Modelling H3.3-G34R mutation *in vitro* and *in vivo*

by

Stephie Hok Yee Leung

A thesis submitted in conformity with the requirements for the degree of Master of Science

Laboratory Medicine and Pathobiology
University of Toronto

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Abstract

Brain tumours are the most common solid tumours in children, with pediatric high-grade gliomas (PHGG) displaying clinically aggressive behaviour and dire survival outcomes. Previous evidence points toward a histone 3 variant $H3F3A$ mutation (H3.3-G34R) in pediatric glioblastomas, which results in redistribution of the histone mark H3K36me3. It has been shown that H3K36me3 is necessary for recruitment of the mismatch repair (MMR) protein complex hMutSalpha (hMSH2-hMSH6). *In vitro*, Western blot and immunofluorescence analysis was performed on immortalized normal human astrocytes (iNHAs) to establish decreased H3K36me3 levels in S phase synchronized H3.3-G34R mutant cells. MSH6 foci formation was unaffected in the absence of a DNA damage inducing agent. *In vivo*, immunohistochemical analysis on H3.3-G34R xenograft tumour showed decreased levels of H3K36me3. Therefore, the H3.3-G34R mutation leads to loss of H3K36me3 levels, highlighting another impact of epigenetic disruption in PHGG.
Acknowledgments

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</tr>
<tr>
<td>γH2AX</td>
<td>Phosphorylated (Ser139) histone variant 2AX</td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>Anaplastic astrocytoma</td>
<td></td>
</tr>
<tr>
<td>ACC</td>
<td>Animal care committee</td>
<td></td>
</tr>
<tr>
<td>AUP</td>
<td>Animal use protocol</td>
<td></td>
</tr>
<tr>
<td>ASH1L</td>
<td>Absent, small, or homeotic-like</td>
<td></td>
</tr>
<tr>
<td>ATM</td>
<td>Ataxia telangiectasia mutated</td>
<td></td>
</tr>
<tr>
<td>ATRX</td>
<td>Alpha-thalassemia/mental retardation syndrome X-linked</td>
<td></td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
<td></td>
</tr>
<tr>
<td>BER</td>
<td>Base excision repair</td>
<td></td>
</tr>
<tr>
<td>BLM</td>
<td>Bloom’s syndrome helicase</td>
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<tr>
<td>BRPF1</td>
<td>Bromodomain and PHD Finger Containing 1</td>
<td></td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
<td></td>
</tr>
<tr>
<td>CAF1</td>
<td>Chromatin assembly factor</td>
<td></td>
</tr>
<tr>
<td>ccRCC</td>
<td>Clear cell renal cell carcinoma</td>
<td></td>
</tr>
<tr>
<td>CENP-A</td>
<td>Centromere protein A</td>
<td></td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
<td></td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
<td></td>
</tr>
<tr>
<td>CtIP</td>
<td>C-terminal binding protein interacting protein</td>
<td></td>
</tr>
<tr>
<td>DAPI</td>
<td>4’,6-diamidino-2-phenylindole</td>
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DAXX  Death-domain associated protein
DIPG  Diffuse intrinsic pontine glioma
DMEM  Dulbecco’s modified eagle media
DNA  Deoxyribonucleic acid
DNA-PKcs  DNA-dependent protein kinase catalytic subunit
DR  Direct repair
DSB  Double stranded breaks
EGFR  Epidermal growth factor receptor
EXO1  Exonuclease 1
FBS  Fetal bovine serum
FITC  Fluorescein isothiocyanate
G1  Gap 1
G2  Gap 2
GAPDH  Glyceraldehyde 3-phosphate dehydrogenase
GBM  Glioblastoma multiforme
GTR  General tumour resection
Gy  Grays
H3K36me3  Histone H3 lysine 36 trimethylation
HeLa  Cell line derived from Henrietta Lacks’ uterine adenocarcinoma
HIRA  Histone chaperone histone regulator A
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<tr>
<td>HMTase</td>
<td>Histone methyltransferase</td>
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<tr>
<td>HBPCC</td>
<td>Hereditary nonpolyposis colon cancer</td>
</tr>
<tr>
<td>HPRT</td>
<td>Hypoxanthine-guanine phosphoribosyltransferase</td>
</tr>
<tr>
<td>HR</td>
<td>Homologous recombination</td>
</tr>
<tr>
<td>hTERT</td>
<td>Human telomerase reverse-transcriptase</td>
</tr>
<tr>
<td>IDH</td>
<td>Isocitrate dehydrogenase</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>iNHAs</td>
<td>Immortalized normal human astrocytes</td>
</tr>
<tr>
<td>IR</td>
<td>Ionizing radiation</td>
</tr>
<tr>
<td>JMJD2A</td>
<td>Jumonji Domain-Containing Protein 2A</td>
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<tr>
<td>KDM4A</td>
<td>Lysine Demethylase 4A</td>
</tr>
<tr>
<td>LEDGF</td>
<td>Lens epithelium-derived growth factor p75 splice variant</td>
</tr>
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<td>M</td>
<td>Mitosis</td>
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<tr>
<td>Mbp</td>
<td>Mega basepairs</td>
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<tr>
<td>MMR</td>
<td>Mismatch repair</td>
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<tr>
<td>MNNG</td>
<td>Methyl-nitro-nitrosoguanidine</td>
</tr>
<tr>
<td>MPG</td>
<td>3-methylpurine-DNA glycosylase</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>MRN</td>
<td>(Mre11)/Rad50/Nibrin1</td>
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<tr>
<td>MSI</td>
<td>Microsatellite instability</td>
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MYCN  V-Myc Avian Myelocytomatosis Viral Oncogene Neuroblastoma Derived Homolog

NER   Nucleotide excision repair

NHEJ  Non-homologous end joining

NSD   Nuclear receptor binding SET domain protein

NSG   NOD/SCID/Gamma immunodeficient mice

PARP  Poly-ADP-Ribose-Polymerase

PBS   Phosphate buffered saline

PCNA  Proliferating cell nuclear antigen

PDGFRA Platelet-derived growth factor receptor, alpha polypeptide

pHGA  Pediatric high-grade astrocytoma

PHGG  Pediatric high-grade glioma

PIP   PCNA-interacting peptide

PTEN  Phosphatase and tensin homolog

PTM   Post-translational modification

PVDF  Polyvinyl difluoride

PWWP  Pro-Trp-Trp-Pro

Rb    Retinoblastoma

RNA   Ribonucleic acid

RNAPII RNA polymerase II holoenzyme

ROS   Reactive oxygen species
<table>
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<tr>
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<tr>
<td>RPA</td>
<td>Replicating protein A</td>
</tr>
<tr>
<td>RT</td>
<td>Radiation therapy</td>
</tr>
<tr>
<td>S</td>
<td>Synthesis</td>
</tr>
<tr>
<td>SCID</td>
<td>Severe combined immunodeficiency</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate – polyacrylamide gel electrophoresis</td>
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<tr>
<td>SET2</td>
<td>Su(var)3-9, Ez, Trithorax domain-containing 2</td>
</tr>
<tr>
<td>SETD2</td>
<td>SET domain containing 2</td>
</tr>
<tr>
<td>SETMAR</td>
<td>SET domain and mariner transposase fusion protein</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>shRNA</td>
<td>Short hairpin RNA</td>
</tr>
<tr>
<td>SMYD2</td>
<td>SET and MYND domain containing 2</td>
</tr>
<tr>
<td>SSA</td>
<td>Single-strand annealing</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered saline with 0.1% Tween 20</td>
</tr>
<tr>
<td>TP53</td>
<td>Tumour protein p53</td>
</tr>
<tr>
<td>TRITC</td>
<td>Tetramethylrhodamine</td>
</tr>
<tr>
<td>UHN</td>
<td>University Health Network</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>V</td>
<td>Volts</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
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</table>
XRCC4  X-ray repair cross-complementing protein 1

ZMYND11  Zinc Finger MYND-Type Containing 11

µg  Microgram

µm  Micrometer
Chapter 1 Introduction

1 Cancer Biology

1.1 Hallmarks of Cancer

Cancer is a disease caused by the uncontrolled and abnormal division of cells in the body. The multistep process of tumorigenesis in humans drives the transformation of normal human cells into a neoplastic and malignant state [1]. The eight biological acquired capabilities of cancer during the development of human tumours include self-sufficiency in growth signals, insensitivity to anti-growth signals, tissue invasion and metastasis, limitless replicative potential, sustained angiogenesis, evading apoptosis, reprogramming of energy metabolism, and evading immune destruction [1, 2]. A normal cell will acquire some or a combination of these complementary traits to allow for tumour growth and metastatic dissemination. Additionally, there are two underlying characteristics of neoplasia that facilitate the acquisition of the listed cancer hallmarks: genome instability and mutation, and tumour-promoting inflammation. The continued study of cancer biology will provide novel therapeutic targeting towards the hallmarks of cancer, which is so necessary for tumour growth and progression.

2 Pediatric High-Grade Gliomas

2.1 Background Information

Brain tumours are the leading group of solid tumours in children and are second behind leukemia as the most commonly diagnosed childhood cancer in Canada [3]. The Canadian Cancer Society has ranked cancers of the central nervous system (CNS) and brain tumours as the most common cause of mortality in children due to cancer under the age of fourteen [3]. Pediatric high-grade gliomas (PHGG) represent 8-12% of all primary CNS tumours in the pediatric population [4]. PHGG arise from astrocytes, oligodendrocytes, oligoastrocytes, and are classified as grade III or IV tumours by the World Health Organization (WHO) based on cytologic and histologic features [5]. Anaplastic astrocytomas (AA) (WHO grade III) are infiltrative tumours with nuclear atypia and high mitotic activity [5]. Glioblastomas (GBM) (WHO grade IV) have the same characteristics as AA in addition to pseudo-palisading necrosis and/or florid microvascular proliferation [5]. PHGG are histologically heterogeneous and
highly malignant tumours that are commonly located in the supratentorial regions of the cortex and thalamus, but are also found in the brainstem and spine [6]. AA and GBM have poor 5-year survival outcomes of 11% and 1.2% respectively [5].

Children diagnosed with PHGG develop the same symptoms as CNS tumours as a result of increased intracranial pressure that includes persistent headaches, behavioural changes, morning nausea or emesis, diplopia, and papilledema [7]. Location-specific symptoms include focal motor deficits, hemiplegia, pyramidal tract findings, dysmetria, and chorea [7]. Various imaging tools are used for diagnosis such as non-contrast computerized tomography scan, or magnetic resonance imaging (MRI). However a biopsy or surgical resection is recommended to diagnose a specific histology and establish a diagnosis. The mainstay of therapy for PHGG is maximal safe tumour resection (gross total resection, GTR) followed by focal radiation therapy (RT) in combination with chemotherapy [7, 8]. Due to the deleterious effects of radiation, only children older than the age of three receive RT. Patients typically receive 50-60 Gy in daily doses of 180-200 cGy over 6 weeks. Several clinical trials (CCG-943, CCG-945, ACNS-0216) have investigated different combinations of chemotherapeutic agents (vincristine, prednisone, lomustine, hydroxyurea, cisplatin, cytarabine, procarbazine, temozolomide) with focal RT, however no survival benefit was observed [9-11]. There is a need for more effective therapeutic regimens to enhance the dismal survival rates of PHGG.

2.2 Genetic and Molecular Alterations

A proposed strategy to improving survival outcome is to better understand tumour biology. There has been an increase of knowledge of the biologic, molecular, and genetic characteristics of PHGG in the past decade. PHGG and adult high-grade gliomas are distinct molecular entities, even though they are indistinguishable under the microscope [12]. These differences are observed through global gene expression and copy number aberrations [13-15].

Amplification of the oncogene epidermal growth factor receptor, (EGFR), is one of the most common genetic alterations in adult high-grade gliomas [16, 17], seen in 20-45% of adults [18, 19]. In contrast, EGFR protein overexpression is present in PHGG, but genetic amplification is rare (6%) [16, 20, 21]. The recurrent, gain of function mutation in isocitrate dehydrogenase 1/2 (IDH1/2) gene, IDH1/2, is prevalent in adult lower grade glioma, while rare in PHGG [14, 22]. Deletions and loss of function mutations in the tumour suppressor phosphatase and tensin
homolog (PTEN) gene, PTEN, are more frequent in adult patients (82%) than in children (35%) [14]. Among the pediatric population, amplification of the platelet-derived-growth-factor-receptor-α (PDGFRA) gene, PDGFRA, was the most common structural genomic event (8-39%) and has prognostic significance [14, 15, 23, 24]. 10% of PHGG show a point mutation in the proto-oncogene B-Raf, BRAF, known as V600E where a valine (V) is substituted by glutamate (E) at codon 600 [25]. This mutation is also associated with tumours that have the PDGFRA amplification [8]. Additionally, deletions and loss of function mutations in TP53 are more common in PHGG at 34-54% frequency compared to 20-32% mutated in adult GBM [18, 21, 24]. In comparison to adult high-grade gliomas, PHGG display many fewer copy number alterations [8]. PHGG show chromosomal gains (1p, 2q, 21q) and losses (6q, 4q, 11q, 16q) [14, 23, 26, 27], with a significantly higher incidence of gains at 1q, and losses at 4q and 16q compared to the adult counterpart [14, 21, 23, 27]. Overall, PHGG contains much fewer focal amplifications and homozygous deletions than adult high-grade gliomas [15]. Altogether, this suggests that there may be an age dependent selection for molecular alterations in various signaling pathways based on gene expression and copy number analysis.

In the first genome-wide sequence analysis of pediatric GBM where 48 PHGG were whole exome sequenced, somatic mutations in the H3.3-ATRX-DAXX chromatin remodeling pathway was found in 44% of all cases [21]. Mutations in the H3F3A gene, which encodes histone H3 variant H3.3 was observed in 31% of tumour samples, and appears to be highly specific to GBM. However the presence of H3F3A mutation was also detected at low frequency in thalamic low-grade pilocytic astrocytoma [28]. Loss of function mutations in the alpha-thalassemia/mental retardation syndrome X-linked (ATRX) and death-domain associated protein (DAXX) were also seen in 31% of tumours. Additionally, TP53 mutations accounted for 54% of samples and were found at a higher percent in tumours with the H3F3A and/or ATRX mutations. This study highlighted the importance of the chromatin structure and how defects in this pathway can contribute to the development of pediatric GBM [21].

3 Epigenetic

3.1 Histone Biology

Epigenetics is commonly defined as inherited and reversible modifications to nucleotide or chromosomes that do not change the genome but can alter gene expression. Therefore, the same
Eukaryotes have lengthy genomic DNA that is packaged into chromatin found inside the nucleus of a cell. Chromatin is made up of basic repeating units of nucleosomes, which consist of two copies each of the four core histone proteins H2A, H2B, H3, and H4, forming an octameric protein complex around which 147 base pairs of DNA are wrapped. This nucleosome core particle then associates with a linker or H1 histone molecule to form a nucleosome. DNA methylation is an epigenetic mechanism that represses gene transcription via the addition of methyl (CH$_3$) groups to DNA in a promoter region. In somatic cells, methylation of the 5-carbon of the cytosine ring (5-methylcytosine) occurs at CpG dinucleotide sites, where cytosine nucleotide is located beside a guanine nucleotide. Methylated CpG sites resulting in silencing of genes. The euchromatin and heterochromatin states can be determined by post translation modifications (PTM) of N-terminal tails of the core histones. PTMs involve covalent attachment of different chemical groups to the histone amino acid residues via protein histone “writers” or removed by histone “erasers”. PTMs are thought to underlie the histone code, which represents the possible combinations of modifications, resulting in unique biological outcomes. PTMs can include DNA methylation, phosphorylation, acetylation, ubiquitylation, sumoylation, biotinylation, and citrulination. Histone “readers” are proteins that recognize these histone modifications to regulate transcription. Chromatin remodelers and chromatin-associated proteins further facilitate the landscape of the chromatin structure.

### 3.2 Histone H3

Higher eukaryotes possess several variants of the core nucleosomal protein histone H3 including H3.1, H3.2, H3.3, and CENP-A, a centromere-specific H3 variant protein. Histone H3.1 and H3.2 are known as the DNA synthesis-coupled histones or replication-dependent histones, because they are synthesized during the S phase of the cell cycle and incorporated into chromatin during de novo synthesis [29]. Histone H3.1 is encoded by ten genes located on chromosome 6, while histone H3.2 is encoded by three genes; $HIST2H3A$, $HIST2H3C$, $HIST2H3D$. Histone H3.3, the replacement histone or replication-independent histone, is expressed throughout the cell cycle, and in quiescent cells [30]. Genes $H3F3A$ on chromosome 1, and $H3F3B$ on chromosome 17 encode this histone. Histone H3.2 is distinguished from H3.1 by a single amino acid substitution from cysteine to serine at position 96, whereas histone H3.3 differs from H3.1 by four additional amino acid substitutions from alanine to serine at position...
31, serine to alanine at position 87, valine to isoleucine at position 89, and methionine to glycine at position 90 [31]. The H3 variants have distinct genome localization patterns based on their DNA synthesis dependency. Histone H3.1 is incorporated universally during S phase by chromatin assembly factor (CAF1) that is recruited by proliferating cell nuclear antigen (PCNA) to newly replicated DNA [32, 33]. Meanwhile, H3.3 is deposited on transient nucleosome-free DNA sequences during transcription and DNA repair, and replaces evicted nucleosomes [34]. H3.3 is enriched in the pericentromeric and subtelomeric regions of the heterochromatin.

Deposition of H3.3 into different regions is determined by binding of histone chaperones: histone chaperone histone regulator A (HIRA) chaperone target genic loci [35], and DAXX in cooperation with ATRX target heterochromatic loci [36]. H3.3 is deposited in transcribed regions more frequently than H3.1 or H3.2. The accumulation of histone H3.3 at actively transcribed regions makes it a marker for areas of high transcriptional activity [37].

3.3 Histone Mutation and Cancer

Genome stability and transcriptional control is highly regulated by chromatin, as such cancer genomes have frequent mutations in chromatin-modifying enzymes [38, 39]. Since histones package DNA into the compacted chromatin, they regulate the accessibility and expression of genetic information, influencing a normal or diseased state [40]. The majority of histone protein sequence is identical in organisms ranging from yeast to humans. Histone proteins are highly conserved in eukaryotes, and its expression is ubiquitous within a cell. Which is why histone-encoding genes were thought to be unlikely targets for mutations. The first report of histone mutations as drivers of human disease was the co-discovery of histone H3 mutations in pediatric GBM in January 2012. Both studies show that patients with non-brainstem pediatric GBM and DIPG have recurrent somatic heterozygous mutations (K27M and G34R/V) in the gene encoding the histone variant H3.3 (H3F3A) after sequencing their tumour cells [21, 41]. These mutations are mutually exclusive in tumours, with distinct gene expression and DNA methylation profiles [21, 42]. One of these groups also found heterozygous mutations in the gene encoding the histone variant H3.1 (HIST1H3B) in DIPG [41]. The mutations result in amino acid substitutions at only two locations on the N-terminal histone tail: K27M in which lysine at position 27 is substituted by methionine, and G34R/V in which glycine at position 34 is substituted by arginine or valine [21, 41]. Wu et al. found at least 78% of DIPGs and 22% of non-brainstem GBM has the K27M mutation in H3F3A or HIST1H3B, while 14% of non-
brainstem GBM has the G34R mutation in *H3F3A* [41]. Sequencing of patient tumour DNA with the histone mutant matched with normal DNA revealed the somatic nature of the mutation in all cases [41]. These mutations are anatomically distinct, which could be explained by different cell types that were affected during brain development. Additionally, H3.3 mutant tumours are mutually exclusive with *IDH1* and *IDH2* mutations [21]. Histone H3 mutation was also found at a high frequency (90%) in almost all cases of chondroblastoma (K36M in *H3F3B*), and (92%) giant cell tumours of the bone (G34W/L in *H3F3A*, glycine to tryptophan or leucine) in adolescents and young adults [43]. The group found that 95% of chondroblastomas contained the K36M mutation, with most occurring in *H3F3B*. This amino acid substitution affected the SETH2 target site and other H3K36 methyltransferases. More than 90% of giant cell tumours of the bone contained the G34W, and to a lesser extent the G34L substitution in *H3F3A*. These skeletal tumours are diploid and Wild-Type (WT) for p53, having a more stable genome compared to PHGG. Low frequency of the H3.3 mutation was also seen in osteosarcoma (G34R in *H3F3A* and *H3F3B*), conventional chondrosarcoma (K36M in *H3F3A*), and clear cell chondrosarcoma (K36M in *H3F3B*) [43]. Surprisingly, the K27M mutant is not found in bone or cartilage tumours, and no K36M mutation is seen in GBM.

### 3.3.1 H3.3-G34R Mutation

15% of cortical high-grade gliomas have the H3.3-G34R mutation, or the less frequent H3.3-G34V mutation [21]. Almost all H3.3-G34R/V mutations are found in *H3F3A*, with a small percentage seen in *H3F3B*. In contrast to the majority of H3.3-K27M mutations found in DIPG, the H3.3-G34R mutations are found exclusively in the cerebral hemisphere, with none appearing in DIPG. Additionally, the age of diagnosis for H3.3-G34R tumours (median age 13-14 years) is older than that of H3.3-K27M (median age 6-7 years) [21, 41, 44]. Survival amongst these patients is also longer (median of 24 months) than those with H3.3-K27M mutant tumours [21, 41, 44]. This highlights a key difference in disease onset and survival between cortical versus mid-line (spinal cord, thalamic, brainstem, pons) HGG. The G34R/V mutation is also seen in adult HGG, with patients 30-40 years old [42]. Additionally, this mutation is associated with global DNA hypomethylation in telomeric regions [42]. Almost 100% of all H3.3-G34R tumours showed concurrent mutations in ATRX/DAXX and p53, and display alternate lengthening of telomeres (ALT) [21].
My thesis work and published reports suggest that H3.3-G34R can have an impact on H3K36 methylation, however, it remains unknown how and why the H3.3-G34R mutation impacts this neighbouring residue. Glycine is the smallest amino acid, and would not be expected to exert any steric effects via charge or size on neighbouring modifications. However, mutation to arginine replaces a small, uncharged amino acid with a larger basic and charged residue that could potentially impact the activity and accessibility of histone writers, erasers, and readers targeting neighbouring K36 (since G34 is not known to be directly posttranslationally modified). Although there is no published data to support this, protein interactome analysis conducted in our laboratory (Figure 2) demonstrates that indeed some H3K36 modifying enzymes have an altered interaction towards H3.3-G34R vs. H3.3-WT. While SETD2 did not appear as having differential associations with H3.3-G34R mutant, NSD1 showed lost interaction with the mutant cells. NSD1 and NSD2 is another HMT that mediates the formation of H3K36me1 and H3K36me2. This demonstrates that binding of K36 HMTs can be indirectly affected by the H3.3-G34R mutant since the catalytic mechanism is highly conserved among SET domain-containing HMTs. Mutation directly at the K36 residue, such as H3.3-K36M mutant protein, causes global reduction of H3K36 methylation by inhibition of two H3K36 methyltransferases MMSET and SETD2 [45]. Methyltransferase assays with peptides or purified nucleosomes of H3.3-K36M/I or H3.1-K36M/I revealed potent inhibition of NSD2 and SETD2 activity [46]. Given that H3-K36 mutations inhibit the activity of H3K36 methyltransferases, it is reasonable to hypothesize that the H3.3-G34R mutation will exert a similar effect to a lesser extent.

Unlike the H3.3-K27M mutation where global levels of H3K27me3 are affected [47], the H3.3-G34 mutation exerts a localized effect on H3K36 methylation where decreased levels are observed on the same and nearby nucleosomes [47-49]. This histone mark was unaffected on total cellular levels. Additionally, it appears that other histone methylation marks like H3K27me1, H3K27me2, H3K27me3, H3K4me3 and H3K9me3 are generally unchanged at a global level [47, 49]. Unlike the three methylation states of H3K9 and H3K27 that have a spreading mechanism by EZH2 to neighbouring nucleosomes, it is possible that H3K36me3 have restricted spreading. As a result, the local decrease in H3K36me3 on a H3.3-G34R mutant nucleosome by decreased SETD2 activity may have no effect on H3.3-WT methylation states. This is supported by the fact that a global decrease in H3K27me3 levels are observed in H3.3-
K27M mutants, which account for 10% of total H3.3 [47]. While global H3K36me3 levels are unaffected in H3.3-G34R mutants, which may be attributed to the lack of a spreading mechanism by SETD2.

The most comprehensive study of the role of H3.3-G34 mutant was completed in the pediatric GBM KNS42 cell line with H3.3-G34V mutation in H3F3A [48]. Bjerke et al. demonstrated that the H3.3-G34 mutation results in altered gene transcription with changes to RNA polymerase II association and levels of H3K36 methylation. The transcriptional program induced reflects a developing forebrain and markers for stem cell maintenance, cell fate decisions, and self-renewal. ChIP-seq analysis revealed more than 150 genes with differentially enriched H3K36me3 peaks in the H3.3-G34 mutant pediatric GBM compared with H3F3A-WT SF188 pediatric GBM. Interestingly, the mutation induced transcript level increase in V-Myc Avian Myelocytomatosis Viral Oncogene Neuroblastoma Derived Homolog (MYCN), a potent oncogene, by two- to threefold over H3.3-WT. This profound upregulation of oncogene transcription may influence the tumorigenic properties of the H3.3-G34 mutant. The oncogenic activity of H3.3-G34R mutation may further be achieved by disrupting H3K36 methylation levels to activate various oncogenes to change gene expression. The altered effect may contribute to chromosomal instability and defects in DNA damage repair pathways such as double strand break (DSB) repair, and mismatch repair (MMR).
Figure 1 – *H3F3A* H3.3-G34R mutation. H3.3-WT histone tail have trimethylation at lysine 36 residue (H3K36me3). H3.3-G34R mutant histone tail have glycine substitution by arginine at position 34. This mutation leads to disruption of the H3K36me3 mark.
Our lab has generated copy number data showing H3.3-G34R mutated PHGG have more genomic instability than other mutations [Siddaway and Hawkins, unpublished data]. Samples from the German Cancer Research Center (DKFZ) [42] and Nada Jabado’s group [50] was combined and used to infer copy number alterations from Illumina 450K array. Patients were sorted into the following groups: H3-K27M, H3-G34R, IDH-mutant, and WT for H3 and IDH, and the absolute number of alterations per sample, and the length in mega basepairs (Mbp) of those alterations were calculated. H3-G34R patients have significantly more copy gains and more Mbp gained than any group (Figure 2A, D). There is no significant difference in the number of copy losses and Mbp deletions between groups, however there is a trend that the H3-G34R group has slightly more losses and deletions than others (Figure 2B, C, E). On top of that, members of the lab have generated interactome data for H3.3-WT and H3.3-G34R, showing the mutant has a lost interaction with several DNA repair pathway proteins (Figure 2F) [Siddaway and Hawkins, unpublished data].

MSH2 is a DNA mismatch repair (MMR) protein which heterodimerizes with MSH6 to form the MutSα mismatch repair complex which recognizes base substitutions and small-loop mismatches. BRCA1 participates in DSB repair through the homologous recombination (HR) mechanism. UIMC1 and FAM175A are both components of the BRCA1-A complex. The BRCA1-A complex recognizes and binds K63-linked ubiquitinated H2A and H2AX, recruiting the BRCA1-BARD1 heterodimer to DNA DSBs for repair [51]. MSH2 and MSH6 are among the many DNA damage repair proteins that associate with BRCA1, forming a large complex named BASC (BRCA1-associated genome surveillance complex) [52]. Through the BASC, BRCA1 may play a role in transcription-coupled repair pathways and a sensor for DNA damage. NSD1 is a HMT that forms H3K36me2, which recruits KU70 and the MRN complex to sites of DNA lesions for DSB repair. Based on the implicated proteins from the BioID experiments, we can speculate that H3.3-G34R tumours will have deficient MMR and DSB recognition abilities. This suggests that these two specific DNA repair pathways are compromised or affected by this mutation, and that H3.3-G34R tumours will have higher incidences of DNA damage. This is described in further detail in Introduction 4.5.1 – 4.5.2.
Figure 2 – H3.3-G34R mutated PHGG is associated with genomic instability. Absolute copy number gains per sample, significance at $p=0.0005$ for G34 vs. all other groups (A). Absolute copy number losses per sample (B, C). Mbp gains per sample, significance at $p=0.0001$ for G34 vs. all other groups (D). Mbp deletions per sample (E). Heatmap illustrating lost interaction between several DNA repair proteins and H3.3-G34R compared to H3.3-WT overexpressing protein identified using BioID protein interactome experiment. Z-scores were calculated to normalize peptide abundance for each DNA repair protein, which have a mean of zero and a standard deviation of one. Relative lost interactions are represented in blue and relative gained interactions are represented in red [Siddaway and Hawkins, unpublished data] (F).
3.3.2 H3K36 Methylation

The lysine residue at the 36th amino acid position of the histone H3 tail (H3K36) can undergo various post-translational modifications including mono-, di-, or tri-methylation (H3K36me1, H3K36me2, H3K36me3), and antagonistic acetylation (H3K36ac). There is one enzyme, the methyltransferase SET (Su(var)3-9, Ez, Trithorax) domain-containing 2 (Set2), that completes all three methylation events at H3K36 in yeast, which is a contrast to the eight different mammalian histone methyltransferases (HMTase) that are responsible for performing methylation of H3K36 [53, 54]. The eight human HMTases, all of which have a catalytic SET domain, include nuclear receptor binding SET domain protein 1, 2, 3 (NSD1, NSD2, NSD3), SETD2, absent, small, or homeotic-like (ASH1L), SET domain and mariner transposase fusion protein (SETMAR), SET and MYND domain containing 2 (SMYD2), and SETD3 [54]. Some of these enzymes have been linked to cancer: SETD2 mutation is found in breast cancer and renal carcinoma [55, 56], NSD2 is a tumour suppressor, and is implicated in myeloma pathogenesis [57].

S-Adenosyl methionine (SAM), or S-Adenosyl-L-methionine, is a cofactor for histone methyltransferases, and a major biological methyl group donor. All three classes of HMTs, SET domain lysine methyltransferases, non-SET domain lysine methyltransferases, and arginine methyltransferases, uses SAM to transfer methyl groups [58]. The chemically reactive methyl group attached to the sulfur atom of SAM is donated to acceptor substrates such as nucleic acids, proteins and lipids. SAM is produced in the cytoplasm from adenosine triphosphate (ATP) bound to the essential amino acid methionine, catalyzed by the enzyme methionine adenosyltransferase (MAT). Although SAM is made in all mammalian cells, the liver is the primary site of synthesis and degradation. The biosynthesis of SAM is controlled by intracellular energy status and nutrient metabolism, which regulates ATP levels. High intracellular energy levels lead to elevated SAM concentrations, which in turn result in high DNA methyl transferase (DNMT) and HMT activity [59]. While SAM has not been examined in brain cancer, altered tumour metabolism is a known hallmark of GBM. Compared to normal brain cells, GBM cells have mitochondrial abnormalities including higher levels of proteins involved in oxidative damage and reduced levels of energy metabolism proteins [60]. Various studies have also identified somatic mitochondrial DNA alterations including point mutations, deletions, copy number changes in the brain and other CNS cancers [61]. The differences in
energy status of brain tumours may alter the ATP available for SAM synthesis, impacting SAM levels for methylation of histone residues but this has not yet been tested. In addition to energy metabolism levels, MAT is also a key regulator of SAM abundance. Nuclear accumulation and distribution of MAT has been shown to correlate with increased H3K27me3 levels [62]. This shows that MAT is required to guarantee SAM availability for specific nuclear methylation. A cell’s doubling time was found to be correlative with MAT activity, where short doubling times have a much higher MAT activity [63]. The same group identified MAT activity to be higher in malignant tissue compared to normal samples. Lung cancer tissue showed a 13-fold increase than the MAT activity of normal lung. Additionally, patients with hepatocellular carcinoma have induced MAT2A and MAT2β, two genes encoding MAT, giving cells a proliferative advantage [64]. Furthermore, the amino acid threonine was found to influence SAM synthesis in mouse embryonic stem cells because it provides the cellular glycine and the acetyl-coenzyme A (CoA) required for SAM formation [65]. The group found that decrease in threonine levels or threonine dehydrogenase resulted in decreased SAM abundance, leading to a reduction in H3K4me3. This could mean that SAM regulation on specific histone methylation marks may impact stem cell fate and the development window. Although this phenomenon has not been studied in pediatric brain tumours, this may partially explain the differential histone mutation localization within the brain and the observed histone methylation levels. Interestingly, SAM was found to be necessary for the competitive inhibition and stable interaction of H3K9 methyltransferase G9a by the mutant histone H3K9M [66]. Tumours containing the lysine to methionine (K-to-M) mutation on histone H3, such as K27M and K36M, display global loss of lysine histone methylation marks due to the accumulation and inhibition of SET domain methyltransferases bound to SAM. This mechanism may contribute to the global decreased methylation of H3K27 and H3K36 observed in pediatric HGG, which remains to be elucidated.

H3K36 methylation is most commonly associated with transcriptional activation. SET2 proteins bind RNAPII in humans to target the H3K36 mark [67, 68]. During elongation, Set2 in yeast and human systems associates with hyperphosphorylated RNAPII to deposit the trimethyl group onto H3K36 [69, 70]. Besides gene activation, H3K36 methylation is also implicated in transcriptional repression, dosage compensation, and DNA replication, recombination, and repair. For dosage compensation, H3K36 methylation is required for coordination between methylation and acetylation in D. melanogaster [71]. Besides that, H3K36 methylation
mediated Set2 transcription repression of the lacZ reporter has been found [72]. This histone mark also regulates replication origin firing in budding yeast, where SET2 deletions delayed Cdc45 loading at origins [73]. The role that H3K36 methylation plays in DNA repair is discussed further in **Introduction 4.5.1 – 4.5.2**.

### 3.3.3 H3K36me3 and SETD2

SETD2, the only H3K36 trimethyltransferase in humans, is mutated in 15% of PHGG and in 8% of adult HGG [74]. SETD2 mutations were initially thought to be mutually exclusive with **H3F3A** mutations, but not with **IDH1** or **IDH2** mutations in HGG [74]. However, it was later discovered alongside H3.1-K27M mutated DIPG [75]. Tumours with SETD2 missense or truncating mutations display a significant decrease in H3K36me3 levels, classifying this mutation as loss-of-function [74]. It is speculated that the loss of the tumour suppressing abilities of SETD2 may lead to defective chromatin template for transcription and DNA repair. Tumours have imitated the loss of SETD2 function by H3.3-K36M mutation, or through H3.3-G34 mutation as a neighbouring residue geographically nearby, altering the binding of histone writers or readers to the H3K36 mark [21, 43, 76]. As previously mentioned, nucleosomes with the H3.3-G34R/V mutant exhibited lowered H3K36me2 and H3K36me3 levels on the same histone tail, with no change on global levels [48, 76]. This was found to be associated with a distinct expression signature and H3K36 methylation profile [48]. This may be due to the aberrant modulation of reader proteins that normally get recruited to sites of H3K36 methylation. Zinc Finger MYND-Type Containing 11 (ZMYND11) is a tumour suppressor protein that specifically reads H3K36me3, but its localization to the histone mark is interrupted in the presence of the H3.3-G34R/V mutation [77]. Another explanation could be attributed to H3K36 methylation status in relation to genomic stability.

# 4 DNA Damage

## 4.1 Sources of DNA Damage

The primary source of DNA damage that leads to the development of human cancer can be classified into two groups: endogenous and exogenous factors. Endogenous metabolic and biochemical reactions include hydrolysis, depurination, depyrimidination, deamination, and alkylation [78]. Free radical species generate reactive oxygen species (ROS) as by-products of
normal metabolic processes modify bases through oxidation. Genomic mutations may also be introduced as DNA replication errors during the S phase. Incorrect nucleotide incorporation, strand slippage, and strand mispairing are examples. Exogenous factors include physical or chemical agents. Physical mutagens include ultraviolet (UV) radiation, ionizing radiations (X-rays) which create ROS. Chemical compounds include platinum-based compounds, intercalating agents, DNA alkylating agents, and psoralens [78].

### 4.2 DNA Damage Repair

There are five main mammalian DNA damage repair mechanisms: direct repair (DR), base excision repair (BER), nucleotide excision repair (NER), double-strand break repair (DSB) which includes homologous recombination (HR), non-homologous end joining (NHEJ), and mismatch repair (MMR) [79]. The two DR mechanisms utilize either DNA photolyase to photoreverse UV-induced pyrimidine dimers, or methylguanine DNA methyltransferase which removes O^6^-methyl group from O^6^-methylguanine. BER repairs chemically altered bases by removing small, non-structural distorting base lesions from the DNA double helix. NER removes larger, bulky helix-distorting lesions. MMR and DSB repair will be discussed further below.

### 4.3 Mismatch Repair

The mechanism of DNA MMR is a highly conserved biological phenomenon that corrects DNA mismatches generated during DNA replication and recombination. This diminishes the number of errors associated with replication and reduces the occurrences of spontaneous mutations from accumulating. The MMR system also plays a role in cellular apoptosis and cell cycle arrest should a cell become so severely damaged beyond repair [80]. DNA MMR is a strand-specific process that consists of three steps: initiation, excision, and finally resynthesis. The newly synthesized daughter strand, or lagging strand will include errors that will need to be repaired. In humans, the DNA mispairs are recognized by hMutSα or hMutSβ complex, which is formed when hMSH2 heterodimerizes with hMSH6 or hMSH3, respectively. These complexes have ATPase activity where hMutSα recognizes base-base mismatches, mispairs of 1-2 nucleotides in length, and single base loops. On the other hand, hMutSβ recognizes insertions and deletion loops of more than one base. hMLH1 heterodimerizes with hPMS2 to form hMutLα which interacts with hMutSα or hMutSβ, enhancing the mismatch recognition. Other proteins
involved in the MMR process are recruited to the site of DNA damage. The replication sliding clamp, PCNA, is responsible for MMR initiation and DNA re-synthesis. It acts as a processivity factor for DNA polymerase δ by encircling the DNA, creating a scaffold to recruit additional proteins. PCNA interacts with MSH3 and MSH6 via the PCNA-interacting motif, PCNA-interacting peptide (PIP) box. The damaged strand is excised by exonuclease 1 (ExoI), removing the incorrect nucleotides from the single-stranded DNA. The new strand of DNA is resynthesized by DNA polymerase δ and ε, responsible for the lagging and leading strand synthesis respectively. Once the new double-stranded DNA is made, DNA ligase I completes the MMR reaction through nick ligation.
Figure 3 – DNA mismatch repair. Initiation of MMR through recognition of mismatches by MutSα or MutSβ complex, and recruitment of MutLα (A). Excision and resynthesis of new DNA strand by additional DNA repair proteins, see text for more detail (B). (Figure adapted from: Helleman et al. Mismatch repair and treatment resistance in ovarian cancer. BMC Cancer. 2006; 6: 201).
Insertion-deletion loops are a result of microsatellite instability (MSI), which are gains or losses of short tandem repeats of one to six base pairs in the DNA sequence. The mutator phenotype caused by MMR deficiency is detected as MSI, and is considered a hallmark of impaired MMR [81]. MMR deficiency is linked to hereditary and sporadic human cancers such as hereditary nonpolyposis colon cancer (HNPCC), and other colorectal cancers [82]. Homozygous mutations in mismatch repair genes leads to constitutional MMR deficiency syndrome and are characterized by early onset malignancies such as lymphoma, leukemia, GBM, supratentorial primitive neuroectodermal tumour, and medulloblastoma [83].

4.4 Double Strand Break Repair – Homologous Recombination & Non-Homologous End Joining

HR or NHEJ are the two principle repair pathways mediating dSBs in eukaryotic cells. HR is a homology driven repair pathway because it uses a sister chromatid or the opposite strand of the DNA as the template to from which to build the new strand. HR is made up of three steps: strand invasion, branch migration, and Holliday junction formation [79]. Bloom’s syndrome helicase (BLM) unwinds the DNA strands upon detection of DSB to allow for 5’ to 3’ endoprocessing by the meiotic recombination 11 (Mre11)/Rad50/Nibrin1 (NBS1) (MRN) complex at the termini of the DSB before strand invasion initiation. The Holliday intermediate is created when the two recombining duplexes covalently join together by single-strand crossovers. Mus81-MMS4 resolvases cleave the Holliday junction to create two duplexes. An alternative pathway in HR is the single-strand annealing (SSA) repair mechanism, which leads to the digestion of the duplex ends by an exonuclease. Homologous regions are exposed on both sides, and the duplex ends are paired and ligated. The MRN complex, Rad52, and replicating protein A (RPA) are involved in SSA. Since HR requires a double-stranded copy of the DNA, this repair pathway occurs during late S or G2 phase.

In contrast, NHEJ does not require a template and tethers the broken DNA strands to one another at random damaged DNA regions of homology. The Ku70/86 heterodimer binds the two broken DNA ends and recruits the catalytic subunit of the DNA-dependent protein kinase (DNA-PKcs) [84]. The ATM-mediated phosphorylation or autophosphorylation of DNA-PKcs leads to a conformational change in which the DNA ends becomes accessible to other repair enzymes. The classical processing resects single-strand overhangs, and a subset of DSB
processing joins one end of the single-strand overhang with the opposite DNA [85]. Finally, DNA ligase 4 and its cofactor X-ray repair cross-complementing protein 1 (XRCC4) ligate the two strands together.

4.5 Histone H3 and Genome Stability

It has been suggested that PHGG are defective in DNA damage repair due to the numerous somatic coding mutations found [24, 86]. PHGG has a median of 15 non-synonymous coding mutations, which is greater than other pediatric cancers such as Ewing’s sarcoma, malignant rhabdoid tumour, or pediatric low-grade glioma [86]. PHGG hypermutator tumours with germline mutations in mismatch repair genes harboured the highest mutational burden with a median of 6810 non-synonymous coding mutations, which is the highest among pediatric and adult cancer types [75, 86]. Histone H3.3 is involved in transcription restoration by being deposited in UVC-damaged regions [87]. As soon as DNA damage is detected through a series of ubiquitylation events, HIRA promotes the restarting of transcription by accumulating at sites of UVC irradiation to deposit newly synthesized H3.3 histones. Furthermore, chicken bursal lymphoma DT40 cells that lack histone H3.3, and cell with H3.3-K27 and H3.3-G34 mutations are hypersensitive to UV light [88]. This may be a defect from an ineffective nucleotide excision repair pathway. On top of that, histone H3.3 depletion is associated with a failure to maintain replication fork progression after UV damage. These deficiencies are restored upon reintroduction of histone H3.3, which requires specific residues in the α2 helix. Many cell types use a HIRA-dependent H3.3 incorporation system as the most dominant form of histone H3 to maintain gene expression [89-91]. HIRA, and consequently histone H3.3, is an important factor for restoring chromatin structure and function due to genotoxic stress. Another study identified H3K36 methylation is a novel regulator of the replication factor Cdc45 and the timing of binding with DNA replication origins during S phase [73]. A model was proposed in which the time of Cdc45 association was sped up by H3K36me1 with histone acetylation, and was slowed down by H3K36me3 and histone deacetylation.

4.5.1 H3K36 methylation and DNA Double-Strand Break Repair

A DNA DSB repair function is associated with transcription-coupled Set2 H3K36 methylation status in yeast [92]. Cells depleted of Set2 and H3K36me are hypersensitive to site-specific DSBs, and was unable to active DNA-damage checkpoint. Set2 and H3K36me3 enrichment
was found at DSBs, and Set2 loss led to altered chromatin structure and abnormal resection near break sites during G1 phase. H3K36 methylation was shown to play a role in HR by binding to the PWWP domain of Lens epithelium-derived growth factor p75 splice variant (LEDGF) [93]. LEDGF preferentially interacted with H3K36me2 and H3K36me3, and to a lesser degree with H3K4me1. The same histone marks also efficiently immunoprecipitated with LEDGF. Depletion of LEDGF was found to impair the recruitment of C-terminal binding protein interacting protein (CtIP) that is involved in DNA-end resection of DSB in the HR repair pathway. SETD2 is also required for HR through the promotion of RAD51 presynaptic filament formation [94]. ccRCC cells mutated for SETD2 showed impaired DNA damage signaling and failed to activate p53. Another study supported the SETD2-dependent H3K36 trimethylation for HR [95]. SETD2 was shown to facilitate the recruitment of CtIP and aid with the binding of RPA and RAD51 by promoting DSB resection. The group also found that HR repair events could be reduced by decreasing H3K36me3 levels through Lysine Demethylase 4A/ Jumonji Domain-Containing Protein 2A (KDM4A/JMJD2A) overexpression, or with a H3.3-K36M transgene. On a different note, H3K36 methylation has also been linked to NHEJ. The generation of H3K36me2 was the major histone methylation event after DSB induction by IR in human cells [96]. To provide further evidence, SETMAR localized to sites of DSB and directly mediated the formation of H3K36me2, which improved the association of NBS1 and Ku70, enhancing overall DSB repair. The type of modifications H3K36 undergoes can regulate the choice of DSB repair pathway in fission yeast as well [97]. NHEJ is favoured when H3K36 methylation occurs by Set2, which reduces chromatin accessibility and resection. On the other hand, HR is promoted when H3K36 acetylation occurs by Gcn5 histone acetyltransferase (HAT), which increases chromatin accessibility and resection. In addition, loss of Set2 increases H3K36 acetylation, and consequently chromatin accessibility and resection. Accordingly, loss of Gcn5 increases H3K36 methylation, and decreases chromatin accessibility and resection. Each DSB repair pathway occurs at a specific stage of the cell cycle; NHEJ happens at G1 phase, HR takes places in S/G2 phase. Interestingly, H3K36 modification was found to be cell cycle dependent and corresponding to the repair pathway is favours. H3K36 methylation peaks in G1 phase, while H3K36 acetylation peaks in S/G2 phase. Altogether, there exists an H3K36 chromatin switch that coordinates whether fission yeast repairs DSB via NHEJ or HR.
4.5.2 H3K36 methylation and Mismatch Repair

The nucleosome-binding Pro-Trp-Trp-Pro (PWWP) domain is a conserved sequence motif present in proteins of nuclear origin, and has been suggested to be involved in protein-protein interactions [98]. The PWWP domain of hMutSα was recently found to be a reader of H3K36me3 [99-101], but whether this histone mark played a role in MMR was unknown. Li et al. demonstrated that H3K36me3 regulates human DNA MMR through direct interaction with hMutSα [102]. Since the only available atomic resolution structure of PWWP domain is that of Bromodomain and PHD Finger Containing 1 (BRPF1) [101, 103], the group superimposed it with H3K36me3 to show that the PWWP domain of hMutSα has three residues that form an aromatic cage surrounding H3K36me3 [104]. Using pull-down assays, they established the interaction between hMSH6 PWWP domain and histone octamers containing H3K36me3. Next, they measured endogenous MSH6 in MMR-proficient HeLa cells with or without short hairpin RNA (shRNA) mediated SETD2 knockdown. Western blot analysis showed overall decreased levels of SETD2, and consequently decreased levels of H3K36me3. Immunofluorescence staining was completed on these cells synchronized to either the S or G2/M phase of the cell cycle to detect chromatin localization of the endogenously expressed MSH6. There were significantly fewer hMSH6 foci formed in S phase shSETD2 cells, where H3K36me3 levels were depleted. In contrast, control cells showed 70% co-localization of hMSH6 foci with H3K36me3. The levels of H3K36me3 expression were found to be cell cycle regulated, as H3K36me3 abundance becomes highest during early S phase, and gradually decrease to very low amounts at late S and G2/M phase, and increase in levels again in G1 phase. Taken together, the group suggested that H3K36me3 recruits hMutSα to chromatin in vivo during G1 to early S phase. Linking this observation to DNA MMR, they discovered that shSETD2 cells displayed a MMR-deficient mutator phenotype by having a higher MSI (28.6% of subclones) and HPRT mutation frequency (18-fold increase). Thus, defective MMR may lead to tumorigenesis in SETD2 deficient clear cell renal cell carcinoma (ccRCC) patients. The same observations in shSETD2 HeLa cells were also seen in the SETD2-deficient ccRCC cell line, UOK143, in an S phase specific manner. The reintroduction of the yeast Set2 gene back into UOK143 cells allowed H3K36 trimethylation, resulting in higher H3K36me3 levels, more hMSH6 foci, and partial co-localization of the two proteins. Overall, the authors showed the importance of the histone code, especially H3K36me3, in maintaining genome stability. In a
separate study that sought to clarify the role of MSI between PHGG and adult tumours, the frequency of MSI was found to be significantly elevated in pediatric cases (14/71, or 19.7%) versus adult cases (5/73, or 6.8%) [105]. Additionally, one pediatric MSI-High tumour was classified as MSH6 absent due to a single base insertion resulting in a truncated protein, which is in line with a MSH6 germline mutation that is associated with an inherited MMR deficiency syndrome. MSI-High occurs when instability is detected at three or more markers out of the five quasimonomorphic mononucleotide repeat markers used. Hence, the presence of MSI and disruption to the MMR pathway may contribute to gliomagenesis.
Trimethyltransferase SETD2 adds a methyl group to H3K36me2 to form H3K36me3. The histone mark H3K36me3 then recruits the hMutSα onto chromatin via direct binding of the MSH6 PWWP domain before DNA replication initiates. (Figure adapted from: Li et al. The histone mark H3K36me3 regulates human DNA mismatch repair through its interaction with MutSα. *Cell.* 2013; 153(3): 590-600).

**Figure 4 – H3K36me3 necessary for recruitment of MMR complex hMutSα (MSH2-MSH6).**
5 Hypothesis

There is a need for better understanding of PHGG tumour biology and identification of additional therapeutic targets to counter the current dismal clinical outcome and prolong patient survival. While several studies have elucidated the downstream consequences of histone H3 mutations, the pathogenic effects of H3.3-G34R mutations remain unclear. Further study of the H3.3-G34R mutation is warranted, as there is a lack of preclinical models investigating its role in gliomagenesis mechanisms. It is my hypothesis that the H3.3-G34R mutation in PHGG results in global changes to H3K36me3 levels and subsequent inability to recruit MSH6 to sites of DNA damage.

Aim 1: Investigate the effects of H3.3-G34R mutation in cell models of PHGG.

Objective 1.1: Characterize H3K36me3 levels and MSH6 foci formation.

Aim 2: Determine the effects of H3.3-G34R mutation in an animal model of PHGG.

Objective 2.1: Evaluate H3K36me3 levels in a xenograft mouse model.
Chapter 2 Methods

6 Methods

6.1 Cell Culture

Immortalized normal human astrocytes (iNHAs) that were derived from normal human fetal brain tissue were obtained from Dr. Russell Pieper [106]. Immortalization was achieved by hTERT overexpression and p53/Rb inactivation by human papillomavirus 16 E6/E7 as previously described [106, 107]. Western blot analysis was used to characterize the expression levels of p53 and E7 [106]. This is a system that is commonly used for studying gliomagenesis and cellular transformation [106, 108, 109]. A pCMV expression plasmid that contained mouse H3f3a cDNA underwent site-directed mutagenesis to generate clones expressing WT, and mutant G34R histone H3.3 (H3F3A). Sanger sequencing confirmed H3.3 was mutated to H3.3-G34R. FLAG- and hemagglutinin (HA) cDNA was added to the end of WT-H3.3 H3F3A cDNA (WT), or mutant H3.3 H3F3A cDNA (H3.3-G34R) and was cloned into the lentiviral plasmid encoding the hygromycin resistance gene. Sanger sequencing confirmed the clones. Lentiviral plasmid containing the H3.3-WT-HA/flag, or H3.3-G34R-HA/flag mutation, or no cDNA empty vector (EV) was transfected into human embryonic kidney-293T (HEK293T) packaging cells with viral packaging plasmids VSVG and viral polymerase Gag/Pol plasmids. FuGENE 6 transfection reagent (Promega) was used for transfection. Lentiviral supernatants were used to infect iNHAs. Infected cell populations were selected in hygromycin (400µg/mL) for one week to generate stably transfected clones from polyclonal expansion of infected cells. Cells were verified by Western blot for expression of the HA flag-tagged protein. Maintenance hygromycin (100µg/mL) was used for selection between cell passages upon reaching 80% confluency. iNHAs were cultured in Dulbecco’s modified eagle media (DMEM) (Gibco) supplemented with 10% Fetal Bovine Serum (FBS) (Invitrogen) and 1% penicillin/streptomycin (Invitrogen). All cells were incubated at 5% CO₂ and 37°C.

6.2 Western Blotting and Densitometry

Cells were washed in PBS, centrifuged, and lysed with hot 2X SDS sample buffer. Samples were boiled at 95°C for 5 minutes, followed by sonication. Protein concentrations were determined using Pierce BCA (Bicinchoninic Acid) Protein Assay Kit (Thermo Scientific).
µg of total protein were loaded onto 10% Sodium dodecyl sulfate – polyacrylamide gels and electrophoresed at 120V in 1X running buffer with SDS for approximately 1.5 hours. Proteins are transferred onto PVDF (polyvinyl difluoride) membranes (BioRad) in a 1X wet transfer buffer running at 100V for 1 hour. Membranes were blocked with 5% non-fat milk diluted in Tris-Buffered-Saline with 0.1% Tween 20 (TBST). Primary antibodies were incubated with the membranes overnight at 4°C, and washed the next day 3 times for 5 minutes each with TBST. Primary antibodies were as follows: HA.11 Epitope Tag Clone 16B12 (1:3000, BioLegend), Histone H3 (1:1000; Cell Signaling), Histone H3 K36 trimethyl (1:100,000; Abcam), GAPDH (1:10,000; Cell Signaling). This was followed by incubation with horseradish peroxidase-conjugated secondary antibodies (1:10,000; BioRad Laboratories) specific to the primary antibodies for 1 hour at room temperature, and washed again three times for 5 minutes each with TBST. GAPDH was used as loading control. Binding of antibodies was detected with Pierce ECL (Chemiluminescence) Western Blotting Substrate (Thermo Scientific). Quantification of images was completed using ImageJ software.

6.3 Cell Synchronization

Cell synchronization was achieved via serum deprivation or double thymidine block. After reaching 25-30% confluency, cells were arrested at G0/G1 stage via serum deprivation by culturing for 48 hours in DMEM containing 0.1% FBS. Cells were released from serum starvation in DMEM containing 20% FBS, and harvested at time points of 0, 8, 12, 16, and 24 hours for cell cycle analysis. Alternatively, cells were arrested at G1/S phase via double thymidine block by culturing in DMEM containing 2 mM thymidine (Sigma-Aldrich) for 18 hours. Cells were placed in thymidine-free media for 9 hours, and then reintroduced into 2 mM thymidine for 15 hours. Cells were released from the block and harvested at time points of 0, 2, and 4 hours for cell cycle analysis.

6.4 Cell Cycle Analysis

Cells were resuspended in PBS and fixed with ice-cold 80% ethanol for 30 minutes. Cells were resuspended with RNase A for 5 minutes at room temperature, then incubated with propidium iodide and NP-40 for 30 minutes at room temperature, protected from light. Cells were filtered through a 35 µm nylon mesh and analyzed by flow cytometry to confirm cell cycle status (Figure 5). The fluorescent activated cell-sorting readout showed cell cycle arrested at G0/G1.
phase (0h) after serum starvation for 48 hours. Cells remained in the G0/G1 phase after reintroduction of FBS for 8, 12, and 16 hours. An accumulation of S phase cells was observed 24 hours after release from serum deprivation, which was the time point chosen for our method of synchronization. This was a robust technique because a large population of cells was observed in the early S phase as shown by the flow cytometry analysis.

**Figure 5 – iNHAs enter S phase 24 hours after serum deprivation.** Cell cycle profiles from flow cytometry confirming cell cycle status. Y-axis: the number of cells. X-axis: intensity of DNA dye. Cells were arrested at G0/G1 phase at time point: 0, 8, 12, 16 hours.

### 6.5 Immunofluorescence

Immunofluorescence was performed as previously described [110]. Cells were seeded on glass coverslips in a 24-well format at 50,000 cells/well, and incubated overnight for adherence. Cells were subjected to cell synchronization before media was removed, washed two times with PBS, and fixed with 4% paraformaldehyde for 10 minutes at room temperature. Cells were nuclear permeated with 0.5% TritonX-100/PBS for 10 minutes at room temperature. Coverslips were blocked with 10% diluent in 1% BSA/PBS with 0.3M glycine for 1 hour at room temperature. Primary antibodies were as follows: Histone H3 K36 trimethyl (1:8000; Abcam), MSH6 (1:250; BD Biosciences). After incubation, coverslips were washed in PBS with 0.05% Tween 20 (PBST), three times. Coverslips were incubated with fluorescent conjugated secondary antibodies specific to the primary antibodies for 1 hour at room temperature in the
dark, then washed with 1% BSA/PBST three times. Secondary antibodies were as follows: Fluorescein isothiocyanate (FITC) conjugated antibody (1:200; LifeTechnologies), Tetramethylrhodamine (TRITC) conjugated antibody (1:200; LifeTechnologies). Double staining was achieved by the combined incubation of the two primary antibodies, followed by the two secondary antibodies. Coverslips were incubated with DAPI (1:100; LifeTechnologies) for 5 minutes at room temperature and washed with PBS once. VectaShield Hard Set Mounting Medium (Vector Laboratories) was used to mount the coverslips onto slides. Images were captured using a spinning disk confocal microscope (Leica DMIRE2; Quorum Technologies) at 100X magnification, and analyzed using Volocity software.

6.6 Xenograft Model of Pediatric High-Grade Glioma

200,000 iNHA EV, H3.3-WT, and H3.3-G34R cells were resuspended in 3 µL of DMEM and injected into the frontal cortex of 10-week-old NOD/SCID/Gamma (NSG) immunodeficient mice via stereotactic-guided implantation method (Coordinates: X=-1, Y=1.5, Z=-2.2, with the reference point set at the Bregma). Mice were monitored weekly for health status and were sacrificed at the first signs of sickness. The University Health Network (UHN) and animal care committee (ACC) approved animal use protocols (AUPs).

6.7 Immunohistochemical Staining and Scoring

Paraffin-embedded blocks from xenografts were cut into 5 µm sections and were dewaxed in xylene and rehydrated in 100%, 95%, and 70% alcohol sequentially. Sections were placed inside a pressure cooker for 20 minutes in citric buffer (pH 6.0) for antigen retrieval. Endogenous peroxidase block was achieved in 3% H$_2$O$_2$ followed by avidin-biotin block (Vector Laboratories), and blocked with 10% serum in TBS with 0.1% Triton-X, 2% BSA at 4°C overnight. The Histone H3 K36 trimethyl (1:500; Abcam) was incubated for 1 hour at room temperature. Antigen detection consisted of incubation with biotinylated secondary antibody for 30 minutes at room temperature, ABC Kit (Vector Laboratories), and DAB Peroxidase Substrate Kit (Vector Laboratories). Hematoxylin (Fisher Scientific Inc.) was used for counterstaining for 2 minutes and treated with Scott’s tap water for 1 minute. Sections were dehydrated in 70%, 95%, 100% alcohol, washed in xylene, and mounted to coverslips with Vectamount (Vector Laboratories). Two observers scored all slides for both intensity (negative, weak, or strong) and distribution (0-25%, 25-50%, or >50%), which was both converted into a
numerical value (0, 1, or 2). Intensity and distribution scores were added for a final value to evaluate H3K36me3 positivity; overall score of 0-2 (H3K36me3 light) or overall score of 3-4 (H3K36me3 strong). Negative staining was observed, however no tumour area had a staining distribution of 0-25%, as a result there were no tumours with an overall score of 0.

6.8 Statistical Analysis

All experiments were completed in triplicate. Kappa coefficient was calculated to measure the agreement between the two observers for immunohistochemical scoring. Results are denoted as mean and Standard Error of the Mean (SEM) for western blot densitometry, immunofluorescence, and immunohistochemical staining. The median and interquartile range was also communicated for immunofluorescence staining. Statistical analysis was completed using GraphPad Prism 6.0. Unpaired students’ t-test, and Kruskal-Wallis one-way analysis of variance were used to determine statistical significance, which was achieved with a p-value of <0.05.
Chapter 3 Results

7 Results

7.1 Uniform H3K36me3 levels in asynchronous iNHAs

H3.3-G34R/V mutation has previously been shown to cause a reduction of the H3K36 methylation mark on the same, and nearby nucleosomes from H3.3 transgenic 293T cells [47, 49]. We hypothesized that the H3.3-G34R mutation will cause changes to H3K36me3 levels. To test this hypothesis, first global H3K36me3 protein levels in EV, H3.3-WT, and H3.3-G34R overexpressing iNHAs, with selection by hygromycin, were characterized by Western blot (Figure 6A). Uniform levels of H3K36me3 were seen in asynchronous iNHAs, and no difference in H3K36me3 expression levels were detected between the three cell lines. Densitometry analysis was used to quantify H3K36me3 expression levels normalized to total H3 (Figure 6B). The results from this initial experiment would suggest that H3K36me3 levels in an unsynchronized population of iNHAs with or without H3.3-G34R mutation, or histone H3.3 overexpression is invariable. Overall amounts of H3K36me3 appear to be unchanged in asynchronous H3.3-G34R iNHAs.

7.2 Decreased H3K36me3 levels in S phase iNHAs with H3.3-G34R mutation

Since Li et al. have demonstrated that global H3K36me3 levels reach maximum abundance in early S phase HeLa control cells [102], we postulated that synchronization of iNHAs to S phase was needed to observe any notable difference in global H3K36me3 levels between cell lines. iNHAs underwent synchronization by serum deprivation, and was collected 24 hours after release from serum starvation. S phase synchronized iNHAs with H3.3-G34R mutation displayed a significant decrease in total H3K36me3 cellular levels by Western blot (Figure 6A). Quantification of H3K36me3 expression levels normalized to total H3 was completed by densitometry analysis (Figure 6B). The H3.3-G34R mutation causes a modest drop in H3K36me3 levels by 19% compared to control iNHAs, as measured by Student’s unpaired t-test (p=0.04). This would suggest that the highest abundance of H3K36me3 in iNHAs is during S phase, and the H3.3-G34R mutation reduces the levels of H3K36me3 at this stage of the cell cycle.
Having previously established the association between H3.3-G34R mutation and a decrease in H3K36me3 levels globally, we next wanted to evaluate H3K36me3 levels on an individual cellular level and correlate any changes to MSH6 recruitment. Immunofluorescence staining was completed on iNHAs synchronized to S phase using an antibody to H3K36me3 (Figure 7). Staining controls were included in which iNHAs were only incubated with the secondary antibody (FITC), without the H3K36me3 antibody. Quantification of immunofluorescence by measuring H3K36me3 intensity per volume of nucleus revealed a significant reduction in H3K36me3 levels in H3.3-G34R mutated iNHAs compared to both iNHAs EV or H3.3-WT overexpression (Figure 8A). These results support the previous findings that the H3.3-G34R mutation results in decreased H3K36me3 expression levels in S phase iNHAs. The percentage drop in mean levels of H3K36me3 in H3.3-G34R mutated iNHAs compared to EV iNHAs, and H3.3-WT iNHAs was 27% and 19%, respectively, as measured by Mann-Whitney U test (p<0.0001). iNHAs with H3.3-G34R mutation also had a significantly decreased median and interquartile range of H3K36me3 levels compared to both control iNHAs (Figure 8B). H3.3-G34R mutated iNHAs had a median H3K36me3 level that is 21% and 19% lower than that of EV iNHAs and H3.3-WT overexpressing iNHAs, as measured by Mann-Whitney U test (p<0.0001). Therefore, these results suggest that the H3.3-G34R mutation decreases H3K36me3 levels in iNHAs synchronized to the S phase.

### 7.3 Decreased H3K36me3 levels in S phase iNHAs with H3.3-WT overexpression

Similar to H3.3-G34R mutant iNHAs, we observed a significant drop in global H3K36me3 levels by Western blot in S phase synchronized iNHAs with H3.3-WT (Figure 6A). Quantification of H3K36me3 expression levels normalized to total H3 was completed by densitometry analysis (Figure 6B). This would suggest that overexpressing H3.3 leads to the same effect on total cellular H3K36me3 levels as H3.3-G34R mutation in S phase synchronized cells. Immunofluorescence staining of individual cells revealed a slight H3K36me3 decrease of 11% in H3.3-WT overexpressing cells compared to EV iNHAs in the S phase, as measured by Mann-Whitney U test (p=0.035) (Figure 7, 8A-B). The reduction in H3K36me3 levels observed in immunofluorescence staining of H3.3-WT overexpressing iNHAs was not as drastic compared to the drop seen in H3.3-G34R mutant iNHAs. Nevertheless, Western blot and immunofluorescence data both propose a H3K36me3 reduction from H3.3 overexpression.
Figure 6 – Uniform H3K36me3 level in asynchronous iNHAs, and decreased H3K36me3 levels in S phase iNHAs with H3.3-G34R mutation. Western blot (A) and densitometry (B) measuring total cellular H3K36me3 levels in a population of unsynchronized iNHAs, and a population of S phase iNHAs. * denotes significance of p=0.01 and p=0.04 calculated using Student’s unpaired t-test. (C) Densitometry analysis measuring total cellular MSH6 levels in unsynchronized vs. S phase iNHAs. *, **, *** denotes significance of p=0.018, p=0.0045, p=0.0009 calculated using Student’s unpaired t-test. Error bars represent SEM. Densitometry analysis was conducted on three biological replicates (n=3).
Figure 7 – Reduction of H3K36me3 levels, and non-significant MSH6 foci per nucleus in S phase synchronized H3.3-G34R mutated iNHAs. Representative confocal laser scanning microscopy images of iNHAs after immunofluorescent staining for H3K36me3 and MSH6 with DAPI as a counterstain after 24 hours release from serum starvation. All images were taken at 100X magnification.
Figure 8 – Decreased H3K36me3 levels in iNHAs with H3.3-G34R mutation synchronized to S phase. H3K36me3 expression levels per nucleus in S phase iNHAs (A, B). ** denotes significance of $p=0.035$ and $p<0.0001$ calculated using Mann-Whitney U test for mean (A), and for median and interquartile range (B). Error bars represent SEM. Three biological replicates including three technical replicates were completed for each cell line. For immunofluorescent quantification analysis at least 100 cells were counted from each replicate (n=900).
Figure 9 – Non-significant MSH6 foci formation in iNHAs with H3.3-G34R mutation synchronized to S phase. Frequency distribution of number of MSH6 foci per nucleus in S phase iNHAs (A). Representative confocal laser scanning microscopy images of S phase iNHAs after immunofluorescent staining for MSH6 after 24 hours release from serum starvation (B). Nuclei from both ends of the frequency distribution spectrum are shown: <10 foci per nuclei, and >100 foci per nuclei. All images were taken at 100X magnification.
7.4 MSH6 foci formation in S phase iNHAs with H3.3-G34R mutation is not significant

Having previously established a decrease in H3K36me3 levels with the H3.3-G34R mutation in S phase iNHAs, we asked whether the mutation had the same effect on MSH6 expression levels. To address this question, global MSH6 protein levels were characterized by Western blot (Figure 6A). Consistent levels of MSH6 were observed in S phase iNHAs between the three cell lines. Densitometry analysis was completed for quantification of MSH6 expression levels normalized to total H3 (Figure 6C). On a total cellular level, changes in H3K36me3 levels in S phase H3.3-G34R mutated iNHAs does not correlate with changes in MSH6 protein. The decrease in histone mark expression does not affect global levels of the mismatch repair protein.

Since H3K36me3 is responsible for recruiting MSH6 to the chromatin, we hypothesized that a decrease in H3K36me3 would induce a drop in MSH6 foci formation as indicative of failure of chromatin localization. To evaluate the chromatin localization ability of MSH6 in S phase iNHAs, immunofluorescent staining for MSH6 protein was completed in tandem with H3K36me3 staining on iNHAs after synchronization to enable counting of MSH6 foci per nucleus (Figure 7). Staining controls were included in which iNHAs were only incubated with the secondary antibody (TRITC), without the MSH6 antibody. Counting of MSH6 foci was completed via a threshold-automated process on Volocity software, in which the threshold was determined visually to include puncta and exclude background noise. A potential concern when employing the user-defined threshold foci counting method is determining the correct threshold to include specific population of spots. The number of MSH6 foci per nucleus we reported is similar to other groups who have also counted MSH6 foci in S phase cells, validating our foci counting method [102, 111]. The majority of iNHAs have nuclei that stained for less than 10 MSH6 foci per nucleus, which was observed in all three cell lines (Figure 9A). However, there is a spectrum in regards to the number of MSH6 foci per nucleus, with the maximum number recorded at more than 150 foci (Figure 9A-B). Altogether, this result suggests that MMR in actively replicating chromatin is correcting a large range of mismatch events, with most cells exhibiting less than 10 mismatch events. iNHAs with H3.3-G34R mutation did not show a significant decrease in MSH6 foci per nucleus compared to both iNHA EV and H3.3-WT overexpressing controls (Figure 9A). Both control and H3.3-G34R mutated iNHAs form a mixture of distinct and evenly distributed foci within the nucleus (Figure 7). These results
suggest that iNHAs form very few foci in the absence of a DNA damage inducing agent, which will need to be added to make any further conclusions regarding the MSH6 foci forming abilities. At the moment, the H3.3-G34R mutation does not impede the distribution of MSH6 in chromatin in iNHAs in the absence of an external DNA stressor.

7.5 Decreased H3K36me3 levels in iNHAs H3.3-G34R xenograft

Using the iNHA xenograft tumours, we next wanted to investigate the levels of H3K36me3 protein expression in vivo. Immunohistochemical analysis was completed for H3K36me3, with each tumour containing tissues from different parts of normal brain as positive controls. Normal cortical brain tissues were positive for H3K36me3 protein expression. In all the xenograft cases (5/5 iNHA H3.3-WT, 3/3 iNHA H3.3-G34R), H3K36me3 showed nuclear localization and protein expression (Figure 10). H3K36me3 displayed a strong nuclear staining with >50% distribution in iNHA H3.3-WT tumours. In contrast, a number of staining patterns were observed for H3K36me3 in iNHA H3.3-G34R tumours. The spectrum of H3K36me3 staining intensity ranged from negative, weak, to mild nuclear staining, and distribution of 25% to >50%. Two independent observers scored all stained slides for intensity and distribution thresholds used for positivity as described in Methods 6.7. iNHA H3.3-WT tumours displayed H3K36me3 strong staining (overall score 3-4), while iNHA-H3.3G34R tumours displayed H3K36me3 light staining (overall score 0-2). The Kappa coefficient, which takes into account the possibility of agreement due to chance, was calculated to be 0.64. The inter-rater agreement was 89% overall. iNHA H3.3-G34R tumours have a significantly lower H3K36me3 score compared to iNHA H3.3-WT tumours (Figure 11). This suggests that the H3.3-G34R mutation plays a role in decreasing H3K36me3 levels in vivo. Moreover, this data further validates the in vitro effects of the H3.3-G34R mutation in lowering H3K36me3 expression levels.
Figure 10 – Staining patterns of H3K36me3. Hematoxylin and eosin stain demonstrating tumour formation from intracranial injection of 200,000 iNHA overexpressing H3.3-WT and H3.3-G34R cells (A,B). Immunohistochemical staining for H3K36me3 with a hematoxylin and eosin counter stain. iNHA H3.3-WT xenograft tumours displayed H3K36me3 staining that was strong and >50% distribution (C,E,G). H3K36me3 staining for iNHA H3.3-G34R xenograft tumours varied in intensity from negative, weak, and strong, with distribution of 25% to >50% (D,F,H). H&E images were taken at 20X magnification, IHC images were taken at 40X magnification.
Table 1: Inter-observer agreement of H3K36me3 antibody scoring

<table>
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<td>0.64</td>
<td>89%</td>
</tr>
<tr>
<td>H3.3-G34R</td>
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**Figure 11 – Decreased H3K36me3 levels in iNHA H3.3-G34R xenograft.** H3K36me3 levels represented as scores from two observers evaluating for intensity and distribution. Significance of p<0.0001 calculated using Student’s unpaired t-test. Error bars represent SEM. Experiments were conducted on at least three biological replicates (n≥3).
Chapter 4 Discussion and Future Directions

8 Discussion and Future Directions

8.1 H3.3-G34R Mutation and Implications to H3K36me3 in vitro

The primary aim of our work was to elucidate the role of H3.3-G34R mutation in PHGG, and to determine whether global changes to levels of H3K36me3 disrupt recruitment of the mismatch repair protein to sites of DNA damage. As described in the introduction, there is a lack of G34 model systems and functional studies for this histone mutation. The most extensive study on G34 mutants to date has been from a pediatric GBM line with the H3.3-G34V mutation [48]. This was the first study to evaluate the association of the G34 mutation on the genomic distribution of H3K36me3 marks. Bjerke et al. demonstrated that total levels of trimethylation at the K36 position in KNS42 cells remained constant. In another study, the G34R substitution reduced H3K36me3 levels only on the mutant allele [47]. Lewis et al. showed that overall amounts of H3K36me3 were unchanged in HEK293T cells carrying the H3.3-G34R/V mutations. However, purified mono- or oligonucleosomes (>95% of four to five nucleosomes in length) of mutant histone H3.2 and H3.3 displayed a marked loss of H3K36me3. Mass spectrometry measured a 2.7-fold and 18.5-fold decrease in H3K36me2 and H3K36me3 respectively, in the H3.3-G34R/V mutants. Our findings agreed with the current literature, in which we showed uniform levels of global H3K36me3 among mutant H3.3-G34R iNHAs compared to control in an asynchronous population of cells through western blot analysis. Interestingly, we observed a significant decrease in total H3K36me3 levels in H3.3-G34R mutant iNHAs when synchronized to the S phase of the cell cycle, as well as a significantly decreased normalized ratio of H3K36me3 to total H3 levels. Furthermore, H3K36me3 levels are highest during the S phase as shown by Western blot analysis. This effect is also seen in HeLa cells where H3K36me3 is at maximum abundance in early S phase and declines to low levels at late S phase and G2/M [102]. Other published data have similarly shown a cell cycle dependency for H3K36me3 abundance [102, 112, 113]. DNA replication occurs in the S phase, where two identical copies of chromosomes are created. H3K36me3 levels may be highest at this stage due to the doubling of histone content and/or to increase DNA repair efficiency in actively replicating chromatin. This offers an explanation to why a difference in H3K36me3 levels was observed in S phase iNHAs, but not in the asynchronous cells. Any variability in
H3K36me3 expression levels, if it exists, is diminished by the heterogeneous population in unsynchronized iNHAs, and would require the homogenous S phase synchronized cells to detect.

Additionally, the global H3K36me3 reduction observed in S phase H3.3-G34R mutant iNHAs phenomenon mimics the discovery of Fontebasso et al., in which western blot analysis of SETD2-mutant gliomas display a significant decrease in overall H3K36me3 levels [74]. The SETD2 missense and truncating mutation is a loss-of-function mutation. Likewise, the H3.3-K36M mutation in 293T cells leads to a dominant loss of global H3K36me3 [47]. Applying this idea to our results, the H3.3-G34R mutation also exerts the same dominant effect over the total cellular pool of histone H3, only when cells are synchronized to the S phase. Taken together, these results indicate that amino acid substitution at position 34 of histone H3.3 tail resulting in a non-glycine residue can decrease SETD2 methylation at H3K36. This effect is most pronounced in vitro during S phase, when cells are actively replicating. This mechanism is different from H3.3-K36M mutation because the lysine to methionine substitution may target the highly conserved active sites of SET domain-containing methyltransferase, therefore competing with substrate binding directly to reduce global methylation at the same residue [47]. While the H3.3-G34R mutation indirectly alters SETD2 binding to H3K36 by the introduction of a charged arginine residue in place of glycine.

Considering the association of H3.3-G34R expression with an increase in copy number alterations (Figure 2), changes in nuclear content when conducting cell cycle assays comparing WT versus mutant H3.3 expressing models must be accounted for. EV iNHAs are included as negative controls for cell cycle analysis to assess for nuclear content in the absence of H3.3 overexpression or mutations. Although flow cytometry is the standard method for cell cycle analysis by measuring cellular DNA levels, an alternative method can be used to ensure S phase synchronized cells. The addition of cyclin staining can provide an accurate method to analyze cell cycle based on protein expression because cyclins are not controlled by changes to copy number alterations. Cyclins are a family of key regulatory proteins that control the progression of cells through the different stages of the cell cycle. The differential expression pattern of cyclins can be used for cell cycle analysis. The presence of Cyclin D marks G1 phase, Cyclin E for G1/S, Cyclin A for G2, and Cyclin B for mitosis. Using immunoblotting techniques, the total levels and phosphorylation of cyclins can be measured to infer cell cycle status.
8.2 H3.3-G34R Mutation and Implications to H3K36me3 in vivo

Having established a relationship between the H3.3-G34R mutation and decreased overall H3K36me3 levels in vitro, we wanted to further explore H3K36me3 level changes in vivo. We implanted NSG mice with EV, H3.3-WT, or H3.3-G34R overexpressing iNHAs and demonstrated that H3.3-G34R xenograft tumours exhibited decreased levels of H3K36me3 levels after immunohistochemical (IHC) analysis. This finding was in accordance with a previous report that found a 20% reduction in H3K36me3-positive nuclei in primary ccRCC with a SETD2 mutation, and 60% reduction in distant metastases relative to the primary tumour [114]. Taken together, the H3.3-G34R mutation mimics the loss of SETD2 function through the loss of H3K36me3, much like in vitro observations.

8.3 H3.3-G34R Mutant Expression Levels

To ensure relevant amounts of H3.3-G34R in the cellular model system, the expression levels of H3.3-G34R versus H3.3-WT can be quantified in patient samples at a RNA or protein level. Using a quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) method, the relative abundance of RNA is measured. RNA from patient samples is reverse transcribed to complementary DNA (cDNA), which is amplified and measured as the reaction progresses after each cycle. Once an estimate of the H3.3-G34R transcript levels in patient samples has been made, the abundance can roughly be replicated in cell culture models by isolating single cell clones from a lentiviral transfection of the mutation, and quantifying mutant transcript levels on a clone by clone basis to identify one which most closely recapitulates levels seen in patients. Alternatively, protein quantification via a Western blot method can assess for total H3 versus H3.3-G34R mutant protein levels. After which the ratios of WT and mutant proteins are recreated in cell models as mentioned previously. David Allis’ group previously reported that the amount of mutant protein is 3.63% to 17.61% of total H3 in human HGG samples [47]. The mutant histone makes up a small portion of total histone, which makes detection by Western blot difficult. The easier method, however, would be to use a CRISPR approach. One copy of the H3.3 gene would be mutated which is reflective of the heterozygous nature of the H3.3-G34R mutation. CRISPR also utilizes the endogenous H3.3 promoter to control expression of the mutant, which will more likely yield expression levels as represented in patient samples.
8.4 H3.3 Overexpression and Implications to H3K36me3 in vitro

H3.3-WT overexpression in S phase iNHAs leads to decreased H3K36me3 levels as observed in Western blot and immunofluorescence staining. Overloading the cell with H3 might be indirectly altering transcription within the cell and therefore levels of H3K36me3, which are associated with active transcription. This effect may be mediated by H3.3 overexpression causing an imbalance in histone stoichiometry. Excess histones have been found to exert deleterious effects by four mechanisms: competing and preventing variant histone assembly, swamping and binding to histone chaperones and histone modifying enzymes, sticking non-specifically to DNA, and sticking to coding and non-coding structural and regulatory RNA [115]. These mechanisms are mediated by electrostatic interactions between the histones’ positive charge and the cellular molecules’ negative charge [115]. Similarly, overexpressing H3.3 may cause inappropriate and non-specific electrostatic interactions, leading to an exaggerated amount of positive charge. Consequently, the reduction of H3K36me3 may be due to the excessive positive charge of H3.3 resulting in altered interactions with K36 methyltransferases and SETD2.

8.5 H3.3-G34R Mutation Does Not Impede DNA Mismatch Repair in the Absence of DNA Damage Inducer

Currently, there have been no studies showing an association between the H3.3-G34R mutation and the DNA mismatch repair protein hMutSα. After obtaining promising results showing that H3K36me3 levels are significantly decreased in S phase iNHAs with the H3.3-G34R mutation, we proceeded to measure the abundance of MSH6 levels and foci formation. Overall amounts of MSH6 are uniform as seen in Western blot analysis. S phase iNHAs were simultaneously immunofluorescent stained with both H3K36me3 and MSH6 antibodies. MSH6 intensity per volume of nucleus was not measured in immunofluorescence as the confocal images were taken with the parameters set towards optimal foci counting. Therefore these images could not accurately assess MSH6 overall expression. We observed that the abundance of MSH6 foci formation in the nucleus of iNHAs is unchanged between EV, H3.3-WT, and H3.3-G34R overexpressing iNHAs. Other groups have shown that MSH6 forms discrete nuclear foci in the presence of DNA damage, which co-localized to the chromatin [116-118]. For this reason, we speculate that iNHAs will need to be stressed with additional DNA damage inducing agents to
accurately count MSH6 foci. This is because endogenous levels of DNA mismatch may not be high enough between control and H3.3-G34R mutant iNHAs to be detected. If this is true, our study may be able to demonstrate that the H3.3-G34R mutation leads to an impaired DNA MMR pathway due to the inability to recruit MSH6 to chromatin, which may consequently lead to genomic instability. Li et al. have shown that hMutSα chromatin localization is made possible by the binding of hMSH6 PWWP domain to H3K36me3, and that HeLa cells depleted of H3K36me3 failed to recruit hMutSα [102]. Others have demonstrated that cells experiencing disruption of MSH6 foci formation during S phase are defective in DNA MMR [102, 111]. Therefore, the H3.3-G34R mutation on its own, without the addition of DNA damage inducing agents, is insufficient to generate nucleotide mismatch incorporations for proper assessment of MSH6 foci formation to deduce MMR efficiency.

8.6 Conclusions

Our study provides *in vitro* and *in vivo* evidence that the H3.3-G34R mutation exhibit decreased levels of H3K36me3 in models of PHGG. Our findings are significant because we have established a global decrease in H3K36me3 levels for H3.3-G34R mutation in S phase iNHAs and H3.3-G34R xenograft tumours. Without the presence of a DNA stressor, we were unable to accurately assess the MSH6 foci forming abilities of iNHAs with the H3.3-G34R mutation. Overall, these findings contribute to our understanding of how a histone methylation mark (H3K36me3) is affected by an adjacent histone residue mutation (H3.3-G34R) in PHGG, and elucidate a possible mechanism for tumorigensis. While our results are encouraging, more work is needed to evaluate the downstream effects of H3K36me3 and MSH6 deficiency and how that links to genomic instability. For example, the varying DNA damage repair capabilities of iNHAs with the H3.3-G34R mutation has not been assessed yet. This includes the ability to repair DNA mismatches and DSB. Finally, since we are proposing that the H3.3-G34R mutation causes mismatch repair protein deficits, MSH6 or H3K36me3 could become a therapeutic target for treating PHGG with this type of mutation. By knocking down the genes that encode these proteins, or introducing a small molecule inhibitor, a cell with the H3.3-G34R mutation may be overwhelmed by DNA mismatches and be forced into cellular senescence or apoptosis.
8.7 Future Directions

To further confirm the DNA damage deficiency phenotype in iNHAs with the H3.3-G34R mutation, several tests could be completed to evaluate which DNA repair pathways are implicated. A MSI test, which was previously optimized, could be used to survey for presence of MMR deficiency. Primers were designed for five mononucleotide repeats to amplify the microsatellite loci of interest (Table 2), and were selected based on their effectiveness in identifying MSI in PHGG [105]. DNA from four different cell lines was used for the optimization process; two negative controls (Neural Stem Cells, Normal Brain), two colorectal carcinomas (HCT-15, HCT-116) with known MMR deficiencies as positive controls [119, 120].

Table 2: Primer designs for five mononucleotide repeats used for MSI Test

<table>
<thead>
<tr>
<th>Name</th>
<th>Gene Chromosome</th>
<th>Genbank #</th>
<th>Repeat</th>
<th>Marker*</th>
<th>Primer sequence 5’ to 3’</th>
<th>Melting Temperature</th>
<th>PCR Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAT-26</td>
<td>hMSH2 (2)</td>
<td>U04045</td>
<td>26 A intron 5</td>
<td>HEX</td>
<td>Ctgcgtaatcaagttttag aaccatcaacatatttaaccc</td>
<td>54.6 57.0</td>
<td>183 bp</td>
</tr>
<tr>
<td>BAT-25</td>
<td>c-kit (4)</td>
<td>XO6182</td>
<td>25 T intron 16</td>
<td>FAM</td>
<td>Tgcctccagaagtaaggt Tgcatattaactatagctc</td>
<td>57.4 54.8</td>
<td>120 bp</td>
</tr>
<tr>
<td>NR-24</td>
<td>Zinc finger 2 (2)</td>
<td>X60152</td>
<td>24 T 3’UTR</td>
<td>FAM</td>
<td>Aaaaactctctctctcggg Atttgctcattgctcacc</td>
<td>60.1 62.2</td>
<td>105 bp</td>
</tr>
<tr>
<td>NR-21</td>
<td>SLC7A8 (14)</td>
<td>XM_033393</td>
<td>21 T 5’UTR</td>
<td>HEX</td>
<td>Gagtgcgtgcaagtteta Ctggtcactggtgcttc</td>
<td>59.2 59.9</td>
<td>109 bp</td>
</tr>
<tr>
<td>NR-27</td>
<td>Inhibitor of apoptosis protein-1 (11)</td>
<td>AF070674</td>
<td>27 A 5’UTR</td>
<td>HEX</td>
<td>Aaccatgtcggcaacacact gaccaataaagcagtcactgtg</td>
<td>60.6 60.9</td>
<td>73 bp</td>
</tr>
</tbody>
</table>

*Fluorescent marker

The controls yielded similar sized microsatellite regions while mutant lines did not (Table 3). From the microsatellite profile, the leftward shifts of microsatellite repeat sequence of HCT-15 and HCT-116 was indicative of repeat numbers loss and identifies them as MMR defective (Figure 12). This affirms that the selected mononucleotide primers are capable of detecting MSI and the test was applied to our iNHA lines. Although no MSI was identified in iNHAs with or without overexpression of H3.3-WT and H3.3-G34R mutation by the selected mononucleotide repeat primers, these cells may require more passages and rounds of replication to accumulate enough mismatches to be detected by this test. It is possible that iNHAs with the H3.3-G34R mutation require additional stress from a DNA damaging agent to induce enough MSI that can be detected. To address this possibility, we would treat iNHAs with varying concentrations of monofunctional methylating agents such as methyl-nitro-nitrosoguanidine...
(MNNG) and test the cells for MSI at the five microsatellite loci as described previously. MNNG treatment has been shown to induce alkylation damage, which rapidly recruits DNA MMR proteins to chromosomal DNA without the need for DNA replication [121]. Western blot would be used to measure H3K36me3 protein expression and correlate low levels of the histone mark with presence of MSI. Genomic DNA from xenograft tumours could also be tested for MSI using this approach to assess MMR deficiency in vivo. Cell viability can also be used as an indirect method to measuring MMR activity; cells proficient in MMR are 100-fold more sensitive to alkylating agents than MMR-deficient cells [122]. We would employ the alamarBlue cell viability assay to measure the cytotoxic effects of MNNG on control iNHAs, and observe a resistance to these agents in H3.3-G34R mutated iNHAs. On a similar note, it would be interesting to measure MSH6 foci recruitment following the introduction of MNNG. The presence of a DNA stressor may reveal a more pronounced difference in MSH6 foci numbers between control and mutant iNHAs.

Table 3: PCR product size in units of base pairs of selected five mononucleotide repeats

<table>
<thead>
<tr>
<th>Cell Lines</th>
<th>Mononucleotide Repeat Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NR-27</td>
</tr>
<tr>
<td>HCT-116</td>
<td>57.3, 62.29</td>
</tr>
<tr>
<td>HCT-15</td>
<td>63.3</td>
</tr>
<tr>
<td>Normal Brain</td>
<td>66.57</td>
</tr>
<tr>
<td>NSC</td>
<td>65.17</td>
</tr>
</tbody>
</table>

To evaluate the effects of the H3.3-G34R mutation on DSB repairs, we could perform a time course on iNHAs with drug incubation or ionizing radiation. This would allow us to gain insight into whether the histone mutation will delay the rate of DNA DSB repair response. We would treat iNHAs with 2Gy of ionizing radiation or 20mM of Etoposide, an agent that causes DNA DSBs. The cells would be cultured for 0, 0.5, 1, 24, 48, and 72 hours following exposure to IR or Etoposide. Levels of DNA DSBs would be measured using phosphorylated (Ser139) histone variant 2AX (γH2AX) as marker of DNA damage. The persistence of DSBs in iNHAs
would be study by the presence of $\gamma$H2AX foci, which is assessed by immunofluorescence. The alamarBlue cell viability assay could also be completed with the drug and IR time course to further confirm the delayed DNA repair response.

Figure 12 – **MSI test optimization with no MSI detection in iNHAs by the selected primers.** A microsatellite profile showing a leftward shift in HCT-15 and HCT-116 peaks compared to controls. The loss in microsatellite repeat numbers identifies HCT-15 and HCT-116 as MMR defective. No MSI was detected in iNHAs with or without overexpression of H3.3-WT and H3.3-G34R mutation by the selected mononucleotide repeat primers.
Chapter 5 References


46. Lu, C., et al., 

47. Lewis, P.W., et al., 

48. Bjerke, L., et al., 

49. Chan, K.M., et al., 

50. Fontebasso, A.M., et al., 

51. Yan, J., et al., 

52. Wang, Y., et al., 

53. Morris, S.A., et al., 

54. Wagner, E.J. and P.B. Carpenter, 

55. Newbold, R.F. and K. Mokbel, 

56. Duns, G., et al., 

57. Kuo, A.J., et al., 

58. Smith, B.C. and J.M. Denu, 

59. Chiang, E.P., et al., 

60. Deighton, R.F., et al., 


