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The Polo-like kinases as recipients and enablers of epigenetic modifications in tumourigenesis

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By

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2014

The Polo-like kinases as recipients and enablers of epigenetic modifications in tumourigenesis

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Declaration of Co-Authorship / Previous Publication

Co-Authorship Declaration

I hereby declare that this dissertation incorporates material that is result of joint research, under the supervision under Dr. JW Hudson as follows:

In all cases, the key ideas, primary contributions, experimental designs, data analysis and interpretation and writing, were performed by the author, and the contribution of co-authors was primarily through the provision of:

Chapter 2. Alan Morettin isolated mouse embryonic fibroblasts and performed methyaltion specific PCR for all human tissues. Dr. David Shum contributed with archived tissue and was consulted for feedback in his area of expertise.

Chapter 4. Several individuals contributed to this chapter. Dr. Anna Kozarova and Dr. Jordan Nantais generated the original mass spectrometry data with input from Dr. Otis Vacratsis, that was the springboard for the rest of this project. Gayathri Sivakumar contributed the kinases assays and co-immunoprecipitation data generated from PRMT5 truncation mutants. Sharon Yong contributed the initial PRMT5 localization images and counts. Brayden Labute performed the Western blot analysis for p53 levels.

Chapter 5. This chapter was done in collaboration with oncologists Dr. Sindu Kanjeekal and Dr. Caroline Hamm. They both contributed with clinical samples, clinical patient data and their expertise where appropriate. Gayathri Sivakumar conducted all the in vitro studies within this chapter.

Chapter 6. I have included work done by Gayathri Sivakumar as part of her undergraduate thesis in the Hudson lab

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II. Declaration of Previous Publication

This dissertation includes 2 original papers that have been previously published/submitted for publication in peer reviewed journals, as follows:

Thesis Chapter	Publication title/full citation	Publication status*
Chapter 2	*Aberrant methylation of the Polo-like kinase CpG islands in Plk4 heterozygous mice. BMC Cancer 2011. 11:71*	*Published*
Chapter 3	*p53-Dependent and cell specific epigenetic regulation of the Polo-like kinases under oxidative stress. PLoS One 2014. e87918*	*Published*

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ABSTRACT

Many highly conserved proteins have evolved specific niches in the cell cycle. For the Polo-like kinases (PLKs), these roles include centrosome duplication and maturation, the interaction with key DNA damage response proteins, cytokinesis, and chromosome separation. The PLKs deliver their effects *via* phosphorylation of their substrates at serine and threonine residues. Due to their importance in the cell cycle, expression of the PLKs is strictly governed. PLK deregulation is ubiquitously associated with malignancy. Elucidating how these proteins are regulated is key to understanding how their proper function can be restored in tumourigenesis. In recent years, the study of epigenetics as an additional mechanism controlling gene expression has come to the forefront. Epigenetic mechanisms include the addition or removal of methyl groups at the DNA and histone levels. My studies describe the regulation of PLK expression at the DNA level through the epigenetic mechanism of DNA methylation, the microenvironmental alteration of PLK epigenetic marks, and how these modifications translate *in vivo* in the context of carcinogenesis. Furthermore, I describe a novel interaction between PLK4, the most structurally divergent of the PLKs, and PRMT5, another evolutionarily conserved protein that is responsible for the methylation of arginine residues. Here I propose that the DNA hypermethylation of *PLK4* promoter, and its subsequent reduction at the protein level, contributes to a tumourigenic state. As a signalling protein, the significant decrease in PLK4 creates a domino effect destabilizing global epigenetic marks, inhibiting the expression of the guardian of the genome, p53, and potentially contributing to the upregulation of the pro-mitotic protein, PLK1, the original member of the PLKs.

DEDICATION

This dissertation is dedicated to the two hardest working, most courageous people I know, without them I would not be here: Edith and Joaquin Vallejos. Thank you for your sacrifice.

Manu militari, magister meus Christus, memores acti prudentes futuri

ACKNOWLEDGEMENTS

Firstly I would like to thank my supervisor John Hudson for taking a chance on me as a graduate student. Thank you for allowing me to be intellectually autonomous and allowing me to explore new avenues and directing my own research. I believe these skills are essential in an independent scientist. A big thank you to Anna Kozarova who trained me to be meticulous, and devoted the time to teaching me the ins-and-outs of a functional and productive lab.

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I want to thank the family and friends that have supported me with their words and prayers the many years I have been a student. I am especially thankful for Brenda and Barry Mason, who have been my second set of parents. My family at Grace Ministries and Reverends Larry and Rose Graham, I couldn't have done this without your stability and the emotional and Spiritual tether that you have provided.

Lastly, I want to thank my one and only, the better AW, my coach, my cheerleader, my best friend, and my more excellent half. Thank you for your strength and for running this race alongside me. You have always seen the forest through the trees when I was myopic. The best is yet to come.

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LIST OF ABBREVIATIONS/SYMBOLS

AML	acute myelogenous leukemia
APC	Anaphase promoting complex
ATM	Ataxia telangiectesia mutated
ATR	Ataxia telangiectesia mutated and Rad3 related
AZA	5'aza-2'-deoxycytidine
BM	bone marrow
Cdc25 proteins	cell division cycle 25 proteins
CEP	centrosomal protein
CML	chronic myelogenous leukemia
CO-IP	co-immunoprecipitation
DAC	decitabine
DLBCL	Diffuse large B-cell lymphoma
DNMT	DNA methylatransferase
EGFR	Epidermal Growth factor receptor
ELISA	enzyme-linked immunosorbent assay
EtOH	ethanol
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GFP	green fluorescent protein
ɣH2AX	phosphorylated histone H2AX
HCC	hepatocellular carcinoma
HDAC	histone deacetylase
HEK 293	human embryonic kidney 293 cells
Hif1α	hypoxia inducible factor 1α
HNSCC	head and neck squamous cell carcinoma
IgG	immunoglobulin G
IHC	immunohistochemistry
JAK2	janus kinase 2
KA	kinase active
KD	kinase dead
kDa	kilodaltons
LEU	leukemia
LOH	loss of heterozygosity
MDS	myelodysplatic syndrome
MEFs	murine embryonic fibroblasts
MGMT	gene O^6-methylguanine DNA methyltransferase
MPO	myeloperoxidase
MS	Mass spectrometry

MSP	methylation-specific polymerase chain reaction
PB	polo box
PCM	Pericentriolar material
Plk (s)	Murine Polo-like kinase (s) at the gene level
PLK(s)	Human Polo-like kinase (s) at the protein level
PLK(s)	Human Polo-like kinase (s) at the gene level
Plk(s)	Murine Polo-like kinase (s) at the protein level
$Plk4^{+/-}$	Murine Plk4 heterozygosity
PRMT5	protein argine methyltransferase
qPCR	quantitatice real-time polymerase chain reaction
ROS	reactive oxygen species
SCF complex	Skp, cullin, F-box containg complex
SET	su(var)3-9, Enhancer-of-zeste, tritothorax
TET2	ten-eleven-translocation 2
TRAIL	TNF-related apoptosis inducing ligand
TSA	trichostatin A
UV	ultra-violet
VEGF	vascular endothelial growth factor
VPA	valproic acid
βTrCP	β transducin repeats containing protein

CHAPTER 1

INTRODUCTION

The *PLKs* and the cell cycle

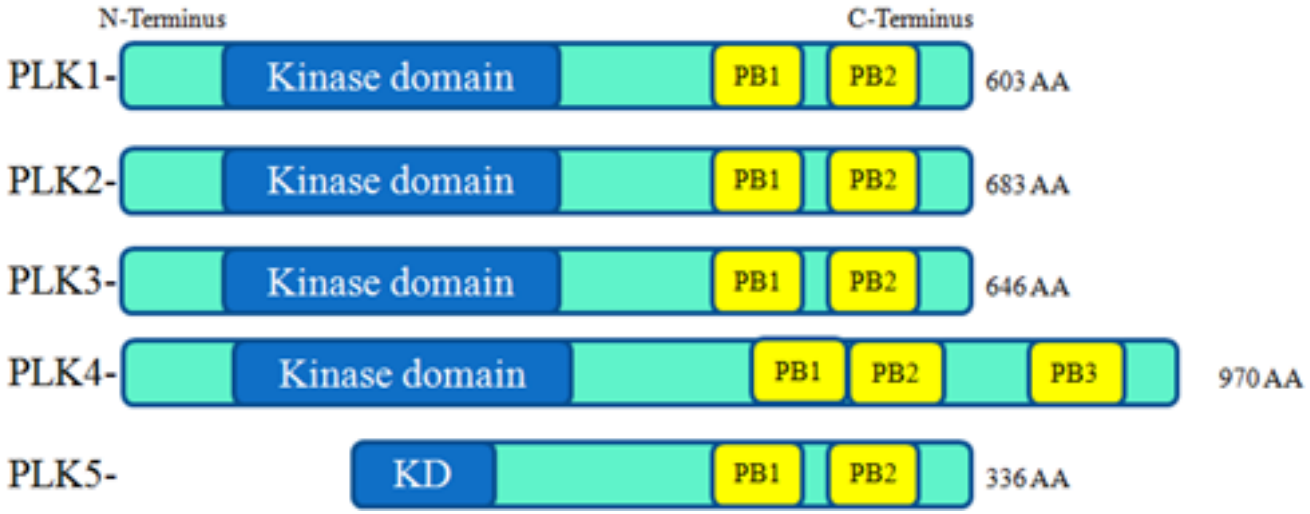
Serine/threonine kinases are essential for cell signalling and progression through the cell cycle. One such group of proteins are the highly conserved Polo-like family of kinases (PLKs) [1]. Initially discovered in fruitflies, orthologs of the *Drosophila* Polo can be found from *S. cerivisiae,* to *Xenopus* and humans [2]. The PLK family consists of five distinct family members in mammalian systems. Evolutionarily, it has been hypothesized that the PLKs were derived from an ancestral member of PLK1, with each PLK becoming more specialized, and crucial cell cycle chores segregated among them [2]. They have a conserved kinase domain that is essential for their activity and two or three polo-box domains which help to bind substrates and aide in PLK localization (Fig. 1.1) [3]. Their individual expression profiles overlap throughout the cell cycle, however, there is no characterized redundancy among them. The PLKs are critical for key cell cycle events which include, but are not limited to: centrosome dynamics, the DNA damage response, mitotic entry, and cytokinesis (Fig. 1.2) [4]. PLK1-4 all interact with p53 with differing outcomes (Fig. 1.3).

PLK1

Polo-like kinase 1 is likely the founding member of the mammalian PLKs from which the remainder of the polo-like kinases evolved from. Its temporal and spatial localization within the cell has been well characterized. In adult tissue it is expressed in

Figure 1.1 PLK protein structure and domains. The polo-like kinases in mammals consist of five members idnetified to-date. PLK1-PLK4 all have a highly conserved kinase domain that exacts phosphorylation on their respective phosphorylation. Human PLK5 has a truncated kinase domain (KD), but remains functional. All the PLKs have at least two polo-box domains that are required for localization and auto-phosphorylation.

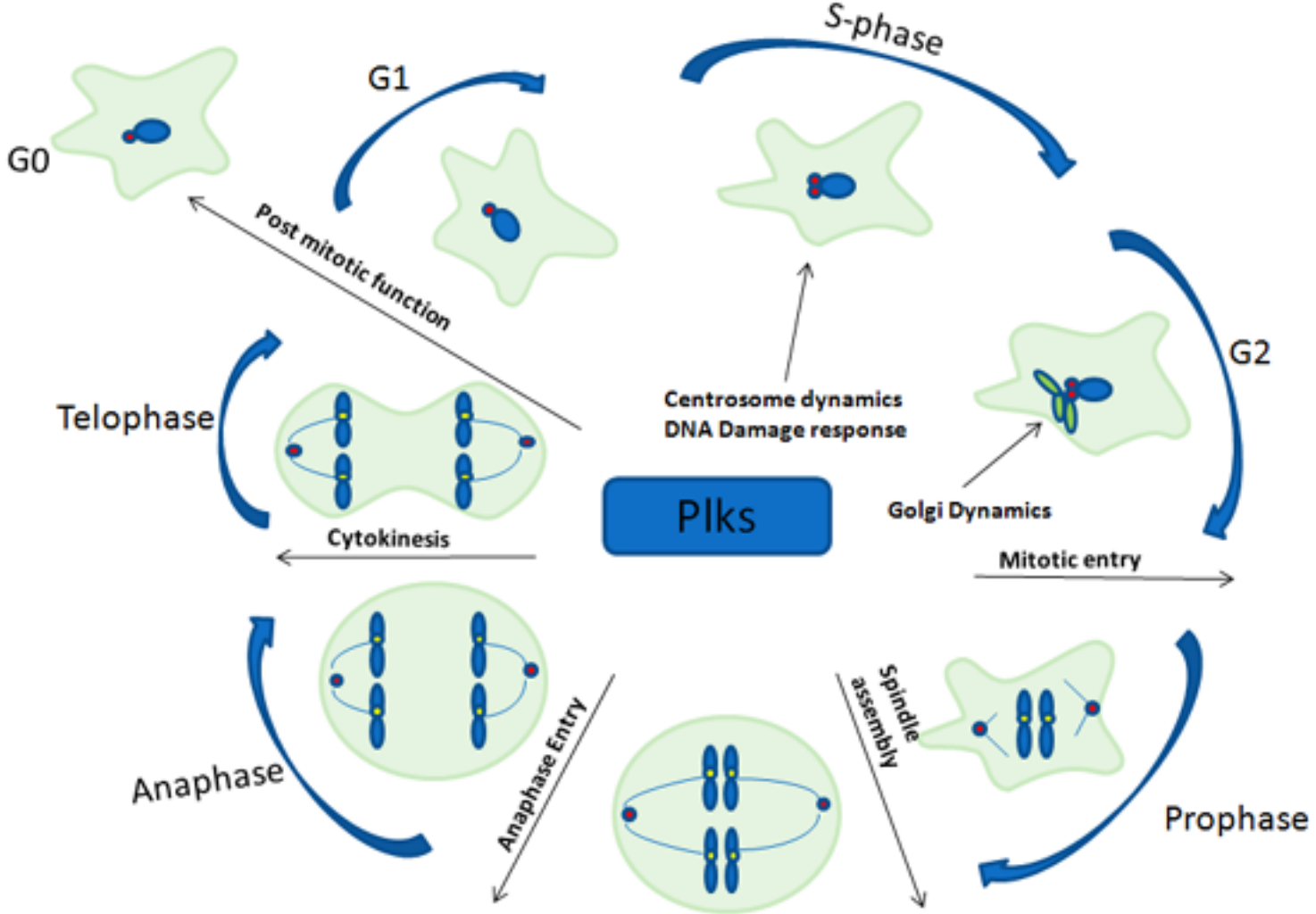
Figure 1.1


N-Terminus
C-Terminus
PLK1-
Kinase domain
PB1
PB2
603 AA
PLK2-
Kinase domain
PB1
PB2
683 AA
PLK3-
Kinase domain
PB1
PB2
646 AA
PLK4-
Kinase domain
PB1
PB2
PB3
970 AA
PLK5-
KD
PB1
PB2
336 AA

actively proliferating cells in the placenta, testes, ovaries, and spleen [5]. PLK1 expression studies have determined that it is least abundant during the G_0 and G_1 phases of the cell cycle, and so, differentiated cells have little to undetectable PLK1 protein levels [5]. The expression of PLK1 increases from late S-phase and peaks in mitosis, when PLK1 activity also reaches its apex [6]. PLK1 has multiple functions prior to- and throughout mitosis, therefore, its localization is dynamically regulated. A requirement for the trasnsition into mitosis is centrosomal maturation, PLK1 plays a crucial role in this process by targeting several key centrosomal proteins including α-,β-, and ɣ-tubulins [7]. Mitotic entry also requires the activation and nuclear localization of Cyclin B/Cdk1, which is accomplished by the simultaneous degradation of Cyclin B/Cdk1 inhibitors, and activation of CDC25C. PLK1 is crucial at multiple stages in this process. CDC25C is a substrate of PLK1 and is phosphorylated at a nuclear export signal, allowing CDC25C to be translocated to the nucleus [8]. Concomitantly PLK1 phosphorylates the Cyclin B/Cdk1 inhibitors Wee1 and MYT1, that allows for the binding of E3 ubiquitin ligases and their degradation [9]. PLK1 also phosphorylates cyclin B at the centrosomes [10]. During mitosis, PLK1 localizes to the bipolar spindles, the kinetochores, and the cytokinetic bridge [6, 11, 12]. During prophase, PLK1 is required for the dissociation of cohesin, a protein complex that regulates the sister chormatid dissociation during cell division, *via* PLK1-dependent phosphorylation, that results in cohesin releasing from chromosomes [13]; PLK1 activates the anaphase promoting complex and the subsequent separation of sister chromatids [14]. Post-mitotically, PLK1 is targeted for degradation by the anaphase-promoting complex *via* ubiquitination [15]. PLK1's essentiality in cell

Figure 1.2. The role of the polo-like kinases throughout the cell cycle. The polo-like kinases are involved in many important events during the cell cycle from centrosome duplication and the DNA damage response to a variety of M-phase events that include spindle assembly, anaphase entry, and cytokinesis. This highlights the importance of tight regulatio of these hihly conserved serine/threonine kinases. Figure adapted from Dai, 2005. Oncogene

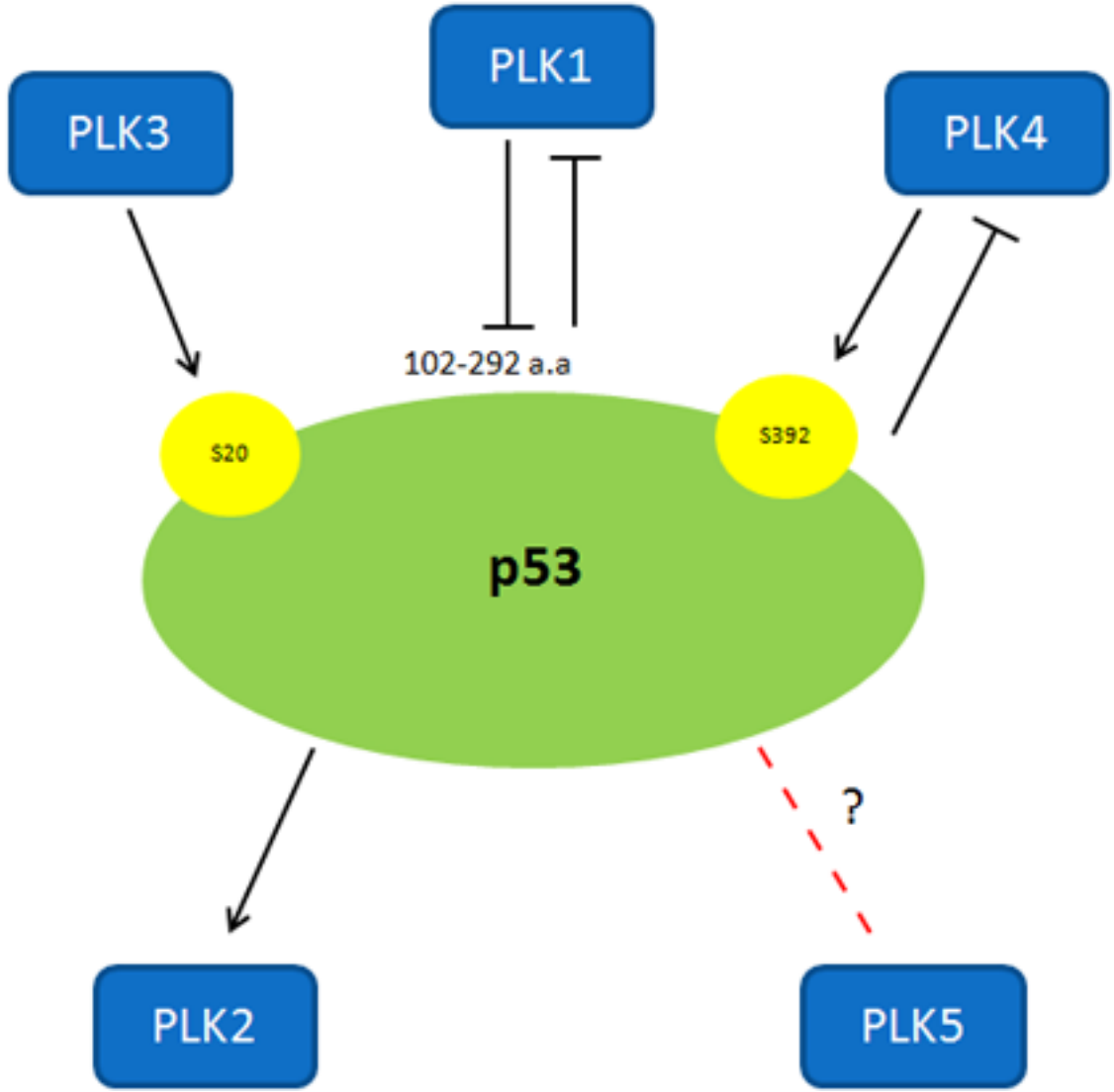
Figure 1.2


S-phase
G1
G0
Post mitotic function
Centrosome dynamics
DNA Damage response
G2
Telophase
Golgi Dynamics
Plks
Cytokinesis
Mitotic entry
Anaphase
Anaphase Entry
Spindle assembly
Prophase

cycle progression is evidenced by the impact of inactivating mutations of PLK1 orthologs in multiple species; in *Drosphila (Polo),*budding yeast (*cdc5),* and fission yeast (*plo1)* all resulting in lethality [16-18]. It comes as no surprise, therefore, that in mammals, *Plk1* $^{-/-}$ murine embryos arrest at embryonic day 3.5 and fail to proliferate past the eight-cell stage [19]). Although these embryos progressed through S-phase and had no defect in DNA synthesis, they did not show markers for mitosis such as phosphorylated H3 or any assembled spindles, both required for the mitotic progression [19]. PLK1 is a pro-mitotic protein, and unlike the other PLKs, its role in the context of DNA damage is not to enhance or activate p53, but in fact to suppress its function. PLK1 suppresses p53 in several direct and indirect ways. By directly binding with p53 in its DNA binding region, PLK1 inhibits p53's transactivation activity [20]. Indirectly, PLK1 also induces p53 degradation by phosphorylating and activating proteins that lead to p53 ubiquitination or sumoylation [20]. These targets include Mdm2 and Cdc25C, whose activation leads to the dephosphorylation of p53 at S15 resulting in p53 degradation [21]. As well, PLK1 phosphorylation of the GTSE1 protein, shuttles it to the nucleus where it displaces p53 into the cytoplasm resulting p53's degradation [21]. However, p53 is not to be underestimated, this repressive relationship is bi-directional. p53 hampers PLK1 expression at the transcriptional, post transcriptional, and the protein levels. The PLK1 promoter has several regions that serve as sites for transcriptional regulation. In these regions, p21, a target of p53, has been known to repress *PLK1* transcription by recruiting complexes that bind upstream of the PLK1 promoter start site, additionally, *PLK1* also has p53 responsive elements where p53 is often recruited after DNA damage [22]. FoxM1, a downstream transcriptional activator of *PLK1*, whose

Figure 1.3. Polo like kinases and the p53 interaction axis. PLKs 1-4 all have important interactive relationships with p53 whose deregulation can result in cell cycle abnormalities. Of the PLKs, only PLK1 downregulates p53 in its DNA binding domain, while PLK3 and 4 play activating roles by phosphorylating S20 and S392 respectively. PLK5 has yet to have a characterized interaction with p53.

Figure 1.3



expression levels mirror that of PLK1's, is also directly repressed by p53 [22, 23]. At the RNA level, p53 activates micro-RNA's that degrade PLK1 transcripts. Lastly, at the protein level, p21 activates the RB family of proteins, which in turn target *PLK1* for transcriptional repression by recruiting the SWI/SNF chromatin remodeling complex that leads the histone deacetylation and a more closed chromatin state [21]. PLK1 is a mitotic regulator which co-ordinates many important events prior to- and during mitotis, and whose relationship with p53 has significant impact on cell cycle progression.

PLK2

Unlike PLK1, PLK2 expression occurs predominantly in G_1 phase of the cell cycle and plays a growth regulatory role at G_1 and in centriole duplication in S phase [5, 6, 24]. In unperturbed cells, PLK2 localizes to the centrosomes and has been implicated in the centrosome duplication process during the late G1/S phase; human cells depleted of PLK2 did not undergo centriole duplication [24]. PLK2 mRNA levels are induced in response to X-ray, UV radiation, and other DNA damaging agents, this stress response is mediated through p53 as PLK2 is a direct target of p53 and [25, 26]. Separate studies in *PLK2* depleted cells demonstrated that with low levels of PLK2, cells are sensitized to stress and there is an increase in apoptotic cells after exposure to genotoxic stress [26]. Although catalytically active, PLK2 has a quick turnover rate with a half life of approximately 15 minutes [27] and PLK2 expression is not segregated to actively proliferating cells, but has a broad tissue distribution with transcripts detected in human brain, uterus, mammary gland, and the trachea [28]. Interestingly, PLK2 is differentially expressed at a variety of brain regions with the highest expression found in the occipital

and putamen lobes [28]. Recently, the focus of PLK2 research has been in a neurological context with several characterized brain-specific targets including α-synuclein, a protein associated with Parkinson's disease and other neurological disorders [29]. Bergeron et al. have shown *in vivo* work demonstrating PLK2 to be directly linked to the phosphorylated α-synuclein accumulation in the brain [30]. In keeping with PLK2 as a stress response protein, PLK2 levels increase rapidly in post-mitotic neurons and downregulate sustain neuronal activity as in the case of seizures [31]. As a result of its activation, PLK2 localizes to the dendrites of these neurons and is involved in the abatement of synaptic strength [32, 33]. However, despite all these roles in the cell cycle and in the brain, PLK2 is not an essential cellular protein. *In vivo*, $Plk2^{-/-}$ cells have a delayed transition into S-phase and a longer cell cycle of 25 hours compared to 22 in the wild type cells, and *Plk2* deficient mice are viable and only display mild growth retardation [34]. It has been suggested that although Plk2 has a distinct role in the progression of the cell cycle, there may be some functional degeneracy among other Plks that may account for the relatively normal phenotype observed in $Plk^{-/-}$ mice [26].

PLK3

Of the PLKs, PLK3 is most similar to PLK2. Like PLK2, PLK3 is an early response gene whose transcripts increase in response to mitogenic stimuli in G_1 [35]. This response has been documented in both murine and a broad spectrum of human cells [5]. Although conflicting reports exist with regard to PLK3's cellular expression, most literature indicates that PLK3 levels are generally constant throughout the cell cycle, except during mitosis when it is degraded during anaphase [5, 15, 36]. Endogenous PLK3

is found in the cytoplasm, but its localization has also been detected at the centrosomes, the nucleus and nucleolus [37, 38]. PLK3 has been implicated in the activation of the DNA damage pathway in response to genotoxic stress. DNA damaging agents like reactive oxygen species (ROS), can trigger an increase in PLK3 levels [38, 39]. PLK3 then helps to activate the p53-dependent DNA damage repair pathway by interacting with Chk2 and phosphorylating p53 at S20 [5, 38, 39]. PLK3 also plays an active role in the G_2/M transition by targeting S191 and S198 within the nuclear export signal (NES) of Cdc25C thus,promoting its accumulation in the nucleus where it activates cyclinB1/Cdk1 [40]. Cdc25A is another key phosphatase targeted by PLK3, in this case, PLK3 phosphorylation of Cdc25A's C-terminus is associated with priming required for its degradation [41]. Although PLK3 has distinct roles in dividing cells, its expression has also been detected in terminally differentiated tissues such as human respiratory organs including the lungs and bronchus, and in neurons [2]. Similar to PLK2, PLK3 also targets α-synuclein and has been implicated in neurological illnesses, though its role in this context is less well characterized compared to PLK2 [30, 32]. Interestingly, two separate labs have developed a PLK3 mouse model. The Dai group developed a viable *PLK3* null mouse that, as it aged, developed tumours in multiple sites: kidney, liver, uterus, and lung at a rate of 60% compared to 7% in the wild type [42]. They also determined that PLK3 imparted its tumour suppressive functions through inhibition of HIF1α, since *PLK3*$^{-/-}$ cells displayed deregulated HIF1α levels and activity after hypoxia treatments. The second *PLK3* mouse model showed no such disparity in tumour formation, and *PLK3* null mice developed tumours at the same rates as their wild type littermates [41]. *PLK3* null MEFs, however, displayed a defect in the G_2/M transition and an increase in the

polyploid population along with an accumulation of Cdc25A, in keeping with the regulatory role PLK3 has on Cdc25A [41]. This data suggests that although PLK3 does have relevant roles in the cell cycle, it is not an essential protein for cell cycle progression, though its primary functions may be more important in the homeostasis of cells such as its increase in activity in response to genotoxic stress.

PLK4

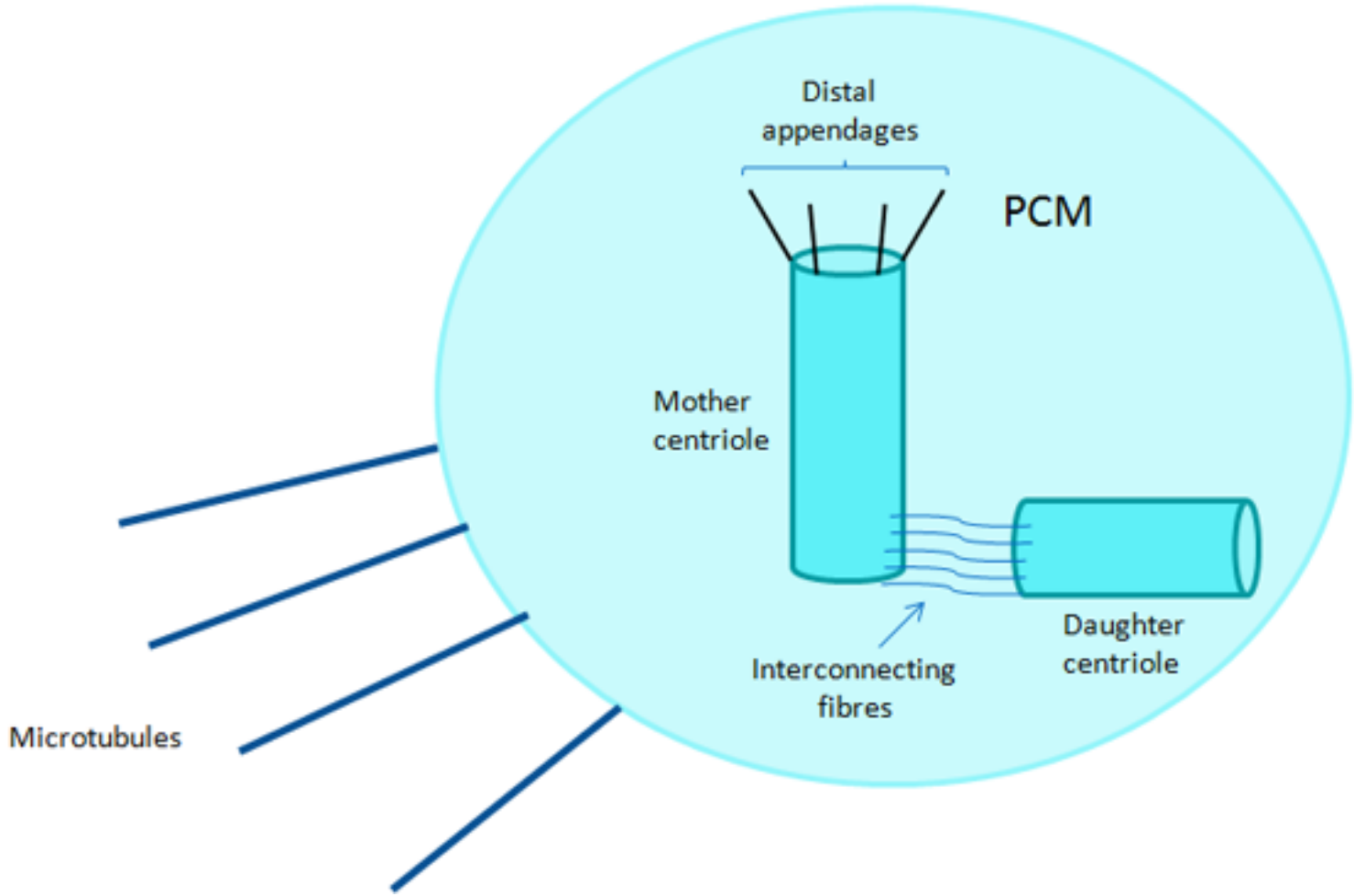
PLK4 is the most structurally anomalous of the PLKs. The N-terminus houses its kinase domain, while its C-terminus accommodates three polo-box domains with PB1 and PB2 contiguous to one another and PB3 located at the more distal end of the C-terminus [43]. It also has three PEST sequences, unique to this PLK, which are required for PLK4 degradation [44, 45]. Like the other PLKs, PLK4 has unique cellular functions. Although PLK4 is expressed throughout the cell cycle, its expression begins to increase during S-phase and peaks at the G_2/M. During this time, PLK4 is multi-tasking by interacting with cell cycle regulators like CDC25C, and localizing to the centrosomes and the cleavage furrow [46-48].

PLK4 is absolutely required in centrosome duplication. The centrosome is the microtubule organization centre (MTOC), preserving cellular polarity and architecture *via* nucleation of microtubules and their subsequent organization [49]. Duplication of the centrosome is licensed to occur once per cell cycle and is intimately coordinated with DNA synthesis, mitosis, and cytokinesis [50]. Centrosomes consist of a mother and daughter centriole perpendicular to one another, surrounded by pericentriolar material (PCM) composed of a variety of proteins like ɣ-tubulin and pericentrin (Fig. 1.4) [51].

Both the microtubule nucleation and pro-centriole biogenesis occurs in the PCM [51]. Centrosomal scaffold proteins Cep192 and Cep152, recruit PLK4 to the centrosome [52]. It localizes to both ends of the centrioles, but at the proximal ends, the site of pro-centriole formation, PLK4 is required for the initiation of centriole duplication [53, 54]. Additionally, once established at the centrioles, PLK4 together with Cep152 recruit PCM and allow the spindle assembly process to begin [55]. With PLK4 being an essential centrosomal protein, its expression must be tightly control, reminiscent of a Goldilocks scenario; where down-regulation or over-expression of PLK4 has deleterious effects on centriole duplication fidelity, its levels have to be just right. In light of this, PLK4 is regulated at multiple levels. At the DNA level, during early G_1, *PLK4* is transcriptionally repressed at cycle-dependent element (CDE) and cell cycle gene homology (CHR) elements upstream of its promoter region [57]. In the case of insufficient Plk4, $Plk4^{+/-}$ murine embryonic fibroblasts (MEFs) display multiple centrosomes and multipolar spindle formation [58]. Likewise, PLK4 over-expression in somatic cells is also associated with supernumerary centrosomes. Centrosome amplification leads to aneuploidy, genomic instability and ultimately, tumourigenesis [59]. This is evidenced in aging $Plk4^{+/-}$ mice, which develop liver and lung tumours at a 15 times higher rate than their wild type counterpart [58].

Figure 1.4. Basic centrosome structure. Centrosomes are made up to two centrioles, mother and daugther centrioles. They are orgonally arranged and have interconecting fibres between them. This is the centre for microtubule organization and determines cell polarity. PCM= pericentriolar material [56].

Figure 1.4


Distal
appendages
PCM
Mother
centriole
Daughter
centriole
Interconnecting
fibres
Microtubules

Adequate Plk4 levels also have important roles during development. *Plk4* null embryos are not viable and arrest at embryonic day 7.5 with a significant proportion of embryos arresting before anaphase [47]. One of PLK4's targets, the transcription factor Hand1, is responsible for placentation, as well as cardiac differentiation and morphogenesis in the developing embryo [60]. PLK4 phosphorylation of Hand1 induces its migration out of the nucleolus and into the cytoplasm, prompting cellular differentiation. Inhibition of PLK4 led to Hand1 accumulation in the nucleolus, lack of differentiation, and continued proliferation in trophoblast stem cells [60]. Furthermore, during early mouse embryogenesis, spindles assemble around chromosomes, since at this time there is an absence of centrioles, Plk4 is required for appropriate spindle assembly [55].

PLK4 has also been implicated in the DNA damage response. Some of the characterized substrates of PLK4 are known DNA damage response inducers including Chk2 and p53 [61]. Cellular exposure to stressors, including etoposide and UV radiation, induced an increase in PLK4 protein and activity *via* phosphorylation by the stress-activated kinase kinase kinases (SAPKKK)[62] and the NFκB transcription factors also function as *PLK4* transcriptional activators during stress [63]. With sustained stress, p53 levels also increased and in turn suppress PLK4 levels [62]. Ko et al 2005 determined that PLK4 interacted with p53. In a liver resection model, p53 failed to become activated in $PLK4^{+/-}$ mice compared to wild type mice, suggesting that under those conditions, Plk4 was essential in the activation of p53. This group also determined that PLK4 phosphorylates p53 at S392, a residue that is phosphorylated in active p53 [64]. Other

than protein-protein interactions, p53 also suppresses PLK4 at the transcriptional level through histone deacetylases, which enable a closed chromatin structure [65].

These studies attest to the essentiality of PLK4 in critical events throughout cell division and in postgastrulative embryonic development.

PLK5

PLK5 is the most recently discovered member of the PLKs. Structurally and functionally, it is most like PLK2 and PLK3, however, in humans, *PLK5* has stop codon in its kinase domain, where mouse *Plk5* retains its kinase domain [66]. Interestingly, both mouse and human PLK5 have almost identical functions and it appears that the kinase domain is not necessary for their functional execution [67]. Plk5 expression is tissue specific, being expressed in the brain, eye, and ovary of the mouse. In humans, PLK5's expression is limited to differentiated cells: acini of the parotid glands, the proximal and distal tubules of the kidney, and neruonal cells to name a few [67]. In neurons, PLK5 depletion resulted in attenuated neurite formation and outgrowth [67], indicating PLK5 is important in neuronal differentiation.

PLK5 primarily localizes to the nucleolus, and has three nucleolar localization signals at its amino-terminus [66]. Removal of any of these motifs resulted in mis-localization of PLK5 [66]. PLK5 also appears to be involved in the response to cellular stress with transcripts becoming elevated with exposure to multiple stressors such as etoposide, nocodazole, and serum starvation [66, 67]. Ectopic expression of Plk5 was also accompanied by elevated levels of p21, G_1 cell cycle arrest, and apoptosis all occurring in a p53-dependent manner [2, 66, 67]. Despite that no relationship has been

established between PLK5 and p53, it is likely that a relationship exists due to the PLKs sharing common interacting partners. Further characterization of PLK5 is required to fully elucidate its role in the cell cycle.

Cell cycle inhibitors and checkpoints

In order to maintain genetic content fidelity, eukaryotic cells have evolved internal monitoring systems to thwart the duplication of faulty and/or damaged DNA. These checkpoints are placed at critical phases of the cell cycle and are composed of proteins that are responsible for halting the cell cycle and initiating, either DNA repair, apoptosis or cellular senescence in response to intra-or extracellular stressors (Fig.1.5) [39].

DNA damage checkpoints

The cell cycle has several DNA damage checkpoints and the spindle assembly checkpoint; these checkpoints collectively safeguard the cell from genomic instability. As part of the checkpoint mechanism, multiple components make up the required sensors, transducers, mediators, and effectors to disseminate the DNA damage response. Ataxia talingiectasia mutated (ATM) and ATR (ATM and rad3 related) proteins act as both sensors and transducers of DNA damage, with DNA damage resulting in their conformational changes and/or changes in their localization. ATM responds to ionzing radiation and double strand breaks, while ATR responds to DNA replication stress [68]. As transducers, ATM/R mobilize p53, a tumour suppressor, and the checkpoint kinases (Chk)1/2, which in turn act as effectors themselves, or activate effector proteins [69].

Chk1/2 inhibit the function of the Cdc25 phosphatases, which are required in Cdk2 and Cyclin B1 activation. p53 in turn, activates the Cdk inhibitor protein (CIP) $p21^{CIP}$, which directly binds and inhibits Cyclin D, Cyclin E/A(Fig. 1.5) [70].

Spindle assembly checkpoint

The spindle assembly checkpoint is made up of several mitotic arrest deficiency proteins (Mad) 1-3 and budding uninhibited by benomyl (Bub) 1-3 proteins which form a large mitotic checkpoint complex. Mad2 and BubR1 work synergistically to sequester Cdc20, an activator of APC/C early in M phase, while Bub1 phosphorylates Cdc20 [71]. This collaboration prevents the early onset of sister chromatid separation prior to the adequate attachment of bivalent spindles [72].

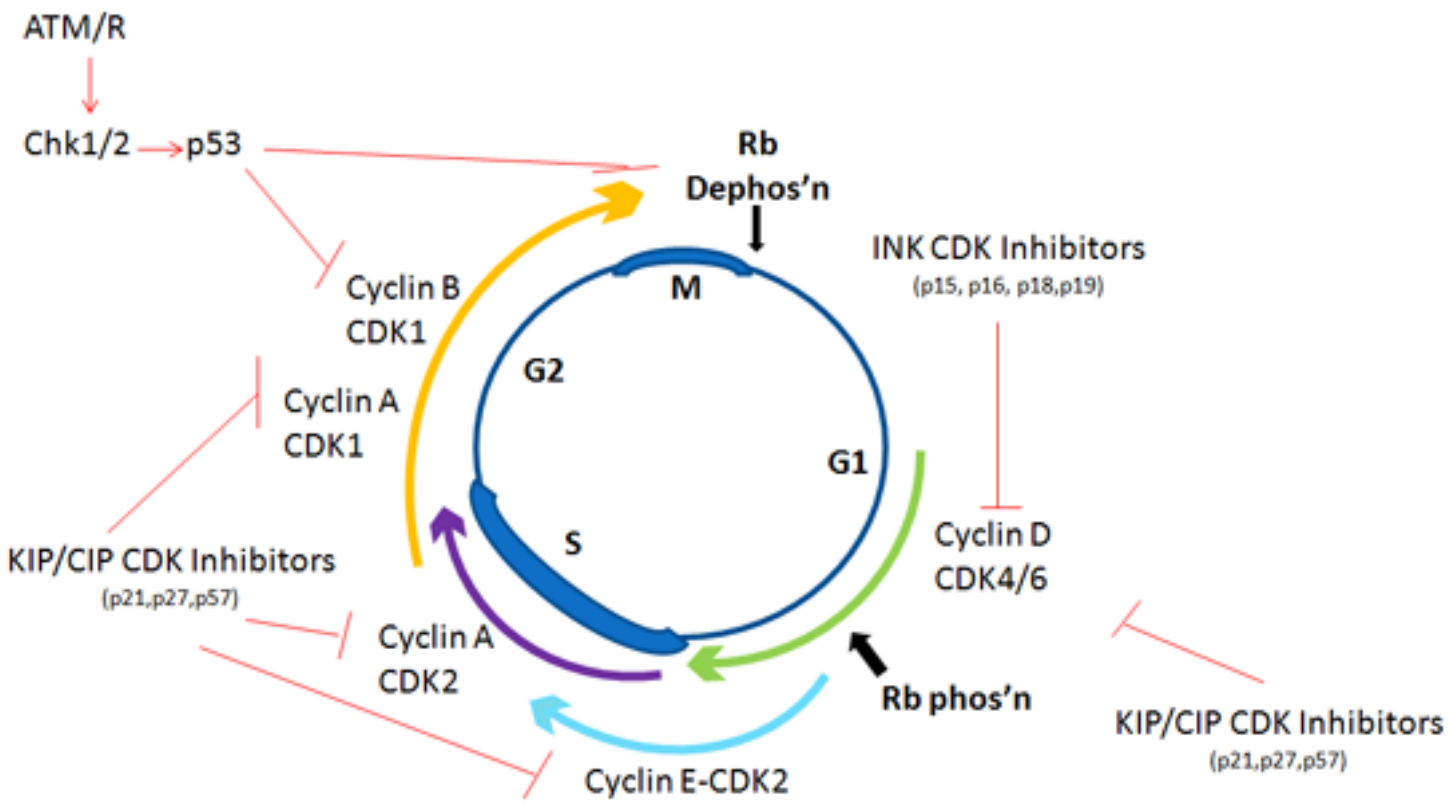
In addition to these checkpoints, there are additional layers of control that can suppress cyclin-cdk activity. Several antagonizers specifically target the Cyclin D-Cdk4/Cdk6 complexes, these are called the inhibitors of kinase 4 family: $p15^{INK4B}$, $p16^{INK4Aa}$, $p18^{INK4C}$, $p19^{INK4d}$ [73]. The CIP family, other than p21, also includes $p27^{KIP1}$ and $p57^{KIP2}$, which have a broadened their inhibitory effects to target multiple cyclin-cdk complexes [73]. Collectively, these proteins are called tumour suppressor proteins, however, in cancer they often deregulated through mutation or inactivation.

The guardian of the genome: p53

Often referred to as the guardian of the genome p53 a key tumour suppressor is defective in approximately 50% of human cancers. The active p53 protein is a

Figure 1.5. Checkpoints. There are multiple proteins that can halt the progression through the cell cycle in the event of an insult. These protein target the cyclin-cdk proteins that are required for cell cycle progression. Modified from Molecular Cell Biology 5th Edition. 2004

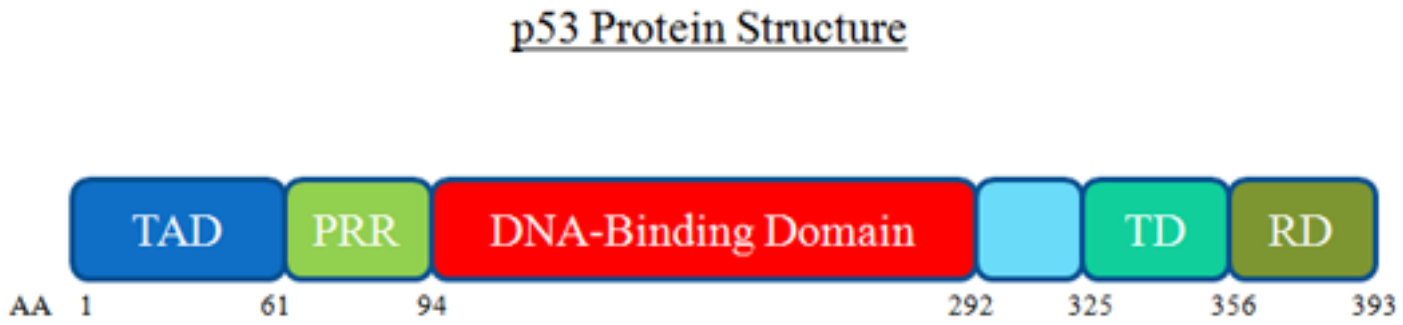
Figure 1.5


ATM/R
Chk1/2
p53
Rb
Dephos'n
INK CDK Inhibitors
(p15, p16, p18,p19)
Cyclin B
CDK1
M
G2
Cyclin A
CDK1
G1
S
KIP/CIP CDK Inhibitors
(p21,p27,p57)
Cyclin D
CDK4/6
Cyclin A
CDK2
Rb phos'n
KIP/CIP CDK Inhibitors
(p21,p27,p57)
Cyclin E-CDK2

homotetrameric transcription factor which is activated in response to a variety of environmental insults and stressors including UV and ionizing radiation, spindle assembly defects, reactive oxygen species, and hypoxia [74] [73, 75]. Levels of p53 are kept at a low but constant level, with a short half life of 20 minutes [73]. Mouse double minute 2(mdm2) is an E3 ubiquitin ligase that binds p53 and targets it for proteolytic degradation by ubiquitination. The most well-studied and primary function of p53 is to create a barrier by which the emergence of transformed and genomically unstable cells is prevented. p53's activation, through the interruption of mdm2 binding and subsequent protein stabilization can result in two basic outcomes: 1. the cell cycle is arrested and the damage is repaired or 2. The damage is too great for cell viability resulting in apoptosis, senesence or autophagy. At the protein level, p53 has important domains that control its activation, oligomerization, and DNA-binding specificity (Fig. 1.6) [73]. The most frequent mutations found in p53 are point mutations, which make up the majority of the 15 000 documented mutations in human tumours, many of them occurring in its DNA binding domain [76, 77]. These mutations result in defective p53 with an inability to bind its downstream targets. Recently, work on mutant p53 has determined that in some cases of breast and malignant skin tumours, p53 mutant proteins go on to form aggregates resulting in amyloid oligomers [78]. These aggregates can bind wild type p53 and the p53 homologues, p63 and p73, creating a dominant negative, prion-like effect on wild type p53 [78]. These studies have revealed that deregulation of p53 does not just affect its downstream targets, but can sequester remaining wild type p53 and its homologues and prevent DNA damage repair.

Figure 1.6. p53 protein structure and domains. The tumour suppressor p53 is a homotetramer composed of six distinct domains. The transactivation domain (TAD), the proline rich region (PRR), the DNA-binding domain, and the tetramerization (TD) and regulatory domains (RD). Portions of this figure have been adapted from Joerger and Fersht, 2010.

Figure 1.6


p53 Protein Structure
TAD
PRR
DNA-Binding Domain
TD
RD
AA 1
61
94
292
325
356
393

Collectively, these checkpoints, along with their respective effector proteins are charged with the genomic intergrity of the cell by responding to DNA damage and other stressors, and activating the adequate pathways for repair, apoptosis, or senescence.

Cancer

Carcinogenesis is a progressive disease consisting of a variety of contributing factors. In their original review, Hanahan and Weinberg proposed that cancer has six hallmarks: sustained cellular proliferation, avoidance of growth suppression, resistance to cell death, induction of angiogenesis, invasive capacity, and unlimited replicative potential [79]. In their most recent review, Hanahan and Weinberg, in addition to their original six hallmarks, have proposed new emerging hallmarks of cancer: deregulated cellular metabolism, and evasion of immune destruction [80] along with underscoring the importance of genomic instability as an enabling characteristic that generates the cellular genotypes required to orchestrate the hallmarks of carcinogenesis.

Genomic instability

Multistep tumourigenesis is achieved through alterations in the genome that confer selective advantages to neoplastic cells. This enables incipient cellular outgrowth and eventual dominance in the surrounding tissue. Genomic instability has been detected at all levels in the progression to cancer, from early, pre-cancerous lesions to end stage cancers, in some cases even prior to p53 mutation [81]. Genomic instability generates mutations through chromosomal re-arrangements and nucleotide sequence alterations.

There are two distinct, and sometimes overlapping types of genomic instability: microsatellite instability (MIN) and chromosomal instability (CIN) and [73].

Microsatellite Instability

Microsatellites are short repetitive elements 1-6 nucelotides in length. They were once considered extra, or "junk" DNA, however, these iterations have surfaced as important biomarkers in the prognosis and diagnosis of cancers, especially gastric and colorectal cancers. Approximately 15% of colorectal cancers present with MIN, though most of these cancers show little to no gross chromosomal aberrations [82]. Although microsatellites are most predominant in introgenic, non-coding regions, they can be hypervariable, harbouring high rates of mutation [73]. These mutations can be single point mutations or deletions. One would imagine that these types of mutations in non-coding regions would be innocuous, however, they can cause frameshift mutations leading to reading errors, loss of function, and translational failures in important cell cycle regulatory genes; such is the case for ATR and Chk1 genes [83]. Other critical genes like *BAX*, a pro-apoptotic gene, harbour mononucleotide repeat mutations in coding regions in more than 25% of gastric cancers [84]. MIN has also been associated with hereditary non-polyposis colon cancer (HNPCC) and endometrial cancers [83]. These mutations can arise from errors in the mismatch repair mechanism (MMR). The MMR mechanism is central to suppressing proof reading errors that arise from DNA replication resulting in spontaneous mutations; MMR it is charged with making the necessary corrections [85]. In cancers, many of the genes required for MMR also undergo mutations related to MIN [83]. Clinically, MIN has been associated with several

pathological features such as heredity, gastric cancer staging, and chemosensivity [83, 85].

Chromosomal Instability

More than 90% of solid tumours present with chromosomal abnormalities, with gains or losses of whole chromosomes, or structural re-arragements, resulting in aneuploidy [82, 86]. Frequently, chromosomal aberrations can target tumour suppressors and known oncogenes, whose expression levels are deregulated due to genomic variations [86]. Aneuploidy is caused by several factors: compromised surveillance and DNA damage response pathways, centrosome amplification, or mis-segration of chromosomes due to SAC or APC/C defects [82]. CIN increases with each successive cell division creating genetically heterogeneous tumour cells. Several pathological indicators are correlated with CIN, such as increase in metastasis, reduced sensitivity to chemothepeutic agents, and poor prognosis [86].

Cells experience DNA damage through endogenous and exogenous forms. Endogenous DNA damage can be a result of normal metabolism, such as the generation of reactive oxygen specicies; whereas exogenous damage can be attributed to UV and IR radiation or chemical exposure. Spontaneous DNA damage is managed through the activation of ATM/ATR and their downstream effectors Chk1/Chk2. Lowered levels of these checkpoint proteins is associated with an increase in chromosomal heterogeneity [86]. Both spontaneous and hereditary forms of cancer can harbour loss of function mutations in DNA repair proteins. ATM mutations are found specifically in lung adenocarcinomas [81]. Breast cancer susceptibility genes 1 and 2 (BRCA1 and BRCA2)

are important in repair of double strand breaks with germline mutations of these genes leading to an inadequate repair mechanism and the generation of breast and ovarian cancers [87]. Unrepaired double strand breaks have deleterious consequences with chromosome loss or chromosomal rearrangements. Mutations can also arise from proofreading errors during the repair process, as such, mutations in the Msh2 gene, which is required for mismatch repair, corresponds with the development of hereditary non-polyposis colorectal cancers (HNPCC) [85].

Likewise, centrosome amplification can create a domino effect, by which the final outcome results in aneuploidy and CIN. Centrosome amplification has been correlated to tumour grade in a broad range of cancer types both solid and hematological [50]. It is well-established that cancer cells often undergo mitosis with supernumerary centrosomes forming multi-polar spindles. Multipolar spindles lead to errors in the kinetochore-microtubule attachment and cause multipolar division. Breast cancer was the first human malignancy in which centrosome abnormalities were studied [50]. These tumours displayed multiple centrosome defects, in addition to centrosome amplification, they had increased centrosome volume, centrosome aggregates, and an accumulation of PCM [88]. There are several defects that can result in centrosome amplification: 1. Improper initiation of centrosome duplication 2. Centrosome re-duplication 3. Failure of cytokinesis. The deregulation of several proteins has been directly linked with these defects resulting in centrosome amplification. p53 and Aurora Kinase A are prime examples. Tarapore and Fukasawa, 2002, showed that p53 is indispensible throughout the centrosome cycle; it is required at the initiation of centrosome duplication and suppression of centrosome re-duplication [89]. *p53*$^{-/-}$ MEFs display centrosome

amplification and defects in cytokinesis [89]. Defects in cytokinesis can be accompanied by lagging chromosomes and incomplete cellular separation, which, in turn also results in multinucleated cells with multiple centrosomes and aneuploidy [89]. Unlike p53, Aurora A, a serine/threonine kinase is required for centrosome maturation, separation and cell progression through mitosis [90]. Its over expression is associated with cytokinetic defects and multinucleated cells, this phenotype is evident in human tumours overexpressing Aurora A [90].

A weakened SAC may allow cells to advance into anaphase with unattached or misaligned chromosomes. Although infrequent, mutations in several SAC proteins have been associated with human cancers. In mice, bi-allelic deletion of many SAC genes, like *Mad2*, *BubR1*, and *Bub3*, all result in embryonic lethality, and many haploinsufficent mouse models for these genes develop multiple tumour types like lung, colon, and hepatocellular carcinomas [82].

In many instances, these mechanisms are not mutually exclusive, but collectively provide several keys pathways making up the portrait of genomic instability throughout human cancers.

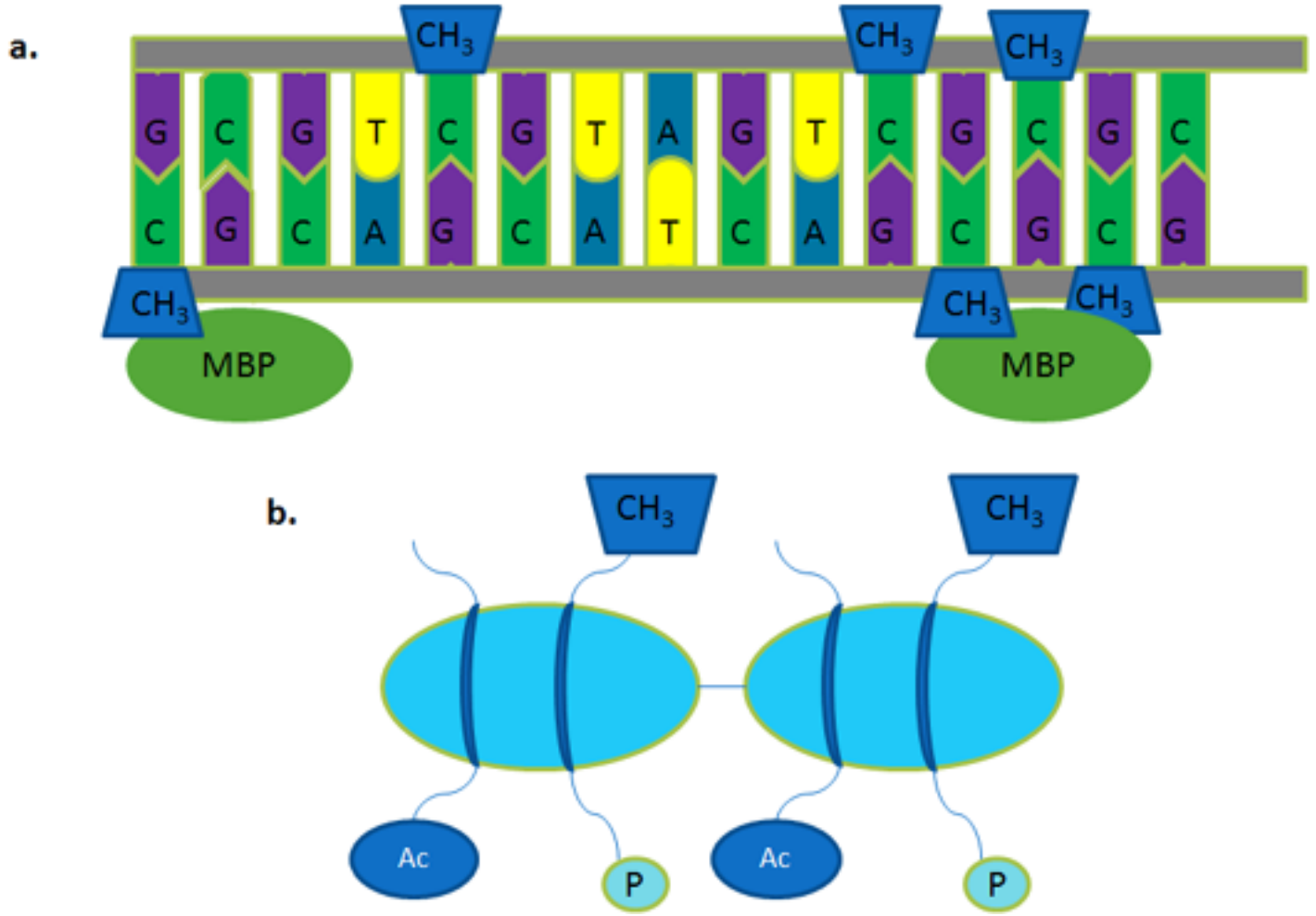
Epigenetics

In recent years, epigenetics has become a bigger focus in cancer research. The term "epigenetics" was coined by Conrad Hal Waddington as a way of describing the interaction between genes and their adaptability to their surrounding environment. More recently, the term is used to describe altered gene expressions that are passed on through somatic and meiotic cell division without modification to the underlying primary

sequence of a gene. Epigenetics encompasses two distinct modifications that greatly impact gene expression. At the DNA level, individual CG sites and regions rich in CG dinucleotides, called CpG islands, are targeted by DNA methyltransferase enzymes (DNMTs) for the addition of methyl groups to the 5' position of cytosines leading to DNA methylation (Fig. 1.7a) [91]. While at the histone level, histone tails undergo post-translation modifications which include methylation, acetylation, and phosphorylation (Fig. 1.7b) [92]. Methyl CpG binding proteins (MBD) act as important liaisons between DNA methylation and histone modification [93]. They bind to hypermethylated promoter regions and block the attachment of transcriptional machinery, while simultaneously recruiting histone deacetylase-containing complexes to reinforce transcriptional repression at the histone level [93].

Figure 1.7. Epigenetic modifications at the DNA and histone level. a) DNA methylation is found at CpG dinucleotide sites. A several CpG sites upstream of a gene promoter region is called a CpG island. Methylation is not found independently, but methyl-binding proteins (MBP) are also attached. b) Histone tail modifications are another form of epigenetic mofication leading to changes in gene expression. Ac=acetylation, p=phosphorylation, CH_3= methylation.

Figure 1.7


a.
CH3
CH3
CH3
G C G T C G T A G T C G C G C
C G C A G C A T C A G C G C G
CH3
CH3
CH3
MBP
MBP
b.
CH3
CH3
Ac
Ac
P
P

DNA methylation in tumourigenesis

DNA methylation was the first epigenetic mark to be linked to tumourigenesis (Lewandowska and Bartoszek, 2011). DNA methylation is also the most well-studied aspect of epigenetics and has several implications in tumourigenesis. In normal tissues, genomes gain global methylation marks with age, consistent with cells achieving a differentiated state and a decrease in cellular activity. In contrast, cancer cells display a global loss of methylation with increased methylation at gene-specific CpG islands, often in tumour suppressor genes [94]. Hypomethylation has been detected in stomach, colon, kidney, liver, lung, and pancreatic cancers [95]. Aberrant DNA methylation marks at CpG islands have been suggested to be an early event on the cellular trajectory towards tumourigenesis. In 1999, Stephen Baylin and his group determined that colon cancers contained a specific DNA methylation profile, they termed this the CpG island methylator phenotype (CIMP), since then, many different cancers have become associated with specific DNA methylation profiles. These modifications can be used as markers for detection of early carcinogenesis, progression of disease, and potential predictors of response to chemotherapy [96]. The epigenetic silencing of the *CDKN2A* gene that codes for $p16^{INK4A}$, has significant clinical value. Hypermethylation of *CDKN2A* was detectable in the sputum of patients that developed lung cancer up to three years prior to onset of clinical symptoms [96]. Interestingly, irregular DNA hypermethylation is detectable in pre-cancerous conditions such as liver cirrhosis due to viral infections, or lung inflammation due to smoking [96, 97]. Deregulated DNA methylation has also been coupled with an increase in tumour aggressiveness and poor patient outcome [97].

DNA methylation in normal cellular development

The importance of DNA methylation is not limited to the tumourigenic context, but has extensive functions in normal mammalian growth and development such as X choromosome inactivation and genomic imprinting, [98]. During development, zygotes undergo large-scale erasure of maternal and paternal DNA methylation (5mC) marks through passive replicative dilution, culminating with the inner cell mass having a globally demethylated genome [99]. Methylation marks are re-established by DNA methyltransferases 3a and 3b (DNMT3a DNMT3b), enzymes responsible for *de novo* DNA methylation along with their co-factor DNMT3L [98], and these new patterns are then preserved in successive cell divisions by DNMT1, which is a maintenance methyltrasnferase and has an affinity for hemimethylated DNA [100]. Maintenance of 5mC patterns are important in the developing embryo, with *Dnmt1* null murine embryos arresting at embryonic day 8.5 [101]. Promoter methylation occurs early in development, potentially as a mechanism of defining lineage types. Such is the case in haematopoiesis, where 5mC defines the myeloid and lymphoid progenitor lineages [102]. DNA methylation is also the final addition to the multi-layer process in the random silencing of the extra X-chromosome in female [91]. DNA methylation is employed in the gene imprinting process by which a specific parental allele is silenced resulting in mono-allelic expression of a gene. In mouse, the gene *insulin-like growth factor 2 receptor* (*Igf2r*) is maternally expressed, and its ligand *insulin-like growth factor 2* (*Igf2*) is paternally expressed [91, 98]. The negative aspects to parent-of-origin monoallelic expression is evident in human disease where the parentally expressed region undergoes a mutation or

a deletion. Both Prader-Willi and Angelman syndromes are two sides of the same coin; Prader-Willi syndrome is characterized by the loss of the paternal chromosome at locus 15q11-13, and converserly, in Angelman syndrome, the 15q11-13 maternal chromosome undergoes deletion [103]. Both of these syndromes are characterized by decreased intellectual abilities.

Epigenetic modifications have indispensable roles in development and DNA methylation profiles in tumourigenesis can give valuable insight and act as biomarkers in cancer.

Polo-like kinases and tumourigenesis

PLK1

PLK1 is a master regulator of mitosis and drives mitotic progression in normal cycling cells. Post DNA damage PLK1 has been shown to re-inititate mitosis by facilitating proteosomal degradation of p53 through GTSE1 activation, which allows the cell to overcome cell cycle arrest and resume division [21]. In the murine cell line NIH 3T3, increased levels of PLK1 conferred a transformed phenotype capable of producing colonies in soft agar assays [104]. Likewise, in normal human fibroblasts, overexpression of PLK1 also produced malignant cell transformation; these transformants were also capable of producing tumours in nude mice [104]. Functionally, PLK1 is expressed differentially between normal and cancer cells. Unlike normal cells, where PLK1 levels begin to rise in S- and peak in G_2 phase, in cancer cells, PLK1 is expressed early in G_1 and is essential for the G_1/S transition [105]. Once in S-phase, PLK1 promotes DNA

synthesis by activating the origin of recognition complex 2 (Orc2), a component of the DNA replication machinery, prompting DNA replication even in the presence of genotoxic stressors [106]. This relationship between PLK1 and Orc2 has translated into drug resistance in the treatment of pancreatic cancer [107]. Patients with head and neck squamous cell carcinoma, esophageal cancers, or melanomas, exhibited increased levels of PLK1 and experienced a shortened 5-year survival compared to those that had low levels of PLK1[108]. Elevated levels of PLK1 have also been positively correlated with an increased tumour grade in ovarian, endometrial cancers, and gliomas [109-111]. In breast cancer, PLK1 levels have been positively associated with estrogen receptors (ER) *in vivo* [112]. While *in vitro*, PLK1 interacts with ER protein and acts as a transcriptional co-factor activating classical ER targets like *Wisp2* [113]. Multiple groups have also reported PLK1 deregulation in colorectal cancers, with increased PLK1 associated with increased age and tumour progression and poor prognosis [47, 114, 115]. Hepatocellular carcinoma and prostate cancers also exhibit a PLK1 over-expression phenotype [23, 116]. It may be that upregulation of PLK1 may trigger downstream effects that push the cell through the cell cycle regardless of completion of DNA replication, DNA damage, or proper microtubule attachments at the kinetochore, generating genomic instability [105]. Of course with PLK1 consistently up-regulated in multiple cancer types and its value as a prognostic indicator, recent research has concentrated on the development of effective PLK1 inhibitors [36, 116-120]. Several small molecule inhibitors targeting PLK1 have advanced onto phase I and phase II clinical trials [105]. Perhaps in the future, PLK1 inhibitors may accompany standard chemotherapy treatments.

PLK2 and PLK3

PLK2 and PLK3 are both genes that become activated during stress in a p53-dependent manner. In contrast to PLK1, PLK2 and PLK3 are consistently downregulated in several tumour types. Downregulation of *PLK2* through epigenetic means is a common event in hematological malignancies including B-cell lymphomas, B-cell chronic lymphocytic leukemias, multiple myeloma, acute myelogenous leukemia and myelodysplastic syndromes (MDS) [121-123]. In addition, two independent groups determined that downregulation of PLK2 is associated with drug resistance in ovarian cancers [124, 125]. Syed et al went on to show that *PLK2* is epigenetically silenced in ovarian cancers with the increase in its promoter methylation associated with chemotherapy resistance. It was proposed that PLK2 may serve as a useful biomarker for chemotherapy insentivity [124, 126]. It is noted that, DNA hypermethylation of *PLK2* is not limited to blood neoplasms and ovarian cancers. Epigenetic downregulation of both *PLK2* and *PLK3* has also been detected in hepatocellular carcinoma [23]. PLK3 levels are also negatively correlated with lung carcinomas and head and neck squamous cell carcinomas, with an low PLK3 levels associated with increased tumourigenicity [108] uterine, bladder [20] and breast cancers [127]. The lowered expression of both of these proteins may confer a growth advantage to cancer cells by avoiding activation of p53

PLK4

Compared to the other PLKs, PLK4's expression profiles in malignancy is not unidirectional. Interestingly both up and down regulation of Plk4 have been implicated in malignancy. This may be related to differences in tissue type and/or the cellular makeup

of these tumours. For example, in the liver, PLK4 levels have been reported to be significantly downregulated in several independent samplings of HCC [23, 58, 128]. Decreased levels of PLK4 in HCC are correlated to increased clinical stage and tumour size along with significantly decreased overall survival [128]. The chromosomal location where *PLK4* resides, 4q.28, frequently undergoes deletion in human HCC, culminating in PLK4 loss of heterozygosity [23, 58]. In multiple myeloma, a large proportion of samples (40%) assayed exhibited centrosomal amplication as well as low levels of PLK4 [129]. However, here, low levels of PLK4 and centrosome amplification were correlated with a better prognosis [129]. Converse to these examples, PLK4 was found to be upregulated in colorectal and breast cancers [114, 130]. In colorectal cancer, similar to PLK1, increased PLK4 levels were linked to increased patient age, but not with tumour size [114]. In breast cancers, overexpression of PLK4 occurs in the more aggressive triple negative breast cancers and its upregulation is associated with poor survival [130]. The latter findings, together with PLK4's unique structural features, has led to the development of selective small molecule inhibitors [130] for PLK4. One of these inhibitors, deemed compound 50 (designated CFI-400437) was particularly effective at suppressing PLK4 and decreasing tumour growth in a xenograft model [130]. Although PLK4 has been clearly implicated in malignancies, its role in tumourigenicity is multi-dimensional and mirrors the protein's ability to be dynamic, with characteristics of a pro-mitotic protein as well as a stress and DNA damage response protein.

PLK5

PLK5 may potentially play a tumour suppressive role in brain tumours. *In vivo*, *PLK5* has been shown to be silenced in astrocytomas and glioblastoma multiforme *via* promoter hypermethylation. While ectopic overexpression in cells derived from glioblastoma multiforme induced their apoptosis [67]. De Carcer et al. have suggested that silencing *PLK5* through promoter hypermethylation may provide glioblastoma cells with a survival advantage that they may not have if PLK5 was active [67]. PLK5 is a newly discovered PLK and has yet to be fully characterized. Although, much of the research to date suggests that it may have tumour suppressive qualities and its function may play a role in deterring the formation of tumours in the brain.

With the PLKs being so conserved across taxa and their function intertwined with indispensable cell dynamics, it is incumbent that their methods of regulation and downstream targets be elucidated. The purpose of my dissertation was to determine if epigenetics played a role in regulating the expression of the *PLKs* and if this was related to a tumourigenic state.

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Chapter 2

Aberrant methylation of *Polo-like kinase* CpG islands in *Plk4* heterozygous mice

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Introduction

The Polo-like kinases (Plks) are a highly conserved family of serine-threonine kinases, found from unicellular eukaryotic organisms to higher multicellular eukaryotes. The mammalian Plks (*Plk1-4*) have been shown to play major roles in cell cycle regulation, centrosome dynamics and the cellular response to stress. Furthermore, perturbations in individual Plk protein levels have been associated with malignancies. For example, high levels of Plk1 are indicative of a poor prognosis in esophageal, non-small cell lung cancer and oropharyngeal carcinomas [1, 2] and have been observed in various forms of cancers including gastric, breast, ovarian, endometrial, gliomas, thyroid and melanomas.[3] In contrast, Plk3 is down-regulated significantly in carcinomas of the lung, head and neck.[4, 5] The *Plk2* gene is downregulated in lymphomas and B-cell malignancies.[6] In the case of Plk4, over 50% of aged *Plk4* heterozygous ($Plk4^{+/-}$) mice develop tumours in comparison to only 3% of their wild-type littermates, the major site of tumour formation being the liver and lung.[7] In mice, *Plk4* is haploinsufficient for tumour suppression, while in humans, loss of heterozygosity (LOH) for the *Plk4* gene was found in 60% of a small sample of human hepatocellular carcinomas (HCC) cases.[7] The increased rate of tumourigenesis likely related to the generation of aneuploidy, as altered Plk4 levels result in abnormal centrosome numbers, [8]

furthermore Plk4 may also play a key role in a DNA damage response pathway consistent with its phosphorylation of p53,[7] and Chk2.[9] In general, overexpression of Plk1 is typically considered to be oncogenic in nature while the remaining Plks likely function as tumour suppressors.

Recently it has become evident that the hypermethylation of CpG islands of tumour-suppressor genes, histone modification and chromatin remodelling are common events in cancers. (for review see [10]) Individual *Plk* gene epigenetic modifications associated with malignancy have previously been documented for *Plk2* where its methylation-dependent silencing was detected at a high rate in B-cell malignancies and Burkitt's Lymphoma as well as in follicular lymphoma.[11, 12] The correlation between the methylation status of the *Plks and* malignancy has not been studied in detail. In this regard, as noted below, we initially identified a gender disparity for the development of HCC in $Plk4^{+/-}$ mice. Previously, the development of HCC was attributed to haploinsufficiency for *Plk4* rather than via loss off heterozygosity. [7] Given that there is accumulating evidence that epigenetic changes are a driving force in the development of HCC, [13] we were interested in determining whether a relationship exists between individual *Plk* epigenetic modifications in the context of *Plk4* haploinsufficiency and the development of HCC.

Results and Discussion

Plk methylation status in ageing mice and HCC samples

Sex specific predisposition to cancer may reflect the underlying effects of the methylation patterns of key cancer genes. While the mechanism remains unclear, gender

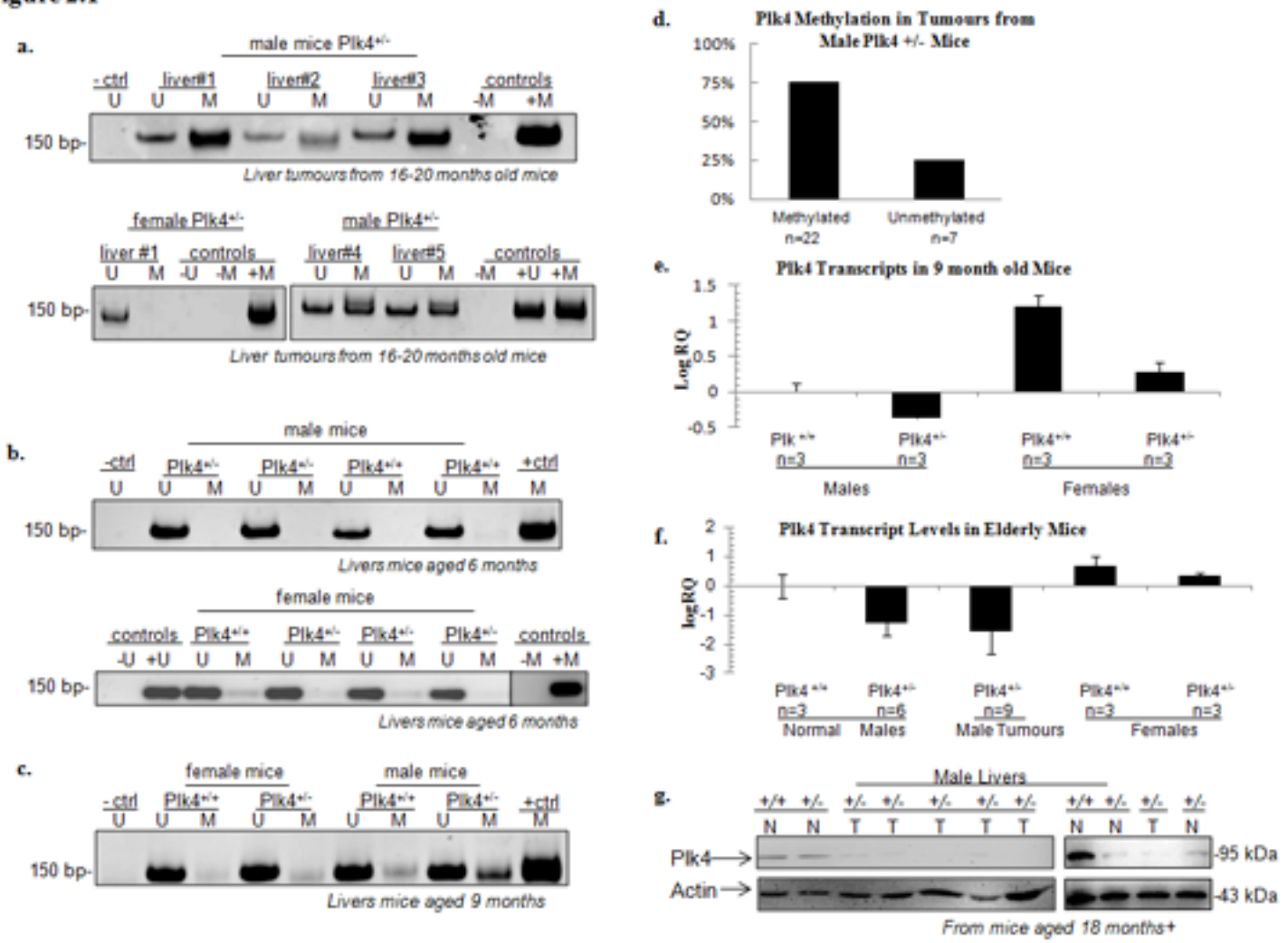
disparity for HCC has previously been established in both humans and mice, where males are 3-5 times more likely to develop HCC than females.[14, 15] Therefore, in the present study, we examined the rate of HCC in female and male *Plk4*$^{+/-}$ mice and found that in females the rate of HCC was approx 12% (n=32) in comparison to 35% (n=60) in male *Plk4*$^{+/-}$ mice, indicative of a gender disparity for HCC development. An analysis of the mouse and human sequence databases revealed that three of four murine and all four human *Plk* genes have CpG rich regions at their 5' termini suggesting they may also be subject to regulation by promoter methylation. We examined the methylation status of the promoter region of the *Plk* genes from DNA extracted from aging mice for normal liver and liver tumours, and detected an increase in methylation status of the *Plk4* gene in 22/29 tumours including 16/22 liver tumours studied in male mice (Fig 2.1a). Methylation status was confirmed *via* bisulfite sequencing of the *Plk4* CpG island, in which 30-40% of the 38 CpG sites analyzed were methylated (Supplementary figure 2.1). In contrast to the situation in males, we detected no *Plk4* methylation in a small number of liver tumours found in females. Interestingly, at 6 months of age, no significant level of *Plk4* CpG island methylation was detected in either male or female livers (Fig. 2.1b). However, at 9 months of age and corresponding to our observed phenotype in aged mice, higher levels of *Plk4* promoter methylation was detected in male mice in comparison to their female littermates (Fig. 2.1c). In total, almost 80% of the HCC samples examined were methylated at *Plk4* (Fig. 2.1d). Similar disparities in the methylation status of individual genes associated with malignancy were previously found for RASSF1A in lung cancer, with males showing higher levels of methylation.[16]

The effect of aberrant Plk methylation on expression

Lower Plk4 levels likely play a role in malignancy by affecting genomic stability through a mechanism related to Plk4's role in centrosome duplication [8] and/or DNA damage pathways. [17] We therefore examined the levels of Plk4 transcripts and found that the levels were substantially lower in males *versus* female mice as early as 9 months of age (Figure 1e) and were greater than 10 fold lower in livers and liver tumours from aged $Plk4^{+/-}$ mice compared to wild type males and females and $Plk4^{+/-}$ females (Fig. 2.1f). Similarly, Plk4 protein was also significantly reduced in tumours (Fig. 2.1g). It is noted that, while livers from $Plk4^{+/-}$ mice were grossly normal, they displayed variable amounts of Plk4 transcripts with an average that is significantly lower than found $Plk4^{+/+}$ mouse livers. Similarly, at the protein level, in $Plk4^{+/-}$, we see varied amounts of protein. It is noted that the Plk4 mice typically develop HCC 18-24 months on with some cases as early as 13 months. We suggest that this likely reflects varying stages of progression towards the development of HCC; suggesting that reduced levels of Plk4 as a result of promoter methylation may precede the appearance of visible tumours. Low levels of Plk4 have been shown to result in the generation of mono-polar spindles and aneuploidy in both cell lines and tissues. [7], [8] This exemplifies the possibility that epigenetic modifications may play a role in gender biases for malignancy and corresponds to our observation that epigenetic modifications of the *Plk4* gene leads to further Plk4 downregulation, particularly in males.

Figure 2.1. *Plk4* CpG island methylation and expression levels in elderly *Plk4*$^{+/-}$ male mice and HCC samples. Shown in each case (a-c) is a representative figure of typical results based on determination of *Plk4* promoter methylation in 6-9 females and males for both *Plk4* wild type and *Plk4*$^{+/-}$ genotypes. (a) Methylation status of *Plk4* promoter regions of genomic DNA extracted from liver tumours in *Plk4*$^{+/-}$ mice as determined by MSP. U=unmethylated, M=methylated. (b) *Plk4* CpG island methylation of liver samples from mice aged 6 months and (c) 9 months. (d) Graphical representation summarizing percentage of *Plk4* promoter methylation in liver tumours from 18-24 month old *Plk4*$^{+/-}$ male mice. (e) Relative levels of Plk4 transcripts as determined by qPCR. RQ values were normalized to the level of Plk4 transcripts in livers from 9 months old *Plk4*$^{+/+}$ animals. The error bars represent the upper and lower limit of the standard error from the mean expression level (RQ). (f) Relative levels of Plk4 transcripts in liver tissue and tumours from elderly mice. (g) Level of Plk4 protein in liver tissue extracts as determined by Western blot analysis. Actin levels were used as a loading control. N= normal tissue, T= tumour tissue.

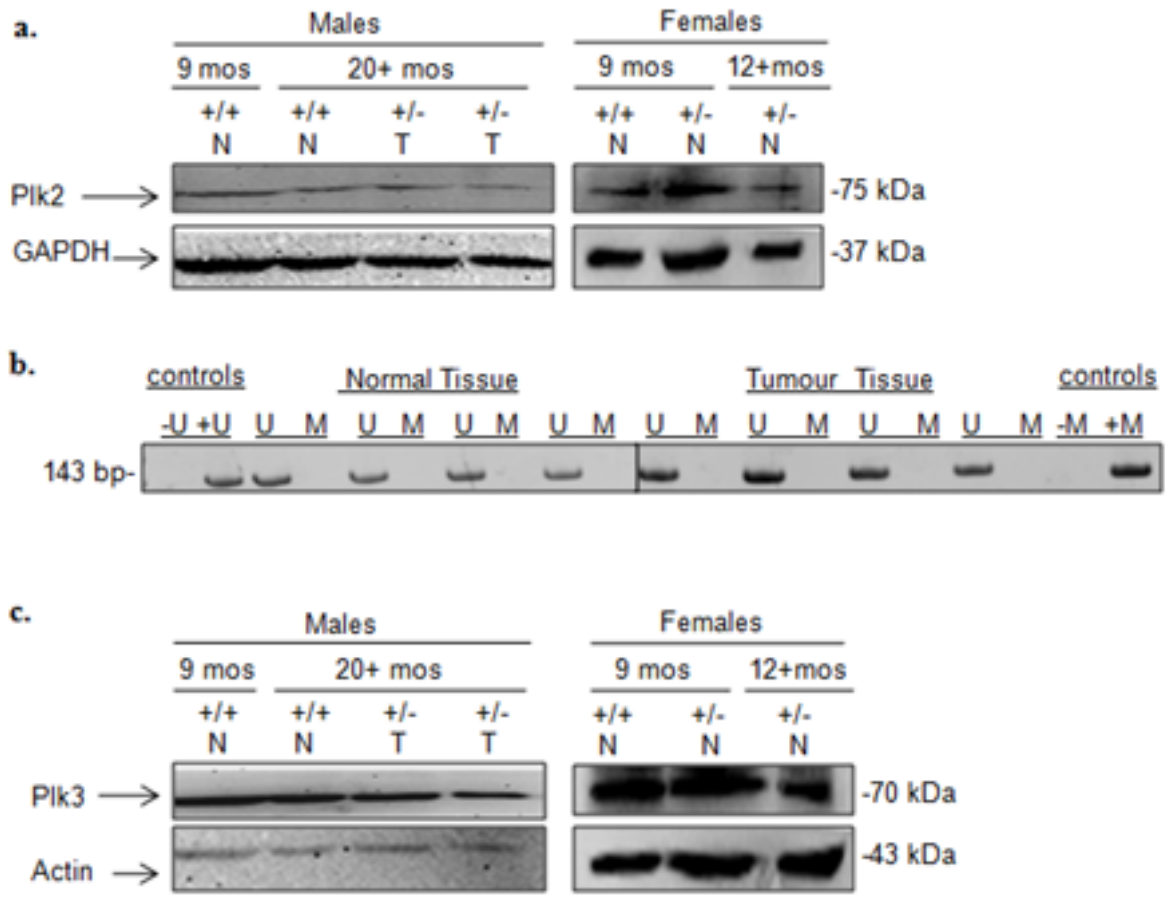
Figure 2.1


a.
male mice Plk4+/-
- ctrl
liver#1
liver#2
liver#3
controls
U U M U M U M -M +M
150 bp-
Liver tumours from 16-20 months old mice
female Plk4+/-
male Plk4+/-
liver #1
controls
liver#4
liver#5
controls
U M -U -M +M U M U M -M +U +M
150 bp-
Liver tumours from 16-20 months old mice
b.
male mice
-ctrl
Plk4+/-
Plk4+/-
Plk4+/+
Plk4+/+
+ctrl
U U M U M U M U M M
150 bp-
Livers mice aged 6 months
female mice
controls
Plk4+/+
Plk4+/-
Plk4+/-
Plk4+/-
controls
-U +U U M U M U M U M -M +M
150 bp-
Livers mice aged 6 months
c.
female mice
male mice
- ctrl
Plk4+/+
Plk4+/-
Plk4+/+
Plk4+/-
+ctrl
U U M U M U M U M M
150 bp-
Livers mice aged 9 months
d.
Plk4 Methylation in Tumours from Male Plk4 +/- Mice
100%
75%
50%
25%
0%
Methylated n=22
Unmethylated n=7
e.
Plk4 Transcripts in 9 month old Mice
Log RQ
1.5
1
0.5
0
-0.5
Plk +/+ n=3
Plk4+/- n=3
Plk4+/+ n=3
Plk4+/- n=3
Males
Females
f.
Plk4 Transcript Levels in Elderly Mice
logRQ
2
1
0
-1
-2
-3
Plk4 +/+ n=3
Plk4+/- n=6
Plk4+/- n=9
Plk4+/+ n=3
Plk4+/- n=3
Normal
Males
Male Tumours
Females
g.
Male Livers
+/+ +/- +/- +/- +/- +/- +/- +/+ +/- +/- +/-
N N T T T T T N N T N
Plk4
Actin
-95 kDa
-43 kDa
From mice aged 18 months+

There is accumulating evidence that the Plk family of proteins often share the same targets or signalling pathways, thereby placing their substrates under tighter or opposing controls.[18] It was therefore of interest to determine whether *Plk4* haploinsufficiency was also correlated with altered CpG island methylation and expression levels for the remaining *Plks*. Unlike the situation found in haematological malignancies, [11] we found no significant change in either the methylation status or expression levels for *Plk2* in tumours, aging mice or association with gender (Fig. 2.2a-b). There were also no discernible changes in Plk3 protein levels (Fig. 2.2c). Interestingly, the methylation status for *Plk1* was opposite to that for *Plk4*. Normal tissue, regardless of age, showed methylation in the *Plk1* promoter region in 80% of the samples tested (Fig. 2.3a-b). However, *Plk1* was found to be hypomethylated in 80% of HCC and other tumours found in $Plk4^{+/-}$ mice (Fig. 2.3a-b). Furthermore, this loss of promoter methylation corresponded to a large increase in Plk1 transcript levels (Fig. 2.3c) and a corresponding increase in Plk1 protein level in HCC samples relative to normal liver tissue (Fig. 2.3d). While the presence of increased Plk1 protein within tumours is by no means novel and is consistent with its potentially oncogenic role in malignancy, our findings indicate a novel

Figure 2.2. *Plk2* methylation of CpG island and protein expression levels for Plk2 and Plk3 in relation to both age and gender in mice. (a) Levels of Plk2 protein in liver tissue extracts as determined by Western blot analysis. (b) *Plk2* CpG island methytlation status as determined by MSP analysis. (c) Levels of Plk3 protein in liver tissue extracts as determined by Western blot analysis. Shown are representative figures of 6-9 females and males for both *Plk4* wild type and $Plk4^{+/-}$ genotypes. N= normal, T= tumour. Note: we did not analyze *Plk3* for methylation status as no CpG islands were detectable for the *Plk3* gene with MethPrimer

Figure 2.2


a.
Males
Females
9 mos
20+ mos
9 mos
12+mos
+/+ +/+ +/- +/-
N N T T
+/+ +/- +/-
N N N
Plk2
GAPDH
-75 kDa
-37 kDa
b.
controls
Normal Tissue
Tumour Tissue
controls
-U +U U M U M U M U M U M U M U M U M -M +M
143 bp-
c.
Males
Females
9 mos
20+ mos
9 mos
12+mos
+/+ +/+ +/- +/-
N N T T
+/+ +/- +/-
N N N
Plk3
Actin
-70 kDa
-43 kDa

mechanism for *Plk1* regulation in that its expression may be influenced by its promoter methylation status, and, our results suggest that the transforming capacity of *Plk4* heterozygosity may be linked to aberrant methylation of *Plk1* and *Plk4*.

Plk methylation status in human HCC samples

In order to determine if *Plk4* methylation status is correlated with the development of HCC in humans, we also examined a limited number of human liver samples (Supplementary figure 2.2). We found that in normal human hepatic tissue the *Plk4* promoter region was not methylated in samples taken from patients with no history of HCC. In the case of HCC samples we detected *Plk4* CpG island hypermethylation and downregulation of *Plk4* transcript levels as well as barely detectable methylation of the *Plk1* promoter region. In 3 of 6 samples we found that the corresponding Plk1 transcript levels were higher than in the normal control (Supplementary figure 2.2e), We did not detect any changes for *Plk2* and *Plk3* promoter methylation (data not shown). Since we began this aspect of our study, Pellegrino *et al.* (2010) examined a large cohort of human HCC samples and reported *Plk2-3* down-regulation in human hepatocellular carcinoma correlated with either promoter hypermethylation and/or loss of heterozygosity at the *Plk2-3* loci. [19] In the case of *Plk4* many of the cases displayed loss of heterozygosity with no methylation within the *Plk4* promoter region. They did not report any analysis for the methylation status of *Plk1*. Their inability to detect methylation changes for *Plk4* and ours for *Plks2-3* may be a reflection of the use of different primers used for methylation specific PCR (MSP) which samples a small subset of the potentially methylated residues

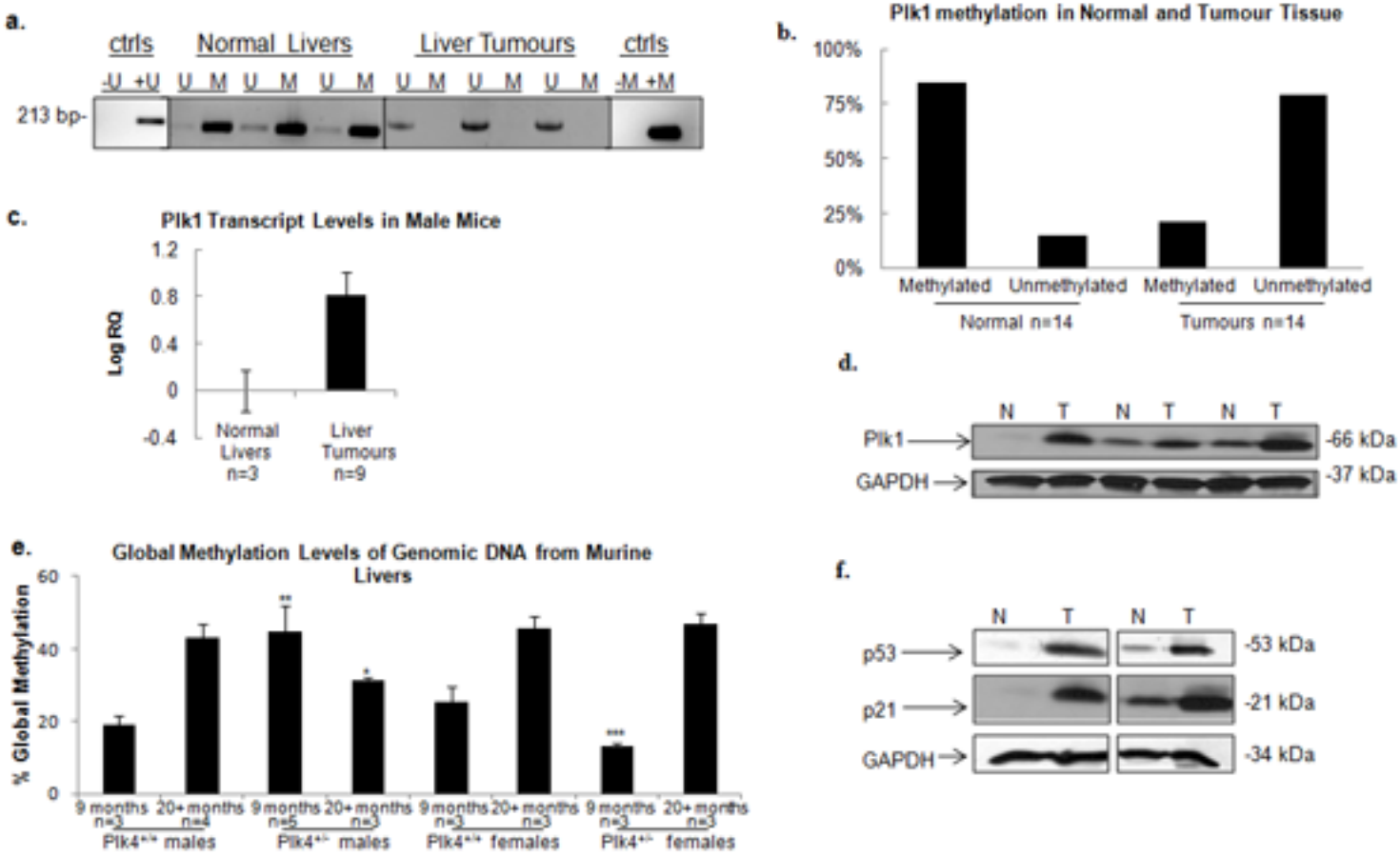
within a CpG island. Together these results suggest that in general, epigenetic changes within the *Plks* may contribute to malignancy in humans.

Global Methylation status and p53 activity

In general, global hypermethylation increases with age; however, studies on aberrant methylation of genes associated with HCC, like in many other malignancies, are characterized by an overall general increase in global hypomethylation along with increased rates of hypermethylation of tumour suppressors.[20] We employed an ELISA-based assay (Epigentek) in order to quantitatively measure genomic methylation. Interestingly, we found no significant difference between the 9 month old wild type males and age-matched wild type and *Plk4* $^{+/-}$ females (Fig. 2.3e). However, consistent with what has been shown with age progression, we found an overall increase in the global methylation of genomic DNA in wild type male mice and both *Plk4* wild type and heterozygous female mice from 9 to 20 months. In contrast, there was a decrease in global methylation in *Plk4*$^{+/-}$ male mice over the same time period (* $p<0.05$). Furthermore, significantly higher levels of global methylation were found in young *Plk4*$^{+/-}$ male mice compared to their wild type littermates (** $p<0.001$), while the opposite is true for the *Plk4*$^{+/-}$ female mice, where they had significantly lower levels of global methylation compared to young wild type females (*** $p<0.05$). . Although, as the females age, both genotypes have similar levels of global methylation. These results suggest that there is an interplay between gender and *Plk4* haploinsufficiency that affects global methylation in liver tissue.

Figure 2.3. Analysis of *Plk1* CpG island methylation status and expression, global methylation and expression levels for p53 and p21 in normal and liver tumour tissue samples (a) *Plk1* CpG island methylation status for HCC samples compared to normal tissue as determined by MSP for aged-matched littermates. U=unmethylated, M=methylated. (b) Graphical representation summarizing the percentage of methylated Plk1 promoters in both normal liver tissue and tumours. (c) Plk1 transcript levels in normal liver and HCC samples as determined by qPCR. RQ values were normalized to the level of Plk1 transcripts in $Plk4^{+/+}$ livers. The error bars represent the upper and lower limit of the standard error from the mean expression level (RQ) (d) Plk1 protein levels were examined by Western blot analysis. GAPDH protein levels were used as a loading control. N = normal, T = tumour. (e) The percent of global methylation of genomic DNA extracted from liver was determined by an ELISA assay specific for methylated DNA. (*$p<0.05$), **$p<0.001$, ***$p<0.05$,) The error bars represent the upper and lower limit of the standard error (f) p53 and p21 protein levels as detected by Western blot analysis. GAPDH levels were used as a loading control. N=normal, T=Tumour.

Figure 2.3


a.
ctrls
Normal Livers
Liver Tumours
ctrls
-U +U U M U M U M U M U M U M -M +M
213 bp-
b.
Plk1 methylation in Normal and Tumour Tissue
100%
75%
50%
25%
0%
Methylated
Unmethylated
Methylated
Unmethylated
Normal n=14
Tumours n=14
c.
Plk1 Transcript Levels in Male Mice
Log RQ
1.2
0.8
0.4
0
-0.4
Normal Livers n=3
Liver Tumours n=9
d.
N T N T N T
Plk1
GAPDH
-66 kDa
-37 kDa
e.
Global Methylation Levels of Genomic DNA from Murine Livers
% Global Methylation
60
40
20
0
9 months n=3
20+ months n=4
9 months n=5
20+ months n=3
9 months n=3
20+ months n=3
9 months n=3
20+ months n=3
Plk4+/+ males
Plk4+/- males
Plk4+/+ females
Plk4+/- females
f.
N T N T
p53
p21
GAPDH
-53 kDa
-21 kDa
-34 kDa

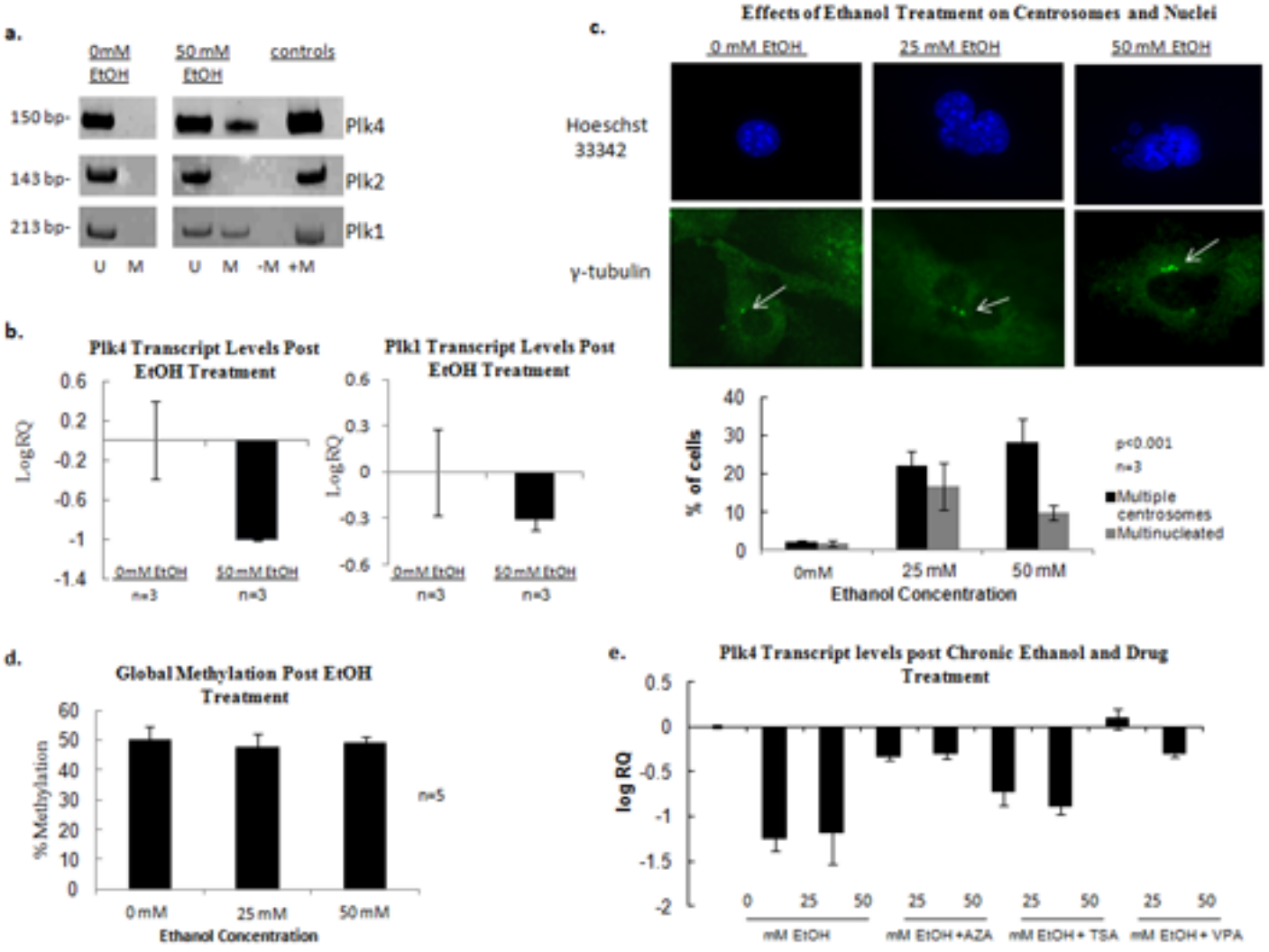
p53 has also been found to be an upstream negative regulator of *Plk4 via* histone deactylation (HDAC).[21] We therefore examined p53 levels in normal and tumour tissue and found that both p53 and p21 were up-regulated in tumour tissue compared to the normal tissue (Fig. 2.3f). p53 is also a substrate for Plk4 [22] and p53 levels/activity are upregulated as a result of haploinsufficiency in MEFs.[17] These observations suggest that increased p53 levels/activity, a consequence of *Plk4* haploinsufficiency, may also contribute to repressive chromosome structure and the reduced transcript profiles seen in aged and tumourigenic *Plk4*$^{+/-}$ mice.

The effect of chronic alcohol exposure on Plk4 Methylation status in MEFs.

Alcohol has become an emerging environmental player in the modification of the epigenome. [23] In humans chronic alcoholism has been shown to increase availability of blood homocysteines, which in turn modifies S'adenosyl methyltransferase (MATs) levels, an enzyme responsible for the transfer of methyl groups to DNA. Furthermore, these patients showed a significant increase in global DNA methylation by up to 10%. [24] There is increasing evidence that alcohol consumption, a known risk for the development of HCC, can increase the methylation status of promoters with a subsequent decrease in gene expression .[24-26] In liver cells, the presence of alcohol results in an increase in the formation of reactive oxygen species, which are in turn responsible for hepatocyte damage, cellular apoptosis, and the tumour promoting effect of ethanol.[27] Interestingly, we have preliminary evidence of increased *Plk4* methylation in human cirrhotic livers with no evidence of viral infection (see supplementary data). This coupled

Figure 2.4. The effect of chronic ethanol exposure on murine embryonic fibroblasts (MEFs) (a) MEFs were exposed to ethanol for a period of 7 days at which time the methylation status of individual *Plk* CpG islands was determined by MSP analysis. U=unmethylated, M=methylated. (b) Plk4 and Plk1 transcript levels in *Plk4* wildtype ($Plk4^{+/+}$) MEFs were determined after 7 days of ethanol exposure by qPCR. RQ values were normalized to the level of transcript found in untreated control MEFs. Standard error was calculated based on the minimum and maximum values from the mean expression levels (RQ) (c) Immunofluorescence analysis $Plk4^{+/+}$ MEFs exposed to 25mM and 50mM ethanol for a period of 7 days. Centrosomes were detected by γ-tubulin staining and DNA by Hoechst staining. A graphical representation of cells exhibiting multiple centrosomes and multinucleation is underneath. Shown are the results of three independent experiments in which more than 200 cells were analyzed each time for each condition. Error bars indicate standard error. (d) Global methylation analysis of genomic DNA from MEFs after 7 days alcohol exposure as determined by MSP analysis of B1 elements. The error bars represent the upper and lower limit of the standard error from the mean.(e) Plk4 transcript levels as determined by qPCR of MEFs exposed to ethanol for 7 days in the presence of 5-aza-2'deoxycytidine (AZA), trichostatinA (TSA) and valproic acid (VPA). RQ values were normalized to the level of Plk4 transcript in untreated control MEFs. The error bars represent the upper and lower limit of the standard error from the mean expression level (RQ)

Figure 2.4


a.
0mM EtOH
50 mM EtOH
controls
150 bp-
143 bp-
213 bp-
Plk4
Plk2
Plk1
U M U M -M +M
b.
Plk4 Transcript Levels Post EtOH Treatment
Plk1 Transcript Levels Post EtOH Treatment
Log RQ
0mM EtOH
50 mM EtOH
n=3
c.
Effects of Ethanol Treatment on Centrosomes and Nuclei
0 mM EtOH
25 mM EtOH
50 mM EtOH
Hoeschst 33342
γ-tubulin
% of cells
p<0.001
n=3
Multiple centrosomes
Multinucleated
0mM
25 mM
50 mM
Ethanol Concentration
d.
Global Methylation Post EtOH Treatment
% Methylation
n=5
0 mM
25 mM
50 mM
Ethanol Concentration
e.
Plk4 Transcript levels post Chronic Ethanol and Drug Treatment
log RQ
mM EtOH
mM EtOH +AZA
mM EtOH + TSA
mM EtOH + VPA

with the associated risk of alcoholism and HCC led us to examine the methylation status and expression of the individual *Plks* in a cell-based model of chronic ethanol exposure

When wild type MEFs were exposed to a 25-50mM dose of alcohol for 7 days we found increased *Plk4* promoter methylation and a significant decrease in corresponding Plk4 transcript levels (Fig. 2.4a-b). (Note, that in MEFs there was no methylation detected for the *Plks* pre-treatment). We also observed an increase in *Plk1* promoter methylation although in this case the change in expression was not significant, displaying a large degree of variation. Furthermore, we found a large increase in the proportion of cells containing multiple centrosomes or multinucleation (Fig. 2.4c), phenotypes correlated with reduced Plk4 levels in $Plk4^{+/-}$ mice.[7] Additionally, this observation mimicked the effect of lower Plk4 levels evident in $Plk4^{+/-}$ MEFs, which display increased centrosome numbers and ploidy with passaging. [28, 29] Unexpectedly, in contrast to the situation found *in vivo* for chronic alcohol exposure, we found no evidence for increased global hypomethylation in MEFs. (Fig. 2.4d). However, these results do suggest that in MEFs that the *Plk4* promoter may be a target for regulation by methylation in response to metabolic stress. This idea is supported by the fact that chronic alcohol exposure of MEFs has been shown to increase levels of reactive oxygen species (ROS), [30] increased levels of p53 and p53 downstream targets such as p21. [31] Interestingly, consistent with this p53 has been shown to indirectly repress Plk4 expression *via* HDAC in response to stress. [21]. Additionally, while ROS have generally been show to induce global hypomethylation,[32] there is increasing evidence that they may also induce promoter hypermethylation. For example, both the E-cadherin and

catalase promoters have been show to become methylated post ROS exposure. [33, 34]This is an area for future consideration.

The effect of concurrent drug treatment on MEFs chronically exposed to alcohol.

Unlike mutations or deletions that lead to the aberrant expression of tumour suppressor genes, epigenetic modifications, like DNA methylation, are reversible using hypomethylating drugs that inhibit DNA methyltransferase activity and/or inhibit histone deacetylases (HDACs). [35] Concurrent alcohol and epigenetic drug treatments revealed that 5-aza-2'-deoxycytidine, a DNA hypomethylator and valproic acid, which has been shown to be an HDAC inhibitor, partially restored Plk4 transcript levels, while no significant differences were seen with trichostatin A (an HDAC inhibitor) treatment (Fig. 2.4e).

Modification of the methylation status and corresponding expression levels of both Plk4 and Plk1 are likely contributing factors in the development of HCC in both mice and humans. This creates interesting possibilities in that epigenetic modifications are potentially reversible through the use of demethylating and HDAC inhibiting drugs as both prophylactic and therapeutic tools. This may lead to the development of novel treatment options for HCC.

Conclusions

We determined that a gender disparity exists for the development of HCC in the *Plk4* mouse model. This disparity was correlated with increased DNA methylation at the *Plk4* locus and higher risk of developing hepatocelluar carcinomas in aged male *Plk4*

heterozygous mice as compared to female mice. In contrast, we discovered the opposite correlation for *Plk1* where in normal liver tissue the *Plk1* promoter is hypermethylated while in tumours, *Plk1* CpG islands become hypomethylated and the gene upregulated. This represents a novel form of regulation for Plk1 that may have implications for its expression in other tumour types. Furthermore, we determined that chronic alcohol exposure, well known to be implicated in the development of cirrhosis leading to HCC, also leads to *Plk4* promoter hypermethylation and downregulation, accompanied by defects in the control of centrosome numbers and by the occurrence of multinucleated cells. Aberrant *Plk4* methylation and expression in chronically exposed MEFs could be rescued by treatment with known hypomethylating and/or HDAC inhibiting drugs.

Material and Methods

Methylation Specific PCR and Global methylation

DNA from tissue was extracted as follows: 20-60 mg of tissue was digested with Pro K at a concentration of 0.5 mg/mL for 48 hrs at 55°C, followed by phenol chloroform extraction. DNA from formalin fixed paraffin embedded tissue was isolated using the FFPE DNA isolation kit following manufacturer's instruction (Qiagen). DNA from cells was isolated by trypsinization for 5 minutes, neutralization with media, the cells were then spun down at 100*g* for 5 minutes, then re-suspended with 200 ul of media, followed by Pro K treatment. Bisulfite modification was preformed as previously described. [36] The DNA was further purified with a Wizard Mini DNA clean up kit (Promega), followed by desulfanation with 2M NaOH for 10 min and ethanol precipitation. MSP was performed after bisulfite treatment of DNA. Mouse fully methylated genomic DNA

(NEB) was used a as a positive control where appropriate. Primers were designed *via* MethPrimer [37] within the CpG islands of each individual *Plk* gene (see Table 1). Global methylation levels for liver tissue were determined by the MethylFlash Methylated DNA Quantification Kit (Epigentek), an ELISA-based colourimetric assay. The assay was done according to the manufacturer's instructions, using 100 ng of genomic DNA. The Wallac Victor3 1420 multilabel counter was used to measure the assay at 450nm. Relative quantification was determined by normalizing the readings to the positive control provided with the kit. In ethanol treated mouse embryonic fibroblasts global methylation was assessed by determining the methylation status of B1 elements with MSP as previously described. [38] Briefly, there are 30, 000 copies of the 163 base pair element dispersed throughout the mouse genome. Each element contains 6 CpG dinucleotides. The methylation status of these elements is also responsive to DNA methyltransferase inhibitors like Azacytidine and therefore they are excellent indicators of global methylation. In order to determine the percentage of B1 element methylation densitometry was performed with analysis *via* the Syngene Gene tools version 3.07 software. Statistical analysis on the normalized results were performed using Statsoft Statistica v7.0.61.0 using a one-way ANOVA t-test where $p < 0.05$ was significant.

Tissue Samples

All murine samples were obtained from our breeding colony, with all protocols with animals were as approved by the University of Windsor Animal Care Committee according to the Canadian Council on Animal Care guidelines. *Plk4*$^{+/-}$ mutant mice on a 129Sv/CD1 background were obtained as described [28] and backcrossed with CD1 mice to establish a colony of *Plk4* wild type and *Plk4* heterozygous littermates. Mice were

maintained under normal light cycle and on regular chow. All tissue samples were obtained from aged matched littermates. For murine hepatocellular carcinoma (HCC) samples, it is noted that *Plk4*$^{+/-}$ mice develop a high rate of liver and lung tumours by 18-24 months of age [7] and thus the analysis was performed on spontaneously occurring hepatocellular carcinomas.

Cell lines

Mouse embryonic fibroblasts (MEFs) were harvested from *Plk4* wild type CD1 mice at day 12.5 post coitum as described previously in [28] and cultured with DMEM supplemented with 20% FBS (Sigma), 1% penicillin G sodium 10 000 U/mL and streptomycin sulphate 10 000 ug/mL, and 0.5% gentamycin 10mg/mL.

Western blot analysis

Protein from fresh tissue was extracted using the Trizol reagent (Invitrogen) according to manufacturer's suggestions. Cell lysates were obtained from cells harvested followed by lysing using lysis buffer (50mM Tris-HCl pH 7.4, 150mM NaCl, 1mM EDTA, 0.5% Triton X) with EDTA free protease inhibitor cocktail tablets (Roche). 20 ug of protein were subjected to immunoblotting. Primary antibodies used were as follows: p53 (Sigma), Plk1 (Abcam), p21 (Santa Cruz), GAPDH (Cell Signaling), and Actin (Sigma). Secondary antibodies used were anti-rabbit (Amersham) and anti-mouse horse radish peroxidase (HRP) (Sigma).

Analysis of gene expression

RNA was extracted from cells and tissues using the RNAeasy kit (Qiagen) according to manufacturer's recommendations. First Strand cDNA synthesis kit using Superscript II according to the manufacturer's instructions. Quantitative real time PCRs (qPCR) were conducted in an ABI 7300 instrument using 250 ng of cDNA with TaqMan Gene Expression Assays (Applied Biosystems) for mouse *Plk1* and *Plk4*. Rodent GAPDH probe was used as an internal control. Relative quantity (RQ) values were generated by the ABI 7300 system SDS software. The error bars represent the upper and lower limit of the standard error from the mean expression level (RQ) as analyzed by the SDS software with the ABI 7300 instrument. The error bars are calculated based on 95% confidence limits.

Immunofluorescence

Post treatment, MEF cells were fixed in 3.7% paraformaldehyde and probed with a mouse γ-tubulin primary antibody (Sigma) followed by an anti-mouse alexa fluor 568 secondary antibody (Invitrogen). The cells were then briefly incubated in Hoescht 33342. Adherent cells were analyzed with a Zeiss Axioskop 2 mot plus microscope and Northern Eclipse imaging software. Conditions for immunofluorescence were as described by Hudson et al, (2001). [28]

Ethanol and Drug treatments

Wild type MEFs were exposed to 25 mM and 50mM of EtOH per day for 7 days. Trichostatin A, 5 aza-2'-deoxycytidine, and valproic acid were administered concurrently at concentrations of 1nM, 10 nM, and 0.5 mM respectively. Plates were sealed with parafilm to prevent evaporation.

Table 1.

Mouse primer sequences:

Target Gene	Sense Primer Sequence
	Antisense Primer sequence
Plk1 U	5'aca aac acc tct ttt ata tct aca tc 3'
	5'tgg ttt gag tat tag ttg att ttg g 3'
Plk1 M	5'acg aac acc tct ttt ata tct acg tc 3'
	5'gtt ggt tcg agt att agt cga ttt c 3'
Plk2 U	5' caa act tta ccc aaa acc tac tcac 3'
	5'ata ggg tta gtt tgg atg ttt gtt t 3'
Plk2 M	5' aaa ctt tac cca aaa cct act cg 3'
	5'ggt tag ttc gga cgt ttg ttc 3'
Plk4 U	5'cac act ctc cac ttc tta aaa aca a 3'
	5' att tta tta tta gtg ttt gtg tta tgg 3'
Plk4 M	5'aca ctc tcc act tct taa aaa cga a 3'
	5' aat tta tta tta gcg ttc gcg tta c 3'
B1 Element U	5'-taa cct caa act caa aaa tcc acc-3'
	5'gtt ggg tgt agt ggt ata tat ttt taa ttt ta 3'
B1 Element M	5'ctcgaactcaaaaatccgcc 3'
	5' gtc ggg cgt agt ggt ata tat ttt t 3'

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Supplementary Materials and Methods:

Human Primers

Primers for methylation specific PCR were determined by MethPrimer and are as shown in Supplementary Table 1 (Human).

Human HCC samples

Human HCC samples were obtained from both the Ontario Tumour Bank and the Alberta Tumour Bank. All protocols were approved by the individual tumour banks and the University of Windsor Research Ethics Board.

Analysis of gene expression

RNA was extracted from tissues using the RNAeasy kit (Qiagen) according to manufacturer's recommendations. First Strand cDNA synthesis kit using Superscript II according to the manufacturer's instructions. Quantitative real time PCRs (qPCR) were conducted in an ABI 7300 instrument using 250 ng of cDNA with TaqMan Gene Expression Assays (Applied Biosystems) for human *Plk1 and Plk4*. Human GAPDH probe was used as an internal control. Relative quantity (RQ) values and error bars were generated by the ABI 7300 system SDS software. The error bars represent the upper and lower limit of the standard error from the mean expression level as analyzed by the SDS software with the ABI 7300 instrument. The error bars are calculated based on 95% confidence limits.

Bisulfite Sequencing PCR

Bisulfite sequencing PCR (BSP) for *Plk4* CpG islands was done by bisulfite converting 2ug of genomic DNA, as described previously (30) followed by PCR

amplification upstream of the *Plk4* promoter region with BSP Primers as follows: 5'GCGCTACGGTCAGTCGTACACTGACC3', 5'GAGGTTGAGGTTTAGTTTGGTT3', 5' AAAT T TTCTAAACTCCCTCCCT 3'. BSP primers were determined with the methyl-primer software package (ABI). PCR products were gel purified using the Qiaquick gel extraction kit (Qiagen) and analysed by direct sequencing or subcloned into the p-GEM-T-easy cloning vector (Promega). Sequence analysis was performed using the ABI 3730 with sequencing chemistry BDT version 3.1 We initially performed direct sequencing of 5 independent PCR amplifications for each tumour sample to identify methylated CpG sites in individual tumours. The sites were confirmed by a cloning method in which 10 subclones were picked for sequencing for each tumour sample. Individual CpG sites were considered to be positive for methylation where a minimum of 60% of the subclones displayed a methylated nucleotide.

Supplementary Table 1.

Human primer sequences for MSP:

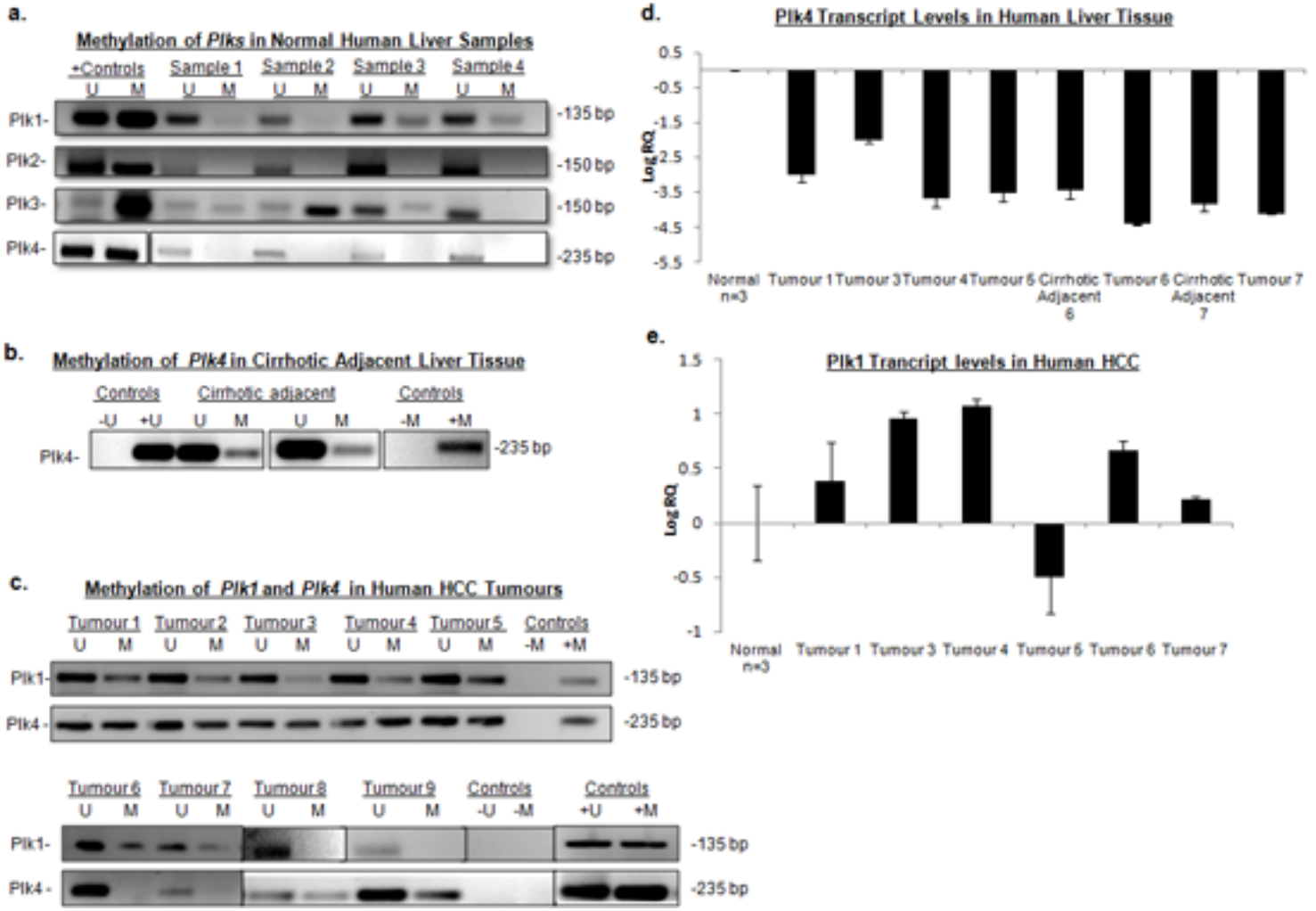
Gene	Sense Primer Sequence
	Antisense Primer Sequence
Plk1 U	5’ tag tga ttg tag gga agt tgg t 3’
	5’aca taa atc cac taa aac ctc c 3’
Plk1 M	5’ gta aat cca cta aaa cct cc 3’
	5’ gta gtg att gta ggg aag ttg 3’
Plk2 U	5’cac ccc aca acc aac caa aca 3’
	5’ gga tgg ttt tga agg ttt ttt t 3’
Plk2 M	5’ ccc acg acc gac cga acg c 3’
	5’ acg gtt ttg aag gtt ttt tcg c 3’
Plk3 U	5’agt aaa ttt agg tag tgt tat 3’
	5’ aaa ccc aac caa aaa aac a 3’
Plk3 M	5’aat tta ggt agc gtt acg cgc 3’
	5’ccg acc gaa aaa acg aac gc 3’
Plk4 U	5’cca aac tct aac cta aat tct cca a 3’
	5’ att att agt tta gtt tgg atg gta agt gg
Plk4 M	5’caa act cta acc taa att ctc cga a 3’
	5’tat tag ttt agt tcg gac ggt aag c 3’

Supplementary figure 2.1. Analysis of the methylation status of the murine *Plk4* CpG promoter CpG island by bisulfite sequencing for genomic DNA isolated from both normal liver and HCC tissue samples. More than 30% of the CpG sites upstream of the *Plk4* promoter region showed methylation in tumour samples (T), while little to no methylation was detected in age-matched wild type controls (WT). Fully methylated 3T3 DNA (NEB) was used as a positive control. Circles represent CG sites within the sequence. Shaded circles depict a methylated state, while open circles lack methylation.

Supplementary figure 2.1

Supplementary Figure 2.2 *Plk4* CpG island methylation and expression levels in human liver samples. (a) Plk methylation status of genomic DNA extracted from normal human liver tissue was determined by MSP. Fully methylated human genomic DNA (NEB) was used as a positive control for methylation. The negative PCR control lacked DNA template. U=unmethylated, M=methylated (b) In contrast the analysis of cirrhotic adjacent tissue by MSP displayed increased levels of *Plk4* methylation suggesting that methylation of *Plk4* may be an early event in the transition of cirrhosis to HCC. (c) Shown is representative image of both the *Plk4* and *Plk1* methylation status in human HCC tumour samples as determined by MSP. Only two of the samples had hypomethylation within the *Plk1* promoter region, suggesting that loss of *Plk1* methylation may be a later event. U=unmethylated, M=methylated (d) Human HCC samples were analysed for Plk expression by qPCR. There was more than a 20 fold decrease in Plk4 transcript levels in tumours in comparison to normal liver tissue, while (e) Plk1 transcript levels were elevated in several of the tumour samples compared to the control. RQ values were normalized to the level of Plk4 transcripts in normal livers. Transcript levels for tumours 2, 8, and 9 were not assessed as they were archival specimens with poor quality RNA. Human GAPDH was used as an internal control.

Supplementary figure 2.2



Chapter 3

p53-dependent and cell specific epigenetic regulation of the *Polo-like kinases* under oxidative stress

Authors: Alejandra Ward and John W. Hudson

Introduction

The polo-like kinases (*PLKs*) have been implicated in a variety of solid and hematopoietic tumours, which include B-cell lymphoma, hepatocellular carcinoma (HCC), head and neck squamous carcinoma, colorectal cancers, and most recently gallbladder cancer, just to name a few [1-5]. Moreover, their deregulation is often associated clinically with poor prognosis, such as the case of PLK1 overexpression in non-small cell lung carcinoma and head and neck squamous carcinoma, or downregulation of Plk4 in HCC [3, 6, 7]. Recently, we and others, have determined that the polo-like kinases, which are cell cycle regulated serine/threonine kinases, are susceptible to aberrant DNA methylation in many of the tumour types described above [1, 8-10]. Aberrant promoter methylation of *PLK1-4* have been implicated in hepatocellular carcinoma [9, 10], while *PLK2* promoter hypermethylation has been detected in hematologic malignancies such as acute myeloid leukemia and B-cell lymphoma, as well as in ovarian cancers [1, 8, 11]. Interestingly, the recently discovered *PLK5*, has tumour suppressor properties, and it is often hypermethylated in glioblastoma [12]. Given that these kinases, which are highly conserved among species, play crucial roles in important cell cycle events such as spindle pole assembly, the DNA damage response, G2/M transitions, and cytokinesis [6, 13, 14], proper regulation of these proteins is essential for the maintenance of genomic integrity and the prevention of

genomic instability. Therefore, the underlying question is what is prompting the aberrant epigenetic regulation of the polo-like kinases in a variety of cancer types?

It has been established that the microenvironment plays a significant role in the initiation and progression of tumourigenesis. The cellular microenvironment provides a platform from which bidirectional molecular cues can be exchanged. This topographical information can direct cellular phenomena which include growth, cellular differentiation, and division. The aberrant alterations in the microenvironment can confer tumourigenicity through direct genetic mutations, but more so *via* epigenetic plasticity [15, 16]. Oxidative stress, in the form of reactive oxygen species (ROS) and hypoxia, are components of the tumour microenvironment, and have been shown to be causative agents of abnormal, epigenetically-induced gene expressions in a variety of tumour types [17-19]. Studies have also revealed that several tumour suppressors and cell cycle regulators such as *p14ARF*, *p16INK4a*, and *BRCA1* are susceptible to epigenetic silencing through DNA hypermethylation or histone modification in the presence of oxidative stress [19, 20]. The purpose of this study was to examine the susceptibility of individual *PLK* regulation through epigenetic modifications in response to oxidative stress in the form of either ROS or hypoxia. Here we have determined that the polo-like kinases are indeed epigenetically modified in the presence of oxidative stress, though in a cell type-dependent and p53-dependent manner. Furthermore, we have determined that *Plk4* heterozygosity may play a role in the epigenetic regulation of *Plk1* in response to oxidative stress.

Results and Discussion

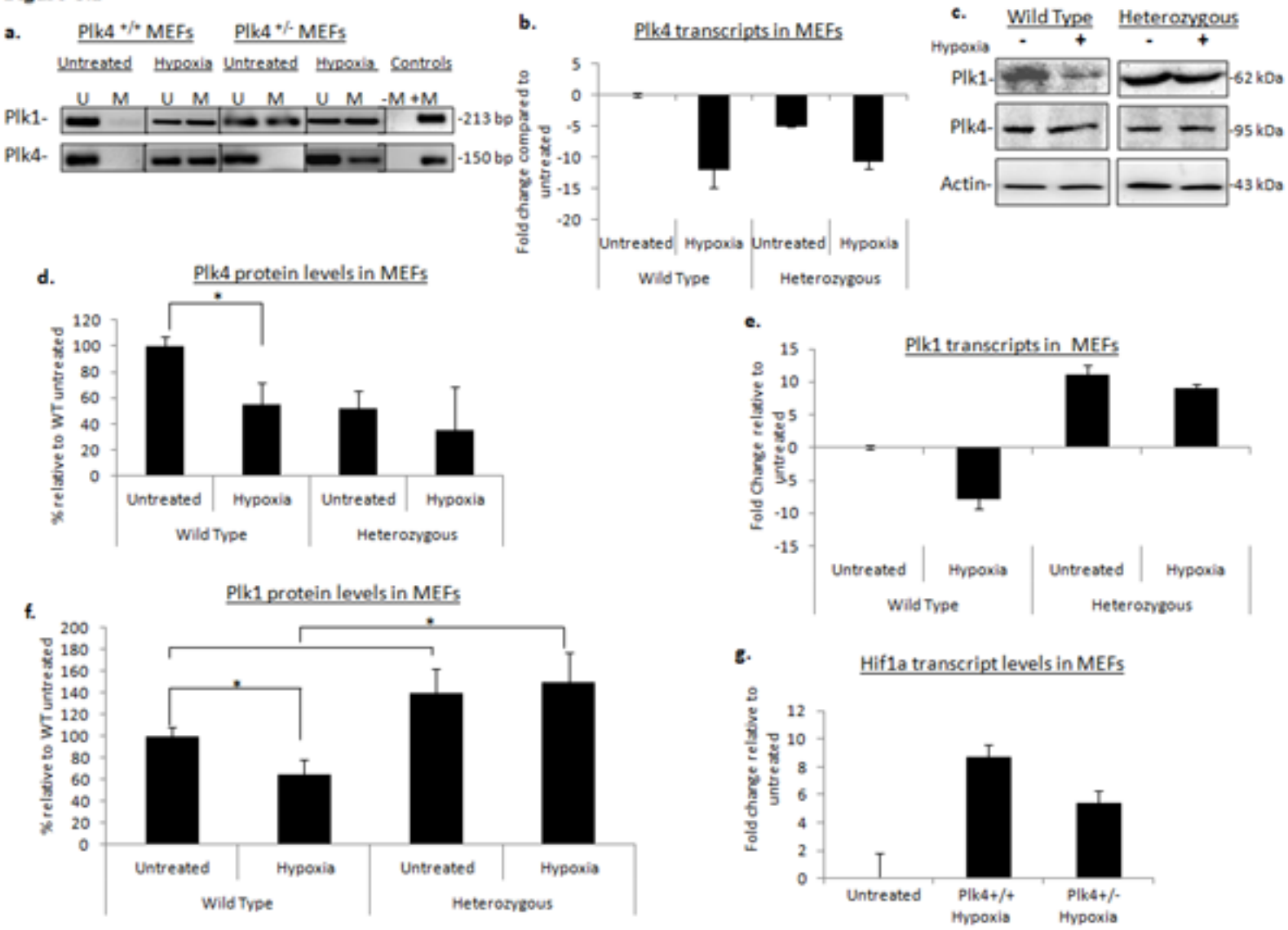
Plks are subject to epigenetic modification under hypoxic conditions in normal and tumour-derived cells in vitro

Hypoxia has been established as a characteristic of the solid tumour microenvironment and has been shown to promote cell migration and cell transformation [21, 22]. The primary mediator of the cellular response to hypoxia is hypoxia inducible factor 1α (Hif1α) which is responsible for the transcriptional regulation of several key genes, such as vascular endothelial growth factor (VEGF) [23] and metabolic components such as nitric oxide (NO) which are important for the cellular adaptation to a hypoxic environment [24]. More recently, Hif1α has been shown to indirectly modify epigenetic marks on histone tails leading to varying levels of transcriptional activation and repression through histone deactylatase (HDAC) recruitment and modification of the H3K9 methylation marks [25].

We have previously shown that *Plk4* heterozygosity increases the susceptibility of *Plk4* promoter methylation in an *in vivo* murine HCC model [10], therefore we wanted to determine whether *Plk4* heterozygosity impacted *Plk* promoter methylation under oxidative stress. First, wild type ($Plk4^{+/+}$) and heterozygous ($Plk4^{+/-}$) murine embryonic fibroblasts (MEFs) were cultured in a hypoxia chamber flooded with 2% oxygen and incubated for 18 hours in order to determine whether the exposure of cells to hypoxia results in the modification of *Plk* gene expression through epigenetic means. After the treatment, methylation specific PCR (MSP) was performed in order to examine the methylation status of the *Plks*. We did observe *Plk4* promoter methylation upon hypoxia treatment, regardless of genotype (Fig. 3.1a). Furthermore, corresponding Plk4

Figure 3.1. Aberrant methylation of *plk1* and *plk4* promoter regions in MEFs under hypoxic stress. (a) DNA extracted from mouse embryonic fibroblasts grown under hypoxic conditions was bisulfite treated and then assessed for promoter methylation of *Plk1* and *Plk4* using methylation specific PCR; U=unmethylated, M=methylated. Fully methylated NIH 3T3 DNA was used as a positive control (+M), no template was added to the negative control (-M). (b) Plk4 transcripts were assessed using qPCR. Transcript levels were normalized to the wild type untreated sample. All qPCR data is representative of the mean value of three independent experiments and error bars represent +/- SD. (c) Western blot analysis to examine protein levels of Plk1 and Plk4 post hypoxic treatment. (-) represents the lysates from untreated cells, (+) lysates from cells were grown in the presence of hypoxia. (d) Densitometric analysis normalized to the levels of the wild-type untreated cells. Error bars represent +/- SD from three independent experiments. (e) The fold change of plk1 transcripts normalized to the respective untreated transcripts. (f) The percent of Plk1 protein expression relative to the untreated wild-type cells. * denotes significance with $p<0.05$. (g) RNA extracted from MEFs along with real-time PCR was used to determine Hif1α transcripts post hypoxia treatment.

Figure 3.1


a.
Plk4 +/+ MEFs
Plk4 +/- MEFs
Untreated
Hypoxia
Untreated
Hypoxia
Controls
U M U M U M U M -M +M
Plk1-
Plk4-
-213 bp
-150 bp
b.
Plk4 transcripts in MEFs
Fold change compared to untreated
5
0
-5
-10
-15
-20
Untreated
Hypoxia
Untreated
Hypoxia
Wild Type
Heterozygous
c.
Wild Type
Heterozygous
Hypoxia
-
+
-
+
Plk1-
Plk4-
Actin-
-62 kDa
-95 kDa
-43 kDa
d.
Plk4 protein levels in MEFs
% relative to WT untreated
120
100
80
60
40
20
0
*
Untreated
Hypoxia
Untreated
Hypoxia
Wild Type
Heterozygous
e.
Plk1 transcripts in MEFs
Fold Change relative to untreated
15
10
5
0
-5
-10
-15
Untreated
Hypoxia
Untreated
Hypoxia
Wild Type
Heterozygous
f.
Plk1 protein levels in MEFs
% relative to WT untreated
200
180
160
140
120
100
80
60
40
20
0
*
*
Untreated
Hypoxia
Untreated
Hypoxia
Wild Type
Heterozygous
g.
Hif1a transcript levels in MEFs
Fold change relative to untreated
12
10
8
6
4
2
0
Untreated
Plk4+/+ Hypoxia
Plk4+/- Hypoxia

transcripts were decreased by approximately 12-fold compared to the untreated in both *Plk4*$^{+/+}$ and *Plk4*$^{+/-}$ MEFs under hypoxic conditions (Fig. 3.1b). Interestingly, Plk4 transcript and protein levels post hypoxia treatment in the *Plk4*$^{+/+}$ MEFs were comparable to the levels normally found in *Plk4* heterozygous cells. Moreover, treated *Plk4* heterozygous MEFs displayed even further depleted Plk4 protein levels by approximately 10% compared to the untreated counterpart (Fig. 3.1c,d). This suggests that the *Plk4* promoter region may be targeted for methylation under hypoxic conditions. Next, we sought to determine whether the modification to the epigenetic marks that we observed were specific to *Plk4*, or if the other *Plks* were also undergoing a similar response. Interestingly, hypoxia treatment of wild-type MEFs resulted in hypermethylation of the *Plk1* promoter region (Fig. 3.1a) with a corresponding seven-fold decrease in transcript levels (Fig. 3.1e) and a 20% decrease in protein levels when compared to non-treated controls (Fig. 3.1f). Considering that *Plk1* was methylated prior to treatment in *Plk4*$^{+/-}$ MEFs, it was not surprising to see that there was no change in the methylation status of *Plk1* promoter with hypoxia (Fig. 3.1a). In contrast, there was a moderate increase in the corresponding *Plk1* transcripts (Fig. 3.1e). Examination of Plk1 protein levels in untreated *Plk4*$^{+/-}$ MEFs revealed almost 40% higher Plk1 levels compared to the wild type cells prior to treatment (Fig.1f). Moreover, post-treatment, *Plk4*$^{+/-}$ MEFs showed approximately a 10% increase in Plk1 protein levels compared to the untreated (Fig. 3.1f). As a positive control, Hif1α transcript levels were assessed post treatment to ensure the cells were responding to hypoxic conditions (Fig. 3.1g).

Previous research has shown that p53 is both necessary and sufficient in transcriptionally repressing Plk1 [26]. In a regenerating liver model, *Plk4* heterozygosity

resulted in decreased p53 protein levels and activity compared to the wild-type model as evidenced by decreased p21 levels and phosphorylated Ser15 on p53 [2], suggesting that *Plk4* heterozygosity is insufficient for proper p53 activation. This also suggests a model in which *Plk1* expression in wild-type MEFs exposed to hypoxia is in part regulated by promoter methylation, resulting in repression of transcription and lower protein levels. The different response for *Plk1* in $Plk4^{+/-}$ MEFs, is likely related to the fact that $Plk4^{+/-}$ MEFs display increased genomic instability along with a lack of active p53 during stress [2]. Thus, the normal regulatory mechanisms necessary to down-regulate Plk1 protein levels are, in part, absent. This combination of lower Plk4 and increased Plk1 likely results in promoting the cellular transition through G2/M, and further propagating genomic instability and aneuploidy resulting in DNA damage caused by *Plk4* haploinsufficiency [2], a contributing factor to tumourigenesis. It also further suggests that Plk4 needs to be at normal levels in order to maintain appropriate Plk1 levels.

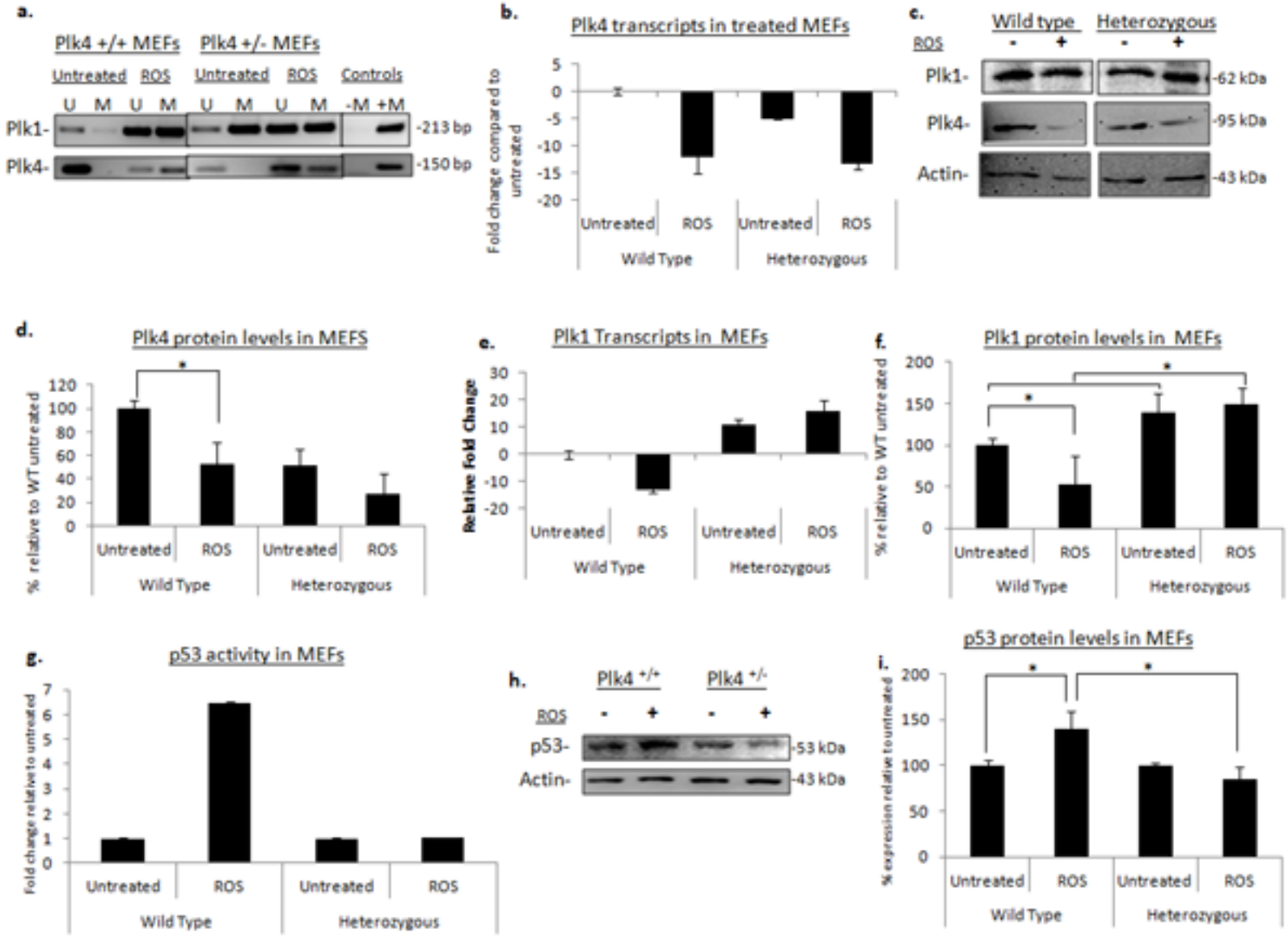
ROS-induced epigenetic downregulation of the Plks in MEFs

Oxidative stress in the microenvironment is not limited to hypoxic conditions. Oxidative stress can also be caused by an increase in free radicals producing reactive oxygen species (ROS). Furthermore, ROS have been shown to promote tumourigenesis through several biological processes which include cell proliferation, metastasis, and evasion of apoptosis [27]. Exposure of cells to high levels of ROS have also been implicated in the hypermethylation of tumour suppressor genes such as runt-related transcription factor 3 (*RUNX3*) [28]. Moreover, ROS exposure, as a result of hydrogen peroxide treatment, has been shown to recruit DNA methyltransferases (DNMT)

complexes to areas in the genome that are CG-rich, which could include the CpG islands upstream of the *Plk* promoter regions [29]. Additionally, in our previous work, we demonstrated that wild type MEFs that were chronically exposed to ethanol (EtOH) treatment, displayed a hypermethylated *Plk4* promoter region resulting in a phenotype that resembles that seen in $Plk4^{+/-}$ cells with multi-nucleation and multiple-centrosome formation [10]. Inherent to ethanol metabolism is the production of high levels of ROS [30] therefore, suggesting that ROS may also impact *Plk* promoter methylation. In order to examine whether *Plk1* and *Plk4* epigenetic marks were susceptible to modification as a result of high levels of ROS, we subjected $Plk4^{+/+}$ and $Plk4^{+/-}$ MEFs to reactive oxygen species (ROS) by exposing them to hydrogen peroxide (H_2O_2) at a 200um dose for a period of 18 hours. This level of ROS is known to induce DNA damage and p53 activity [31]. Methylation specific PCR (MSP) revealed that the *Plk4* promoter became hypermethylated in the presence of ROS (Fig. 3.2a). Both $Plk4^{+/+}$ and $Plk4^{+/-}$ MEFs displayed a decrease in *Plk4* transcripts of more than 10-fold (Fig. 3.2b) and subsequent Western blot analysis revealed a significant decrease in Plk4 protein in both MEF genotypes by approximately 50% ($p<0.05$) relative to the untreated cells (Fig. 3.2c,d). These results are similar to what we observed under hypoxic conditions, and suggest that as part of the stress and DNA damage response, *Plk1 and Plk4* may normally become downregulated *via* promoter methylation likely in order to arrest cell division. It is noted previous work by Ko et al. revealed that low levels of Plk4 results in a delay in cell cycle progression [2], and we have shown that lower levels of *Plk4* results in cells aggregating at the G2/M transition of the cell cycle [32].

Figure 3.2. Modification of *plk1* and *plk4* epigenetic marks with ROS exposure in MEFs. (a) MSP analysis shows the promoter methylation of *plk1* and *plk4* pre- and post-ROS treatment; U=unmethylated, M=methylated. Fully methylated NIH 3T3 DNA was used as a positive control (+M), no template was added to the negative control (-M). (b) Plk4 transcript levels determined by qPCR. All transcripts were normalized to the wild type untreated control. All qPCR data is representative of the mean value of three independent experiments and error bars represent +/- SD. (c) Plk1 and plk4 protein levels examined *via* Western blot analysis, actin was used as a loading control. (-) represents the lysates from untreated cells, (+) lysates from cells grown in the presence of ROS (d) Plk4 protein expression levels determined by densitometry. All densitometry data is representative of three independent experiments and the error bars represent +/- SD. * denotes significance with a $p<0.05$. (e) Plk1 transcripts of cells treated with ROS, the transcripts were normalized to the respective untreated samples. (f) The relative plk1 protein levels post treatment was normalized to the wild-type untreated samples. Levels determined by densitometric analysis of Western blot images. (g) An ELISA-based p53 activity assay. Relative activity was determined by normalizing values to the untreated samples. This data represents the mean value obtained over three independent experiments and error bars denote the +/- SD. (h) p53 protein levels in MEFs post treatment as determined by Western blot analysis. (i) Densitometry was performed on three independent experiments and all data has been normalized to the respective untreated. The mean expression is presented with error bars denoting +/- SD. * denotes significance with a $p<0.05$.

Figure 3.2


a.
Plk4 +/+ MEFs
Plk4 +/- MEFs
Untreated
ROS
Untreated
ROS
Controls
U M U M U M U M -M +M
Plk1-
Plk4-
-213 bp
-150 bp
b.
Plk4 transcripts in treated MEFs
Fold change compared to untreated
5 0 -5 -10 -15 -20
Untreated
ROS
Untreated
ROS
Wild Type
Heterozygous
c.
Wild type
Heterozygous
ROS
- + - +
Plk1-
Plk4-
Actin-
-62 kDa
-95 kDa
-43 kDa
d.
Plk4 protein levels in MEFS
% relative to WT untreated
120 100 80 60 40 20 0
*
Untreated
ROS
Untreated
ROS
Wild Type
Heterozygous
e.
Plk1 Transcripts in MEFs
Relative Fold Change
30 20 10 0 -10 -20
Untreated
ROS
Untreated
ROS
Wild Type
Heterozygous
f.
Plk1 protein levels in MEFs
% relative to WT untreated
200 150 100 50 0
* *
Untreated
ROS
Untreated
ROS
Wild Type
Heterozygous
g.
p53 activity in MEFs
Fold change relative to untreated
7 6 5 4 3 2 1 0
Untreated
ROS
Untreated
ROS
Wild Type
Heterozygous
h.
Plk4 +/+
Plk4 +/-
ROS
- + - +
p53-
Actin-
-53 kDa
-43 kDa
i.
p53 protein levels in MEFs
% expression relative to untreated
200 150 100 50 0
* *
Untreated
ROS
Untreated
ROS
Wild Type
Heterozygous

Plk1 promoter methylation and levels in *Plk4*$^{+/+}$ MEFs were responsive to increased ROS in a similar manner to that seen with hypoxia, in which *Plk1* was downregulated (Fig. 3.2a). *Plk1* transcripts were decreased by approximately 12-fold, which was reflective of the promoter hypermethylation (Fig. 3.2e). This was correlated with visibly reduced protein levels post ROS exposure by almost 40% (Fig. 3.2c,f). Although there appeared to be no visible change at the promoter region *via* MSP analysis, *Plk1* transcripts were elevated in the heterozygous MEFs in the presence of ROS with transcripts almost 15-fold higher compared to the untreated (Fig. 3.2a,e). Moreover, Plk1 protein expression levels were also 10% higher in ROS-treated heterozygous MEFs compared to the untreated counterparts and 100% higher compared to the treated wild-type MEFs ($p<0.05$) (Fig. 3.2c,f). In contrast to *Plk4 and Plk1*, *Plk2* promoter methylation as well as Plk2 and Plk3 protein levels displayed no detectable changes in either cell type in response to hypoxia and upon exposure to reactive oxygen species (Supplementary figure 3.1a,b). Note that, we did not examine *Plk3* promoter methylation as the gene in mouse lacks CpG islands.

The experimental results observed for *Plk1* and *Plk4* epigenetic regulation in MEFs as a response to ROS were similar to those obtained under hypoxic conditions, suggesting that an adequate response to stress and the DNA damage may be impaired in *Plk4*$^{+/-}$ MEFs and that lower Plk4 protein levels have an indirect impact on the epigenetic regulation of *Plk1*. This model is supported by the observations that upon DNA damage, p53 is activated and subsequently represses Plk1 [33, 34]. Previous work has determined that p53 interacts with and is a substrate of Plk4; and in the *Plk4*$^{+/-}$ mouse model, partial

hepatectomy failed to activate p53 within the first 24 hours post-surgery, unlike the wild-type counterparts which displayed p53 activation almost immediately [2, 35].

Given these observations, it was therefore of interest to determine whether p53 was activated in *Plk4*$^{+/-}$ MEFs post ROS treatment. We performed an ELISA-based p53 activity assay with MEF nuclear extracts post H_2O_2 treatment. *Plk4*$^{+/+}$ cells had an increase in p53 protein levels by almost 50% and an increase in p53 activity by almost 6-fold relative to the untreated cells (Fig. 3.2g-i). Unexpectedly, in *Plk4*$^{+/-}$ MEFs, p53 activity was not elevated, but was comparable to the untreated counterparts (Fig. 3.2g). This corresponded to the lack of a significant change in p53 protein levels for the *Plk4*$^{+/-}$ MEFs (Fig. 3.2h,i). Our observations suggest that *Plk4* heterozygosity and the subsequent low Plk4 protein levels are insufficient to activate p53 during genotoxic stress caused by ROS, resulting in an upregulation in the pro-mitotic protein, Plk1. Interestingly, in our previous examination of HCC in *Plk4*$^{+/-}$ mice, we also observed elevated Plk1 protein in tumours, but not in normal liver tissue [10]. Human studies have found that loss of heterozygosity for *PLK4* occurs in 45-60% of HCC cases examined together with an increase in Plk1 protein levels [2, 9]. *PLK4* LOH may be an early event in the progression to carcinogenesis. Here we show that a combinatorial effect of *Plk4* heterozygosity, together with micro-environmental stressors such as hypoxia and ROS, result in the upregulation of Plk1.

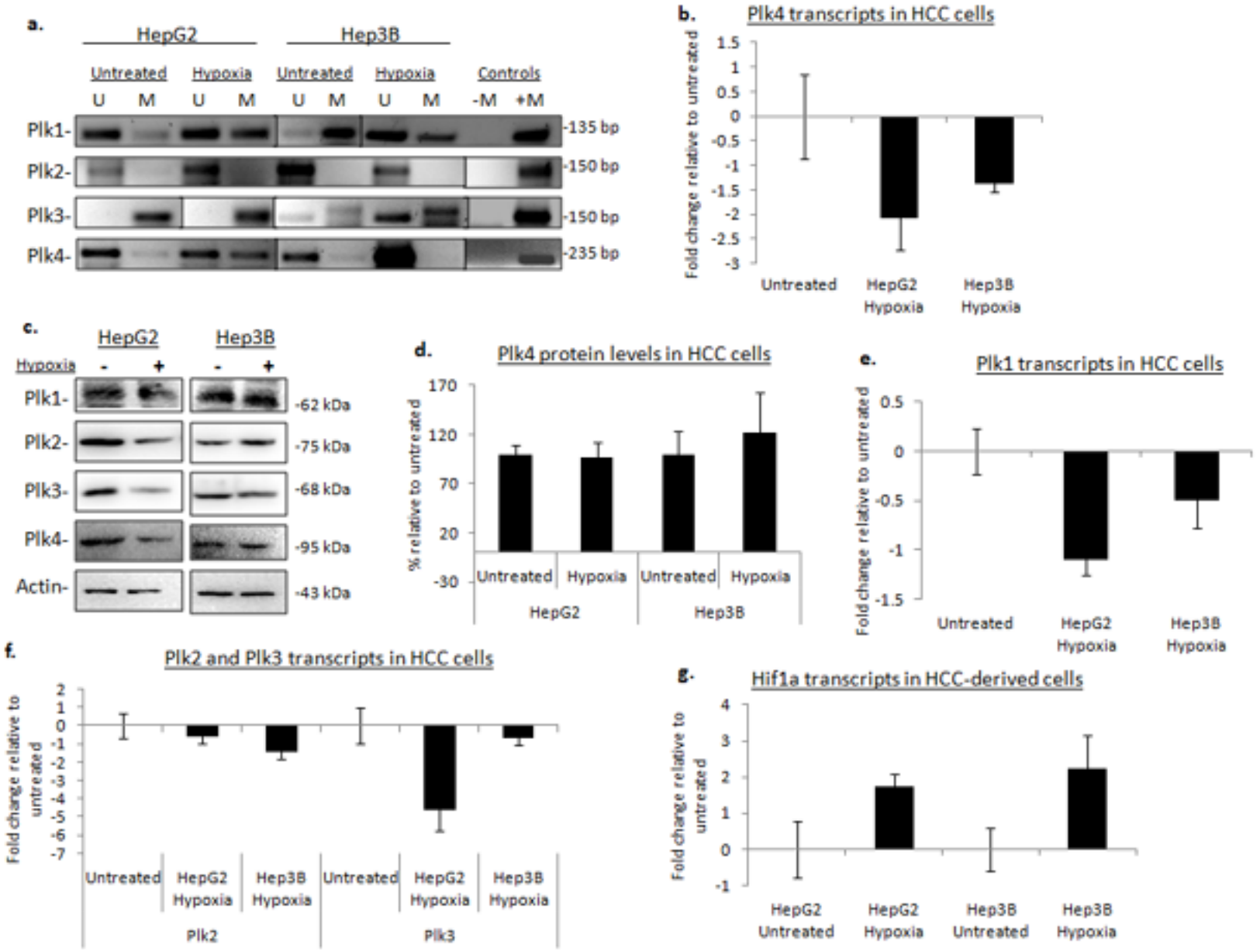
Promoter methylation of the Plks in HCC tumour cells

Li et al. 2005 demonstrated that PLK4 mRNA is regulated in a p53-dependent manner in lung carcinoma cells and osteosarcoma-derived cells exposed to etoposide

[36]. The levels of *PLK4* transcripts were most affected at 6 and 24 hours post treatment [36]. Thus, p53 plays a role in the transcriptional downregulation of *PLK4* through histone deacetylation upon exposure to DNA damaging agents [36]. Recently, Nakamura et al. also showed that sustained genotoxic stress *via* etoposide and UV resulted in the attenuation of PLK4 in a p53-dependent manner [37]. In addition, p53 is known to be an important player in the epigenetic downregulation of another tumour suppressor, ras-associated domain family 1 (*RASSF1A*), by directly binding to the promoter of *RASSF1A* and recruiting DNA methyltransferase 1(DNMT1) along with accessory proteins to the promoter region [38]. Moreover, p53 interacts and cooperate with DNMT1 in the methylation of the PLK4 target, CDC25C, in the presence of DNA damage [39] and also interacts with DNMT3a, which is responsible for *de novo* methylation [40]. This suggests that p53 likely also regulates the *Plks* through an epigenetic mechanism. We were therefore interested in determining whether the promoter methylation of the *Plks,* which we observed in MEFs under hypoxia and ROS treatment, was dependent on the presence or absence of p53. We employed the hepatocellular carcinoma (HCC) derived cell lines, HepG2 and Hep3B to answer this question. HepG2 cells exhibited an increase in *PLK4* promoter methylation post hypoxia, but not Hep3B cells (Fig. 3.3a). In the case of HepG2 cells there was an increase in the detectable level of methylation accompanied with a corresponding 2-fold decrease in *PLK4* transcripts (Fig. 3.3b) compared to the untreated as well as a 5% decrease in protein levels (Fig. 3.3c,d) . For Hep3B cells, under hypoxic conditions, the increase in promoter methylation did not translate into significant changes at transcript and proteins levels (Fig. 3.3c,d). In this case, protein levels of PLK4 did not show a significant difference, although transcript levels were slightly decreased

Figure 3.3. Hypoxia-induced modification of *PLK* promoter methylation in HCC cells. (a) Promoter methylation status of the plks examined in HCC-derived cells HepG2 and Hep3B; U=unmethylated, M=methylated. Fully methylated HeLa DNA was used as a positive control (+M), no template was added to the negative control (-M). (b) Post hypoxia, PLK4 transcripts were assessed *via* qPCR in RNA extracted from HCC cells. All qPCR data is representative of the mean value of three independent experiments and error bars represent +/- SD. (c) PLK protein levels were examined post treatment from whole cell lysates. Actin was used as a loading control. (-) represents lysates from untreated cells, (+) lysates from cells grown in the presence of hypoxia. (d) Quantification of protein levels using densitometry. Levels have been normalized to the respective untreated controls. Data is representative of the mean value of three independent experiments and error bars represent +/- SD. (e) The fold change of PLK1 transcripts as determined by qPCR. Values normalized to the respective untreated sample. (f) PLK2 and PLK3 analyzed and fold changed determine by normalization to the respective untreated samples. (g) Hif1α transcripts post hypoxia were determine by real-time PCR using a Taqman probe.

Figure 3.3


a.
HepG2
Hep3B
Untreated
Hypoxia
Untreated
Hypoxia
Controls
U M U M U M U M -M +M
Plk1-
Plk2-
Plk3-
Plk4-
-135 bp
-150 bp
-150 bp
-235 bp
b.
Plk4 transcripts in HCC cells
Fold change relative to untreated
Untreated
HepG2 Hypoxia
Hep3B Hypoxia
c.
HepG2
Hep3B
Hypoxia - + - +
Plk1-
Plk2-
Plk3-
Plk4-
Actin-
-62 kDa
-75 kDa
-68 kDa
-95 kDa
-43 kDa
d.
Plk4 protein levels in HCC cells
% relative to untreated
Untreated
Hypoxia
Untreated
Hypoxia
HepG2
Hep3B
e.
Plk1 transcripts in HCC cells
Fold change relative to untreated
Untreated
HepG2 Hypoxia
Hep3B Hypoxia
f.
Plk2 and Plk3 transcripts in HCC cells
Fold change relative to untreated
Untreated
HepG2 Hypoxia
Hep3B Hypoxia
Untreated
HepG2 Hypoxia
Hep3B Hypoxia
Plk2
Plk3
g.
Hif1a transcripts in HCC-derived cells
Fold change relative to untreated
HepG2 Untreated
HepG2 Hypoxia
Hep3B Untreated
Hep3B Hypoxia

(Fig. 3.3c,d). As HepG2 cells contain a functional p53 whereas as Hep3B cells lack a functional p53 [41], these results once again suggest the involvement of p53 in the epigenetic regulation of *PLK4*.

Likewise, for *PLK1,* the change in methylation status was similar to that seen with hypoxia treatment in MEFs. Before treatment, HepG2 cells displayed some methylation for the *PLK1* promoter (Fig.3a). Post hypoxia, the *PLK1* promoter region became hypermethylated (Fig. 3.3a). In addition, transcript levels were decreased by almost 2.5-fold (Fig.3e) and accompanied by a slight decrease in protein levels (Fig. 3.3c). Hep3B cells, on the other hand, showed no distinct change in the methylation status of *PLK1* promoter region compared to the untreated (Fig. 3.3a). Moreover, PLK1 transcript and protein levels in treated Hep3B cells were not significantly impacted by hypoxia treatment (Fig. 3.3c,e).

Human *PLK3*, unlike its murine homolog has two CpG islands in its promoter region. We used two sets of primers in order to assay for any changes in methylation status for *PLK3*. With both, MSP published primers based on the first 200 base pairs of the upstream CpG island [1] and an additional set of MSP primers downstream, we detected no overt change in promoter methylation for *PLK3* in either HepG2 or Hep3B cells (Fig. 3.3a). This suggests that the regulation of *PLK3* under hypoxic conditions is not p53 dependent and is likely not regulated by an epigenetic mechanism in this context.

Likewise, for *PLK2*, there was no dramatic change in promoter methylation, for either HepG2 and Hep3B cell lines. This indicates that *PLK2* and *PLK3* do not undergo aberrant changes to their promoter methylation in response to hypoxia.

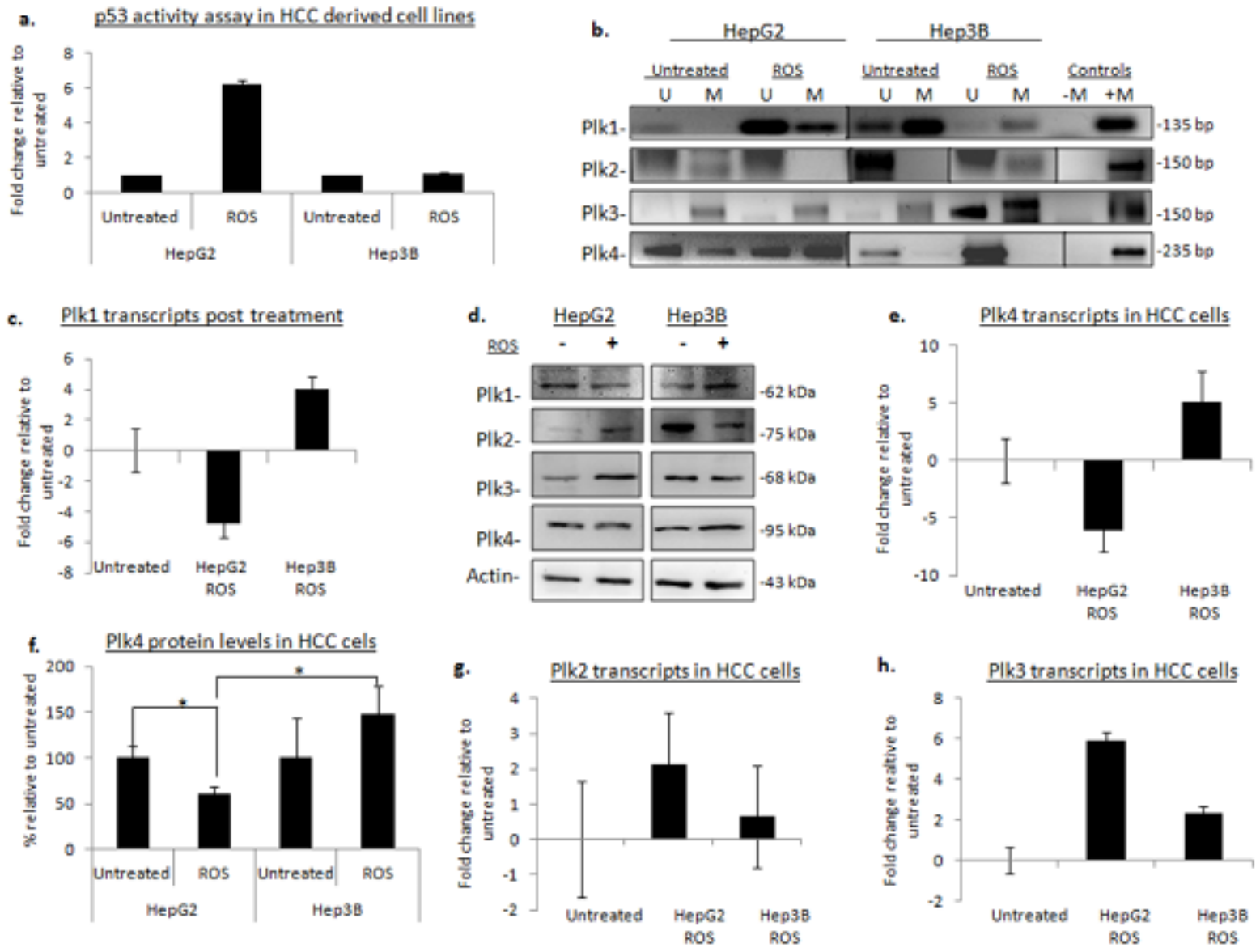
As an experimental control, we assessed the transcript levels of HIF1α to determine whether these cells were responding to hypoxic stress under the same hypoxic conditions as used with the MEFs. With hypoxia, *HIF1α* transcripts were elevated by more than 1.5 times in both cell lines (Fig. 3.1g), indicating that the cells were indeed responding to low oxygen levels and the change in *HIF1α* transcript levels were similar to previously reported hypoxia treatments in HCC cells [42].

Plk promoter methylation in HCC with ROS treatment

HepG2 and Hep3B were cultured in the presence of hydrogen peroxide at a concentration of 200um and activation of p53 by ROS was confirmed via an ELISA-based p53 activity assay and Western blot analysis. As expected, we found a 6-fold increase in p53 activity in HepG2 cells in the presence of ROS, while no change in activity was detected for Hep3B (Fig. 3.4a). The increase in activity also corresponded to an increase in p53 protein levels in HepG2 cells, while in agreement with Hep3B p53 status, no p53 protein was detected in Hep3B cells (Supplementary figure 3.1c).

Figure 3.4. Modification of *PLK* promoter methylation marks in HCC cells exposed to ROS. (a) A p53 activity assay was performed to confirm activation of p53 with genotoxic stress caused by ROS. The percent activity is the average of three independent experiments with error bars representing the +/- SD. (b) MSP analysis of *plk* promoter methylation; U=unmethylated, M=methylated. Fully methylated HeLa DNA was used as a positive control (+M), no template was added to the negative control (-M). (c) Plk1 transcript levels were examined and normalized to the respective untreated samples. All qPCR data is representative of the mean value of three independent experiments and are normalized to the untreated samples. Error bars represent +/- SD. (d) Western blot analysis of PLK protein levels. Actin was used as a loading control. (-) represents the lysates from untreated cells, (+) lysates from cells were grown in the presence of ROS. (e) The fold change in plk4 transcripts from cells exposed to ROS. (f) Quantification of PLK4 protein levels. Data is representative of three independent experiments and the error bars represent +/- SD. * denotes significance with a $p<0.05$. (g,h) PLK2 and PLK3 change in transcripts as determined by real time PCR.

Figure 3.4


a.
p53 activity assay in HCC derived cell lines
Fold change relative to untreated
Untreated
ROS
Untreated
ROS
HepG2
Hep3B
b.
HepG2
Hep3B
Untreated
ROS
Untreated
ROS
Controls
U M U M U M U M -M +M
Plk1-
Plk2-
Plk3-
Plk4-
-135 bp
-150 bp
-150 bp
-235 bp
c.
Plk1 transcripts post treatment
Fold change relative to untreated
Untreated
HepG2 ROS
Hep3B ROS
d.
HepG2
Hep3B
ROS - + - +
Plk1-
Plk2-
Plk3-
Plk4-
Actin-
-62 kDa
-75 kDa
-68 kDa
-95 kDa
-43 kDa
e.
Plk4 transcripts in HCC cells
Fold change relative to untreated
Untreated
HepG2 ROS
Hep3B ROS
f.
Plk4 protein levels in HCC cels
% relative to untreated
Untreated
ROS
Untreated
ROS
HepG2
Hep3B
g.
Plk2 transcripts in HCC cells
Fold change relative to untreated
Untreated
HepG2 ROS
Hep3B ROS
h.
Plk3 transcripts in HCC cells
Fold change realtive to untreated
Untreated
HepG2 ROS
Hep3B ROS

PLK1 became hypermethylated in HepG2 post ROS exposure, while in Hep3B the level of detectable methylation decreased in comparison to that initially present in untreated cells (Fig. 3.4b). Subsequent examination of the transcript and protein expression for PLK1 were correlated with their respective promoter methylation status. Specifically, in HepG2, PLK1 transcripts and protein were significantly reduced, whereas in Hep3B, *PLK1* transcripts were almost 4-fold higher compared to the untreated control and protein expression was also elevated (Fig. 3.4c,d). Here we show that *PLK1* downregulation in response to DNA damage in p53-wild type cells is also accompanied by promoter hypermethylation and this hypermethylation can be induced by ROS whereas the opposite scenario is observed for the p53 null cells.

PLK4 promoter methylation patterns also paralleled what we have observed with *PLK1*, where HepG2 had a qualitative gain in *PLK4* promoter methylation (Fig. 3.4b) accompanied by a 6-fold decrease in transcripts and a 40% decrease in protein expression (Fig. 3.4d-f). This is in direct opposition to what we observed in Hep3B cells, which had no observable gain of methylation for *PLK4*, but more importantly, there was an increase in transcripts and protein by 5-fold and 30% respectively compared to the untreated cells (Fig. 3.4d,e).

This data indicates that *PLK1* and *PLK4* promoter methylation is p53-dependent and that ROS may play an important role in the regulation of both of these genes. This correlates with recent work by Nakamura et al. which determined that under stress and DNA damage in colorectal cells, PLK4 is initially activated, but its expression is abrogated over time in p53-wild type cells followed by an increase in p53 levels. In p53-null cells, PLK4 protein levels persisted over the same period of time [37].

Previous examination of *PLK2* expression has shown that it can be induced by p53 during DNA damage and stress *via* p53 directly binding to its consensus sequence within the *PLK2* promoter [43, 44]. More recently, *PLK2* transcript levels have been used as predictors in determining the genotoxicity of potential hepatocarcinogens [45]. So, it was not surprising to see that post ROS treatment of HepG2 cells, *PLK2* lost promoter methylation (Fig. 3.4b) along with a 2-fold increase in *PLK2* transcript (Fig. 3.4g) and protein levels (Fig. 3.4d). In Hep3B cells, *PLK2* displayed a gain of methylation at its promoter region after ROS exposure (Fig.4b), correlated with decreased protein levels, suggesting that in the absence of p53, the *PLK2* promoter region becomes hypermethylated in HCC in the presence of ROS (Fig. 3.4d).

PLK3 activity is also known to become upregulated in the presence of H_2O_2. This increase in activity leads to the phosphorylation of p53 at serine 20 in human fibroblast cells [31]. Therefore, we would expect PLK3 levels to increase in response to ROS treatment. Although *PLK3* promoter methylation remained largely unchanged between the untreated and the ROS exposed cells (Fig. 3.4b), *PLK3* transcripts (Fig. 3.4h) and protein levels (Fig. 3.4d) were elevated in ROS treated HepG2 cells. However, in the absence of p53, *PLK3* transcripts and protein levels were not significantly changed with ROS treatment (Fig. 3.4h,d).

Here we show that in HCC cells, *PLKs* 1,2, and 4 become epigenetically modified in the presence of ROS, and that this regulation is in part, p53 dependent. Moreover, in Hep3B cells, which lack p53, the upregulation of the *PLKs* needed for DNA damage repair, PLK2 and PLK3, are impaired in the presence of ROS. This is also accompanied by an increase in PLK1 and PLK4 in p53 null cells. In the clinical setting, PLK1 and

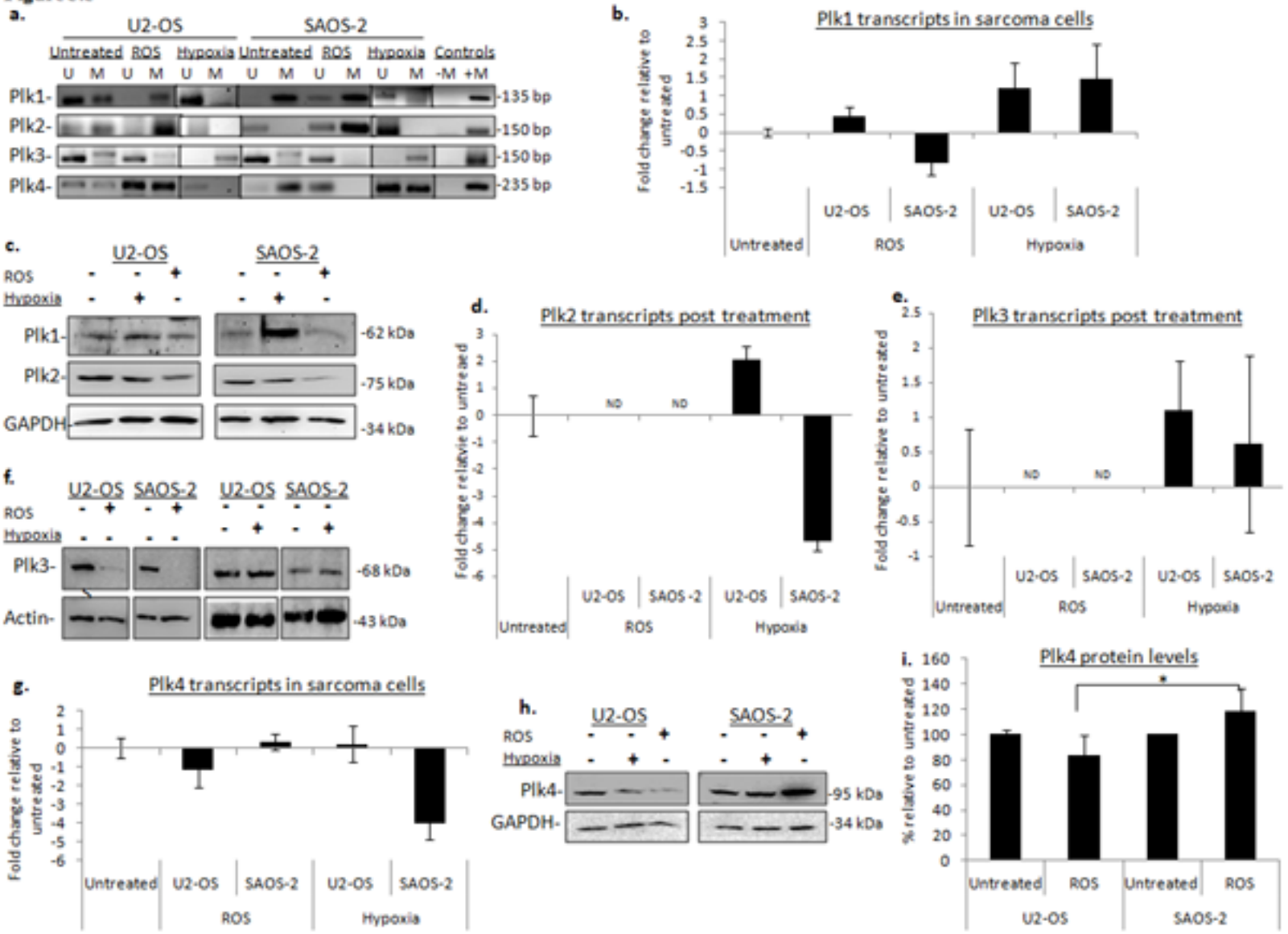
PLK4 have been found to be jointly upregulated in colorectal cancers compared to the normal mucosa in almost 80% of the cases examined [4]. Furthermore, upregulation of PLK4 leads to centrosome amplification and multipolar spindle formation resulting in aneuploidy, which is a signature of many solid tumours [46]. In addition, it is important to note that more than 50% of colorectal cancers harbour p53 mutations [47]

Plk promoter methylation in osteosarcoma-derived cells

These results raised the question whether these modifications were a general phenomenon or were these epigenetic modifications specific to tissue or cell type? Previous literature suggested that certain gene-signatures that are found in HCC cells are not found in other cell types such as colon carcinomas [42]. We chose to replicate our experiments with hypoxic conditions and in the presence of ROS using osteosarcoma derived cells within the same p53 context. We employed the p53-wild type cells U2-OS and the p53 null cells Saos-2 [41]. First, we examined the promoter methylation and expression of the *PLKs* in the sarcoma-derived cells under hypoxic conditions. Interestingly, in osteosarcoma cells, *PLK1* promoter regions became hypomethylated in both U2-OS and Saos-2 cells (Fig. 3.5a) followed by upregulation of the accompanying transcripts and protein levels compared to the untreated cells (Fig. 3.5b,c). This suggests that hypoxia-induced modifications to the promoter methylation of *PLK1* in the above mentioned cell lines is not p53 dependent. Conversely, when examining the *PLK2* promoter methylation under hypoxic conditions, U2-OS cells displayed a loss of

Figure 3.5. Examination of *PLK* promoter methylation in sarcoma-derived cells grown in the presence of oxidative stress. (a) *PLK* promoter methylation was determined by methylation-specific PCR; U=unmethylated, M=methylated. Fully methylated HeLa DNA was used as a positive control (+M), no template was added to the negative control (-M). (b) Fold change in plk1 transcripts. All qPCR values have been normalized to the respective untreated samples. Here the mean value of three independent experiments are depicted with error bars representing the +/- SD. (c) PLK1 and PLK2 protein levels in U2-OS and SAOS-2 cells treated with hypoxia and ROS. GAPDH was used as a loading control. (-) indicates lysates extracted from untreated samples, (+) represents lysates extracted from cells exposed to either hypoxia or ROS. (d,e) PLK2 and PLK3 transcripts as determined by qPCR. ND= not detectable. (g) Transcript changes for PLK4 in cells exposed to ROS and hypoxia. (h) PLK4 protein levels in sarcoma cells treated with hypoxia and ROS (+) compared to the untreated counterpart (-). GAPDH was used as a loading control. (i) PLK4 protein levels quantified with densitometry analysis of the Western blot images. The histogram is representative of the mean from three independent experiments with error bars showing the +/- SD. * denotes significance with a $p<0.05$.

Figure 3.5


a.
U2-OS
SAOS-2
Untreated ROS Hypoxia Untreated ROS Hypoxia Controls
U M U M U M U M U M U M -M +M
Plk1-
Plk2-
Plk3-
Plk4-
-135 bp
-150 bp
-150 bp
-235 bp
b.
Plk1 transcripts in sarcoma cells
Fold change relative to untreated
Untreated
ROS
Hypoxia
U2-OS
SAOS-2
c.
U2-OS
SAOS-2
ROS
Hypoxia
Plk1-
Plk2-
GAPDH-
-62 kDa
-75 kDa
-34 kDa
d.
Plk2 transcripts post treatment
Fold change relative to untreated
ND
e.
Plk3 transcripts post treatment
Fold change relative to untreated
f.
U2-OS SAOS-2 U2-OS SAOS-2
ROS
Hypoxia
Plk3-
Actin-
-68 kDa
-43 kDa
g.
Plk4 transcripts in sarcoma cells
Fold change relative to untreated
h.
U2-OS
SAOS-2
ROS
Hypoxia
Plk4-
GAPDH-
-95 kDa
-34 kDa
i.
Plk4 protein levels
% relative to untreated
*

promoter methylation (Fig. 3.5a) followed an almost 2-fold increase in transcripts (Fig. 3.5d), while a only a slight change in protein level was observed (Fig. 3.5c); Saos-2 cells on the other hand, had no distinct change in promoter methylation (Fig. 3.5a), however, qPCR analysis revealed a decrease in *PLK2* transcripts by almost 5-fold resulting in a slight decrease in protein (Fig. 3.5c,d). A study by Matthew et al. (2009) revealed that PLK2 has an active and p53-dependent role in the cellular response to hypoxia by indirectly restraining the mTOR signaling pathway during hypoxia, so it was expected that we would see an increase in PLK2 in U2-OS and not Saos-2 [48]. When examining the remaining *PLKs, PLK3*'s promoter region did not appear to change in response to hypoxia, in either cell type and transcript and protein levels did not differ from the untreated (Fig. 3.5a,e,f), similar to what we have seen in the MEFs and HCC cells. In Saos-2 cells, the *PLK4* promoter region became hypermethylated in the presence of hypoxia (Fig. 3.5a) followed by a decrease in *PLK4* transcripts by nearly 4-fold compared to the untreated (Fig. 3.5g), which resulted in a moderate decrease in protein levels (Fig. 3.5h). In U2-OS, the *PLK4* promoter region was initially methylated prior to treatment, but with hypoxia treatment, there was a loss of detectable methylation, though this did not translate into significant changes at the transcript or protein levels (Fig. 3.5a,g-i). The examination of sarcoma cells illustrates that hypoxia can differentially impact the *PLK* promoter methylation patterns between cell types, and that p53 may not have the same impact on the epigenetic regulation of the *PLKs* in all cells. *HIF1a* transcript levels were examined and were found to be elevated by 1.5-2 fold in both cell types (Supplementary figure 3.2a).

ROS treatment of sarcoma cells resulted in very different pattern of methylation than that seen in HCC cell lines. Confirmation of ROS-induced increased in p53 activity was carried out *via* Western blot analysis and with a p53 activity assay , which showed an increase in p53 activity in U2-OS cells by almost 9-fold, whereas no change was detected with SAOS-2 (Supplementary figure 3.2b,c). Unlike HCC cells, in both osteosarcoma cell lines, *PLK2* became hypermethylated (Fig. 3.5a) accompanied by undetectable transcripts and significantly decreased protein levels (Fig. 3.5c,d). Although *PLK3* promoter methylation did not increase with treatment, transcripts and protein levels were also undetectable in either cell type (Fig. 3.5e,f). This suggests that *PLK2* and *PLK3* are differentially regulated in osteosarcoma cell lines compared to HCC cell lines. The *PLK1* promoter region also did not display a change in promoter methylation, remaining hypermethylated in both cell lines similar to our observations in HCC and MEFs (Fig. 3.5a). Real-time PCR did reveal a slight decrease in *PLK1* transcripts (Fig. 3.5b) and protein levels in SAOS-2 cells, but not in U2-OS cells (Fig. 3.5c). However, when examining *PLK4*, we noticed a dramatic loss of promoter methylation in Saos-2 cells in response to ROS, but not in U2-OS cells (Fig. 3.5a). Along with promoter hypomethylation in Saos-2 there was a minor increase in transcripts (Fig. 3.5a,g). PLK4 protein levels were also elevated in treated Saos-2 cells by more than a 10%; whereas U2-OS cells displayed a decrease in PLK4 protein by almost 20% compared to the untreated, similar to the response observed in HCC cells (Fig. 5h,i). This suggests that regardless of cell type, *PLK4* continues to be sensitive to ROS-induced promoter hypermethylation within a functional p53 context.

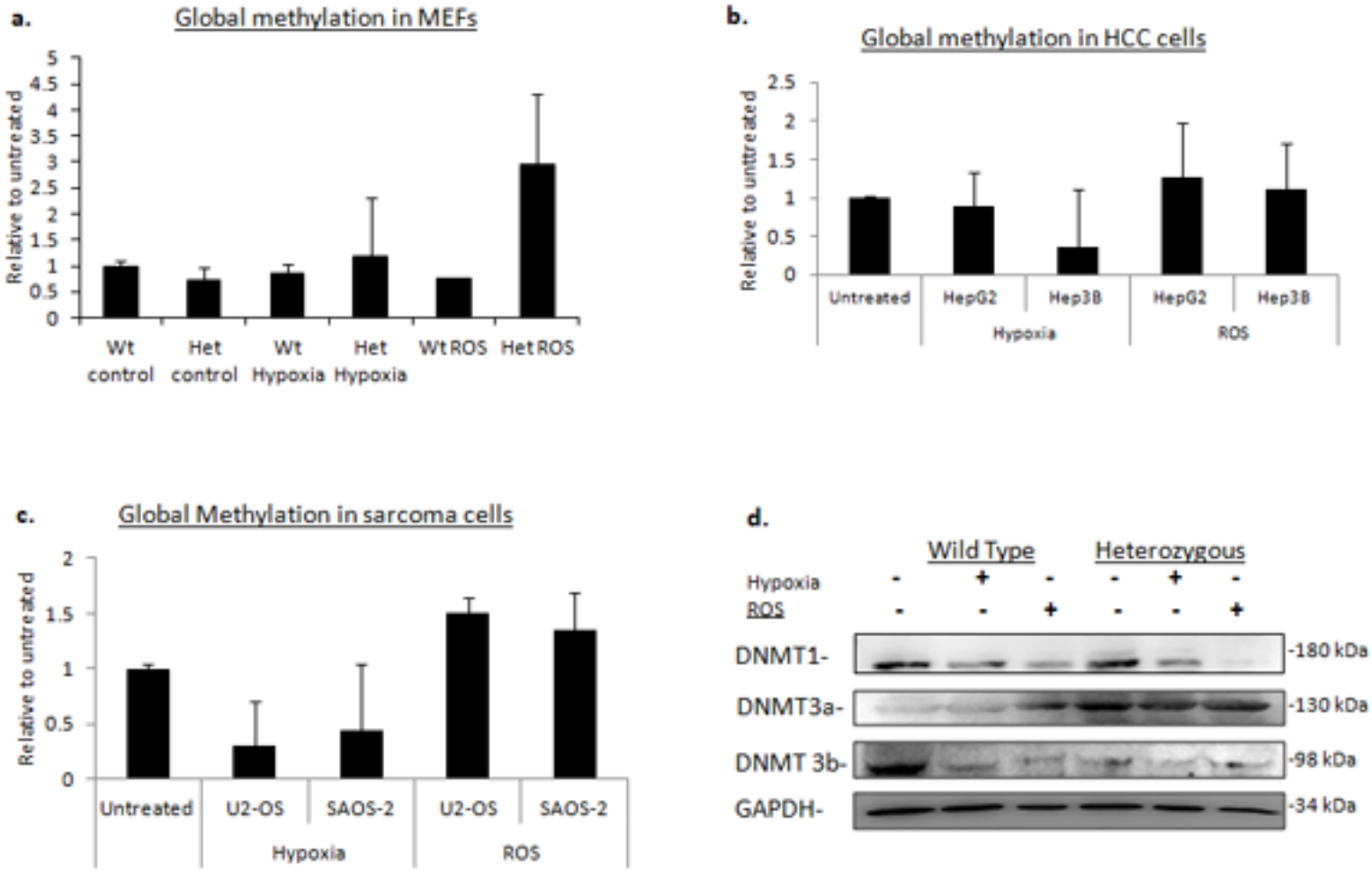
Global methylation and DNMT levels

In general, cells exposed to oxidative stress also experience shifts in global methylation patterns that can be associated with modifications to local methylation patterns at gene promoter regions [49, 50]. As part of our epigenetic analysis of the *Plks*, we wanted to determine if the modifications we observed at *Plk*-specific promoter regions were associated with a general increase in global methylation and whether any change varied between p53 wild type and p53 null cells. Here we examined the whole genome methylation of DNA from cells subjected to either ROS or hypoxia treatment. With hypoxia, both *Plk4* wild type and heterozygous MEFs had a slight decrease in global methylation compared to the untreated samples by approximately 15% (Fig. 3.6a). This is similar to what Shahrzad et al. demonstrated in melanoma cells, under anoxia, global methylation decreased between 15-20% [49]. We also observed a similar trend with HCC and osteosarcoma cells, with a 15-40% decrease in global methylation (Fig. 3.6b,c). There was little difference in global methylation between the hypoxia treated p53 wild type and p53 null cells although, in three independent experiments, Hep3B cells displayed a greater loss of global methylation in comparison to HepG2 (Fig. 3.6b). DNA methylation is maintained by DNA methyltransferases (DNMTs) which are enzymes that catalyze the transfer of methyl groups to cytosines which are 5' to guanine [51]. DNMT1 is responsible for maintenance methylation during replication, and DNMT3a and DNMT3b drive *de novo* methylation [51]. It was therefore of interest to determine whether the changes in global methylation were also accompanied by differences in protein levels of the DNMTs.

DNMT1 and 3b protein levels have both been shown to become downregulated with hypoxia along with a decrease in DNMT activity which would lead to an overall decrease in global methylation marks [52]. We examined the levels of the DNMT's in both wild type and *Plk4* heterozygous MEFs and found that this was also the case, where DNMT1 and DNMT3b protein levels decreased with hypoxia (Fig. 3.6d). When examining DNMT3a, protein levels were elevated in *Plk4* heterozygous MEFs prior to treatment and remained elevated post hypoxia treatment, but the wild type MEFs did not display this change in DNMT3a levels (Fig. 3.6d). It was previously reported that p53 wild type and p53 null colorectal cells, post hypoxia exposure, have increased transcript levels of DNMT3a, with a greater increase observed in p53 null colorectal cells [52]. Also, in an *in vivo* study done by Park et al., a p53 heterozygous and null mouse model revealed elevated levels of DNMT3a compared to the wild type littermates prior to any tumour development [53]. This suggests that DNMT3a is deregulated in $Plk4^{+/-}$ MEFs in a manner similar to that seen in p53 null cells. This also correlates to the decrease in p53 activity that we have observed in $Plk4^{+/-}$ MEFs and re-enforces the importance of the Plk4-p53 relationship and interaction axis.

Figure 3.6. Analysis of global methylation in MEFs, HCC and osteosarcoma cells and DNMTs levels in MEFs. An ELISA-based global methylation assay was performed to determine changes in global methylation levels due to oxidative stress as a result of hypoxia and ROS exposure. The histograms are representative of three independent experiments and the error bars depict the +/- SD. (a) In MEFs the values have been normalized to the untreated wild-type cells. (b,c) The values have been normalized to the respective untreated samples. (d) Western blot analysis was used to determine the levels of the DNMTs from whole cell lysates extracted from untreated (-) and treated (+) MEF cells.

Figure 3.6


a.
Global methylation in MEFs
Relative to untreated
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Wt control
Het control
Wt Hypoxia
Het Hypoxia
Wt ROS
Het ROS
b.
Global methylation in HCC cells
Relative to unttreated
2.5
2
1.5
1
0.5
0
Untreated
HepG2
Hep3B
HepG2
Hep3B
Hypoxia
ROS
c.
Global Methylation in sarcoma cells
Relative to untreated
2
1.5
1
0.5
0
Untreated
U2-OS
SAOS-2
U2-OS
SAOS-2
Hypoxia
ROS
d.
Wild Type
Heterozygous
Hypoxia
ROS
DNMT1-
-180 kDa
DNMT3a-
-130 kDa
DNMT 3b-
-98 kDa
GAPDH-
-34 kDa

ROS treated *Plk4*$^{+/-}$ MEFs also displayed an increase in global methylation (Fig. 3.6a), similar to what we observed in the HCC and osteosarcoma cancer cells (Fig. 3.6b,c). This was in contrast to global methylation levels in the *Plk4* wild type MEFs which decreased with ROS (Fig. 3.6a). This once again suggests that *Plk4* heterozygosity results in deregulation of the response to oxidative stress.

Conclusions

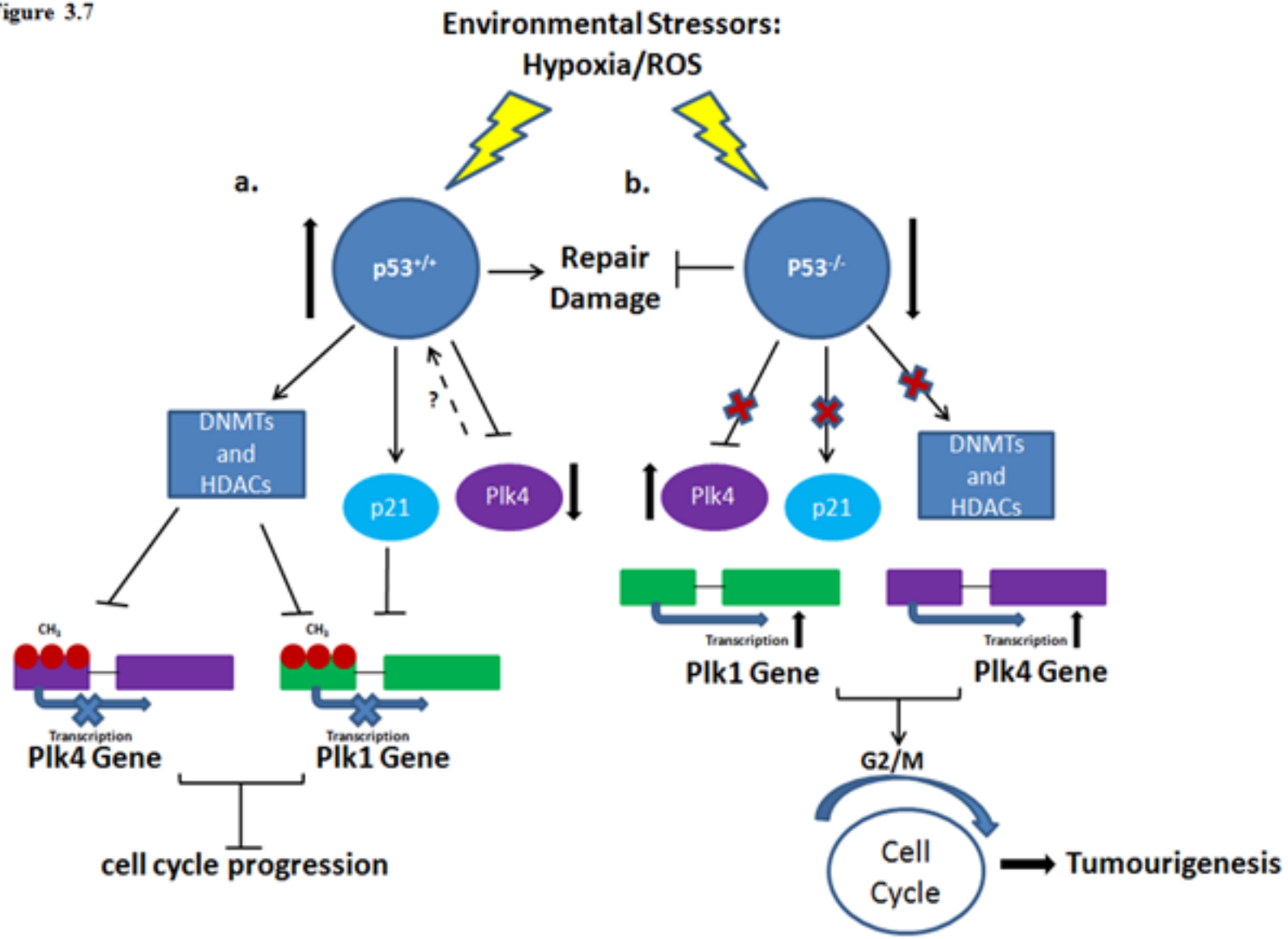
The contributions to tumourigenesis are complex and multi-factorial. Oxidative stress has been acknowledged as one such contributor in the path to carcinogenesis. Previous studies have shown that the *PLKs* are subject to regulation through post-translational modifications [54, 55]. Our observations here show that the *Plks*, whose proper regulation is essential for cell cycle fidelity, become deregulated in the presence of both hypoxia and ROS through epigenetic modifications to their respective promoter regions. However, the deregulation that we have observed is cell-specific, resulting in methylation patterns that are similar, like those between MEFs and HCC, and patterns that differ like those observed in sarcoma-derived cells. The promoter methylation of *PLK4* is also correlated with the status of p53 in the cell. *Plk4* haploinsufficiency, together with oxidative stress-induced epigenetic deregulation can inadvertently lead to the upregulation of *Plk1*. Based on our observation and the current literature, we propose a model in which p53 likely leads to downregulation of transcription for *PLK1* and *PLK4* in the presence of cellular stress by either recruiting or cooperating with DNMT1, DNMT3a and/or histone deacetylases (HDACs); this leads to an increase in promoter hypermethylation and hence changes in expression [36-40] (Fig. 3.7a). In the absence of p53, cellular stress would lead to the upregulation of pro-mitotic PLKs (PLK1 and PLK4)

resulting in a push through the G2/M checkpoint that would contribute to genomic instability and tumourigenesis (Fig. 3.7b)

The methylation status of the *PLKs* could also be used as an indicator of oxidative stress at the cellular level. These modifications to *PLK* epigenetic marks may even be an early event in the multi-stage process leading to tumourigenesis, given that we have observed detectable changes 18 hours post-treatment. Furthermore, promoter hypermethylation of the *PLKs* is a common event in a variety of cancers, including blood neoplasms, hepatocellular carcinoma, and ovarian cancer. Aberrant promoter methylation, induced specifically *via* microenvironemtal cues, could be another contributor to carcinogenesis [1, 8-11]. Currently, PLK1 has been the most targeted PLK for drug development [56-58], however, promoter hypermethylation is a reversible phenomenon for which there are drugs already in clinical use [59] that could be used as prophylactic agents or could help reverse hypermethylation-induced downregulation of the remaining four tumour suppressing PLKs in combination with traditional therapies.

Figure 3.7. A potential role for p53 in the silencing of the *PLKs* as a result of oxidative stress. Previous data has established that p53 can regulate both PLK1 and PLK4 expression through protein-protein interactions. Here we have incorporated our observations into the known mechanisms of the p53-PLK regulatory axis (a.) Our data suggests that when oxidative stress upregulates p53 activity, this can lead to downstream effects that can potentially induce the epigenetic silencing of the *PLKs*. In wild type p53 cells, these mechanisms can include the recruitment and/or collaboration with epigenetic modifiers such as DNMT1, DNMT3a or histone deacetylases (HDACs). (b) However, oxidative stress in the absence of p53, these vital inhibitory interactions carried out through the p53 pathway are abolished. PLK1 and PLK4 expression thus carries on unhindered, potentially pushing the cell through the G2/M transition point with unrepaired DNA damage, resulting in genomic instability and aneuploidy, both of which are hallmarks of cancer.

Figure 3.7


Environmental Stressors:
Hypoxia/ROS
a.
b.
p53+/+
Repair
Damage
P53-/-
DNMTs
and
HDACs
?
p21
Plk4
Plk4
p21
DNMTs
and
HDACs
CH3
CH3
Transcription
Plk4 Gene
Transcription
Plk1 Gene
Transcription
Plk1 Gene
Transcription
Plk4 Gene
G2/M
Cell
Cycle
Tumourigenesis
cell cycle progression

Materials and Methods

Ethics Statement

All animal procedures were carried out in accordance with animal care protocols approved by the University of Windsor Animal Care Committee under the guidelines of the Canadian Council on Animal Care.

Tissue Culture

Mouse embryonic fibroblasts (MEFs) were harvested from *Plk4*$^{+/+}$ and *Plk4*$^{+/-}$ embryos at day 12.5 *post coitus*, as described in Ko et al, 2005 [2]. The procedure was carried out in accordance with animal care protocols approved by the University of Windsor Animal Care Committee under the guidelines of the Canadian Council on Animal Care. The MEFs were maintained in DMEM supplemented with 20% fetal bovine serum and 1% penicillin G sodium/streptomycin sulphate at 10,000ug/mL, and 0.5% gentamycin 10mg/mL. Cell lines were purchased from ATCC, U2-OS and Saos-2 cells were maintained in McCoy's media supplemented with 10% FBS. Hep3G2 and Hep3B cell lines were grown in MEM with 10% FBS. All the cell lines were kept in a 37˚C incubator with 5% CO_2. During hypoxic treatment, cells were grown in a hypoxia incubator chamber (STEMCELL Technologies Inc.) flooded with 2% CO_2 at a rate of 10L/min for 8 minutes then incubated for 18 hours at 37˚C. Reactive oxygen species were generated using 200um H_2O_2 for 18 hours; treated cells were grown in standard culture conditions.

Methylation Specific PCR

DNA was extracted from cells prior and post treatment using ProK digestion buffer (0.5mg/mL) followed by phenol chloroform extraction. Genomic DNA was subjected to bisulfite conversion as described in Herman et al. [60]. Post-bisulfite treatment, the DNA was purified using the Wizard mini DNA clean-up kit (Promega), desulfanated with NaOH and ethanol precipitated. MSP was performed with primers designed for individual *Plks* using the MethPrimer program [61]. For sequences please see Ward et al. [10]. Positive controls of fully methylated NIH 3T3 mouse DNA and HeLa human DNA (NEB) were also included in all experiments.

Western blot analysis

Whole cell lysates were extracted using a lysis buffer (50mM Tris-HCl pH 7.4, 150mM NaCl, 1mM EDTA, 0.5% Triton X) with an EDTA-free protease inhibitor cocktail (Bio Basics Inc.) 20ug of total protein was used to perform Western blot analysis. Primary antibodies were purchased accordingly, anti-PLK2, anti-PLK3, and anti-DNMT3b (from Santa Cruz), anti-PLK1, anti-PLK4, anti-GAPDH, and anti-DNMT3A (from Cell Signalling), and anti-DNMT1 and anti-Actin (from Sigma). For secondary antibodies, anti-rabbit (from Cell Signalling) and anti-mouse HRP (from Sigma) were used. Bands were visualized by ECL (Thermo Scientific) and blots were acquired on an Alpha Innotech Multimage™ Light Cabinet and densitometry analysis was carried out using OptiQuant software Version 5.0.

Real time PCR

RNA from treated cells was extracted using the RNeasy® mini kit (Qiagen). RNA extraction was performed according to manufacturer's protocols. Reverse transcription was carried out using the First Strand cDNA synthesis kit (Invitrogen) according to manufacturer's instructions. Real time PCRs were carried out on an ABI 7300 machine using Taqman gene expression probes for mouse *Plk1*, *Plk4*, and *HIF1α*; and human *PLK1-PLK4*, and *HIF1α* (Applied biosystems). GAPDH was used as an internal control in ROS qPCR, but not in hypoxia qPCR due to GAPDH transcripts also being affected by hypoxia treatment [62]. Hypoxia transcript values were normalized by addition of 100ng of total cDNA to each reaction and ΔCT for treated samples were calculated from the untreated CT values as per [57]; additional calculations were performed according to the Taqman assay manual.

p53 activity assay

The Human/Mouse Active p53 DuoSet® IC (R&D Systems) assay was carried out according to the manufacturer's protocol. Briefly, cells were grown to 80-90% confluence and the nuclear fraction of protein was extracted and sandwich ELISA assay was used to determine p53 activity.

Global methylation assay

The global methylation of genomic DNA was determined by using 100ng of ProK extracted gDNA in a sandwich ELISA colourimetric assay (Epigentek). The assay was carried out according to manufacturer's instructions.

Statistical analysis

All Western blot analysis, transcript levels, and global methylation assays are represented as the mean +/- standard deviation. These data were evaluated using Statsoft Statistica software version 7.1 using One-way ANOVA analysis. Significance represents a $p<0.05$. All results are representative of three independent experiments.

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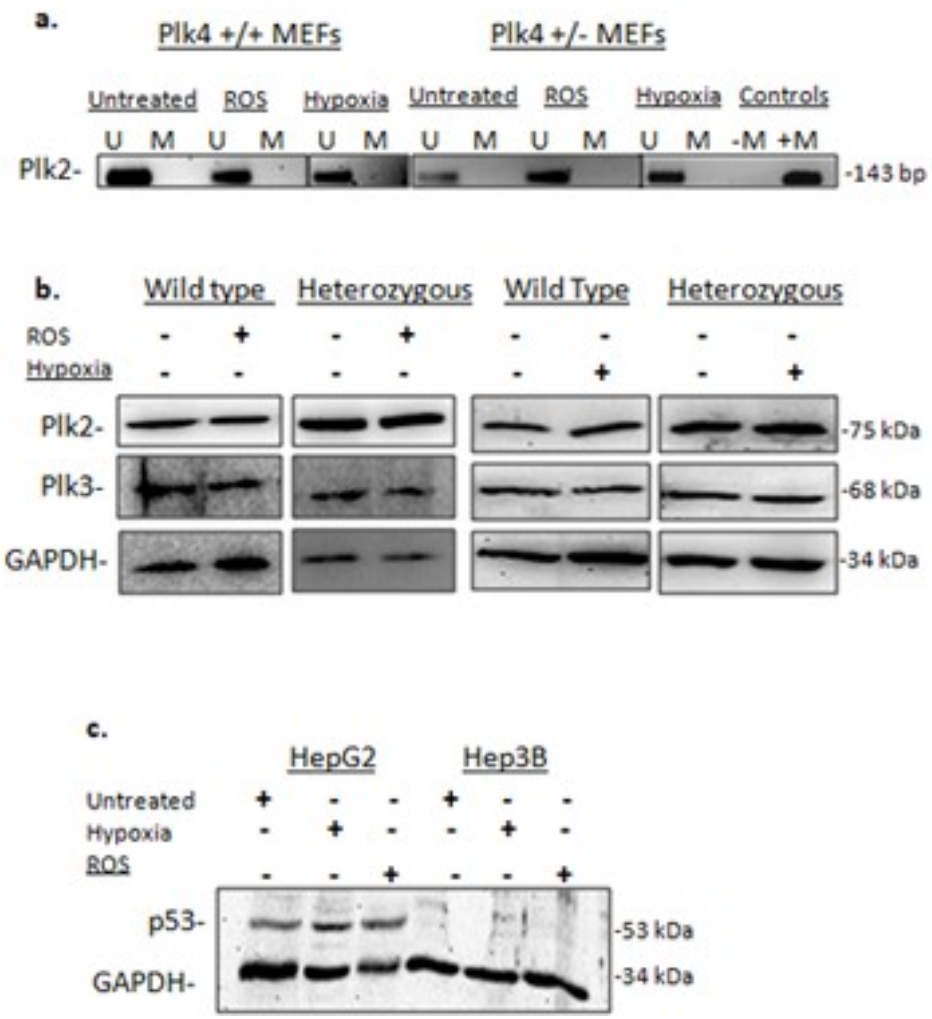
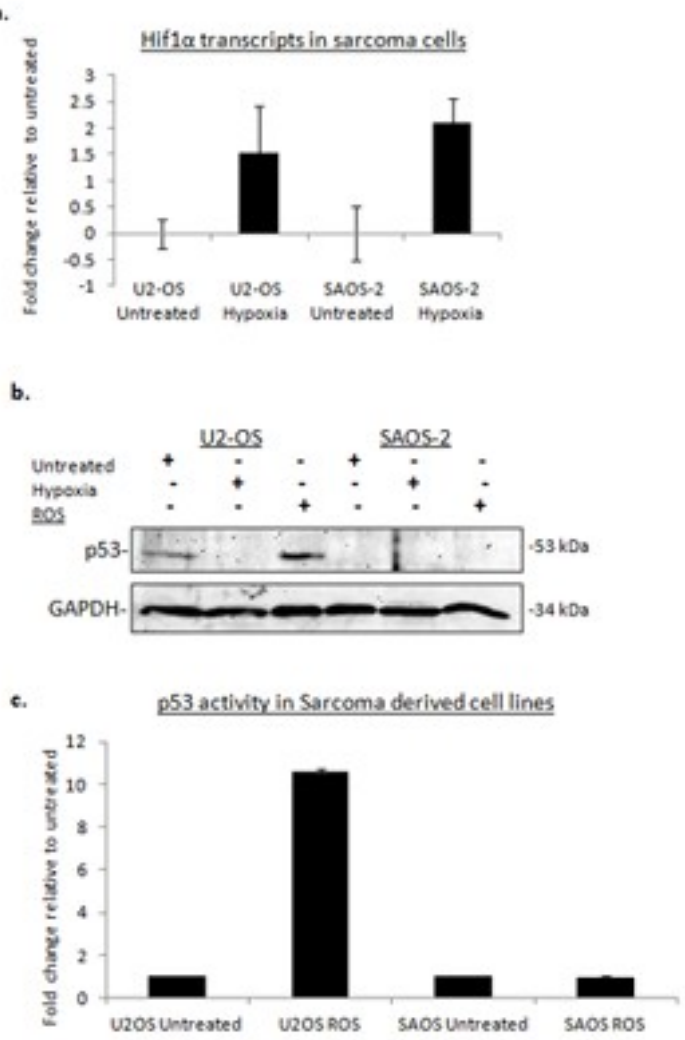
Supplementary figure 3.1. Assessment of plk2 and plk3 levels in treated MEFs and p53 levels in HCC cells. (a) Methylation status of *Plk2* in treated MEFs was determined by MSP. (b) Western blot analysis of Plk2 and Plk3 protein in untreated (-) and treated (+) MEFs. GAPDH was used as a loading control. (c) p53 protein levels determine *via* Western blot analysis in untreated (-) and treated (+) HCC cells. GAPDH was used as a loading control.

Supplementary figure 3.1



Supplementary figure 3.2. Examination of Hif1α transcripts along with p53 levels and activity in treated osteosarcoma cells. (a) Transcript levels of Hif1α were determined by qPCR and normalized to the respective untreated samples. The histogram is representative of the mean from three independent experiments with errors bars showing +/- SD. (b) p53 protein levels in untreated (-) and treated (+) U2-OS and SAOS-2 cells. (c) The activity of p53 pre- and post-treatment from nuclear extracts of osteosarcoma cells. The values were normalized the respective untreated samples. Error bars represent the +/- SD from three independent experiments.

Supplementary figure 3.2



Chapter 4

PRMT5, a novel PLK4 interacting partner is deregulated in *Plk4* heterozygous MEFs

Authors: **Alejandra Ward**, Gayathri Sivakumar, Sharon Yong, Anna Kozarova, Jordan Nantais, Otis Vacratsis, John W. Hudson

Introduction

The highly conserved polo-like kinase family is essential for a variety of cellular phenomena which include centrosome dynamics, spindle pole formation, mitotic and anaphase entry, as well as involvement in the DNA damage response [1-4]. Of the five members discovered to-date, PLK1-4 are the most well characterized of these serine/threonine kinases. Congruent with the promiscuity of these types of kinases, multiple interacting proteins have been identified for PLK1-4. Among these interacting partners, PLK1 and PLK3, and PLK4 have been shown to interact with key cell cycle proteins such as CDC25C [5-8] and cyclin B1[9]. As well, PLK1-PLK4 all interact with DNA damage response proteins Chk2 and p53 [6, 8, 10, 11] with varying biological outcomes [12, 13]. Most recently, PLK2 has been implicated in a variety of neurological pathways including the phosphorylation of α-synuclein in yeast and mammals [14], and regulating the activity of the guanosine triphosphatases, Ras and Rap, in neurons [15]. PLK4's primary function is in centrosome duplication with several of its identified targets involved in this process. This includes GCP6, a member of the γ-tubulin ring complex, an essential component of the centrosome, and the SCF-FBOXW5 E3 ubiquitin ligase which helps prevent centrosome overduplication [16, 17]. Deregulation of PLK4 protein levels in both humans and mice leads to multiple centrosomes and multipolar

spindle formation resulting in genomic instability and is associated with tumourigenesis [18, 19]. During development, murine embryos which are *Plk4*$^{-/-}$ fail to progress past embryonic day 7.5 [20]. PLK4 also has been implicated in cell-fate determination through its regulation of Hand1, which, when phosphorylated by PLK4, signals for trophoblast stem-cell differentiation [21]. *Plk4*$^{+/-}$ mice develop hepatocellular carcinomas and lung tumours at a rate 15 times higher than their wild type littermates as they age [19]. Deregulation of PLK4 has been reported in several human tumour types including colorectal cancers, hepatocellular carcinoma, and most recently, the highly aggressive triple negative breast cancers [13, 22-24]. Recent studies from our lab have revealed that downregulation of Plk4 levels has downstream impacts on normal epigenetic modifications *in vitro* and *in vivo* [23, 25]. *Plk4*$^{+/-}$ MEFs have higher levels of DNA methyltransferase 3A (DNMT3A), an enzyme responsible for *de novo* methylation, before and after oxidative stress [25]. Furthermore, *Plk4*$^{+/-}$ mice display significantly higher global methylation when they are young compared to their wild type counterparts, and eventually go on to develop hepatocellular carcinoma, where *Plk4* itself is further downregulated through DNA hypermethylation [23]. It is therefore of importance to identify PLK4 downstream targets in order to fully understand the essential functions of PLK4 and how this may impact epigenetic modifications. Here we report the characterization of a novel interaction between PLK4 and protein arginine methyltransferase 5 (PRMT5), an epigenetic modifier. In addition, we also show *Plk4* heterozygosity directly impacts the levels, activity, and localization of PRMT5.

Results and Discussion

Characterizing the interaction between Plk4 and PRMT5

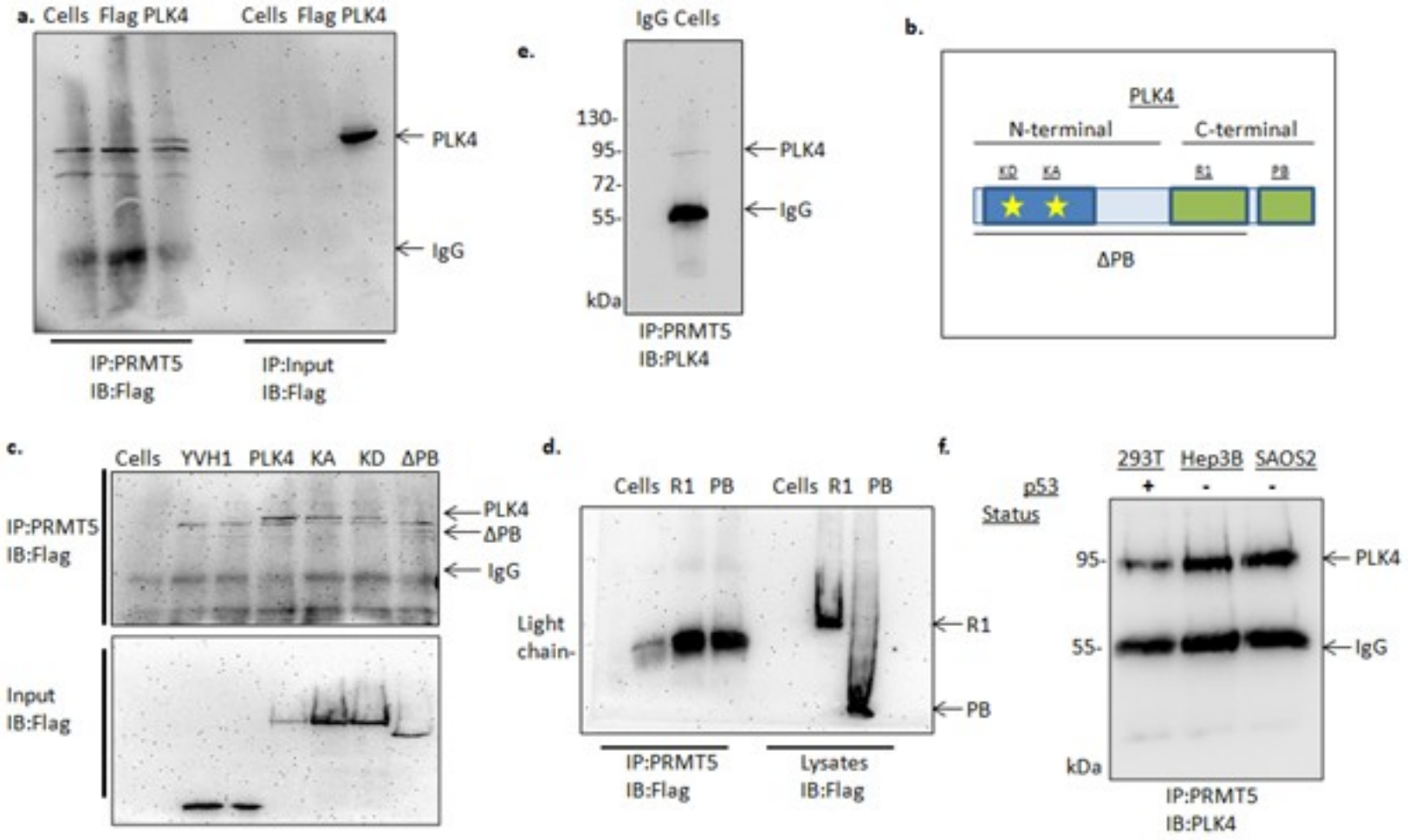
In order to enhance the detection of novel PLK4 interacting partners, we used a Flag-tagged mass spectrometry (MS) approach. The resulting mass spectra were analyzed for any peptide peaks which had particularly strong signals and these were chosen for analysis by tandem-MS (MS-MS). PRMT5 was identified in these peaks. Concerns with non-specific binding of PRMT5 in co-immunoprecipitations have been previously documented in the literature [26]. Nishioka and Reinberg noted that Flag-M2 agarose beads could non-specifically bind PRMT5 in pull down assays [26]. Therefore, to validate our mass-spectrometry findings, we performed a co-immunoprecipitation with a PRMT5 antibody against the endogenous protein using G-sepharose beads. Our co-immunoprecipitation indeed showed Flag-PLK4 was interacting with PRMT5 under these conditions (Fig. 4.1a). To further eliminate the possibility that this interaction may be due to non-specific Flag binding, the experiment was repeated using endogenous protein from whole cell lysates of HEK 293T cells. The interaction still persisted in unsynchronized cells (Fig. 4.1b). PLK4 is the largest of the PLKs with an N-terminal domain housing its kinase activity, a unique central region termed the cryptic polo box which encompasses two tandem polo-box domains, and the C-terminus with a third polo-box domain [27]. This tri-polo-box architecture facilitates PLK4 activities such as oligomerization, autophosphorylation and substrate targeting [27]. Cep152, an essential centrosome protein, recruits PLK4 to the centrioles and binds to PLK4's cryptic polo-box domain, though it does not interact at the N-terminal domain and is not a substrate of PLK4 [28]. However, PLK4's interaction with GCP6 and Ect2 only requires the N-

terminal domain of PLK4 and both are substrates of PLK4 [16, 29]. As a first step in determining the nature of the interaction between PLK4 and PRMT5, we used various PLK4 mutants. Initially, we examined the interaction at the N-terminal domain of PLK4 by using Flag-PLK4K41M kinase dead (KD), Flag-PLK4T170D kinase active (KA), and Flag-PLK4ΔPB (lacking polo-box domains) mutants (Fig. 4.1c). Based on our co-immunoprecipitations, all of the N-terminal mutant constructs interacted with PRMT5 (Fig. 4.1d) while there was no detectable interaction for the C-terminal domains, Flag-PLK4R1 (only the cryptic polo-box) and Flag-PLK4Pb (only the polo-box domain) (Fig. 4.1d). Flag-YVH1, a phosphatase, was used as an additional control for non-specific binding (Fig. 4.1c). Note, we had previously determined that this protein did not interact with Plk4 [7, 8].

PRMT5 is an epigenetic modifier which can methylate both histone and non-histone proteins at arginine residues, and as a type II arginine methyltransferase, it catalyzes the symmetric transfer of methyl groups onto arginine [30, 31]. PRMT5 can mediate transcriptional repression at the histone level by methylation of histone tails at H3R8 and H4R3, [32] which in turn, recruit the DNA methylating proteins, DNA methyltranferase 3A (DNMT3A), and histone deacetylase 1 (HDAC1) to increase transcriptional downregulation at the DNA level. The function of PRMT5 is not limited to histone modification, but it has also been shown to play a role in the regulation of cell growth proteins like Epidermal Growth Factor Receptor (EGFR) and TNF-related apoptosis inducing ligand (TRAIL), by suppressing their functions [33, 34]. PRMT5 is essential for embryonic development as PRMT5 null mouse embryos are embryonic lethal between E3.5-E6.5 [35].

Figure 4.1. PRMT5 interacts with PLK4's N-terminal domain. (a) PRMT5 Co-immunoprecipitations using HEK 293T cells were conducted in order to determine if PLK4 interacts with PRMT5. The cells lane represents un-transfected whole cell lysates; Flag represents cells transfected with Flag empty vector. (b) Schematic diagram showing the sites modified on PLK4 to obtain mutants. KD represents the kinase dead mutant where lysine 41 was converted to methionine. KA is the kinase active mutant with threonine converted to aspartic acid. ΔPB encompasses the entire kinase domain without the polo box domains. R1 represents what was once called the cryptic polo-box domain, but now is known to harbour 2 polo-box domains. PB is just the third and C-terminal polo-box domain only. (c) To characterize the interaction between PRMT5 and PLK4, transiently transfected N-terminal mutants were pulled down with PRMT5 antibody. (d) A PRMT5 co-immunoprecipitation with lysates from transiently transfected C-terminal domain truncation mutants. (e) The co-immunoprecipitation was performed on endogenous PLK4 proteins using the PRMT5 antibody. The first lane has a negative control with beads immunoprecipitated with mouse IgG only. IgG label at 55 kDa represents the heavy chain of the antibody. (f) Endogenous PLK4 co-immunoprecipitation was performed with PRMT5 antibody with lysates from p53 null cells.

Figure 4.1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Cells Flag PLK4
PLK4
IgG
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IB:Flag
IP:Input
IB:Flag
e.
IgG Cells
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95-
72-
55-
kDa
PLK4
IgG
IP:PRMT5
IB:PLK4
b.
PLK4
N-terminal
C-terminal
KD
KA
R1
PB
ΔPB
c.
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IP:PRMT5
IB:Flag
PLK4
ΔPB
IgG
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IB:Flag
d.
Cells R1 PB
Cells R1 PB
Light
chain-
R1
PB
IP:PRMT5
IB:Flag
Lysates
IB:Flag
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PLK4
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Additionally, PRMT5 has a role in the DNA damage response by activating p53 during genotoxic stress *via* arginine methylation [36, 37]. Interestingly, Ko et al., determined that PLK4 interacts with p53 [19] an observation that suggested to us that perhaps the PLK4 and PRMT5 interaction required p53, or p53 may be acting as an intermediary in binding. We thus performed endogenous co-immunoprecipitation of PRMT5 from cell extracts of the p53-null cell lines Hep3B which is derived from a hepatocellular carcinoma, as well as with Saos-2, an osteosarcoma-derived cell line [38]. In these p53 null cells, the PLK4-PRMT5 interaction persisted and was evident in both cell types (Fig. 4.1f), indicating that p53 was not required for the interaction.

Prmt5 is a bonafide substrate of Plk4

Since PRMT5 interacted with PLK4 specifically at its N-terminal region, which houses PLK4's kinase domain, we next sought to determine whether PRMT5 may be a potential substrate of PLK4. Flag-tagged versions of human wild-type (Flag-Plk4), kinase active (Flag-Plk T170D) and kinase dead (Flag-Plk4 K41M) were expressed in HEK-293 cells and after affinity purification were subsequently used in in vitro kinase assays to test the ability of PLK4 to phosphorylate bacterially expressed GST-PRMT5 fusion protein. Our results demonstrated PRMT5 was targetted by both wild type and kinase active forms of PLK4, thus indicating that PRMT5 is a substrate of PLK4 (Fig. 4.2a). Moreover, our kinase assay also indicated that kinase activity of PLK4 is required for the phosphorylation of PRMT5 since the kinase-inactive mutant of PLK4 did not phosphorylate PRMT5 (Fig. 4.2a). As expected auto-phosphorylation of both full length and kinase active forms of PLK4 were also observed (Fig. 4.2a) and Plk4 did not

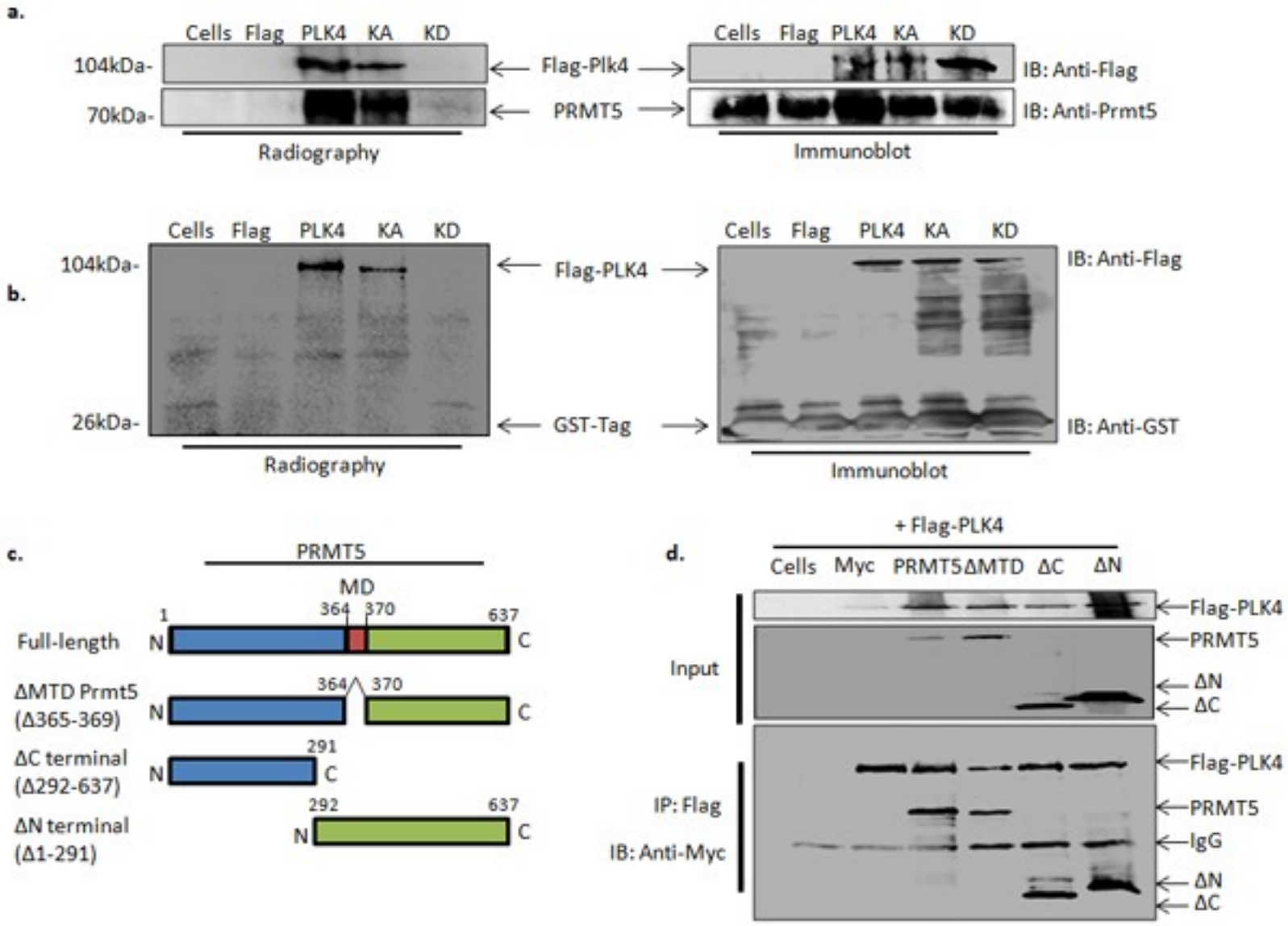
phosphorylate the residual GST-tag or purified GST protein, thus confirming that the phosphorylation of PRMT5 by PLK4 was specific for PRMT5 (Fig. 4.2b).

Identification of the Plk4 interacting region within Prmt5

PRMT5 exists as a tetramer and the oligomeric interactions between dimer pairs are propelled by comprehensive associations between the Triosphosphate isomerase (TIM) barrel at the amino-terminal lobe of one monomer and the carboxy-terminus β-barrel of another [39]. In addition, the N-terminus of PRMT5 is essential for the binding of the co-factor methylosome protein 50 (MEP50) to form the integral unit of the protein methyltransferease complex. This moiety can in turn collaborate with a myriad of partner proteins, creating a pool of multimeric complexes with unique substrate selectivity and functionalities [40, 41]. Gu et al. (2012) have previously identified the presence of three nuclear exclusion signals in PRMT5 with one in the N-terminal and two in the C-terminal region. Depending on the cell type, PRMT5 has discrete subcellular localizations and consequently, altered functions. It is speculated that the dynamic regulation of its localization may be dependent on post-translational modifications [42]. The amino-terminal of PRMT5 provides a platform for substrate interaction through MEP50, while the carboxy-terminal domain encompasses the salient catalytic core possessing the S-adenosylmethionine (AdoMet)-dependent arginine methyltransferase activity of PRMT5 [40]. Undoubtedly, the active site of PRMT5 is key to its functions in several cellular processes, including the modulation of signaling pathways and gene expression.

Figure 4.2. PRMT5 is a novel substrate of PLK4. (a) Kinase assays were performed to ascertain whether PLK4 phosphorylates PRMT5. HEK 293T cells were transiently transfected with Flag empty vector and Flag-tagged PLK4 constructs, including wild type (PLK4), kinase active (KA), and kinase dead (KD) mutants. 293T lysates transfected with the indicated constructs were immunoprecipitated with anti-Flag antibody and incubated with bacterially expressed, GST-cleaved PRMT5 in the presence of [ɣ-^{32}P]. The assay was visualized by autoradiography. Immunoblots show the loading of PLK4 constructs and PRMT5. (b) In vitro kinase assay conducted with GST protein alone is shown as control. (c) A schematic representation of PRMT5 full-length protein and truncation mutants. (d) Co-immunoprecipitation of truncation mutants with Flag-PLK4 identifies the PRMT5 domains which interact with PLK4.

Figure 4.2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104kDa-
70kDa-
Flag-Plk4
PRMT5
IB: Anti-Flag
IB: Anti-Prmt5
Radiography
Immunoblot
b.
Cells Flag PLK4 KA KD
104kDa-
26kDa-
Flag-PLK4
GST-Tag
IB: Anti-Flag
IB: Anti-GST
Radiography
Immunoblot
c.
PRMT5
MD
1
364
370
637
Full-length
N
C
ΔMTD Prmt5
(Δ365-369)
ΔC terminal
(Δ292-637)
291
ΔN terminal
(Δ1-291)
292
d.
+ Flag-PLK4
Cells Myc PRMT5 ΔMTD ΔC ΔN
Input
IP: Flag
IB: Anti-Myc
Flag-PLK4
PRMT5
ΔN
ΔC
IgG

In order to map the region in PRMT5 that interacts with PLK4, we generated Myc-tagged deletion mutants for human PRMT5. The truncation mutants were generated to target three distinct domains on PRMT5. The first is a ΔN-terminal (amino acids 1-291)-PRMT5, a deleted arginine methyltransferase domain ΔMTD (amino acids 365-369)-PRMT5, which causes the protein to lose its enzymatic activity, and a ΔC-terminal (amino acids 292-637)-PRMT5 (Fig. 3.2c). Surprisingly, results from the immunoprecipitations displayed PLK4 interaction with all three mutated constructs of PRMT5 (Fig. 3.2d). As a way to exclude the possibility of non-specific binding of Myc tag with Flag-PLK4, we also performed a Co-IP assay where HEK 293Ts were co-transfected with Myc-SPY1 and Flag-PLK4. Again, Flag antibody was used to co-immunoprecipitate proteins and no interaction was observed between Myc-tagged SPY1 and PLK4 (data not shown). To dissect the implication of PLK4's association with multiple domains of PRMT5, we performed an *in silico* analysis using NetPhos 2.0 server [43] to predict Serine/Threonine phosphorylation sites on PRMT5. Nine Ser and two Thr residues have potential for such post-translation modifications within the N-terminus, while the C-terminus possesses four Ser and four Thr phosphorylatable sites. It is widely known that many substrates need to be phosphorylated at multiple sites in order to modulate localization or function [44]. It may be possible that distributive phosphorylation events may be essential for the nature of the signaling network between PLK4 and PRMT5. We are currently using a mass spectrometry-based approach to determine the site(s) targeted by PLK4. As both domains of PRMT5 contain putative nuclear exclusion sequences, all of which are in close proximity to phosphorylatable Ser/Thr residues, it is possible that phosphorylation of these specific regions by PLK4 is

an impetus to produce the changes in localization patterns innate to PRMT5. Moreover, distal binding interactions between the kinase and substrate can further alter substrate activity through allosteric regulation and induce changes in sub-cellular localization [45].

Prmt5 does not arginine methylate Plk4

PRMT5 orchestrates the arginine methylation of a repertoire of proteins to modulate important biological processes such as growth, proliferation, and differentiation. We assessed whether the interplay between PRMT5 and PLK4 could potentially be bi-directional. *In silico* analysis revealed that PLK4 had a putative site for arginine methylation between residues 353-361 [46]. Using a symmetric arginine dimethylation antibody, we performed a co-immunoprecipitation to determine whether PLK4 is able to co-purify with this dimethyl arginine antibody. PLK4 was undetectable in the immunocomplex, suggesting that under these conditions PRMT5 does not confer any post-translational arginine methylation marks on PLK4 (data not shown). This suggests that the interaction between PLK4 and PRMT5 may be unilateral. While PLK4 is able to phosphorylate PRMT5, PRMT5 may not necessarily mediate arginine methylation of PLK4.

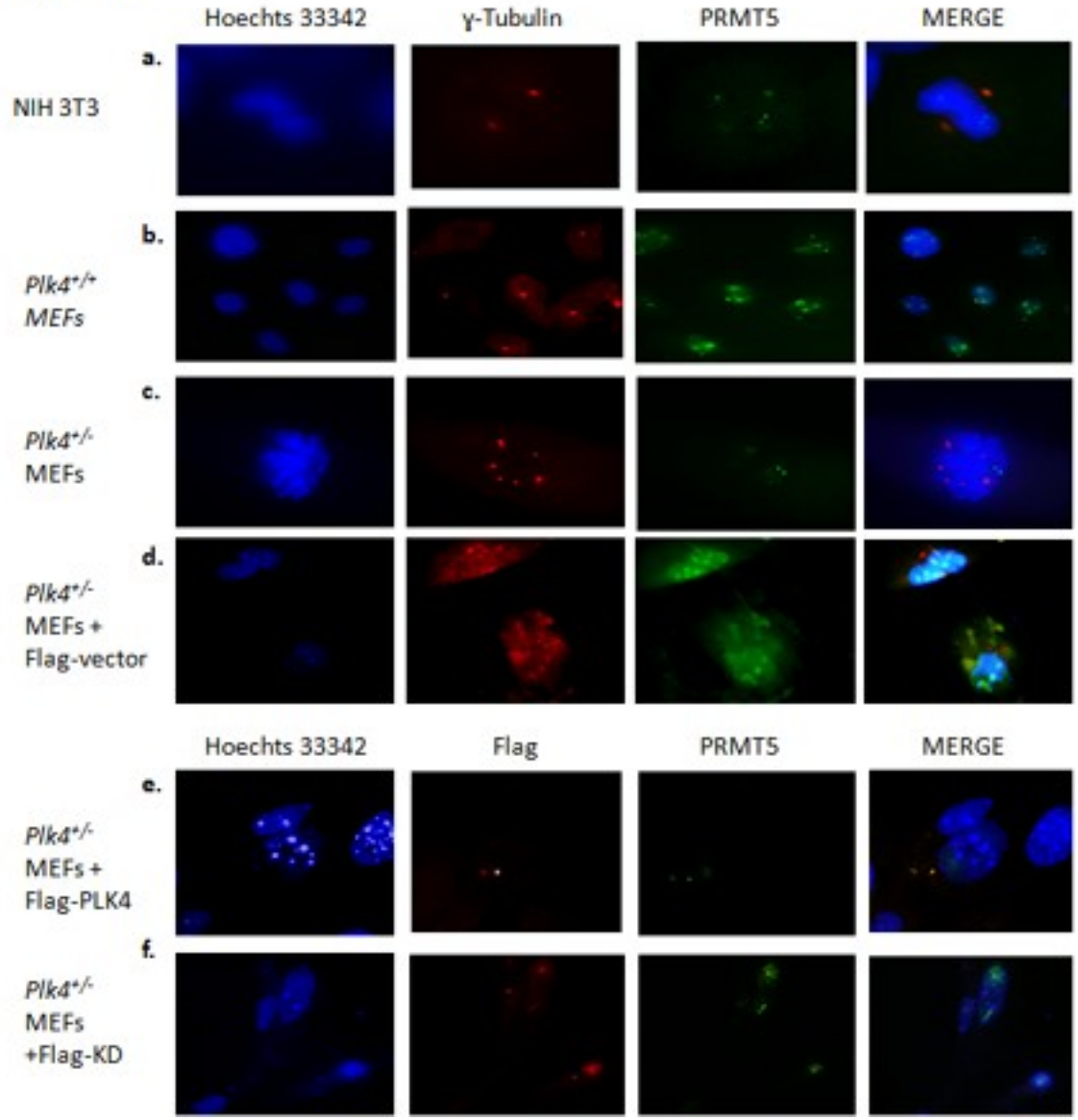
Plk4 heterozygosity results in aberrant localization of PRMT5

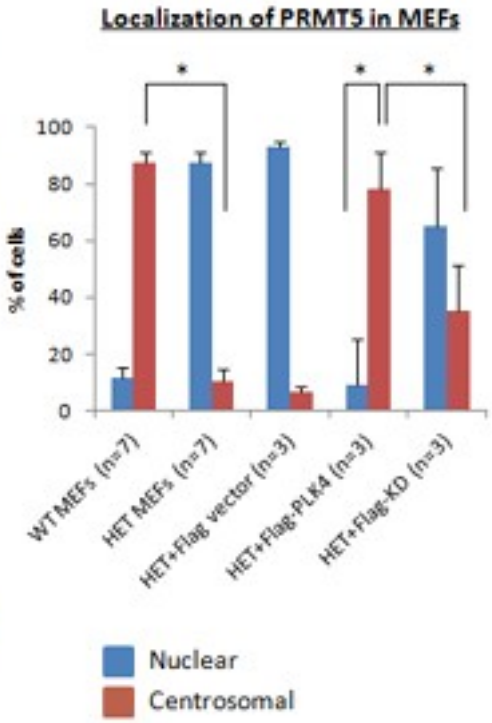
Plk4 has been well established as a master regulator of centrosome duplication, and localizes to this and other important mitotic structures including the midbody and the cleavage furrow [29, 47]. Therefore, it was important to determine to which of these subcellular areas PRMT5 localized to. Previous literature has established that PRMT5

localizes both to the cytoplasm and the nucleus, however, ectopic expression of GFP-PRMT5 in HEK 293 cells resulted in PRMT5 being expressed predominantly in the cytoplasm with regions of increased staining [42, 48], which were suggestive of centrosomal localization. The cellular compartment in which PRMT5 inhabits has a direct impact on its function and activity. Distinct partitioning of PRMT5 results in a cellular dichotomy, where nuclear localization of PRMT5 has been associated with a decrease in arginine methyltransferase activity, while cytoplasmic localization is associated with an increase in cellular proliferation [42]. This has been documented in several cell types: in colorectal tumour tissue, there is a greater proportion of PRMT5 localizing to the nucleus of tumour cells compared to the normal cells, while in prostate cancer cells, PRMT5 localization in the cytoplasm was associated with an increase in cellular proliferation [42, 49]. Initially, since the predominant site of localization for PLK4 is the centrosomes [47] we performed immunofluorescence on NIH 3T3 cells with γ-tubulin to stain for centrosomes and anti-PRMT5 staining for endogenous PRMT5. We found that in these cells, PRMT5 was detected in dot-like patterns in the nucleus, with some generalized staining in the cytoplasm, and, more specifically, PRMT5 was also found to have a strong signal on the centrosomes (Fig. 3.3a). We examined this phenotype further in primary cells in the context of Plk4 levels by utilizing both wild type and heterozygous Plk4 mouse embryonic fibroblasts (MEFs). Perhaps, low levels of Plk4 could perturb the centrosomal localization of PRMT5? For example, one of PLK4's substrates, Ect2 is required for appropriate cytokinesis; in the context of *Plk4* heterozygosity, during late mitosis, Ect2 fails to localize to the midbody and does not activate RhoA, a necessary protein for completion of cytokenesis [29]. In wild type

Figure 4.3. Plk4 heterozygosity impacts Prmt5 localization. Mouse fibroblasts were used to examine the endogenous localization of Prmt5. Hoechts 33342 was used to stain the nucleus, ɣ-tubulin to examine the centrosomes, and Prmt5 antibody was used to examine endogenous localization of Prmt5. (a) Initially NIH 3T3 mouse fibroblasts were employed to examine the localization of Prmt5. In some instances, Prmt5 also localized to the centrosomes. (b) Primary wild type (WT) mouse embryonic fibroblasts (MEFs) show the co-localization of Prmt5 to the centrosomes. (c) *Plk4* heterozygous MEFs were used to determine if varying levels of Plk4 also impacted Prmt5 localization. (d-e) $Plk4^{+/-}$ MEFs were transiently transfected with Flag empty vector, full length PLK4, and the KD PLK4 mutant. (f) The distribution of Prmt5 localization was quantified in WT and HET MEFs by counting a minimum of 200 cells. The cell counts for the transfected cells were obtained by counting a minimum of 100 cells positive for Flag staining. Error bars represent the standard deviation from three independent experiments. * represents a $p<0.001$.

Figure 4.3


Hoechts 33342
γ-Tubulin
PRMT5
MERGE
a.
NIH 3T3
b.
Plk4+/+ MEFs
c.
Plk4+/- MEFs
d.
Plk4+/- MEFs + Flag-vector
Hoechts 33342
Flag
PRMT5
MERGE
e.
Plk4+/- MEFs + Flag-PLK4
f.
Plk4+/- MEFs +Flag-KD


Localization of PRMT5 in MEFs
% of cells
100
80
60
40
20
0
*
*
*
WT MEFs (n=7)
HET MEFs (n=7)
HET+Flag vector (n=3)
HET+Flag-PLK4 (n=3)
HET+Flag-KD (n=3)
Nuclear
Centrosomal

MEFs, PRMT5 was also detected in the nucleus and the cytoplasm, but PRMT5 also co-localized to the centrosomes in more than 85% of the cells examined (Fig. 4.3b,g). By contrast, in *Plk4*$^{+/-}$ MEFs, we found that on average, that approx. 10% of the cells examined had PRMT5 localizing to the centrosome (Fig. 4.3c,g) with the majority of PRMT5 localized to the nucleus in *Plk4*$^{+/-}$ MEFs (Fig. 4.3c,g). In order to further establish a role for Plk4 levels in PRMT5's localization patterns, we examined whether Plk4 over-expression in Plk4 heterozygous MEFs would alter or rescue expression the pattern. We observed that PRMT5 co-localized with ectopically expressed PLK4 in *Plk4*$^{+/-}$ MEFs at the centrosomes in approximately 80% of the cells examined, suggesting that re-introducing normal PLK4 levels is sufficient to rescue the predominant nuclear localization of PRMT5 in *Plk4*$^{+/-}$ MEFs (Fig. 4.3e,g). Furthermore, transfection with the kinase dead-PLK4 mutant resulted in a partial rescue, though not to the same degree as the full length PLK4 rescue (Fig. 4f,g). The PRMT5 localization in *Plk4*$^{+/-}$ MEFs transfected with Flag-vector alone remained primarily in the nucleus similar to un-transfected cells (Fig. 4.3d,g).

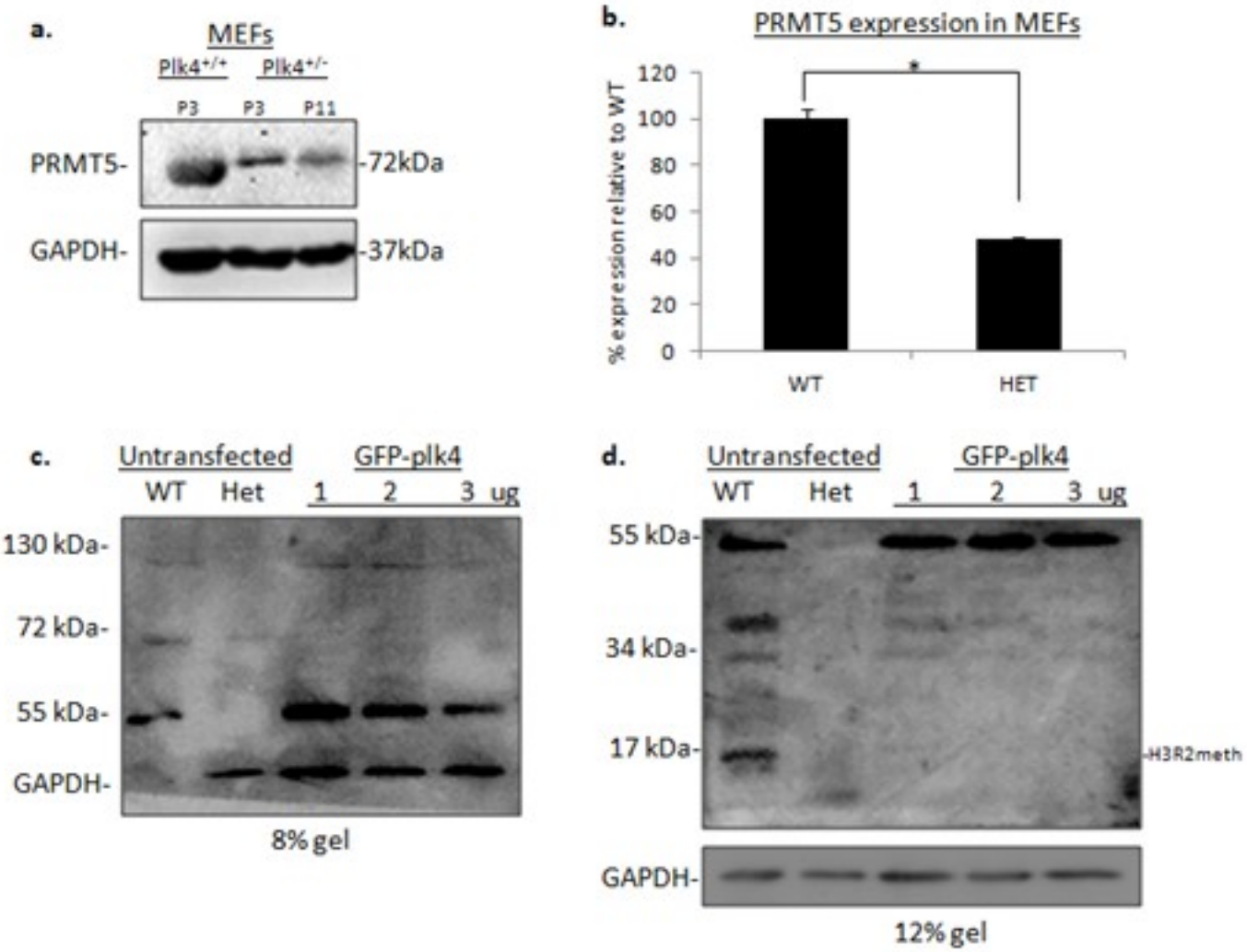
Plk4 heterozygosity is insufficient for normal PRMT5 activity

It is known that cellular localization impacts PRMT5 function. With the aberrant localization of PRMT5 in *Plk4*$^{+/-}$ MEFs, it raised the question of how else does *Plk4* heterozygosity impact PRMT5? To answer this question, we examined protein levels of PRMT5 in *Plk4*$^{+/+}$ and *Plk4*$^{+/-}$ MEFs *via* Western blot analysis. Interestingly, we found that in the *Plk4*$^{+/-}$ MEFs, PRMT5 levels were dramatically decreased compared to the wild-type cells regardless of whether the MEFs were from an early or late passage (Fig.

4.4a). This indicates that PRMT5 down-regulation is correlated with lower levels of Plk4. Decreased levels of PRMT5 that persist through later passages indicates that this is an inherent phenotype of *Plk4* heterozygous cells and it is not merely a transitory event (Fig. 4.4a). PRMT5 protein levels were 50% lower in *Plk4* heterozygous MEFs than Plk4 wild type MEFs (Fig. 4.4b), which is directly proportional to the Plk4 protein levels found in these Plk4 genotypes [13]. This suggests PRMT5 levels are tethered to that of Plk4 protein levels and that Plk4 may have an upstream regulatory role on PRMT5 levels. Previous studies have shown that PRMT5 levels are correlated with its activity, significantly effecting the arginine methylation of its downstream targets [49-51]. In agreement with this, the examination of whole cell lysates for symmetric arginine methylation revealed that $Plk4^{+/-}$ MEFs had almost no detectable arginine methylated proteins (Fig. 4.4e,f) [52]. PRMT5 catalyzes the formation of symmetric dimethyl arginine residues in proteins), indicating that along with depletion of PRMT5 proteins, $Plk4^{+/-}$ MEFs also had a decrease in PRMT5 activity. *Plk4* heterozygosity indirectly results in insufficient global symmetric protein arginine methylation patterning, due to Plk4 interacting with and potentially playing a role in both the localization of PRMT5 and its levels.

Figure 4.4. *Plk4* heterozygosity decreases Prmt5 protein level and activity. Whole cell lysates from $Plk4^{+/+}$ and $Plk4^{+/-}$ MEFs were extracted and examined for Prmt5 levels. (a) Prmt5 levels from $Plk4^{+/+}$ MEFs at passage 3 (p3) were used as a normal comparison for the Prmt5 protein levels obtained from $Plk4^{+/-}$ MEFs at passage 3 and 11 (P3, P11). (b) Prmt5 levels from MEFs treated with reactive oxygen species in the form of H_2O_2 were examined for changes in protein levels between genotype and treatment. (c) Densitometry was used to quantify the differences in Prmt5 protein between $Plk4^{+/+}$ and $Plk4^{+/-}$ MEFs. * represents a $p<0.001$. The error bars represent the standard deviation obtained from three independent experiments. (d,e) Whole cell lysates from $Plk4^{+/+}$ and $Plk4^{+/-}$ MEFs were examined *via* Western blot analysis for global symmetric arginine methylation marks. GAPDH was used a loading control.

Figure 4.4

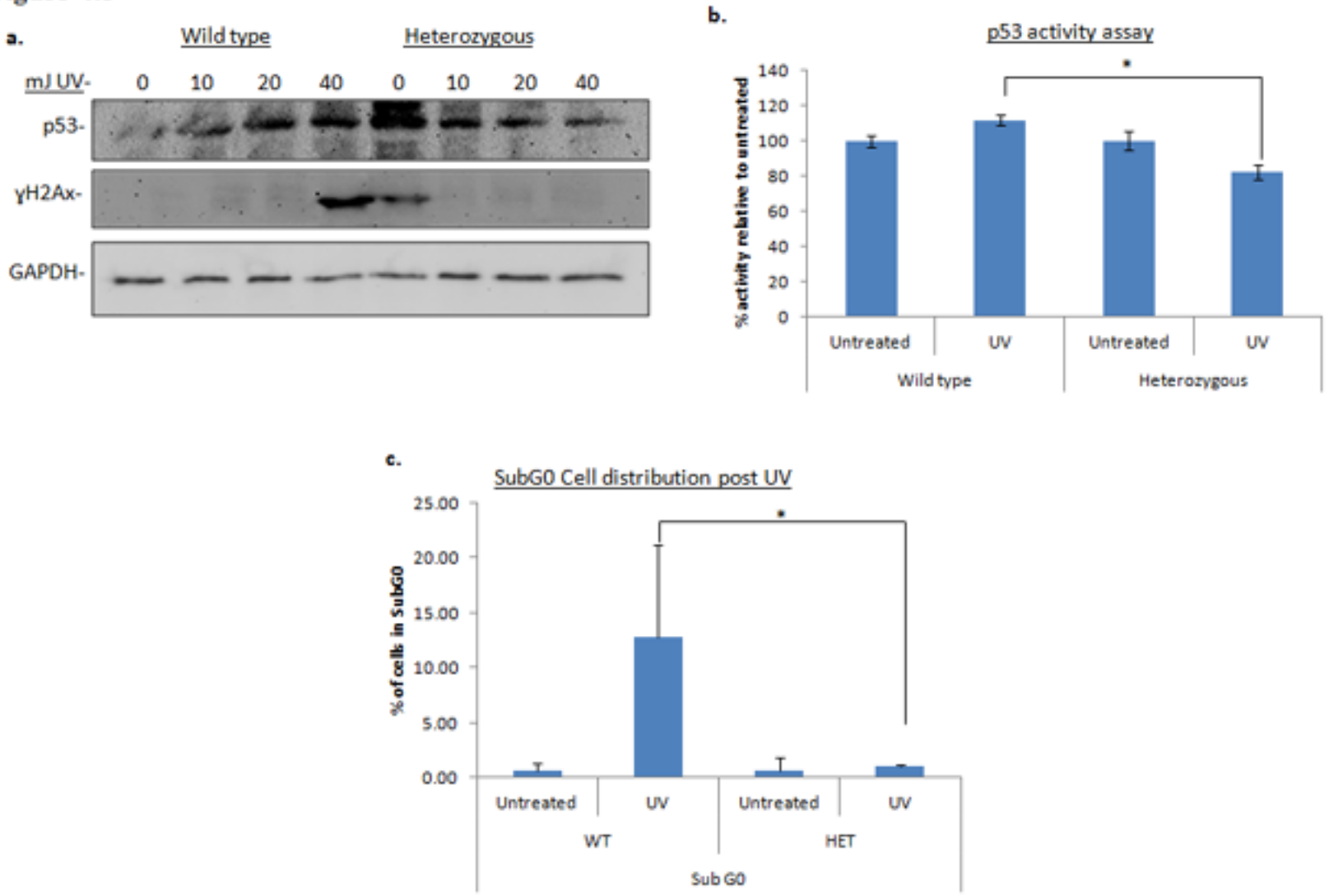

a.
MEFs
Plk4+/+
Plk4+/-
P3
P3
P11
PRMT5-
-72kDa
GAPDH-
-37kDa
b.
PRMT5 expression in MEFs
% expression relative to WT
120
100
80
60
40
20
0
*
WT
HET
c.
Untransfected
GFP-plk4
WT
Het
1
2
3 ug
130 kDa-
72 kDa-
55 kDa-
GAPDH-
8% gel
d.
Untransfected
GFP-plk4
WT
Het
1
2
3 ug
55 kDa-
34 kDa-
17 kDa-
-H3R2meth
GAPDH-
12% gel

Plk4 heterozygosity impacts PRMT5's role in DNA damage: a model for PLK4 and PRMT5 cooperation in the activation of p53

In order to further characterize the impact of *Plk4* heterozygosity on PRMT5 activity, we examined the activity of one of PRMT5's targets, p53. PRMT5 methylates several arginine residues on p53, which, during genotoxic stress, are necessary for p53 stability, activity, and specificity [36, 37]. In addition, ectopic knockdown of PRMT5, at similar levels to what we have observed in *Plk4*$^{+/-}$ MEFs, resulted in decreased p53 stability and activity [36, 37]. Previously, we have shown that the DNA damage induced by ROS in *Plk4*$^{+/-}$ MEFs did not translate into an increase in p53 activity compared to the wild type counterparts [25]. We therefore sought to examine p53 levels post-DNA damage using UV as the damaging agent since it is a genotoxic stressor that activates the p53 response and previous PRMT5 studies have used UV to examine the p53 response [37]. p53 levels increased with increased UV dosage in the wild-type MEFs as one would be expect (Fig. 4.5a). On the other hand, in *Plk4*$^{+/-}$ MEFs, p53 levels started out high, as previously reported (Morettin et al, 2009) and then, decreased with increased dosages of UV (Fig. 4.5a), indicating a loss of p53 stability. The p53 activity of nuclear extracts from treated MEFs also displayed a 20% decrease in p53 activity in *Plk4*$^{+/-}$ MEFs in comparison to untreated MEFs, while the wild-type counterparts displayed a 15% increase in p53 activity with UV exposure (Fig. 4.5b). The role of p53 during DNA damage is to either mediate DNA damage repair pathways, or, if the damage is too great, to activate the apoptotic pathway in order to prevent DNA lesions from being propagated in the next round of cell division (Reviewed in Yoshida and Miki, 2009).We next looked at levels of phosphorylated histone H2AX (γH2AX), which become

Figure 4.5. Lowered levels of Prmt5 inhibit p53 activation during DNA in a *Plk4* heterozygous context. MEFs were subjected to 0-40 mJ/UV and the DNA damage response was assessed. (a) Western blot analysis for p53 and ɣ-H2AX post DNA damage in $Plk4^{+/+}$ and $Plk4^{+/-}$ MEFs. (b) An ELISA-based p53 activity assay was used to determine p53 activity with cellular exposure to UV radiation. Nuclear extracts from MEFs were used. (c) Flow cytometry was conducted to determine the distribution of cells throughout the cell cycle post UV damage of MEFs. (d) The distribution of cell populations in the Sub-G0 phase of the cell cycle. The data is representative of three independent experiments and error bars are the standard deviation of the mean. *represents a $p<0.001$.

Figure 4.5


a.
Wild type
Heterozygous
mJ UV-
0
10
20
40
0
10
20
40
p53-
γH2Ax-
GAPDH-
b.
p53 activity assay
% activity relative to untreated
140
120
100
80
60
40
20
0
*
Untreated
UV
Untreated
UV
Wild type
Heterozygous
c.
SubG0 Cell distribution post UV
% of cells in SubG0
25.00
20.00
15.00
10.00
5.00
0.00
*
Untreated
UV
Untreated
UV
WT
HET
Sub G0

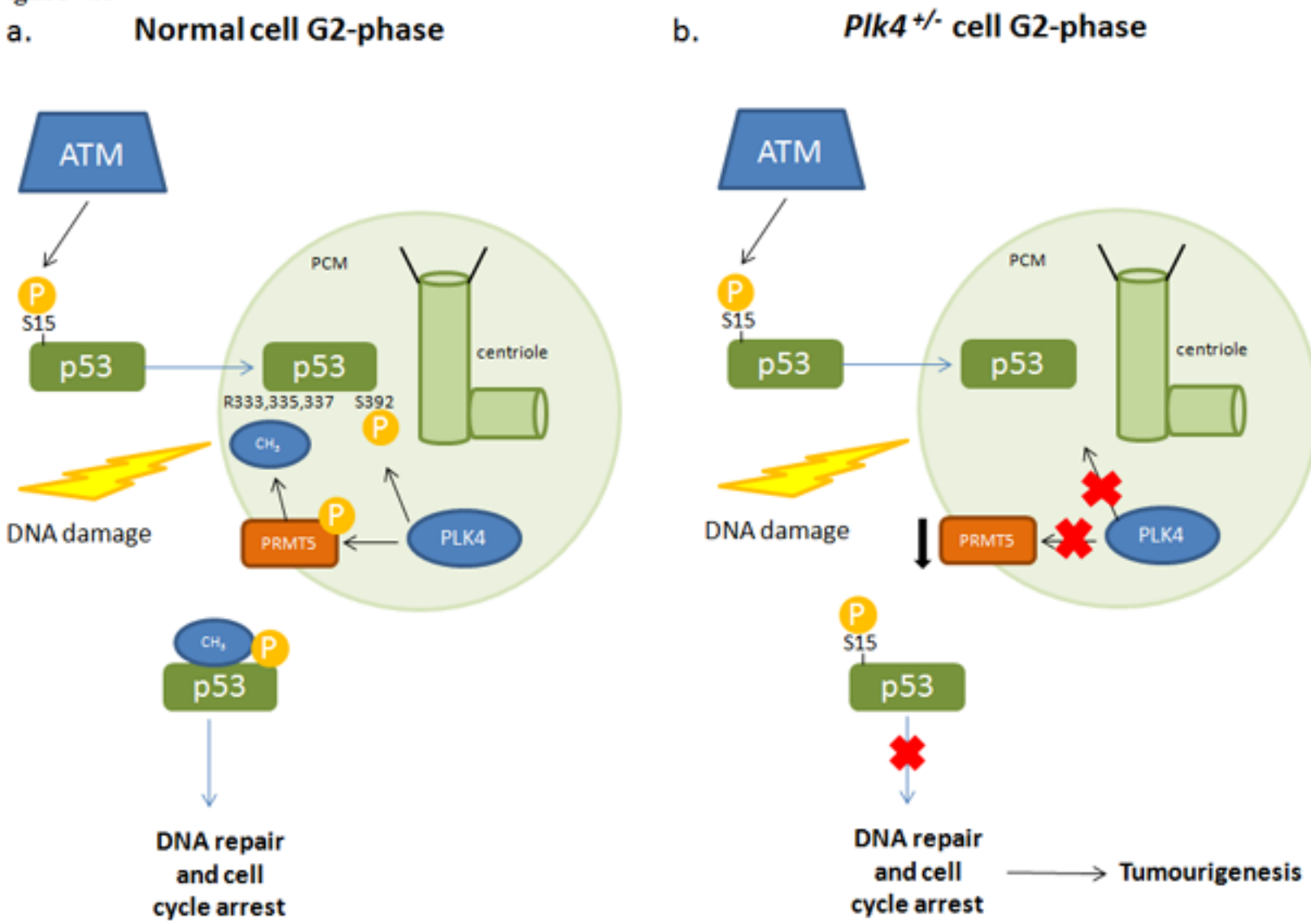
generally increases in response to DNA damage [53, 54]. In *Plk4*$^{+/+}$ MEFs, γH2AX levels peaked at 40mJ/UV dose, while in *Plk4*$^{+/-}$ MEFs, γH2AX was initially high and protein levels became depleted with increasing dosage of UV (Fig. 4.5a), suggesting that *Plk4*$^{+/-}$ MEFs have an impaired DNA damage repair pathway. We examined the proportion of MEFs post-UV treatment undergoing apoptosis using flow cytometry. As expected, after 8 hours post 40 mJ/UV exposure, *Plk4*$^{+/+}$ MEFs displayed an increase in the sub-G0 population compared to the untreated cells by 12%, suggestive of apoptosis (Fig. 4.5c), whereas, *Plk4*$^{+/-}$ MEFs showed no such increase in the sub-G0 population compared to the untreated counterparts, indicating that there may be an inadequate p53-mediated apoptotic response to DNA damage (Fig. 4.5c). These results are similar to those obtained by Michael Ko, where UV treated MEFs were examined *via* flow cytometry [55]. He observed that with 100 J/m^2 UV, *Plk4*$^{+/-}$ MEFs displayed half the number of apoptotic cells compared to the wild type cells [55]. PRMT5 methylation of p53 arginine residues 333, 335, 337 has been shown to impact p53 activity and localization during DNA damage [37]. With *Plk4*$^{+/-}$ MEFs displaying a decrease in PRMT5 protein and a general decrease in global PRMT5-mediated arginine methylation marks, this may result in a lack of the arginine dimethylation required for appropriate p53 activity during DNA damage. Furthermore, PLK4 has been also been shown to interact with and phosphorylate p53 at S392 [19, 55] and in liver resection studies in *Plk4*$^{+/-}$ mice, p53 failed to become activated, providing evidence that normal levels of Plk4 are also necessary for the activation of p53. Given the dynamic nature of the centrosome, where a variety of proteins either reside, or transitionally inhabit the pericentriolar material, it has been proposed that the centrosome may be a control centre for the initiation of the DNA

damage response [56]. Indeed, several DNA damage response proteins localize to the centrosome, including Chk2, BRCA1 and p53 [57-59]. During the normal progression of the cell cycle, at the G2/M transition, p53 localizes to the centrosomes in an ATM dependent manner *via* phosphorylation of S15 and monitors the integrity of the mitotic spindles [58]. With PLK4 levels peaking at the G2/M transition and PRMT5 interacting and co-localizing to the centrosomes, this now provides a spatial and temporal platform by which PLK4, PRMT5 and p53 could interact. We surmise that this tripartite grouping could be acting collaboratively to disseminate the appropriate response to DNA damage prior to the onset of mitosis (Fig. 4.6).

Here, we demonstrated that PLK4 is required for the normal function of PRMT5 through direct interaction, phosphorylation, and co-localization at the centrosomes as well as maintenance of normal PRMT5 levels. Interestingly, in data from our lab obtained from human hematological malignancies, PLK4 and PRMT5 followed a comparable pattern, where downregulation of PLK4 *via* promoter methylation, was also associated with lower levels of PRMT5 (Ward et al, Chapter 5 dissertion). Moreover, it may be that the opposite scenario is also true; over-expression of PLK4 has been detected in breast and colon cancers and is associated with poor prognosis [18, 24]; Independent studies have, likewise, determined that PRMT5 overexpression in these same tumour types is also associated with poor outcome [49, 60]. Together with our results, these studies suggest that PRMT5 expression may be proportionally tethered to PLK4 levels and this directly impacts normal PRMT5 activity. Some of the processes that are

Figure 4.6. Potential mode of action by which PLK4 haploinsufficency results in an altered DNA damage response. (a) During the normal cell cycle ATM (Ataxia telangiectesia mutated) phosphorylates p53 at S15 where it then shuttles to the centrosome, acting as an overseer of the mitotic spindle assembly, and promptly dephosphorylated [59]. If DNA damage occurs during this time, PLK4 together with PRMT5 act collaboratively to arginine methylate and phosphoryate the respective residues that will allow p53 to shuttle into the nucleus and activate the appropriate downstream targets. (b) In the context of *Plk4* haploinsufficiency, although p53 is still phosphorylated by ATM, it does not remain at the centrosome and phosphorylation of S15 is not lost. Together with low levels of Plk4 and Prmt5, p53 cannot have the post-translational modifications on the R333,335,337 and S392, required for it to exert its cellular protective function, allowing the cell through mitosis with damaged DNA and into the next cycle of cell division, which can ultimately result in tumourigenesis.

Figure 4.6


a.
Normal cell G2-phase
b.
Plk4 +/- cell G2-phase
ATM
P
S15
p53
PCM
centriole
R333,335,337
S392
CH3
DNA damage
PRMT5
PLK4
DNA repair
and cell
cycle arrest
Tumourigenesis

regulated by arginine methylation include transcriptional regulation by histone modifications, RNA processing, DNA repair, and signal transduction [30]. PRMT5's downstream targets include E2F-1, Hif1α, and NF-κβ. [49-51]. The deregulation of all of these proteins along with their associated pathways, confers pro-survival effects at the cellular level and have all been implicated in tumourigenesis. The regulatory role of PLK4 on PRMT5 reinforces the importance of PLK4's upstream role as an anti-tumour protein. Furthermore, in previous studies, the *PLKs* have been the recipients of epigenetic modifications displaying aberrant promoter methylation in a variety of tumour types including ovarian cancer, hepatocellular carcinoma, and hematological malignancies [22, 23, 61, 62]. However, this is the first study that shows a role reversal, in which PLK4 acts as an upstream regulator of epigenetic modifications. Much of the investigation surrounding Plk4 deregulation in malignancy has been associated with centrosomal aberrancies and the ensuing aneuploidy and genomic instability [19, 23]; here we demonstrate that deregulation of PLK4 is not limited to centrosome abnormalities, but in fact, has downstream impacts on critical epigenetic modifications and the DNA damage response.

Currently, the prevailing research suggests that PRMT5 may be an ideal therapeutic target in cancers since its expression or localization is deregulated in many malignancies [33, 63-65]. Although, much is known about its structure and function, other than as a JAK2 substrate, little is known about its upstream regulators. As a future initiative, in tumours where PRMT5 is de-regulated, perhaps examining the upstream PLK4 levels may provide more insight into PRMT5 deregulation and subsequent therapeutic design efforts can be more refined.

Materials and Methods

SDS-PAGE stains and Mass spectrometry

Silver-Stain Protein Staining Procedure for SDS-PAGE Gels

Following SDS-PAGE, the gel containing samples was treated in de-staining solution (25% ethanol, 10% glacial acetic acid) for one hour. The gel was then washed three times in 50% ethanol and subsequently, the gel was pre-treated in thiosulfate solution (1.3 mM sodium thiosulfate) and washed. Next, the gel was impregnated with silver nitrate solution (11.8 mM silver nitrate, $7.5x10^{-4}$% v/v formaldehyde). Developer solution (28.3 mM sodium carbonate, $5.0x10^{-4}$% v/v formaldehyde, $2.6x10^{-2}$ mM sodium thiosulfate) was then used to develop the solution for anywhere between one and ten minutes. After incubation, the gel was washed and the developing reaction was stopped with de-staining solution. Post de-stain, the gel was maintained in 1% glacial acetic acid. The visible bands were excised and rehydrated in siliconized microcentrifuge tubes (please note that all water used in this section was mass spectrometry grade). Water was then removed from all excised bands and replaced with 100 µL of de-stain solution (15 mM potassium ferrocyanide, 50 mM sodium thiosulfate). After de-staining, the bands were incubated in 50 mM ammonium bicarbonate and subsequently, charged with 200 µL of acetonitrile repeatedly until the pieces reached a white opacity. The samples were then dried using a Savant Speed Vac Plus SC110 A then re-hydrated in trypsin digestion buffer (50 mM ammonium bicarbonate, 13 ng/µL Promega modified trypsin) to cover each gel piece (≈20µL). The samples were incubated for 30 minutes on ice. If after 30 minutes the gel pieces had absorbed all of the digestion buffer, additional ammonium bicarbonate (50 mM stock) was added until the original volume was re-established. Each

microcentrifuge tube was then sealed using Parafilm, and incubated at 37^{o}C and 220rpm shaking for 30-60 minutes. After digestion, the remaining supernatant was incubated in protein extraction solution (60% acetonitrile, 1% formic acid) at 37^{o}C and 220rpm shaking for 45 minutes. The resulting digestion buffer and protein extractions were then concentrated by volume reduction to ≈10μL using the Savant Speed Vac Plus SC110 A at room temperature. In order to perform analysis by MALDI-TOF, 1μL of sample was spotted into each well of the MALDI plate, followed by 1μL of matrix solution (53 mM alpha cyano-4-hydroxy cinnaminic acid) which was then spotted on top of the sample. The samples were allowed to dry for several hours, followed by MALDI-TOF analysis. The resulting mass spectra were analyzed for any peptide peaks which had particularly strong signals and these were chosen for analysis by tandem-MS (MS-MS). Through the use the bioinformatic program Protein Prospector (The Regents of the University of California) as well as the SwissProt database it was possible to estimate the identity of PRMT5.

Cell culture

Human embryonic kidney 293T cells were cultured in Dulbecco's modified Eagle's media (DMEM) supplemented with 10% fetal bovine serum (FBS). Primary Mouse embryonic fibroblasts (MEFs) were harvested at embryonic day 12.5 under sterile conditions and in accordance with the University of Windsor's and the Canadian Animal Care guidelines. MEFs were cultured in DMEM supplemented with 20%FBS, 1% penicillin/streptomycin, and 0.5% gentamicin. MEFs used for experiments were between passages 2-5 unless otherwise stated. All cells were maintained at 37˚C with 5% CO_2. Transient transfections in HEK 293T cells were carried out using 10 μg of respective

purified plasmid DNA (Qiagen Maxiprep kit) and 1 mg/mL polyethylenimine (Sigma). MEFs were transfected using the Qiagen Effectine™ transfection kit. The transfections were carried out according to manufacturer's recommendations. In order to establish inducible cell lines, T-REx™ HeLa cells were used and the standard growth media composed of Minimum Essential Medium (MEM), 2 mM L-glutamine, and 10% FBS, was supplemented with 5 μg/mL blasticidin 24 hours post-transfection. Following this, inducible colonies were established by maintaining transfected cells in growth medium with 5 μg/mL blasticidin and 500 μg/mL Zeocin™. Once inducible cell lines were established, Flag-PLK4 expression was induced using tetracycline at a final concentration of 1 μg/mL.

To carry out the co-transfection of two plasmids, a total of 13 μg of combined plasmid DNA was introduced to cells along with polyethylenimine in serum-free media. After 4-6 hours post-transfection, serum-free media was replaced with complete media. Protein lysates were collected 24 hours post-transfection.

Western blot analysis

Protein analysis was carried out using Western blot analysis. Whole cell protein was extracted using a lysis buffer (50mM Tris-Cl, 150mM NaCl, 1% Triton-X, 0.1% SDS) supplemented with EDTA-free protease inhibitor cocktail (Roche). 40 μg of total protein was used for Western blot analysis. The primary antibodies used were obtained accordingly, anti-PLK2 and anti-PLK3, (Santa Cruz); anti-ɣ-H2AX, anti-PLK4, anti-PRMT5 and anti-GAPDH (Cell Signalling); anti-flag (Sigma-Aldrich); anti-symmetric arginine methylation (Novus biologicals). For secondary antibodies, anti-rabbit HRP

(Cell Signalling) and anti-mouse HRP (Sigma) were used. Bands were visualized by ECL using the SuperSignal West Femto maximum sensitivity detection kit (Thermo Scientific), blots were acquired on an Alpha Innotech Multimage™ Light Cabinet and densitometry analysis was carried out using ImageJ software Version 1.47.

Co-immunoprecipitations

M2-agarose with anti-flag antibody (Sigma-Aldrich) and G-sepharose (GE Healthcare and Life Sciences) beads for immunoprecipitations were prepared as follows: 15 µL of beads for each plate of cells lysed were washed 2x using wash buffer (50mM Tris-Cl pH 7.4, 150mM NaCl, and 0.025% Triton-X) and equilibrated with lysis buffer followed by the addition of the respective cell lysates. In some cases, stringent washes with 50 mM Tris-Cl pH 7.4, 500 mM NaCl, 0.1% SDS, and 0.1% Triton-X were required to reduce any non-specific interactions during the co-immunoprecipitation process. Immunocomplexes were flushed of protein contaminants by washing at least 5 to 7 times. For cell lysates obtained from inducible cell lines the lysates and beads were incubated at 4^{o}C for 2-3 hours with gentle rocking. Protein from transient transfections was incubated with beads at 4^{o}C for 16 hours.

p53 activity assay

An enzyme-linked immunosorbent assay (ELISA) was performed in order to determine p53 activity. The ELISA was carried out according to manufacturer's protocol (R&D Systems) using 30 ug of nuclear extracts from MEFs grown to 90% confluency.

UV treatments and Flow cytometry

MEFs were UV treated using a Spectrolinker™ XL-1000 UV Crosslinker (Spectronics Corporation) at specified doses ranging from 10-40 mJ/UV and cell lystaes collected directly after treatment. For flow cytometry analysis, UV treated cells were collected at 8 hours post UV exposure and fixed in ice cold 80% ethanol and incubated on ice for an hour. Cells were permeabilized with PBS+ 0.1% triton X-100 solution and stained with propidium iodide. Cell cycle analysis was performed on a Cytomic FC 500 flow cytometer (Beckman Coulter).

Plasmid Clones

Wild-type human PRMT5 was cloned into the pCMV Myc vector by PCR amplification of PRMT5 from pANT7_cGST vector (DNASU) using forward primer 5'-TAGAATTCGGATGGCGGCGATGGC-3' and reverse primer 5'-ATAAGATCTCTAGAGGCCAATGGTATATGAGC-3' which also introduced EcoRI and BglII restriction sites, respectively. Myc-tagged PRMT5 deletion expression vectors were created by mutagenesis of the full length Myc PRMT5 vector using QuikChange multi-site directed mutagenesis kit (Stratagene). The following primers were used for ΔMethyltransferase domain, ΔC-terminal, and ΔN-terminal Prmt5, respectively: 5'-CTG ATG GTG CTG CCA CTA GTG AAC GCT TCC C-3', 5'–CAG AAC CGT CCT TGA GAG ATC TCT CGA GGT-3', and 5' –GAG GCC CGA ATT CGG CCA CCT AAT GCC TAT-3'. For kinase assay experiments, recombinant GST-PRMT5 was cloned from pANT7_cGST vector (DNASU) using forward primer 5'-TAG GAT CCA TGG CGG

CGA TGG C-3' and reverse primer 5'-ATG AAT TCC TAG AGG CCA ATG GTA TAT GAG C-3' containing BamHI and EcoRI restriction sites, respectively.

Expression and Purification of GST-Fusion Protein

GST-PRMT5 was expressed in *E.coli* BL21 DE3 (Stratagene) cells by induction with 1 mM of IPTG (isopropyl-β-D-1-thiogalactopyranoside) at 30ºC for 4 hours. After collecting bacterial pellets, cells were lysed in a buffer containing 50 mM Tris-HCl pH 7.5, 100 mM NaCl, 0.1 mM EDTA, 0.1% Triton X-100, 2 mM DTT, and protease inhibitor pellet (Roche). After incubation on ice for 20 min, lysates were subjected to sonication. Following centrifugation at 12,000 rpm for 30 min, the supernatant was incubated with glutathione *S*-transferase beads on a nutator for 2h at 4ºC. The beads were washed once with five column volumes of cell lysis buffer and twice with wash buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM dithiothreitol). Glutathione-agarose bead-conjugated GST fusion proteins were washed with five column volumes of cleavage buffer (100 mM NaCl, 50 mM Tris pH 7.5, 2.5 mM $CaCl_2$, 1 mM dithiothreitol) in order to prepare the protein for GST cleavage by thrombin. After this washing step, 10 units of thrombin (Sigma Aldrich) were added per mg of fusion protein into one column volume of fresh cleavage buffer and left on a nutator overnight at 4°C. Stock solutions of thrombin (1 unit/µl) were prepared in sterile PBS and stored in a -80°C freezer. Soluble cleaved fusion protein was eluted from the filtrate by washing with elution buffer (50 mM Tris HCl pH 7.5, 100 mM NaCl, 10 mM glutathione). Following purification, eluted protein was cleansed of contaminating salts and proteins and concomitantly concentrated using Amicon Ultra centrifugal filters (Millipore). As a negative control for kinase assays, empty GST protein was also expressed by inducing at room temperature

overnight with the addition of 1 mM IPTG. GST protein was purified and concentrated as mentioned above.

Kinase Assays

Following lysis of cells 24 hours post-transfection, whole cell lysates were collected and incubated with 1 µg of anti-Flag antibody (Sigma) and 20 µl of calibrated Protein G Sepharose beads 4 Fast Flow (GE Healthcare Life Sciences) at 4°C overnight. Immuno-complexes were washed twice with wash buffer (50 mM Tris HCl pH 7.5, 150 mM NaCl, 0.1% Triton X-100), twice with wash buffer supplemented with 500 mM LiCl and lastly, once with kinase buffer (60 mM Hepes pH 7.5, 3 mM $MgCl_2$, 3 mM $MnCl_2$, 50 mM NaF, 25 mM dithiothreitol, 125 µM cold ATP and Roche protease inhibitor pellet). Bacterial-purified, thrombin cleaved GST-PRMT5 protein was incubated with purified immuno-complexes of Flag-PLK4 constructs. For each kinase reaction, 8 µg of GST-PRMT5 and 10 µCi of [γ-^{32}P] [Perkin Elmer] were supplemented to the kinase buffer. Reactions were incubated at 30°C for 30 min. Protein samples were resolved by sodium dodecyl sulphate polyacrylamide gel electrophoresis and subjected to autoradiography. Phosphorylated bands were visualized using Cyclone Plus Phosphor Imager (Perkin Elmer) and the Optiquant software Version 5.0.

Immunofluorescence

Mouse embryonic fibroblasts were grown on glass slides in 6-well plates to 80-90% confluency. Transfections in MEFs were carried out at 60% confluency and incubated for 24hours before collection. Cells on the slides were fixed in 3.7% pFa + 0.1% triton X-

100. The cells were then incubated with PRMT5 antibody at a 1:50 dilution for 16 hours at 4˚C. Anti-flag and anti-γ-tubulin antibodies (Sigma) staining was incubated for an hour at room temperature at a dilution of 1:100. Secondary antibodies Alexa Fluor® 568 (Invitrogen) and FITC (Vector laboratories) staining was also conducted at room temperature for 1 hour at a dilution of 1:500. Cells were then stained using Hoescht 33342 at a dilution of 1:10,000 for 2 minutes at room temperature. Images were resolved on a Zeiss Axioskop 2 mot plus using Northern Eclipse software.

Statistical anaylsis

Statisical analysis was conducted using Statsoft Statistica software Version 7. A One-way ANOVA was carried out to determine p-value. * represent a $p<0.01$. The results are the mean values obtained from three independent experiments. The error bars represent the standard deviation of the mean (SD) from three independent experiments.

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Chapter 5

The deregulated methylation of the *PLKs* in hematological malignancies as a potential biomarker

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Introduction

In a myriad of tumour types, hypermethylation of promoter associated CpG islands, histone modifications, and dynamic reassembly of chromatin architecture seem to be common mechanisms triggered to deregulate critical genes, including tumour suppressors [1]. B-cell lymphomas, along with other hematological malignancies including myelodysplastic syndromes and associated leukemias, for example, acute myelogenous leukemia (AML), are clinically and molecularly heterogeneous and harbour a variety of genetic and epigenetic abnormalities [2-4]. Gene expression profiling helps t identify novel biomarkers and potential prognostic indicators [5, 6]. Recently, the importance of epigenetics in the genesis of blood neoplasms has come to the forefront. The deregulation of gene expression is a hallmark of tumourigenesis and increasing evidence suggests that there are DNA methylation signatures that are highly associated with specific hematological malignancies [7-10]. All of the members of the Polo-like kinases (Plks), proteins with critical functions in cellular processes, often display aberrant methylation at their respective gene promoter regions, culminating in transcriptional modifications and abnormal gene expression in malignant cells [10-13]. More specifically, in the context of hematological disorders, Syed et al. (2006) previously reported aberrant cytosine methylation at the promoter region of *PLK2*, followed by

transcriptional silencing, as a common epigenetic phenomenon that may be driving the development and pathogenesis of B-cell neoplasms [14]. In addition, *PLK2* promoter hypermethylation has also been detected in almost 70% of AML and approximately 90% of MDS cases studied [10]. PLK2 is one of five known members of the highly conserved family of serine/threonine kinases, the Polo-like kinases. The PLKs are essential in specific cell cycle events which include centrosome maturation, spindle-pole formation, the DNA damage response, and cytokinesis [15, 16]. Abnormal expression of these proteins has been associated with tumourigenesis, for example, over-expression of PLK1 has been detected in head and neck squamous carcinoma [17], hepatocellular carcinoma [12, 13] and colorectal cancers [18]. More importantly, aberrant promoter methylation of *PLK1, PLK4*, and *PLK5* along with *PLK2,* have been implicated in a variety of tumour types such as ovarian cancer, hepatocellular carcinoma, and glioblastoma [10, 12, 13, 19, 20]. The epigenetic profiling of *PLK2* promoter methylation has revealed clinically valuable patterns in MDS, AML, and ovarian cancers [14, 19]. In ovarian cancer, an epigenetic mark on *PLK2* was associated with a greater risk of relapse for post-operative patients [19]. While, in MDS and AML cases, *PLK2* hypermethylation trended towards a correlation with longer overall survival [10]. These studies indicate that examining the promoter methylation of the *PLKs* can have clinical applications. Thus far, the PLK methylation studies in hematologic malignancies have been limited to *PLK2* and *PLK3* and have not been inclusive of the other Polo-like kinases. Given their importance in cell cycle regulation and their implication in carcinogenesis, we sought to expand on these studies and examine the methylation status of the remaining *PLKs* within the context of hematologic malignancies. The overarching aim of our study was to investigate the extent

of epigenetic deregulation of the polo-like kinase family in a variety of blood neoplasms such as myelodysplasia, leukemia, and lymphoma and understand whether these lesions play a role in the development and progression of blood neoplasm. Here we show that the *PLKs* are differentially methylated between normal and neoplastic samples and these changes in methylotype are detectable in standard bone marrow aspirates which are routinely collected as part of the diagnostic procedures for hematological malignancies In addition, we demonstrate the impact of the physiologic microenvironment and the effect of common epigenetic therapies on PLK expression using a variety of patient-derived MDS and lymphoma cell lines.

Results/Discussion

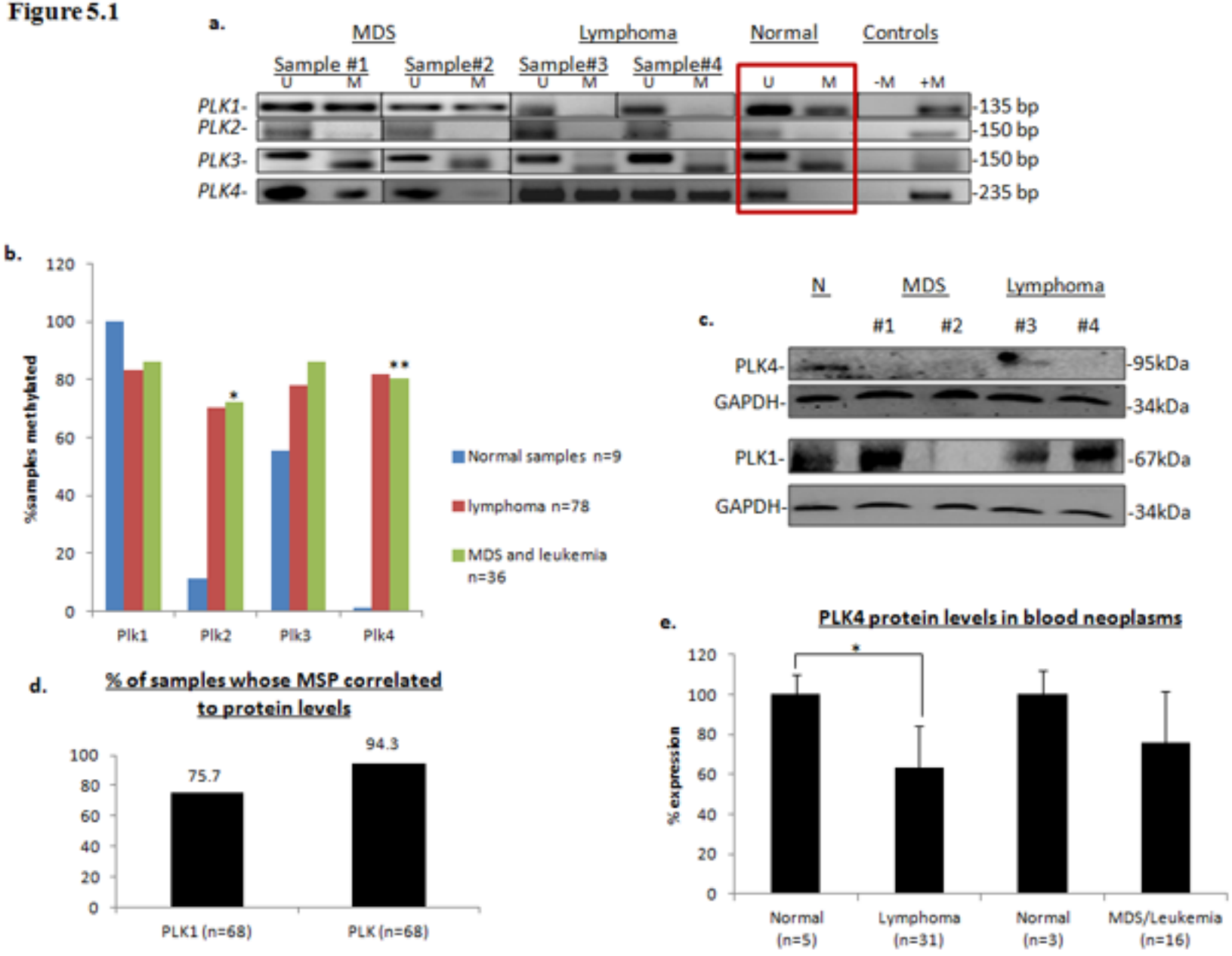
PLK promoter methylation in hematological malignancies

Epigenetics is defined as the stable modifications of gene expression at the DNA and histone level without modification to the primary sequence of a gene. There are two distinct mechanisms whose end result greatly impact gene expression: DNA methylation and histone modifications. At the DNA level, dense regions of CG dinucleotides upstream of gene promoters, called CpG islands, are targeted by DNA methyltransferase (DNMTs) enzymes for the addition of methyl groups to the 5' position of cytosines, leading to DNA methylation. Using *PLK*-specific primers we embarked on the screening of the methylation status of the *PLKs* in human bone marrow aspirates using methylation specific PCR (MSP). In normal samples, of those bone marrow aspirates which did not exhibit any detectable pathologies, the methylation pattern for *PLK1*promoter region was methylated, while the *PLK2* and *PLK4* CpG islands remained largely unmethylated. The

methylation status for *PLK3* was undetectable in approximately 50% of these samples (Figure 5.1a,b). Interestingly, when examining samples from patients with hematological malignancies, this pattern was reversed. For *PLK1,* approximately 15% of malignancies displayed a loss of *PLK1* promoter methylation (Fig. 5.1a,b). In our previous studies, we determined that loss of *PLK1* promoter methylation is associated with an increase in protein levels [13]. Recently, Plk1 over-expression was observed in both acute myeloid leukemia (AML) and chronic myelogenous leukemia (CML) [21, 22]. Importantly, increased PLK1 protein is a poor prognostic indicator in several malignancies [23]). It was therefore of interest to determine whether individual Plk promoter methylation status was correlated with protein levels in a spectrum of blood neoplasms. We thus examined individual PLK protein levels in whole cell lysates from bone marrow aspirates. In the case of Plk1, in the majority (80%) of the cases, where there was a loss of promoter methylation there was a corresponding increase at the protein level (Fig. 5.1 c,d). We saw an inverse pattern for Plk4 where 82.0% and 80.5% of lymphoma and MDS/Leukemia samples displayed hypermethylation at the *PLK4* promoter region respectively (Fig. 5.1 a,b). *PLK4* hypermethylation was consistently associated with a decrease in its protein levels [13, 24]. On average, in lymphoproliferative neoplasms, there was a significant decrease in PLK4 protein by 35% (Fig. 5.1e). A similar trend was observed in MDS/Leukemia samples (Fig. 5.1e). Strikingly, in more than 90% of the cases, *PLK4* promoter methylation status correlated to protein expression (Fig. 5.1d), suggesting that in blood neoplasms aberrant promoter methylation of Plk4 is an important method of regulating

Figure 5.1 Promoter methylation analysis of the *PLKs* in hematological malignancies. (a) *PLK* promoter methylation profiles in MDS and lymphoma samples as determined by MSP. This is representative data. U=unmethylated, M=methylated. Genomic, fully methylated HeLa cell DNA was used as a positive control. (b) Graphically representation of the data obtained from all MSPs conducted in a many types of blood neoplasms. * 4 samples did not show amplication for *PLK2* with either methylated or unmenthylated primers. **2 samples did not show amplification for *PLK4* with either methylated or unmethylated primers. (c) PLK4 and PLK1 protein analysis from whole cell lysates prepared from bone marrow aspirates. This is representative data. (d) The correlation between methylation status and the protein expression of PLK1 and PLK4 in all samples analysed. (e) Graphical representation of the densitometry quantification of PLK4 protein levels among those samples that displayed down-regulation of PLK4. * represents statistical significance $p<0.05$. Error bars represent the standard deviation from the raw values obtained from densitometry analysis.

Figure 5.1


a.
MDS
Lymphoma
Normal
Controls
Sample #1
Sample#2
Sample#3
Sample#4
U
M
-M
+M
PLK1-
PLK2-
PLK3-
PLK4-
-135 bp
-150 bp
-150 bp
-235 bp
b.
%samples methylated
120
100
80
60
40
20
0
Plk1
Plk2
Plk3
Plk4
*
**
Normal samples n=9
lymphoma n=78
MDS and leukemia n=36
c.
N
MDS
Lymphoma
#1
#2
#3
#4
PLK4-
GAPDH-
PLK1-
GAPDH-
-95kDa
-34kDa
-67kDa
-34kDa
d.
% of samples whose MSP correlated to protein levels
100
80
60
40
20
0
75.7
94.3
PLK1 (n=68)
PLK (n=68)
e.
PLK4 protein levels in blood neoplasms
% expression
120
100
80
60
40
20
0
*
Normal (n=5)
Lymphoma (n=31)
Normal (n=3)
MDS/Leukemia (n=16)

its expression. Aberrant levels of Plk4 may have profound effects on a cell as PLK4 is an important mitotic regulator with reduced levels of PLK4 directly impacting centrosome maturation resulting in centrosome abnormalities, aneuploidy and thus contributing to genomic instability in dividing cells [25, 26]. Consistent with our observations, PLK4 down-regulation along with centrosome amplification was previously described in multiple myeloma [27] and in plasma cell neoplasms, centrosome amplification was correlated with a poor prognosis and shortened survival [28]. Lower Plk4 levels may also have an effect on the cellular response to cellular stress and DNA damage as reduced PLK4 levels are associated with decreased p53 activity, a factor which likely contributes towards the progression of carcinogenesis [24, 25].

For *PLK2* we found that approximately 70% of lymphoma samples were positive for promoter hypermethylation, similar to the findings of Syed et al (2006) for primary B-cell neoplasms and cell lines. We obtained a slightly lower proportion of *PLK2* hypermethylation in our combined samples of MDS and leukemia with 72.2% methylation detected, compared to 88.4% methylation in MDS samples as previously reported [10, 11]. This difference is attributed to the heterogeneity of MDS, but more likely, due to the MDS subtype examined. The vast majority of the samples examined by Benetatos *et al.* (2011) were MDS classified as refractory cytopenia with multi-lineage dysplasia (RCMD) or refractory anemia with excess blasts-1 or -2 (RAEB-1, or RAEB-2) with intermediate to very high risk, suggestive of a more aggressive MDS subtypes. The available clinical data from our samples classified the majority of our cases as low risk (data not shown). In MDS, abnormal DNA methylation present in low-risk cases only increases with disease aggressiveness or progression [10]. Likewise, in ovarian

carcinoma cells, *PLK2* hypermethylation proportionally increased with increased drug resistance to paclitaxel and carboplatin [19].

Previous reports indicated that in B-cell malignancies, the *PLK3* promoter region remained unmethylated [11]. Interestingly, we observed changes in *PLK3* promoter methylation and in contrast to Syed et al. (2006) we detected *PLK3* promoter methylation in 55% of normal samples. This proportion increased in both lymphoma and MDS/Leukemia samples, where 78.2% and 86.1% were positive for methylation (Fig. 5.1a,b). This is the first report of aberrant *PLK3* promoter methylation in hematological malignancies. Our study differed from Syed et al. (2006) in that they conducted their analysis primarily *in vitro* with several B-cell neoplasm-derived cell lines and a limited number of clinical samples consisting of primarily of mantle cell lymphomas [11]. Our DNA samples were obtained directly from 78 bone marrow aspirates which included several lymphoma subtypes: B-cell, non-Hodgkins, Hodgkins, and follicular lymphomas.

PLK promoter methylation in familial MDS

We also examined the *PLK* promoter methylation profile in a family that had all been diagnosed with MDS. This small group consisted of three females: twin sisters with one of them having a daughter. We obtained peripheral blood samples and the bone marrow biopsy for the mother, along with bone marrow biopsies for the other family members. In the blood sample from the mother, *PLK1* was demethylated and the *PLK4* promoter was hypermethylated (Fig. 5.2a). Bone marrow biopsies for all three family members revealed the same promoter methylation profile in all three individuals: a

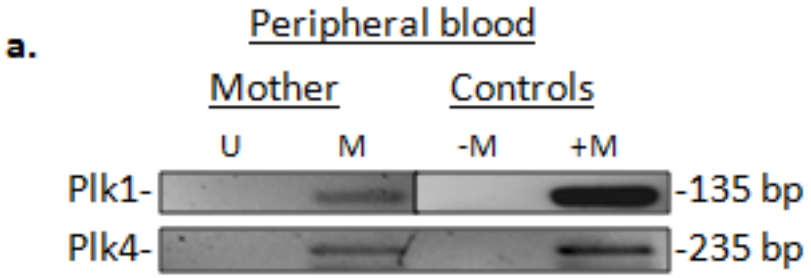
hypomethylated *PLK1* promoter and an increase in *PLK4* promoter methylation (Fig. 5.2b), the same methylation profile as the initial peripheral blood sample.

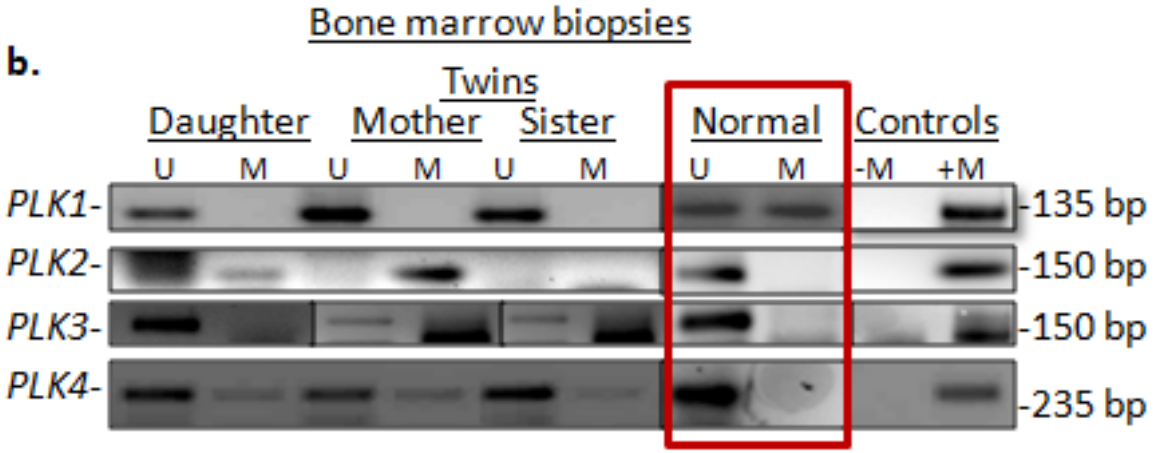
PLK levels with respect to current protein markers of hematological malignancies

Presently, a number of biomarkers have emerged as important indicators of prognosis and pathogenicity in hematological malignancies. Examining the extent to which *PLK4* hypermethylation and expression relates to these biomarkers may provide some insight into its translational value. *Janus kinase 2* (*JAK2*) overexpression dovetails disease entities and is deregulated in both myeloid and lymphoid neoplasias [29-32]. JAK2 is a tyrosine kinase integrated into signalling pathways that have a wide-breadth of cellular effects which include the proliferation, survival and normal functioning of hematopoietic cells [33]. JAK2 undergoes several activating mutations, including a gain of copy number at chromosomes 9p.24 [31], an oncogenic point mutation at residue 617 and several mutations within exon 12 at residues 537-543, all of which generate a constitutively active JAK2 [32]. We examined JAK2 protein levels, and overall, JAK2 was elevated in malignant versus normal samples in lymphoproliferative disorders (Fig. 5.3a, b). However, we noticed there was a great deal of variation in expression levels, with some samples displaying JAK2 overexpression and others displaying reduced JAK2 levels (Fig. 5.3b). We divided the polarized levels of JAK2 into elevated or down-regulated expression and paired them with corresponding PLK4 levels. PLK4 and JAK2 appear to have an inverse relationship: when JAK2 was overexpressed, PLK4 levels were significantly reduced ($p<0.05$), and inversely, when JAK2 levels were at their lowest, PLK4 levels were comparable to- or exceeded the levels found in normal samples (Fig. 5.3a,c). This is suggestive of a regulatory relationship between these two proteins. We

Figure 5.2 *PLK* promoter analyses of familial MDS cases. (a) Individual *PLK* promoter methylation status as determined by MSP for peripheral blood. U=unmethylated, M=methylated. (b) Individual *PLK* promoter methylation profile as determined by MSP of genomic DNA extracted from bone marrow biopsies. The comparative normal samples are highlighted by a red box. U=unmethylated, M=methylated. Genomic, fully methylated HeLa cell DNA was used as a positive control.

Figure 5.2


a.
Peripheral blood
Mother
Controls
U
M
-M
+M
Plk1-
-135 bp
Plk4-
-235 bp

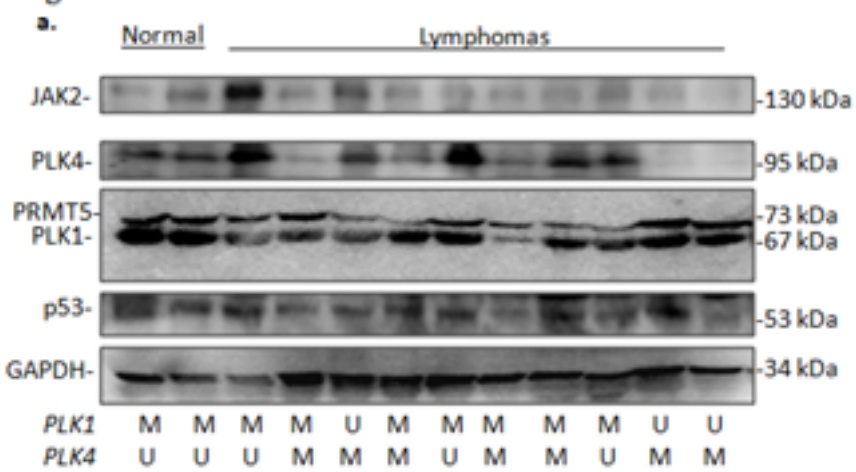

b.
Bone marrow biopsies
Twins
Daughter
Mother
Sister
Normal
Controls
U
M
-M
+M
PLK1-
-135 bp
PLK2-
-150 bp
PLK3-
-150 bp
PLK4-
-235 bp

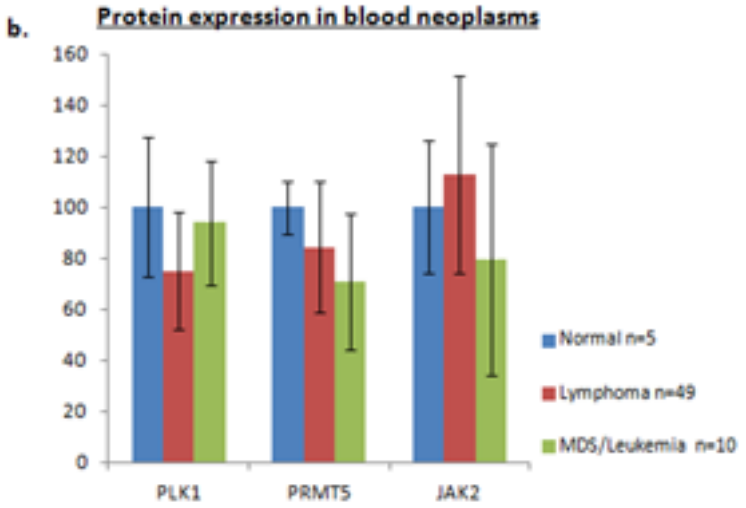
also examined a validated substrate of JAK2, protein arginine methyltransferase 5 (PRMT5). PRMT5 is often down-regulated in hematological malignancies as a result of constitutively active JAK2 [34]. On average, there was a trend of PRMT5 being down-regulated by almost 20% in neoplastic samples in comparison to the normal (Fig. 5.3b). JAK2 down-regulation of PRMT5 in hematological malignancies has been linked to the promotion of cellular proliferation and colony formation *in vitro* [34]. Recent work from our lab has shown that PRMT5 is also a substrate of PLK4 (Ward et al, Chapter 4 dissertation) and perhaps JAK2 and PLK4 are antagonistic to each other in the context of blood neoplasias.

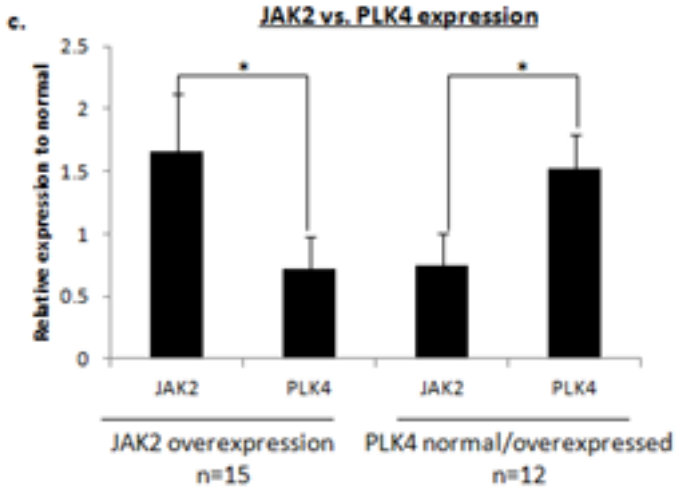
We also examined the levels of myeloperoxidase (MPO) and ten-eleven-translocation 2 (TET2) due to their association with myeloproliferative neoplasms [29, 30, 35]. TET2, a homologue of the TET family of proteins, along with TET1 and TET3 are responsible for converting 5-methylcytosine to 5-hydroxymethylcytosine thus resulting in DNA demethylation[36]. TET2 frequently undergoes inactivating mutations or chromosomal deletions at location 4q.24 in myeloid neoplasias [37] and is down-regulated in approximately 10-15% of MDS and acute myelogenous leukemias (AML) [29]. Interestingly, we found that several MDS and leukemia samples had lowered or depleted levels of TET2 (Fig. 5.4a,b) This was also evident in the familial cases of MDS (Fig. 5.4b). The lowest levels of PLK4 were associated with the most depleted levels of TET2 (Fig. 5.4a-c). The mutations and subsequent reduction of TET2 has been linked to an increase in global methylation and genomic methylation errors in regions of genes involved in hematopoietic differentiation and cell cycling, suggesting that deregulation of methylation marks are not random but are specifically targeted [37]. Perhaps , *PLK4* may

Figure 5.3 Expression of the PLKs, JAK2, and PRMT5 in blood neoplasms. (a) Representative Western blot analysis for protein levels from several lymphoproliferative malignancies. Normal lysates were obtained from bone marrow aspirates with no detectable pathology. U=unmethylated, M=methylated representing the methylation state of respective *PLK* for that sample. (b) Protein levels for PLK1, JAK2, and PRMT5. Densitometry was conducted on a total of 64 samples for each of the proteins noted. GAPDH was used as an internal loading control and all samples were normalized to the ratio of the normal protein value/GAPDH. Error bars represent the standard deviation from raw densitometry values. (c) A comparative analysis of JAK2 expresion with respect to corresponding PLK4 levels. *represents the statistical significance with $p<0.05$. Error bars represent the standard deviation for the raw values generated from densitometry analysis.

Figure 5.3


a.
Normal
Lymphomas
JAK2-
PLK4-
PRMT5-
PLK1-
p53-
GAPDH-
130 kDa
95 kDa
73 kDa
67 kDa
53 kDa
34 kDa
PLK1 M M M M U M M M M M U U
PLK4 U U U M M M U M M U M M


b.
Protein expression in blood neoplasms
160
140
120
100
80
60
40
20
0
PLK1
PRMT5
JAK2
Normal n=5
Lymphoma n=49
MDS/Leukemia n=10


c.
JAK2 vs. PLK4 expression
Relative expression to normal
2.5
2
1.5
1
0.5
0
*
*
JAK2
PLK4
JAK2
PLK4
JAK2 overexpression
n=15
PLK4 normal/overexpressed
n=12

be a recipient of these methylation errors in conjunction with increases in methylation at the genome level. To examine this, we performed an ELISA-based global methylation assay and determined that all malignant samples assayed displayed at least a two-fold increase in global methylation compared to the normal (Fig. 5.4c). We previously demonstrated that initial *Plk4* CpG island hypermethylation is also associated with an increase in global methylation in young *Plk* $4^{+/-}$ mice [13]. This may be indicative of an arms- length relationship between TET2 and PLK4.

MPO is an enzyme specific to the myeloid lineage of cells and is often dergulataed in MDS and acute myelogenous leukemia (AML) and used as a diagnostic marker [38]. It has been associated with disease-free survival and post-transplant prognosis in AML [38]. However, we found no distinct pattern between the normal and the diseased state (Fig. 5.4a,b). We did have a limited number of MDS and AML samples, perhaps with a larger samples size, and a greater variability in disease stages, a pattern may have emerged.

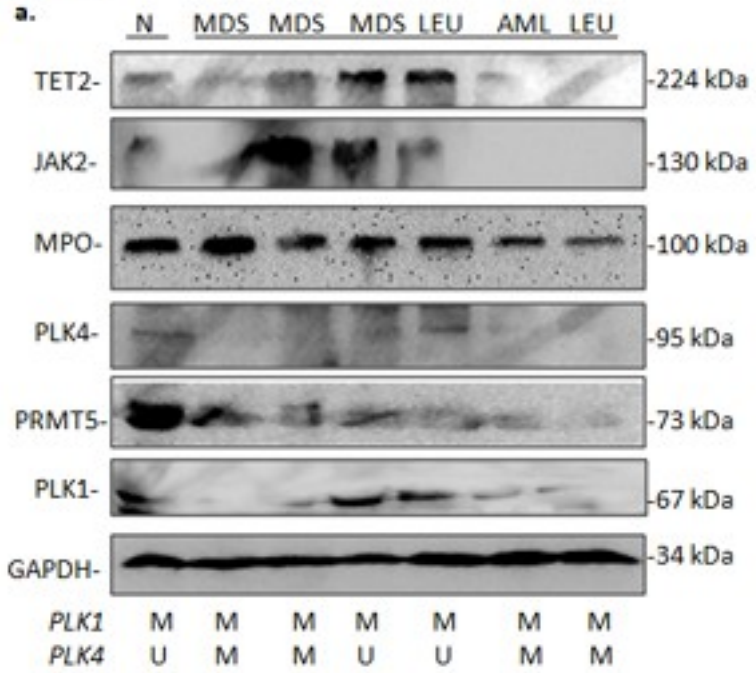
Our results show that PLK4 levels may be tethered to important biomarkers of lymphoid and myeloid malignancies, JAK2 and TET2 respectively. However, to further determine the impactof this, statistical analysis using correlation co-efficients may determine the extent of correlation between PLK4 and JAK2 and TET2 protein levels. This suggests that PLK4 expression may be impacted in the progression of hematological neoplasms.

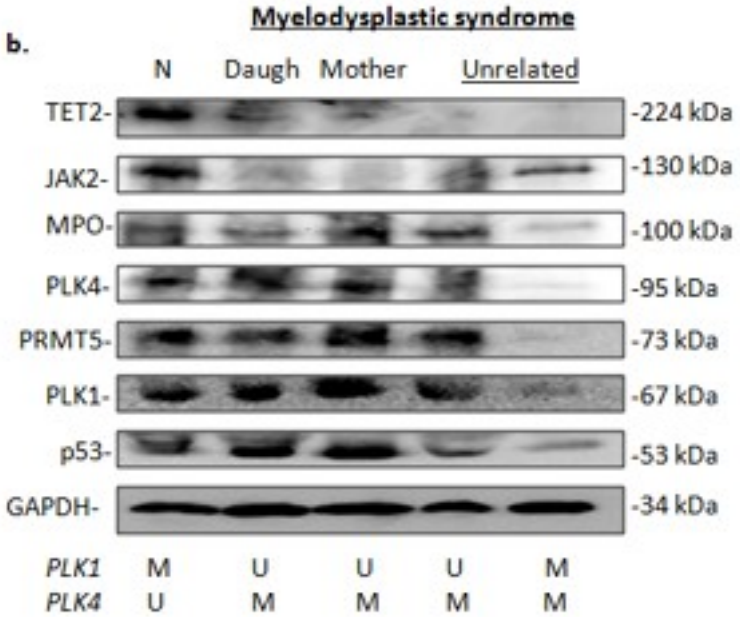
Hypermethylation of the PLK promoter regions in cell-lines derived from hematological malignancies

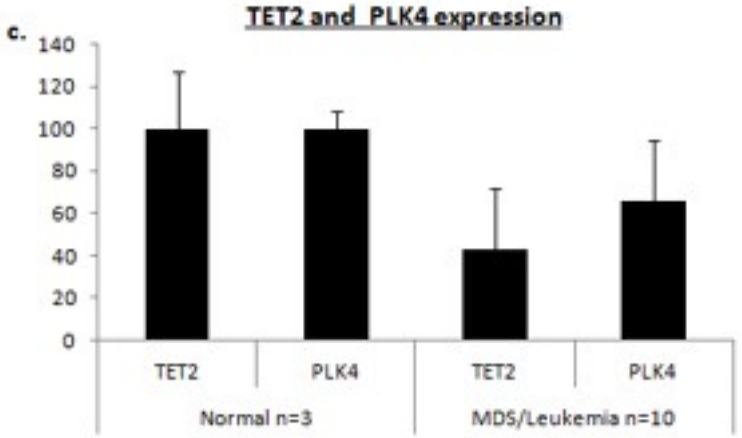
To further understand the dysregulation of Polo-like kinases in hematological malignancies, we considered the significance of the low oxygen tension innate to the bone marrow (BM) microenvironment in both normal and diseased conditions. While the

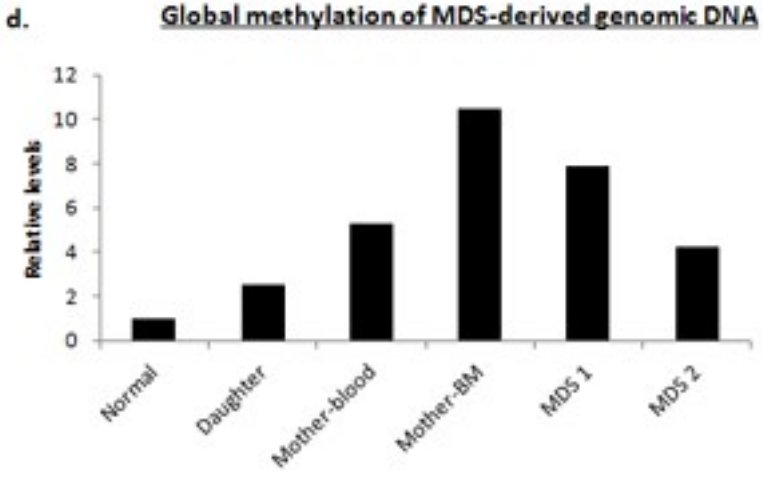
Figure 5.4 Analysis of protein levels from MDS/Leukemia-derived bone marrow aspirates. (a) Representative Western blot analysis for key markers of myeloid disorders U=unmethylated, M=methylated representing the methylation state of respective *PLK* for that sample (b) Western blot analysis of familial MDS samples compared to unrelated MDS samples. U=unmethylated, M=methylated representing the methylation state of respective *PLK* for that sample (c) Quantitative levels of TET2 protein compared to PLK4 protein expression in normal and MDS/leukemia samples. Error bars represent standard deviation. (d) An ELISA-based global methylation assay of genomic DNA from familial MDS samples compared to unrelated MDS samples. Relative levels are represented with normal global methylation given the arbitrary value of 1.

Figure 5.4


a.
N MDS MDS MDS LEU AML LEU
TET2-
JAK2-
MPO-
PLK4-
PRMT5-
PLK1-
GAPDH-
224 kDa
130 kDa
100 kDa
95 kDa
73 kDa
67 kDa
34 kDa
PLK1 M M M M M M M
PLK4 U M M U U M M


b.
Myelodysplastic syndrome
N Daugh Mother Unrelated
TET2-
JAK2-
MPO-
PLK4-
PRMT5-
PLK1-
p53-
GAPDH-
224 kDa
130 kDa
100 kDa
95 kDa
73 kDa
67 kDa
53 kDa
34 kDa
PLK1 M U U U M
PLK4 U M M M M


c.
TET2 and PLK4 expression
140
120
100
80
60
40
20
0
TET2
PLK4
TET2
PLK4
Normal n=3
MDS/Leukemia n=10


d.
Global methylation of MDS-derived genomic DNA
Relative levels
12
10
8
6
4
2
0
Normal
Daughter
Mother-blood
Mother-BM
MDS 1
MDS 2

BM hypoxia is important for normal marrow hematopoiesis and stem cell maintenance, the unique milieu of the BM provides a reserve rich with growth factors and cytokines, augmenting proliferation, survival, and malignancy of blood-derived neoplastic cells [39]. Although many have described the methylation-dependent silencing of genes involved in cell cycle regulation, DNA damage repair, or apoptosis using *in vitro* study models, very few have examined the methylation status under biologically relevant conditions. A number of studies have revealed the microenvironment as a causative factor for inducing alterations to the epigenetic landscape, consequently, modulating the expression of gene products. Considering that previous work from our lab has demonstrated the ability of environmental stimuli to mediate epigenetic marks of the *PLKs* in a cell type-dependent manner [24], we sought to determine whether the hypoxic nature of the BM could confer similar aberrant epigenetic features in malignant cells *via* changes in *PLK* promoter methylation.

Initially, we screened the methylation status of nine patient-derived MDS/leukemia and lymphoma cell lines under standard cell culture conditions *via* methylation-specific PCR analysis (Fig. 5.5a) (Supplementary Table 5.1). Remarkably, examination of patient-derived leukemia and lymphoma cell lines *in vitro* largely identified the *PLKs* as genes subject to methylation-dependent deregulation, seemingly a prevalent epigenetic signature in various subtypes of blood neoplasia. All MDS and lymphoma cell lines displayed hypermethylation of the *PLK2* promoter-associated CpG islands in accordance with the previous findings by Benetatos *et al.* (2011)[10]. However, uncharacteristic of most malignant cells, *PLK1* promoter methylation was detectable in these *in vitro* models of neoplasia. Moreover, 7/9 and 5/9 of neoplastic cell lines had

Figure 5.5 Methylation profiles of MDS/leukemia- and lymphoma-derived cell lines at the *PLK* promoter regions pre- and post-hypoxia. (a) MDS and lymphoma cell lines were screened for basal levels of methylation at the *PLK* promoter CpG islands under standard conditions *via* methylation-specific PCR (MSP) analysis. U = unmethylated, M = methylated. Fully methylated HeLa DNA was used as a positive control. (b) Blood neoplastic cells were exposed to hypoxia to determine whether *PLK* promoter regions were susceptible to changes in methylation status as a result of the low oxygen environment. The effect of hypoxia on mRNA levels was determined by real-time PCR to examine HIF1α transcript levels post-hypoxic treatment. (c) Profiling of *PLK* methylation marks was performed *via* MSP to determine whether hypoxia induced hypermethylation in promoter regions in cell lines derived from blood neoplasms. Con = normoxic conditions Hyp = hypoxic conditions; U = unmethylated, M = methylated. Fully methylated HeLa DNA was used as a positive control.

Figure 5.5

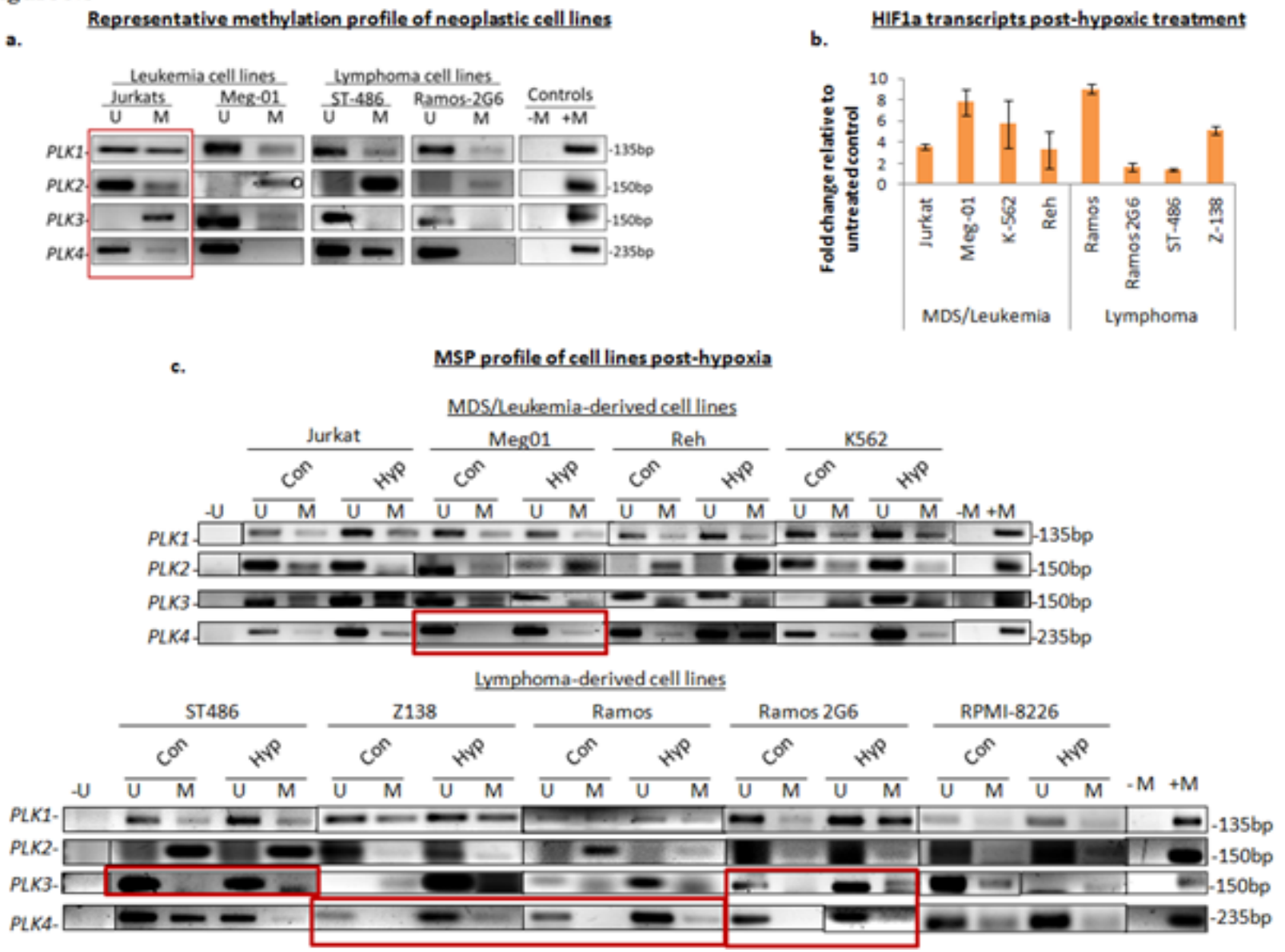
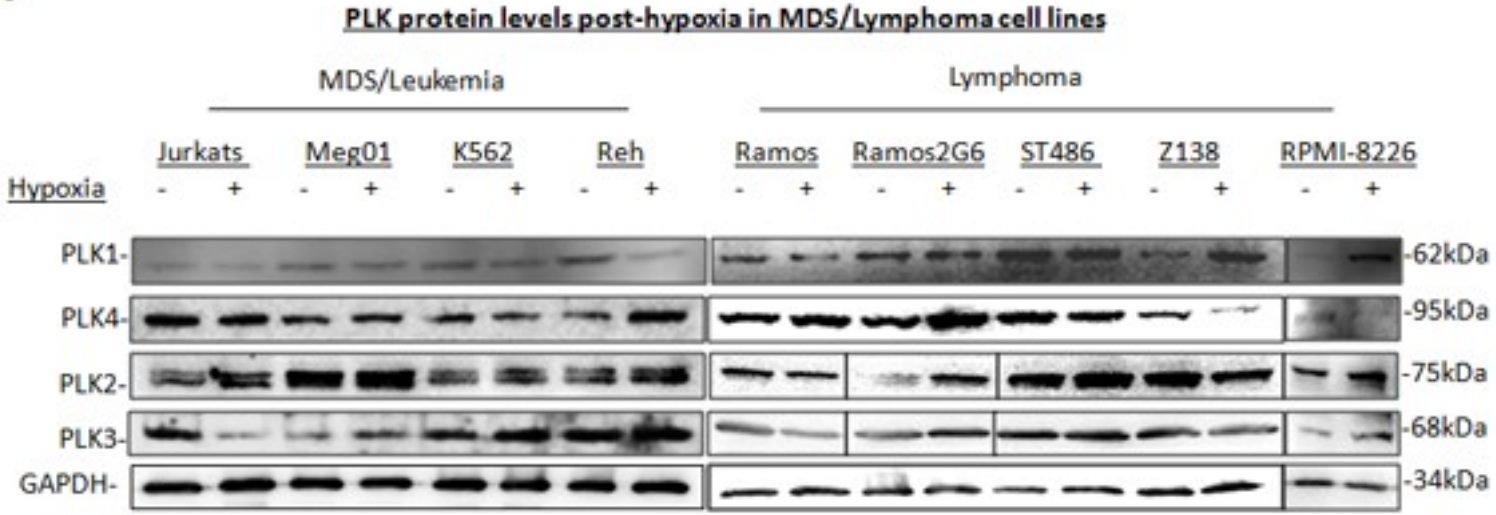

a.
Representative methylation profile of neoplastic cell lines
Leukemia cell lines
Lymphoma cell lines
Jurkats
Meg-01
ST-486
Ramos-2G6
Controls
U M U M U M U M -M +M
PLK1
PLK2
PLK3
PLK4
-135bp
-150bp
-150bp
-235bp
b.
HIF1a transcripts post-hypoxic treatment
Fold change relative to untreated control
10
8
6
4
2
0
Jurkat
Meg-01
K-562
Reh
Ramos
Ramos 2G6
ST-486
Z-138
MDS/Leukemia
Lymphoma
c.
MSP profile of cell lines post-hypoxia
MDS/Leukemia-derived cell lines
Jurkat
Meg01
Reh
K562
Con
Hyp
-U U M U M U M U M U M U M U M U M -M +M
PLK1
PLK2
PLK3
PLK4
-135bp
-150bp
-150bp
-235bp
Lymphoma-derived cell lines
ST486
Z138
Ramos
Ramos 2G6
RPMI-8226
Con
Hyp
-U U M U M U M U M U M U M U M U M U M U M -M +M
PLK1-
PLK2-
PLK3-
PLK4-
-135bp
-150bp
-150bp
-235bp

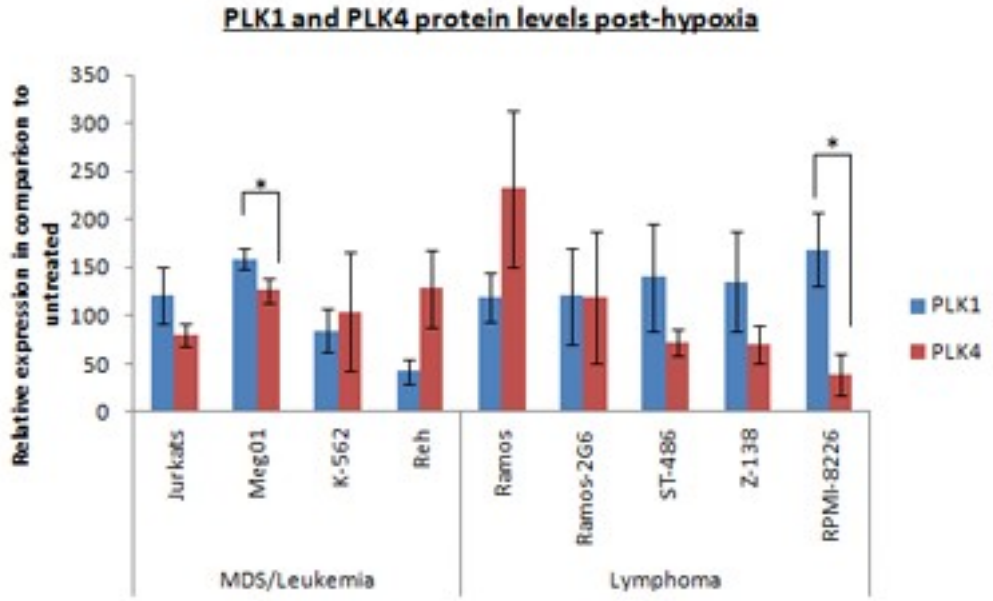
Figure 5.6 Hypoxia-induced changes in PLK protein levels. (a) To ascertain whether the hypoxic conditions of the BM affect individual PLK protein levels, proteins extracted from hypoxia-treated cells were analyzed for changes in PLK protein levels. Western blot analysis revealed changes in PLK expression. GAPDH is used as a loading control. (b) Changes in PLK1 and PLK4 expression were quantified using densitometry. Error bars represent the standard deviation of three independent experiments. * represents statistical significance with $p<0.05$.

Figure 5.6

a.


PLK protein levels post-hypoxia in MDS/Lymphoma cell lines
MDS/Leukemia
Lymphoma
Jurkats
Meg01
K562
Reh
Ramos
Ramos2G6
ST486
Z138
RPMI-8226
Hypoxia
- + - + - + - + - + - + - + - + - +
PLK1-
PLK4-
PLK2-
PLK3-
GAPDH-
-62kDa
-95kDa
-75kDa
-68kDa
-34kDa

b.


PLK1 and PLK4 protein levels post-hypoxia
Relative expression in comparison to untreated
350
300
250
200
150
100
50
0
*
*
Jurkats
Meg01
K-562
Reh
Ramos
Ramos-2G6
ST-486
Z-138
RPMI-8226
MDS/Leukemia
Lymphoma
PLK1
PLK4

detectable *PLK3* and *PLK4* methylation, respectively. To determine whether low oxygen tension can target *PLK* promoter regions, cells were grown in a hypoxia chamber flooded with 5% O_2, the biologically relevant oxygen level of the bone marrow, for a period of 48 hours. Post-hypoxia treatment, HIF1a transcript levels were examined to confirm cellular response to hypoxic conditions (Fig. 5.5b). Subsequently, the methylation statuses of *PLK* promoter regions were re-evaluated using MSP analysis. Interestingly, all promoter areas of *PLK3* and *PLK4* that were unmethylated prior to hypoxia exposure acquired abnormal methylation post-treatment (Fig. 5.5c). Out of all the samples that gained promoter methylation post-hypoxia, Meg-01 and Z-138 lymphoma cells were the only ones whose gain of *PLK4* methylation translated into decreased PLK4 protein levels. Intriguingly, a strong inverse relationship between PLK1 and PLK4 protein levels was seen in a number of cell lines post-hypoxia (Fig. 5.6a, b). This antagonistic correlation between the notorious oncogene and tumor suppressor has been previously reported, as aforementioned [13].

While it is uncertain whether the collective epigenetic lesions of the Polo-like kinases is an early event associated with the development and progression of these disorders or possibly, a downstream result of transformation which further propagates carcinogenesis, insight into *PLK* methylotype profile can produce valuable information to effectively monitor and treat cancer patients. This data, in combination with our *in vivo* bone marrow study, provides evidence that the *PLKs* are epigenetically deregulated and consequently, have perturbed expression levels in hematological malignancies. In addition, we also show that upon changes to oxygen tension, the *PLK* promoter associated CpG islands become sensitive to DNA methylation. More than likely, the *PLK*

promoter hypermethylation associated with hypoxia is a progressive process during the transformation and pathogenesis of cancer cells. Our experimental results emphasize the importance of performing studies under physiologically relevant conditions and suggest that hypoxia, a micro-environmental stressor, may prompt changes to *PLK* promoter methylation profile.

In previous work, we have demonstrated that the *PLKs* become down-regulated with exposure to oxidative stress, whether in the form of hypoxia or reactive oxygen species [24]. In our *in vitro* studies, short term exposure to these environmental stressors was sufficient to induce promoter methylation in the *PLKs*. The life history of a subset of B-cell lymphoma patients, with a mean age of 64.8 years, was collected. We examined the smoking history and oxygen saturation data of these individuals and noticed that 47% of the patients had a history of heavy smoking or were current smokers (Supplementary Table 5.2). In addition, there were several non-smokers that also exhibited low oxygen saturation rates (<95%). Smoking results in lowered oxygen saturation rates [40] and decreased oxygen saturation has been associated with physiologic hypoxic conditions. The metabolites generated from smoking can also create reactive oxygen species [41]. Interestingly, a large-scale study with a cohort of over one million women demonstrated chronic cigarette consumption to significantly augment the risk of Hodgkin's lymphoma, mature T-cell malignancies, and myelodysplastic diseases [42]. Perhaps, reduced oxygen levels and ROS in these individuals mediate aberrant epigenetic modifications of the *PLKs*.

*Effect of epigenome-targeting drugs on the methylotype and expression of the Polo-like kina*ses

Blood syndromes, in particular myelodysplasia, are speculated to be epigenetically-driven in nature [7]. This paradigm stems in part from the serendipitous response, recovery, and improved survival rates seen in MDS patients administered with therapies aimed at the reversal of aberrant epigenetic marks [43]. Naturally, considering the epigenetic plasticity of cells, large efforts have been directed towards demonstrating the efficacy of epigenome-modifying drugs in reversing epigenetic defects and restoring expression of specific anti-tumour proteins [44]. Hypomethylating agents have been used to reverse aberrantly methylated promoters of the tumour suppressors $p15^{INK4B}$, p73, and E-cadherin in myeloid leukemia cells [Farinha et al 2004 Activation of expression of p15, p73, and E cadherin in leukemic cells]. Because of the therapeutic benefits associated with these drugs, chemical agents targeting the epigenetic machinery are being used concurrently with conventional chemotherapy, and importantly, are the mainstay treatment regimen for high-risk MDS and lymphoma patients with poor prognosis [43].

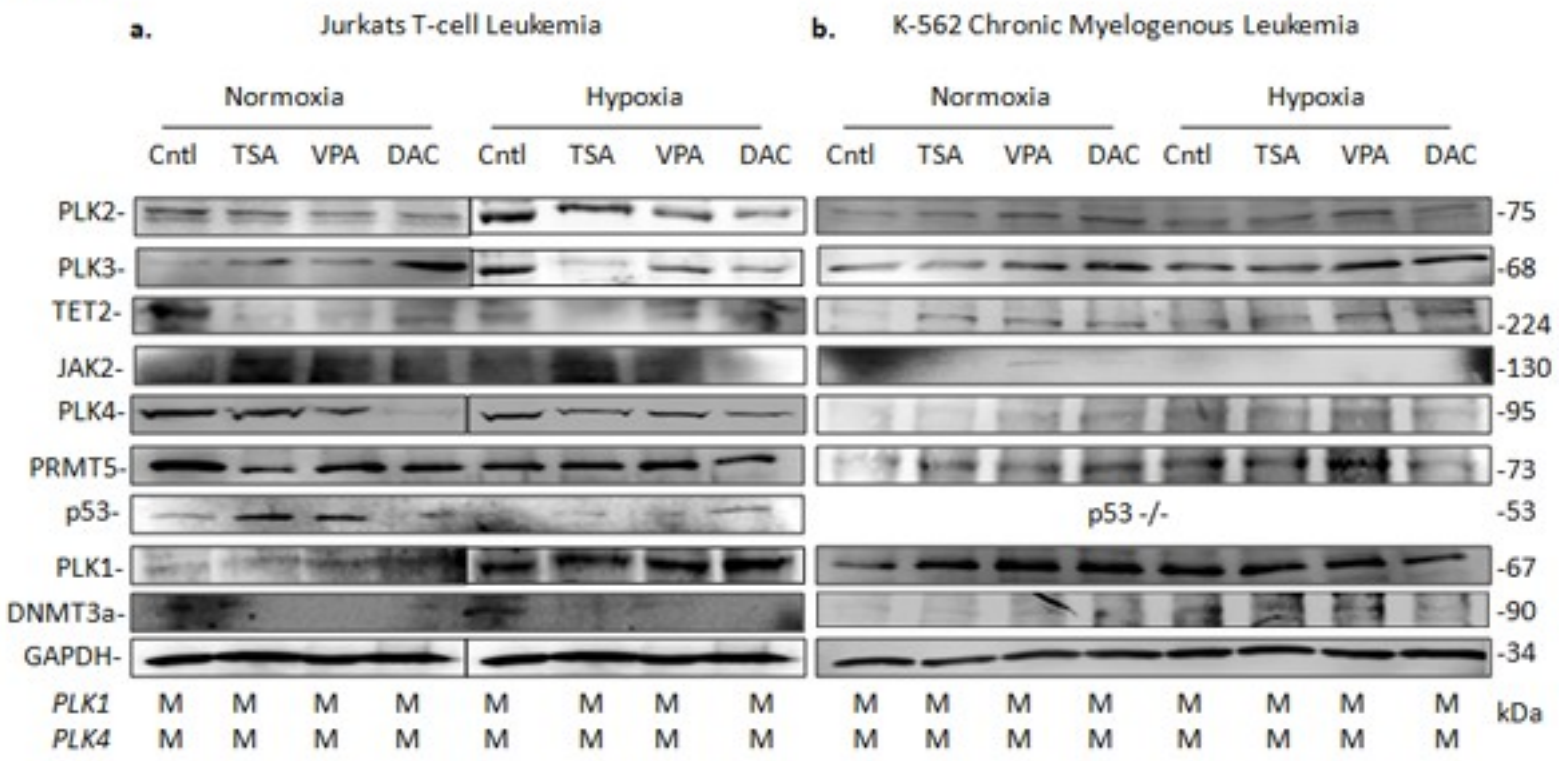
Low-dose decitabine (5-aza-2'-deoxycytidine) has been approved for the treatment of myelodysplastic patients. A cytosine analogue, decitabine (DAC), is able to reverse epigenetic mutations by incorporating itself into the newly synthesized daughter strand during replication. When DNA methyltransferases (DNMTs) interact with the DNA to place methyl groups on CpG dinucleotides, decitabine is able to covalently bind and arrest these enzymes [45]. With subsequent rounds of replication, DNMTs become depleted within the cell, allowing for a genome-wide decrease in methylation levels and reactivation of methylated genes. In contrast, trichostatin A (TSA) and valproic acid (VPA) potentiate gene re-expression by inhibiting histone deacetylases (HDACs) which

remove acetyl groups from histone tails to repress transcription [46]. By allowing for greater persistence of these acetyl groups on histones, HDACs can remodel the chromatin structure into a relaxed, open state and initiate gene transcription. Although it may seem that DNMT and HDAC inhibitors work independently of another, methylation and histone modifications synergistically impact gene expression. Inhibiting DNA methylation prevents methyl-binding protein 2-dependent recruitment of histone deacetylases and restores gene expression. Sarkar *et al.* (2011) have also shown inhibitors of HDACs to revert promoter CpG methylation *via* suppression of MAPK1/ERK signalling pathway which ultimately results in reduced DNMT1 levels [47].

It was therefore of interest to determine whether such therapy could potentially modulate the epigenetic and transcriptional state of the Polo-like kinases during therapy through an *in vitro* model. MDS/leukemia and lymphoma-derived cells were treated with DAC, TSA, and VPA chronically for a period of 5 days under both standard and biologically relevant oxygen conditions. In the clinical setting, DAC is administered over a period of several months, with clinical symptoms abating by the second round of treatment (Anecdotal, Dr. Kanjeekal) Drugs were administered chronically in order to characterize the long-term effects of such treatments. Cell viability and global methylation assays were performed throughout the period of drug treatment to optimize drug dosage and ensure that changes in global methylation take place before cell death (Supplementary Fig. 5.1a-c). To highlight the heterogeneity of blood-derived neoplasms, drug treatment data from two leukemia and two lymphoma cell lines have been shown here.

Figure 5.7 Epigenome-targeting drugs affect PLK expression in leukemia-derived cells. (a) Leukemic cell lines, Jurkats and K-562, were chronically treated with histone-modifying drugs, Trichostatin A (TSA) and Valproic Acid (VPA) and a hypomethylating agent, Decitabine (DAC) under standard and hypoxic conditions. Expression levels of the PLKs, PLK4-associated proteins p53, PRMT5, and DNMT3a, and biomarkers of hematological malignancies, TET2 and JAK2, were analysed post-drug treatment. GAPDH is used as a loading control. DNA methylation marks on *PLK1* and *PLK4* promoters were also examined *via* MSP. U = unmethylated, M = methylated. Fully methylated HeLa DNA was used as a positive control.

Figure 5.7


a.
Jurkats T-cell Leukemia
b.
K-562 Chronic Myelogenous Leukemia
Normoxia
Hypoxia
Normoxia
Hypoxia
Cntl TSA VPA DAC Cntl TSA VPA DAC Cntl TSA VPA DAC Cntl TSA VPA DAC
PLK2-
PLK3-
TET2-
JAK2-
PLK4-
PRMT5-
p53-
PLK1-
DNMT3a-
GAPDH-
p53 -/-
-75
-68
-224
-130
-95
-73
-53
-67
-90
-34
PLK1 M M M M M M M M M M M M M M M M
PLK4 M M M M M M M M M M M M M M M M
kDa

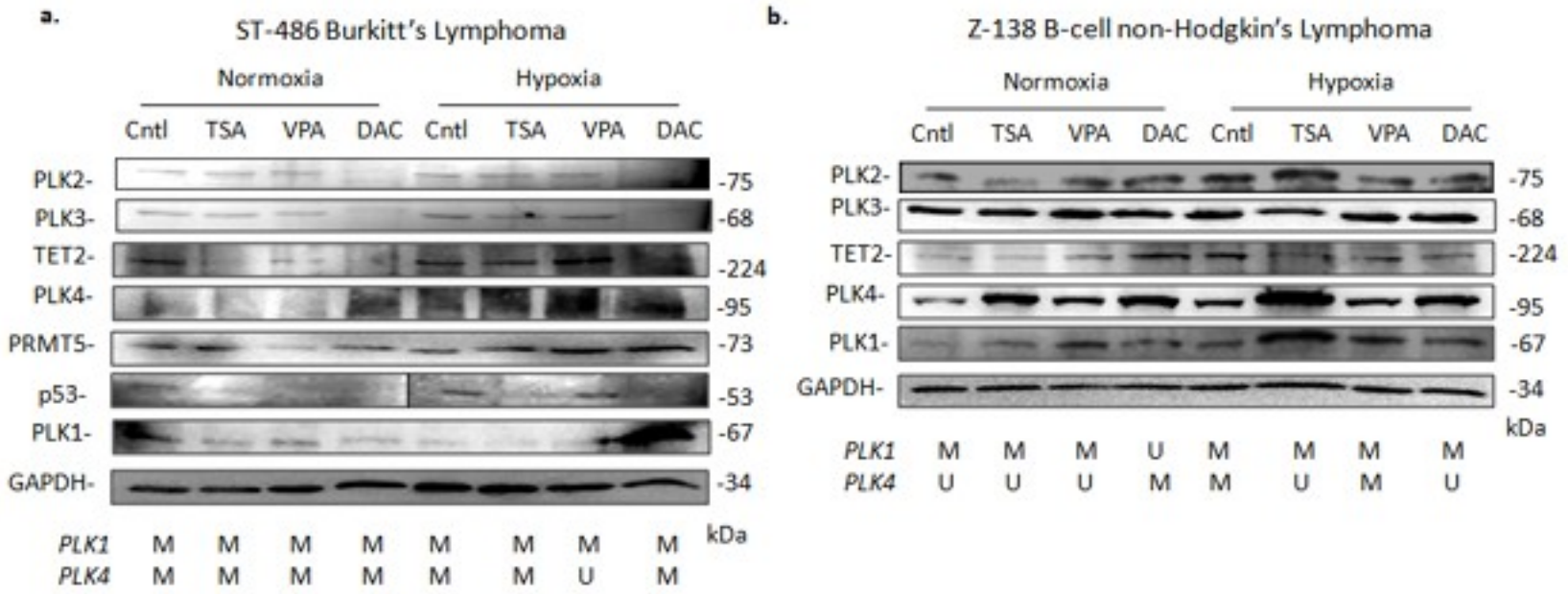
Post-drug administration, there was a remarkable sensitivity of the polo-like kinases to these drugs largely at the functional protein level. Treatment of Jurkats, a T-cell leukemia cell line, under normoxic conditions with TSA, VPA, or DAC resulted in decreased PLK4 protein levels; in fact, PLK4 is depleted with chronic exposure to decitabine (Fig. 5.7a). A similar trend is observed in hypoxia where treatment with epigenome-targeting drugs results in lower levels of endogenous PLK4. Interestingly, the decrease in PLK4 protein levels also corresponds with the expression of its known target, PRMT5, as reported previously (Ward *et al.* Chapter 4 dissertation). We have also previously shown a converse relationship between p53 and PLK4 (Ward *et al*. Chapter 4 dissertation). p53 has been implicated in the transcriptional repression of PLK, potentially by recruitment of HDACs and DNMT3a*,* and consequently, high levels of p53 are associated with low levels of PLK4 [48]. Interestingly, this pattern is observed in drug- treated Jurkats under both normoxic and hypoxic conditions: upon exposure to any drug, while PLK4 protein levels become reduced in comparison to its untreated counterpart, p53 levels increase noticeably. As aforementioned, TET2 and JAK2 are important biomarkers of hematological malignancies. In accordance with results obtained from our *in vivo* study, high levels of the demethylating enzyme, TET2, is associated with increased levels of PLK4 while JAK2 up-regulation is correlated with suppression of PLK4. Derived from chronic myelogenous leukemia, K-562 cells, displayed a similar trend with TET2 and JAK2 expression in relation to PLK4 levels. As seen in the Jurkats, K-562 had reduced PLK4 levels with TSA, VPA, and DAC treatment under hypoxia (Fig. 5.7b). In contrast to the Jurkats, under normoxia, PLK4 protein expression in K-562 was up-regulated with administration of each drug. The differed response to drugs *via* PLK4 expression in these

leukemic cells is indicative of the heterogeneity of blood disorders. K-562 cells are p53-deficient and in this case, perhaps, the varied *PLK4* response induced by drugs is tethered to the p53 status of the cell line.

The heterogenicity of these blood diseases is further reiterated by the unique responses to DNA-modifying drugs in lymphoma-derived cell lines. ST-486 is a cell line derived from a patient with Burkitt's Lymphoma. Chronic treatment of these cells with TSA, VPA, and DAC did not alter PLK4 transcriptional activity under hypoxia as PLK4 expression was comparable to the untreated control (Fig. 5.8a). Intriguingly, TET2 levels were also stable throughout the duration of drug treatment in the low oxygen environment. Under standard conditions, however, PLK4 protein levels increased specifically with decitabine treatment. Moreover, the same inverse relationship between PLK1 and PLK4 protein levels was observed in the ST-486 line under normoxia (Fig. 5.8a). PLK1 is down-regulated when PLK4 levels are the elevated and vice versa. A mantle cell lymphoma, Z-138, was the only cell line examined which responded significantly to treatment with trichostatin-A in an oxygen-independent manner. The efficacy of TSA in increasing acetylation marks and promoting transcription in *in vitro* models has been established previously [3, 49]. In fact, a TSA-analog, vorinostat, is currently in phase I and II of clinical trials for the treatment of acute myeloid leukemias and lymphomas. Although it has shown tremendous anti-tumor activity with minimal side effects in patients [50], perhaps, the efficacy of TSA is augmented in the treatment of distinct subtypes of blood neoplasms. In the examined leukemia and lymphoma cell lines, PLK2 and PLK3 protein levels seem largely unperturbed by these epigenetic drugs. The most notable response

Figure 5.8 Impact of DNA-modifying drugs on PLK protein levels in lymphoma cells. (a) *PLK4* responded to the long-term five-day treatment with epigenome-modifying drugs in ST-486 and Z-138 lymphoma cell lines as indicated by changes in protein levels. Varied responses to drugs were observed in hypoxia in comparison to normoxia. p53, PRMT5, TET2, and JAK2 levels were also examined. MSP analysis was performed to determine whether changes in PLK expression occurred *via* modifications to the *PLK* epigenetic status. U = unmethylated, M = methylated. Fully methylated HeLa DNA was used as a positive control.

Figure 5.8


a.
ST-486 Burkitt's Lymphoma
Normoxia
Hypoxia
Cntl TSA VPA DAC Cntl TSA VPA DAC
PLK2-
PLK3-
TET2-
PLK4-
PRMT5-
p53-
PLK1-
GAPDH-
-75
-68
-224
-95
-73
-53
-67
-34
kDa
PLK1 M M M M M M M M
PLK4 M M M M M M U M
b.
Z-138 B-cell non-Hodgkin's Lymphoma
Normoxia
Hypoxia
Cntl TSA VPA DAC Cntl TSA VPA DAC
PLK2-
PLK3-
TET2-
PLK4-
PLK1-
GAPDH-
-75
-68
-224
-95
-67
-34
kDa
PLK1 M M M U M M M M
PLK4 U U U M M U M U

was seen in ST-486 where administration of decitabine resulted in gene silencing of *PLK2* and *PLK3*, as noted by depletion of these proteins under standard and hypoxic conditions (Fig. 5.8a). It is interesting that a drug is able to evoke either a robust expression or complete suppression of PLK4 in a particular subtype of blood disorder but not in another. In this study, we also sought to determine whether these DNA-modifying drugs targeted the epigenetic status of the *PLKs*. Surprisingly, MSP analysis of the *PLK* promoter region showed little to no change in the methylation status, even with exposure to the DNA methyltransferase inhibitor, decitabine. As our results show distinct changes to PLK4 expression under varying treatments, more than likely, it may be that changes in methylation of the *PLK* promoter regions are taking place outside of the region amplified by the MSP primers. Bisulfite sequencing may be necessary to identify changes in the methylation marks of CpG dinucleotides. There is also potential for modulation of PLK expression to be driven through histone modifications rather than DNA methylation. Another possibility may be that the baseline transcription of PLK4 has not changed, but there may be a change in the protein's stability. Epigenetic modifying treatments are not targeted to specific genes, but rather confer their effects at a global level. Perhaps, as a by-product of treatment, an upstream regulator of PLK4 may be activated. This, in conjunction with DNA hypermethylation, may further contribute to changes in PLK4 at the protein level.

Our results demonstrate that hypomethylating and histone deacetylase inhibitors indeed modulate PLK expression, however, the extent of this regulation seems to be dependent on oxygen conditions and likely, on the molecular background of these cell lines. Specifically, it is important to recognize that these cells have aberrant cytogenetics

and a myriad of biochemical imbalances (Supplementary Table 5.1). For instance, a majority of the cell lines used in this study are polyploid, harbour multiple chromosomal deletions and translocations, and have irregular molecular behaviours such as perpetual secretion of cytokines and inappropriate expression of numerous antigens (ATCC) (Supplementary Table 5.1). In consideration of the nature of these neoplasms, crosstalk between genetic alterations and epigenetic state may play an important role in dictating the efficacy of these drugs in re-activating tumor suppressors.

Conclusions

MDS, leukemia, and lymphoma cells are derived from stem cells of the bone marrow and it is thought that the heterogeneity seen in these disorders is likely a reverberation of the genetic, epigenetic, and molecular heterogeneity within the resulting cancer cell population [51]. Inter- and intra-tumor heterogeneity has been extensively studied in several tumor types to understand phenotypic and functional differences among cancer cells and as well as provide insight into the complex clinical outcomes seen during therapy [52, 53]. Our *in vitro* drug study identifies the *PLKs* as responsive to these epigenome-targeting agents, however, the ability of methyltransferase and HDAC inhibitors to target CpG island hypermethylation of the *PLKs* and the subsequent reactivation of these genes varies between subtypes of blood disorders, but more so, between normoxia and hypoxia. The diversity of the patients' profiles presages the unique range of responses observed in cell lines at the protein level. This observation is also in consistent with the well-described model which posits that many cancers undergo clonal evolution and tumorigenic cell differentiation that independently or synergistically

contribute to heterogeneity within a cancer population [51]. While this enigma continues to be a hindrance in the effective treatment of patients with blood disorders, understanding the effect of DNA-modifying drugs on the epigenetic profile of *PLKs* and their expression may provide some insight as to how these highly conserved and essential cell cycle-dependent kinases are impacted during therapy. As shown here, under particular conditions, drugs have a negative impact on the tumor suppressors PLK2-4 and promote the elevation of the oncogenic PLK1. Understanding how epigenetic therapy can influences the underlying epigenetic lesions of the *PLKs* in leukemia and lymphoma-diseased patients may be a valuable biological predictor in the clinical setting Though more studies need to be carried out at this point.

At the methylome level, there are distinct signatures that are specific to the tumour phenotype. In diffuse large B-cell lymphomas (DLBCL), 15 differentially methylated genes were identified, whereas in another subset of aggressive B-cell lymphomas, 56 hypermethylated genes were discovered using epigenomic profiling [54, 55]. At the individual gene level for example, the gene O^6-methylguanine DNA methyltransferase (*MGMT*), important for genomic stability, is methylated in 39% of cases of DLBCLs [56]. Epigenetic studies in blood neoplasms have also been important in the identification of DLBCL and AML subtypes, which other than through DNA methylation signatures, had no other molecular means of distinction [9, 57]. Interestingly, the most well-studied methylation-regulated genes in hematologic malignancies are tumour suppressors and cyclin-dependent kinase inhibitors like $p15^{INK4B}$ and $p16^{INK4A}$, which are hypermethylated at a rate of 60% in leukemia and lymphoma respectively (reviewed in Esteller 2003). Prognostic models of MDS methylotype have

been proposed in an effort to predict important clinical outcomes like disease-free- and overall survival [58]. Our data suggests that the methylation phenotype of the *PLKs*, is associated with a tumourigenic state in hematological malignancies. Moreover, with deregulated PLK4 expression affiliated with proteins commonly deregulated in blood neoplasms, JAK2 and TET2, the *PLK* methylation and expression profile may have some clinical value as a biomarker.

Materials and Methods

Bone marrow aspirates

The collection of bone marrow aspirates from patients was conducted under the approval of the ethics committee at Windsor Metropolitan Hospital and the research ethics committee at the University of Windsor.

DNA Extraction

DNA from bone marrow aspirates was extracted under sterile conditions. Several washes of 1XSSC buffer were used to remove the serum from the blood cells after which samples were incubated in proteinase K enzyme for 90 min at 55˚C, followed by a standard phenol/chloroform extraction. Similarly, DNA extractions from cells grown *in vitro* also employed the use of proteinase K, however, samples were incubated at 55˚C for 24 hours prior to phenol/chloroform extractions. DNA concentrations were quantified using the NanoDrop® ND-1000 spectrophotometer (Thermo Scientific).

Methylation specific PCR

Initially, 2ug of DNA were bisulfite treated as per Herman et al, 1996 [59]. In the case where low yields of DNA were recovered from bone marrow aspirates, 200-500ng of DNA was used as the starting material and the EZ DNA Methylation-Gold™ kit was used to perform the bisulfite conversions (Zymo Research). The bisulfite converted DNA was then PCR amplified using PLK-specific primers. PLK1 and PLK4 primer sequences are found in [13]. For PLK2 and PLK3 primers sequences, these are the same sequences used by Syed et al. 2006 and Ward et al. 2011[13, 14].

Cell culture

The following cell lines were used in the study: chronic myelogenous leukemia: K-562 and Meg-01; acute lymphocytic leukemia: Reh; B cell non-Hodgkin's lymphoma: Z-138; and Burkitt's lymphoma: ST-486, Ramos, Ramos-2G6. The aforementioned cell lines were purchased from ATCC (Manassas, VA). Jurkats (T-cell leukemia) were also employed in the study and was kindly donated to us by S. Pandey (U of Windsor). The cells were cultured in cell-line specific growth media, as recommended by ATCC, supplemented with 10% fetal bovine or horse serum at 20% O_2/5% CO_2 (normoxia) or at 5% O /5% CO_2 (hypoxia).

Hypomethylation and histone-modifying treatments

To perform treatments with epigenome-targeting agents, cells were treated with either 0.5 μM 5-aza-2'deoxycytidine (Decitabine -DAC; Sigma), 1 mM valproic acid (VPA; Sigma), or 0.5 μM trichostatin A (TSA; Sigma) for a period of 5 days to observe

their effects on *PLK* promoter methylation status. TSA was administered to cells every other day, while cells were treated with DAC and VPA every day of the treatment period. Cells were passaged every 24 hours with the supplementation of fresh media and if necessary, the drugs. After the treatment period, cells were harvested for DNA and protein extraction.

Western blot analysis

Whole cell lysates from both bone marrow aspirates and *in vitro* cultured cells were used to quantify protein expression. Samples and cells were lysed using lysis buffer (50mM Tris-Cl, 150mM NaCl 1% Triton-X, 0.1% SDS) supplemented with *complete* EDTA-free protease inhibitor cocktail tablets at a ratio of 1:10000 (Roche). The following primary antibodies were used to examine respective protein levels PLK4, GAPDH, PRMT5 (Cell Signalling); PLK2, PLK3, TET2 (Santa Cruz), PLK1 (Abcam), MPO (OWL), and JAK2 (Millipore). Secondary antibodies used were anti-rabbit HRP at a ratio of 1:10000(Cell Signalling) and anti-mouse HRP at a ratio of 1:50000 (Sigma)

Global methylation assay

Genomic DNA from samples was subjected to global methylation analysis using the Methylamp Global Methylation quantification kit (Epigentek). Experiments were carried out according to manufacturer's recommendations using 30 ug of DNA. Colourimetric analysis was carried out on a Wallac Victor3 140 multilabel counter at 450nm.

Statistical analysis

Statistical analyses were carried out using Statistica software version 7.1. Error bars represented here are reflective of the standard deviation from the sample size indicated or from three independent experiments. * denotes statistical significance $p<0.05$.

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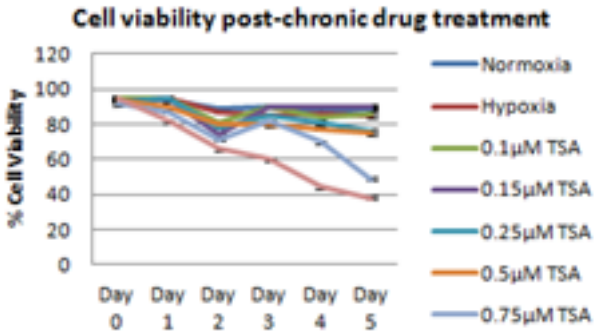
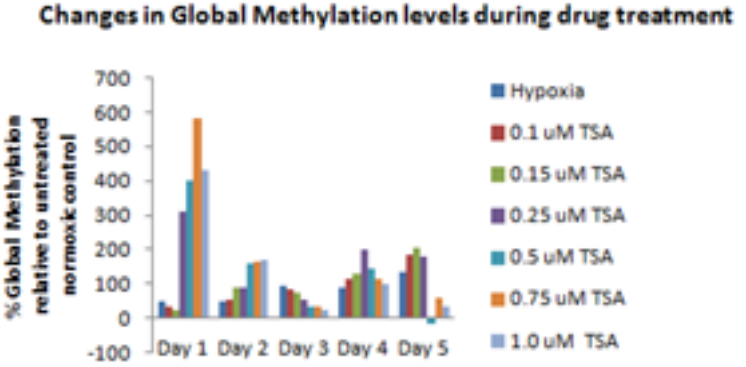
Supplementary figure 5.1. Cell viability and global methylation changes take place in cells upon drug treatment in a dose- and time-dependent manner. Jurkat cells were chosen as the standard cell line to determine optimal drug dosage for TSA, VPA, and DAC for all cell lines. Cell viability and an ELISA-based global methylation assays were performed to ensure that changes in methylation marks were accumulating prior to drug-induced apoptosis. After 24 hours of each drug treatment, cells were collected for Tryphan Blue staining to measure cell death and DNA was extracted for methylation assays. Viability and changes in global methylation were assessed each day for a period of five days for a) TSA, b) VPA, and c) DAC treatment.

Supplementary Figure 5.1

a.





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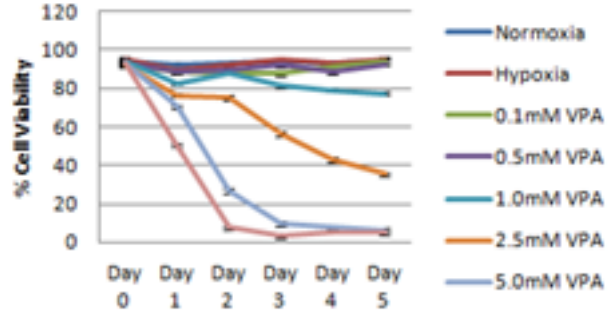

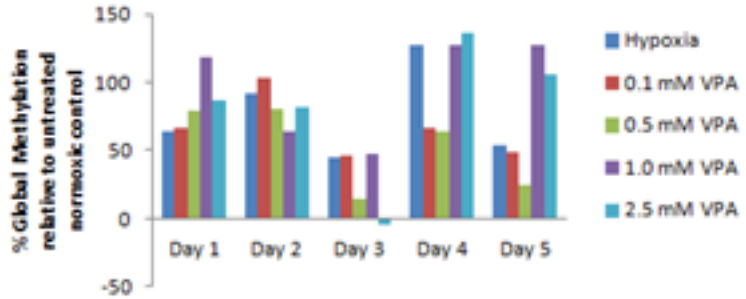

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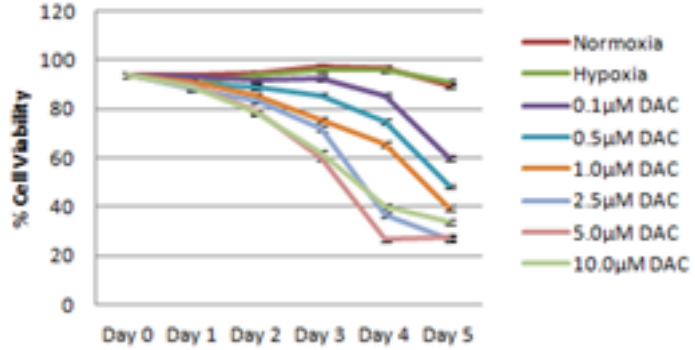
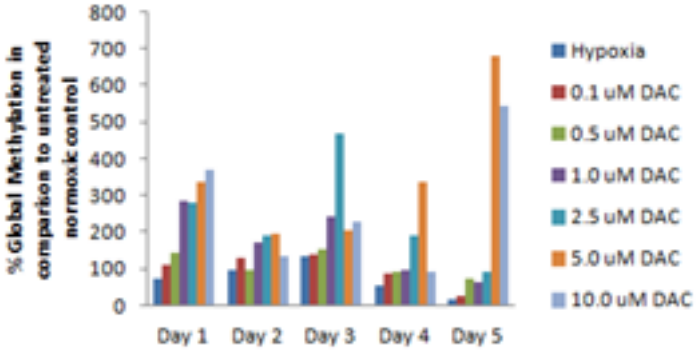




Supplementary Table 5.1. Description of MDS/leukemia and lymphoma cell lines. Background information of patient-derived MDS/leukemia and lymphoma cell lines (ATCC) used in the study including description of disease type, gender, and abnormal cell characteristics. All information provided here was obtained from ATCC.

Supplementary Table 5.1

a.

	Cell line Description	Disease type	Age/Gender	Characteristics
MDS/ Leukemia	Jurkat	Acute T-cell Leukemia	14 year old male	Karyotype: pseudodiploid; 46,XY,-2,-18,del(2) (p21p23),del(18) (p11.2); Normal X and Y chromosome Antigen expression: CD3 Receptor expression: T-cell antigen receptor
	Meg-01	Chronic myelogenous leukemia	55 year old male	Karyotype: hyperdiploid; the Philadelphia chromosome is present Antigen expression: CD41 +; CD61 +; CDw14 +
	K-562	Chronic myelogenous leukemia	53 year old female	Karyotype: triploid; occurrence of spontaneous dicentrics; X chromosome is disomic Tumorigenic: Yes; tumors developed in nude mice at 100% frequency with inoculation Antigen expression: CD7
	Reh	Acute lymphocytic leukemia (non-T; non-B)	None provided	Antigen expression: CD3 A (17%) B (17%) C (20%), CD4 (15%), CD10 (55%)
Lymphoma	Ramos	Burkitt's lymphoma	3 year old male	Antigen expression: CD23+ Receptor expression: interleukin 4 (IL-4); low affinity IgE (Contain about 1500 IL-4 binding sites per cell) Genes expressed: immunoglobulin (surface and secreted) Tumorigenic: Yes; tumor formation in nude mice
	Ramos-2G6	Burkitt's lymphoma	3 year old male	Antigen expression: CD23+ Receptor expression: interleukin 4 (IL-4); low affinity IgE (Contain about 1500 IL-4 binding sites per cell) Genes expressed: immunoglobulin M (surface and secreted) Tumorigenic: Yes; tumor formation in nude mice
	ST-486	Burkitt's lymphoma	Female	Derived from parent line Ramos; Antigen expression: CD23+ Receptor expression: interleukin-4 (IL-4); low affinity IgE (Contain about 1500 IL-4 binding sites per cell) Genes expressed: Immunoglobulin (surface and secreted)
	Z-138	Mantle cell lymphoma (B cell non-Hodgkin's lymphoma)	70 year old male	Karyotype: hyperdiploid; t(11;14)(q13;q32); del(5)(p15), der(9)t(9;?), der(14)t(8;14)?, and add(17p) Antigen expression: CD3-, CD5-, CD10-, CD19+, CD20+, CD23+, FMC7- Overexpression of cyclin D1.
	RPMI-8226	plasmacytoma; myeloma	61 year old male	Karyotype: triploid; evidence of terminal centromeres Antigen expression: HLA Aw19, B15, B37, Cw2; CD19 -; CD20 -; CD28 +; CD38 +; CD49e +

Supplementary Table 5.2 Summary of smoking history and oxygen saturation of patients. A summary of the patients with lymphoproliferative disorders and their smoking history and oxygen saturation levels. The bone marrow for all these patients were included in our methylation analysis for the *PLKs*

Population information	**n=51**	**%**
Avearage age	64.75	
Number of patients with a smoking history	24	47.0
Patients with low O_2 Saturation	9	17.6

Supplementary Materials and Methods

Cell Viability Assay

Various dosages of demethylating and histone-modifying drugs were administered for a period of 5 days to determine the optimal as well as toxic doses. Tryphan Blue staining was performed to discriminate between viable and non-viable cells post-drug treatment. To do this, a sample of the cell suspension was re-suspended in filter-sterilized Tryphan Blue stain at 1:1 ratio. From this mixture, 10 µl of the sample was added to the BioRad cell counting chamber slides and placed into the BioRad TC10 Automated Cell Counter to measure cell viability. This assay was performed each day of the drug treatment to monitor changes in cell survival over time.

Chapter 6

Towards a model for the polo-like kinases and epigenetic regulation: Overall summary of conclusions and future directions

This chapter includes data generated by Gayathri Sivakumar (Plk1 BSP)

In the last decade, the role of epigenetic modifications as contributors of malignancy has been cemented. These reversible modifications have become the protagonists in the narrative of cancer research and treatment, with the epigenome developing into a liaison of sorts between the environment and the changes in gene expression that lead to tumourigenesis. Specific epigenomic aberrancies are often used as biomarkers and are paving the way towards personalized medicine, with deregulated DNA methylation as the most commonly used epigenetic indicator. The DNA hypermethylation profile found in cancers has been dubbed the "hypermethylome" by the likes of Manel Esteller and Stephen Baylin [1, 2]. These hypermethylomes include several commonly methylated genes in cancers like E-cadherin, p16, and BRCA1; hypermethylomes have been assessed within many of the most prevalent cancer types [2]. Although these analyses included many genes encoding known tumour suppressors and other cell cycle regulators, they are not exhaustive. The aberrant DNA methylation of the Plk family of cell-cycle regulated serine/threonine kinases is emerging as a new and potentially important indicator and contributor to tumourigenesis. Here I outline the current understanding for the *PLKs* and epigenetic regulation. The information, based on both my results, and the current literature, leads towards a model in which epigenetics and the *PLKs* have a bi-directional exchange, a key relationship that has impact at the both a basic research and clinical level. This chapter thus includes a systematic overview

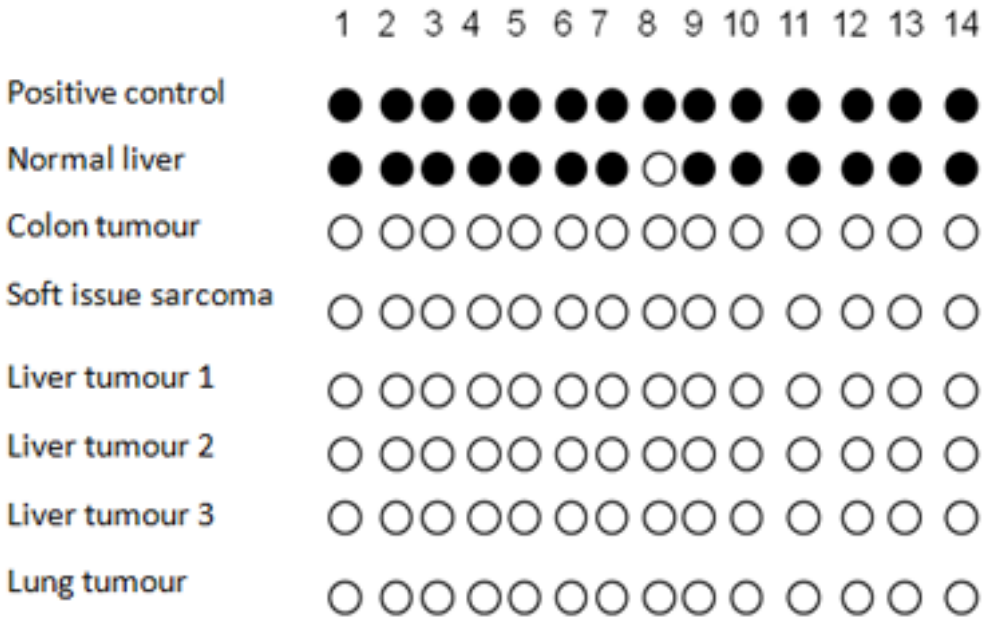
of the individual PLKs and their association with specific epigenetic marks, with a focus on PLK4 as the crux between PLK signalling and the dissemination of epigenetic marks.

PLK1

Recent evidence supports a model in which Plk1 levels in normal tissue from mice and humans, are maintained at relatively low levels by an epigenetic mechanism as noted by the presence of detectable methylation within its promoter [3, 4] (Ward et al. unpublished Chapter 4). PLK1 is commonly augmented in a myriad of cancer types. More specifically, PLK1 becomes upregulated in both human and mouse hepatocellular carcinoma (HCC) [4, 5] and in hematological malignancies (Ward et al. Chapter 5 dissertation). We were the first to identify the loss of promoter methylation of *PLK1* correlated to an increase in its expression. Specifically, in human HCC, samples that displayed hypomethylation of *PLK1* had almost a 10-fold increase in PLK1 transcripts [4]. Using a $Plk4^{+/-}$ mouse model with an inherently high rate of developing HCC, we also observed DNA hypomethylation of *Plk1* in more than 70% of the HCC samples assayed, this was in contrast to normal liver samples which were strongly methylated [4]. This loss of methylation directly correlated to Plk1 overexpression at the protein level in HCC [4]. In addition, these mice develop several different tumour types other than liver cancer, such as soft tissue sarcoma, along with lung and colon tumours. We examined these tumours for promoter methylation of Plk1 by MSP and BSP. These analyses revealed a loss of methylation in lung and colon tumours, and soft tissue sarcoma (data not published), with 100% of the 14 CpG sites examined within the *Plk1* CpG island completely hypomethylated. In contrast, in normal tissue and in the normal positive control a loss of methylation was not detected (Fig 1).

Figure 6.1 Plk1 distribution of methylation marks in 3T3, normal liver and tumor samples. Both the 3T3 mouse fibroblast cell line and normal mice liver samples exhibited 100% methylation of CpG sites in the *Plk1* promoter region. In contrast, Plk1 promoter CpG sites were unmethylated in all tumour samples

Figure 6.1


1 2 3 4 5 6 7 8 9 10 11 12 13 14
Positive control
Normal liver
Colon tumour
Soft issue sarcoma
Liver tumour 1
Liver tumour 2
Liver tumour 3
Lung tumour

We also observed *PLK1* promoter hypomethylation in blood neoplasms with almost 20% of malignant human bone marrow aspirates analyzed displaying *PLK1* promoter hypomethylation with a subsequent increase in PLK1protein levels (Ward et al, Chapter 5 dissertation). Brauninger et al. (1995) identified the 2.3 kb region immediately upstream of the transcriptional start site of *PLK1* as sufficient for its transcriptional regulation [6]. Within this region, *PLK1* harbours a 909 base pair CpG island identified *in silico* [7]. This CpG island also overlaps a recognition sequence for the forkhead transcription factor, FoxM1 [8]. p53 targets FoxM1 for downregulation, and in cells where there was a loss of p53 function, FoxM1 was elevated [9, 10]. FoxM1 is a known transcriptional activator for PLK1[5] and its overexpression is followed by PLK1 amplification in human HCC, esophageal, and other cancers [5, 11, 12]. At the protein level, PLK1 phosphorylates FoxM1, increasing its activity, creating a positive feedback loop that ultimately promotes the cellular transition through G2/M [13]. It therefore will be of interest to examine the FoxM1 levels in tumours that display hypomethylation of the *PLK1* promoter region.

PLK1 also has two distinct p53 responsive elements within this region that p53 binds to, thus, directly leading to the transcriptional repression of *PLK1* [14]. Interestingly, *PLK1* promoter methylation responds to oxidative stress in a p53-dependent manner. Cells exposed to reactive oxygen species harbouring a defective p53 response displayed promoter hypomethylation with a subsequent increase in PLK1 protein [3]. This suggests that p53 in conjunction with promoter hypermethylation help to downregulate PLK1 expression. In a scenario where there is both promoter hypomethylation of *PLK1* along with a defective p53 response, the natural consequence

would inevitably be the upregulation of PLK1. We also examined the effect of *Plk4* heterozygosity on Plk1 promoter methylation. *Plk4* heterozygosity results in a deficiency in the p53 response [3, 15] (Ward et al, Chapter 5 dissertation) and in tumours derived from $Plk4^{+/-}$ mice, there is a loss of *Plk1* promoter methylation [4]. This suggests that *Plk4* heterozygosity provides the scaffold necessary for the inadvertent upregulation of Plk1.

PLK2

PLK2 was the first of the PLKs to be characterized with promoter methylation aberrancies in malignancies. Its hypermethylation is a potentially valuable clinical marker in that approximately 60-90% of B-cell lymphoma, myelodysplastic syndromes, acute myelogenous leukemia, and multiple myeloma display *PLK2* DNA hypermethylation [16] (Ward et al. Chapter 5 dissertation). *PLK2* hypermethylation has been associated with better over-all survival in hematological malignancies, more specifically, in the case of multiple multiple myeloma this translated to a 48% lowered risk of death [17]. In a broad spectrum of hematological malignancies, we were able to detect *PLK2* methylation in bone marrow aspirates, a procedure which is performed routinely in bone marrow biopsies. We were fortunate in that there is more material in aspirates than actual bone marrow biopsies and more importantly the aspirates display the same methylation profile. The use of the aspirate negates the need for additional bone marrow samples and may be of use clinically in that there is less of a burden on patients (Ward et al, Chapter 5 dissertation). Consistent with this, others have detected *PLK2* promoter hypermethylation in the patient's sera for ovarian cancer cases [18]. *PLK2*'s methylation status as a clinical indicator varies with tumour type. For example, unlike the case for

hematological malignancies, in ovarian cancer *PLK2* hypermethylation is a poor prognostic indicator and is associated with a shorter time to relapse and resistance to the commonly used chemotherapeutic agent, paclitaxel [19]. Epigenetically reduced levels of PLK2 abrogate the paclitaxel-induced blockade of the transition from the G2 to M phases of the cell cycle [18, 19].

Oxidative stress, like hypoxia and reactive oxygen species are known to be causative agents that induce aberrant epigenetic marks in several tumour types [20-22]. With the PLKs frequently deregulated by promoter hypermethylation in malignancies, we sought to determine if these micro-environmental factors did in fact contribute to PLK expression changes. My research found that *PLK2* CpG island methylation status is subject to changes in the microenvironment in a p53-dependent manner. When HCC and osteosarcoma cells with an intact p53 response were exposed to oxidative stress, *PLK2* underwent promoter demethylation paired with an increase in transcripts. However, this was not observed in cells where p53 was abrogated [3]. In p53 null cells, like Hep3b and Saos-2, *PLK2* had a detectable gain in promoter methylation accompanied by a corresponding decrease at the protein level [3]. *PLK2* hypermethylation has also been described in human HCC, an organ that is often subjected to high levels of oxidative stress [5]. In these cases, PLK2 downregulation was associated with increased cell growth and shorten survival [5].

PLK3

Several CpG islands exist in human *PLK3* within the first 2000 base pairs of the gene, and the largest is 687 bp in length [3, 7]. However, the mouse *Plk3* gene lacks a

CpG island within its promoter region [4]. Based on the current literature available to date and data generated from our lab, irregular patterns of human *PLK3* promoter methylation are not as prevalent compared to *PLK2*. The initial studies implicating changes in Plk3 methyaltion status associated with malignancy were conducted by Pellegrino et al. (2010). Here they found that in human HCC that 37.3% of HCCs had an increase in *PLK3* promoter methylation, which, like PLK2, also correlated to poorer patient outcome [5]. An analysis of *PLK3* promoter methylation profiles in normal human liver tissue and bone marrow aspirates determined that *PLK3* promoter methylation is detectable in a subset of samples [4, 5] (Ward et al. Chapter 5 dissertation). These studies suggest that *PLK3* promoter methylation may be tissue specific.

Our initial investigation of potentially aberrant *PLK3* methylation patterns was conducted in B-cell malignancies, with no promoter hypermethylation detected *in vitro* or in primary tumour samples [16]. More recently, we found that *PLK3* was hypermethylated in almost 80% of lymphoid and myeloid derived neoplasms (Ward et al, Chapter 5 Dissertation). This represented a proportional increase of 30% detectable promoter methylation in malignancies compared to the methylation patterns observed in normal tissue. This difference could be attributed to the larger sample size that we examined (n=114) and a greater number of hematological subtypes (Ward et al. Chapter 5 dissertation).

The microenvironment also has an effect of Plk3 promoter methylation status. Previously, we determined that *PLK* promoter methylation marks are dynamic and are susceptible to change upon exposure to oxidative stress [3]. *In vitro*, cells derived from

human HCC and osteosarcoma display *PLK3* promoter methylation, however, unlike the other *PLKs*, post treatment with ROS and hypoxia, no difference in promoter methylation was detected, [3]. . This was also the case in cells derived from blood neoplasms, which showed no significant change in presence of hypoxia (Ward et al Chapter 5 dissertation). Across a variety of cellular types and with different stressors, little change in the promoter methylation of *PLK3* has been observed when comparing samples prior to- and post-treatment. Even with primers designed to examine different CpG islands, the methylation status of *PLK3* remained constant [3]. Although there were no detectable differences in promoter methylation between treated and untreated cells, the expression at the transcript and protein levels was significantly different. Under oxidative stress, dramatic changes in PLK3 expression are observed [3]. PLK3 transcript and protein levels with cellular exposure to reactive oxygen species in osteosarcoma cells were almost undetectable, regardless of the p53 status of the cells. Whereas, in HCC cells with intact p53, ROS exposure triggered an increase in PLK3 levels, regardless of promoter methylation status [3]. This suggests that *PLK3*'s expression may not be tightly tied to its promoter methylation, but in fact may be regulated through other mechanisms.

Another important epigenetic mechanism that may play a role in Plk3 regulation is via microRNAs. MicroRNAs are short, non-coding oligos composed of 21-25 nucleotides. They mediate the post-transcriptional repression of mRNAs in a sequence-specific manner [23]. Interestingly, *in silico* analysis of the PLK3 mRNA sequence, using the database Target Scan Human, revealed a very high likely-hood that PLK3's RNA product may be targeted for degradation by microRNAs. One of the micro-RNAs with a

high context score percentile (82%) was miR-24. This miRNA has been associated with the silencing of other DNA repair proteins during DNA damage, such as H2AX [24].

PLK4

We were the first to describe the regulation of PLK4 expression *via* promoter methylation with age, and in the context of carcinogenesis [4]. We found that, not only is *Plk4* promoter region targeted for hypermethylation with age in mice, but $Plk4^{+/-}$ mice displayed an even further increase in hypermethylation at 9 months of age compared to their wild type littermates [4]. Furthermore, 75% of HCC tumours extracted from $Plk4^{+/-}$ mice were hypermethylated at the CpG island upstream of the *Plk4* promoter region with an corresponding decrease in Plk4 transcripts and protein compared to age-matched wild type mice [4]. This phenotype was also observed in a small sample of human HCC samples [4]. Interestingly, at 9 months of age, $Plk4^{+/-}$ mice, displayed a significantly higher level of global methylation compared to the wild type mice (Ward et al. 2011) and in general *Plk4* heterozygosity pre-disposes these mice to abnormal epigenetic modifications. For example, When examining the methyltransferases in $Plk4^{+/-}$ MEFs, DNMT3a levels were elevated compared to wild type MEFs [3]. DNMT3a is a methyltransferase responsible for the establishment of *de novo* methylation marks. Other PLK4 interacting partners, like PRMT5 and p53, which can directly or indirectly mediate epigenetic changes, are also deregulated in *Plk4* heterozygous cells [3, 4, 15, 25](Ward et al, Chapter 4 dissertation). These will be discussed in more detail later on.

PLK4 methylation in hematological malignancy

Haematological malignancies often display aberrant promoter methylation of key genes. In the context of the present study, this includes tumour suppressors such as *PLK2*. This along with our results for *Plk4* and Plk1 in HCC, led us to examine the methylation status for *PLK4* in hematological malignancies. We found that *PLK4* was hypermethylated in almost 80% of lymphomas, myelodysplastic syndromes (MDS), and leukemias, while in normal bone marrow aspirates, no promoter methylation was observed (Ward et al. Chapter 5 dissertation). *PLK4* hypermethylation was accompanied by a significant overall decrease in PLK4 protein. This striking shift in *PLK4* downregulation in these malignancies indicated that perhaps the *PLK4* promoter methylation status may be of value as a clinical or prognostic biomarker. If so, one would expect that *PLK4* methylation status and levels may be correlated with some of the known markers. We therefore screened these samples and assessed the levels of some proteins commonly deregulated in hematological malignancies. This included the clinically relevant proteins, JAK2 and TET2. JAK2 expression was inversely correlated with PLK4 levels, while TET2 and PLK4 follow similar patterns of downregulation in blood neoplasms (Ward et al, Chapter 5 dissertation). These observations support our notion that *PLK4* may have merit as a biomarker in hematological malignancies.

PLK4 promoter hypermethylation in vitro and in response to environmental stressors

PLK4 hypermethylation has been detected in cell lines from diverse cancer types including leukemia, lymphoma, hepatocellular carcinoma, and osteosarcoma [3](Ward et al, Chapter 5 dissertation). Within these cell types, we have found that the cellular

microenvironment plays a significant role in the promoter methylation status of *PLK4*. The *PLK4* promoter region is targeted for hypermethylation in response to oxidative stressors commonly found in tumourigenesis, such as hypoxia and ROS [3]. This hypermethylation of *PLK4* is dependent on the p53 status of cells with the p53 null sarcoma cell line Saos-2 and the hepatocellular carcinoma cell line Hep3B, displaying a loss of *PLK4* methylation and an increase at the protein level [3]. These results are indicative of the importance of p53's regulatory effect on PLK4. We thus explored the relationship between PLK4 and p53.

PLK4 is dynamic, and has a role in the DNA damage response and as a pro-mitotic protein. Acute stress temporarily stabilizes PLK4, however, sustained stress triggers p53 activation accompanied by p53-dependent downregulation of PLK4 at the protein level along with promoter hypermethylation at the DNA level [3, 26](Sepal Bonni, 2007 Master's thesis). In normal cells, mechanisms that regulate PLK4 at the DNA and protein level provide an efficient approach by which to delay the transition to mitosis until the stressor has abated, prompting the repair of DNA damage, or triggering the apoptotic response if the damage is too great. Indeed, flow cytometric analysis of *Plk4* heterozygous MEFs reveal a higher proportion of cells accumulated at the G2/M phase of the cell cycle and in an even greater accumulation in response to UV damage [27] (Sepal Bonni Master's thesis; Ward unpublished).

PLK4 and its novel interacting partner PRMT5

In chapter 4, I described the characterization of a novel PLK4 interacting partner, PRMT5. PRMT5 is evolutionarily conserved and is the only methyltransferase that

mediates the symmetric dimethylation of arginine residues at histone and non-histone proteins [28]. These methylation marks regulate the expression and activity of many proteins, including the master guardian of the genome, p53 [28, 29]. We determined that PRMT5 is a substrate of PLK4 and that in normal MEFs, Plk4 and Prmt5 co-localize to the centrosomes (Ward et al. Chapter 4 dissertation). Plk4 levels directly impact Prmt5 localization, levels, and activity in *Plk4*$^{+/-}$ MEFs (Ward et al. Chapter 4 dissertation). Prmt5's deregulation in *Plk4*$^{+/-}$ MEFs leads to mislocalization of Prmt5 and loss of arginine methylation marks. This loss of symmetric arginine methylation marks potentially could contribute to an insufficient p53 response in *Plk4*$^{+/-}$ MEFs exposed to DNA damaging agents (Ward et al. Chapter 4 dissertation). In order for p53 to be properly activated, it requires the symmetric methylation of arginine residues at its C-terminal domain; p53 is also targeted for activation by PLK4 at S392 [25] [29]. My findings revealed that *in vitro, Plk4*$^{+/-}$ MEFs exposed to a variety of stressors have depressed levels of p53 along with decreased p53 activity [3, 15] (Ward et al. Chapter 5 dissertation). In the context of *Plk4* heterozygosity, both PRMT5 and p53 are aberrantly expressed, suggesting that PLK4 may be a liaison between PRMT5 and p53. The normal expression and activity of all three of these proteins is often compromised in a multitude of cancers.

PLK4 at the intersection of epigenetics and cell signalling: a model

In order to gain a better understanding of how *Plk4* heterozosity impacts global epigenetic patterns, we should consider PLK4's known and potential interacting partners. Thus far it has been determined that PLK4 phosphorylates several cell cycle regulatory proteins such as Cdc25C, Chk2, and p53 [15, 30, 31]. Work presented here also suggests

that PLK4 may have a relationship with JAK2 and TET2 (Ward et al. Chapter 5 dissertation), but there are also several epigenetic modifiers that are targets of PLK4. We propose a model where PLK4 is central to the dissemination of several important epigenetic modifications through its interaction with PRMT5 and DNMT3a (Fig. 6.2). Thus far we have characterized the interaction between PLK4 and PRMT5 (Ward et al. Chapter 4 dissertation). In addition, we know *Plk4*$^{+/-}$ MEFs harbour deregulated levels of PRMT5 and DNMT3a [3](Ward et al. Chapter 4 dissertation). Although we have established a relationship with PRMT5 and PLK4, we still need to elucidate the specific phase of the cell cycle when this interaction occurs. PLK4 is expressed throughout the cell cycle, and its abundance is low in early G_1, and peaks in late G_1, remaining elevated through S and G_2/M [32]. It could be that PRMT5 interacts with PLK4 when PLK4 levels are at their highest. Beyond the PLK4 and PRMT5's interaction, the question then becomes how are symmetric arginine methylation marks impacted by PLK4 phosphorylating PRMT5? With Prmt5 primarily localizing to the nucleus in *Plk4* heterozygous MEFs, but more so at the centrosomes and cytoplasm in wild type MEFs, it suggests that its interaction with Plk4 may be required for proper cellular localization. This can in turn impact its activity and the deposition of arginine methylation (Ward et al. Chapter 4 dissertation). Lastly, PLK4 and PRMT5 are involved in the DNA damage response and both PLK4 and PRMT5 mediate post-translational modifications on p53 at sites associated with p53 activation (Nakamura, et al. Ko et al dissertation). This prompts us to ask whether the nature of their interaction changes with DNA damage?

Plk4 heterozygous MEFs display elevated levels of DNMT3a compared to wild type MEFs [3], raising the question of whether this increase is merely a symptom of *Plk4*

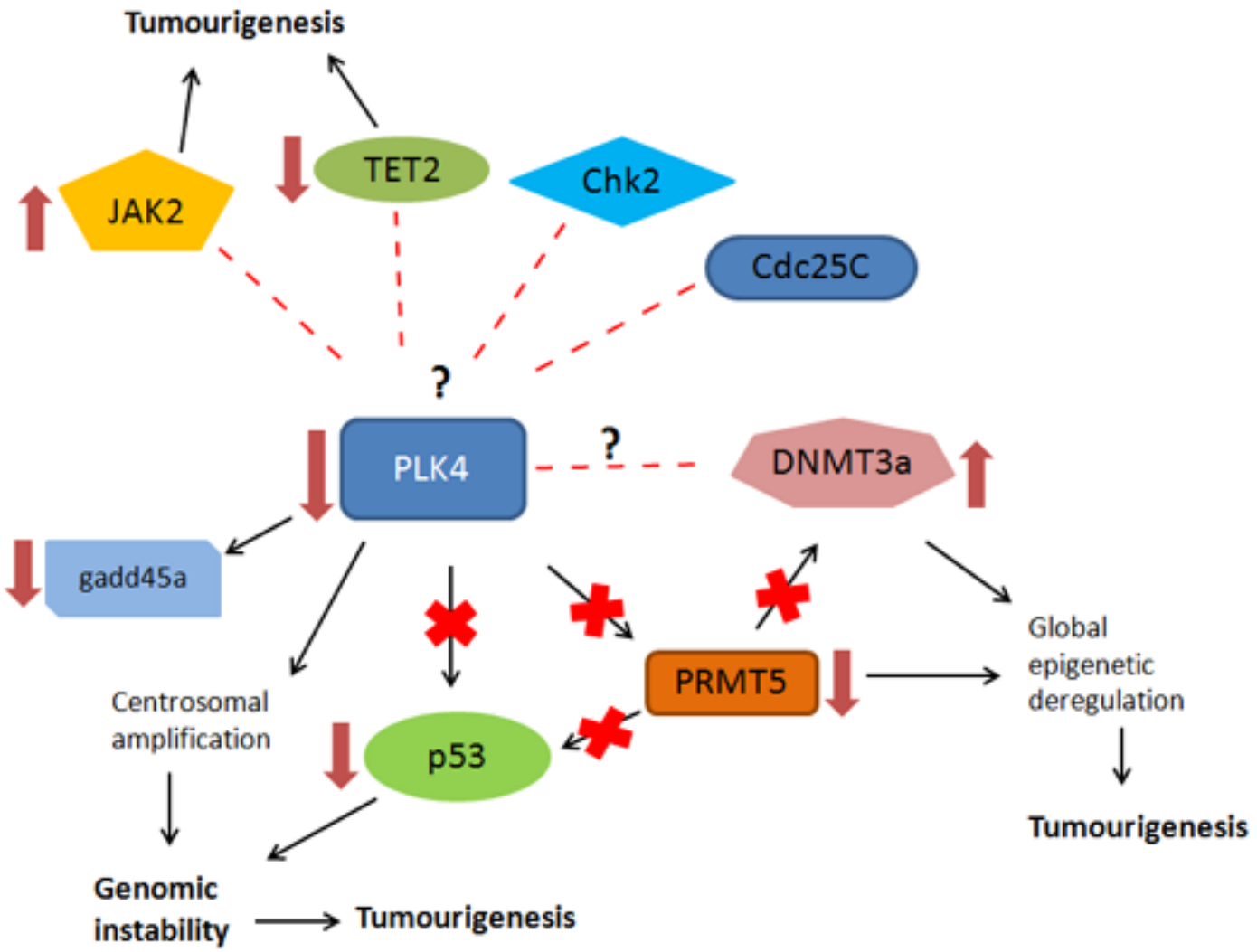
heterozygosity, or whether Plk4 and DNMT3a have a direct relationship? Preliminary work from our lab has established that PLK4 and DNMT3a are interacting partners as indicated in their co-immunoprecipitation from cellular extracts (Ward and Cerghet unpublished). However, we have not as yet, determined the nature of this interaction; whether DNMT3a is a substrate for phosphorylation by PLK4.

Future directions

To address the above questions and scenarios, I propose several experiments. To further elucidate the PLK4-DNMT3a interaction, one would employ approaches including kinase assays to determine if DNMT3a is a substrate of PLK4; as well as using DNMT3a truncation mutants in co-immunoprecipitation experiments to determine the regions required for PLK4 and DNMT3a to interact; ChIP assays with DNMT3a and *PLK4* may help determine if DNMT3a is responsible for the increase in *PLK4* promoter hypermethylation seen in malignancy [3, 4]. It will also be valuable to examine the extent of DNMT3a deregulation in *Plk4* heterozygous MEFs. Is the protein elevation also accompanied by an increase in activity, as well as broad-spread changes in sites of methylation? Initially, to rule out any false results due to interference from the other DNMTs, cells would be treated with mahanine, a plant-derived carbazole alkyloid which specifically inhibits DNMT1 and DNMT3b, but not DNMT3a [33]. Post treatment, an analysis of genome-wide methylation using a non-isotopic cytosine extensions assay. This technique takes advantage of methylation sensitive restriction enzymes, which cleave genomic DNA strictly at unmethylated sites, creating 5'guanine overhangs. Then, using a single primer extension, Cy5 fluorescently labeled dCTP is incorporated into the cleaved site [34]. Recognition sites which have methylated cystosine do not get cleaved

Figure 6.2. Downstream implications of downregulation of PLK4 *via* promoter methylation. This diagram incorporates several of the known targets of PLK4 and those proteins whose interaction have yet to be fully elucidate (Represented by a dotted line and a question mark). It is evident that aberrant downregulation of PLK4 has a domino effect that ultimately ends in tumourigenesis. Of most profound interest is PLK4's interaction with p53, PRMT5 and DNMT3a. The deregulation of these specific pathways have global implications resulting in improper DNA damage response, aberrant global epigenetic marks and the subsequent deregulation of gene expression.

Figure 6.2


Tumourigenesis
JAK2
TET2
Chk2
Cdc25C
?
PLK4
?
DNMT3a
gadd45a
Centrosomal amplification
p53
PRMT5
Global epigenetic deregulation
Tumourigenesis
Genomic instability
Tumourigenesis

and labeled dCTP is not incorporated. This technique could provide an overall view of the global methylation between *Plk4* heterozygosity, compared to *Plk4* wild type MEFs. Dimethylation of histone 3 at arginine 4 (H3R4me2) by PRMT5 acts as a recruiting mark for DNMT3a to mediate *de novo* methylation [35]. In PRMT5 knockdown experiments, loss of H3R4me2 resulted in the loss of DNMT3a binding [35]. It is of note that since *Plk4*$^{+/-}$ MEFs lack symmetric dimethylation marks due to reduced Prmt5 levels (Ward et al. Chapter 4 dissertation), it is likely that DNMT3a activity may not be guided to specific sites of histone methylation resulting in irregular DNA methylation patterns, potentially deregulating the expression of important cell cycle regulatory genes. This could collectively contribute towards tumourigenesis. Interestingly, both PRMT5 and DNMT3a jointly function in regulating gene expression in erythroid progenitor cells [35]. PRMT5 levels are generally reduced in blood neoplasms whose *PLK4* promoters are hypermethylated (Ward et al, chapter 5 dissertation) and DNMT3a is often deregulated in a broad spectrum of hematological malignancies from both lymphoid and myeloid origins [36]. It may therefore be prudent to examine DNMT3a levels in hematological malignancies that displayed aberrant PLK4 methylation patterns.

Further elucidating the nature of the interaction triangle among PLK4, PRMT5, and p53 would be of scientific and clinical interest. In the case of Prmt5, it is mislocalized in *Plk4*$^{+/-}$ MEFs. Therefore, at a more functional level, determining their spatial and temporal association in the cell cycle, with the use of co-immunoprecipitation, flow cytometry, and in the context of *Plk4* heterozygosity would provide insight into the necessity of this pairing as part of normal cell functioning. With regards to the PLK4-PRMT5-p53 interaction axis, a tri-fluor immunofluorescence approach using *Plk4*$^{+/+}$

MEFS would provide an *in vitro* snapshot of how these proteins interact. This assay, in combination with cell cycle synchronization and DNA damage, would use antibodies specific for phosphorylated residues on p53, such as S15 and S392. Additionally, in the presence of DNA damaging agents (UV), immunoprecipitation with an anti-arginine methylated p53 antibody in lysates from MEFs, would determine whether *Plk4* heterozygosity does indeed impede Prmt5-mediated p53 activations *via* arginine methylation.

Additionally, in reviewing the genes deregulated by insufficient Plk4, an interesting pattern is emerging. Recent work from out lab has also identified decreased levels of gadd45a, another epigenetic modifier (Sivakumar, G, Master's thesis). In human cells, gadd45a has been validated as a substrate of PLK4, although the full extent of its biological significance still remains at large (Sivakum G Master's thesis and Wu B Master's thesis). Moreover, microarray data also revealed that *Plk4* heterozygosity was associated with the upregulation of 143 genes, many of which have p53 responsive elements in their respective promoter regions and several are also epigenetic modifiers like sap30 (Sin3) and setdb1[27]. These genes were increased by more than 2-fold compared to wild type MEFs, and both function as transcriptional regulators in conjunction with histone modifying proteins [27, 37, 38]. This suggests that PLK4 has a direct impact on the expression of several important disseminators of epigenetic marks, and one may even be so bold as to suggest that PLK4 may be a master regulator of epigenetic modifications. Conceivably, CpG island hypermethylation of *PLK4* and its accompanied depression in tumourigenesis trigger broad-reaching undulations that could result in the destabilization of many key epigenetic regulatory pathways. Here we

provide a model by which downregulation of PLK4 can have a potentially devastating results on cellular integrity.

Collectively, these studies highlight the importance of the regulatory pathways of the polo-like kinases, and how epigenetic modifications to their respective promoters can impact downstream proteins and ultimately, conventional cell cycle dynamics.

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APPENDICES: Appendix A

Letters of consent

Date: 12 April 2014

I, Sharon Yong, hereby give my consent to R. Alejandra Ward to use data I generated in Chapter 4-PRMT5, A novel substrate of PLK4 is deregulated in *Plk4* heterozygous MEFs in her dissertation. I am aware that she has given me credit for my contributions.

Specifically, I contributed the following data: Chapter 4 MEF localization studies in Wild type and Plk4 heterozygous MEFs. Figure 5.3 panels a-c.

Sincerely,

Sharon Yong
Research Assistant
University of Windsor
Room 300, Biological Sciences
yongs@uwindsor.ca

Date: 11/04/2014

I, Gayathri Sivakumar, hereby give my consent to R. Alejandra Ward to use data I generated in (Chapter 4-PRMT5, A novel substrate of PLK4 is deregulated in *Plk4* heterozygous MEFs or Chapter 5- The deregulated methylation of the *PLK*s in hematological malignancies as a potential clinical biomarker) in her dissertation. I am aware that she has given me credit for my contributions.

Specifically, I contributed the following data:

Chapter 4 Kinase assays with PRMT5 and PLK4; Co-immunoprecipitations of PRMT5 truncation mutants with PLK4

Chapter 5 *In vitro* analysis of *PLK* methylation profile and protein levels in neoplastic cell lines (pre- and post-hypoxia); effect of epigenome-targeting drugs on PLK expression in MDS/leukemia and lymphoma cells in standard and hypoxic conditions

Sincerely,

Gayathri Sivakumar
MSc. Candidate
University of Windsor
sivakum@uwindsor.ca

Date: 12 April 2014

I, Alan Morettin, hereby give my consent to Rosa Alejandra Ward to use data I generated in Chapter 2-Aberrant methylation of Polo-like kinase CpG methylation islands in Plk4 heterozygous mice in her dissertation. I am aware that she has given me credit for my contributions.

Specifically, I performed the experiements which are included in Chapter 2 of her PhD dissertation. I performed all the MSP analysis in human tissue and isolated murine embryonic fibroblasts for the experiments performed.

Sincerely,

Alan Morettin
PhD Candidate
University of Ottawa
1535 Alta Vista Dr. Apt. 614
Ottawa ON
K1G 3N9
amore091@uottawa.ca

Date: April 14, 2014

I, Jordan Nantais, hereby give my consent to R. Alejandra Ward to use data I generated in "Chapter 4 - PRMT5, A novel substrate of PLK4 is deregulated in *Plk4* heterozygous MEFs" of her dissertation. I am aware that she has given me credit for my contributions.

Specifically, I contributed the following data: Chapter 4 mass spectrometry analysis.

Sincerely,

Dr. Jordan Nantais
PGY-2 General Surgery
Dalhousie University
Nantaisj@gmail.com

WINDSOR REGIONAL HOSPITAL

INTEGRATED HOSPITAL LABORATORIES SERVICE WINDSOR - ESSEX

LDMH

Medical Director: Dr. David Shum

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Dr. X. Nguyen, Dr. P.M. Ra, Dr. R. Saad, Dr. S. Shukoor

Our Mission: To provide quality patient centered Laboratory Medicine as an essential member of the health care team

Date: April, 14, 2014

To Whom it may concern:

I, Dr. David Shum, hereby give my consent to R. Alejandra Ward to include my contributions in Chapter 2- Aberrant methylation of *Polo like kinase* CpG islands in *Plk4* heterozygous mice as part of her dissertation.

Specifically, I contributed the following:

In chapter 2, I contributed clinical samples for analysis, and provided consultation and my professional insight where applicable.

Sincerely,

Dr. David Shum
Chief and Medical Director
Department of Pathology and Laboratory Medicine
Windsor Regional Hospital
1995 Lens Avenue| Windsor, Ontario, N8W 1L9
519.254.5577 ext. 52360 | 519.254.6861 (fax) | david.shum@wrh.on.ca

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April 14, 2014

To: Rosa Alejandra Ward,

I, Dr. Sindu Kanjeekal hereby give my consent to R. Alejandra Ward to use data I contributed in Chapter 5- The deregulated methylation of the PLKs in hematological malignancies as potential clinical biomarkers her dissertation. I am aware that she has given me credit for my contributions.

Specifically, I contributed the following:

In chapter 5, I contributed clinical samples for analysis, clinical data and patient life history, and provided consultation and my professional insight where applicable.

Sincerely,

Sindu Kanjeekal, MD
Medical Oncologist/Hematologist
Adjunct Professor, University of Western Ontario
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WINDSOR REGIONAL HOSPITAL
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April 14, 2014

To: Rosa Alejandra Ward,

I, Dr. Caroline Hamm hereby give my consent to R. Alejandra Ward to use data I contributed in Chapter 5- The deregulated methylation of the PLKs in hematological malignancies as potential clinical biomarkers her dissertation. I am aware that she has given me credit for my contributions.

Specifically, I contributed the following:

In chapter 5, I contributed clinical samples for analysis, clinical data and patient life history, and provided consultation and my professional insight where applicable.

Sincerely,

Caroline Hamm, MD
Clinical Director Windsor Cancer Research Group
Assistant Professor, Western
Adjunct Professor, University of Windsor
Clinical Research Director, Windsor Cancer Research Group
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Date: 11/04/2014

I, Anna Kozarova, hereby give my consent to R. Alejandra Ward to use data I generated in (Chapter 4-PRMT5, A novel substrate of PLK4 is deregulated in *Plk4* heterozygous MEFs) in her dissertation. I am aware that she has given me credit for my contributions.

Specifically, I contributed the following data:

Chapter 4 Mass spectrometry data that first identified PRMT5 as a potential substrate of PLK4

Sincerely,

Anna Kozarova, PhD
University of Windsor
kozarova@uwindsor.ca

Date: 11/04/2014

I, Brayden Labute, hereby give my consent to R. Alejandra Ward to use data I generated in Chapter 5- The deregulated methylation of the *PLK*s in hematological malignancies as a potential clinical biomarker in her dissertation. I am aware that she has given me credit for my contributions.

Specifically, I contributed the following data:

Chapter 5 – Western blot analysis of p53 protein levels in human bone marrow aspirates

Sincerely,

Brayden Labute
MSc. Candidate
University of Windsor
labuteb@uwindsor.ca

VITA AUCTORIS

NAME:	Rosa Alejandra Ward
PLACE OF BIRTH:	Tangolona, San Miguel, El Salvador
YEAR OF BIRTH:	1982
EDUCATION:	Stratford Central Secondary School Stratford, ON, 2000
	University of Windsor, B.Sc. with Honours with Thesis, Windsor, ON, 2006
	University of Windsor, PhD Biological Sciences, Windsor, ON, 2014