Polyploidy and Mitotic Cell Death are Two Distinct HIV-1 Vpr-Driven Outcomes in Renal Tubule Epithelial Cells

by

Emily Harman Payne

Department of Pathology
Duke University

Date:_____________________
Approved:

______________________________________
Mary Klotman, Supervisor

______________________________________
Donald Fox

______________________________________
David Howell

______________________________________
Micah Luftig

______________________________________
Robin Bachelder

Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy the Department of Pathology in the Graduate School of Duke University

2016
ABSTRACT

Polyploidy and Mitotic Cell Death are Two Distinct HIV-1 Vpr-Driven Outcomes in Renal Tubule Epithelial Cells

by

Emily Harman Payne

Department of Pathology
Duke University

Date: ____________________________

Approved:

___________________________
Mary Klotman, Supervisor

___________________________
Donald Fox

___________________________
David Howell

___________________________
Micah Luftig

___________________________
Robin Bachelder

An abstract of a dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Pathology in the Graduate School of Duke University

2016
Abstract

Given the emerging epidemic of renal disease in HIV+ patients and the fact that HIV DNA and RNA persist in the kidneys of HIV+ individuals despite therapy, it is necessary to understand the role of direct HIV-1 infection of the kidney. HIV-associated kidney disease pathogenesis is attributed in large part to viral proteins. Expression of vpr in renal tubule epithelial cells (RTECs) induces G2 arrest, apoptosis and polyploidy. The ability of a subset of cells to overcome the G2 block and progress to polyploidy is not well understood. Polyploidy frequently associates with a bypass of cell death and disease pathogenesis. Given the ability of the kidney to serve as a unique compartment for HIV-1 infection, and the observed occurrence of polyploid cells in HIV+ renal cells, it is critical to understand the mechanisms and consequences of Vpr-induced polyploidy.

Here I determined the effects of HIV-1 Vpr expression in renal cells using highly efficient transduction with VSV.G pseudotyped lentiviral vectors expressing vpr in the HK2 human tubule epithelial cell line. Using FACS, fluorescence microscopy, and live cell imaging I determined that G2 escape immediately precedes a critical junction between two distinct outcomes in Vpr+ RTECs: mitotic cell death and polyploidy. Vpr+ cells that evade aberrant mitosis and become polyploid have a substantially higher survival rate than those that undergo complete mitosis, and this survival correlates with enrichment for polyploidy in cell culture over time. Further, I identified a novel role for ATM kinase in promoting G2 arrest escape and polyploidy in Vpr+ RTECs. In summary, my work identifies ATM-dependent override of Vpr-mediated G2 arrest as a critical determinant of cell fate Vpr+ RTECs. Further, my work highlights how a poorly understood HIV mechanism, ploidy increase, may offer insight into key processes of reservoir establishment and disease pathogenesis in HIV+ kidneys.
Dedication

This dissertation is lovingly dedicated to my mother, Kimberly Armstrong Camp. The impact of her love, patience and support on my life is immeasurable, and I will be forever grateful that she is my mom.
# Contents

Abstract......................................................................................................................................................... iv

List of Tables ...................................................................................................................................................... xii

List of Figures .................................................................................................................................................... xiii

Acknowledgements ............................................................................................................................................. xviii

1. Introduction .................................................................................................................................................. 1

1.1 Introduction to HIV-Associated Nephropathies ....................................................................................... 1

1.1.1 Epidemiology .......................................................................................................................................... 1

1.1.1.1 HIV Infection ................................................................................................................................. 1

1.1.1.2 HIV-Associated Nephropathy (HIVAN) ....................................................................................... 2

1.1.1.3 HIV-Associated Renal Disease in the Era of cART ..................................................................... 2

1.1.2 Clinical Aspects of HIVAN and Other HIV-associated Renal Diseases ............................................ 4

1.1.2.1 Overview of Kidney Function ......................................................................................................... 4

1.1.2.2 Clinical Presentation and Histopathology of HIVAN .................................................................... 5

1.1.2.3 Diagnosis of HIVAN ..................................................................................................................... 6

1.1.2.4 Treatment of HIVAN ................................................................................................................... 7

1.1.3 Mechanisms of HIVAN Pathology ....................................................................................................... 9

1.1.3.1 Direct HIV Infection of Kidney Cells ........................................................................................... 9

1.1.3.2 Viral Factors of HIVAN Pathology ............................................................................................... 10

1.1.3.3 Mechanisms of Nef-induced Podocyte Dysregulation ................................................................ 11

1.1.3.4 Mechanisms of Vpr-induced Tubulointerstitial Injury .................................................................. 12
1.1.3.5 Genetic Factors Associated with HIVAN Pathogenesis ......................... 13
1.1.4 The Kidney as an HIV-1 Compartment and Possible Reservoir ..................... 13
1.2 Overview of HIV-1 Acessory Protein VPR ........................................ 15
  1.2.1 Proposed *In Vivo* Functions of Vpr ......................................... 15
  1.2.2 Proposed *In Vitro* Functions of Vpr ......................................... 16
1.3 Vpr and G2 Cell Cycle Arrest ................................................................ 18
  1.3.1.1 ATR and ATM: Key Mediators of the DNA Damage Response .............. 20
  1.3.2 Overview of Vpr-induced G2 Arrest .............................................. 22
    1.3.2.1 The Relationship Between DNA Damage and Vpr-induced Cell Cycle
          Arrest ......................................................................................... 23
    1.3.2.2 Vpr Interaction with the Ubiquitin Proteosome System
          Induces Cell Cycle Arrest ......................................................... 25
  1.3.3 Biological Significance of Vpr-induced G2 Arrest ................................ 27
  1.3.4 Mechanisms of Vpr-induced G2 Arrest in the Kidney ............................ 27
1.4 VPR and Apoptosis ............................................................................. 30
  1.4.1 Overview of Apoptosis ................................................................. 30
  1.4.2 Overview of Vpr-induced Apoptosis .............................................. 31
    1.4.2.1 The Relationship Between Vpr-induced Cell Cycle Arrest and
          Apoptosis ................................................................................. 33
    1.4.2.2 The Relationship Between Vpr-induced Aberrant Mitosis and
          Apoptosis ................................................................................. 34
  1.4.3 Mechanisms of Vpr-induced Apoptosis in the Kidney ............................ 35
1.5 VPR and Polyploidy ........................................................................... 37
  1.5.1 Overview of Polyploidy ............................................................... 37
  1.5.2 Polyploidy in the Setting of HIV-1 Infection and Vpr Expression .......... 41
1.5.3 Polyploidy in the Setting of HIV-1 Infection and Vpr Expression in RTECs ... 42

1.6 Research Aims Outlined in this Dissertation................................................................. 45

1.6.1 Characterize HIV-1 Vpr-mediated G2 Arrest in Renal Tubule Epithelial Cells 46

1.6.2 Identify the Mechanism of HIV-1 Vpr-induced Polyploidy in Renal Tubule Epithelial Cells .......................................................................................................................... 47

1.6.3 Characterize the Physiological Implication of Mitotic Entry and Polyploidy Following Vpr Expression in Renal Tubule Epithelial Cells.......................................................... 47

1.6.4 Assess the Role of ATM Kinase in the Emergence of Polyploidy in Vpr+ RTECs .................................................................................................................................................. 48

2. Materials and Methods..................................................................................................... 49

2.1 Cell Culture........................................................................................................................ 49

2.2 Generation of pseudotyped virus, transfection and infection .................................... 49

2.3 Cell Cycle Analysis.......................................................................................................... 49

2.4 Drug Treatments ........................................................................................................... 50

2.4.1 ATR Inhibition ........................................................................................................... 50

2.4.2. Thymidine G1 Cell Cycle Synchronization ............................................................... 50

2.4.3. ATM Inhibition ....................................................................................................... 50

2.5 Western Blot Analysis ................................................................................................... 50

2.5.1 Whole-cell Extract Preparation ............................................................................... 50

2.5.2 Standard Western Blot ............................................................................................. 51

2.5.3 Western Blot for Large Proteins .............................................................................. 51

2.5.4 Western Blot Densitometry Analysis ....................................................................... 52

2.6 Immunofluorescence Flow Cytometry ......................................................................... 52
2.7 Immunofluorescence Microscopy .............................................................. 52
2.8 Renal Biopsy Specimens and Immunohistochemistry .................................. 53
2.9 FUCCI Probes and Live Cell Imaging .......................................................... 53

3. Characterize HIV-1 Vpr-mediated G2 Arrest in Renal Tubule Epithelial Cells .... 54
   3.1 Introduction .................................................................................................. 54
   3.2 Results ........................................................................................................ 55
      3.2.1 Vpr Expression Induces an ATR dependent G2 Arrest in Vpr+ RTECs .... 55
      3.2.2 A Subset of HIV-1 Vpr+ Renal Tubule Epithelial Cells Escape ATR Dependent G2 Arrest to Become Polyploid ......................................................... 60
      3.2.3 G2 Arrest and Polyploidy in Vpr+ RTECs Does Not Require Virus Integration or de novo Synthesis of Vpr Protein ......................................................... 61
      3.2.4 Vpr-mediated G2 Arrest Differs from Genotoxic-induced G2 Arrest, and is Required for Progression to Polyploidy ......................................................... 62
   3.3 Discussion ................................................................................................... 64

4. Identify the mechanism of HIV Vpr-induced Polyploidy in Renal Tubule Epithelial Cells .......................................................... 67
   4.1 Introduction .................................................................................................. 67
   4.2 Results ........................................................................................................ 67
      4.2.1 Vpr+ Renal Tubule Cells Express the Mitotic Marker Phospho-Histone H3.... 67
      4.2.2 A Subset of Vpr+ RTECs Enter Mitosis Following a Prolonged G2 Arrest....... 69
      4.2.3 Polyploid Vpr+ RTECs Replicate DNA Without Undergoing Mitosis .......... 71
      4.2.4 Renal Tubule Cells in Murine and Human HIVAN are Positive for Mitotic Marker PH3 ......................................................................................... 73
5. Characterize the Physiological Implications of Mitotic Entry and Polyploidization Following Vpr Expression in Renal Tubule Epithelial Cells .................................................. 76
5.1 Introduction ..................................................................................................................................... 76
5.2 Results ............................................................................................................................................. 76
5.2.1 Vpr Promotes Aberrant Mitosis in Renal Tubule Epithelial Cells ................................. 76
5.2.2 Polyploidy Represents an Alternative to Mitotic Cell Death in Vpr-expressing Renal Tubule Epithelial Cells ..................................................................................... 78
5.2.3 Polyploid Vpr+ RTECs Become Enriched in Cell Culture Over Time....................... 82
5.3 Discussion ....................................................................................................................................... 83
6. Assess the Role of ATM Kinase in the Emergence of Polyploidy in Vpr+ RTECs ...... 86
6.1 Introduction ..................................................................................................................................... 86
6.2 Results ............................................................................................................................................. 87
6.2.1 ATM is Required for Escape from Vpr-mediated G2 Arrest and Polyploidy Accumulation in Renal Tubule Epithelial Cells ................................................................. 87
6.2.2 Activation of ATM in Vpr+ Renal Tubule Cells is Restricted to Cells in the G2/M phase of the Cell Cycle and Polyploid cells................................................................. 89
6.2.3 Inhibition of ATM Prevents Progression From G2 to Mitosis in Vpr+ Renal Tubule Cells, but not in Control Cells ................................................................. 91
6.2.4 Activated ATM is Present in Renal Tubule Cells in Human HIVAN Biopsies, but Not in Control Kidneys................................................................. 94
6.3 Discussion ....................................................................................................................................... 95
7. Conclusions and Implications ........................................................................................................... 99
7.1 Escape From G2 Arrest is a Critical Determinant of Cell Fate in Vpr+ RTECS ...... 99
7.2 ATM is Required for G2 Arrest Escape in Vpr+ Kidney cells ............................................. 101
7.3 The Role of Vpr-induced Polyploidy in the Kidney Following HIV Infection... 103

References........................................................................................................................................ 107

Biography ......................................................................................................................................... 129
List of Tables

Table 1: Spectrum of HIV-Associated Kidney Diseases in the Era of cART.................... 3
List of Figures

Figure 1: Classic Histopathological Features of HIVAN. (A) Occlusion of glomerular capillary lumina by collapse of glomerular basement membranes with accompanying hypertrophy and hyperplasia of overlying podocytes (Jones methenamine silver, ×400). (B) Distended tubules with microcysts containing proteinaceous casts. Adjacent cortical tubules display degenerative changes (H&E, 200×). Adapted from Wyatt et al. 2012[25].

Figure 2: Activation of the DNA Damage Response in Murine and Human HIVAN. Kidneys from littermate controls or Tg26 mice with HIVAN (upper panel) and normal human kidney and a biopsy from a HIVAN patient were stained with anti-γH2AX antibody. Fields are 200X and inserts are 400X.

Figure 3: Schematic of Polyploidy Generated Through Endoreplication. Polyploidy can be generated by endoreplication, non-canonical cell cycles characterized by multiple rounds of genome replication without complete cell division. Endoreplication cell cycles are characterized as either endocycling, in which cells undergo successive rounds of DNA replication without an intervening mitotic phase, or as endomitosis, in which genome replication is accompanied by incomplete mitosis. Modified from Fox and Duronio 2013[168].

Figure 4: Hypertrophy and Multinucleation in a Murine Model of HIVAN. Examples of control murine tubules (top left) compared to HIVAN murine tubules. PAS stain at x600 original magnification. HIVAN tubules are dilated, and exhibit cellular hypertrophy and multinucleation (arrows)[60].

Figure 5: Increased Chromosome Number in Tubule Cells in Human HIVAN biopsies. Renal FISH staining on a female human control (upper panel) and male human HIVAN biopsy (lower panel). L, tubular lumen; arrows, cell of normal ploidy (control) or increased ploidy (HIVAN). Centromeric enumeration probes (CEP) are Aqua, chromosome 8; red, chromosome X; and green, chromosome Y. The original magnification of control and HIVAN sections was x1000 [60].

Figure 6: Inhibition of DDR Kinase ATR Alleviates G2 arrest in Vpr+ RTECs. Analysis of HK2 cell cycle 24 hours after transduction with TY2-Vpr-GFP +/- ATR inhibitor VE821. The X-axis shows DNA content, as measured by Propidium Iodide (PI), and the Y-axis indicates relative cell number. The percentage of cells within the G1 and G2 gates is indicated in each panel.
Figure 7: Activation of DDR Kinase ATR in Vpr+ RTECs Corresponds with Accumulation of Cells in the G2 Phase of the Cell Cycle. Left panel: Time course of ATR phosphorylation (western blot) in HK2 cell +/- expression of pHR-HA-VPR-GFP (HA-Vpr). Densitometric analysis of phospho-ATR band was adjusted to ATR loading control and normalized to corresponding HK2 time point. Right panel: Corresponding cell cycle phase analysis from the same populations of cells in left panel. Cell cycle phase was identified using FACS analysis of DNA content (PI). The Y-axis indicates the percentage of cells within the G1 (Green), S (Red) and G2 (Blue) phases of the cell cycle for each time point. ..............................

Figure 8: Inhibition of DDR Kinase ATR Alleviates CHK1 Activation in Vpr+ RTECs. Top panel: HK2 cell cycle analysis (FACS) 72 hours after transduction with TY2-GFP, and TY2-Vpr-GFP +/- ATR inhibitor VE821 or ATM inhibitor KU55933. The X-axis shows DNA content, as measured by Propidium Iodide (PI), and the Y-axis indicates relative cell number. The percentage of cells within the G1 and G2 gates is indicated in each panel. Lower panel: Corresponding western blot analysis for phospho-CHK1 for cell populations in upper panel. Densitometric analysis of phospho-CHK1 band was adjusted to CHK1 loading control and normalized to HK2 sample. ..............................

Figure 9: A Subset of HIV-1 Vpr+ RTECs Escape ATR Dependent G2 Arrest to Become Polyploid. FACS cell cycle analysis of HK2 cells showing the emergence of polyploidy in TY2-Vpr-GFP+ RTECs over time. The X-axis shows DNA content, as measured by Propidium Iodide (PI), and the Y-axis indicates relative cell number. The percentage of cells within the G1, G2 and polyploid gates is indicated in each panel. ..............................

Figure 10: Progression to Polyploidy Does Not Require Viral Integration or De Novo Synthesis of Vpr Protein. HK2 Cell cycle analysis 72 ours after transduction with TY2-Vpr-GFP +/- IN or Vpr VLP. The X-axis shows DNA content, as measured by Propidium Iodide (PI), and the Y-axis indicates relative cell number. The percentage of cells within the G1, G2 and polyploid gates is indicated in each panel. ..............................

Figure 11: G2 Arrest is Required, but Not Sufficient, for the Emergence of Polyploidy in Vpr+ RTECs. Right Panel: FACS cell cycle analysis of HK2 cells 48 hours following transduction with: TY2-VPR-GFP (VPR48H), TY2-Q65R-GFP (VPR Q65R 48H), or following treatment with Doxorubicin or Cisplatin. The X-axis shows DNA content, as measured by Propidium Iodide (PI), and the Y-axis indicates relative cell number. The percentage of cells within the G1, G2 and polyploid gates is indicated in each panel. Left panel: Cell cycle analysis of HK2 cells 48 hours after transduction with TY2-VPR-GFP or TY2-VPR-GFP + VE821. ..............................
Figure 12: Vpr+ RTECs Express Mitotic Marker Phospho-Histone H3. FACS analysis of global PH3 staining in asynchronous HK2 cells 72 hours following transduction with TY2-VPR-GFP (orange) or a TY2-GFP control (green). As a control, non-transduced HK2 cells were stained with PH3 + secondary antibody (blue), and secondary alone (red). The X-axis shows phospho-Histone H3 staining fluorescent intensity, and the Y-axis indicates relative cell number.

Figure 13: A Subset of Vpr+ RTECs Enter Mitosis Following a Prolonged G2 Arrest. (A) Experimental schematic for transduction of HK2 cells following G1 thymidine synchronization. (B) Time course monitoring cell cycle progression and mitotic entry in synchronized HK2 control cells and cells transduced with HR-HA-VPRΔGFP. Graphical representation of FACs analysis of the % of cells with 4C DNA content at each time point from T=4 to T=26 (top panel) and mitotic pH3+ cells within that 4C population at each time point (bottom panel).

Figure 14: Polyploid Vpr+ RTECs Replicate DNA Without Undergoing Mitosis. FACS analysis of BrdU incorporation (Y axis) and DNA content (Propidium Iodide- PI, X axis) in control HK2 cells (HK2) and HK2 cells transduced with pH-HA-VPRΔGFP (Vpr48H).

Figure 15: Renal Tubule Cells in Murine and Human HIVAN are Positive for Mitotic Marker PH3. Kidneys from littermate controls or Tg26 mice with HIVAN (upper panel) and normal human kidney and a biopsy from HIVAN patients (lower panel) were stained with anti-PH3 antibody.

Figure 16: Vpr+ RTECs Undergo Aberrant Mitosis Following a Prolonged G2 Phase. Immunofluorescence microscopy depicting mitosis in synchronized HK2 cells 18 hours post-transduction with HR-HA-VPRΔGFP. Cells were analyzed for centrosome formation (γ-Tubulin, Red), mitotic spindle formation (α-Tubulin, Green) and DNA (DAPI, Blue). VPR+ cells exhibited mitotic defects, including centrosome amplification and bipolar spindles with more than 2 spindle poles (Vpr1) and tripolar spindles with more than 2 spindle poles (Vpr2).

Figure 17: Schematic of FUCCI Probe Function. Expression of FUCCI vectors allows visualization of cell cycle progression through G1 phase (red fluorescence), S/G2 (cytoplasmic green fluorescence), and M phases (nuclear green fluorescence).

Figure 18: Live Cell Imaging of Vpr+ HK2 cells with FUCCI vectors. Upper panel: Live cell imaging of HK2 cells transduced with FUCCI vectors (HK2 FUCCI), showing representative cell undergoing mitosis (arrow). Middle panel: Live cell imaging...
of HK2 cells co-transduced with HR-HA-VPRΔGFP and FUCCI vectors (VPR FUCCI). Vpr+ cells were counted and characterized as either mitosis (arrow head) or no mitosis (arrow). Bottom Panel: Table on right indicates total cell counts for three separate trials. Graph on right indicates average survival rates for cells that undergo mitosis vs. cell that do not complete mitosis in control and Vpr+ cells in 3 separate trials. Vpr+ cells that bypassed mitosis had higher average rates of survival (79.3%) than Vpr+ cells that attempted mitosis (32.7%). Control cells that underwent mitosis had 87% of cells surviving, while none of the control cells that bypassed mitosis survived.

Figure 19: Polyploidy Becomes Enriched in Vpr+ RTECs Over Time. HK2 cells transduced with TY2-VPR-GFP at 0, 3 and 9 days post-transduction. The X-axis shows DNA content, as measured by Propidium Iodide (PI), and the Y-axis indicates relative cell number. Distribution of cell cycle population indicated in each panel.

Figure 20: The ATM Kinase is Required for the Emergence of Polyploidy in HIV-1 Vpr+ RTECs. HK2 cell cycle analysis 48 hours following transduction with TY2-VPR-GFP +/- ATM inhibitor KU55933. The X-axis shows DNA content, as measured by Propidium Iodide (PI), and the Y-axis indicates relative cell number. The percentage of cells within the G1, G2 and polyploid gates is indicated in each panel.

Figure 21: The Activation of ATM Corresponds with the Emergence of Polyploidy in HIV-1 Vpr+ RTECs. Left panel: Time course of ATM phosphorylation (western blot) in HK2 cell +/- expression of HR-HA-VPR-GFP (HA-Vpr). Densitometric analysis of phospho-ATM band was adjusted to ATM loading control and normalized to corresponding HK2 time point. Right panel: Corresponding cell cycle phase analysis from the same populations of cells in left panel. Cell cycle phase was identified using FACS analysis of DNA content (PI). The Y-axis indicates the percentage of cells within the G1 (Purple), S (Green), G2 (Red) and polyploid (Blue) phases of the cell cycle for each time point.

Figure 22: ATM is Activated in G2/M and Polyploid Populations of Vpr+ RTECs. Upper Panel: FACS analysis of pATM and PI co-staining 44 hours following transduction with HR-HA-VPR-ΔGFP. The X-axis shows DNA content, as measured by Propidium Iodide (PI), and the Y-axis indicates pATM fluorescent intensity. Gated area indicates the percent of total cells that are positive for pATM. Lower Panel: Analysis of the fluorescent intensity of pATM in distinct cell cycle phases Vpr+ cells. The same population of Vpr+ RTECs from the upper panel was gated based on DNA content, and the fluorescent intensity of pATM (lower right, X-axis) was determined for the G1 (Green), G2/M (Blue) and polyploid (Red) cell populations.
Figure 23: Inhibition of ATM Decreases Mitosis in Vpr+ Cells. Analysis of the fold change in pH3+ control and Vpr-expressing cells following treatment with ATM inhibitor KU55933. Synchronized cells were analyzed via FACS for pH3 18 hours following transduction with HR-HA-VPR-ΔGFP +/- ATMi. The Y-axis indicates the fold change in pH3+ cells for control HK2 (HK2 vs. HK2+ ATMi) and Vpr-expressing cells (Vpr vs. Vpr + ATMi). Each data point represents the fold change in a separate experimental trial. .................................................................................................................... 92

Figure 24: Inhibition of Polyploidy is Reversible in Vpr+ RTECs. Upper panel: HK2 cell cycle analysis 72 and 120 hours following transduction with TY2-VPR-GFP. Lower Panel: HK2 cell cycle analysis 72 hours following transduction with TY2-VPR-GFP +ATMi. ATMi was removed at 72 hours, and cells were left in culture until 120 hours post-transduction, at which point cell cycle analysis was performed. The X-axis shows DNA content, as measured by Propidium Iodide (PI), and the Y-axis indicates relative cell number. The percentage of cells within the G1, G2 and polyploid gates is indicated in each panel. .................................................................................................................... 94

Figure 25: Activated ATM is Present in Renal Tubule Cells in Human HIVAN Biopsies. Kidney biopsies for normal human kidney and HIVAN patients were stained with anti-PATM antibody. .................................................................................................................... 95

Figure 26: Proposed Model For Polyploidy Acquisition in Vpr+ RTECs ................. 106
Acknowledgements

I would like to express my appreciation and thanks to my advisor, Dr. Mary Klotman. Her candor, guidance and support, both professionally and personally, have been instrumental to the successful completion of my dissertation project. As a woman in science, I am inspired not only by Dr. Klotman’s accomplishments as a physician-researcher, but also her dedication to maintaining a fulfilling family life, and I aspire to achieve similar successes going forward.

I would like to thank my committee member and unofficial co-mentor, Dr. Donald Fox, for introducing me to the fascinating phenomenon of polyploidy. Dr. Fox’s enthusiasm for science is unparalleled, and his mentorship and expertise were invaluable to the development and execution of my project. I also want to thank, Dr. David Howell, Dr. Micah Luftig and Dr. Robin Bachelder for serving on my committee. Their support, questions and feedback have made me a stronger scientist and contributed greatly to the completion of my dissertation.

I especially want to thank the other members of the Klotman lab. Dr. Bala Balakumaran has helped me with everything from experimental design to microscopy, and I can truly say I could not have done this without his willingness to help on many fronts. I want to thank Dr. Maria Blasi for her guidance and support; she is one of the most gifted scientists I know, and I am so grateful I had the opportunity to work along
side her. Thank you to Dr. Andrea Cara and Dr. Donatella Negri who were instrumental in my early training and project design. Thank you to the two newest members of the lab, Dr. Ian Belle and Erich Baker. And lastly, thank you to Donna Salvo, who always helped me find time in Mary’s busy schedule for a meeting. The assistance, collaboration and friendship I received from other members of the Klotman lab were invaluable, and I am grateful that I had such a wonderful team of people to support me during graduate school.

I want to thank the members of ‘Can I Get a PhD in Trivia’; our trivia nights were always the highlight of my week, and I am so grateful for their friendship over the past six years. Thank you to all of my Durham friends, and to the friends that have supported me from across the miles. I cannot thank my family enough for their love and support. I want to express a special thanks to my Dad, who supported me throughout countless years of school. Thank you to my mom and all of my family in Flagstaff; they have stood by me throughout the years, and I cannot express how much their love and support has meant. Thank you to Buckley and Zoë, who sat with me every day while I wrote my dissertation, and snuggled with me when it was time to take a break. And lastly, I have to thank my husband Stephen. His unconditional love and support changed my life, and his encouragement sustained me through the many highs and lows of graduate school. I am grateful every day for such a wonderful husband, and I can’t wait to start the next chapter of our life together.

xix
1. Introduction

Widespread use of combined antiretroviral therapy (cART) has dramatically altered the landscape of HIV-1 associated morbidity and mortality. While deaths attributed to traditional AIDS-defining illnesses have decreased, HIV deaths associated with chronic non-AIDS defining disease of the liver, heart and kidney have become more prevalent. Acute kidney injury and chronic kidney disease, including HIV-associated nephropathy (HIVAN) occur at high rates in HIV-1 infected individuals, and in particular, HIV patients of African descent. The emergence of this epidemic of HIV-1 related renal disease necessitates further investigation of the role of direct HIV-1 kidney infection in reservoir establishment and disease pathogenesis.

1.1 Introduction to HIV-Associated Nephropathies

1.1.1 Epidemiology

1.1.1.1 HIV Infection

By 2015, 36.9 million people, including 25.8 million people in sub-Saharan Africa, were infected with HIV worldwide. Since the onset of the HIV epidemic over 78 million people have been infected, and over 39 million have died from AIDS-related illnesses. While new HIV infections have fallen by 35% since 2000, there were still 2 million new infections reported in 2014. Globally, 1.2 million people died from AIDS-related illness, representing a 42% decrease in AIDS-related deaths from the peak in 2004. Advances in antiretroviral drugs and improved access to care are major factors in the changing
landscape of HIV-associated morbidity and mortality, with over 15.8 million HIV+ patients receiving antiretroviral therapy[1].

1.1.1.2 HIV-Associated Nephropathy (HIVAN)

Classic HIV-associated nephropathy (HIVAN) is an aggressive form of kidney disease that was first reported in groups of African-Americans and Haitian immigrants in the United States in 1984[2]. Prior to the introduction of combined antiretroviral therapy, the incidence of HIVAN was reported in 3%-10% of HIV+ patients in the United States, and occurred primarily in patients of African descent[3, 4]. Lacking cART intervention, HIVAN progresses rapidly to end-stage renal disease (ESRD), and was identified as a leading driver of ESRD in African Americans by the early 1990s[5]. Although combinatorial antiretroviral therapy has reduced the impact of HIVAN in the United States, continued growth of the HIV epidemic in susceptible African populations may have important global health implications. Renal disease, with varying criteria for diagnosis, has been reported in as many as 48.5% of HIV+ individuals in Africa[6], and in South Africa, 24-83% of cases of HIV-associated renal disease are classified as classic HIVAN[7-9].

1.1.1.3 HIV-Associated Renal Disease in the Era of cART

While the widespread use of cART has dramatically decreased AIDS-related deaths and ESRD attributed to HIVAN, the spectrum of other HIV-related renal diseases
continues to evolve. Several studies in which HIV+ patients underwent clinically indicated biopsies, revealed diverse histological findings indicative of renal disease other than HIVAN (Table 1)[5, 10].

Table 1: Spectrum of HIV-Associated Kidney Diseases in the Era of cART

<table>
<thead>
<tr>
<th>Category</th>
<th>Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Classic HIVAN</td>
<td></td>
</tr>
<tr>
<td>Non-collapsing focal segmental glomerulosclerosis</td>
<td></td>
</tr>
<tr>
<td>HIV-immune-complex-kidney disease</td>
<td></td>
</tr>
<tr>
<td>Mesangial proliferative</td>
<td></td>
</tr>
<tr>
<td>Membranoproliferative glomerulonephritis +/- HCV co-infection</td>
<td></td>
</tr>
<tr>
<td>Lupus-like glomerulonephritis</td>
<td></td>
</tr>
<tr>
<td>IgA nephropathy</td>
<td></td>
</tr>
<tr>
<td>Membranous nephropathy +/- HBV co-infection</td>
<td></td>
</tr>
<tr>
<td>Exudative proliferative or crescentic</td>
<td></td>
</tr>
<tr>
<td>Immunotactoid or fibrillary glomerulonephritis</td>
<td></td>
</tr>
<tr>
<td>HIV thrombotic thrombocytopenic purpura or haemolytic uraemic syndrome</td>
<td></td>
</tr>
<tr>
<td>Minimal change nephropathy</td>
<td></td>
</tr>
<tr>
<td>Diabetic nephropathy</td>
<td></td>
</tr>
<tr>
<td>Arterionephrosclerosis</td>
<td></td>
</tr>
<tr>
<td>Other glomerular and tubular diseases</td>
<td></td>
</tr>
</tbody>
</table>
Other indications of chronic kidney disease, including estimated glomerular filtration rates (eGFR) and proteinuria, indicate between 2.4-17% of HIV+ patients suffer from CKD[11-17]. Additionally, HIV+ patients remain at an increased risk for developing ESRD, particularly if they are of African descent[18]. Several factors have been linked to HIV-associated CKD, including race, HCV co-infection, comorbid diabetes, protracted use of cART and age[14, 15]. Prolonged cART induced nephrotoxicity, primarily caused by protease inhibitors and tenofovir therapy, is becoming an increasing clinical burden in aging HIV individuals[19, 20]. Thus, while the incidence of HIVAN has decreased, the emerging spectrum of HIV-associated chronic kidney disease demands further investigation of the contribution of direct HIV-1 kidney infection in disease pathogenesis in aging HIV+ patients. Further, understanding the mechanisms that contribute to HIVAN pathogenesis will likely offer insight into viral and host factors governing other forms of HIV-associated renal disease.

1.1.2 Clinical Aspects of HIVAN and Other HIV-associated Renal Diseases

1.1.2.1 Overview of Kidney Function

To understand the impact of HIV on kidney disease, it is important to understand the basic architecture and function of the kidney. The functional unit of the kidney is a nephron, which includes a renal corpuscle, or filtration unit, and associated tubules. The renal corpuscle is comprised of a network of capillaries, or the glomerulus,
and the surrounding Bowman’s capsule. Blood is filtered through glomerulus, and the remaining fluid passes along the tubule before being excreted as urine. Injury to either the glomerulus or epithelial cells of the tubule can results in kidney dysfunction. In the context of HIVAN, injury to renal tubule epithelial cells and podocytes, visceral epithelial cells that wrap around glomerular capillaries, are most often associated with disease pathogenesis. Podocyte injury disrupts the filtration of blood in the renal corpuscle, while injury to tubule epithelial cells compromises tubule function. Thus, HIV promotes renal disease by damaging two critical components of kidney function.

1.1.2.2 Clinical Presentation and Histopathology of HIVAN

Acute phase HIVAN is an aggressive form of focal segmental glomerulosclerosis (FSGS) with injury to both glomeruli and renal tubules. HIVAN patients present with enlarged kidneys, heavy proteinuria and rapidly progressive renal failure. Definitive HIVAN diagnosis requires renal biopsy to identify the distinguishing collapse of the glomerular basement membrane, and accompanying hypertrophy and hyperplasia of surrounding podocytes (Figure 1A). Characteristic tubulointerstitial pathology includes tubular atrophy, interstitial fibrosis, microcyst formation and inflammation (Figure 1B). Renal tubule epithelial cells exhibit hypertrophy with enlarged hyper-chromatic nuclei, and focal apoptosis [21-24].
1.1.2.3 Diagnosis of HIVAN

Currently available non-invasive diagnostic testing for HIV-related kidney disease includes measurement of proteinuria, eGFRs, CD4 counts, circulating HIV RNA, and kidney size. However, these conventional diagnostic methods lack the sensitivity and specificity necessary to differentiate between the myriad of diagnoses on the HIV-related renal disease spectrum. While several studies indicate HIVAN associates with high viremia, low CD4 counts and heavier proteinuria, these outcomes are not specific to a diagnosis of HIVAN[26-28]. Therefore, kidney biopsy remains the gold standard for diagnosing HIVAN and distinguishing between other renal disease in HIV+ patients, including comorbid kidney disease, nephrotoxicity from cART and other HIV-specific kidney disease[25].

Figure 1: Classic Histopathological Features of HIVAN. (A) Occlusion of glomerular capillary lumina by collapse of glomerular basement membranes with accompanying hypertrophy and hyperplasia of overlying podocytes (Jones methenamine silver, ×400). (B) Distended tubules with microcysts containing proteinaceous casts. Adjacent cortical tubules display degenerative changes (H&E, 200×). Adapted from Wyatt et al. 2012[25].
Given the reliance on kidney biopsy for a differential diagnosis in kidney disease, there is a need to develop alternative non-invasive diagnostic tools that are both sensitive and specific. One potential unique biomarker for HIVAN is neutrophil gelatinase- associated lipocalin (NGAL), which is excreted by tubular cells following damage to the nephron. NGAL was previously identified as being upregulated in the Tg26 mouse model of HIVAN, and a subsequent study confirmed upregulated urinary NGAL levels were associated with the diagnosis of HIVAN, but not other chronic kidney diseases[29]. While NGAL needs to be validated in future studies, these results indicate that novel biomarker identification holds potential as a non-invasive diagnostic tool to differentiate between diagnoses of HIV-associated renal disease[25].

1.1.2.4 Treatment of HIVAN

Following the introduction of cART incidence of HIVAN and HIV-associated ESRD in the United States fell dramatically[30, 31]. Given the efficacy of cART in controlling HIVAN disease progression, antiretroviral therapy remains the standard of care for cases of HIVAN. While several studies noted improved renal function in HIV patients with other forms of CKD following adherence to a cART regimen[10], others concluded cART therapy offered no definitive benefit to patients with non-HIVAN HIV-related kidney disease[32]. The current guidelines set by The HIV Medicine Association of the Infectious Diseases of Society of America recommend cART for treatment of any renal disease in HIV+ patients, regardless of CD4 count[33]. Angiotensin-converting
enzyme (ACE) inhibitors, angiotensin receptor blockers (ARBs) and prednisone can be prescribed for patients that do not improve following the implementation of cART therapy. Standard therapies for chronic kidney disease, including weight loss, smoking cessation, nephrotoxin avoidance and control of blood pressure, are also recommended[33].

In cases where cART does not effectively control HIVAN or ESRD progression, kidney transplant may be recommended. While patient and graft survival was dismal pre-ART, a case series of 150 kidney transplants in HIV+ patients on cART reported three year patient and graft survival rates of 88.2% and 73.7%, respectively. Per the Patient Scientific Registry of Transplant Recipients (SRTR), this patient survival rate was similar to non-HIV patients, while allograft survival was slightly lower in HIV+ individuals. Of note, 31% of HIV+ renal transplant patients experience acute rejection events, compared to 12.3% in other populations[34]. This study noted 2 cases of HIVAN recurrence in kidney allografts, which had also been reported in 3/40 allografts in a previous study[35], suggesting HIV infection was re-established in the kidney, despite adherence to a cART regimen.
1.1.3 Mechanisms of HIVAN Pathology
1.1.3.1 Direct HIV Infection of Kidney Cells

Direct HIV-1 infection and gene expression in renal parenchymal cells is a critical mediator of HIVAN pathology[10, 24, 36]. In 2000 the Klotman lab identified renal epithelial cells as a unique HIV-1 infection site. HIV-1 mRNA (Nef and Gag) and DNA (Env) were detected in glomerular and tubular epithelial cells of HIV+ patients, including patients with undetectable viral loads[37]. Canonical HIV-1 infection of CD4+ T cells requires HIV-1 Env binding to the CD4 cell surface receptor. Subsequent interaction between the HIV-1 envelope glycoprotein gp-120 and either the CXCR4 or CCR5 co-receptors initiates membrane fusion and virus entry into the cell[38]. Because renal epithelial cells do not traditionally express CD4 and CXCR4/CCR5 receptors[39-41], there have been multiple studies to determine an alternate mechanism of viral entry in these cells. HIV-1 interacts with C-type lectin receptor DEC-205 to promote virus internalization in renal tubule epithelial cells; however, this mode of entry ultimately results in a nonproductive infection[41]. Alternatively, heparan-sulfate proteoglycans have also been suggested as alternate receptors for envelope-independent HIV-1 entry into CD4-negative cells [40, 42-44]. However, the rates of infection of RTECs using cell-free HIV are low, indicating this mechanism of viral entry is an inefficient mechanism to establish an HIV-1 compartment in the kidney [42, 45].
Infiltrating lymphocytes are a hallmark characteristic of HIVAN renal biopsies[24], suggesting a possible role for T cells in HIV-1 spread within the kidney. The role of T-cells in propagating HIV infection in the kidney was first demonstrated in a study that used video microscopy to show transfer of replication-competent virus from T-cells to renal epithelial cells through virologic synapses. Further, subsequent de novo synthesis of viral proteins indicates this mode of transfer results in a productive infection of RTECs[42]. In a subsequent study, the Klotman lab confirmed cell-to-cell contact between infected T cells and uninfected renal epithelial cells results in a productive HIV-1 infection in renal epithelial cells. This contact-dependent transfer of HIV-1 is bidirectional, allowing infected renal epithelial cells to propagate the infection and transfer the virus to uninfected T cells[45]. Taken together, these studies suggest a mechanism for establishing an intrarenal HIV-1 compartment and offer insight into the virus life cycle in the kidney.

1.1.3.2 Viral Factors of HIVAN Pathology

The pathogenic consequences of direct HIV-1 infection of renal epithelial cells were first noted in an early HIV-1 transgenic mouse model (Tg26). HIV-1 encodes nine gene products, including structural proteins Gag, Pol, and Env, essential viral regulatory factors Tat and Rev, and accessory proteins Vpu, Vif, Nef, and Vpr. To assess the role of viral proteins in disease pathogenesis, a replication incompetent transgene, lacking gag and pol genes, was expressed in mice. Despite ubiquitous expression of the HIV-1
transgene, renal disease characteristic of HIVAN was the sole phenotype that emerged[46]. As the transgene lacks gag and pol, two genes critical viral replication, this model suggests HIVAN pathogenesis requires HIV-1 protein expression, but occurs independently of viral replication. Subsequent transgenic mouse modeling determined expression of accessory proteins Nef and Vpr are key determinants of HIVAN pathogenicity, as expression of these proteins is sufficient to recapitulate the full spectrum of HIVAN pathology. Specifically, Nef drives glomerulopathy through podocyte dysregulation, while Vpr mediates both glomerular and tubulointerstitial injury [47-50].

1.1.3.3 Mechanisms of Nef-induced Podocyte Dysregulation

Podocyte dysregulation disrupts the architecture of the renal corpuscle, which promotes glomerular injury and kidney dysfunction. Therefore, research into HIVAN glomerular pathology has largely focused on Nef-induced podocyte proliferation, and multiple promising pathways have been identified. Nef-mediated activation of MAPK1,2 and Stat3 pathways drives podocyte dedifferentiation and proliferation[51, 52], and podocyte-specific knockdown of the Stat3 pathway prevents HIVAN development in transgenic mice[53]. Further, Nef-induced podocyte proliferation is also attenuated through activation of cAMP-regulated pathways using retinoic acid receptor-\(\alpha\) agonists[54, 55], and inhibition of Notch signaling[56]. Several studies indicate HIV gene expression sensitizes podocytes to angiotensin II injury[57], and suggest blocking
the renin angiotensin system is protective to the kidney in the setting of HIV infection[58, 59].

1.1.3.4 Mechanisms of Vpr-induced Tubulointerstitial Injury

Despite being a major component of HIVAN pathology, Vpr-mediated tubule degeneration and interstitial injury is not as well characterized as podocyte and glomerular dysfunction. Tubulointerstitial histologic findings characteristic of HIVAN include tubular atrophy, interstitial fibrosis, microcyst formation, inflammation. Additionally, tubule epithelial cells exhibit hypertrophy with enlarged hyperchromatic nuclei, and focal apoptosis[24, 60]. Tubules in HIVAN biopsies also exhibit high levels of DNA damage marker γH2AX, indicating activation of the DNA damage response in these cells[61]. While not specific to Vpr, interstitial inflammation is likely attributed to persistent activation of pro-inflammatory nuclear factor kappa B (NF-κB) signaling pathways in HIV+ renal epithelial cells[62]. Many other aspects of HIV-mediated tubule pathology have been recapitulated by Vpr expression in vitro, including activation of the DNA damage response, apoptosis, hypertrophy and increased DNA content. A comprehensive analysis of the mechanisms associated with these phenotypes is provided later in this dissertation.
1.1.3.5 Genetic Factors Associated with HIVAN Pathogenesis

Early case studies made it clear that HIV patients of African descent are disproportionately at risk for developing HIVAN. In fact, individuals of African descent are more likely to develop renal disease than individuals of human European descent, regardless of HIV status. Genetic analysis reveals this increased susceptibility is associated with high frequencies of two APOL1 variants, G1 and G2, in these populations[63]. 35% of African-Americans carry one of the risk alleles (G1 or G2), while 12-14% of African-Americans are homozygous or compound heterozygous[10]. It is estimated that, without treatment, 50% HIV+ patients that harbor two APOL1 risk alleles will develop HIVAN[64]. However, HIVAN infrequently occurs in patients without risk alleles or with one risk allele, suggesting other factors contribute to disease pathogenesis. Little is known about the mechanisms by which variant APOL1 promotes renal dysfunction, but considerable research is currently under way to identify relevant pathways.

1.1.4 The Kidney as an HIV-1 Compartment and Possible Reservoir

HIV plasma viremia can be successfully suppressed for prolonged periods of time using antiretroviral therapy. However, due to the persistence of HIV-1 compartments and latent reservoirs in multiple tissues throughout the body, patients experience rapid rebound of HIV once cART is removed[65]. HIV-1 compartments are defined as infected cells or tissues that restrict HIV trafficking and gene flow, thereby
promoting viral evolution and divergence from HIV-1 isolates in the peripheral blood. Characterizing an HIV-1 compartment as a reservoir requires proof that the anatomical site harbors latent, replication competent HIV that can stably persist despite antiretroviral therapy. Further, in a reservoir, latent HIV is reactivated following removal of cART, thereby propagating viral rebound and infection of new cells[66]. Identifying HIV compartments and reservoirs, and understanding the lifecycle of HIV in these unique anatomical sites, is critical to designing effective strategies for reservoir eradication and cure. HIV-1 compartmentalization has been documented in various lymphoid tissues, breast milk and the central nervous system[65, 66]. The Klotman lab identified the kidney as a unique HIV-1 compartment in 2000. HIV-1 mRNA and DNA were detected in renal biopsies from seropositive kidney disease patients, and further, viral sequences within renal epithelial cells diverge from those in peripheral blood cells[37, 67]. Consistent with HIV-1 compartmentalization in the kidney, a recent phylogenetic analysis of urine-derived env sequences from four HIV+ patients showed that the majority of urine-derived sequences clustered independently of those sequences derived from peripheral blood mononuclear cells and plasma[68]. Taken together, the compartmentalization of HIV-1 in the kidney, and the persistence of HIV-1 DNA and RNA in the kidney despite antiretroviral therapy[37] strongly suggest the kidney is a unique long-term HIV reservoir. Therefore, it is imperative to identify the unique
mechanisms that govern renal HIV-1 replication, reservoir establishment, and latency in the kidney.

**1.2 Overview of HIV-1 Acesory Protein VPR**

HIV-1 is a lentivirus that encodes nine gene products, including structural proteins Gag, Pol, and Env, essential viral regulatory factors Tat and Rev, and accessory proteins Vpu, Vif, Nef, and Vpr. The vpr gene is a highly conserved lentiviral protein that exhibits little sequence variation across HIV-1 isolates[69-71]. De novo expression of the 96 amino acid (14 kDa) protein occurs late in the viral life cycle[72], and Vpr is incorporated into nascent virion particles via interaction with Gag p6 (reviewed in [73]). In addition to being found intracellularly and incorporated into the virion, extracellular Vpr has been detected in serum of HIV+ individuals, and in cerebrospinal fluid of AIDS patients with neurological disorders[74]. Detection of Vpr in the virion, circulating serum and infected cells suggests a diverse role for Vpr across different stages of the HIV-1 life cycle, in both an intracellular and extracellular manner.

**1.2.1 Proposed In Vivo Functions of Vpr**

Despite years of research, pinpointing the precise role of Vpr in HIV-1 disease pathogenesis in vivo has proven challenging. Early in vitro studies showing that mutation of the vpr gene results in impaired virus replication was the first indication that Vpr plays an important part in the HIV-1 life cycle[75, 76]. Subsequent SIV studies
in primate models were the first to identify Vpr function as critical for natural HIV infection and AIDS progression. HIV-1 vpr has evolved into two genes in HIV-2/SIV, vpr and vpx. Deletion of both vpr and vpx in a SIV model of HIV infection inhibits disease progression in vivo[77-79]. Further, several primate vaccine studies show that viruses containing mutated vpr often revert to wild-type vpr over time, indicating a considerable selective pressure to maintain functional Vpr in vivo[80-82].

Recently, a study demonstrated Vpr-mediated G2 cell cycle arrest and apoptosis in vivo using a human hematopoietic stem cell-transplanted humanized mouse model. Cell cycle perturbations and apoptosis primarily affected regulatory CD4+ T cells, resulting in CD4+ T cell depletion and enhanced virus production[83]. A 2004 study isolated lymphocytes from recent HIV-1 seroconverters and analyzed the cells for DNA content. Researchers determined a majority of the lymphocytes were arrested in the G2 phase of the cell cycle[84], and while these results are not Vpr-specific, they do support an in vivo role for HIV-mediated G2 arrest. Taken together, these studies suggest Vpr-induced G2 arrest and apoptosis play a role in HIV-1 disease pathogenesis in vivo.

1.2.2 Proposed In Vitro Functions of Vpr

A number of in vitro functions for Vpr have been identified, including facilitation of nuclear import of the HIV Pre-Integration Complex (PIC), transactivation of the HIV-1 long terminal repeat (LTR) and other promoters, immunosuppression, and induction of G2 arrest and apoptosis[73, 85].
After entry into the host cell, the HIV-1 capsid shell dissociates and releases viral RNA and proteins, including Vpr, into the cytoplasm. The HIV single strand RNA-genome is reverse transcribed into cDNA, which is then incorporated in the PIC in preparation for relocation to the nucleus. In addition to double-strand viral DNA, the PIC includes several viral proteins including the matrix protein MA, Integrase and Vpr. The PIC is targeted to the nucleus via an interaction between Vpr and importin-α, resulting in nuclear import through nuclear pore proteins. Once inside the nucleus HIV-1 Integrase enables integration of double-strand HIV-1 DNA into the host genome, which is a critical step in the HIV-1 life cycle[86-88]. The ability of Vpr to mediate nuclear localization and entry via importin-α is essential for productive HIV infection of non-dividing cells, including macrophages and primary CD4+ T-cells[75, 89-91].

Following virus integration, Vpr functions as a transactivator of the HIV-1 long terminal repeat (LTR) and other host promoters. Early observations that vpr expression drives reactivation of latently infected HIV+ cells suggested Vpr played an important role in virus replication[74, 92]. Vpr was later shown to increase transcriptional activity through the HIV-1 LTR and other promoters[93-98], and this HIV-1 LTR-driven gene expression was highest in the G2 phase of the cell cycle[94].

Vpr has also been shown to modulate several host immune responses in vitro. Following antigenic stimulation and activation, antigen presenting cells, macrophages, and dendritic cells (DCs) produce Interleukin-12 (IL-12). IL-12 plays a role in the
maturation of Th1 cells, and also stimulates the production of interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α). Vpr has been shown to inhibit IL-12 production in these cells, while also up-regulating production of the anti-inflammatory IL-10 cytokine in DCs[99]. Further, Vpr inhibits monocyte and DC maturation in vitro by down-regulating CD80, CD83, and CD86 expression in these cells[99, 100]. Vpr has also been implicated as a negative regulator of type1 IFN production, ISG induction, and NF-kB activation in HeLa and 293T cells[101]. Taken together these in vitro studies suggest Vpr may play a key role in HIV-1 mediated immune modulation in vivo.

Of all the in vitro phenotypes ascribed to vpr expression, two of the earliest and most striking observations were that Vpr arrests cells in the G2 phase of the cell cycle[102-105] and induces apoptosis in multiple cell types[106]. Given the capacity of Vpr to induce cell cycle arrest and apoptosis in vitro, many researchers speculated Vpr was driving the depletion of CD4+ lymphocytes characteristic of HIV-1 infection, and therefore the functional analysis of these mechanisms became the center of copious amounts of research.

1.3 Vpr and G2 Cell Cycle Arrest

One of the earliest noted phenotypes associated with in vitro expression of HIV-1 Vpr in human cells was arrest in the G2 phase of the cell cycle[102-105]. Subsequent studies determined that Vpr-mediated cell cycle arrest is a conserved property among primate lentiviruses, suggesting a critical role for Vpr-mediated G2 arrest in vivo[70, 107].
The G2 arrest activity of Vpr appears to be cell type specific and is not maintained across species. For example, Vpr isolates from SIVAGM and SIVSYK arrest African green monkey cells, but not human cells. Vpr isolates from HIV-1 and HIV-2 maintain functionality in both simian and human cells. Similarly, SIVSM Vpr arrests simian cells, and to a lesser degree, human cells[107]. This species specificity suggests that the precise mechanism by which Vpr exerts cell cycle arrest has evolved to include host factors unique to particular cell types and primate species. While Vpr-induced G2 arrest is a striking phenotype and conserved property among lentiviruses, the biological implications of cell cycle arrest during natural HIV-1 infection are poorly understood.

1.3.1 Overview of the DNA Damage Response and G2 Arrest

Cells employ multiple surveillance systems to ensure high fidelity DNA replication and proper cell division and growth. One such surveillance system, the DNA Damage Response (DDR), maintains genomic integrity through rigorous checkpoint controls. After sensing DNA damage, the DDR initiates a complex signaling cascade to activate checkpoints and facilitate repair before allowing the cell cycle to progress. Because the DDR is a key regulator of the G2/M checkpoint, much of the research surrounding Vpr-mediated G2 arrest has been in the context of DDR checkpoint activation. Therefore, understanding the basic principals and pathways involved in DDR check point arrest will offer key insights into Vpr-mediated manipulation of the cell cycle.
1.3.1.1 ATR and ATM: Key Mediators of the DNA Damage Response

The DNA Damage Response is a complex network of signaling pathways coordinated primarily by members of the phosphatidylinositol 3-kinase (PI3K)-like kinase (PIKK) family, ATM (ataxia-telangiectasia-mutated), and ATR (ataxia telangiectasia and Rad3-related protein). ATM primarily responds to double strand breaks (DSBs), whereas ATR responds to stalled replication forks and regions of single-strand DNA (ssDNA). While the initiating stimuli differ for ATM and ATR activation, the principles of the signaling pathway are similar, and in fact, exhibit significant overlap in substrates. Following the detection of DNA damage by sensor proteins, apical kinases ATM and ATR phosphorylate mediator proteins, which amplify the DDR by recruiting ATM/ATR substrates. From there, the signal is transduced through effector kinases and proteins to coordinate a complex cellular response that includes checkpoint maintenance, DNA repair, transcription and metabolic signaling (reviewed in [108-110]).

Canonically, G2 arrest in response to DSBs is mediated through ATM and its effector kinase, Chk2. Following induction of double strand breaks, changes in chromatin structure prompt inactive, dimerized ATM to autophosphorylate in trans and dissociate into catalytically active monomers[111]. Independent of ATM activation, the MRN complex, which consists of MRE11, RAD50, and NBS1 proteins, assembles and binds to DNA at the site of the DSB. Assembly of the MRN complex is critical for DSB signaling, as it initiates recruitment of DDR signaling molecules, including ATM, to the
break site[112]. Recruitment of activated ATM to the MRN complex further propagates the signal by amplifying phosphorylation of dimerized ATM molecules (reviewed in [108, 110, 113]).

Once activated, ATM initiates a phosphorylation cascade through an intricate network of substrates. One of the principal substrates in the ATM DDR is histone protein H2AX. γH2AX, the phosphorylated form of H2AX, helps amplify ATM signaling by recruiting several other DDR factors, including components of the MRN complex, to the site of the DSB. Given its role as a core mediator of DDR signaling, γH2AX is often used as a marker of DDR activation. Other ATM substrates include p53, which induces G1 arrest through p21 up regulation, and effector kinase Chk2. Once activated, Chk2 phosphorylates multiple substrates including p53, Cdc25 family phosphatases, BRCA1 and multiple transcription factors. Most often, ATM activation of Chk2 associates with a signaling cascade resulting in G2/M checkpoint activation. Thus, ATM can mediate multiple cellular responses including cell cycle progression, apoptosis, and gene transcription (reviewed in [108-110, 113]).

In contrast to ATM signaling, the ATR DDR response is activated in response to ssDNA generated at stalled replication forks and resected DSBs. Because of its critical role in resolving stalled replication forks, ATR maintains kinase activity even in the absence of direct DNA damage. Therefore, activation of the ATR DDR signaling cascade requires relocation of ATR to the site of DNA damage. To this end, ATR is recruited via
interaction with binding partner ATRIP, which directly interacts with replication protein A (RPA) complexed with ssDNA at stalled replication forks and DSBs. Recruitment of mediator proteins TopBP1 and Claspin to sites of ssDNA-RPA is also required for efficient ATR activation (Reviewed in [108-110]).

Following activation, ATR phosphorylates many substrates implicated in cell cycle control, including H2AX, Rad17, BRCA1, p53 and Chk1. Effector kinase Chk1 plays a central role in G2 cell cycle arrest, as Chk1-mediated phosphorylation inhibits the activity of phosphatases Cdc25A and Cdc25C. Cdc25c inhibition promotes Wee1 kinase activity, which in turn, inhibits Cdk1. Phosphorylation of Cdk1 inhibits the mitotic Cdk1/cyclin B complex, and arrests cells at the G2/M checkpoint (Reviewed in [108-110]. Thus, the ability of both the ATM and ATR DDR signaling pathways to arrest cells during G2 phase of the cell cycle drove copious amounts of research to determine a possible role for these kinases in Vpr-mediated G2 arrest.

1.3.2 Overview of Vpr-induced G2 Arrest

Early studies on Vpr-induced cell cycle arrest determined that Vpr inhibits mitotic progression via inhibition of the Cdk1 kinase, as indicated by phosphorylation at Tyr15[102, 104]. Hyper-phosphorylation of Cdk1 is also associated with G2 arrest induced by DNA damaging agents, leading to speculation that Vpr mediates cell cycle arrest via activation of the DDR[114]. In an effort to clarify the role of the DDR in Vpr-induced G2 arrest, one study expressed Vpr in cells from patients with Ataxia-
telangiectasia (A-T). A-T patients lack functional ATM, and therefore, cells from patients with A-T are incapable of arresting the cell cycle in response to DNA damage. Their observation that Vpr+ A-T cells maintain G₂ arrest capabilities indicated ATM was dispensable for this phenotype,[115] and shifted focus to elucidating the involvement of the other apical kinase of the DDR, ATR.

Subsequent studies identified ATR and several of its downstream substrates as being critical for Vpr-mediated cell cycle arrest. Vpr expression in HeLa and fibroblast cell lines results in the activation of ATR and its effector kinase, Chk1[116-118]. Further, inhibition of either ATR or Chk1 largely ameliorates Vpr-induced G₂ arrest and Cdk1 inhibition in lymphocytes and other cell lines[116, 118]. Consistent with a model of DDR activation through the ATR-Chk1-Cdk1 pathway, one study found that kinase Wee1, which negatively regulates Cdk1 activity via phosphorylation, is also required for Vpr-induced G₂ arrest[119]. Other components of the ATR DDR pathway, including BRCA1, are also activated following vpr expression[84, 120], however, Vpr-induced cell cycle arrest occurs independently of p53 status[115]. While activation of the DDR has been observed in multiple vpr-expressing cell systems, the initiating stimulus for DDR signaling was not readily identified.

1.3.2.1 The Relationship Between DNA Damage and Vpr-induced Cell Cycle Arrest

Vpr unambiguously activates components of the DNA Damage Response, however, whether or not Vpr causes direct DNA damage is less clear. Multiple studies
show Vpr robustly induces γH2AX foci formation in vitro[84, 120], and one study noted the accumulation of RPA in Vpr+ primary lymphocytes, indicating the presence of ssDNA. Induction of ATR-dependent G2 arrest involves direct binding of Vpr to chromatin, suggesting Vpr may directly cause DNA damage [117]. However, analysis of Vpr-induced DNA damage using pulse field gel electrophoresis (PGFE) proved inconclusive, as one study found evidence of DSBs[121], while another did not[117]. While the presence of RPA-coated ssDNA can be indicative of either stalled replication forks or DSB repair intermediates, the dispensability of ATM for Vpr-mediated G2 arrest suggests this phenotype is not induced by ATM-dependent DSB repair[115, 117, 122]. Thus, given the stringent requirement for components of the ATR DDR signaling pathway and the presence of RPA foci, a prevailing model for Vpr-mediated G2 arrest is activation of the ATR DNA Damage Response by replication stress.

Interestingly, several studies speculate that Vpr-induces G2 arrest using components of the DDR, but that this response differs from a canonical DNA damage response. Early work in Jurkat cells noted that irradiated cells remain arrested in G2, while Vpr+ Jurkat cells can bypass G2 arrest to increase in size and ploidy[115]. A later study found hydroxyurea and ultraviolet light (UV) induce Chk1 activation in a similar manner to Vpr. However, HU and UV-treated cells arrest in S phase, whereas vpr-expressing cells progress through S phase and arrest at the G2/M border[118]. Indeed, even the manner in which ATRi abrogates G2 arrest in Vpr+ cells suggests a mechanism
fundamentally different from the DDR. If cells are arrested during the cell cycle due to DNA damage, releasing them back into the cell cycle without adequate DNA repair results in genomic instability, and often death[123]. However, inhibition of the DDR, and specifically ATR, in Vpr+ cells results in abrogation of the $G_2$ arrest phenotype, but does not correlate with an increase in cell death. Rather surprisingly, ATR inhibition associates with cell survival in the context of $vpr$-expression[120]. Thus, the mechanisms by which Vpr modulates the cell cycle appear to be fundamentally different from the canonical DDR signaling pathways, suggesting Vpr has evolved mechanisms of cell cycle control that are unique to this virus-host relationship.

1.3.2.2 Vpr Interaction with the Ubiquitin Proteosome System Induces Cell Cycle Arrest

Over the years several cellular proteins have been reported to bind to Vpr. An early study identified a cellular binding partner of unknown function, originally called Vpr Binding Protein (VprBP)[124]. Subsequent studies identified VprBP as the member of a family of proteins that confers substrate specificity to Cullen 4-and DDB1-based E3 ubiquitin ligase systems[125-127]. Briefly, E3 ubiquitin ligases catalyze the attachment of ubiquitin protein chains to cellular proteins, thus targeting them for destruction by the proteosome. While cells use this ubiquitin proteasome system (UPS) to regulate processes such as DNA repair, DNA replication and transcription, multiple viruses are known to hijack the UPS to manipulate host responses and cellular environments
Multiple studies determined that proteasome-mediated protein degradation via the VPRBP-DDB1-CUL4 E3-ligase complex is critical to Vpr-mediated ATR activation and G2 arrest [129-134]. However, the identity of the cellular protein(s) targeted for degradation by the VPRBP-DDB1-CUL4 E3-ligase complex remained elusive for many years.

A recent study identified structure-specific endonucleases (SSEs) Mus81 and Eme1, and SSE regulator SLX4 (as part of the SLX4 complex, SLX4com) as novel Vpr interacting partners [101]. During normal cell cycle, activation of the SLX4com and associated SSEs occurs during the G2/M transition. Activation of the SLX4com promotes the resolution of DNA replication intermediates found in collapsed replication forks, such as Holliday Junctions (HJs), thereby enabling the transition into mitosis. Vpr is shown to concurrently interact with the SLX4 scaffold protein, the VPRBP-DDB1-CUL4 E3-ligase complex, and mitotic regulator Plk1. This association promotes Mus81 ubiquitination, and phosphorylation of Eme1 by p-PLK1, resulting in early activation of SLX4com during S phase. Aberrant activation of the SLX4com hampers progression of the replication fork, resulting in replication stress and G2 arrest. In support of the model, inhibition of VPRBP/DCAF-1 or any SLX4com components abrogates Vpr-mediated G2 arrest [101]. Thus, Vpr modulates cell cycle progression through direct interaction with the VPRBP-DDB1-CUL4 E3-ligase complex and subsequent activation of an ATR-dependent signaling cascade.
1.3.3 Biological Significance of Vpr-induced G₂ Arrest

The biological significance of Vpr-mediated G₂ cell cycle arrest in the context of HIV-1 infection in vivo is not fully appreciated. HIV-1 LTR activation and transcription is most active in the G₂ phase of the cell cycle, suggesting G₂ arrest may confer a more favorable environment for HIV replication[81, 135]. In support of this model, inhibition of Vpr-induced G₂ arrest using siRNA against ATR also abrogates transactivation of the LTR [116]. Other groups speculate that G₂ arrest is a secondary, unintended consequence of Vpr function. Per this model, Vpr-mediated activation of the SLX4com and associated endonucleases functions to suppress a host immune response. Premature initiation of SLX4com exonuclease activity is proposed to prevent accumulation of viral DNA, thereby preventing the activation of type 1 IFN response. In support of this model, previous studies report Vpr tempers IFN production during in vitro infection with HIV-1 [136, 137]. However, given that Vpr-mediated G₂ arrest is highly conserved and cell-specific[107], it is possible that cell cycle manipulation represents the converging of multiple redundant or synergistic mechanisms to modulate virus propagation in a host environment.

1.3.4 Mechanisms of Vpr-induced G₂ Arrest in the Kidney

Vpr-mediated arrest in the G₂ phase of the cell cycle is well established in both immortalized and primary renal tubule epithelial cells[60, 138, 139]. Previous work using pseudotyped lentiviral particles expressing single-gene constructs, pHR-Vpr,
pHR-Tat, or pHR-Vif, confirm that the G2 arrest phenotype is Vpr-specific in RTEC model systems[60]. Further, activation of the DNA Damage Response, as indicated by accumulation of the DNA damage marker γH2AX, occurs in both cultured Vpr+ RTECs and in tubule cells in murine and human HIVAN renal biopsies (Figure 2)[61].
Figure 2: Activation of the DNA Damage Response in Murine and Human HIVAN. Kidneys from littermate controls or Tg26 mice with HIVAN (upper panel) and normal human kidney and a biopsy from a HIVAN patient were stained with anti-γH2AX antibody. Fields are 200X and inserts are 400X.
1.4 VPR and Apoptosis

The mechanisms of G2 arrest and apoptosis in lymphocytes have been studied in detail as a possible mechanism of CD4+ lymphocyte depletion characteristic of HIV-1 infection. Vpr-induced apoptosis has also been shown to be a major driver of glomerular and renal disease pathogenesis in the context of HIV infection of the kidney. Therefore, understanding the basic principals and pathways involved in Vpr-induced apoptosis offer key insights into this critical component of HIV-1 disease progression.

1.4.1 Overview of Apoptosis

Multicellular organisms use apoptosis as a homeostatic mechanism to modulate cell populations in order to maintain tissue architecture and function. Apoptosis is triggered by varieties of stimuli, both physiological and pathological, which transduce a pro-apoptotic signal through two distinct pathways: intrinsic or extrinsic.

Initiation of the extrinsic apoptotic signaling pathway occurs following stimulation of receptors from the tumor necrosis factor (TNF) receptor superfamily, including TNF receptor and Fas. Stimulation of cell surface death receptors recruits caspase-8 and death domain-containing adaptor proteins, such as FADD, to intracellular death receptor domains. Caspase-8 is cleaved and activated, at which point it propagates the apoptosis signal by cleavage of downstream effector caspases, such as caspases-3 and -7. Alternatively, cleaved caspase-8 can activate Bid, a pro-apoptotic member of the Bcl-2 family. Active Bid, or tBid, translocates to the mitochondria to promote Bax
oligomerization, which in turn promotes cytochrome c release. Cytochrome c triggers caspase-3 activation through formation of the cytochrome c/Apaf-1/caspase-9-containing apoptosome complex (reviewed in [140-142]).

Intrinsic apoptotic signaling originates from intracellular stimuli, including DNA damage, and promotes caspase activation through mitochondrial membrane permeabilization. Once intrinsic signaling is activated by intracellular stress, proapoptotic members of the Bcl-2 family, including Bad, Bim and Puma, promote Bax oligomerization and release of cytochrome c and other pro-apoptotic proteins from the mitochondria. As previously discussed, cytochrome c binds Apaf-1, promoting the activation of caspase-9, and ultimately, caspase-3. Thus, the defining differences between the extrinsic and intrinsic pathways are the origins of the proapoptotic signal, and the participation of caspase-8, which is exclusive to the extrinsic pathway (reviewed in [140-142]).

1.4.2 Overview of Vpr-induced Apoptosis

While vpr expression associates with cell death in numerous contexts, the mechanisms of Vpr-induced apoptosis are complex and cell-type specific. In one study, expressing Vpr in various human tumor cell lines resulted in cytochrome c release and caspase-9 activation. Importantly, the authors did not observe activation of caspase-8 or expression of Fas or its ligand, indicating activation of the intrinsic apoptotic signaling pathway[143]. Subsequent studies confirmed caspase-9 dependent apoptosis in HeLa
cells, rodent cells, neuronal cells and peripheral blood mononuclear cells[144-146]. Another group showed virion-associated Vpr concurrently activates caspase-9 and caspase-8 in Jurkat cells, but that that the caspase-8 activation is independent of Fas stimulation[147]. This study also reports that virion-associated Vpr enhances Fas-induced apoptosis, possibly through caspase-8 dependent amplification and mitochondrial injury[147].

An alternate model of Vpr-induced cell death suggests Vpr can induce apoptosis via direct interaction with the adenine nucleotide translocator (ANT), a component of the permeability transition pore complex at the inner mitochondrial membrane. Together with ANT, Vpr triggers mitochondrial membrane permeabilization (MMP), and release of pro-apoptotic proteins into the cytoplasm. Vpr-induced MMP is suppressed by over-expression of bcl-2 or by inhibition of Vpr/ANT binding [148-150]. However, a subsequent study showed siRNA-mediated knockdown of ANT had little effect on Vpr-induced apoptosis in HeLa cells. Instead, inhibition of Bax, another mitochondrial pore protein associated with intrinsic apoptotic signaling, abrogated cell death in HeLa and primary CD4+ T cells. Taken together, these studies indicate Vpr evokes apoptosis via multiple pathways, and suggest possible redundancies and synergistic pathways in Vpr-induced cell death.
1.4.2.1 The Relationship Between Vpr-induced Cell Cycle Arrest and Apoptosis

The earliest indication of a functional link between Vpr-mediated cell cycle arrest and apoptosis was the temporal relationship between the two phenotypes: cells arrest in G\(_2\) before progressing to apoptosis\[114, 151\]. Later studies supporting these observations show that inhibition or knockdown of proteins that regulate G\(_2\) arrest in Vpr\(^+\) cells, including ATR and Wee1, also abrogates apoptosis\[119, 120, 152\]. ATR inhibition in Vpr\(^+\) cells also negatively regulates the activity of the growth arrest and DNA damage protein 45\(\alpha\) (GADD45\(\alpha\)), an essential mediator of the Vpr-induced cell death\[120\]. Vpr also initiates ATR-dependent phosphorylation of BRCA1, a p53-independent regulator of GADD45\(\alpha\)\[152\]. BRCA1 foci have been identified in Vpr\(^+\) cells\[84\], suggesting a mechanism for Vpr-induced apoptosis, independent of p53 status. Taken together, these studies suggest a model in which Vpr-mediated G\(_2\) arrest and apoptosis are temporally and mechanistically linked through shared signaling pathways.

Other studies suggest a model by which Vpr-induced G\(_2\) arrest and apoptosis occur independently of one another. Constructs with specific C-terminal mutations fail to induce apoptosis, but retain cell cycle arrest capabilities \[153, 154\]. In contrast, certain Vpr-GFP fusion proteins were reported to induce apoptosis, but not cell cycle arrest\[154\]. However, subsequent work in immortalized T-cells contradicts these findings\[152\]. One groups suggests the coupling of Vpr-mediated G\(_2\) arrest and apoptosis is species specific. In their system, Vpr\(^+\) HeLa cells underwent both cell cycle
arrest and apoptosis, while rodent cells underwent apoptosis independent of G2 arrest[145]. Taken together with the observation that Vpr directly induces apoptosis through direct binding with mitochondrial pore proteins[148-150], these studies suggest the mechanisms governing Vpr-mediated cell cycle arrest and cell death can be decoupled in specific cell types.

1.4.2.2 The Relationship Between Vpr-induced Aberrant Mitosis and Apoptosis

Several in vitro model systems in mammalian and yeast cells have reported severe mitotic defects in vpr-expressing cells. Following Vpr expression, fission yeast and human cell lines exhibit supernumerary centrosomes, multipolar spindle formation, misaligned chromosomes, defects in cytokinesis and multi-nucleation[155-157]. Mitotic progression in Vpr+ cells occurs after an extended G2 phase, indicating Vpr-induced cell cycle arrest may be associated with subsequent mitotic defects[156, 158]. Further, progression through mitosis in the presence of multiple Vpr-induced defects can result in cell death[156]. Centrosomal amplification and spindle multipolarity often result in apoptosis[123, 159, 160], and therefore, the contribution of Vpr-induced mitotic defects to HIV-1 related cell death warrant further investigation in other biologically relevant cell systems.
1.4.3 Mechanisms of Vpr-induced Apoptosis in the Kidney

Apoptotic death of renal epithelium is a long-established hallmark of HIVAN pathogenesis[24]. In vitro expression of HIV-1 in renal epithelial cells drives apoptosis through NF-κB-mediated Fas upregulation and caspase activation[161, 162]. These in vitro findings are supported by subsequent demonstrations of constitutively active NF-κB activity and upregulation of the Fas receptor and ligand proteins in renal epithelial cells in biopsies from HIVAN patients[62, 162]. Mitogen Activated Protein Kinase (MAPK) signaling pathways have also been shown to be important mediators of HIV-associated pathogenesis in the kidney[52].

There were several early indications that Vpr plays a central role in HIV-1 induced apoptosis in renal epithelium. Expression of Vpr in transgenic mice is sufficient to drive apoptosis and disease development [47]. Further, Vpr is a potent inducer of cell death in multiple in vitro model systems, including in renal tubule epithelial cells[60, 106]. Characterization of apoptotic pathways associated with cell death in Vpr+ RTECs indicates a role for components of both the extrinsic and intrinsic pathways. Expression of Vpr activates caspases-8 and 9 in cultured RTECs, and inhibition of either caspase is equally protective against apoptosis[139]. Inhibition of either Bax or BID prevents Vpr-mediated apoptosis in RTECs, however, co-suppression of both Bax and BID do not synergistically increase cell survival. Further blocking Fas, TNF-α and TRAIL does not
ameliorate apoptosis in Vpr+ RTECs, suggesting the observed caspase-8 activation is not the result of extrinsic death receptor signaling[139, 161].

Given the known role of MAPK/ERK signaling in Nef-mediated podocyte death, the role of this signaling pathway was also examined in Vpr+ RTECs. While ERK is most often associated with cellular proliferation, ERK signaling can also regulate cell cycle arrest and apoptosis, particularly following cell stress or damage (reviewed in [163]). As with Nef-mediated cell death, ERK activation is required for caspase-8 activation and cell death in VPR+ RTECs. In support of this, inhibition of ERK kinases MEK1 and MEK2 reduces caspase-8 activation and cleavage of BID. Phospho-ERK was also detected in renal tubule cells in HIVAN biopsies, confirming ERK activation in the setting of HIV-1 infection in vivo. The mechanism of Vpr-mediated ERK activation in RTECs is currently unknown. Fat10, a ubiquitin like protein, has also been implicated in HIV/Vpr mediated cell death via activation of NFκB signaling pathways[138, 164, 165]. Thus, several pathways mediating death in Vpr+ renal tubule cells have been identified, however, the initiating stimulus for apoptosis has yet to be identified. Further, as in other cell systems, Vpr evokes apoptosis via multiple pathways in RTECs, suggesting possible overlap of or synergy between these cell death mechanisms.
1.5 VPR and Polyploidy

Expression of vpr in RTECs induces diverse phenotypic changes, including activation of the DNA damage response, G2 arrest, apoptosis, cellular hypertrophy and polyploidy[61]. Polyploid cells, which contain multiples of the typical diploid genome number, have also been reported in other vpr-expression model systems in vitro[114, 115, 118, 157, 166, 167]. Thus, while Vpr-associated polyploidy is not a novel observation, the physiological implications of increased ploidy for the viral life cycle and disease pathogenesis are underappreciated and poorly understood. Further, in vivo demonstrations of polyploidy in renal tissue from the TG26 mouse line and in biopsies from individuals with HIVAN confirm this phenomenon is a physiologically relevant, novel aspect of HIV pathology[60].

1.5.1 Overview of Polyploidy

Polyploid cells, which contain multiple copies of a typical diploid genome, are prevalent in many different organisms throughout nature. To understand the significance of polyploidy in normal physiology and disease, it is first important to understand the mechanism generating polyploidy. Polyploidy can be generated by endoreplication, non-canonical cell cycles characterized by multiple rounds of genome replication without complete cell division, or by cell-cell fusion. Endoreplication cell cycles can be further characterized as either endocycling, in which cells undergo successive rounds of DNA replication without an intervening mitotic phase, or as
endomitosis, in which genome replication is accompanied by incomplete mitosis (Figure 3). The serine/threonine cyclin-dependent kinases (CDKs) that regulate canonical G₁-S-G₂-M cell division also govern DNA replication and cell cycle during endoreplication. As in the canonical cell cycle, CDK activity oscillates between low levels in G₁ and high levels in S-G₂-M to ensure complete replication of genomic DNA before mitosis occurs. This overlap between the regulators of canonical cell cycle and endoreplication provides a unique opportunity for targeted investigation of the previously characterized protein function (Reviewed in[168-171]).
Figure 3: Schematic of Polyploidy Generated Through Endoreplication. Polyploidy can be generated by endoreplication, non-canonical cell cycles characterized by multiple rounds of genome replication without complete cell division. Endoreplication cell cycles are characterized as either endocycling, in which cells undergo successive rounds of DNA replication without an intervening mitotic phase, or as endomitosis, in which genome replication is accompanied by incomplete mitosis. Modified from Fox and Duronio 2013[168].

Within the human body, polyploidy can occur in the context of normal physiology, aging and disease. Platelet-producing polyploid megakaryocytes exist in healthy bone marrow, while polyploid giant trophoblasts are a critical component of
placenta. Polyploid cells accumulate with age in hepatocytes and vascular smooth muscle cells in vivo, while fibroblasts and endothelial cells become tetraploid in in vitro models of aging. Polyploidy and its associated genomic instability is also associated with many diseases, most notably, cancer. In fact, a recent analysis of over 4,000 cancer genomes indicated 37% of the samples exhibited whole-genome doubling[172](Reviewed in [168-171]).

Endoreplication and polyploidy have also been described as a response to physiologic stress in multiple mammalian tissues. Endoreplication is often induced after cellular stresses, as polyploidy can promote transcriptional/metabolic changes that confer stress tolerance. Additionally, polyploidy is a mechanism of compensatory cell growth and tissue repair. Following telomere dysfunction, which normally results in apoptosis or senescence, murine hepatocytes undergo endoreplication as a compensatory growth mechanism to maintain and regenerate liver function. Similarly, cardio-myocytes undergo endoreplication following acute myocardial infarction. p53 mutated tumor cells are capable of switching to an endoreplication cell cycle following genotoxic drug induced mitotic catastrophe, thereby enabling cell survival and drug resistance (Reviewed in [168-171]).

Further, genomic instability generated through polyploidy generates a mechanism to select for fitness at a single cell level. Acquiring specific gene amplifications or gene deletions enables cells to modulate metabolic and transcriptional
outputs that may make that cell more fit in particular environment. Among polyploid liver cells, a sub-population of cells harboring a chromosome-specific aneuploidy was recently shown to confer a survival advantage in a liver disease model[171, 173]. According to this model polyploidy may promote long-term HIV survival/propagation through 1) increasing virus transcription, or 2) altering the host cell karyotype/genome to select for cell survival viral maintenance in the kidney. Further, polyploid RTECs may function differently than diploid RTECs, thereby contributing to HIVAN pathogenesis and kidney dysfunction. Thus, understanding the mechanisms and physiologic consequences of Vpr-induced polyploidy in the kidney is essential to elucidating the role in possible HIV reservoir establishment and disease pathogenesis.

1.5.2 Polyploidy in the Setting of HIV-1 Infection and Vpr Expression

The presence of polyploidy in the context of HIV-1 infection has been described in various reports over the past two decades. In one early study, analysis of DNA content via flow cytometry was performed on Feulgen-stained lymph node smears from nine AIDS patients. Lymphocyte tetraploidy was identified in three cases, while octoploidy was observed in two cases [174]. One of the most striking examples of HIV-associated ploidy increase is the occurrence of multinucleated giant cells (MNGCs) in patients with HIV-encephalitis and myelopathy. Resulting from cell-to-cell fusion between macrophages and microglia, MNGCs, or syncytia, actively produce virus before undergoing apoptosis[175]. Similar MNGCs are also present in tonsil and adenoid
lymphoid tissues of HIV patients, and have been documented in the colonic mucosa, nodal lymphoma associated with HIV, and cysts of the parotid gland [176-178]. Further, HIV-1 Env induces T-cell syncytia, which associates with CD4+ T-cell depletion [179]. Thus, in some instances, HIV-1-associated polyploidy occurs in the context of cell-cell fusion.

Vpr-induced ploidy increase was first noted in an in vitro T-cell model system. Both wild-type and p53-deficient T cells lines progressed past the G2 checkpoint to accumulate 8N DNA content within a single nucleus, while irradiated cells arrested at the G2/M border[115]. Multiple in vitro studies in yeast, fibrosarcoma cell lines, and HeLa cells also note polyploid 8N Vpr+ cells in culture[118, 156, 157, 166, 167]. The observation that many individual polyploid Vpr+ cells are hypertrophied with a single, enlarged nucleus indicates endoreplication, rather than cell fusion, produces polyploid cells in the context of Vpr-expression[115, 118]. Of note, multinucleated polyploid Vpr+ cells are also present in cell culture, suggesting endoreplication accompanied by an abortive mitotic phase[166].

1.5.3 Polyploidy in the Setting of HIV-1 Infection and Vpr Expression in RTECs

HIV-1 associated polyploidy in the kidney was first appreciated in an in vitro setting. Proximal tubule epithelial cells were transduced using a pseudotyped lentivirus vector expressing HIV-1 vpr and control genes[60]. Following vpr expression, both
mononuclear cells with large nuclei and hypertrophied multinucleated cells were noted. Analysis via flow cytometry confirmed the presence of cells with 8N DNA content in Vpr+ samples, but not in controls. These *in vitro* findings were first confirmed *in vivo* in Tg26 mouse renal biopsies. Close examination of murine HIVAN biopsies revealed multiple hypertrophied, multinucleated tubule cells ([Figure 4][60]).

![Figure 4: Hypertrophy and Multinucleation in a Murine Model of HIVAN.](image)

Examples of control murine tubules (top left) compared to HIVAN murine tubules. PAS stain at x600 original magnification. HIVAN tubules are dilated, and exhibit cellular hypertrophy and multinucleation (arrows)[60].
As in murine HIVAN samples, human HIVAN biopsies revealed individual tubule cell hypertrophy and enlarged nuclei. Multinucleation could not be conclusively identified in histologic human HIVAN samples, however, fluorescence in situ hybridization (FISH) to the centromeric regions of chromosomes 8, X and Y indicated an increased chromosome number in tubule cells of HIVAN patients (Figure 5)[60]. This in vivo demonstration polyploidy in HIV+ renal cells was the first to associate in vitro demonstrations of Vpr-induced polyploidy with a known disease phenotype. Thus, understanding the mechanisms and implications of Vpr-mediated polyploidy may offer crucial insight into the role of polyploidy in HIVAN pathogenesis and more broadly, the role of polyploidy in the HIV-1 life cycle in the kidney.
Figure 5: Increased Chromosome Number in Tubule Cells in Human HIVAN biopsies.
Renal FISH staining on a female human control (upper panel) and male human HIVAN biopsy (lower panel). L, tubular lumen; arrows, cell of normal ploidy (control) or increased ploidy (HIVAN). Centromeric enumeration probes (CEP) are Aqua, chromosome 8; red, chromosome X; and green, chromosome Y. The original magnification of control and HIVAN sections was x1000 [60].

1.6 Research Aims Outlined in this Dissertation

HIV-1 associated kidney disease is an emerging epidemic in the era of cART, and represents an increasing clinical burden in the United States and worldwide. Direct HIV-1 infection and expression of viral proteins in the kidney drives disease pathogenesis, and a growing body of research suggests the kidney is another HIV reservoir site. Early
transgenic mouse modeling indicated that HIV-1 vpr associates with HIVAN pathogenesis in RTECs and podocytes[10, 11]. Subsequent in vitro studies showed that expression of vpr in RTECs induces diverse changes in the infected cell population, including hypertrophy, DNA damage response activation, G2 arrest, apoptosis and polyploidy[12-15]. While G2 arrest and apoptosis are hallmarks of Vpr expression in multiple cell types, polyploidy and its contribution to the HIV-1 life cycle are not well characterized. In vivo demonstrations of polyploid renal tubule epithelial cells in both murine and human HIVAN biopsies confirm this phenomenon is a physiologically relevant, novel aspect of HIV pathology. Therefore, this dissertation aims to identify the mechanism and consequence(s) of polyploidy following Vpr expression, in order to better understand the role of polyploidy in HIVAN pathogenesis and possible reservoir establishment.

1.6.1 Characterize HIV-1 Vpr-mediated G2 Arrest in Renal Tubule Epithelial Cells

A long established characteristic of in vitro expression of HIV-1 Vpr in human cells is cell cycle arrest during the G2 phase of the cell cycle via the ATR-Chk1 DNA Damage Response pathway [84, 106, 116-119, 122]. However, given the ability of RTECs to progress past the G2 phase of the cell cycle to become polyploid, it is unclear if the Vpr-mediated cell cycle arrest signals through the ATR-Chk1 pathway in RTECs.
Understanding the mechanism of G2 escape will offer key insights into polyploidy generation in the context of HIV infection.

1.6.2 Identify the Mechanism of HIV-1 Vpr-induced Polyploidy in Renal Tubule Epithelial Cells

To appreciate the significance of polyploidy HIV+ kidneys, it is first important to understand the mechanism generating polyploidy in renal tubule epithelial cells. Therefore, I will distinguish between 3 possible models of polyploidization: endocycles (repeated S-phases but no M phase), endomitosis (truncated M-phase), and cell-cell fusion. By uncovering the mechanism of Vpr-induced of ploidy increase, I may uncover key insights into the physiological implications of polyploidy in an HIV+ kidney.

1.6.3 Characterize the Physiological Implication of Mitotic Entry and Polyploidy Following Vpr Expression in Renal Tubule Epithelial Cells

Vpr expression promotes multiple mitotic defects, including supernumerary centrosomes, multipolar spindle formation, misaligned chromosomes, defects in cytokinesis and multi-nucleation[155-157]. Given that centrosomal amplification and spindle multipolarity often result in apoptosis[123, 159, 160], it is possible that renal cells may undergo mitotic cell death following vpr expression. Further, polyploidy associates with cell survival in multiple contexts, including in the context of mitotic stress, suggesting a mechanism for the induction of endoreplication in Vpr+ cells. Therefore, understanding the contribution of Vpr-induced mitotic defects to cell death and
polyploidy may offer key insights into the physiological role of polyploidy during HIV infection.

1.6.4 Assess the Role of ATM Kinase in the Emergence of Polyploidy in Vpr+ RTECs

ATM has been suggested to play a role in various aspects of the HIV-1 life cycle[180-182]. The varied functions attributed to ATM suggest this kinase may have multiple, cell-type specific roles in the HIV-1 life cycle, and therefore, examining the possible role of ATM in Vpr–mediated pathology in RTECs may uncover novel pathways for disease pathogenesis in this unique cell system.
2. Materials and Methods

2.1 Cell Culture

HK2 and the HEK 293T Lenti-X cell lines were obtained from American Type Culture Collection (ATCC). HK2 cells were cultured in serum-free keratinocyte media with growth supplement (ThermoFisher). HEK 293T Lenti-X cells were cultured in DMEM with 10% FBS, penicillin/streptomycin and L-glutamine.

2.2 Generation of pseudotyped virus, transfection and infection

Viral particles pseudotyped with the vesicular stomatitis virus glycoprotein (VSV-G) were produced by co-transfection (Polyplus jetPRIME Transfection Kit) of 293T Lenti-X cells with three plasmids: the VSV envelope plasmid pMD2.G, the packaging plasmid psPAX2, and the desired expression plasmid (pTY2-VPR-GFP, pHР-VPR-GFP, pHР-Q65RVPR-GFP, pHР-HA-Vpr-GFP, pHР-HA-Vpr-ΔGFP). Supernatant was collected and filtered using a 0.45 mm filter at 48 and 72 hours post-transfection. Virus was titrated in 293T cells via flow cytometry. An MOI of 1 was used to infect HK2 cells.

2.3 Cell Cycle Analysis

Cell cycle analysis was performed by flow cytometry (FACSCalibur, BD Biosciences, San Jose, CA, USA). Cells were fixed in 1% PFA, then permeabilized with 70% ethanol. The fixed cells were treated with 100 mg/ml of propidium iodide and 0.5mg/ml of RNAse A prior to cytometry. FloJo was used for all analysis.
2.4 Drug Treatments

2.4.1 ATR Inhibition

Small molecule inhibitor VE821 (Axon 1893) was dissolved in DMSO to a stock solution of 10mM. Cells were treated with a working concentration of 10μM VE821 in serum-free keratinocyte media with growth supplement (ThermoFisher) for indicated times.

2.4.2. Thymidine G₁ Cell Cycle Synchronization

Thymidine (Sigma T9250) was dissolved in H₂O to a stock solution of 100mM. Cells were treated with a working concentration of 2mM Thymidine in serum-free keratinocyte media with growth supplement (ThermoFisher) for 18-24 hours.

2.4.3. ATM Inhibition

Small molecule inhibitor KU55933 (Tocris 3544) was dissolved in DMSO to a stock solution of 10mM. Cells were treated with a working concentration of 5μM KU55933 in serum-free keratinocyte media with growth supplement (ThermoFisher) for indicated times.

2.5 Western Blot Analysis

2.5.1 Whole-cell Extract Preparation

Cells were lysed in NP40 lysis buffer (Invitrogen FNN0021) containing 10mM PMSF (Thermo-Fisher 36978), protease inhibitors (cOmplete Mini Protease Inhibitor Cocktail Tablets, Roche 11836153001) and phosphatase inhibitors (Pierce Phosphatase
Inhibitor Mini Tablets, Thermo Fisher 88667) for 30 minutes on ice. Cell lysates were run through a QIAshredder column (Qiagen 79654) at 14000 RPM for 2 min.

2.5.2 Standard Western Blot

Whole-cell extracts were run on a 4–20% Tris-glycine gel (Thermo Fisher EC6025) under denaturing conditions and transferred to PVDF membranes (Bio-Rad 1620177). Primary antibodies were to beta actin (Cell Signaling 3700), HA-Tag (Cell Signaling 3724), Chk1 (Cell Signaling 2360), phospho-Chk1 (Cell Signaling 2348). Secondary antibodies were horseradish peroxidase–conjugated goat anti-rabbit (Cell Signaling 7074), or anti-mouse (Cell Signaling 7076), and detection was by chemiluminescence (Thermo Fisher 34080). Antibody dilutions were prepared to manufacturer’s specifications.

2.5.3 Western Blot for Large Proteins

Whole cell extracts were run on a 3–8% Tris-Acetate gel (Thermo-Fisher EA0375) under denaturing conditions and transferred to PVDF membranes (Bio-Rad 1620177) overnight at 4C. Primary antibodies were against ATR (Cell Signaling 2790), phospho-ATR (Cell Signaling 2853), ATM (Cell Signaling 2873) and phospho-ATM (Epitomics 21-512). Secondary antibodies and detection were as described above. Antibody dilutions were prepared to manufacturer’s specifications.
2.5.4 Western Blot Densitometry Analysis

Densitometry of Western Blot bands were analyzed using ImageJ software. Phospho protein densitometry levels were adjusted for loading control using total non-phospho protein densitometry levels. Loading control adjusted densitometric values for Vpr samples were normalized to densitometric value of corresponding HK2 time point.

2.6 Immunofluorescence Flow Cytometry

Staining and cell cycle analysis was performed by flow cytometry (FACSCalibur, BD Biosciences, San Jose, CA, USA). Cells were fixed in 4% PFA then permeabilized with 90% methanol. The fixed and permeabilized cells were stained with primary antibodies against Phospho-Histone H3 (Cell Signaling 3377) or ATM (Cell Signaling 13050). The secondary antibody was fluorescent 488-conjugated anti-rabbit (Thermo Fisher A-11034). Cells were treated with 100 mg/ml of propidium iodide and 0.5mg/ml of RNase A prior to cytometry. FloJo was used for all analysis.

2.7 Immunofluorescence Microscopy

Cells were fixed in 4% PFA and permeabilized in ice-cold 100% methanol. Primary antibodies were to γ-tubulin (Sigma T3559) and α-tubulin (Cell Signaling 3873). The Secondary antibody was fluorescent 488-conjugated anti-mouse (Cell Signaling 4414) or fluorescent 647-conjugated anti-rabbit (Cell Signaling 4408). Cells were stained with DAPI (Thermo Fisher 62248), and coverslips were placed on slide using antifade
mountant (Thermo Fisher P36930). Representative fields were captured using a Leica SP5 inverted confocal microscope.

2.8 Renal Biopsy Specimens and Immunohistochemistry

HIV transgenic (Tg26 murine line) FVB/N mice with 3-plus proteinuria were killed and examined. Comparisons from 3 different HIVAN biopsies were made to wild-type FVB/N mice. Human HIVAN biopsies were compared with normal control biopsies. Heat mediated antigen retrieval was performed using Tris/EDTA buffer (Abcam 97051) or Citrate buffer (Abcam 93678), per antibody specifications. Primary antibodies were against Phospho-histone H3 (Cell Signaling 9701) or phospho-ATM (Epitomics 21-512). HRP-conjugated goat anti-rabbit IgG (H+L) was used as the secondary antibody (Cell Signaling 8114). Slides were developed using an AEC peroxidase substrate kit (Vector SK-4205. Representative areas were chosen for publication.

2.9 FUCCI Probes and Live Cell Imaging

Cells were transduced with FUCCI Cell cycle sensors per manufacturer’s instructions at a concentration of fifty particles per cell (Thermo Fisher P36238). Cells were imaged using the Olympus VivaView FL Incubator Microscope at 10-minute intervals. Total cell counts and corresponding survival rates were determined for three separate trials.
3. Characterize HIV-1 Vpr-mediated G₂ Arrest in Renal Tubule Epithelial Cells

3.1 Introduction

A long established characteristic of in vitro expression of HIV-1 Vpr in human cells is cell cycle arrest during the G₂ phase of the cell cycle[102-105]. The capacity for G₂ cell cycle arrest is a highly conserved property of Vpr isolates from human HIV and in other primate lentiviruses, suggesting a critical role for Vpr-mediated G₂ arrest in the HIV-1 lifecycle[70, 107]. Therefore, the observation that some Vpr+ cells, including renal tubule epithelial cells, are able to circumvent the G₂/M checkpoint to become polyploid is unexpected. Two possible mechanisms to explain the divergent phenotypes are (1) Vpr-mediated G₂ arrest pathways are inherently different among different cell types, or (2) certain cell types have developed mechanisms to overcome Vpr’s inhibitory effects on the cell cycle.

Because canonical HIV-1 disease progression primarily affects CD4+ T cells, much of research into the mechanisms of Vpr-induced cell cycle arrest is conducted in lymphocyte cell lines. Other commonly available, but not necessarily physiologically relevant, cell lines, including fibroblasts and HeLa cells, are often used as well. Using these cell systems, researchers have identified the ATR–Chk1 DNA Damage Response pathway as a critical mediator of G₂ arrest in Vpr+ cells[84, 106, 116-119, 122]. Activation of the DDR, as indicated by DNA damage marker γH2AX, has been noted in cultured
Vpr+ RTECs and tubule cells of murine and human HIVAN biopsies. However, given the ability of RTECs to progress past the G₂ phase of the cell cycle to become polyploid, it is unclear if the Vpr-mediated cell cycle arrest results from signaling through the ATR-Chk1 pathway in RTECs. Here I confirm that the ATR-Chk1 signaling pathway mediates Vpr-induced G₂ arrest, suggesting other host factors contribute to G₂ escape and polyploidy progression.

3.2 Results

3.2.1 Vpr Expression Induces an ATR dependent G₂ Arrest in Vpr+ RTECs

To determine whether ATR’s role in G₂ arrest is conserved in Vpr+ renal tubule epithelial cells I used the Klotman lab’s well established model system of highly efficient transduction with VSV.G pseudotyped lentiviral vectors expressing vpr (HA-tagged or with a GFP reporter) in the HK2 human tubule epithelial cell line (see Methods). To assay for G₂ arrest, I performed FACS analysis of DNA content. Indeed, by 24 hours after transduction, I detected a strong G₂ arrest, as indicated by a roughly 3-fold increase of cells in G₂ (63.4% of vpr+ cells in the G₂ phase of the cell cycle, compared to 20.4% of control cells, VPR vs. HK2 24H, Figure 6). This G₂ arrest is dependent on ATR, as inhibiting ATR with the small molecule VE821, a potent and selective inhibitor of ATR (hereafter- ATRi), partially relieves this arrest (32.9% of vpr+ cells in G₂, VPR + ATRi 24H
vs. HK2 + ATRi 24H, Figure 6). While ATRi does not fully abrogate Vpr-mediated G\textsubscript{2} arrest in RTECs, these results are consistent with other published data.

I concurrently monitored levels of the active, phosphorylated version of ATR via Western Blot and cell cycle phase via FACS over a 24-hour period. While control cells had little variation of P-ATR levels and distribution of cell cycle phases, vpr-expressing cells underwent a concomitant increase in P-ATR levels and G\textsubscript{2} accumulation (Figure 7).

To determine whether ATR is signaling through its canonical DDR effector kinase, Chk1, I monitored cell cycle phase distribution and corresponding levels of the active, phosphorylated version of Chk1 via Western Blot. At 72 hours post-transduction, P-Chk1 was upregulated in cells expressing Vpr compared to control HK2 cells (Western Blot, Lanes 1 and 3, Figure 8). While there was a slight increase in P-Chk1 in the GFP-control, the corresponding FACS analysis indicated this activation has minimal effect on the cell cycle (Western Blot, Lane 2, Figure 8). Chk1 activation is dependent on ATR, as inhibiting ATR abrogated Chk1 phosphorylation (Western Blot, Lanes 3 and 5, Figure 8). Further, inhibition of ATM using the small molecule KU55933 had no effect Chk1 phosphorylation or accumulation of cells in the G\textsubscript{2} phase of the cell cycle (ATMi, Figure 8). These results indicate the canonical Vpr-mediated ATR-Chk1 signaling pathway initiates G\textsubscript{2} arrest in vpr-expressing RTECs.
Figure 6: Inhibition of DDR Kinase ATR Alleviates G2 arrest in Vpr+ RTECs. Analysis of HK2 cell cycle 24 hours after transduction with TY2-Vpr-GFP +/- ATR inhibitor VE821. The X-axis shows DNA content, as measured by Propidium Iodide (PI), and the Y-axis indicates relative cell number. The percentage of cells within the G1 and G2 gates is indicated in each panel.
Figure 7: Activation of DDR Kinase ATR in Vpr+ RTECS Corresponds with Accumulation of Cells in the G2 Phase of the Cell Cycle. Left panel: Time course of ATR phosphorylation (western blot) in HK2 cell +/- expression of pHR-HA-VPR-GFP (HA-Vpr). Densitometric analysis of phospho-ATR band was adjusted to ATR loading control and normalized to corresponding HK2 time point. Right panel: Corresponding cell cycle phase analysis from the same populations of cells in left panel. Cell cycle phase was identified using FACS analysis of DNA content (PI). The Y-axis indicates the percentage of cells within the G1 (Green), S (Red) and G2 (Blue) phases of the cell cycle for each time point.
Figure 8: Inhibition of DDR Kinase ATR Alleviates CHK1 Activation in Vpr+ RTECs.

Top panel: HK2 cell cycle analysis (FACS) 72 hours after transduction with TY2-GFP, and TY2-Vpr-GFP +/- ATR inhibitor VE821 or ATM inhibitor KU55933. The X-axis shows DNA content, as measured by Propidium Iodide (PI), and the Y-axis indicates relative cell number. The percentage of cells within the G₁ and G₂ gates is indicated in each panel.

Lower panel: Corresponding western blot analysis for phospho-CHK1 for cell populations in upper panel. Densitometric analysis of phospho-CHK1 band was adjusted to CHK1 loading control and normalized to HK2 sample.
3.2.2 A Subset of HIV-1 Vpr+ Renal Tubule Epithelial Cells Escape ATR Dependent G\textsubscript{2} Arrest to Become Polyploid

By conducting a longer-term analysis of DNA content, I found that for a substantial subset of Vpr+ RTECs, the ATR-mediated G\textsubscript{2} arrest is only transient and precedes a doubling of genome content. To determine the duration of G\textsubscript{2} arrest, I analyzed DNA content in Vpr+ and control cells at not 24 hours, 36 and 48 hours. While at 24 hours post-transduction a majority of Vpr+ cells were in the G\textsubscript{2} phase of the cell cycle, by 48 hours 25% of the total cell population exhibited clear polyploidy (HK2 48H vs. VPR 48H, Figure 9), as evidenced by having greater than 4C DNA content.
Figure 9: A Subset of HIV-1 Vpr+ RTECs Escape ATR Dependent G2 Arrest to Become Polyploid. FACS cell cycle analysis of HK2 cells showing the emergence of polyploidy in TY2-Vpr-GFP+ RTECs over time. The X-axis shows DNA content, as measured by Propidium Iodide (PI), and the Y-axis indicates relative cell number. The percentage of cells within the G1, G2 and polyploid gates is indicated in each panel.

3.2.3 G2 Arrest and Polyploidy in Vpr+ RTECs Does Not Require Virus Integration or de novo Synthesis of Vpr Protein

Integration of lentiviral vector DNA introduces breaks in host cell chromatin that require repair of this damage to complete the integration process[183]. To ensure DNA damage introduced during integration does not play a role in G2 arrest or polyploidy in Vpr+ RTECs, I infected cells with integrase (IN) defective lentiviral particles. Without integrase activity, the vpr transgene is expressed via unintegrated circular forms of the vector genome. At 72 hours post infection IN- and IN+ Vpr expression resulted in similar cell cycle profiles (IN- Vpr 72H and IN+ VPR72H, Figure 10), indicating DNA
damage introduced through integration does not contribute to the observed phenotype. Further, Vpr Virus-like Particles (VLPs), empty particles that integrate Vpr protein into the capsid, but do not contain a Vpr-expression vector, also potentiated cell cycle arrest. These results indicate that Vpr-mediated G₂ arrest and polyploidy do not require virus integration or de novo synthesis of Vpr protein.

**Figure 10: Progression to Polyploidy Does Not Require Viral Integration or De Novo Synthesis of Vpr Protein.** HK2 Cell cycle analysis 72 ours after transduction with TY2-Vpr-GFP +/- IN or Vpr VLP. The X-axis shows DNA content, as measured by Propidium Iodide (PI), and the Y-axis indicates relative cell number. The percentage of cells within the G₁, G₂ and polyploid gates is indicated in each panel.

### 3.2.4 Vpr-mediated G₂ Arrest Differs from Genotoxin-induced G₂ Arrest, and is Required for Progression to Polyploidy

Two known causes of polyploidy are DNA damage and a prolonged G₂ phase[168-170]. To distinguish between these possibilities, I treated HK2 cells with the DNA damaging agents Cisplatin, which induces replication stress, and Doxorubicin, a potent inducer of double stranded DNA breaks. Multiple doses and treatment times
were tested (data not shown), but in all instances cells either arrested in G2 and/or underwent cell death without progressing to polyploidy (Figure 11, Doxorubicin, Cisplatin). These results indicate that DNA damage is not sufficient to promote Vpr-induced polyploidy in RTECs. In contrast, we find that an extended G2 is required for Vpr-induced polyploidy. Specifically, neither cells expressing the G2 arrest-deficient Vpr mutant Q65R, nor Vpr+ ATRi cells showed an increase in polyploidy by 48 hours post-transduction (Figure 11 VPR Q65R, VPR + ATRi). Together, these results indicate that DNA damage is not sufficient to promote Vpr-induced polyploidy in RTECs. Taken together, I found that G2 arrest/extension is required, but not sufficient, to promote polyploidy in renal cells. These results also indicate that Vpr-mediated alteration of the cell cycle extends beyond induction of G2 arrest. These results are consistent with a previously published report that irradiated A549 and Jurkat cells arrest in G2/M, but continue to cycle and become tetraploid following vpr expression [115]. Further, the capacity for, and magnitude of Vpr-mediated polyploidization appears to be dependent on cell type[118, 152].
Figure 11: G2 Arrest is Required, but Not Sufficient, for the Emergence of Polyploidy in Vpr+ RTECs. Right Panel: FACS cell cycle analysis of HK2 cells 48 hours following transduction with: TY2-VPR-GFP (VPR48H), TY2-Q65R-GFP (VPR Q65R 48H), or following treatment with Doxorubicin or Cisplatin. The X-axis shows DNA content, as measured by Propidium Iodide (PI), and the Y-axis indicates relative cell number. The percentage of cells within the G1, G2 and polyploid gates is indicated in each panel. Left panel: Cell cycle analysis of HK2 cells 48 hours after transduction with TY2-VPR-GFP or TY2-VPR-GFP + VE821.

3.3 Discussion

A long established hallmark of Vpr expression is cell cycle arrest during G2/M [102, 103, 115, 116, 184] via activation of the ATR–Chk1 DDR signaling pathway [84, 106, 116, 118, 119, 122]. This study confirms that Vpr initiates an ATR-dependent G2/M arrest in RTECs. Further, I identified ATR-dependent phosphorylation of the effector kinase...
Chk1, indicating the ATR-Chk1 signaling pathway is conserved in my in vitro model system. However, the presence of polyploidy in Vpr+ RTECs suggests either the absence or failure of the canonical Vpr-mediated G₂ arrest in these cells.

In general, the mechanisms by which Vpr modulates the cell cycle appear to differ from cell cycle arrest pathways governed by traditional DDR signaling. My observation that DNA damaging agents Cisplatin and Doxorubicin robustly induce cell cycle arrest indicate an intact G₂ checkpoint in RTECs; however, Vpr+ RTECs continue to cycle and become polyploid. Similarly, irradiated Jurkat cells fully arrest in G₂, while Vpr+ Jurkat cells can bypass G₂ phase of the cell cycle to become polyploid[115]. Another study found that DNA damage introduced with hydroxyurea (HU) or ultraviolet light (UV) induces Chk1 activation in a similar manner to Vpr. However, HU and UV-treated cells arrest in S phase, whereas Vpr+ cells arrest at the G₂/M border[118]. Further, ATR inhibition sensitizes cells with genotoxin-induced damage to cell death, while ATRi associates with cell survival in the context of Vpr-expression. Thus, ATR-Chk1 activation in the setting of Vpr expression initiates G₂ arrest, but does so via mechanisms that are fundamentally different from canonical activation of the DDR[120].

Strict maintenance of Vpr-mediated G₂ arrest appears to be dependent on cell type[118, 152]. Indeed, a previous study reported that Vpr isolates from different primate species exert cell cycle control in a species specific manner, suggesting Vpr-mediated G₂ arrest has evolved to include host factors unique to particular cell
types\cite{107}. Overcoming Vpr-mediated G$_2$ arrest is a critical requirement for progression to polyploidy in RTECs, and identifying the mechanisms that control this process in renal cells is paramount to understanding this unique facet of cell cycle manipulation in the kidney.
4. Identify the mechanism of HIV Vpr-induced Polyploidy in Renal Tubule Epithelial Cells

4.1 Introduction

To understand the significance of polyploidy after HIV infection, it is first important to understand the mechanism generating polyploidy. Polyploidy can be generated by endoreplication, non-canonical cell cycles characterized by multiple rounds of genome replication without complete cell division, or by cell-cell fusion. Endoreplication cell cycles can be further characterized as either endocycling, in which cells undergo successive rounds of DNA replication without an intervening mitotic phase, or as endomitosis, in which genome replication is accompanied by incomplete mitosis. Here I show that, following an extended G2 arrest, a subset of Vpr+ RTECs enter mitosis. A second subset of these cells also bypass complete mitosis and enter a second S-phase to become polyploid, suggesting that Vpr+ RTECs become polyploid through endomitosis.

4.2 Results

4.2.1 Vpr+ Renal Tubule Cells Express the Mitotic Marker Phospho-Histone H3.

The distinguishing characteristic between endocycling and endomitosis is the presence of an intervening mitotic phase between cycles of genome replication. Mitotic markers such as phospho-histone H3 (PH3) can be used to detect mitosis and differentiate between these cell cycles. As a preliminary experiment to determine if Vpr+
RTECs exit undergo mitosis, I transduced asynchronous HK2 cells with lentiviral vectors expressing either GFP alone, or GFP-tagged Vpr. 72 hours post-transduction, cells were stained with a primary PH3 (pSer28) antibody and fluorescently labeled secondary antibody and analyzed via FACs. FACS analysis revealed that vpr-expressing cells have increased levels of PH3 compared to both GFP transduced cells and HK2 control cells (Figure 12). The presence of PH3 in Vpr+ cells indicates ongoing mitosis, and also suggests that endomitosis drives ploidy increase in these cells. Further, PH3 staining in Vpr+ RTECs has a higher mean fluorescent intensity than staining in either control, suggesting an enrichment of mitosis in the Vpr+ cell population.
**Figure 12: Vpr+ RTECs Express Mitotic Marker Phospho-Histone H3.** FACS analysis of global PH3 staining in asynchronous HK2 cells 72 hours following transduction with TY2-VPR-GFP (orange) or a TY2-GFP control (green). As a control, non-transduced HK2 cells were stained with PH3 + secondary antibody (blue), and secondary alone (red). The X-axis shows phospho-Histone H3 staining fluorescent intensity, and the Y-axis indicates relative cell number.

**4.2.2 A Subset of Vpr+ RTECs Enter Mitosis Following a Prolonged G₂ Arrest**

To better understand the kinetics of G₂ exit and mitotic entry in Vpr+ RTECs, I monitored thymidine synchronized RTECs for both DNA content (PI) and PH3 every hour, for 26 hours post transduction with pHR-HA-Vpr-ΔGFP. To represent progression through cell cycle over time, the percent of cells with 4C DNA content,
indicating G₂/M phase, was charted for each time point. I also charted PH3+ cells within the G₂/M population over time to identify mitotic cells (Figure 13A). Once released from the thymidine block, control HK2 cells progressed through S-phase to accumulate 4C DNA content. A peak in mitotic cells 16-18 hours post-release corresponded with a subsequent decrease in the percentage of cells with 4C DNA content, indicating that cells began to undergo successful mitosis by this time point. Consistent with the extended G₂ following Vpr expression, Vpr+ RTECs with 4C DNA content had a greater delay between arrival at 4C DNA content and the appearance of PH3+ cells (Figure 13B). Following a prolonged G₂ phase many Vpr+ cells entered mitosis. Within the G₂/M population, Vpr+ cells exhibited higher levels of PH3+ cells than the control, suggesting that Vpr either increases the number of mitotic cells, or causes a longer mitotic phase (Figure 13B).
Figure 13: A Subset of Vpr+ RTECs Enter Mitosis Following a Prolonged G2 Arrest. (A) Experimental schematic for transduction of HK2 cells following G1 thymidine synchronization. (B) Time course monitoring cell cycle progression and mitotic entry in synchronized HK2 control cells and cells transduced with HR-HA-VPRΔGFP. Graphical representation of FACs analysis of the % of cells with 4C DNA content at each time point from T=4 to T=26 (top panel) and mitotic pH3+ cells within that 4C population at each time point (bottom panel).

4.2.3 Polyploid Vpr+ RTECs Replicate DNA Without Undergoing Mitosis

Given accumulation of polyploidy in many Vpr+ RTECs after G2, I next examined if surviving cells undergo an additional S-phase to increase DNA content. I synchronized HK2 cells as previously described with thymidine, and following release
from the thymidine block, I transduced cells with HR-HA-Vpr-ΔGFP. I added BrdU to culture media 18 hours post-transduction, when a majority of Vpr+ cells are in the G2/M phase of the cell cycle. At 48 hours post-transduction I harvested the cells, and analyzed them via FACS for BrdU incorporation and DNA content. Nearly all of the 4C Vpr+ cells incorporated BrdU, further illustrating that RTECs enter a second S-phase after escaping Vpr-mediated G2 arrest (Figure 14). The progression through a second S-phase without intervening mitosis is indicative of endoreplication, a known cell cycle modification that generates polyploid cells[168, 170].

![Figure 14: Polyploid Vpr+ RTECs Replicate DNA Without Undergoing Mitosis. FACS analysis of BrdU incorporation (Y axis) and DNA content (Propidium Iodide- PI, X axis) in control HK2 cells (HK2) and HK2 cells transduced with pHR-HA-VPRΔGFP (Vpr 48H).](image)
4.2.4 Renal Tubule Cells in Murine and Human HIVAN are Positive for Mitotic Marker PH3

Having demonstrated that Vpr drives mitotic re-entry from G2 arrest \textit{in vitro}, I next wanted to investigate whether HIV-1 promotes mitosis \textit{in vivo}. The normally quiescent renal tubule epithelium has the capacity to re-enter the cell cycle to regenerate following injury\cite{185-187}, and the Klotman group has previously demonstrated that a high number of renal tubule epithelial cells from HIV transgenic mice stain positive for Ki-67, a cellular proliferation marker \cite{188}. Because Ki-67 expression occurs in all phases of an active cell cycle and thus does not directly indicate mitosis, I performed immunohistochemistry on both murine and human HIVAN biopsies using the mitosis specific marker PH3. Tubule epithelial cells in biopsies from both the Tg26 HIVAN murine model (containing a gag/pol deleted HIV transgene) and renal biopsy material from a patient with HIVAN stained positive for PH3 compared to the respective controls (\textbf{Figure 15}), indicating that RTECs undergo increased mitosis in the context of HIV infection \textit{in vivo}. Given my \textit{in vitro} observations, and the previous demonstrations that RTEC hypertrophy and polyploidy are characteristic of HIVAN \cite{60}, it is likely that HIV induced mitosis and endoreplication/polyploidy are two distinct outcomes in human HIV+ kidneys.
Figure 15: Renal Tubule Cells in Murine and Human HIVAN are Positive for Mitotic Marker PH3. Kidneys from littermate controls or Tg26 mice with HIVAN (upper panel) and normal human kidney and a biopsy from HIVAN patients (lower panel) were stained with anti-PH3 antibody.

4.2.3 Discussion

Polyploidy frequently results from endoreplication, non-canonical cell cycles characterized by multiple rounds of genome replication without complete cell division, or by cell-cell fusion. Here I show that, following an extended G2 arrest, a subset of Vpr+ RTECs enter mitosis. A second subset of these cells also bypass complete mitosis and
enter a second S-phase to become polyploid. This data suggests that Vpr+ RTECs become polyploid through endoreplication, not cell-cell fusion.

Endoreplication can be further characterized as either endocycles, repeated S-phase without mitosis, or endomitosis, abbreviated mitosis without cell division. Endomitotic cells exit mitosis at varying points in the cell cycle phase, and thus, can become either mononuclear or binucleate polyploid cells. Further studies are necessary to confirm the mode of Vpr-induced endoreplication. However, given the high levels of mitotic marker PH3 in Vpr+ RTECS, and the presence of both large mononuclear and binucleate tubule cells in biopsies from murine HIVAN kidneys[60], I favor endomitosis as the mechanism of Vpr-mediated polyploidy.

Vpr-mediated G₂ arrest is evolutionarily conserved[70], but is also cell-type dependent[152]. This data is supported by work in other in vitro model systems that note mitotic progression in Vpr+ cells following G₂ arrest. However, mitosis in Vpr-expressing yeast and mammalian cells is associated with numerous defects, including centrosome amplification, multipolar spindle formation, misaligned chromosomes, defects in cytokinesis and multi-nucleation[155-158]. Thus, escape from Vpr-mediated G₂ arrest and progression into mitosis may have unintended consequences for cell survival in the setting of HIV infection.
5. Characterize the Physiological Implications of Mitotic Entry and Polyploidization Following Vpr Expression in Renal Tubule Epithelial Cells

5.1 Introduction

Mitotic progression in Vpr+ cells, including renal tubule epithelial cells, is preceded by an extended G2 phase [156, 158]. The consequences of escape from the G2 phase of the cell cycle and entry into mitosis in RTECs are not fully appreciated. However, multiple in vitro model systems in mammalian and yeast cells have reported severe mitotic defects in vpr-expressing cells, including centrosome amplification, multipolar spindle formation, misaligned chromosomes, defects in cytokinesis and multi-nucleation[155-157]. Defective mitosis caused by centrosome amplification and spindle multipolarity often results in apoptosis[123, 159, 160]. Therefore, the contribution of Vpr-induced mitotic defects to HIV-1 related cell death warrants further investigation. Here I show that Vpr induces aberrant mitosis in RTECs. Further I show that, following escape from a prolonged G2 phase, mitotic cell death and polyploidy are two distinct outcomes for Vpr+ RTECs.

5.2 Results

5.2.1 Vpr Promotes Aberrant Mitosis in Rental Tubule Epithelial Cells

Because Vpr has been previously associated with mitotic defects in both yeast and HeLa cells [157], I closely examined mitosis in Vpr+ RTECs via immunofluorescence microscopy. To ensure the first round of mitosis following vpr expression was captured,
HK2 cells were synchronized as previously described and harvested 18 hours post-transduction with pHR-HA-Vpr-ΔGFP. While 95% of mitotic control cells displayed normal bipolar spindles, over 66% of Vpr+ cells exhibit multiple spindle poles, a hallmark of abnormal mitosis (Figure 16, Vpr1, Vpr2). Vpr is known to cause centrosome amplification in some contexts [155-157]. I therefore examined centrosomes by γ-tubulin staining in Vpr+ cells, and found that many cells exhibited centrosome amplification. Thus, it is clear that Vpr promotes centrosome amplification and formation of supernumerary spindle poles in RTECs, and that these mitotic defects likely play a role in Vpr-mediated cell death and polyploidy accumulation.
Figure 16: Vpr+ RTECs Undergo Aberrant Mitosis Following a Prolonged G2 Phase.

Immunofluorescence microscopy depicting mitosis in synchronized HK2 cells 18 hours post-transduction with HR-HA-VPRΔGFP. Cells were analyzed for centrosome formation (γ-Tubulin, Red), mitotic spindle formation (α-Tubulin, Green) and DNA (DAPI, Blue). VPR+ cells exhibited mitotic defects, including centrosome amplification and bipolar spindles with more than 2 spindle poles (Vpr1) and tripolar spindles with more than 2 spindle poles (Vpr2).

5.2.2 Polyploidy Represents an Alternative to Mitotic Cell Death in Vpr-expressing Rental Tubule Epithelial Cells

Aberrant mitosis from cells with multiple spindle poles was recently shown to be lethal in vivo (reviewed in [160]). To follow the cell fate of Vpr+ RTECs during mitosis in
real-time, I used fluorescent ubiquitination-based cell cycle indicator (FUCCI) probes, which permit live visualization of G₁ phase (red fluorescence), S/G₂ (green fluorescence), and M phases (nuclear green fluorescence) ({Figure 17})[189].

{Figure 17: Schematic of FUCCI Probe Function. Expression of FUCCI vectors allows visualization of cell cycle progression through G₁ phase (red fluorescence), S/G₂ (cytoplasmic green fluorescence), and M phases (nuclear green fluorescence).

I imaged HK2 cells for 36 hours post-transfection with HR-HA-Vpr-ΔGFP. In both control (HK2) and Vpr+ cells, progression through G₁, S, and into G₂ is similar ({Figure 18}). Upon exit from G₂, however, my imaging revealed two distinct phenotypes in Vpr+ RTECs (VPR FUCCI, {Figure 18}). As expected, I found that HK2 control cells were capable of successful mitosis (HK2 FUCCI, 87% of HK2 cells, ‘mitosis’, {Figure 18}).
In contrast, a substantially decreased fraction of Vpr+ cells (63%) progressed through mitosis. The remaining cells (37%) displayed morphological signs of early mitosis, including slight rounding of the cell, but stayed attached to the plate and did not complete mitosis (‘no mitosis’, Figure 18). These Vpr+ cells that bypass a full mitotic phase had substantially higher survival rate than those that attempted mitosis (79.3% vs. 32.7%, Figure 18). Thus, bypass of mitosis favors cell survival in Vpr+ RTECs that escape G2 arrest.

Further, I did not record any instances of cell-cell fusion during the live imaging experiments in either control HK2 or Vpr+ RTECs. Together with the observation that Vpr+ RTECs undergo second S-phase without undergoing mitosis (Figure 14), these data are indicative of endoreplication, a known cell cycle modification that generates polyploid cells.
Figure 18: Live Cell Imaging of Vpr+ HK2 cells with FUCCI vectors. Upper panel: Live cell imaging of HK2 cells transduced with FUCCI vectors (HK2 FUCCI), showing representative cell undergoing mitosis (arrow). Middle panel: Live cell imaging of HK2 cells co-transduced with HR-HA-VPRΔGFP and FUCCI vectors (VPR FUCCI). Vpr+ cells were counted and characterized as either mitosis (arrow head) or no mitosis (arrow). Bottom Panel: Table on right indicates total cell counts for three separate trials. Graph on right indicates average survival rates for cells that undergo mitosis vs. cell that do not complete mitosis in control and Vpr+ cells in 3 separate trials. Vpr+ cells that bypassed mitosis had higher average rates of survival (79.3%) than Vpr+ cells that attempted mitosis (32.7%). Control cells that underwent mitosis had 87% of cells surviving, while none of the control cells that bypassed mitosis survived.
5.2.3 Polyploid Vpr+ RTECs Become Enriched in Cell Culture Over Time

In earlier BrdU experiments, I noticed that BrdU continues to accumulate in the emerging 8C polyploid population (Figure 14). To determine the longer-term fate of polyploid Vpr + RTECs, I maintained cells in culture for nine days. By day 9 polyploid cells account for 38.2% of surviving cultured cells, compared to 28.6% of the population at day 3. Additionally, day 9 cultures show the emergence of another polyploid peak with 16C DNA content, indicating a subset of Vpr+ polyploid cells are able to continue endoreplication (Figure 19). The enrichment of polyploid cells in long-term cell culture is consistent with the observation that bypassing Vpr-mediated aberrant mitosis favors cell survival and subsequent polyploidy in RTECs.

Figure 19: Polyploidy Becomes Enriched in Vpr+ RTECs Over Time. HK2 cells transduced with TY2-VPR-GFP at 0, 3 and 9 days post-transduction. The X-axis shows DNA content, as measured by Propidium Iodide (PI), and the Y-axis indicates relative cell number. Distribution of cell cycle population indicated in each panel.
5.3 Discussion

Using FACS, fluorescence microscopy, and live cell imaging I show that G2 escape immediately precedes a critical junction between two distinct outcomes in Vpr+ RTECs: mitotic cell death and polyploidy (Figure 18). Following G2 exit, Vpr+ cells that evade mitosis and become polyploid have a substantially higher survival rate than those that undergo complete mitosis.

While defective mitosis and mitotic cell death have been noted in several in vitro model systems[155-157], to my knowledge, mitosis-induced cell death has not been previously attributed to HIV pathogenesis in renal tubule epithelial cells. Vpr-mediated apoptosis is complex and appears to invoke cell death via multiple pathways. Previous studies in have identified caspase activation and mitochondrial injury as a key component cell death in Vpr+ RTECs[138, 139]. Thus, mitotic cell death is a novel mechanism of Vpr-induced apoptosis in RTECs in vitro, and my demonstration of high levels of mitotic marker PH3 in murine and human HIVAN kidney biopsies suggest mitotic progression may be relevant to HIV pathogenesis in vivo.

For many years, cell death resulting from aberrant mitosis was designated as mitotic catastrophe. Recently, mitotic catastrophe was reclassified as a mechanism to sense mitotic failure, which subsequently initiates either senescence, or cell death via apoptosis or necrosis[160, 190]. While mitotic catastrophe induces cell death via multiple, heterogeneous pathways, there are specific characteristics maintained during
these processes. First, all mitotic catastrophe results from insults to key components of the mitotic process, including chromosomes, the Spindle Assembly Checkpoint (SAC) or the Anaphase-Promoting complex (APC/C). Further, some degree of mitotic arrest is required to initiate mitotic catastrophe. Per this definition, Vpr-mediated mitotic death and apoptosis via caspase activation and mitochondrial injury would not be mutually exclusive. Therefore, I propose a model in which Vpr-induced mitotic stress activates mitotic catastrophe signaling, which then initiates apoptosis via the previously characterized mechanism of caspase activation.

In addition to cell death, mitotic stress has also been shown to induce endoreplication and polyploidy in multiple situations. p53-mutant cancer cells can avoid apoptosis by inducing endoreplication after treatment with radiation or microtubule-depolymerizing drugs such as colcemid. Mitotic stress induced by anti-mitotic drugs can also drive endoreplication in some non-neoplastic tissues, including plants cells and keratinocytes (reviewed in [168-170]). In the context of Vpr-induced mitotic stress, abortive mitosis and endoreplication also represent a pro-survival mechanism, and polyploidy becomes enriched in Vpr+ cell culture over time (Figure 19).

If mitotic stress induces both mitotic catastrophe and polyploidy, then all Vpr+ cells enter the mitotic phase of the cell cycle following G2 arrest. Cells that become polyploid would therefore, initiate the switch to endoreplication during mitotic arrest. As depicted in the live imaging data, Vpr+ cells designated as ‘no mitosis’ remain
attached to the plate, but exhibit slight rounding as is characteristic of early stage mitosis (Arrow, Figure 18). Taken together, the high levels of PH3 staining in Vpr+ cells, the presence of multinucleated cells in murine HIVAN biopsies, and the observed morphology of Vpr+ RTECs during live imaging support the hypothesis that Vpr cells become polyploid via endomitosis.

Based on my proposed mode, mitotic stress represents a previously unrecognized initiating stimulus for both Vpr-induced apoptosis and polyploidy. Given that polyploidy may play a role in HIV disease pathogenesis and reservoir establishment, identifying the molecular signals that regulate the switch from mitotic cycles to endoreplication is a critical next step in understanding and circumventing this facet of the HIV-1 life cycle in the kidney.
6. Assess the Role of ATM Kinase in the Emergence of Polyploidy in Vpr+ RTECs

6.1 Introduction

Given its canonical role as an apical activator of a DDR signaling cascade in response to double strand breaks (DSBs), ATM was first investigated as a possible mediator of Vpr-induced cell cycle arrest. While it was determined that ATM is dispensable for Vpr-mediated G2/M arrest [84, 115, 120], subsequent studies suggest that ATM-dependent DNA repair is critical following Vpr-mediated DSBs [121, 181], and after integration of the full HIV-1 provirus [180, 191, 192]. However, neither the ability of Vpr to induce DSBs, nor the requirement for ATM-dependent DNA repair following virus integration, is maintained in all cell systems [117, 193]. ATM has also been implicated in the activation of HIV-1 transactivator rev, and in apoptotic death of HIV-1 Env induced syncytia [182, 194]. The varied functions attributed to ATM suggest this kinase may have multiple, cell-type specific roles in the HIV-1 life cycle, and therefore, I chose to examine the possible role of ATM in Vpr-mediated pathology in RTECs. In this study, I show that ATM is required for both Vpr-mediated polyploidy and mitotic cell death in Vpr+ RTECs, thus uncovering a novel role for ATM in Vpr-mediated pathogenesis.
6.2 Results

6.2.1 ATM is Required for Escape from Vpr-mediated G2 Arrest and Polyplody Accumulation in Renal Tubule Epithelial Cells

To examine the possible role of ATM in Vpr-mediated pathology in RTECs, I transduced HK2 cells with TY2-Vpr-GFP, and followed with treatment with small molecule KU55933, a potent and selective inhibitor of ATM, for 48 hours (hereafter-ATMi). Interestingly, by analyzing DNA content via FACS, I found that ATM inhibition substantially reduced polyplody in Vpr+ cells (from 16.6% to 2.65%, VPR 48H vs. VPR + ATMi 48H, Figure 20). ATMi did not affect the cell cycle profile of control HK2 cells (Figure 20), suggesting that inhibition of polyplody was not due to overall alterations in cell cycle progression.
Figure 20: The ATM Kinase is Required for the Emergence of Polyploidy in HIV-1 Vpr+ RTECs. HK2 cell cycle analysis 48 hours following transduction with TY2-VPR-GFP +/- ATM inhibitor KU55933. The X-axis shows DNA content, as measured by Propidium Iodide (PI), and the Y-axis indicates relative cell number. The percentage of cells within the G₁, G₂ and polyploid gates is indicated in each panel.

I next concurrently monitored cell cycle phase and levels of the active, phosphorylated form of ATM via Western Blot over a 48-hour period. While control cells have little variation of P-ATM levels and distribution of cell cycle phases, Vpr+ cells undergo a concomitant increase in P-ATM levels and polyploidy between 36 and 48 hours (Figure 21).
Figure 21: The Activation of ATM Corresponds with the Emergence of Polyploidy in HIV-1 Vpr+ RTECs. Left panel: Time course of ATM phosphorylation (western blot) in HK2 cell +/- expression of HR-HA-VPR-GFP (HA-Vpr). Densitometric analysis of phospho-ATM band was adjusted to ATM loading control and normalized to corresponding HK2 time point. Right panel: Corresponding cell cycle phase analysis from the same populations of cells in left panel. Cell cycle phase was identified using FACS analysis of DNA content (PI). The Y-axis indicates the percentage of cells within the G1 (Purple), S (Green), G2 (Red) and polyploid (Blue) phases of the cell cycle for each time point.

6.2.2 Activation of ATM in Vpr+ Renal Tubule Cells is Restricted to Cells in the G2/M phase of the Cell Cycle and Polyploid cells

To determine whether ATM activation occurs preferentially in cells that progress to polyploidy, I next analyzed p-ATM levels within each cell cycle phase. Cells were transduced with HR-HA-Vpr-ΔGFP and analyzed via FACS for both pATM and DNA content (PI). At 44 hours post-transduction, 29.4% of Vpr+ cells stained positive for pATM, compared to only 1.92% of the control cells (Figure 22, top panel). To determine
when ATM is expressed in Vpr+ cells, I next gated Vpr+ cells based on cell cycle phase, and analyzed the mean fluorescent intensity of pATM staining (FL-1) within each population. While fluorescent intensity was low overall, pATM staining intensity in the 8C polyploid cell population was more than twofold higher than that of the 2C G1 population. Cells in the 4C population, which includes cells in G2, mitotic cells, and cells with 4C DNA content re-entering S phase to become polyploid, also have mean pATM staining intensity nearly double that of 2C cells in G1 (Figure 22, bottom panel). The presence of activated pATM in polyploid and G2/M cell populations, but not in G1 cells, coupled with my ATMi data, suggests that ATM activation is required for polyploidy emergence during/following exit from G2. Further, these data suggest that ATM remain active in the polyploid state.
Figure 22: ATM is Activated in G₂/M and Polyploid Populations of Vpr+ RTECs.

**Upper Panel:** FACS analysis of pATM and PI co-staining 44 hours following transduction with HR-HA-VPR-ΔGFP. The X-axis shows DNA content, as measured by Propidium Iodide (PI), and the Y-axis indicates pATM fluorescent intensity. Gated area indicates the percent of total cells that are positive for pATM. **Lower Panel:** Analysis of the fluorescent intensity of pATM in distinct cell cycle phases Vpr+ cells. The same population of Vpr+ RTECs from the upper panel was gated based on DNA content, and the fluorescent intensity of pATM (lower right, X-axis) was determined for the G₁ (Green), G₂/M (Blue) and polyploid (Red) cell populations.

### 6.2.3 Inhibition of ATM Prevents Progression From G₂ to Mitosis in Vpr+ Renal Tubule Cells, but not in Control Cells

One possible mechanism by which ATM may promote polyploidy, is that ATM promotes mitosis. While mitotic cell counts varied across three experiments, my data
consistently showed that ATMi decreased the incidence of mitosis in Vpr+ RTECs (Figure 22). In contrast, control HK2 cells underwent an increase in mitosis, as would be expected in situations where cell cycle checkpoints were inhibited. These data suggest a key role for ATM in overcoming Vpr-mediated G2 arrest, leading to either Vpr-induced mitotic catastrophe or the acquisition of polyploidy.

Figure 23: Inhibition of ATM Decreases Mitosis in Vpr+ Cells. Analysis of the fold change in pH3+ control and Vpr-expressing cells following treatment with ATM inhibitor KU55933. Synchronized cells were analyzed via FACS for pH3 18 hours following transduction with HR-HA-VPR-ΔGFP +/- ATMi. The Y-axis indicates the fold change in pH3+ cells for control HK2 (HK2 vs. HK2+ ATMi) and Vpr-expressing cells (Vpr vs. Vpr + ATMi). Each data point represents the fold change in a separate experimental trial.

To assess whether ATM-mediated inhibition of mitosis is reversible, I transduced HK2 cells with TY2-VPR-GFP +/- ATMi. Cells were maintained in culture for 72 hours, at
which point I removed the media from TY2-Vpr-GFP + ATMi, and added fresh, drug-
free media to the wells. Cells were cultured for an additional 48 hours, at which point
samples were harvest and analyzed for DNA content via FACS. As expected, at 72 hours
post transduction ATM inhibition reduced polyploidy in Vpr+ cells (from 29% to 5.2%,
VPR 72H vs. VPR + ATMi 72H, **Figure 24**). Strikingly, more than half of Vpr+ cells
became polyploid within 48 hours of removing ATMi (56.8%, VPR - ATMi 120H, **Figure
24**). By 120 hours post transduction, Vpr+ RTECs exhibited a cell cycle profile that
consisted of a combination of G₁, G₂, polyploid and apoptotic cells. This data is
consistent with long-term Vpr cultures in another cell system[139]. In contrast, Vpr+
cells that were cultured in the presence of ATMi exhibited few apoptotic and G₁ cells,
but high levels of polyploidy, at 120 hours post-transduction (**Figure 24**).
**Figure 24:** Inhibition of Polyploidy is Reversible in Vpr+ RTECs. Upper panel: HK2 cell cycle analysis 72 and 120 hours following transduction with TY2-VPR-GFP. Lower Panel: HK2 cell cycle analysis 72 hours following transduction with TY2-VPR-GFP +ATMi. ATMi was removed at 72 hours, and cells were left in culture until 120 hours post-transduction, at which point cell cycle analysis was performed. The X-axis shows DNA content, as measured by Propidium Iodide (PI), and the Y-axis indicates relative cell number. The percentage of cells within the G1, G2 and polyploid gates is indicated in each panel.

### 6.2.4 Activated ATM is Present in Renal Tubule Cells in Human HIVAN Biopsies, but Not in Control Kidneys

To ask whether activation of ATM occurs *in vivo*, I performed immunohistochemistry on human renal biopsies. Renal tubule epithelial cells in HIVAN biopsies stained positive for pATM, while cells in the control kidney did not (Figure 25).
Thus, activation of ATM occurs in vivo in the setting of HIV infection, and represents a novel pathway in Vpr-mediated renal pathology.

![Image of activated ATM in renal tubule cells in human HIVAN biopsies.](image)

**Figure 25: Activated ATM is Present in Renal Tubule Cells in Human HIVAN Biopsies.** Kidney biopsies for normal human kidney and HIVAN patients were stained with anti-PATM antibody.

### 6.3 Discussion

While previous work concluded that the DNA damage-responsive kinase ATM is dispensable for Vpr-mediated G2 arrest [115, 120] other groups determined that activation of an ATM pathway plays a role in Vpr-associated pathology [181]. Overcoming Vpr-mediated G2 arrest is a critical requirement for both Vpr-induced mitotic catastrophe and polyploidy. My demonstration that inhibition of ATM precludes G2/M escape in Vpr+ RTECs, and thereby prevents polyploidy and mitotic catastrophe, suggests a novel role for ATM during HIV-1 infection.
One intriguing observation is that ATMi inhibition of G2 arrest escape is reversible, and further, this prolonged G2 arrests associates with increased levels of polyploidy, and decreased cell death. Based on this data, I propose that ATMi synchronizes cells in G2; without a cellular signal from ATM Vpr+ cells cannot overcome G2 arrest to enter mitosis. Removing ATMi ‘opens the flood gates’, and Vpr+ cells are free to enter mitosis, at which point they switch to an endomitotic cell cycle. However, the concurrent decrease in apoptosis suggests fewer cells are undergoing the alternate cell fate, mitotic cell death. Perhaps this extended G2 arrest primes a switch to endoreplication, and therefore, fewer cell progress with the mitotic cell cycle. Additionally, cell death observed in Vpr+ cultures may also be from a combination of mitotic cells and polyploid cells that are not refractory to the multiple cytopathic consequences of Vpr expression. Polyploidy can confer survival through adaptation[195], making certain polyploid cells more fit for survival than others in a particular environment. In this context, 48 hours may not be long enough to detect apoptosis in polyploid cells that would eventually undergo apoptosis.

While future studies are necessary to determine the mechanism by which ATM drives Vpr+ RTECs past the G2/M checkpoint, there are several known functions of ATM that may offer insight into this novel pathway. In its canonical role as an apical kinase in the DDR, ATM is most often associated with G2 arrest via Cdc25 inhibition, which will impair full activation of the mitotic kinase Cdk1 [196]. However, ATM is dispensable for
G2 arrest in Vpr+ RTECs, and Vpr has previously been shown to inhibit Cdc25, and thus Cdk1, via alternate signaling pathways[118, 119, 197]. However, a study in Arabidopsis leaf development suggests that activation of the ATM-mediated DNA damage response pathway leads to the promotion of endocycles and compensatory cell growth[198]. Given that activation of ATM in Vpr+ RTECs is not detected until 36 hours post transduction, and that this activation corresponds with the accumulation of polyploid cells (Figure 5B), it is possible that ATM drives endoreplication through its role as a DDR regulator.

Independent of its function in the DDR, ATM has recently been identified as a critical regulator of mitosis[199-201]. Following mitotic activation by the Aurora-B kinase, ATM phosphorylates kinetochore protein Bub1 to activate the spindle checkpoint and inhibit defective mitosis[199]. ATM knockdown in Nocodazole treated HeLa cells abrogates the spindle checkpoint and allows mitotic progression[199]. Surprisingly, ATM inhibition in vpr+ RTECs suppresses, rather than promotes, mitotic progression. This result contradicts the current dogma that ATM acts as a negative regulator of mitotic catastrophe, either through the DDR G2/M checkpoint[202, 203] or the mitotic spindle checkpoint. However, vpr has previously been shown to induce other non-canonical cell signaling pathways. For example, ATR inhibition sensitizes HeLa cells to genotoxin-induced apoptosis, but relieves cell death induced by Vpr[152]. Further studies to identify the mechanism governing ATM-dependent G2/M arrest
override, and subsequent polyploidy or mitotic cell death, are vital to understanding this novel aspect of Vpr manipulation of the cell cycle.
7. Conclusions and Implications

The current epidemic of HIV-1 related renal disease necessitates further investigation of the role of HIV-1 in renal epithelial cell infection. HIV-1 in the kidney may promote disease pathogenesis and may also enable viral reservoir establishment. We have previously shown that the HIV-1 accessory protein Vpr drives HIVAN-associated RTEC pathology, including G2/M arrest, apoptosis, and polyploidy[60, 138, 139]. Despite being reported in multiple in vitro systems[115, 118], the temporal progression, molecular mechanism, and physiological significance of vpr-mediated polyploidy is poorly understood. In this study, we use an in vitro model system to demonstrate that polyploidy is associated with cell survival following escape from G2 arrest in Vpr-expressing RTECs. Further, we identify an important role for ATM kinase in promoting polyploidy in this context.

7.1 Escape From G2 Arrest is a Critical Determinant of Cell Fate in Vpr+ RTECS

A long established hallmark of vpr expression is cell cycle arrest during G2/M [102, 103, 115, 184]. This study confirms that, unlike genotoxin-induced G2/M checkpoint activation, Vpr-mediated G2/M arrest in renal cells is transient. Using FACS, fluorescence microscopy, and live cell imaging I show that G2 escape immediately precedes a critical junction between two distinct outcomes in Vpr+ RTECs: mitotic cell death and polyploidy. Following G2 exit, Vpr+ cells that evade mitosis and become
polyploid have a substantially higher survival rate than those that undergo complete mitosis.

To my knowledge, mitosis-induced cell death has not been previously attributed to HIV pathogenesis in renal tubule epithelial cells. While previous studies identified caspase activation and mitochondrial injury as key components of cell death in Vpr+ RTECs[138, 139], there is little known about the stimulus that initiates these pathways. Therefore, apoptosis initiated by mitotic catastrophe sensing systems, represents a novel, alternative mechanism for Vpr-induced apoptosis in RTECs in vitro. Further, the demonstration of high levels of mitotic marker PH3 in murine and human HIVAN kidney biopsies suggests mitotic progression may be relevant to HIV pathogenesis in vivo. One intriguing link between Vpr-induced mitotic catastrophe and the previously identified apoptotic pathways is the ubiquitin like protein, FAT10. Overexpression of FAT10 is a known mediator cell death in Vpr+ and HIV+ RTECs[138, 165]. In other cell systems, overexpression of FAT10 has been associated with chromosome instability and inhibition of the spindle assembly checkpoint[204-207]. Therefore, future studies to interrogate the relationship between FAT10-induced apoptosis and FAT10-induced chromosome instability, may offer mechanistic insight into Vpr-induced mitotic stress and cell death.

The demonstration that mitotic cell death follows G2 escape also offers insight into the poorly understood relationship between Vpr-mediated cell cycle arrest and
apoptosis. While some studies suggest cell cycle arrest and cell death can occur independently[153, 154], many others indicate a temporal and casual relationship between vpr-mediated G2/M arrest and apoptosis[119, 120, 151, 156, 184]. In our model system, the accumulation of extranumerary centrosomes following vpr expression is a likely driver of this mitotic cell death, as multipolar mitotic spindles are often catastrophic in cell division[208]. Centrosome biogenesis is a previously recognized component of vpr-associated pathology[155-157], and interestingly, only occurs in cells arrested in G2 by vpr, but not by irradiation[156]. Under this model, centrosome amplification occurs during or just prior to vpr-dependent G2 arrest, and subsequently drives mitotic death in cells that progress through full mitosis. Thus, our work suggests a novel link between vpr-dependent cell cycle manipulation and cell death in RTECs.

7.2 ATM is Required for G2 Arrest Escape in Vpr+ Kidney cells

Overcoming Vpr-mediated G2 arrest is a critical requirement for both Vpr-induced mitotic catastrophe and polyploidy. Here I demonstrate that inhibition of ATM prevents G2 arrest escape and subsequent mitotic catastrophe and polyploidy in Vpr+ RTECs. Control of G2 exit and progression to polyploidy and apoptosis represents a previously unappreciated role for ATM during HIV-1 infection. In fact, to the best of my knowledge, ATM-mediated G2 arrest escape represents a novel role for ATM in any context. Interestingly, a previous report in Arabidopsis leaf developments shows that the ATM DDR triggers a switch to compensatory growth via endoreplication in the
presence of specific gene mutations[198]. Thus, ATM has a previously identified role in endoreplication, and future studies to assess the relationship between ATM-dependent G2 escape and endoreplication may offer key insights into its mechanism of action in Vpr+ RTECs.

ATM-dependent G2 arrest override and mitotic catastrophe in RTECs could represent an innate immune response to HIV-1 infection. In a previous study, ATM was shown to mediate centrosome amplification during a prolonged G2 arrest, thereby ensuring any cells that escape G2 arrest and enter mitosis are killed via mitotic catastrophe [209]. To support this hypothesis, my data show that activation of ATM coincides with G2 arrest escape in Vpr+ RTECs, and that centrosome amplification likely plays a role in mitotic cell death. Expression of viral genes has been reported to be enhanced during the G2 phase of the cell cycle[60], which could provoke an antiviral response by the host cell. If ATM drives G2 arrest escape to eradicate HIV+ renal cells via mitotic catastrophe, then polyploid cells that abort mitosis and switch to an endoreplication cell cycle represents an alternative outcome with many physiological implications for disease pathogenesis and maintenance of the putative HIV renal reservoir. Future work can determine the precise mechanism of ATM in Vpr-induced mitotic death and polyploidy.
7.3 The Role of Vpr-induced Polyploidy in the Kidney Following HIV Infection

While eradication of HIV+ renal cells via mitotic catastrophe benefits the host, the physiological consequences of polyploidy in an HIV+ kidney are less definitive. Identifying the mechanisms by which vpr+ cells commit to either mitosis or endoreplication following G2 arrest escape is critical to characterizing polyploidy as specific viral response or an unintended consequence of aberrant mitosis. However, whether or not polyploidy is the intended consequence of viral cell cycle manipulation, there are clear benefits of polyploidy in the HIV life cycle. In addition to enabling cell survival by circumventing mitotic cell death, polyploidy may promote long-term HIV survival/propagation through 1) increasing virus production, or 2) altering the host cell karyotype/genome to select for viral maintenance. Polyploidy and associated gene amplification has been previously shown to increase rates of transcription per cell[210-212]. Amplified transcription rates may correlate with increased virus production in surviving polyploid cells, thereby promoting efficient infection and virus propagation through neighboring T cells. In addition to increasing viral output, an alternative possibility is that polyploidy enables re-configuration of the infected cell’s genome, which in turn facilitates long-term survival and expansion of an HIV-1 infected cell. Indeed, among polyploid liver cells, a sub-population of cells harboring a chromosome-specific aneuploidy was recently shown to confer a survival advantage in a liver disease model[171, 173]. Therefore, polyploidy may promote genomic alterations that enable
HIV+ RTECs to withstand viral toxicity, enabling maintenance of a HIV renal compartment.

In addition to promoting cell survival and reservoir maintenance, the accumulation of vpr-mediated polyploidy may exacerbate renal dysfunction in aging HIV+ kidneys. Polyploidy associates with disease pathogenesis in numerous contexts, including aging, cancer, and drug resistance through aneuploidy (Reviewed in [168]). Given the known role of polyploidy in disease pathogenesis, the presence of polyploid cells in HIV+ renal biopsies, and the high incidence of renal disease in aging HIV+ patients, the role of polyploidy in HIV-associated renal pathology warrants further investigation. One possible mechanism by which vpr-induced polyploidy aggravates HIV-associated renal disease is by impeding the ability of normally quiescent renal tubule epithelium to re-enter the mitotic cell cycle to regenerate tissue following injury[185-187]. In the context of HIV infection, surviving polyploid RTECs could mire compensatory proliferation following cell death. Alternatively, the functionality of polyploid renal cells may differ from diploid RTECs. Thus, understanding the physiological implications of vpr-mediated polyploidy is a critical next step in uncovering the broader consequences of HIV-1 infection in the kidney.

By uncovering the mechanisms and implications of ploidy increase that associate with HIV+ cell survival, we may uncover key strategies to prevent renal disease or to alter the reservoir. ATM-dependent exit from Vpr-mediated G2 arrest is a critical
requirement for both mitotic catastrophe and polyploidy, and therefore represents a novel therapeutic target for disease prevention and reservoir establishment. ATMi would effectively eliminate polyploid cells, thereby ameliorating polyploidy-associated renal pathology and preventing the long-term survival of polyploid HIV+ RTECs. However, ATMi would also inhibit host-mediated clearance of HIV+ cells through mitotic catastrophe. Therefore, future studies to identify which downstream factors govern the junction between mitotic cell death and polyploidy is critical to effectively manipulate both outcomes in HIV+ renal cells.

In summary, this work identifies ATM-dependent override of Vpr-mediated G2 arrest as a critical determinant of cell fate Vpr+ RTECs (Figure 26). Further, our work highlights how a poorly understood HIV mechanism, ploidy increase, may offer insight into key processes of reservoir establishment and disease pathogenesis in HIV+ kidneys.
Figure 26: Proposed Model For Polyploidy Acquisition in Vpr+ RTECs
References


Biography

Emily Harman Payne (née Camp) was born on December 2, 1983 in Fort Worth, Texas. She received a Bachelor of Arts in Journalism and a minor in Cinema Studies from New York University in January 2005. Throughout college Emily fostered her passion for media by working as an editorial intern at CNN during the 2004 presidential election, and as a production assistant on the short-lived FOX reality show, ‘Trading Spouses: Meet Your New Mommy’. After graduation Emily worked as an associate producer and video editor for a corporate communications firm in New York City. She was accepted to the University of Texas at Austin’s Master’s Program in Advertising, but before enrolling she read a book about the 1918 Influenza Pandemic and decided to transition to a new career. Emily graduated from UT-Austin in May 2010 with a Bachelor’s of Science in Microbiology. That fall, Emily enrolled in the Duke Graduate School Department of Pathology as a Chancellor’s Scholar, and she joined the lab of Dr. Mary Klotman in December 2011. Her dissertation project was the basis for a CFAR Creative and Novel Ideas in Research grant entitled “The impact of polyploidy on establishing an HIV-1 reservoir in the kidney”. Emily’s paper, “Polyploidy and Mitotic Cell Death are Distinct Outcomes in Vpr+ Renal Cells” is currently being submitted to the journal AIDS. Emily currently lives in Boston with her husband and two cats.