, we did not detect any differences in cell cycle in medulloblastoma mice treated with pVE-822 (Fig. 4.S2B). Similarly, analyzing the cell cycle after subsetting for tumor
cells with DNA damage using γH2A.X staining failed to reveal any significant changes to cell cycle with pVE-822 treatment (Fig. 4.S2C). In ATR-disrupted CGNPs, we had previously found increased inappropriate mitotic entry (Lang et al., 2016). However, the proportion of tumor cells with DNA damage in M-phase appeared to be unaffected by ATR inhibition with pVE-822 (Fig. 4.S2D). Finally, in order to account for non-apoptotic or acute cell death that might have been missed by quantification of immunostained brain sections from medulloblastoma mice treated with pVE-822 for consecutive days, we analyzed the percentage of γH2A.X+ tumor cells with sub-G1 DNA content from mice treated with pVE-822 or Vehicle and sacrificed after 6 hr. Although there were minimal levels of sub-G1 DNA content in tumors from animals of both genotypes in both treatment groups (data not shown), we did find that tumors, regardless of treatment status, generally had detectable >4N DNA content, suggesting possible mitotic defects (Fig. 4.S2E).

**No transcriptional changes are seen in Atr-deleted medulloblastoma mice**

In a preliminary study to understand the genetic consequences of ATR disruption in medulloblastoma, we generated medulloblastoma-prone M-SmoM2 mice with and without conditional Atr deletion (M-SmoM2 and Math1-Cre;SmoM2;Atr<sup>loxP/loxP</sup> (SmoM2;Atr<sup>M-cre</sup>)) and harvested tumors at P18 for microarray analysis. Our prior work in Atr-deleted CGNPs demonstrated strong induction of the p53 pathway, and almost exclusively the p53 pathway, as a consequence of proliferation-associated DNA damage in CGNPs with ATR disruption (Lang et al., 2016). However, using the same thresholds for differential expression – false discovery rate (FDR) < 0.01 and fold-change (FC) of |log<sub>2</sub>(SmoM2;Atr<sup>M-cre</sup>/M-SmoM2)| > 1.5 – we failed to detect any differentially expressed genes induced by Atr deletion in medulloblastoma (data not shown). Analysis of principal components using four samples of each genotype showed overlap between the two groups without strong separation (data not shown).
Thus, we were unable to determine the transcriptional changes associated with ATR disruption in medulloblastoma using our genetic mouse model.

**VE-822 has a combined anti-proliferative effect with Vismodegib or Etoposide on CGNPs**

To ascertain if ATR inhibition in medulloblastoma might provide a greater therapeutic benefit in combination with other treatments, we performed an exploratory study by culturing CGNPs freshly isolated from P5 WT mice in the presence of VE-822 and various compounds. Synergy between VE-822 and the tested compounds was determined by comparing the effect of VE-822 alone on CGNPs, as we have previously reported (Lang et al., 2016), to the effect of the compound alone to the effect of VE-822 and the compound in combination. Vismodegib is an antagonist of the SMO receptor that has shown good anti-tumor effect against the SHH medulloblastoma subgroup (Robinson et al., 2015; Gajjar et al., 2013). However, we saw minimal effect of Vismodegib on WT CGNPs, as measured by changes in proliferation by Cyclin D2 (CD2), DNA damage by $\gamma$H2A.X, and apoptosis by cC3 (Fig. 4.S3A, left). Adding VE-822 decreased CD2 levels but did not increase levels of $\gamma$H2A.X or cC3 (Fig. 4.S3A, right). Cisplatin is a DNA cross-linker commonly used to treat medulloblastoma, including as a part of the eight-in-one regimen (Gentet et al., 1995) and the POG regimen (Packer et al., 2006). VE-822 plus Cisplatin has shown synergy in lung cancer cells *in vitro* (Hall et al., 2014) and the combination is in clinical trials for various non-brain malignancies. Cisplatin alone reduced proliferation and increased apoptosis in CGNPs, but adding VE-822 failed to provide any further benefit (Fig. 4.S3B). Gemcitabine is a nucleoside analog that has shown efficacy against Group 3 medulloblastoma, which is associated with MYC amplification (Taylor et al., 2012; Morfouace et al., 2014). ATR inhibition seems to sensitize various cancer cells *in vitro* to the effects of Gemcitabine (Hall et al., 2014;
Prevo et al., 2012; Vendetti et al., 2015; Fokas et al., 2012), and VE-822 is currently in clinical trials with Gemcitabine for urothelial and fallopian tube cancers. Similarly, the topoisomerase inhibitor Irinotecan (bioactive as SN-38) has shown synergy with VE-822 in lung cancer cells (Hall et al., 2014) and the combination is being tested in clinical trials for metastatic solid tumors. In cultured CGNPs, Gemcitabine had no marked effect while SN-38 reduced proliferation and increased cell death (Fig. 4.S3C). Addition of VE-822 did not heighten these effects.

Like Irinotecan, Etoposide is a topoisomerase inhibitor and it is regularly used to treat medulloblastoma as a part of the POG regimen (Packer et al., 2006). Etoposide has also shown efficacy in combination with VE-822 in lung cancer cells (Hall et al., 2014), and topoisomerase inhibitors like Irinotecan and Topotecan are in clinical trials with VE-822 for various non-brain cancers. Used alone, Etoposide dramatically reduced proliferation while increasing DNA damage and apoptosis in WT CGNPs (Fig. 4.S3D, left). We have previously shown that CGNPs exposed to VE-822 alone exhibit a similar response (Lang et al., 2016). Interestingly, combining the two agents was apparently cytoprotective despite possible synergy in reducing proliferation (Fig. 4.S3D, right). SB-743921 is an investigational mitotic inhibitor that targets EG5 and has a similar mechanism of action to Vincristine and Paclitaxel, but with increased specificity for mitosis (Grisold et al., 2012; Marcus et al., 2005). Mitotic inhibitors like Vincristine are standardly used in most medulloblastoma treatment regimens, including eight-in-one (Gentet et al., 1995), VCP (Mulhern et al., 1998), and POG (Packer et al., 2006). Moreover, the ATR inhibitor AZD-6738 is in clinical trials with Paclitaxel for metastatic cancers, suggesting therapeutic benefit in combining ATR inhibition with mitotic disruption. In cultured CGNPs, SB-743921 reduced proliferation and increased DNA damage and apoptosis in time- and dose-dependent manners (Fig. 4.S3E, left top and bottom). However, as with Etoposide, adding VE-822 to SB-743921-treated cells...
appeared to have a cytoprotective effect that was more pronounced with shorter drug exposure (Fig. 4.S3E, right top and bottom). In a final test of synergy, VE-822 was given alone or in combination with 2 Gy. X-ray radiation therapy (XRT) to cultured CGNPs (Fig. 4.S3F). Here, again, VE-822 seemed to reduce the levels of γH2A.X and cC3 induced by radiation. To verify these effects in vivo in mice with medulloblastoma, we exposed M-SmoM2 and SmoM2;AtrM-cre mice to a single dose of 10 Gy. XRT at P12 and followed animals for event-free survival. No difference in survival was noted between XRT-treated medulloblastoma mice with and without Atr deletion (Fig. 4.S3G).

**Discussion**

Here, we have shown that ATR is required by medulloblastoma tumor cells to prevent toxic accumulation of DNA damage. Increased DNA damage from ATR inhibition by pVE-822 was associated with tumor regression in two mouse models of primary, spontaneous medulloblastoma. Actual tumor regression is suggested by gross observation that the primary tumor in P16 pVE-822-treated medulloblastoma mice is even smaller in cross-sectional area than the tumor in P12 Vehicle-treated medulloblastoma mice. We noted decreased proliferation in tumors treated with pVE-822 but did not observe differences in the mitotic rate, p53 activation, or caspase-mediated apoptosis. Absence of cell cycle effects was confirmed by FACS analysis, which did, however, detect aneuploidy in tumor cells, but regardless of drug treatment status. ATR disruption was not found to increase survival times in mice with medulloblastoma. We postulated that combining ATR disruption with other treatments might produce an enhanced anti-tumor effect, but our preliminary studies in cultured CGNPs did not reveal any remarkable combinations, and, if anything, suggested that ATR inhibition may be cytoprotective against various agents (Table 4.S1). Indeed, in vivo testing of ATR disruption combined with XRT in
a mouse genetic model of Atr-deleted medulloblastoma showed no survival benefit to radiation in mice with Atr deletion versus Atr intact.

Despite the significant anti-tumor effect of pVE-822 treatment in mice with medulloblastoma, the question remains as to how ATR inhibition promotes tumor regression. We have previously shown in Atr-deleted CGNPs from neonatal mice that accumulation of proliferation-associated DNA damage primarily induces p53-mediated, caspase-dependent apoptosis, but can also promote p53- and caspase-independent cell death as well as p21-induced cell cycle arrest (Lang et al., 2016). With earlier developmental deletion of Atr in neural stem cells, including the cells that give rise to CGNPs, endogenous DNA damage led to reduced proliferation rather than frank cell death in the nascent cerebellum (Lee et al., 2012). In medulloblastoma mice treated with pVE-822, we observed DNA damage that was associated with reduced proliferation rather than increased cell death, as seen in Atr-deleted neural stem cells. This could suggest that our tumor cells behave more like neural stem cells than CGNPs, but the tumors are themselves derived from CGNPs and other studies have demonstrated the similarity between CGNPs and CGNP-derived tumor cells (Gibson et al., 2010; Schuller et al., 2008; Yang et al., 2008). Another possibility is that the anti-tumor effect from ATR inhibition was not due to decreased proliferation as measured by PCNA since PCNA is also recognized as a marker of DNA damage (Balajee and Geard, 2001; Strzalka and Ziemienowicz, 2011; Essers et al., 2005; Gamper et al., 2012). Indeed, we saw no differences in mitotic rate or p53 activation in pVE-822-treated medulloblastomas compared to Vehicle-treated and concordantly, there were no apparent cell cycle alterations associated with ATR inhibition in medulloblastoma cells. This possibility, however, would make interpretation of tumor regression even more difficult. In any case, reduced proliferation, as measured by PCNA, could explain failed tumor expansion but not actual tumor regression, as we observed.
Although our FACS findings were congruent with our immunohistochemical data on the absence of cell cycle alterations associated with pVE-822 treatment in medulloblastoma cells, FACS did not detect the high level of DNA damage in tumors seen by immunohistochemistry. This inconsistency could be explained by the differing treatment regimens and pVE-822 pharmacokinetics. For the immunohistochemistry studies, tumors were analyzed from mice that had been treated with 60 mg/kg pVE-822 delivered by IP injection once per day for five consecutive days. For FACS, we treated tumor-bearing mice with a single 60 mg/kg dose of pVE-822 and sacrificed 6 hr. later. Therefore, it is likely that pVE-822 takes longer than 6 hr. to produce an effect in medulloblastoma mice in vivo. Even with our prior experiments using pVE-822 in neonatal WT mice, we treated animals with three doses of drug spaced 12 hr. apart, which allowed for detection of increased DNA damage and apoptosis in CGNPs (Lang et al., 2016). The FACS experiment will be repeated in the future under different treatment conditions. It is also likely that pVE-822 treatment induces tumor cell apoptosis in vivo between 6 hr. and 12 hr. after administration since cC3 has a short half-life and apoptotic cells are cleared rapidly by phagocytosis, which could have thus impaired our detection by immunostaining at five days post-initial treatment (Elliott and Ravichandran, 2010; Walsh et al., 2011). Therefore, apoptosis could account for actual tumor regression while reduced proliferation would explain failed tumor expansion. Moreover, we have previously shown that CGNPs with disrupted ATR can experience non-apoptotic cell death, so this form of cell death could also be operational in ATR-inhibited medulloblastoma cells (Lang et al., 2016). TUNEL analysis of brain sections from medulloblastoma mice treated with pVE-822 should resolve this possibility.

While it is possible that tumor regression from ATR inhibition could be due to tumor cell apoptosis early in the course of the pVE-822 regimen, the lack of p53 activation and Caspase-3 cleavage 12 hr. after drug administration on the fifth and
final consecutive day of treatment suggests that at least some cells developed resistance to apoptosis. That is, levels of DNA damage from ATR inhibition were still elevated at this time point, and proliferation as measured by PCNA was reduced, but there may have been clonal selection for tumor cells that can evade DNA damage-induced apoptosis – a well-recognized phenomenon in cancer (Schimmer, 2004; Srinivasula and Ashwell, 2008; Fulda and Vucic, 2012; Deveraux et al., 1999). Clonal selection could potentially also explain our microarray results. In this case, Cre-mediated Atr deletion in early development could have been associated with incomplete recombination, as we have previously described with Cre expression under the control of the Math1 promoter (Lang et al., 2016), and subsequent selection for CGNPs with functional ATR that then transform into medulloblastoma cells. Selection for cells with intact ATR following Atr mutation has also been previously described by others looking at the testes and lungs of mice (Murga et al., 2009). Thus, to better understand the transcriptional changes induced by ATR disruption in medulloblastoma, we will perform studies in the future where we inject pVE-822 into mice with established medulloblastoma and isolate tumor RNA between 6 and 12 hr. after administration.

pVE-822 treatment had a clear anti-tumor effect in two mouse models of spontaneous medulloblastoma. Yet, there was no survival benefit to ATR inhibition in these animals. In fact, G-SmoM2 mice treated with pVE-822 had the same median survival as untreated or Vehicle-treated G-SmoM2 mice, which is also the same median survival for mice with Atr deletion driven by the hGFAP promoter (Gershon et al., 2013; Lang et al., 2016). This observation suggests the possibility that failure of ATR inhibition to extend tumor mouse survival despite tumor regression could be due to the systemic toxicity of pVE-822, especially given that the drug was administered by IP injection. Atr-null mice are embryonic lethal, perishing by E8.5 due to DNA damage and chromosomal abnormalities that lead to cell death.
Hypomorphic mutation of Atr in mice is also unsustainable for life, as animals perish by 35 weeks postnatally (median survival 20 weeks) due to multiple organ failure (Murga et al., 2009). In postnatal life, mice with global deficiency of ATR that was induced embryonically appear progeric, with osteoporosis, greying hair, kyphosis, and pancytopenia. When Atr was deleted in adult mice, animals again displayed these signs of progeria as well as thymic involution, alopecia, and organ fibrosis (Ruzankina et al., 2007). In mature Drosophila, ATR is required to maintain intestinal stem cells (Park et al., 2015). As such, systemic ATR inhibition by IP pVE-822 delivery may be overly toxic to show a survival benefit in medulloblastoma despite tumor regression. Even with a short course of five consecutive days of pVE-822 administration, we observed treatment-associated side effects in mice such as growth impairment, lethargy, and diarrhea that were more severe in pVE-822-treated compared to Vehicle-treated animals. Direct delivery of pVE-822 through intracranial injection may bypass some of these negative systemic effects and is an attractive therapy strategy given the lack of obvious neurotoxicity from pVE-822, and given the recent research suggesting that ATR inhibition may actually protect quiescent cells like post-mitotic neurons from genomic stress (Kemp and Sancar, 2016).

This suggested cytoprotective effect of ATR inhibition was observed in our experiments combining various therapeutics with VE-822 in cultured CGNPs. The only tested compounds that may have had an additive or synergistic effect with VE-822 were Vismodegib and Etoposide, and only in reducing proliferation, not in increasing DNA damage or apoptosis. Curiously, Etoposide, SB-743921, and XRT may have a combined cytoprotective effect with VE-822 in CGNPs. On their own, increasing concentrations of Etoposide, SB-743921, and VE-822 led to dose-dependent increases in DNA damage and apoptosis with concomitant decreases in proliferation in CGNPs from P5 WT mice (Lang et al., 2016). However, in the
presence of VE-822, increasing concentrations of Etoposide failed to increase DNA
damage and apoptosis and increasing concentrations of SB-743921 actually led to
dose-dependent decreases in DNA damage and apoptosis. With XRT, 2 Gy. caused
substantial DNA damage and apoptosis in cultured CGNPs after 2 hr., but pre-
treatment with VE-822 led to attenuation of these effects in a dose-dependent
manner. These results were unexpected given prior research showing that ATR
inhibition, and even VE-822 treatment specifically, sensitizes various proliferating
cells \textit{in vitro} to the effects of the same therapeutics that we tested here in CGNPs for
the first time (Hall et al., 2014; Fokas et al., 2012). Further research is needed to
elucidate possible mechanisms leading to these cytoprotective effects arising from
combinatorial therapy in a highly proliferative, primary neuronal progenitor cell
population like CGNPs.

A final consideration is in using pVE-822 to treat \textit{TP53}-mutated
medulloblastoma. Mutations in \textit{TP53} are most common in the SHH subgroup of
medulloblastoma and \textit{TP53}-mutated medulloblastoma is particularly aggressive and
unresponsive to current treatments (Zhukova et al., 2013). Similarly, in mice with
SHH subgroup medulloblastoma, loss of \textit{p53} accelerates tumorigenesis (Wetmore
et al., 2001). Combined deletion of \textit{p53} and various DNA repair pathway genes has
been shown to induce medulloblastoma in animal models (Frappart et al., 2007,
2009; Holcomb et al., 2006; Lee and McKinnon, 2002). Interestingly, when we
conditionally co-deleted \textit{Atr} and \textit{p53} in CGNPs, no mice developed tumors (Lang
et al., 2016). Rather, this combination led to extremely high levels of DNA damage,
severe chromosomal abnormalities, and ultimately caspase-independent cell death.
Importantly, widespread chromosome damage is also found in tumor cells from
patients with \textit{TP53}-mutated, SHH subgroup medulloblastoma (Rausch et al., 2012).
Thus, for a cancer that is resistant to most current therapies, \textit{TP53}-mutated
medulloblastoma may respond well to ATR inhibition since an anti-tumor effect
could still potentially be achieved through an as yet uncharacterized form of caspase-independent cell death.

Materials and Methods

Mice

We generated G-SmoM2 and M-SmoM2 SHH subgroup medulloblastoma mice by crossing hGFAP-Cre (Jackson Labs, Bar Harbor, ME; Stock 012886) (Zhuo et al., 2001) and Math1-Cre (Jackson Labs, Stock 011104) (Matei et al., 2005) animals with SmoM2loxP/loxP mice, in which tumorigenesis is induced by deletion of a loxP-flanked Stop cassette between the mutant, constitutively active, Rosa26-driven SmoM2 promoter and coding region (Jackson Labs, Stock 005130) (Schuller et al., 2008). Medulloblastoma-prone mice with Atr deletion, SmoM2;AtrM-cre, were born from the cross between Math1-Cre;AtrloxP/+ and SmoM2loxP/loxP;AtrloxP/loxP, as previously described (Lang et al., 2016). Animals were determined to have tumors based on PCR genotyping and phenotypic observation for symptoms such as ataxia, lethargy, growth impairment, reduced grooming, and/or posterior skull deformity. All mice were of species Mus musculus and crossed into the C57BL/6 background through at least five generations. We used equal numbers of male and female mice, as we did not observe any differences based on sex. Numbers (n) indicated in each figure (biologic replicates) were determined so as to measure a 25% difference in means with Power = 80% and $\alpha = 0.05$. Animal use was in keeping with the policies of the University of North Carolina at Chapel Hill Institutional Animal Use and Care Committee.

Genotyping

PCR primers for Cre recombinase were: Forward = 5’ - GCG GTC TGG CAG TAA AAA CTA TC - 3’ and Reverse = 5’ - GTG AAA CAG CAT TGC TGT CAC TT - 3’.
Each reaction contained 0.2 μM of each primer, 4% DNA from toe lysates, and 42% nuclease-free water in Apex Red Taq DNA polymerase master mix (Genesee Scientific, San Diego, CA; 42-137). Cycling conditions were 95°C for 3 min. followed by 40 cycles of 1.5°C/s. to 95°C, 95°C for 30 s., 1.5°C/s. to 51.7°C, 51.7°C for 30 s., 1.5°C/s. to 72°C, and 72°C for 30 s. Primers to detect mutant SmoM2 were: Forward = 5’ - AAG TTC ATC TGC ACC ACC G - 3’ and Reverse = 5’ - TCC TTG AAG ATG GTG CG - 3’. Each reaction contained 0.5 μM of each primer and 4% DNA from toe lysates in Platinum blue PCR SuperMix (ThermoFisher Scientific (TFS), Waltham, MA; 12580015). Cycling conditions were 94°C for 2 min., 1.5°C/s. to 94°C, 94°C for 30 s., 1.5°C/s. to 64°C, 64°C for 30 s., 1.5°C/s. to 68°C, 68°C for 1 min., 1.5°C/s. to 94°C, 94°C for 30 s., 1.5°C/s. to 63.5°C, 63.5°C for 30 s., 1.5°C/s. to 68°C, 68°C for 1 min., 1.5°C/s. to 94°C, 94°C for 30 s., 1.5°C/s. to 62.5°C, 62.5°C for 30 s., 1.5°C/s. to 68°C, 68°C for 1 min., 1.5°C/s. to 94°C, 94°C for 30 s., 1.5°C/s. to 61.5°C, 61.5°C for 30 s., 1.5°C/s. to 68°C, 68°C for 1 min., and 1.5°C/s. to 94°C followed by 24 cycles of 94°C for 30 s., 1.5°C/s. to 60°C, 60°C for 30 s., 1.5°C/s. to 68°C, and 68°C for 1 min. To detect Atr^{loxP/loxP}, we used the following primers: Forward = 5’ - TAC ATT TTA GTC ATA GTT GCA TAA CAC - 3’ and Reverse = 5’ - CTT CTA ATC TTC CTC CAG AAT TGT AAA AGG - 3’. Each reaction contained 0.5 μM of each primer, 4% DNA from toe lysates, and 42% nuclease-free water in Apex Red Taq DNA polymerase master mix. Cycling conditions were 94°C for 4 min. followed by 34 cycles of 1.5°C/s. to 94°C, 94°C for 1 min., 1.5°C/s. to 62°C, 62°C for 2.5 min., 1.5°C/s. to 72°C, and 72°C for 2.5 min. All primers were obtained from Invitrogen (TFS, Waltham, MA) and all PCR-amplified products were resolved on a 1.4% agarose gel run at 170V for 50 min.
**pVE-822 formulation and *in vivo* administration**

pVE-822 was formulated as previously described (Lang et al., 2016). For immunohistochemical studies, medulloblastoma-bearing mice were injected IP with 60 mg/kg pVE-822 or an equal volume of Vehicle once per day for five consecutive days starting either at P8 or P12 and animals were sacrificed 12 hr. following the fifth and final administration.

**Immunostaining brain sections**

Mouse brains were processed and immunostained as previously described (Lang et al., 2016). Primary antibodies used are listed in the Supplemental Materials and Methods. Alexa 647 secondary antibody for PCNA staining was used at 1:2000 (TFS Z25108). DAPI and hematoxylin were used for nuclear counterstains.

**Quantification of immunostaining**

Stained slides were digitally imaged and positively stained cells were counted using Aperio Software (Aperio Technologies, Vista, CA) for chromogen-stained slides or Tissue Studio (Definiens, Munchen, Germany) for fluorescence, as previously described (Lang et al., 2016). The entire posterior fossa region in each section was manually annotated and used for staining quantifications, which were normalized to the total number of nucleated cells in the designated region. Tumor density was defined as the total number of nuclei in the manually annotated posterior fossa region, in units of number/mm$^2$. The entire posterior fossa was used for quantifications so as to avoid bias in determining which regions contained tumor. We measured statistical significance using the two-sided independent samples t-test assuming unequal variances in SPSS Statistics 23 (IBM, Armonk, NY).
Microarray analysis

For microarray comparison of SmoM2;Atr<sup>M-cre</sup> versus M-SmoM2, total RNA was purified using the RNeasy Mini Kit (Qiagen, Hilden, Germany; 74104) from the tumors of four P18 animals of each genotype. RNA quality and quantity were assessed by spectrophotometry and capillary gel electrophoresis. 500 ng of RNA from each sample was run on an Affymetrix GeneChip Mouse Transcriptome Assay 1.0 (Affymetric, Santa Clara, CA). Differential gene expression was analyzed in Partek Genomics Suite (Partek Inc., Chesterfield, MO) using RMA normalization and significance thresholds of FDR < 0.01 and |Log<sub>2</sub>(FC)| > 1.5.

Competing Interests

The authors are both co-inventors on a patent that includes pVE-822: Composition and methods using poly(oxazoline) micelles for delivery of poorly soluble drugs (US 62/395,202).

Author Contributions

P.Y.L. and T.R.G. conceived and designed the experiments, which were carried out by P.Y.L. The manuscript was written by P.Y.L.

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Supplemental Materials and Methods

Tumor size quantification

The number of nuclei in the posterior fossa was counted in a manually-annotated region on H&E-stained, sagittal brain sections from P16 mice of the indicated genotypes. Similarly, the size of the posterior fossa was determined in a manually-annotated region on H&E-stained, mid-sagittal brain sections from P16 mice of the indicated genotypes. Quantification of cortical staining was performed in manually-annotated regions of the anterior cerebral cortex. We measured statistical significance using the two-sided independent samples t-test assuming unequal variances in SPSS Statistics 23 (IBM, Armonk, NY).

Survival analysis

pVE-822- and Vehicle-treated G-SmoM2 and M-SmoM2 mice and XRT-treated M-SmoM2 and SmoM2;Atr
M-cre mice were followed for event-free survival. XRT was given as a single dose of 10 Gy. to the cranium and spine at P12. Tumor-bearing mice were identified by PCR and phenotypic observation for symptoms such as ataxia, lethargy, growth impairment, reduced grooming, and/or posterior skull deformity. Animals were sacrificed either at the censure age of 300 days-old or at observation of severe weight loss, marked difficulty ambulating, onset of seizures, and/or significantly diminished physical activity. We measured statistical significance using the log-rank test in SPSS Statistics 23 (IBM, Armonk, NY).

Tumor cell isolation

Tumor cells were isolated as previously described (Lang et al., 2016) from the posterior fossa of P16 G-SmoM2 and M-SmoM2 mice treated with 60 mg/kg pVE-822 or Vehicle for 6 hr. Briefly, we separated the tumor from the rest of the brain,
removed the meninges, and dissociated the tissue in 20 units/mL of papain (Worthington Biochemical Corporation, Lakewood, NJ; PDS) at 37°C for 15 min. Tumor cells were then purified from the dissociated tissue by successive rounds of centrifugation, discontinuous density gradient, and mesh filtering.

**Flow cytometry**

Flow cytometry on isolated tumor cells was performed by fixing and permeabilizing (TFS GAS-004) the cells suspended in HBSS with 6 g/L glucose, as previously described (Lang et al., 2016). Tumor cells were then stained successively for DNA damage with e660-conjugated γH2A.X Ser139 (eBioscience, San Diego, CA; 50-9865) at 1:10, for M-phase with 488-conjugated pH3 (Cell Signaling Technology (CST), Danvers, MA; 9708) at 1:25, and for DNA content with FxCycle Violet (TFS F-10347) at 1:50. Technical controls included no stain, single-stained, and fluorescence-minus-one samples. FACS was performed on a LSR Fortessa (BD Biosciences, Franklin Lakes, NJ). For all experiments, 10k-50k cells were counted. Analysis of FACS data was done using FlowJo V10.0.8 (FlowJo LLC, Ashland, OR).

**Western blot analysis**

For Western blot analysis, CGNPs were isolated from the cerebella of P5 WT mice in the same manner as tumor cells, described above. Purified CGNPs were cultured in plates coated with poly-L-lysine (Sigma-Aldrich, St. Louis, MO; P4707) and in media containing 0.5 mg/mL SHH (R&D Biosystems, Minneapolis, MN; 464SH025). Cells were treated with the indicated drugs, harvested after the indicated times, and lysates were probed for γH2A.X Ser139 at 1:50 (CST 9708, Batch 10), cC3 at 1:100 (Biocare Medical, Concord, CA; CP229C), CD2 at 1:300 (CST 3741, Batch 14), and β-actin at 1:5000 (CST 3700, Batch 3). HRP-conjugated secondary antibodies were used at 1:2000. VE-822 (MedKoo Biosciences, Chapel Hill, NC; 406258) was
dissolved in DMSO, Vismodegib (SelleckChem, Houston, TX; GDC0449) was dissolved in DMSO, Cisplatin (SelleckChem S1166) was dissolved in DI water, Gemcitabine HCl (SelleckChem S1149) was dissolved in DI water, SN-38 (SelleckChem S4908) was dissolved in DMSO, Etoposide (SelleckChem S1225) was dissolved in DMSO, and SB-743921 (SelleckChem S2182) was dissolved in NMP. Synergistic effect of combination therapies was approximated as greater than additive effect of single agents.

**Primary antibodies**

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Dilution</th>
<th>Source&lt;sup&gt;a,b&lt;/sup&gt;</th>
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</thead>
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<td>060115</td>
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<tr>
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<td>CST 9284</td>
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<td>1:100</td>
<td>CST 9706</td>
<td>9</td>
</tr>
<tr>
<td>γH2A.X Ser139</td>
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<td>CST 9718</td>
<td>10</td>
</tr>
<tr>
<td>CD2</td>
<td>1:300</td>
<td>CST 3741</td>
<td>14</td>
</tr>
<tr>
<td>β-actin</td>
<td>1:5000</td>
<td>CST 3700</td>
<td>3</td>
</tr>
</tbody>
</table>

<sup>a</sup>BM = Biocare Medical, Concord, CA, USA.

Antibody validated on brain sections from WT mice at P0, P3, P7, P12, and P20 (n = 10 for each) and WT mice exposed to 10 Gy XRT at P0, P3, P7, P12, and P20 (n = 10 for each).

<sup>a</sup>CST = Cell Signaling Technology, Danvers, MA, USA.

 Antibody validation information:

http://www.cellsignal.com/common/content/content.jsp?id=ourApproach-validation-principles
Inhibition of KIF11/EG5 in CGNPs results in transient mitotic arrest without DNA damage or apoptosis

Introduction

Microcephaly and brain cancer represent opposite neurodevelopmental phenotypes that can arise from shared dysregulation of the same processes. As a result, insight from microcephaly may provide novel therapeutic strategies for brain cancer. Primary autosomal recessive microcephaly has been described as essentially a mitotic disorder (Faheem et al., 2015). Mitotically important proteins whose genes are mutated in primary microcephaly include the centrosome assembler SAS6 (Spindle assembly abnormal protein 6 homolog) (Khan et al., 2014; Rodrigues-Martins et al., 2007) and the spindle organizers ASPM (Abnormal spindle microtubule assembly) (Bond et al., 2002; Kouprina et al., 2005; Roberts et al., 2002; Zhong et al., 2005), WDR62 (WD repeat domain 62) (Bastaki et al., 2016; Bhat et al., 2011; Kousar et al., 2011; Nicholas et al., 2010), and CDK6 (Cylin-dependent kinase 6) (Hussain et al., 2013), which is canonically recognized for G₁-S cell cycle progression (Sherr et al., 2016; Tigan et al., 2016). Disruption of mitosis has also been linked to other microcephalic disorders. Seckel syndrome is characterized by intrauterine growth restriction, postnatal growth impairment, characteristic cranio-facial deformities, and microcephaly (Goodship et al., 2000; O’Driscoll et al., 2003). This disorder can be caused by mutation of ATR (Ataxia-telangiectasia and Rad3-related), which codes for a DNA damage response protein that has also been shown to regulate mitotic entry in proliferative cells (Lang et al., 2016; Brown and Baltimore, 2003). CENPE (Centromere protein E) is a microtubule-associated motor protein that aligns chromosomes in prometaphase (Cai et al., 2009; Kim et al., 2008), and mutation of its coding gene has been identified in two siblings with microcephalic primordial dwarfism (Mirzaa et al., 2014). On the other hand, mutations in NIPBL
(Nipped-B-like protein) are the most common cause of Cornelia de Lange syndrome – a complex congenital disorder that presents with hearing impairment, visual defects, cardiac abnormalities, growth failure, seizures, and microcephaly (Ireland et al., 1993; Jackson et al., 1993; Krantz et al., 2004; Tonkin et al., 2004). The NIPBL protein is a cohesin that binds sister chromatids together after replication, and its disruption results in mitotic arrest (Peters et al., 2008; Watrin et al., 2006). Finally, KIF11 (Kinesin family member 11) mutation has been found in one family with primary, autosomal dominant microcephaly (Ostergaard et al., 2012). This gene codes for the protein EG5, which, like CENPE, is a microtubule-associated motor protein (Sawin et al., 1992; Sawin and Mitchison, 1995). EG5 activity is limited to mitosis and it acts in prophase and prometaphase to establish a bipolar mitotic spindle that is required to pull apart sister chromatids (Kashina et al., 1997, 1996).

Importantly, while expression of these genes may be decreased in microcephalic disorders, their expression is increased or otherwise vital in brain cancers. Upregulation of ASPM and CDK6 has been reported in both glioblastoma (Bikeye et al., 2010; Costello et al., 1997; Hagemann et al., 2008; Horvath et al., 2006; Lam et al., 2000), the most common malignant brain tumor in adults, and medulloblastoma (Mendrzyk et al., 2005; Vulcani-Freitas et al., 2011), the most common malignant brain tumor in children. While ATR expression may not be increased in medulloblastoma, we have recently shown that its protein product is essential for medulloblastoma tumorigenesis (Lang et al., 2016). Overexpression of KIF11 has been seen in glioblastoma, and inhibition of EG5 led to mitotic arrest and reduced proliferation of glioblastoma cells in vitro and in a mouse xenograft model (Venere et al., 2015). These observations suggest that mitotic disruption may be particularly toxic to brain tumor cells in the same manner of toxicity experienced by neural stem and progenitor cells in the genesis of microcephaly. Indeed, medulloblastoma is highly sensitive to the effects of mitotic disruption and
Vincristine, which irreversibly binds microtubules to prevent mitotic spindle formation, is used in three common medulloblastoma treatment regimens: eight-in-one (Gentet et al., 1995), VCP/CCG (Mulhern et al., 1998), and POG (Packer et al., 2006). However, Vincristine, and vinca alkaloids and taxanes in general, also disrupt non-mitotic microtubules, which regulate diverse cellular processes such as axonal transport, and therefore their use is associated with dose-limiting neurotoxicity (Conde and Caceres, 2009; Grisold et al., 2012; Marcus et al., 2005). As such, targeting a molecule like KIF11/EG5, whose downregulation produces microcephaly through reduced neural stem/progenitor cell proliferation and whose activity is limited to mitosis, may produce a strong anti-tumor effect in medulloblastoma with reduced side effects.

Medulloblastoma is a tumor of the cerebellum that can arise from mutations in cerebellar granule neuron progenitors (CGNPs) (Schuller et al., 2008; Yang et al., 2008). Molecular and genomic analyses have shown that medulloblastoma tumor cells with mutations in the Sonic hedgehog (SHH) signaling pathway are similar in many aspects to CGNPs (Gibson et al., 2010; Gilbertson and Ellison, 2008; Taylor et al., 2012; Wechsler-Reya and Scott, 2001). Indeed, mutation in CGNPs of a single gene in the SHH pathway that increases pathway activity can produce medulloblastoma in mice (Schuller et al., 2008; Yang et al., 2008). Here, we have tested the effect of EG5 inhibition on CGNPs using the specific small molecule inhibitor SB-743921 in neonatal mice. CGNP proliferation in mice occurs in the external granular layer (EGL) of the developing cerebellum and is maximal between postnatal day (P) 3 and P7 (Wechsler-Reya and Scott, 1999, 2001; Hatten and Heintz, 1995). We found that in SB-743921-treated P5 mice, EG5 inhibition dramatically increased the number of CGNPs in mitosis between 2 hr. and 12 hr. following treatment. Mitotically arrested CGNPs were seen to be specifically in prometaphase and prolonged delay in mitosis was associated with increased phosphorylation of the
translational repressor 4EBP1 (EIF4EBP1) (Gingras et al., 1999). To our surprise, mitotic arrest was not associated with increased DNA damage or apoptosis, and arrested CGNPs appeared to re-enter the cell cycle 24 hr. after SB-743921 administration. These results suggest that SB-743921 may not have a strong anti-tumor effect as single-agent therapy in CGNP-derived medulloblastoma, at least at the concentrations that we tested.

**Results**

To investigate the effect of KIF11/EG5 inhibition on CGNPs, we treated P5 wild-type (WT) mice with 7.5 mg/kg SB-743921 by IP injection and then sacrificed animals 2 hr., 4 hr., 6 hr., 8 hr., 12 hr., or 24 hr. later. Immunohistochemical analysis of brain sections stained for the mitotic marker phospho-Histone H3 (pH3) demonstrated that SB-743921 induced mitotic arrest of CGNPs in a time-dependent manner, with an apparent effect by as early as 2 hr. post-treatment and a maximal effect 12 hr. post-treatment (Fig. 4.2A). By 24 hr. after treatment with SB-743921, pH3 staining appeared almost back to baseline levels, and the EGL did not seem to be diminished. The effects of mitotic arrest were not limited to the cerebellum, as increases pH3+ cells were also seen in the hippocampus and the subventricular zone (SVZ). On quantification, we found that the proportion of CGNPs in the EGL significantly increased with each successive length of time after treatment with SB-743921, from 2 hr. to 12 hr. (Fig. 4.2B). Between 12 hr. and 24 hr. post-treatment, the fraction of pH3+ CGNPs in the EGL decreased dramatically, but at 24 hr., the proportion of pH3+ was still significantly greater than with no treatment and was equivalent to the proportion at 2 hr. Interestingly, mitotic arrest was not accompanied by induction of DNA damage, as seen by phospho-Histone H2A.X (γH2A.X) (Fig. 4.4A) or apoptosis, as seen by cleaved Caspase-3 (cC3) (Fig. 4.4B).

Dual-staining for pH3 and Survivin allows for distinguishing between the
different stages of mitosis, as we have previously demonstrated in CGNPs (Williams et al., 2015). Using this technique, we saw that CGNPs in the EGL of SB-743921-treated mice arrested predominantly in prometaphase, beginning at 2 hr. post-injection and maximally at 12 hr. (Fig. 4.2C). Arrest in prometaphase due to EG5 disruption, and specifically by SB-743921, has been previously described (Blangy et al., 1995; Chen et al., 2014). One study has reported that transient arrest at prometaphase increases inhibitory phosphorylation of the translational repressor 4EBP1 through metaphase (Velasquez et al., 2016). By double-labeling for pH3 and phospho-4EBP1 (p4EBP1), we saw that prolonged mitotic arrest from EG5 inhibition was associated with increased levels of p4EBP1 (Fig. 4.2D). Mitotic CGNPs had low levels of p4EBP1 at 2 hr. following SB-743921 treatment that increased dramatically through 12 hr. post-treatment and then declined again by 24 hr. p4EBP1 was detected almost exclusively in pH3+ CGNPs.

**Discussion**

We report here that EG5 inhibition by SB-743921 in neonatal mice induces widespread, transient mitotic arrest in CGNPs in the EGL of the cerebellum. A single dose of SB-743921 at 7.5 mg/kg delayed cells for up to 12 hr. in prometaphase, but by 24 hr., it appeared that arrested CGNPs had escaped the mitotic block without consequence. That is, we did not detect any changes in CGNP DNA damage or apoptosis over a 24 hr. time course of *in vivo* EG5 inhibition. However, we did see increasing levels of p4EBP1 as CGNPs were delayed in M-phase, and these levels declined again once cells began to progress through the cell cycle (Table 4.S2).

Cell cycle arrest is a physiological response to endogenous or exogenous sources of stress that acts to prevent DNA damage that may result from attempted duplication and redistribution of the genetic material, which could otherwise lead to neoplastic transformation or cell death (Elledge, 1996; Levine, 1997; Pietenpol and Stewart, 2015).
Even in a normal, unperturbed cell, delayed progression through the cell cycle can occur due to natural phenomenon such as oxidation-induced DNA damage (Willis et al., 2013), transcription - replication collision (Deshpande and Newlon, 1996; Ivesa et al., 2003; Takeuchi et al., 2003), or absence of growth factors (Braun-Dullaeus et al., 1998; Fingar and Blenis, 2004; Gross and Rotwein, 2016; McCubrey et al., 2007). Once the source of stress has been resolved, the cell cycle block can be lifted and cells are able to progress through the cycle normally (Agarwal et al., 1995, 1998; Cortez et al., 2004; Murray, 1992; Yoo et al., 2004). However, prolonged cell cycle arrest is often indicative of a serious underlying problem and triggers permanent cell cycle exit or apoptosis (Borel et al., 2002; Orth et al., 2012; Colin et al., 2015; Di Leonardo et al., 1994; Leontieva et al., 2010). How a cell determines whether cell cycle arrest should lead to cell cycle re-entry, cell cycle exit, or cell death is still being investigated. One possibility is that duration of arrest may dictate outcome (Charp et al., 1983; Hoffman et al., 1991; Lalande, 1990; Kaur et al., 1992; Linke et al., 1996; DiGregorio et al., 2001). Here, we have shown that the dose of SB-743921 that we administered to neonatal mice induced mitotic arrest for up to 24 hr. and that this length of mitotic arrest did not cause rapidly-proliferating CGNPs to experience increased DNA damage or apoptosis.

CGNPs were specifically arrested in prometaphase by SB-743921. In this stage of mitosis, the nuclear envelope breaks down and microtubules attach to chromosomes in order to later arrange and separate them (Magidson et al., 2011). EG5 directs microtubule activity in prophase and prometaphase to push apart duplicated centrosomes and establish a bipolar mitotic spindle that can organize chromosomes (Sawin et al., 1992; Kashina et al., 1997, 1996; Blangy et al., 1995). As such, EG5 inhibition by SB-743921 disrupts normal prophase and prometaphase mechanics, which could lead to activation of the spindle assembly checkpoint that induces mitotic arrest (Musacchio and Salmon, 2007; Peters, 2006; Cleveland et al., 2003).
Indeed, other microtubule poisons like Vincristine (O'Connor et al., 2002; Steen et al., 2008), Paclitaxel (Ikui et al., 2005; Snyder and Mullins, 1993; Uetake and Sluder, 2010), Nocodazole (Uetake and Sluder, 2010; Velasquez et al., 2016; Han et al., 2014), and Monastrol (Steen et al., 2008; Kapoor et al., 2000) have been reported to increase the proportion of cells arrested in prometaphase. However, the spindle assembly checkpoint is thought to prevent entry into anaphase, thus blocking cells at metaphase, so how microtubule poisons promote prometaphase arrest is a bit unclear, but could possibly relate to blurred distinctions between prometaphase and metaphase.

At the concentration of SB-743921 that we tested (single dose of 7.5 mg/kg by IP injection), mitotic arrest was transient. This observation is likely due to reversible binding of SB-743921 to EG5, which has been shown with its related compound SB-715992 (Ispinesib) (Lad et al., 2008). Thus, over time, SB-743921 dissociates from EG5 and is cleared from cells, allowing EG5 to resume normal activity and thereby clearing the block to mitotic progression. Nevertheless, during this period of SB-743921-induced mitotic arrest in prometaphase, we observed an increase in levels of p4EBP1 specifically in arrested CGNPs. 4EBP1 is a translational repressor that sequesters the EIF4 translation initiation factors, which are required to bring mRNA to the small ribosomal subunit (Gingras et al., 1998; Rousseau et al., 1996). Phosphorylation of 4EBP1 is inhibitory and increases the rate of protein translation (von Manteuffel et al., 1996; Beretta et al., 1996). Therefore, increased p4EBP1 in M-phase-arrested CGNPs may be indicative of hyperactivation of translation. On the one hand, increased translation could be a mechanism to enforce mitotic arrest. One study found that Cyclin A levels peak in prometaphase and that Cyclin A degradation was required for mitotic progression (den Elzen and Pines, 2001). Increasing Cyclin A induced mitotic arrest that was independent of the spindle assembly checkpoint, although the nature of this separate mitotic checkpoint has yet
to be elucidated. Thus, EG5 inhibition may allow a cell to sense disrupted prophase/prometaphase dynamics and increase 4EBP1 phosphorylation to promote translation of Cyclin A for prometaphase arrest. On the other hand, prolonged arrest in mitosis could exert a differential energy strain on cells. One report has demonstrated that mitotically arrested cells lose mitochondria, activating AMPK (Adenosine monophosphate-activated protein kinase) and increasing glycolysis over oxidative respiration (Domenech et al., 2015). Increased AMPK production was attributed to increased translation of the protein. Therefore, our observed increases in p4EBP1, which were specific to cells in mitosis, could be due to greater cellular requirements for glycolytic proteins such as AMPK. In either case, increasing translation is an important mechanism for responding to changing cellular demands in mitosis since transcription is suppressed during mitotic arrest (Taylor, 1960; Prescott and Bender, 1962; Prescott, 1964; Segil et al., 1996; Martinez-Balbas et al., 1995; Long et al., 1998; Gottesfeld and Forbes, 1997).

Arrest of CGNPs in prometaphase for 12 hr. - 24 hr. by SB-743921 did not induce DNA damage or apoptosis. The lack of cytotoxicity raises the question of the therapeutic value of SB-743921 for CGNP-derived medulloblastoma. In cultured leukemia cells, SB-743921, even at low concentrations, was found to cause mitotic arrest that led to apoptosis (Yin et al., 2015). Recent research on the SB-743921-related EG5 inhibitor Ispinesib has also been promising. Ispinesib reduced proliferation and increased cell death in HeLa cells, although DNA damage was not apparently increased and the cell death was described as p53-independent (Ohashi et al., 2015). In human breast cancer cell lines and mouse xenograft models, Ispinesib enhanced mitotic arrest that led to increased apoptosis (Purcell et al., 2010). For the in vivo studies, Ispinesib given at 5, 7.5, 8, or 10 mg/kg every four days for three total doses all reduced tumor growth, and with the 10 mg/kg treatment was found to increase caspase-dependent apoptosis. Similarly, in glioblastoma cell lines
and mouse xenograft models, Ispinesib, at 10 mg/kg for the in vivo studies, had a significant anti-tumor effect through mitotic arrest and induction of apoptosis (Venere et al., 2015). Unfortunately, clinical trials with EG5 inhibitors like Ispinesib have shown more attenuated efficacy (Huszar et al., 2009; Khoury et al., 2012; Lee et al., 2008). Although neurotoxicity has generally been absent with trials of EG5 inhibitors, in contrast to use of non-specific microtubule inhibitors like vinca alkaloids and taxanes, the anti-tumor effect of several inhibitors tried as monotherapy in various cancers has been limited, which may be related to the short half-life of these EG5 inhibitors. In addition, there have been no clinical trials for EG5 inhibitors in primary brain tumors, few studies have explored the efficacy of EG5 inhibition for treating brain tumors in animal models, and thus the ability of EG5 inhibitors like SB-743921 and Ispinesib to reach brain tumors in sufficient concentrations and remain active long enough to induce cell death is uncertain. In neonatal mice, a single IP injection of 7.5 mg/kg SB-743921 was insufficient to cause DNA damage or apoptosis in CGNPs of the proliferative cerebellum. Increased drug concentration and/or more frequent drug dosing may lead to CGNP cytotoxicity, and CGNP-derived medulloblastoma may be more sensitive to EG5 inhibition than CGNPs. We will explore these possibilities in our future research.

**Materials and Methods**

**Mice**

WT C57BL/6J mice were used for all experiments (Jackson Labs, Bar Harbor, ME; Stock 000664). All mice were of species *Mus musculus*. We used equal numbers of male and female mice, as we did not observe any differences based on sex. Numbers (n) indicated in each figure (biologic replicates) were determined so as to measure a 25% difference in means with Power = 80% and \( \alpha = 0.05 \). Animal use was in keeping with the policies of the University of North Carolina at Chapel Hill.
Institutional Animal Use and Care Committee.

**SB-743921 formulation and in vivo administration**

SB-743921 ([SelleckChem, Houston, TX; S2182](https://www.selleckchem.com)) was dissolved in NMP and diluted in PEG-200. All animals were treated with a single dose of 7.5 mg/kg SB-743921 by IP injection and then sacrificed after the indicated lengths of time.

**Immunostaining of brain sections**

Mouse brains were processed and immunostained as previously described ([Lang et al., 2016](https://www.selleckchem.com)). Primary antibodies used are listed in the Primary antibodies table at the end. Alexa 555 secondary antibody for pH3 staining was used at 1:2000 ([ThermoFisher Scientific (TFS), Waltham, MA; Z25005](https://www.thermofisher.com)), Cy5 secondary antibody for Survivin staining was used at 1:2000 ([TFS A10523](https://www.thermofisher.com)), and Cy5 secondary antibody for p4EBP1 staining was used at 1:2000 ([TFS A10523](https://www.thermofisher.com)). DAPI and hematoxylin were used for nuclear counterstains.

**Quantification of immunostaining**

Stained slides were digitally imaged and positively stained cells were counted using Aperio Software (Aperio Technologies, Vista, CA) for chromogen-stained slides or Tissue Studio (Definiens, Munchen, Germany) for fluorescence, as previously described ([Lang et al., 2016](https://www.selleckchem.com)). The entire EGL region in each section was manually annotated and used for staining quantifications, which were normalized to the total number of nucleated cells in the designated region. We measured statistical significance using the two-sided independent samples t-test assuming unequal variances in SPSS Statistics 23 (IBM, Armonk, NY).
Primary antibodies

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Dilution</th>
<th>Source&lt;sup&gt;a,b&lt;/sup&gt;</th>
<th>Batch</th>
</tr>
</thead>
<tbody>
<tr>
<td>cC3</td>
<td>1:50</td>
<td>BM CP229C</td>
<td>060115</td>
</tr>
<tr>
<td>pH3 Ser10</td>
<td>1:100</td>
<td>CST 9706</td>
<td>9</td>
</tr>
<tr>
<td>γH2A.X Ser139</td>
<td>1:2000</td>
<td>CST 9718</td>
<td>10</td>
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<td>Survivin</td>
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<td>p4EBP1 Thr37/46</td>
<td>1:500</td>
<td>CST 2855</td>
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</tbody>
</table>

<sup>a</sup>BM = Biocare Medical, Concord, CA, USA.

Antibody validated on brain sections from WT mice at P0, P3, P7, P12, and P20 (n = 10 for each) and WT mice exposed to 10 Gy XRT at P0, P3, P7, P12, and P20 (n = 10 for each).

<sup>a</sup>CST = Cell Signaling Technology, Danvers, MA, USA.

Antibody validation information:

http://www.cellsignal.com/common/content/content.jsp?id=ourApproach-validation-principles

Competing Interests

The authors have no competing financial interests.

Author Contributions

P.Y.L. and T.R.G. conceived and designed the experiments, which were carried out by P.Y.L. The manuscript was written by P.Y.L.

Funding

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### TABLES

**Table 4.S1:** In CGNPs, VE-822 combines with Vismodegib or Etoposide to produce an enhanced anti-proliferative effect, but combines with Etoposide, SB-743921, or XRT to decrease DNA damage and apoptosis.

<table>
<thead>
<tr>
<th>Compound</th>
<th>CD2</th>
<th>cC3</th>
<th>γH2A.X</th>
<th>+VE-822†</th>
</tr>
</thead>
<tbody>
<tr>
<td>VE-822</td>
<td>-</td>
<td>↑</td>
<td>↑</td>
<td></td>
</tr>
<tr>
<td>Vismodegib</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>↓CD2</td>
</tr>
<tr>
<td>Cisplatin†</td>
<td>↓</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Gemcitabine</td>
<td>-</td>
<td>-</td>
<td>↓</td>
<td></td>
</tr>
<tr>
<td>SN-38</td>
<td>↓</td>
<td>↑</td>
<td>↓</td>
<td></td>
</tr>
<tr>
<td>Etoposide§</td>
<td>↓</td>
<td>↑</td>
<td>↑</td>
<td>↓CD2, ↓cC3, ↓γH2A.X</td>
</tr>
<tr>
<td>SB-743921, 6hr</td>
<td>-</td>
<td>↑</td>
<td>↑</td>
<td>↓cC3, ↓γH2A.X</td>
</tr>
<tr>
<td>SB-743921, 12hr</td>
<td>↓</td>
<td>↑</td>
<td>↑</td>
<td></td>
</tr>
<tr>
<td>XRT</td>
<td>-</td>
<td>↑</td>
<td>↑</td>
<td>↓cC3, ↓γH2A.X</td>
</tr>
</tbody>
</table>

†Indicates increased (↑), decreased (↓), or unchanged (-) protein levels with single-agent treatment, as determined by Western blot, when compared with vehicle-treated.
§Indicates the apparent combined effect on protein levels of the indicated compound plus VE-822, as determined by Western blot. Only changes beyond the effects of either compound as single-agent are indicated.
Part of the eight-in-one medulloblastoma treatment regimen.
Part of the POG medulloblastoma treatment regimen.

**Table 4.S2:** Mitotic arrest of CGNPs in prometaphase increases over time following *in vivo* administration of SB-743921†, accompanied by increased p4EBP1.

<table>
<thead>
<tr>
<th>Time post-SB (hrs)†:</th>
<th>0</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>8</th>
<th>12</th>
<th>24</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH3§</td>
<td>-</td>
<td>↑</td>
<td>↑↑</td>
<td>↑↑↑</td>
<td>↑↑↑</td>
<td>↑↑↑</td>
<td>↑</td>
</tr>
<tr>
<td>Survivin‡</td>
<td></td>
<td>↑</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p4EBP1‡</td>
<td></td>
<td>↑</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>γH2A.X†</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>cC3‡</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

†SB-743921 given once at 7.5 mg/kg by IP injection to P5 WT mice.
‡Hour 0 is taken to be untreated mice.
§Indicates increased (↑) or unchanged (-) protein levels in the EGL, as determined by IHC/IF, when compared with untreated mice. Gray boxes indicate that staining was not performed/ measured for the indicated time point.
Figure 4.1: ATR inhibition by pVE-822 induces medulloblastoma tumor shrinkage. (A) Representative, H&E-stained, sagittal brain sections from mice of the indicated genotypes and ages treated with 60 mg/kg pVE-822 or Vehicle QDx5. (B) Quantification of posterior fossa density in P16 mice of the indicated genotypes treated with 60 mg/kg pVE-822 or Vehicle QDx5. (C-D) P16 brains in sagittal section from mice of the indicated genotypes, treated with 60mg/kg pVE-822 or Vehicle QDx5, stained for PCNA and DAPI (C) or γH2A.X (D). (E) Quantification of staining as in C, D, and Fig. 4.S1. Average is shown with s.e.m. P-values by two-sided independent samples t-test assuming unequal variances. All stainings were repeated on at least three biological replicates (n). Scale bars: 2 mm in A; 2 mm in C; 2 mm in D.
Figure 4.2: SB-743921 induces transient cell cycle arrest in prometaphase in CGNPs. (A) Sagittal brain (left) and cerebellar (right) sections, stained for pH3, from P5 WT mice injected with SB-743921 and sacrificed after the indicated lengths of time. Non-injected animals used as staining controls. (B) Quantification of pH3 staining as in A. Average shown with s.e.m. and trend line. P-values by two-sided independent samples t-test assuming unequal variances. All pH3 stainings were repeated on at least three biological replicates (n).
(C) The EGL in cross-section from P5 WT mice treated with SB-743921 and sacrificed after the indicated lengths of time, stained for pH3 (green), Survivin (red), and DAPI (blue). White arrowheads indicate cells in prometaphase, red arrowheads indicate cells in metaphase, and yellow arrowheads indicate cells in anaphase. (D) The EGL in cross-section from P5 WT mice treated with SB-743921 and sacrificed after the indicated lengths of time, stained for pH3 (green), p4EBP1 (red), and DAPI (blue). White arrowheads indicate cells positive for all three stains and yellow arrowheads indicate cells positive only for pH3 and DAPI. Scale bars: 2 mm in A (left); 0.5 mm in A (right, inset); 50 μm in C; 50 μm in D.
Supplemental Figure 4.1: ATR inhibition by pVE-822 produces no survival benefit in mice with medulloblastoma. (A) Quantification of the number of nuclei in the posterior fossa in P16 mice of the indicated genotypes treated with 60 mg/kg pVE-822 or Vehicle QDx5.
(B) Quantification of the posterior fossa size in P16 mice of the indicated genotypes treated with 60 mg/kg pVE-822 or Vehicle QDx5. (C-E) P16 brains in sagittal section from mice of the indicated genotypes, treated with 60 mg/kg pVE-822 or Vehicle QDx5, stained for pH3 (C), p-p53 (D), or cC3 (E). (F) Quantification of the percentage of cortical cells staining positive for PCNA, pH3, γH2A.X, p-p53, or cC3 in P16 mice of the indicated genotypes treated with 60 mg/kg pVE-822 or Vehicle QDx5. (G-H) Kaplan-Meier survival curves of G-SmoM2 (F) or M-SmoM2 (G) mice treated once daily, five times per week, with 60 mg/kg pVE-822 or Vehicle starting at P12. Average is shown with s.e.m. P-values in A, B, and F by two-sided independent samples t-test assuming unequal variances. P-values in G and H by log-rank test. All stainings were repeated on at least three biological replicates (n). Scale bars: 2 mm in C; 2 mm in D; 2 mm in E.
Supplemental Figure 4.2: pVE-822 treatment does not alter tumor cell cycle dynamics in mice with medulloblastoma. (A) FACS analysis without subsetting for γH2AX on tumor cells from P16 medulloblastoma mice of the indicated genotypes treated with 60 mg/kg pVE-822 or Vehicle for 6 hr. M-phase was determined by pH3 labeling.
(B) FACS analysis with subsetting for γH2A.X⁺ tumor cells from P16 medulloblastoma mice of the indicated genotypes treated with 60 mg/kg pVE-822 or Vehicle for 6 hr. M-phase was determined by pH3 labeling. (C) Quantification of γH2A.X⁺/pH3⁺ tumor cells from P16 medulloblastoma mice of the indicated genotypes treated with 60 mg/kg pVE-822 or Vehicle for 6 hr. (D) Live tumor cells from the posterior fossa of P16 G-SmoM2 mice treated with 60 mg/kg pVE-822 or Vehicle for 6 hr. can be found in all phases of the cell cycle by staining for DNA content with FxCycle Violet. γH2A.X⁺ cells are found in low abundance in both drug- and vehicle-treated tumors. Equivalent proportions of γH2A.X⁺ tumor cells in G₂/M from pVE-822-treated G-SmoM2 brains are pH3⁺ compared to γH2A.X⁺ tumor cells in G₂/M from Vehicle-treated G-SmoM2 brains. (E) Quantification of >4N DNA content detected by FACS in γH2A.X⁺ tumor cells from P16 medulloblastoma mice of the indicated genotypes treated with 60 mg/kg pVE-822 or Vehicle for 6 hr. Individual data points are plotted. n = sample size.
Supplemental Figure 4.3: VE-822 combined with Vismodegib or Etoposide enhances reduction of CGNP proliferation. (A) CGNPs isolated from P5 WT mice treated in vitro with Vismodegib alone or in combination with VE-822 at the indicated concentrations for 24 hr. and then collected for Western blot analysis of CD2, cC3, and γH2A.X, with β-actin loading control. (B) CGNPs isolated from P5 WT mice treated in vitro with Cisplatin alone or in combination with VE-822 at the indicated concentrations for 24 hr. and then collected for Western blot analysis of CD2, cC3, and γH2A.X, with β-actin loading control. (C) CGNPs isolated from P5 WT mice treated in vitro with VE-822, Gemcitabine, or SN-38 alone or in combination with VE-822 at the indicated concentrations for 24 hr. and then collected for Western blot analysis of CD2, cC3, and γH2A.X, with β-actin loading control. (D) CGNPs isolated from P5 WT mice treated in vitro with Etoposide alone or in combination with VE-822 at the indicated concentrations for 6 hr. and then collected for Western blot analysis of CD2, cC3, and γH2A.X, with β-actin loading control. (E) CGNPs isolated from P5 WT mice treated in vitro with SB-743921 alone or in combination with VE-822 at the indicated concentrations for 6 hr. (top) or 12 hr. (bottom) and then collected for Western blot analysis of CD2, cC3, and γH2A.X, with β-actin loading control. (F) CGNPs isolated from P5 WT mice treated in vitro with VE-822 for 2 hr. at the indicated concentrations and then continued with VE-822 alone or exposed to 2 Gy. XRT and then collected after another 2 hr. for Western blot analysis of CD2, cC3, and γH2A.X, with β-actin loading control. (G) Kaplan-Meier survival curve of M-SmoM2 and SmoM2;AtrM-cre mice given one dose of 10 Gy. cranio-spinal XRT at P12. P-values by log-rank test. Vis = Vismodegib, Cis = Cisplatin, VE = VE-822, Gem = Gemcitabine, SN = SN-38, Eto = Etoposide, SB = SB-743921.
Supplemental Figure 4.4: SB-743921 does not induce DNA damage or apoptosis in CGNPs. (A) Sagittal brain (left) and cerebellar (right) sections, stained for γH2A.X, from P5 WT mice injected with SB-743921 and sacrificed after the indicated lengths of time. Non-injected animals used as staining controls. (B) Sagittal brain (left) and cerebellar (right) sections, stained for cC3, from P5 WT mice injected with SB-743921 and sacrificed after the indicated lengths of time. Non-injected animals used as staining controls. All stainings were repeated on at least three biological replicates. Scale bars: 2 mm in A (left); 0.5 mm in A (right, inset); 2 mm in B (left); 0.5 mm in B (right, inset)
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234


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The role of ATR in the developing cerebellum

Chapter 1 of this dissertation provided an overview of the function of ATR (Ataxia telangiectasia and Rad3-related) in normal physiology and in the pathology of Seckel syndrome. On the other hand, Chapter 3 specifically investigated the role of ATR in cerebellar granule neuron progenitors (CGNPs) and delineated the consequences of ATR disruption in the developing cerebellum. Yet, the question remains as to why ATR is disproportionately important in the cerebellum compared to other brain structures. Two non-mutually exclusive possibilities will be briefly explored here. One possibility is that the proliferative nature of the cerebellum, both in terms of how long the cerebellum remains proliferative over developmental time and how much proliferation actually occurs during that window, creates a unique dependence on ATR, which mitigates replicative stress and prevents proliferation-associated DNA damage. The other possibility is that ATR has a different function or set of functions in CGNPs compared to other brain cells, which is plausible given the numerous posited and described functions of ATR, some of which differ by cell type and by situation. Indeed, a combination of these two possibilities may ultimately explain cerebellar reliance on ATR.

The proliferative nature of CGNPs could explain cerebellar dependence on ATR

In one study, early embryonic conditional deletion of Atr in the mouse central nervous system (CNS) using the neural stem cell-expressed driver Nestin (Atr\textsuperscript{N-cre}), which is expressed starting around embryonic day (E) E9.5 and induces Cre-
mediated deletion by E10.5, led to disruptions only in the ganglionic eminence and rhombic lip/nascent cerebellum (Lee et al., 2012). ATR loss from the ganglionic eminence resulted in high levels of DNA damage and apoptosis by E15.5, whereas ATR loss from the rhombic lip/nascent cerebellum caused low levels of DNA damage and apoptosis at E15.5 but significant reduction in proliferation by E17.5. These defects combined to generate postnatal microcephaly and cerebellar hypoplasia and the effects were thought to be p53-independent. In Atr^{N-cre} mice, neither DNA damage nor apoptosis were detected at significant levels in other parts of the brain through E17.5, including highly proliferative regions such as the cerebellar ventricular zone (CVZ), the hippocampus, and the cortical subventricular zone (SVZ). Even earlier conditional deletion of Atr from cortical progenitors using Emx1 (Empty spiracles homeobox 1) (Atr^{E-cre}), which is expressed around E8.5 and leads to Cre-mediated deletion by E9.5 (Gorski et al., 2002; Chou et al., 2009), induced decreased proliferation and increased DNA damage and apoptosis in the neocortex that produced a diminished hippocampus and cortex later in development (Lee et al., 2012).

The authors of the studies using Atr^{N-cre} and Atr^{E-cre} mice suggested that the selective effect of Atr deletion on specific cell populations in the developing brain could be attributed to a critical role for ATR in proliferation. Part of this assertion stems from the observation that significant cortical defects are absent from Atr^{N-cre} mice but exacerbated in Atr^{E-cre} mice in which Atr is deleted slightly earlier in development (E9.5 versus E10.5), at what the authors propose might be a period of more rapid cortical proliferation. However, the peak period of proliferation for most neural stem and progenitor populations occurs after E10.5 in mice (Mody et al., 2001; Finlay and Darlington, 1995; Dehay and Kennedy, 2007; Huang et al., 2010). Moreover, the authors remarked upon the surprising finding that not all rapidly-proliferating areas of the embryonic brain were affected by Atr deletion. Indeed, our
own research has supported the observation that Atr deletion does not affect all areas of the brain. Conditional deletion of Atr in mice using the hGFAP (human Glial fibrillary acidic protein) promoter (Atr\textsuperscript{G-cre}), which is expressed by neural stem cells around E11.5 (Andrae et al., 2001), caused widespread DNA damage and apoptosis almost exclusively in CGNPs at postnatal day (P) P0, with relative sparing of other proliferative regions such as the SVZ and the hippocampus (Lang et al., 2016). Similar results were seen upon administration of the ATR inhibitor pVE-822 (nano-formulated VE-822) to P5 WT mice. Thus, ATR is clearly critically important in some proliferative structures but less vital in others.

In a separate study released concurrently with the Atr\textsuperscript{N-cre} and Atr\textsuperscript{E-cre} experiments (Lee et al., 2012), the authors found that E17.5 Atr\textsuperscript{N-cre} mice had reduced proliferation and increased cell death in the SVZ, the cortical ventricular zone (VZ), and the rhombic lip/nascent cerebellum (Zhou et al., 2012). Specifically, in the E17.5 cerebellum, cell death was limited to TUJ1\textsuperscript{+} (Neuron-specific class III \(\beta\)-tubulin) non-progenitor cells. In addition, cultured primary mouse neurons experienced cell death without external stimulus following induced Atr deletion. These post-mitotic neurons were \(\gamma\)H2AX\textsuperscript{-} but enriched for RPA (Replication protein A), which the authors interpreted as evidence of DNA single-strand breaks (SSBs) but not DNA double-strand breaks (DSBs). Without external stimulus, neuronal cell death from ATR loss did not involve p53 activation, although upon irradiation, p53 was activated in ATR-depleted neurons. It is unclear why disparate results were obtained by different authors both investigating the effect of Atr deletion on E17.5 mouse brains using Atr\textsuperscript{N-cre} mice. That is, why was cell death in the SVZ seen to be elevated in one study but not the other? On the other hand, the findings in the cerebellum may be parallel, since both studies found decreased proliferation in the stem/progenitor region of the nascent cerebellum and minimal cell death in that area. Both results are in contrast to our own findings, however, of increased
cerebellar progenitor cell death and relatively unaffected proliferation with deletion of *Atr* later in development ([Lang et al., 2016](#)). The observation of increased cell death in post-mitotic neurons following *Atr* deletion was also interesting and again in contrast to our own findings with *Atr* deletion and inhibition. Indeed, a recent study using non-cycling keratinocyte-derived HaCaT cells has suggested that ATR inhibition has a cytoprotective effect in post-mitotic cells ([Kemp and Sancar, 2016](#)). The authors who described neuronal cell death from *Atr* deletion speculated that the loss of viability in differentiated neurons could have been due to accumulation of DNA damage associated with ATR loss in progenitor cells prior to neuronal differentiation, which has previously been suggested ([Ruzankina et al., 2007](#)).

One possible explanation for why ATR is necessary for the development of some brain structures but not others could be differences in proliferation rate and/or duration. With regards to the cerebellum in particular, CGNPs proliferate over a long period spanning from mid-embryonic development through the third week of postnatal life in mice, and peaking between P5 and P7 ([Carletti and Rossi, 2008](#); [Chizhikov and Millen, 2003](#); [Goldowitz and Hamre, 1998](#); [Hatten and Heintz, 1995](#)). This extended period of rapid proliferation ultimately gives rise to the largest population of neurons in the entire brain. Interestingly, in three mouse models of conditional *Atr* deletion from the brain (*Atr^{N-cre}, Atr^{G-cre}, and with the Math1 promoter in Atr^{M-cre} *), the cellular consequence of ATR loss on the cerebellum, whether in the form of reduced proliferation or increased apoptosis, occurred prior to the period of peak CGNP proliferation ([Lee et al., 2012](#); [Lang et al., 2016](#)). Moreover, maximal DNA damage and cell death from *Atr* deletion in the neocortex and ganglionic eminence were also detected before the periods of maximal proliferation for those structures ([Lee et al., 2012](#); [Finlay and Darlington, 1995](#); [Dehay and Kennedy, 2007](#)). Therefore, it seems unlikely that the special cerebellar role of ATR is exclusively a consequence of the proliferation rate of CGNPs. Likewise, the length
of the CGNP proliferative period in development may not by itself fully explain the
particular importance of ATR in the cerebellum since other neurons, such as found in
the olfactory bulbs, the SVZ, and the hippocampus, are known to proliferate over long periods of time through development (Finlay and Darlington, 1995; Ming and Song, 2011; Gage, 2000; Lledo et al., 2006; Lim et al., 2000). If still the proliferative nature of CGNPs is to be thought of as responsible for the cerebellar dependence on ATR, then perhaps this importance could be due to a combination of the proliferative index and proliferation duration of CGNPs.

**ATR may function in a unique manner in CGNPs**

ATR is thought to have many possible functions. In a large-scale proteomic analysis, ATR was found to respond to DNA damage by putatively regulating hundreds of targets encompassing numerous biological processes such as cell cycle control, signal transduction, cell proliferation and differentiation, immune function, protein metabolism and modification, mRNA transcription, and DNA replication, recombination, and repair (Matsuoka et al., 2007). Moreover, the functions of ATR are not limited to responding to DNA damage, suggesting that ATR may potentially regulate thousands of molecules spanning dozens of categories of biological functions. Therefore, it is conceivable that ATR does not display its full range of possible activity in all cells at all times but rather functions differently in specific cell types under specific conditions. In addition, beyond ATR, there can exist remarkable heterogeneity in the transcriptional profile of disparate cells, such that certain molecules like ATR may be more important in some cells than others. For example, in the cerebellum, DNA damage from deficiency of the DNA repair protein XRCC1 (X-ray repair cross complementing 1) leads to cell cycle arrest in cerebellar interneuron progenitors but apoptosis in CGNPs, suggesting that XRCC1 may function differently in these cell types or that the transcriptional profile differs.
between these cells to cause variable dependence on XRCC1 or some other DNA damage response molecule that operates in the absence of XRCC1 (Lee et al., 2009). Our group has reported that CGNPs maintain the key apoptotic regulator BAX (BCL2 (B-cell lymphoma 2)-associated X) in an active state at mitochondria in order to be able to induce rapid cell death, which may be another unique feature of CGNPs and supports the idea that ATR too could function in a special manner in CGNPs (Crowther et al., 2013).

Most of the research into the function of ATR has been performed in immortalized human cell lines and mouse embryonic fibroblasts (MEFs). As such, it is unclear whether these functions are retained in other cell types and especially in primary cells. Indeed, there have sometimes been conflicting reports of ATR behavior and function and some of these differences could be attributed to variable roles for ATR in specific cell types or on exposure of the same cell type to distinct stimuli that then induce altered ATR responses. A selection of prior findings will be reviewed here in brief to illustrate both the multitude of processes that can be regulated by ATR and to suggest that ATR may perform a unique function in CGNPs compared to other brain cells.

The most widely studied function of ATR has been in its response to DNA damage and Chapter 1 presents a summary of what may be considered the canonical DNA damage response of ATR. Additional roles for ATR in various other aspects of DNA damage and repair have also been described, but it is unclear whether all of these functions are always all active in every cell type. In cultures of human fibroblasts, ATR has been found to directly phosphorylate and activate the DNA repair protein BRCA1 (Breast cancer 1), which has previously been proposed to rely predominantly on ATM (Ataxia-telangiectasia-mutated) for activation (Cortez et al., 1999; Gatei et al., 2000; Tibbetts et al., 2000). Also in studies using human fibroblasts, the DNA damage response protein Nibrin facilitated ATR phosphorylation of targets like
CHK1 (Checkpoint kinase 1) and p53 (Stiff et al., 2005). Interestingly, in a separate study using a human lymphoblast cell line, Nirbin did not interact with ATR, but rather with ATM (Marceilain et al., 2005).

In primary human melanocytes, PKA (Protein kinase A) was found to directly activate ATR, inducing recruitment of the nucleotide excision repair molecule XPA (Xeroderma pigmentosum group A) to repair ultraviolet (UV) light damage (Jarrett et al., 2014). In turn, XPA has been found to directly activate ATR in MEFs and cervical cancer-derived HeLa and osteosarcoma-derived U2OS cell lines (Bomgarden et al., 2006; Shell et al., 2009). Other studies using MEFs have found that phosphorylation of the DNA damage marker H2A.X (Histone H2A, member X) is ATR-dependent and ATM-independent following exposure to UV light and hydroxyurea (Ward and Chen, 2001), which is in contrast to prior suggestions that H2A.X phosphorylation is primarily ATM-dependent (Burma et al., 2001). In MEFs, the multifunctional tyrosine kinase c-ABL (Abelson murine leukemia viral oncogene homolog 1) has also been found to directly activate ATR (Wang et al., 2011).

Apart from nucleotide excision repair, ATR has also been implicated in mediating mismatch and DSB repair. In HeLa and the human embryonic kidney HEK293 cell lines, ATR has been found to activate the mismatch repair program (Stojic et al., 2004; Yamane et al., 2004). Similarly, mismatch repair molecules like MutS (Mutator S) and MutL (Mutator L) have been shown to activate ATR in HeLa cells (Yoshioka et al., 2006; Liu et al., 2010). This type of mismatch repair positive feedback loop may also involve the mismatch repair protein HEXO1 (β-hexosaminidase 1), which has been shown to both activate ATR and be activated by ATR in HEK293, HeLa, U2OS, and MEF cell lines (El-Shemerly et al., 2008; Sertic et al., 2011). With regards to DSB repair, FANCM (Fanconi anemia complementation group M) was seen to respond to the presence of DSBs by mediating ATR pathway signaling, which in turn activated FANCM in HEK293, HeLa, and U2OS cells (Collis et al., 2008;
Singh et al., 2013). Using these same cell types, other authors have further shown ATR involvement in the Fanconi anemia repair pathway through ATR activation of FANCD2 and FANCJ (Andreassen et al., 2004; Sakasai et al., 2012). Additional ATR-mediated regulation of DSB repair may be through the helicase WRN (Werner syndrome RecQ-like helicase), which has exonuclease activity and has been shown to form a complex with ATR and require ATR activation for DNA repair in HeLa cells (Pichierri et al., 2003; Otterlei et al., 2006).

HeLa cells have also demonstrated an interesting self-regulatory mechanism for ATR inactivation in which the ATR-activated kinase CHK1 phosphorylates the phosphatase PP2A (Protein phosphatase 2A), which then opposes ATR activation of CHK1 (Leung-Pineda et al., 2006). Another protein phosphatase, PP5, may regulate ATR activity in a similar way, as has been shown in HeLa, HEK293, and U2OS cell lines (Kang et al., 2011; Zhang et al., 2005). The SRC (Rous sarcoma) family of protein kinases can provide additional negative control over ATR signaling in HeLa cells to limit the DNA damage response (Fukumoto et al., 2014). In terms of positive regulation, studies in HeLa and breast cancer-derived MCF7 cell lines found ATR recruitment to sites of DNA damage by the protein MDC1/NFBD1 (Mediator of DNA damage checkpoint 1/Nuclear factor with BRCT (BRCA1 C-terminus) domains 1) (Peng and Chen, 2005). In response to replication fork stalling, a primary ATR activating event, ATR was shown to phosphorylate and inactivate SMARCAL1 (SWI (Switch)/SNF (Sucrose-non-fermentable)-related, matrix-associated, actin-dependent regulator of chromatin, subfamily A-like 1) in U2OS cells, thereby preventing SMARCAL1-mediated regression of stalled forks, which would otherwise generate DSBs (Couch et al., 2013). To promote the recovery of stalled replication forks, ATR may activate the DNA repair protein XRCC3, as has been seen in HEK293, HeLa, U2OS, and MCF7 cell lines (Somyajit et al., 2013).

Following the initial response to DNA damage, one possible outcome is cell cycle
arrest to potentially allow for additional time to repair DNA. As highlighted in Chapter 1, ATR predominantly mediates cell cycle arrest in S-phase or at the G₂/M checkpoint. Indeed, in primary human fibroblasts and HeLa cells, ATR has been shown to inactivate the serine/threonine kinase PLK1 (Polo-like kinase 1) so as to inhibit entry into M-phase by preventing activation of the Cyclin B - CDK1/CDC2 (Cyclin-dependent kinase 1/cell division cycle 2) complex (van Vugt et al., 2001; Deming et al., 2002). ATR has similarly been seen to regulate mitotic entry in MEFs, with inappropriate entry in the absence of ATR leading to chromosome breaks (Brown and Baltimore, 2003). Curiously, in a separate study employing MEFs, forced hyperactivation of ATR in the absence of DNA damage induced p53-mediated cell cycle arrest in G₁-phase rather than at G₂/M (Toledo et al., 2008). In support of the hypothesis that ATR can have varied function in different cell types, forced overexpression of Atr in the C2C12 mouse myoblast cell line resulted in inhibition of the muscle differentiation factor MYOD1 (Myogenic differentiation 1), duplication of centrosomes, and failed cell cycle arrest at G₁-phase, all in the absence of induced DNA damage (Smith et al., 1998). As mentioned, though ATR can induce S-phase arrest, inhibition of ATR was recently found to similarly promote S-phase arrest in human breast cancer cells that subsequently experienced apoptosis (Kim et al., 2017).

Should DNA damage be extensive, cell death may result following or independent of cell cycle arrest and ATR can mediate this process through activation of p53. In U2OS and the H1299 non-small cell lung cancer (NSCLC) cell lines, ATR phosphorylates MDM2 (Mouse double minute 2) following DNA damage to allow for p53 activation and apoptosis (Shinozaki et al., 2003). Although ATR can either directly activate p53 or indirectly through its downstream kinase CHK1, a study in HEK293 and U2OS cells exposed to DNA damaging agents also found that ATR phosphorylated DBC1 (Deleted in breast cancer 1), which inhibited the p53 negative
regulator SIRT1 (Silent mating type information regulation 2 homolog 1), inducing apoptosis (Zannini et al., 2012). As another indirect mechanism for activating p53-mediated apoptosis, ATR can prevent degradation of the p53-activator HIPK2 (Homeodomain-interacting protein kinase 2) by inhibiting SIAH2 (Seven in absentia homolog 1) in HEK293, U2OS, and MCF7 cell lines (Winter et al., 2008). Apart from promoting cell death through p53, ATR has been suggested to promote autophagy, through an as yet uncharacterized mechanism, when hyperactivated in HeLa and U2OS cells (Mori et al., 2013). While the above studies have all implicated a pro-cell death role for ATR, one recent report has actually revealed that outside of the nucleus in MEFs and HEK293 cells, ATR antagonizes the apoptotic function of tBID (truncated BH3-interacting domain death agonist) at the mitochondrion in order to suppress Cytochrome C release following DNA damage (Hilton et al., 2015). This anti-apoptotic function of ATR is apparently normally suppressed by the isomerase PIN1 (Peptidylpropyl cis/trans isomerase, NIMA (never-in-mitosis gene A)-interacting 1), which alters the conformation of ATR, but ATR was found to be freed from PIN1 in the presence of DNA damage. In a separate study, nuclear BID promoted the necessary, activating ATR-ATRIP (ATR-interacting protein) interaction with RPA following DNA damage in U2OS cells (Liu et al., 2011). Taken together with the previous study, these results suggest a complex relationship between ATR and BID that may differ by cell type. Another component of the classic apoptotic pathway, MCL1 (Myeloid cell leukemia 1), which normally suppresses apoptosis, has been seen to promote ATR activation of CHK1 in HeLa cells (Jamil et al., 2008). In contrast, the p53 negative regulator PPM1D/WIP1 (Protein phosphatase 1D/Wild-type p53-induced phosphatase 1), which, like MCL1, suppresses apoptosis, reduced the activity of ATR and its substrates in PC3 prostate cancer cell lines but not in CHO Chinese hamster ovary cell lines, again suggesting that ATR can behave in non-identical ways in different cell types (Song et al., 2013;
Since ATR has a demonstrated role in responding to DNA damage in almost all cell types investigated, the absence of ATR should have significant consequences. As we have revealed with our experiments in CGNPs, loss of ATR can lead to the accumulation of severe chromosome aberrations (Lang et al., 2016). Earlier experiments with Atr deletion in mice found that ATR loss was similarly associated with widespread chromosome breaks in blastocysts that led to embryonic lethality (Brown and Baltimore, 2000). In a study using human lymphocytes, ATR disruption was seen to induce chromosome damage through allowing the expression of common fragile sites (Casper et al., 2002). Hypomorphic Atr mutation had a similar effect in primary mouse fibroblasts, which displayed increased chromosome damage and fragile site expression (Ragland et al., 2009). However, a separate study described the ATR pathway as actually being inactive in human lymphocytes, putting into question why the absence of ATR would promote chromosome damage in these cells (Jones et al., 2004). Through yet another set of experiments in human lymphocytes, Atr amplification paradoxically increased chromosomal instability (Mossafa et al., 2004). Other relationships between ATR and chromosomes have also been reported in various types of cells. In HeLa cells, ATR cooperates with UPF1 (Up-frameshift suppressor 1 homolog), which regulates nonsense-mediated decay, to degrade histone mRNAs upon cessation of DNA synthesis (Kaygun and Marzluff, 2005).

Furthermore, chromatin remodeling in HeLa cells could involve ATR association with HDAC2 (Histone deacetylase 2) (Schmidt and Schreiber, 1999). ATR’s binding to chromatin, however, was seen in another study to occur only in S-phase and not in M-phase in HeLa cells (Dart et al., 2004). Association of ATR with chromatin, as suggested by one group using MEFs, HeLa, U2OS, and the NIH3T3 MEF cell lines, regulates chromatin condensation and this regulation requires that ATR be tethered to the nuclear envelope, which occurs in S- and M-phases, where ATR also modulates
envelope plasticity in response to mechanical stress and mediates envelope breakdown in mitosis (Kumar et al., 2014). Chromatin condensation prohibits transcription, and ATR may employ chromatin condensation as a mechanism to inactivate the extra X-chromosome in female cells, as seen in MEFs (Ouyang et al., 2005). ATR-mediated chromosome silencing has also been described by several groups as operative in human and murine meiotic cells of the seminiferous tubules, where ATR phosphorylated H2A.X on unpaired and un-synapsed chromosomes to mark them for degradation (Keegan et al., 1996; Perera et al., 2004; Royo et al., 2013). Clearly then, ATR may either directly or indirectly control chromosome stability, with variable function by cell type.

The association of ATR with chromosomes has been further elaborated in terms of its interaction with telomeres and telomeric molecules. In HEK293 and HeLa cells, ATR recruited telomerase, the ribonucleoprotein that adds DNA repeat sequences to the end of telomeres (Tong et al., 2015). Atr-knockout studies using MEFs and primary human fibroblasts have also demonstrated a role for ATR in protecting telomeres (McNees et al., 2010; Pennarun et al., 2010). At the same time, however, MEFs were found to paradoxically utilize the telomere-protecting protein POT1 (Protection of telomeres 1) to prevent ATR association with telomeres (Gong and de Lange, 2010). Indeed, other components of the Shelterin telomere-protecting complex, which includes POT1, have shown activity in preventing ATR-mediated DNA resection at telomeres in MEFs (Kibe et al., 2016). Even the non-coding RNA component of telomerase has been implicated in directly inhibiting ATR activity in HEK293, U2OS, and MCF7 cell lines (Kedde et al., 2006). As such, it is unclear whether ATR actively protects telomeres or is excluded from telomeres for its detrimental effect since reports have been conflicting, and even in one cell type used by two groups under similar conditions, both roles have been reported for ATR.

In the immune system, ATR can facilitate immunoglobulin class switching and
somatic hyper-mutation, as seen in human B-cells (Pan-Hammarstrom et al., 2006). Studies performed in HeLa and HCT116 human colon cancer cell lines have found that ATR activates the V(D)J recombination complex Artemis (Zhang et al., 2004). An additional role for ATR may be in responding to tissue oxygenation since ATR has been found to activate p53 following both hypoxia and hyperoxia in HEK293 and HCT116 cell lines (Das and Dashnamoorthy, 2004; Hammond et al., 2002, 2003). To further add to the complexity of ATR activity, various unrelated transcription factors have been shown to regulate ATR. In mouse kidneys, the purine hydrolase and transcriptional regulator FHIT (Fragile histidine triad) modulated ATR activity and promoted p53 signaling following DNA damage (Hu et al., 2005b,a). The transcriptional activator SNIP1 (SMAD (Mothers against decapentaplegic) nuclear-interacting protein 1) has also demonstrated the ability to enhance ATR-mediated activation of p53 and CHK1 in U2OS cell lines in response to DNA damage (Roche et al., 2007). Finally, MEFs lacking the transcription factor CUX1 (Cut-like homeobox 1) have reduced ATR pathway signaling upon induction of DNA damage (Vadnais et al., 2012). These studies combine to further demonstrate how ATR can have a unique role in a specific cell type, such as generating immune diversity in lymphocytes and sensing oxygen levels in kidney cells.

Another putative cell-specific function of ATR has been described in primary mouse retinal photoreceptors, where ATR disruption led to shorter cilia and photoreceptor degeneration (Valdes-Sanchez et al., 2013). ATR’s role in maintaining ciliary length has also been seen in human fibroblasts, and in these cells, ATR additionally controlled secretion of factors like Sonic hedgehog (SHH) – a potent mitogen that coincidentally is critical for CGNP proliferation (Stiff et al., 2016). In the gastrointestinal (GI) system, ATR has been described, using in vivo fruit fly and mouse studies, as necessary for maintaining the proliferation of intestinal stem cells by preventing proliferation-associated DNA damage – a well-recognized function of
ATR (Ruzankina et al., 2007; Park et al., 2015). Recently, work using HEK293 cells has suggested another possible role of ATR in the GI system: negative regulation of the appetite-stimulating molecule Leptin, which can be secreted by adipose tissue and cells in the stomach (Ericson et al., 2015). In a different part of the GI system, primary mouse hepatocytes apparently down-regulate ATR upon becoming senescent, suggesting that ATR is not even active in all cell types (Panda et al., 2008). Likewise, other tissues may also experience low levels of ATR activity as the hormone Estrogen has been found to inhibit ATR pathway signaling in studies employing human breast cancer cell lines (Pedram et al., 2009).

Several miscellaneous ATR functions and associations have been described in specific cells and these descriptions help complete the picture of the known complexity of ATR regulation and activity. For instance, experiments in MCF7 and NIH3T3 cell lines have suggested that the MEK/ERK (Mitogen-activated protein kinase kinase/Extracellular signal-related kinase) pathway can regulate ATR activation in response to DNA damage (Wei et al., 2011; Wu et al., 2006). ATR activation following DNA damage could also require the putative oxidoreductase WWOX (WW domain-containing oxidoreductase), as seen in MEF, HEK293, HeLa, and MCF7 cell lines (Abu-Odeh et al., 2016). Moreover, the SUMO (Small ubiquitin-like modifier) ligase PIAS3 (Protein inhibitor of activated STAT3 (signal transducer and activator of transcription 3)) activated ATR in response to DNA damage in HEK293, HeLa, and U2OS cell lines (Wu and Zou, 2016). Research using HeLa cells further demonstrated that the acetyltransferases P300 and CBP (CREB (cAMP (Cyclic adenosine monophosphate) response element binding)-binding protein) interact with ATR to activate CHK1 (Stauffer et al., 2007). Another interesting method utilized by HEK293 and HeLa cells to promote ATR activity is to increase ATRIP binding to RPA through association with the mRNA splicing factors PRPF19 (Pre-mRNA processing factor 19) and BCAS2 (Breast carcinoma-amplified sequence...
2) (Wan and Huang, 2014). Most recently, the newly identified ETAA1 (Ewing’s tumor-associated antigen 1) protein was shown to directly activate ATR independent of the key ATR regulator TOPBP1 (Topoisomerase DNA II-binding protein 1) in HeLa, U2OS, and HCT116 cell lines (Haahr et al., 2016). Finally, ATR pathway activity has even been suggested to follow a circadian rhythm in primary MEFs and mouse livers due to CRY1 (Cryptochrome circadian clock 1) regulation of the circadian factor TIM (Timeless circadian clock), which can mediate ATR phosphorylation of CHK1 (Kang and Leem, 2014; Yoshizawa-Sugata and Masai, 2007; Chou and Elledge, 2006).

Given this vast sum of work on ATR, again only presented in brief here, that demonstrates a multitude of possible roles for this protein and that suggests yet many more potential roles, with significant variability by cell type and even within the same cell type under similar or different conditions, it becomes easy to imagine that ATR could have a unique and special function in CGNPs. However, it must be noted that, as summarized in Chapter 1, specific disturbances to the cerebellum of greater severity than in other brain regions are not commonly associated with the ATR-mutated human condition Seckel syndrome. Moreover, in mice, pan-CNS Atr deletion from neural stem cells using Nestin-Cre disrupted both the ganglionic eminence and nascent cerebellum according to one report (Lee et al., 2012), and targeted additional proliferative regions beyond the cerebellum like the SVZ and the VZ according to another study (Zhou et al., 2012). In our own research, we have observed that Atr deletion from neural stem cells using hGFAP-Cre almost exclusively perturbed the neonatal cerebellum (Lang et al., 2016). Despite the disparity between these three reports on CNS Atr deletion in mice, the one common tissue that is clearly severely affected is the proliferative cerebellum. This discrepancy in specific cerebellar pathology associated with ATR disruption between man and mouse could be attributed to the fact that Seckel syndrome is caused by
hypomorphic mutations in $ATR$ whereas the described murine experiments utilized complete $Atr$ deletion. As has been highlighted in this section, comparison of several studies can lend support to the idea of interspecies variations in ATR function and regulation, and indeed, the hypomorphic $ATR$ mutation that has been identified in several cases of Seckel syndrome fails to produce a phenotype in mice (Ragland et al., 2009). Therefore, whether ATR has a unique and special function in CGNPs remains an open question, and although it is an interesting consideration, it might be more important to focus on the fact that ATR clearly has a vital pro-survival function in CGNP-derived medulloblastoma and that ATR disruption seems to be relatively-well tolerated by other normal cells in the postnatal brain. Future research can reveal whether, beyond surveillance of DNA damage, any of the spectrum of ATR activities described in other cells make ATR indispensable to the cerebellum.

**ATR inhibition as treatment for medulloblastoma**

Regulation of proliferation controls both normal development and oncogenesis. In the cerebellum, CGNPs proliferate maximally in early postnatal life to generate the largest neuronal population in the brain. Failed CGNP expansion, such as with disruption of the DNA damage response protein ATR, produces cerebellar hypoplasia, as demonstrated in mouse models in Chapter 3. Excessive CGNP expansion promotes formation of medulloblastoma, the most common malignant brain tumor of childhood. Chapter 2 explored a possible role for ATR in tumorigenesis and outlined therapeutics for inhibiting ATR that are being explored in cancer treatment. Meanwhile, Chapter 4 specifically addressed the possibility of targeting ATR to treat medulloblastoma. This section will briefly explore the benefits and pitfalls of pursuing ATR inhibition for the treatment of medulloblastoma and present possible future directions for improving medulloblastoma treatment using ATR inhibition.
ATR inhibitors with good in vivo activity and their uses in cancer treatment

Given the pivotal role of ATR in maintaining the viability of various cell types, there has been longstanding interest in probing the therapeutic potential of ATR inhibition. Caffeine was among the first described non-specific ATR inhibitors, with additional activity against ATR-related molecules like ATM (Sarkaria et al., 1999; Blasina et al., 1999). Although caffeine sensitizes cultured cells to DNA damage, the concentrations required for effective ATR/ATM inhibition in vivo are toxic. Cell-based studies and compound screens have since identified several other more selective ATR inhibitors such as Torin2, NU6027, ETP-46464, and NVP-BEZ235, but these chemicals still exhibited non-trivial disruption of related kinases like ATM, mTOR (mammalian Target of rapamycin), and PI3K (Phosphoinositide 3-kinase) (Nishida et al., 2009; Peasland et al., 2011; Mukherjee et al., 2012; Liu et al., 2013; Toledo et al., 2011). VE-821 was the first discovered ATR inhibitor with very high selectivity over ATM, mTOR, PI3K, and DNA-PK (DNA protein kinase) (Charrier et al., 2011). Addition of VE-821 to various cancer cell lines in vitro increased sensitivity to radiation and different chemotherapeutics (Prevo et al., 2012; Pires et al., 2012; Huntoon et al., 2013; Vavrova et al., 2013). Unfortunately, as we have found through our own experience, VE-821 is poorly soluble, preventing its use in vivo, and indeed, shortly after reporting VE-821, Vertex Pharmaceuticals developed the related compound VE-822/VX-970, which has better pharmacodynamic properties and even greater ATR selectivity (Fokas et al., 2014, 2012).

In the initial study using VE-822, addition of VE-822 shortly before X-ray radiation therapy (XRT) significantly reduced the viability TP53-mutant/KRAS-mutant PSN-1 and MiaPaCa-2 pancreatic ductal adenocarcinoma cell lines (Fokas et al., 2012). Similar reductions in the viability of these cell lines was observed when VE-822 was added after pre-treatment with Gemcitabine. Interestingly, VE-822 demonstrated no cytotoxic effect against normal HFL-1 fibroblasts or human dermal
microvascular endothelial cells, suggesting a tumor-specific effect. For the in vivo experiments, mice with PSN-1 xenografts had reduced tumor growth when treated with 60 mg/kg VE-822 by oral gavage once per day over five days and either concurrently with 2 Gy XRT once per day over five days or with a single dose of 6 Gy XRT on the first day of VE-822 treatment. Mice bearing MiaPaCa-2 xenografts responded with great variability to the five days of VE-822 plus five days of XRT regimen, with some mice failing to grow tumors at all and others showing minimal response. In both experiments, VE-822 mono-therapy had no significant effect. The addition of 100 mg/kg Gemcitabine, given once by intraperitoneal (IP) injection on the day before VE-822 and XRT treatment, to the regimen of a single dose of 6 Gy XRT further restricted tumor xenograft growth. Importantly, there was no observed intestinal pathology from use of VE-822 alone or in combination with XRT, which is salient given the proliferative nature of the intestinal tract and its sensitivity to various chemotherapeutics.

In H2009 lung cancer cells, VX-970 alone only mildly increased DNA damage, but this effect was markedly increased in concurrent combination with Cisplatin (Hall et al., 2014). Moreover, in a panel of thirty-five lung cancer cell lines, VX-970 significantly reduced cell viability in over 40% of these cell lines when combined with Cisplatin, Oxaliplatin, Gemcitabine, Etoposide, or SN-38. VX-970 combination with Cisplatin or Gemcitabine actually reduced the viability of more than 75% of the cell lines. Here, again, VX-970 produced no cytotoxic effect against normal cultured HFL-1 human fibroblasts, but cell viability was decreased upon the addition of Gemcitabine. Interestingly, knockdown of TP53 in A549 human lung cancer cells greatly enhanced the combined anti-tumor effect of VX-970 paired with Cisplatin or Gemcitabine. Just as in H2009 cells, VX-970 and Cisplatin combined to produce a cytotoxic effect in cultured, primary human NSCLC samples. In vivo, VX-970 alone, given by oral gavage at either 30 or 60 mg/kg once per day for four times per week,
failed to reduce the growth of mice with human NSCLC xenografts, but addition of Cisplatin, given by IP injection at 3 mg/kg once per week, led to diminished tumor burden. Similarly, in mice bearing OD26749 NSCLC xenografts, the combination of VX-970 at 60 mg/kg and Cisplatin, given as above, produced nearly complete tumor regression. VX-970 has also been found to have a synergistic cytotoxic effect when combined with SN-38 in cultured COLO205 colorectal cancer cells (Josse et al., 2014). This anti-tumor effect was also seen in mice with COLO205 xenografts, where VX-970 was given by oral gavage at 60 mg/kg on the first three days of a four-day cycle and Irinotecan, which is metabolized into SN-38, was given at 20 mg/kg by IP injection on the first day of the cycle. Active clinical trials for VX-970, all of which VX-970 is delivered by intravenous (IV) injection, in the United States are summarized in Chapter 2.

In 2013, one year after the first publication on VE-822/VX-970 and two years after the discovery of VE-821, AstraZeneca described the compound AZ-20 as another highly selective ATR inhibitor, with similar activity to VE-821 (Foote et al., 2013). As with VE-821, AZ20 had poor solubility, limiting its translational applications, so AZD-6738, as with VE-822/VX-970, was developed, demonstrating better solubility and pharmacodynamics. In one study, cultured LoVo human colon adenocarcinoma cells treated with AZD-6738 experienced dose-dependent apoptosis (Checkley et al., 2015). Mice bearing LoVo xenografts were treated with variable doses of AZD-6738 by oral gavage once per day for up to twenty-one consecutive days, and 80 mg/kg was found to be the optimal dose in producing an anti-tumor effect as monotherapy. Addition of two rounds of five consecutive days of 2 Gy. XRT enhanced these anti-tumor effects from ATR inhibition.

Unlike VX-970, AZD-6738 alone significantly reduced the viability of four different NSCLC cell lines in vitro through increased p21-mediated cell cycle arrest and apoptosis (Vendetti et al., 2015). Specifically, AZD-6738-treated cells were found
to arrest at \( G_1/S \), with depletion from \( G_2 - M \)-phases. Here again, addition of Gemcitabine, and especially Cisplatin, enhanced with cytotoxic effect of AZD-6738, particularly in the ATM-deficient H23 cell line. Cell death from AZD-6738 and Cisplatin treatment could similarly be elevated in non-ATM-deficient NSCLC cell lines that had \( ATM \) knocked down. For \textit{in vivo} studies, mice bearing ATM-competent H460 NSCLC xenografts were treated with 50 mg/kg AZD-6738 by oral gavage once daily for fourteen consecutive days and/or with 3 mg/kg Cisplatin by IP injection once on days 1 and 8. Single-agent therapy failed to reduce tumor growth whereas combination therapy had a significant anti-tumor effect. Moreover, using the same dosing schedule but with an even lower amount of 25 mg/kg AZD-6738, mice bearing ATM-deficient H23 xenografts experienced near-complete tumor regression.

ATR inhibition through AZD-6738 has also shown efficacy in chronic myeloid leukemia (CLL), which can present with mutations in \( ATM \) and \( TP53 \) (Kwok et al., 2016). AZD-6738 treatment greatly reduced the viability of ATM-deficient or p53-deficient human-derived CLL cell lines compared to CLL cells with intact ATM or p53 and compared to non-CLL cells. Administration of AZD-6738 for two weeks in mice with \( ATM \)- or \( TP53 \)-mutated CLL xenografts led to significant reductions in tumor load compared to Vehicle. Molecular studies revealed that AZD-6738 induced cell cycle arrest at \( G_1/S \) in ATM/p53-proficient CLL cells, with minimal activation of apoptosis, but that in cells with mutated \( ATM \) or \( TP53 \), cell cycle checkpoints failed and cells proceeded into mitosis, ultimately undergoing what the authors described as mitotic catastrophe. Combining AZD-6738 with chemotherapeutic agents like Chlorambucil, Fludarabine, and 4-hydroperoxycyclophosphamide reduced the viability of normally chemo-resistant ATM- or p53-deficient CLL cell lines. \textit{In vivo}, the combination of AZD-6738 plus Chlorambucil produced a significant anti-tumor effect in mice xenografted with primary, human, ATM-deficient CLL cells.

AZD-6738 has also been tested in human breast cancer cell lines, where it was
shown to attenuate the proliferation of six different cell lines (Kim et al., 2017). On further analysis, administration of AZD-6738 alone in the SK-BR-3 breast cancer cell line increased both S-phase arrest and apoptosis. Moreover, in SK-BR-3 cells, but not in the related BT-474 cell line, AZD-6738 increased DNA damage while reducing the levels of DNA repair mediators like RAD51, MRE11 (Meiotic recombination 11), and ERCC1 (Excision repair cross-complementation group 1). Proliferative signaling was thought to be reduced by AZD-6738 due to dose-dependent decreases in the phosphorylation of AKT (Protein kinase B) and ERK. Addition of Cisplatin, as in other studies with AZD-6738 and with VE-822/VX-970, produced a synergistic cytotoxic effect in all six breast cancer cell lines through inhibiting proliferation, promoting apoptosis, and suppressing other DNA repair mechanisms. Clinical trials for AZD-6738 are currently underway and a summary of these is provided in Chapter 2.

Synthesis of the available data on VE-822/VX-970 and AZD-6738 can inform our own research on nano-formulated pVE-822 in medulloblastoma preclinical studies, as presented in Chapter 4. For instance, the in vivo studies using VX-970 reported to date all seem to find that 60 mg/kg given once daily on consecutive days provides the maximum therapeutic index, which informed our choice for using 60 mg/kg of pVE-822 given once daily for five consecutive days in treating mice with medulloblastoma. Notably, however, in vivo VX-970 experiments all utilized oral gavage for drug delivery while clinical trials for VX-970 use IV injection. Our strategy of IP pVE-822 administration was based on the contingency that medulloblastoma develops at a very early age in our mouse models, making IV injection difficult due to the diminutive size of the animals. But, perhaps, oral gavage could be attempted in our medulloblastoma-bearing animals, especially given that the reports to date have described no systemic toxicity from oral administration of VX-970 whereas we have noted considerable extra-CNS adverse side effects with
IP delivery of pVE-822. Other routes of pVE-822 administration should also be explored so as to minimize undesired reactions, including intracranial (IC) injection and intranasal (IN) delivery, both of which we are actively investigating. Inclusion of inhibitors against the blood-brain barrier efflux regulator PGP (P-glycoprotein) into VE-822-bearing polymeric micelles could potentially further increase drug bioavailability in the brain and tumor, again reducing systemic toxicity. Use of PGP inhibitors to increase the potency of cancer therapeutics in the brain has been well-studied (Abdallah et al., 2015; Kemper et al., 2004, 2003; Amin, 2013; Agarwal et al., 2011), and encapsulation of PGP inhibitors into nanoparticles is an active area of research (Singh and Lamprecht, 2016, 2015; Singh et al., 2015). In order to better understand systemic toxicity from pVE-822 administration, whether by IP, IV, IC, IN, or oral delivery, various tissues, such as intestine, liver, heart, kidneys, spleen, and bone marrow, as well as blood can be harvested from mice at different time points (i.e.: 2, 4, 6, 8, 12, and 24 hours) following a single treatment with pVE-822 at 60 mg/kg. High performance liquid chromatography and mass spectrometry can ascertain the presence of VE-822 in these tissues and immunohistochemical staining can reveal whether specific cells are adversely affected. Not only will such experiments provide valuable information for future clinical trials in human patients, but they will also inform on how to better formulate and deliver pVE-822 so as to minimize specific toxicities.

Another important observation taken from these published studies is that VX-970 failed to provide an anti-tumor effect as monotherapy in preclinical mouse xenograft experiments, and, indeed, most clinical trials with VX-970 employ at least one adjunct form of therapy. AZD-6738 demonstrated efficacy as single agent therapy in the context of ATM- or p53-deficient tumors and is thus being investigated as monotherapy in clinical trials with relapsed/refractory CLL, prolymphocytic leukemia (PLL), or B-cell lymphoma, all of which frequently demonstrate ATM or
TP53 mutations. It is thus informative that pVE-822 alone achieved a significant anti-tumor effect in ATM- and p53-proficient medulloblastoma, suggesting that this tumor is critically dependent on ATR. Nevertheless, other therapeutics could be tried in combination with pVE-822 in preclinical models of medulloblastoma. Cisplatin, Gemcitabine, and XRT were the most commonly used adjunct therapies in preclinical experiments using either VX-970 or AZD-6738, showing effective synergy with ATR inhibition. Moreover, clinical trials with VX-970 and AZD-6738 are also utilizing addition of Cisplatin, Gemcitabine, or XRT. As discussed in Chapter 4, however, Cisplatin and Gemcitabine failed to show a combinatorial effect with VE-822 in cultured CGNPs, and VE-822 actually possibly provided a cytoprotective effect against XRT in these cells, putting into question the likely efficacy of these combinations in treating CGNP-derived medulloblastoma. Nevertheless, medulloblastoma cells in vivo may ultimately demonstrate a disparate response to pVE-822 plus Cisplatin, Gemcitabine, and/or XRT than CGNPs in vitro. But again, we found treating Atr-deleted medulloblastoma-prone mice with XRT failed to improve survival. Importantly, we did note possible synergy between VE-822 and Etoposide as well as the SHH pathway antagonist Vismodegib in reducing CGNP proliferation, albeit not in increasing DNA damage or apoptosis. Thus, future experiments should try combining pVE-822 with either of these compounds in medulloblastoma-bearing mice.

Further considerations for future experiments are that besides optimizing the route of pVE-822 delivery, the dose and schedule of pVE-822 should also be optimized for treating young mice with medulloblastoma. Again, we chose 60 mg/kg once per day for five consecutive days for most of our experiments, but this dosage and schedule were based on reports of efficacy in much older mice with non-primary, non-brain tumors. As such, it is conceivable that a different dose could provide a better therapeutic index. For example, we have tried dosing 30 mg/kg once per day
for five consecutive days, and this dose seems to provide some, albeit diminished, anti-tumor effect with reduced peripheral toxicity. Perhaps, as with AZD6738 in CLL, 50 mg/kg of pVE-822 given once daily is ideal. Then again, the route of drug delivery will conceivably impact the optimal dose of pVE-822 and therefore several concentrations should be tried with each mode of delivery. To add further complexity to the consideration of optimizing the pVE-822 therapeutic index in treating medulloblastoma, drug delivery once per day for consecutive days may not be ideal. Toxicity might be reduced if pVE-822 were given once per day every other day for a certain period of time, or maybe given in two smaller doses in a day either every day or every other day. This then begs the question of how long pVE-822 should be administered. Five total days? Ten? Until evidence of complete tumor regression? Or, upon observation of discontinued tumor response to drug? It is currently unclear which, if any, of these possibilities might improve the therapeutic index of pVE-822 for medulloblastoma treatment, but these prospects should certainly be explored.

Moreover, as a part of determining the optimal pVE-822 dosing schedule for treating mice with medulloblastoma, the age at which mice begin receiving treatment must be decided. In our studies, we chose to initiate therapy either at P8 or at P12 since SmoM2^{G-cre} mice typically perish before P20, but commencing treatment at a different age, such as P6, P10, or P14, might lead to improved outcomes.

Once the optimal route of delivery, dose, and dosing schedule for pVE-822 have been established, the same must be done for any adjunct therapies that will be tried. There is evidence that the proper sequence of agent administration is imperative: that is, whether ATR inhibitor should be given before, concurrent with, or after another therapeutic like Gemcitabine or XRT (Karnitz and Zou, 2015). In the wrong sequence, pVE-822 combined with another therapy may either fail to demonstrate an anti-tumor effect or may in fact provide a cytoprotective effect, both of which we have encountered in our own experiments, as highlighted in Chapter 4. Indeed, in the
initial study reporting VE-822 efficacy in pancreatic cancer, VE-822 produced cytotoxicity only when given after Gemcitabine and before XRT both in vitro and in vivo (Fokas et al., 2012). In that same study, fractionated radiation – 2 Gy. once per day over five consecutive days – induced less of an effect in combination with VE-822 – also given once daily over five consecutive days – than a single dose of 6 Gy. given after the first of five doses of VE-822. In clinical trials, VX-970 is given one day after Cisplatin in treatment cycles for patients with refractory/metastatic solid malignancies or locally-advanced head and neck squamous cell carcinoma. Similarly, VX-970 is given one day after Gemcitabine in a treatment regimen for patients with recurrent ovarian, primary peritoneal, or fallopian tube cancers. Even with combined administration of Gemcitabine and either Cisplatin or Carboplatin in patients with recurrent ovarian, primary peritoneal, fallopian tube, or metastatic urothelial cancers, VX-970 is given one day after the concurrent administration of the other two drugs. Interestingly, Irinotecan, Topotecan, or Veliparib are administered concurrent with VX-970 in patients with refractory/metastatic solid tumors, small cell lung cancer, or refractory/advanced solid tumors, respectively. Similarly, XRT is administered before, on the same day, and after VX-970 dosing in patients with locally-advanced head and neck squamous cell carcinoma or brain metastases from NSCLC, unlike the most effective regimen in mice with pancreatic tumor xenografts (Fokas et al., 2012). Surprisingly, unlike the variability in and consideration for dosing sequence seen with VX-970 plus adjunct therapies, AZD-6738 is given concurrently with Paclitaxel, XRT, or Carboplatin in patients with metastatic/refractory/advanced cancers. Combinatorial studies using pVE-822 in medulloblastoma mice must therefore carefully consider in what sequence each additional therapy is given with relation to pVE-822, besides also determining the optimal synergistic dose and route of delivery.

Taken together, our own findings and the published data on ATR inhibition in
tumor-bearing mice suggest continued investigation of pVE-822 for the treatment of medulloblastoma. As detailed here, many more experiments must be performed in order to ascertain the optimal delivery strategy for pVE-822 and any combinatorial therapies in order to maximize the therapeutic index. Furthermore, molecular analyses should then be performed so as to describe in detail the mechanism by which pVE-822-mediated ATR inhibition, alone or in combination with another therapy, effects medulloblastoma cytotoxicity and/or attenuated proliferation. Our experiments with disrupting ATR in CGNPs have already hinted at some of the likely operative mechanisms, but these should be confirmed with in vivo medulloblastoma models (Lang et al., 2016).

**ATR inhibition for the treatment of TP53-mutated medulloblastoma**

In our initial studies, we had hypothesized that given the importance of ATR in preventing DNA damage in the cerebellum (Lee et al., 2012; Zhou et al., 2012), conditional *Atr* deletion in CGNPs might actually promote tumor formation through accumulation of mutations. Instead, we found that loss of ATR led to widespread p53-mediated, BAX/BAK-dependent apoptosis in CGNPs in the postnatal period, which has not been previously reported (Lang et al., 2016). *Tp53* mutation has been shown to induce medulloblastoma in combination with deletion of various DNA repair pathway genes in mice (Frappart et al., 2009, 2007; Holcomb et al., 2006; Lee and McKinnon, 2002), so we predicted that co-deletion of *Atr* and *p53* would result in mice with medulloblastoma, especially given that pathology in ATR-deficient CGNPs was almost exclusively p53-driven. That is, since ATR prevents proliferation-associated DNA damage that activates p53-mediated apoptosis, or, to a lesser extent, cell cycle arrest, deletion of *Atr* should lead to increased DNA damage and co-deletion of *p53* should allow for the accumulation of that DNA damage, leading to mutation and tumorigenesis. As presented in Chapter 3, we instead found that loss
of both ATR and p53 resulted in persistent cerebellar pathology through caspase-independent CGNP cell death, emphasizing the critical importance of ATR to CGNP survival.

We were surprised that tumors did not develop in Atr/p53-co-deleted mice. Although Atr-knockout is embryonic lethal, Atr\(^{+/-}\) mice variably develop tumors such as mixed follicular center cell lymphoma, hepatocellular adenoma, fibrous histiocytoma, ovarian cystadenoma, and ovarian fibroma (Brown and Baltimore, 2000). Interestingly, increased tumor incidence is not associated with Seckel syndrome, as summarized in Chapter 1. Indeed, there have been very few reports on ATR mutations in human cancers. In one study, a subset of stomach tumors with microsatellite instability demonstrated ATR mutations (Menoyo et al., 2001). Another study found that a very small subset of endometrioid endometrial cancers harbored ATR mutations and that these mutations were associated with tumor aggressiveness as measured by worse disease-free and overall survival times (Zighelboim et al., 2009). Finally, the most convincing evidence for the association of ATR with cancer formation has come from a report where twenty-four related individuals across a five-generation pedigree were found to have an autosomal dominant disorder caused by a missense mutation spanning the region containing ATR, which presented with oropharyngeal cancer (Tanaka et al., 2012). Thus, while ATR hypomorphism may sporadically induce various tumors in mice, neither decreased, increased, nor altered ATR expression appears to have a strong correlation with the development of cancer in humans. As such, and based on our own findings, inhibition of ATR should not have a pro-tumor effect, but rather, as we have demonstrated in mice with medulloblastoma, produce an anti-tumor effect.

Chapter 4 and the preceding subsection provided data and speculation on the use of pVE-822 for the treatment of medulloblastoma. However, our observation of persistent cerebellar pathology in the context of combined Atr and p53 deletion due
to caspase-independent cell death suggests the additional therapeutic potential for ATR inhibition in treating intractable TP53-mutated medulloblastoma, as discussed in Chapter 4. VE-822 reduced tumor growth in mice bearing TP53- and KRAS-mutated PSN-1 pancreatic adenocarcinoma xenografts, but only with adjunct XRT (Fokas et al., 2012). Similarly, AZD-6738 produced a significantly stronger anti-tumor effect as monotherapy in mice bearing TP53-mutated CLL xenografts compared to TP53-intact xenografts (Kwok et al., 2016). These results have led to suggestions for the use of ATR inhibition in cancers with TP53 mutation (Murga et al., 2009; O’Driscoll, 2009). Indeed, it is well-known that TP53 pathway mutation is a hallmark of many cancers and that these mutations can confer therapeutic resistance, at least partially through attenuated cell death programs (Muller and Vousden, 2013, 2014; Shetzer et al., 2014; Hanahan and Weinberg, 2011, 2000; Weinberg, 2013). We have provided evidence that, at least in CGNPs, ATR disruption can still induce cell death even in the absence of p53 (Lang et al., 2016). Whether this observation can be reproduced in other cell types, including TP53-mutant cancers, remains to be tested, but at least recommends experiments in ATR inhibition in p53-mutated medulloblastoma.

For these experiments, p53-mutated, medulloblastoma-prone mice can be generated by crossing SmoM2loxP/loxP;p53loxP/loxP with Math1-Cre;p53loxP/loxP or hGFAP-Cre;p53loxP/loxP to generate Math1-Cre;SmoM2;p53loxP/loxP or hGFAP-Cre;SmoM2;p53loxP/loxP mice in which p53-deficient CGNPs initiate formation of p53-deficient medulloblastoma. Tumor-bearing mice can then be treated with pVE-822 or Vehicle and followed for tumor burden by magnetic resonance imaging (MRI) and for survival. As outlined in the previous subsection, pVE-822 administration must be optimized in these animals to determine the route of delivery, dose, and dosing schedule that provides the maximum therapeutic index. To start, 60 mg/kg of pVE-822 given by IP injection once a day for five consecutive
days per week over two weeks starting at P12 could be attempted. Additionally, should several combinations of delivery, dose, and scheduling of pVE-822 monotherapy fail to provide a therapeutic benefit in mice with p53-mutated medulloblastoma, supplementation with adjunct therapies like XRT, Vismodegib, or Etoposide may lead to an anti-tumor effect. Apart from measuring therapeutic response by following tumor burden and survival, some animals should be sacrificed after a shorter course of pVE-822 for molecular analyses. Immunostaining will reveal whether pVE-822 compared to Vehicle treatment increases DNA damage (γH2A.X) and cell death (TUNEL) in p53-deleted medulloblastomas, as the case in CGNPs. Flow cytometry can provide information on disturbed cell cycle mechanics, which could potentially inform the selection of adjuvant therapies. Transcriptional profiling of tumors from p53-deleted medulloblastoma mice treated with pVE-822 would also reveal the genetic response of these tumor cells to acute ATR inhibition, offering further information for selecting potential combinatorial therapeutics.

A remaining consideration in treating p53-mutated medulloblastoma mice with pVE-822 is the precise nature of cell death that occurs from ATR disruption in the absence of p53. In CGNPs, we found that co-deletion of Atr and p53 led to the abrogation of cleaved Caspase-3 (cC3) positivity in the EGL, which was present with just Atr deletion (Lang et al., 2016). Yet, CGNPs in mice of both genotypes were TUNEL-reactive, suggesting persistent caspase-independent cell death. We confirmed that TUNEL positivity was a valid marker for frank cell death rather than simply DNA damage by showing that CGNPs with deletion of Atr and the key apoptotic mediators Bax and Bak were TUNEL+ despite very high levels of DNA damage. Further examination of Atr/p53-deleted CGNPs in the EGL revealed the presence of cells with micronuclei and giant multinucleated cells, suggestive of necrosis (Vakifahmetoglu et al., 2008). Curiously, the EGL in these mice failed to show positive staining for the marker of regulated necrosis, or necroptosis, p-MLKL
(phosphorylated Mixed lineage kinase domain-like) (Cai et al., 2014). Therefore, it remains an open question as to how CGNPs undergo cell death in the absence of ATR and p53 if not through apoptosis or necrosis, and better understanding of this mechanism will also be important in understanding the anti-tumor mechanism of ATR inhibition in p53-mutated medulloblastoma.

Chapter 1 provided a detailed outline of the apoptotic pathway. In brief, Caspase-3 is considered the primary executioner caspase both because it is able to activate the other executioner Caspases, 6 and 7, and because Caspases-6 and -7 perform many roles redundant to those of Caspase-3. Thus, absence of cC3 positivity in the TUNEL+ EGL of Atr/p53-deleted mice argues against engagement of the apoptotic program. However, the possibility must be explored that Atr/p53-deleted CGNPs are simply cC3+ earlier in development, such as at P0, P1, or P2, and that TUNEL positivity, as a marker for apoptosis, is retained longer than cC3 positivity. Immunohistochemical analyses of cerebellar sections from these double-deletion mice at earlier ages will resolve this possibility. Immunostaining for cC6 and cC7 should also be done with sections from the double-knockout mice at all of these ages, including P3, in order to determine if CGNPs can utilize intrinsic pathway apoptosis independent of Caspase-3. While it is conceivable that Caspase-6/7-dependent, Caspase-3-independent apoptosis could occur in cells with deletion of Atr and p53 since cC6 can promote nuclear degradation and cC7 can further inhibit DNA repair, this possibility remains unlikely due to limited reports on such a mechanism (Hirata et al., 1998; Kaufmann et al., 1993; Boulares et al., 1999). It is further possible that apoptosis in ATR/p53-deficient CGNPs proceeds through the extrinsic pathway mediated by either TNFR1 (Tumor necrosis factor receptor 1) or CD95 (Cluster of differentiation 95) (McIlwain et al., 2013). Caspase-8 is activated through these two receptors, so immunostaining could be performed for cC8 on Atr/p53-deleted cerebellar sections. However, signaling downstream of cC8 ultimately involves the
same executioner caspases as the intrinsic apoptotic pathway, so cC3-negativity suggests absence of apoptosis in general.

Necroptosis is most commonly associated with an inflammatory response (Kanduc et al., 2002). In this pathway, TNF-α (Tumor necrosis factor α) acts as the apical signal (Vandenabeele et al., 2010). This pro-inflammatory molecule is primarily secreted by immune cells such as macrophages, but in the brain, TNF-α is released predominantly by microglia in response to infection (Welser-Alves and Milner, 2013; Hanisch, 2002), although it can also be made by astrocytes and neurons (Chung and Benveniste, 1990; Gahrng et al., 1996; Breder et al., 1993). TNF-α binds to TNFR1, which recruits the adaptor TRADD (TNFR1-associated Death domain protein) complex that allows the docking and activation of RIPK1 (Receptor-interacting serine/threonine kinase 1) (Declercq et al., 2009; Zhao et al., 2015; Hitomi et al., 2008). Activated RIPK1 recruits and activates RIPK3, which in turn phosphorylates/activates MLKL (Newton et al., 2014; Wang et al., 2014; Li et al., 2012). RIPK3 has been shown to mediate the release of reactive oxygen species from the mitochondria, which contributes to organelle and DNA damage (Moriwaki and Chan, 2013; Cai and Liu, 2014; Dondelinger et al., 2013). Activated MLKL, on the other hand, relocates to the plasma membrane to promote calcium influx, which leads to plasma membrane rupture (Cai et al., 2014). As a result, a response to inflammation ultimately promotes further inflammation (Davidovich et al., 2014; Pasparakis and Vandenabeele, 2015). In our initial analyses, we did not observe either an increase pMLKL or in signs of gross inflammation in the cerebella of Atr/p53-deleted mice. In future experiments, immunostaining for other markers of necroptosis like phosphorylated RIPK1 or RIPK3 could confirm whether necroptosis occurs in these double-knockout CGNPs. A more careful investigation as to the presence of an inflammatory response and frank necrosis in the EGL of ATR/p53-deficient mice should also be performed.
A final recognized form of cell death is by autophagy (Diaz et al., 2005). Cells experiencing autophagic cell death display cytosolic double membrane vacuoles and fused autophagosomes with lysosomes (Tasdemir et al., 2008). Increased expression of genes like ATG5 (Autophagy-related 5), ATG12, and MAP1LC3A (Microtubule-associated protein 1 light chain 3) are also considered hallmarks of autophagy (Shintani and Klionsky, 2004). We did not specifically look for or observe signs of autophagy in Atr/p53-deleted CGNPs. High-power microscopy on H&E-stained cerebellar sections from double-knockout animals, which are presently already available, may reveal the presence of morphological signs of autophagy. However, in our RNA-Seq experiments, described in Chapter 3, we did not find increased expression of Atg5, Atg12, or Map1lc3a in CGNPs from ATR/p53-deficient mice. In the end, more experiments should be performed in order to parse out the true mechanism – whether by intrinsic or extrinsic apoptosis, necroptosis, autophagy, or necrosis – of cell death, as seen by TUNEL reactivity, that is operant in CGNPs with co-deletion of Atr and p53. These same experiments must then also be performed with Atr-deleted medulloblastoma mice treated with pVE-822, should ATR inhibition lead to cell death in the tumors.
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295


1 The contents of this chapter are currently being prepared for publication as a review article.

2 This chapter previously appeared as an article in the journal *Development*: [http://dev.biologists.org/content/143/21/4038](http://dev.biologists.org/content/143/21/4038). The original citation is as follows: P.Y. Lang, G.J. Nanjungud, M. Sokolsky-Papkov, C. Shaw, D. Hwang, J.S. Parker, A.V. Kahanov, and T.R. Gershon. ATR maintains chromosomal integrity during postnatal cerebellar neurogenesis and is required for medulloblastoma formation. *Development*, 143(21): 4038-52, 2016.

3 The contents of this chapter are currently being prepared for submission as a research-in-brief article.