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# Polo-like kinase 4 at the nexus of epigenetic modifications and the DNA damage signaling network

By

## Gayathri Sivakumar

A Thesis Submitted to the Faculty of Graduate Studies through the Department of **Biological Sciences** in Partial Fulfillment of the Requirements for the Degree of **Master of Science** at the University of Windsor

Windsor, Ontario, Canada

2014

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## Polo-like kinase 4 at the nexus of epigenetic modifications and the DNA damage signaling network

by

Gayathri Sivakumar

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## **Declaration of Co-Authorship**

## Co-Authorship Declaration

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

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

Chapter 2. Dr. Anna Kozarova and Dr. Jordan Nantais contributed to the mass spectrometry data and subsequent analysis in collaboration with Dr. Otis Vacratsis. Alejandra Ward generated the data characterizing the PRMT5 interaction with PLK4 including localization studies. She also performed experiments to determine p53 levels and activity in wild-type and heterozygous MEFs. Sharon Yong was responsible for preliminary work with PRMT5 localization studies in NIH3T3 cells and MEFs.

Chapter 3. Dr. Sindu Kanjeekal and Dr. Caroline Hamm, oncologists from Windsor Regional Hospital, were contributors of this chapter. They provided all clinical samples, patient history and data, and their professional expertise. Alejandra Ward was responsible for generating all the in vivo bone marrow studies. Brayden Labute contributed to analysis of p53 levels in clinical samples.

Chapter 4. This chapter includes data generated by Bing Wu during her Master's studies. She performed co-immunoprecipitation assays to determine the region of PLK4 that was important for GADD45a association.

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#### ABSTRACT

The evolutionarily conserved polo-like kinases (PLKs) are pivotal cellular proteins that govern mitotic progression, DNA damage, centrosome replication, and neuronal processes. Appropriate expression of these serine/threonine kinases is essential for cellular homeostasis as perturbations to *PLK* expression often results in oncogenic transformation and tumor development. In addition to loss of heterozygosity, the *PLKs* are also susceptible to aberrant epigenetic modifications in tumorigenic states. My studies describe the deregulation of the PLKs in the context of hematological malignancies and provide support that the methylationdependent dysregulation may possess diagnostic and prognostic value. Moreover, I have characterized novel interacting substrates (PRMT5 and GADD45a) and interacting partners (Nucleophosmin and JAK2) of PLK4, a polo family member with tumor suppressive functions. These novel interactions implicate PLK4 in epigenetic and DNA damage regulatory pathways that may be essential to preserve and maintain centrosome and genomic integrity.

## DEDICATION

I dedicate this dissertation to the two most wonderful people I have been blessed to have in my life. My sister, Jay Sivakumar, has been my rock, my pillar of support, and my comfort throughout my life. Thank you for always standing by my side to be my strength through the hardest of times, for never letting me fall, and for never failing to bring a smile to my face. You are the light to my life. I am grateful to have Jaz Takhar as one of my best friends. Your belief in me has been unwavering. Your words of encouragement and support will never be forgotten. Thank you for showing me the true meaning of friendship. I truly would not be where I am in life without you both.

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Last, but not least, I want to say a huge thank you to all the wonderful HL'ers of past and present. I would like to first say a special thank-you to Alex Ward. You have been a mentor and a great friend to me throughout my master's. I am forever indebted to you for all the times you've helped me and guided me during my rocky start as a graduate student. To Natalie, Candace, Brayden, Andrei, and Vaishali, you have all been more than just lab-mates to me. I am blessed to have you amazing people as friends. I have wonderful memories filled with laughter, coffee-o'clocks, and Eros which I will always treasure. I love you all and I look forward to our future adventures.

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## LIST OF IMPORTANT ABBREVIATIONS/SYMBOLS

Plk (s)	Murine Polo-like kinase (s) at the gene level
	Human Polo-like kinase (s) at the protein
PLK(s)	level
PLK(s)	Human Polo-like kinase (s) at the gene level
	Murine Polo-like kinase (s) at the protein
Plk(s)	level
<i>Plk4</i> <sup>+/-</sup>	Murine Plk4 heterozygosity

#### Chapter 1

## Introduction

Since the discovery of the mutant of the mitotic *polo* gene in *Drosophila melanogaster* by Sunkel and Glover, a principal family of cell cycle regulators known as the polo-like kinases (PLKs) have come to light [1]. These enzymes function as mitotic serine/threonine kinases and have a broad-spectrum of roles in cell cycle regulation, DNA damage response, and neuronal processes (Reviewed in [2]). The PLKs are notably conserved in the simplest to the most complex of eukaryotes, emphasizing their importance in biological regulatory pathways (Reviewed in [3]). While S. cerevisiae and S. pombe possess a single plk gene named Cdc5 and Plo1, respectively, multiple polorelated kinases have been identified in higher eukaryotes. X. laevis and C. elegans possess four PLKs, while humans and mice have known five members, PLKs1-5. Regulated by temporal and spatial determinants, each member of the polo family has a dynamic expression and subcellular localization profile, unique substrate specificities, and specialized functions in cellular processes (Reviewed in [4]). From development to differentiation, these kinases are involved in many stages of cell cycle progression including mitotic entry, spindle formation, chromosome segregation, mitotic exit, and cytokinesis. Moreover, the PLKs are indispensable for centrosome regulation and stress response pathways (Reviewed in [4]). While it is not well understood whether simple organisms like yeast employ a single PLK to achieve the same functions as PLKs1-5, it seems very likely that additional homologues have evolved in advanced eukaryotes to emplace stringent controls on cell cycle progression and facilitate cells during environmental or physiological stress.

#### General structure and regulation of the Polo-like kinases

The PLKs feature a signature catalytic N-terminal kinase domain and a C-terminal region with conserved amino acid sequences known as the polo-box [5] (Fig. 1.1). The serine-threonine kinase domain is generally thought to be highly conserved amongst the *PLK* genes; however, the most recently discovered PLK family member, PLK5, retains important functions despite having a truncated kinase domain-. At the C-terminus, PLKs 1-3 and 5 have strong homologies in two distinct polo-box modules. As the odd member of the family, PLK4 was recently discovered to have three separate poloboxes, making it the most divergent member. In comparison to the kinase domain, the polo-box domain has been less conserved through evolution as more than half the amino acid sequence of this region varies between species [6].

## Polo-box mediates PLK functions

The non-catalytic C terminal region containing the polo-boxes is a distinct functional entity collectively known as the polo-box domain (PBD). Each of the two polo-boxes of PLKs1-3 and 5 are comprised of ~80 amino acids [7] and are connected by a linker region of ~20 amino acids [8]. The Plks are dependent on the unique structure of the PBD to mediate their localization, kinase activity, and substrate targeting. Mutation of the polo-box domain of PLK1, for instance, was adequate to disrupt its localization to spindle poles, centrosomes, and kinetochores [9, 10]. In addition, both the depletion of PLK1 or overexpression of its polo-box domain led to cell cycle arrest and incomplete cytokinesis which ultimately results in mitotic catastrophe [10, 11].

**Figure 1.1. General structure of the Plks.** This is a representation of the conserved domains and key residues common to the Polo-like kinases, specifically PLKs1-3 and 5. The N-terminal domain contains the kinase domain that includes its activation site. Polobox 1 and 2 reside within the C-terminal domain. This domain also possesses a pSer/Thr ligand binding pocket.



Figure 1.1

Evidence of the PBD as a director of localization signals has been observed in the context of PLK3. Jiang *et al.* (2006) have demonstrated that both of the polo-box motifs of PLK3 are sufficient for localization to its subcellular structures [12]. Similar to PLK1, ectopic expression of PLK3 in U-2 OS cells causes mitotic arrest and failure of cytokinesis [12].

The polo-box binding domain also modulates PLK activity. The PBD naturally acts as an auto-inhibitory domain by binding to the unstimulated kinase domain which diminishes its activity by three-fold (Fig. 1.2a) [13, 14]. Moreover, interaction between the PBD and kinase domain substantially reduces phospho-peptide binding by ten-fold and also decreases the phospho-selectivity of substrates [13-15]. In contrast, direct phosphorylation of the kinase at Thr210 frees the kinase domain from C-terminal interaction [15]. Interaction of the optimal phospho-peptide with full-length PLK1 alone was also sufficient to promote kinase activity by over two-fold. Although the mechanism is undefined, this suggests that PBD binding to a 'primed' substrate must prompt changes in PLK conformation resulting in kinase activation and concomitant substrate phosphorylation.

## Models for PBD-mediated substrate phosphorylation

Yaffe and his colleagues developed a proteomic approach to identify proteins that bind phosphoserine/threonine (pSer/Thr) peptides [16], allowing for the identification of the pSer/Thr binding motif in polo-like kinase 1 [17]. From this study, it became evident that the polo-box domain, including the linker region in between the polo-boxes and a portion of the region in between the kinase domain and the first polo-box, functioned as a phospho-peptide binding module [17]. Cheng *et al.* (2003) further determined that the

phosphorylated ligand-binding site was located specifically at the interface of the two polo-boxes [7]. Elia et al. (2003) proposed that phospho-peptide is likely to be phosphorylated by an upstream priming kinase, such as CDKs/MAPKs, prior to PBD binding [17]. Currently, there are two proposed models for PBD-mediated PLK functions and substrate targeting. The first mode, known as 'processive phosphorylation', requires the pSer/Thr-binding pocket to recognize and interact with a protein that has already received 'priming' phosphorylation by a mitotic kinase. Then, the binding of this protein by the PBD relieves the kinase domain from basal inhibition and most importantly, situates the substrate such that the kinase domain can easily phosphorylate the protein at a nearby site (Fig. 1.2b). This validity of this model has been verified in a number of PLK1 substrates, including Cdc25C [18]. Cdk1 is known to generate priming phosphorylation on Cdc25C and subsequently, bind to the PBD in a phospho-dependent manner [19]. Another substrate of Plx1 in frogs, claspin, has also been verified to use this processive phosphorylation model. In fact, the timing of the two separate events was well-defined where binding of a pre-phosphorylated claspin to PBD was essential for Plx1 to phosphorylate claspin on a proximate site [20]. This processive phosphorylation has also been noted in PLK2 where CDK5 acts as the priming kinase for the PLK2 substrate, SPAR, a spine-associated Rap GTPase activating protein, in neurons [21].

Alternatively, the 'distributive phosphorylation' model suggests that PBD binding of a phosphorylated docking/scaffold protein directs PLK localization to specific subcellular structures where the PLK kinase domain can target proximate substrates or other molecules bound to the scaffold or docking protein itself (Fig. 1.2c) [6]. Although many docking proteins and scaffolds have yet to be discovered, PLK1 is known to have two, Cut23 and Mklp2. Binding PBD, Cut23 localizes to PLK1 to the anaphase promoting complex where PLK1 can target other proteins [22, 23]. Similarly, Mklp2, a kinesin-like protein, moves PLK1 to the spindle to phosphorylate proteins to allow for cytokinesis [24, 25]. This model is consistent with the dynamic subcellular localization patterns and functional complexity of the PLKs which are mediated by the polo-box domain during cell cycle progression.

**Figure 1.2. Models for PBD-facilitated substrate-targeting of the Plks.** a) The PBD naturally represses Plk kinase domain function in the absence of substrates, docking proteins, or scaffolds. b) The processive phosphorylation model requires the pSer/Thr binding motif to bind to a primed substrate. Subsequently, the close proximity to the substrate allows the kinase domain to phosphorylate the substrate on a nearby site. c) Distributive phosphorylation model requires the PBD to be bound to a phosphorylated docking protein or scaffold which alters Plk localization to vicinal substrates at subcellular structures for targeting. *Figure adapted from Lowery et al.*, 2005 [6].





Much remains to be characterized whether known PLK substrates use the processive or distributive phosphorylation mechanism. Although, PLK4 has a ligand binding pocket in its polo-box domain [8], it is unknown whether PLK4 employs either of these proposed models of substrate targeting. Regardless, it is apparent that the PBD has a dual role in regulating PLK kinase activity. Not only does it function as an auto-inhibitory module, but the PBD temporally regulates PLK activity by assimilating the activity of the mitotic kinases with that of PLKs (Reviewed in [6]). Consequently, distinct priming phosphorylation events facilitated by kinases like CDKs and MAPKs are a prerequisite for PLK-mediated functions during mitotic progression (Reviewed in [6]). Moreover, the polo-box domain spatially controls kinase activity. PLK-mediated phosphorylation takes place if and only if the docking proteins bound by PBD positions the kinase domain intimately with the substrate, which may be located at subcellular structures or may bind to the docking protein upon localization.

#### Activation of the Polo-like kinases

In general, protein levels can be regulated at the transcriptional or posttranscriptional level. However, in face of changing external or internal stimuli, activity of protein kinases also needs to be tightly regulated throughout the cell cycle by posttranslational modifications to dictate appropriate cellular responses. Interestingly, protein kinases which phosphorylate proteins are regulated by upstream phosphorylation events; ultimately, this regulation can modify localization, functional capacity, and stability of kinases. Strict control of PLK levels and activity during cell cycle progression is critical for cell viability and proliferation. Dysregulation of their activity causes mitotic and cytokinetic defects which culminate in mitotic infidelities. The intricate control of these important cell cycle regulators are achieved by direct phosphorylation of these enzymes at a conserved activation site, a process similar to other kinases [3, 4, 14, 26]. The PLKs possess several conserved Ser/Thr residues within their kinase domain that includes T210. These residues are critical for kinase function as mutating these amino acids abrogates PLK function. T210 is found within the activation segment in the T-loop of the kinase domain [27]. Phosphorylation of this residue stabilizes the loop and alters the conformation of the domain into an open state for substrate binding. This is generally associated with stimulated kinase activity and this has been seen in many kinases including Aurora A/B, PKA, Cdk1/2, Erk/2, Akt/PKB, and MEK1 [28]. While many of the upstream PLK activating kinases involved in T210 phosphorylation is unknown for PLKs 2-5, human SLK and STK10 was identified as the T210 phosphorylating factor for PLK1 [29, 30].

#### Polo-like kinases: Overview of their functions

## <u>PLK1</u>

The polo-like kinases have emerged as important elements of cell cycle progression, centrosome replication, and genotoxic stress response *via* p53-mediated pathways. The best-characterized member of the family, Polo-like kinase 1, and its orthologues, polo and Cdc5, are known to be putative governing entities of cell division. Transcription and stability of PLK1 is tightly regulated in a cell-cycle dependent manner; PLK1 levels are maintained at low levels in G<sub>1</sub> phase and gradually increase to its peak in G<sub>2</sub>/M phase [31, 32]. The kinase activity of PLK1 is tethered to its phosphorylation status and is most active during mitosis [26, 33, 34]. It interacts with numerous substrates

throughout cell cycle to promote cell cycle progression, centrosome maturation, spindle formation, chromosome segregation, and cytokinesis (Reviewed in [2]). PLK1 localizes to cytoplasmic and centrosomal regions during G0/G1 and to kinetochores, spindle poles, midbody, and cytokinetic bridge in M phase [33]. Loss of PLK1 activity *via* RNA interference or inhibitors prevents spindle formation and microtubule attachment to kinetochores to initiate normal chromosomal segregation. Indeed, knockdown mutants of PLK1, Cdc5, or polo in organisms cause lethality (Reviewed in [2]). Moreover, *Plk1-/*mice embryos undergo cellular arrest at the morula stage and die [2, 35, 36]. Albeit PLK1 is essential for viability, stringent controls to regulate its levels and activity are of utmost importance. As deregulated PLK1 activity supports uncontrolled growth and proliferation, PLK1 functions are inhibited in an ATM-dependent fashion in vulnerable circumstances, such as exposure to genotoxic stress [37].

As enzymes that govern a wide-breadth of cellular processes, perturbations to the expression of the Polo-like kinases can contribute to malignant transformation and tumourigenesis. Oncogenic transformation of cells requires unregulated proliferation-fueling signals. Given that PLK1 promotes the G2/M transition and encourages cellular proliferation, a myriad of tumors and cancer cell lines exhibit elevated mRNA and protein levels of PLK1. It has been speculated that up-regulation of PLK1 allows unregulated cellular proliferation and culminates in malignant transformation and tumor development [38]. In addition, cells displaying aberrantly high PLK1 levels circumvent cell cycle checkpoints and harbour chromosomal abnormalities that contribute to genomic instability and genesis of cancer [39]. Consistent with these findings, PLK1 overexpression has been observed in non-small-cell lung cancer [40], carcinomas

originating in head/neck squamous cells [41], esophagus [42], oropharynx [43], papillary thyroid [44], and endometrium [45], ovarian cancer [46], colorectal cancer [47, 48], pancreatic cancer [49], prostate cancer [50], and melanomas [51]. Moreover, high levels of PLK1 serve as an indicator of poor clinical outcomes in patients. Particularly, PLK1 expression correlates with disease aggressiveness and higher tumor grade in a number of cancers [46, 47, 52]. In clear cell renal cell carcinomas, over-expression of PLK1 had prognostic value and was associated with a greater degree of malignancy in patients [52]. Depletion of PLK1 activity *via* small-molecule inhibitors of PLK1 was sufficient to significantly reduce cellular proliferation in renal cell carcinoma-derived cell lines and cause substantial tumor regression in xenograft nude mouse models [52].

Questions of whether PLK1 overexpression is a causative factor or a consequence of malignant transformation were answered through a number of studies. Ectopic expression of Plk1 in murine fibroblasts by Smith *et al.* (1997) resulted in morphological transformation including high frequency of colony formation on soft agar and ability to induce tumours in nude mice [53]. Correspondingly, down-regulation of PLK1 in osteosarcoma cells inhibited colony formation [54]. Ito *et al.* (2004) has also shown that PLK1 up-regulation in thyroid carcinomas is an early event that drives cellular transformation and malignancy [44]. The mechanism behind how PLK1 might mediate this process has been the focus of many researchers.

P53 tumor suppressor, the master protector of our genome, is indispensable for processes such as DNA repair, cellular arrest, and apoptosis, to name a few [55]. It is also known to be mutated or altered in at least fifty-percent of human cancers [56]. Interestingly, accumulating evidence suggests that p53 and PLK1 directly and indirectly

antagonize one another in signaling networks. P21/Waf1, a p53-effector gene, inhibits PLK1 expression by targeting the repressive elements of the PLK1 promoter [57]. Transcriptional activity of *PLK1* is also negatively regulated by p53-mediated inhibition of *PLK1*'s transcription factor, FoxM1 [58]. P53 is able to directly interact with the p53 response elements at the *PLK1* promoter and disrupt gene expression [59]. Reciprocally, PLK1 physically associates with p53, phosphorylates it, and disrupts its transactivation and apoptotic activity [60]. Activity of MDM2, a E3 ubiquitin ligase, which is responsible for the inhibition and degradation of p53, is further stimulated by PLK1 [61]. Additionally, high levels of PLK1 in cells correlates with decreased activation of p53 at Ser15 during DNA damage [62]. Taken together, these findings suggest that when the appropriate balance between p53 and PLK1 levels is lost as a result of non-functional p53 or disruption to the p53 signaling network, PLK1 levels become unregulated to promote oncogenesis.

The amalgamation of studies from neoplastic cell lines and patient specimens has helped to identify PLK1 as a hallmark of cellular proliferation and consequently, PLK1 has proven to be a useful diagnostic and prognostic indicator, suggesting that it has merit as a potent therapeutic target for cancers.

## <u>PLK2</u>

Polo-like kinase 2 has been implicated in centrosome duplication and in the DNA damage response pathways [63-65]. In the cell cycle, PLK2 is activated in the G1/S transition and its' levels and kinase activity peak during S phase, likely to monitor the replication of centrioles. During the centrosome cycle, concerted localization of PLK2

takes place at the centrosomes as kinase activity of PLK2 is critical for procentriole formation. Ectopic expression of kinase-deficient PLK2 as well as silencing PLK2 with small hairpin RNAs halted centriole duplication in mammalian cells [64]. Upon exposure to mitotic poisons such as paclitaxel or nocodazole, *PLK2* is also known to be activated in a p53-dependent manner to avert spindle damage-associated mitotic aberrancies [65]. While the importance of PLK2 function has been recognized, Ma *et al.* (2003) revealed that Plk2-null mice were viable although the embryos exhibited growth retardation and slightly delayed development [66]. Interestingly, as the expression and localization pattern of Plk3 is similar to Plk2, it has been speculated that Plk3 might serve to balance the functions of Plk2 in Plk2 knockdown mutants [66]. As a transcriptional target of p53 and an interacting partner of Chk1 and Chk2, PLK2 is involved in the cellular response to DNA damage and S-phase checkpoints [67]. There are also unique, emerging roles for PLK2 in synaptic homeostasis, neuronal plasticity, and differentiation [21, 68].

Given that PLK2 is implicated in DNA- and spindle-damage response to facilitate cell cycle arrest and prevent mitotic catastrophe, it is largely known to have tumor suppressive roles. Expression of PLK2 is down-regulated in blood-related neoplasms including Burkitt's lymphomas, acute myeloid leukemias, and B-cell malignancies and ovarian cancers [69-71]. Specifically, the deregulation of PLK2 in tumors seems to occur through an epigenetic mechanism.

Expression of PLK3 has been observed in all stages of the cell cycle although it is most abundant during mitosis [72]. The kinase activity of PLK3 peaks during late S and G<sub>2</sub> phase and is also stimulated during DNA damage [73]. Localization of PLK3 has largely been concentrated to the nucleolus [74]. PLK3 is implicated in the transition from G<sub>1</sub> to S phase for DNA replication and entry into mitosis *via* PLK3-dependent phosphorylation and nuclear translocation of Cdc25C phosphatase [74, 75]. Similar to PLK2, PLK3 responds to signals that produce synaptic plasticity and prompts long term potentiation [76]. More notably, PLK3 is known as a stress response protein that is activated in an ATM- and Chk2-dependent manner to propagate p53 activity to coordinate cell cycle arrest and apoptosis [77]. Evidence for PLK3 as a tumor suppressor is substantiated by its ability to promote p53 stabilization *via* phosphorylation at Ser20 [77]. The importance of PLK3 as a mediator of cellular response to environmental and physiological stress is also emphasized by the high susceptibility of *Plk3-/-* mice for tumor development with old age and angiogenesis in comparison to normal mice [78].

Analysis of PLK3 in tumor specimens shows down-regulation of this kinase in head, neck, lung, and colon cancers, suggesting that lower levels of PLK3 may be associated with tumor development [79-81]. Exogenous expression of Plk3 in NIH3T3 mice fibroblasts induces apoptosis and abrogates cell growth, which are collectively suggestive of its tumor suppressive qualities [82, 83].

#### <u>PLK5</u>

Recently, a fifth member, PLK5, was added to the family of polo-like kinases [84]. Interestingly, although this protein has a truncated kinase domain and a stop codon

in the middle of the protein, the PLK5 protein is still functional. Andrysik *et al.* (2010) demonstrated activation of PLK5 following exposure to wide range of cellular insults, however, the mechanism behind this unclear [84]. It is speculated that ATM, ATR or DNA-dependent protein kinases may be involved in the phosphorylation and activation of PLK5 as SQ/TQ cluster domains have been identified in the PBD. Ectopic expression of PLK5 causes cellular arrest at G<sub>1</sub> phase, decreased DNA replication, and apoptotic cell death, suggesting it may function as a tumour suppressor [84]. Similar to PLK2, PLK5 has also been noted to participate in neuronal differentiation [85]. Interestingly, PLK5 is widely transcriptionally silenced *via* DNA methylation in brain cancers such as astrocytomas and glioblastoma multiforme [85].

## Expression of PLKs in tissues

The polo-like kinases are differentially regulated and expressed in a tissuespecific manner. Given the essential roles of PLK1 and PLK4 in cell survival and cellular division, they are expressed abundantly in embryonic tissues which are highly proliferative (Reviewed in [2]). PLK1 and 4 have also been abundantly detected in the testis, spleen, and bone marrow [31, 32, 86]. Naturally, adult tissue specimens including liver, kidney, brain, and heart displayed undetectable levels PLK1 and 4 considering their low levels of cell turnover [31, 32, 86].

PLK2 and PLK3 have a wide-breadth of expression in both proliferative- and non-proliferative tissues. In humans, polo-like kinase 2 has been detected in testis, mammary gland, spleen, brain, heart, uterus, and trachea [87]. Provided that roles of PLK2 have been delineated in the neuronal network, expression of this protein has been reported in the central nervous system, cerebral cortex, hippocampus, occipital, temporal and parietal lobes, putamen, and ganglion (Reviewed in [2]). PLK3 mRNA is expressed in skin, lungs, trachea, bronchus, intestinal mucosa, brain, placenta, and ovary [76, 79, 88]. Oddly, out of all the PLKs, PLK5 is the only polo member that is expressed solely in non-proliferated, highly differentiated tissues. This includes the central nervous system, particularly the neurons of the brain cortex and glia cells, and the cerebellum [85].

## Polo-like kinase 4: Background of its structure and functions

#### The architecture of Polo-like kinase 4 (Sak/Plk4)

As aforementioned briefly, Polo-like kinase 4 (Plk4) is the most divergent member of the family with many structural variations and weak sequence homology with the other PLKs. While PLK4 has the canonical kinase domain, it was thought to possess only one polo-box at the carboxy-terminal. This polo-box appeared to mediate homo-dimerization and allows for weak centrosome localization [8]. PLK4 features a unique structural component termed the "cryptic polo-box" (CPB) which was a large central module implicated in centriole localization and binding the kinase domain in *trans* [8]. Recently, Slevin and her colleagues (2012) made a striking discovery that the CPB was actually composed of two distinct polo-boxes (PB1 and PB2), rendering PLK4 to have *three* tandem PBs in the C-terminal domain (Fig. 1.3) [89]. The PB1-PB2 cassette was found to be important for homo-dimerization with another PLK4, Asterless and Cep152 binding, robust recruitment of PLK4 to centrosomes, and PB1-PB2 homodimerization-mediated *trans* autophosphorylation of PLK4 for subsequent ubiquitination and degradation by the SCF<sup>Slimb</sup> ubiquitin ligase complex (Fig. 1.4) [89, 90]. In addition,

structural alignment of PLK1 PB1 bound to a phospho-peptide with PLK4 PB1 revealed that the ligand is positioned at the interface of PLK4 PB1 and PB2 [89]. It remains to be determined whether substrates or docking proteins bind to this region for targeting by PLK4.

**Figure 1.3. Structural architecture of Polo-like kinase 4.** The most divergent member of the PLK family, PLK4 contains three unique polo-box motifs. These polo-boxes mediate important functions of PLK4 in centrosome biology and auto-regulation. Unlike the other PLKs, PLK4 possesses three different PEST sequences that regulate protein stability and turnover rate of the kinase. The multiphospho-degron region refers to sites targeted by PLK4 during the *trans*-autophosphorylation event.




Centrosome localization; Plk4 trans-autophosphorylation

## The functional significance of Plk4 in a mouse model

Polo-like kinase 4, also known as SAK, is a master regulator of centriole formation and more recently, evidence has been accumulating for possible roles in damage control during stress [91][Ward et al., 2014 Chapter 4 dissertation]. PLK4 exhibits a dynamic pattern of subcellular localization that presages its functional intricacy during cell cycle: it is found at the nucleolus in  $G_2$ , at the centrosomes in  $G_2/M$ , and at the cleavage furrow during cytokinesis [92]. Plk4-/- mouse embryos are non-viable as they display severe mitotic defects and increased apoptosis after gastrulation at E7.5 [92]. Plk4 deficient cells also had high levels of cyclin B1 in late stages of anaphase and telophase, suggesting that Plk4 is essential for degradation of cyclin B1 by the anaphase-promoting complex and that post-gastrulation, Plk4 was necessary for mitotic exit [92]. Mice with only one functioning allele of *Plk4* were generated as a model to further dissect the roles of Polo-like kinase 4 [92]. Interestingly, despite the presence of one functioning allele, Plk4+/- mouse embryonic fibroblasts (MEFs) displayed mitotic aberrancies including improper spindle formation, centrosome amplification, and aneuploidy. In addition, the Plk4 haploinsufficiency in these mice ultimately led to the development of spontaneous lung and liver tumors at a rate that was 15 times higher than what was observed in wildtype mice [93].

Partial hepatectomy of Plk4 heterozygous mice liver caused delayed entry into and exit out of mitosis in regenerating cells [93]. Ko *et al.* (2005) suggested that the diminished control of cell cycle transitions seen with reduced Plk4 levels may promote aberrant spindle pole formation and chromosome abnormalities in *Plk4*+/- regenerating liver [93]. The mitotic defects, supernumerary centrosomes, and chromosome instability associated with *Plk4+/-* MEFs has also been demonstrated to be an outcome of cytokinetic failure [94]. Reduced dosage of Plk4 in heterozygous MEFs impairs the localization of Rho GEF Ect2 to the cleavage furrow which prevents RhoA activation, culminating in poor, unstable organization of the actomyosin contractile ring, incomplete cytokinesis, mitotic errors, and loss of chromosomal integrity [94]. Taken together, these studies highlight the importance of the tumor suppressive roles of PLK4 in preserving mitotic integrity and that dysregulation of PLK4 levels can contribute to tumourigenesis.

### PLK4 as an important protein in stress response

While PLK4 has been predominantly associated with centrille reproduction, accumulating evidence suggest PLK4 may be a key player in the signal transduction pathways in response to cellular insults. Co-immunoprecipitation and *in vitro* kinase assays confirmed Chk2 to be an interacting partner and substrate of PLK4 [95]. Chk2 is activated by ATM and ATR in response to genotoxic stress to target important cell cycle proteins like Cdc25C to cease cell cycle progression [96, 97]. Implication of PLK4 in DNA damage pathways is further underscored by the interaction of PLK4 with p53, the guardian of the genome [98]. Ko et al. (Dissertation) has demonstrated that p53 is a substrate of PLK4 and PLK4-dependent phosphorylation at Ser392 may further propagate p53-mediated DNA damage signal transduction [91]. In Plk4+/+ MEFs, p53 levels and activity are initially low but eventually rise with increasing duration of UV dosage and peak at 8 h post-damage [91]. Additionally, when p53 levels peak during damage response, in a negative feedback loop, p53 inhibits PLK4 through recruitment of DNA methyltransferase 3A (DNMT3a) and histone deacetylases (HDACs) which synergistically facilitate transcriptional repression [99]. This critical event safeguards

cells against PLK4 hyperactivity and associated centrosome amplification. Conversely, *Plk4+/-* MEFs initially have higher levels of p53 and with increasing measures of UV treatment, p53 levels and activity become substantially reduced in comparison to the wild-type counterparts (Ward *et al.*, 2014 Chapter 4 dissertation).

Nakamura *et al.* (2013) recently discovered novel regulation of PLK4 through the stress-activated protein kinase kinase kinases (SAPKKK), MTK1/MEKK4 pathway [100]. MTK1 activates PLK4 activity *via* phosphorylation at T170 residue with exposure to stress stimuli. This specific phosphorylation event of PLK4 triggers an increase in AKT (protein kinase B) phosphorylation at its catalytic site, T308, to promote cell survival during stress [100]. However, unregulated activity of PLK4 can lead to centrosome amplification. Specifically, regulation of cell cycle progression and centrosome duplication during DNA damage is of paramount importance to prevent mitotic catastrophes which can potentiate malignant transformation in cells. As a way to balance these conflicting outcomes under stress cues, MTK1 inhibits the centrosomal functions of PLK4 and prevents centrosome amplification through an unknown mechanism [100]. Moreover, to further substantiate the PLK4-suppressing role of p53, unabated stress from exposure to etoposide and ultra-violet radiation mitigated PLK4 levels in a p53-dependent manner.

### The self-governing capacities of PLK4 and other modes of PLK4 regulation

While being short-lived with a half-life of 2-3 hours, PLK4 is a master regulator of centrosome dynamics. Depletion of PLK4 as well as ectopic expression of PLK4 results in supernumerary centrosomes, improper chromosome segregation, and aneuploidy in cells [93, 101]. Naturally, it becomes important for cells to maintain appropriate levels and activity of PLK4 to prevent mitotic aberrancies. Likely to safeguard cells from its own activity, PLK4 self-regulates its kinase activity. Auto-regulation of PLK4 is directed by three PEST sequences; these peptide sequences are rich in proline (P), glutamine (E), serine (S), and threonine (T) [102]. One PEST sequence is located near the kinase domain while the other two are near at the tail-end of the carboxyterminal domain. Mutation of these regions results in increased protein stability; early studies specifically revealed that loss of the first PEST sequence confers a greater amount of stability in comparison to the others. Utilizing chemical genetics, Holland et al. (2010) demonstrated that a kinase-active mutant of PLK4 is destabilized and quickly targeted for proteolytic degradation [103]. Conversely, expression of the kinase-dead version of PLK4 increased protein stability and caused the formation of supernumerary centrosomes in cells. Co-expression of the kinase-dead PLK4 with the kinase-active PLK4 substantially diminished the stability of kinase-dead PLK4 and its associated phenotypes. Holland and colleagues (2010) also provided evidence for kinase-mediated selfdestruction of PLK4 via autophosphorylation of numerous sites, specifically a 13-site multi-phosphodegron that encompasses the first PEST sequence [103]. Intriguingly, this region contains a  $\beta$ -*TrCP* binding site which mediates substrate recognition for the SCF<sup> $\beta$ -</sup> <sup>TrCP</sup> E3 ubiquitin ligases via F-box protein  $\beta$ -TrCP. As briefly mentioned above, this

occurs specifically during homo-dimerization of PLK4 that is facilitated by PB1-PB2 [89]. This in turn triggers PLK4 to *trans* auto-phosphorylate itself, leading to loss of protein stability, ubiquitination, and proteosomal degradation (Fig. 1.4) [89, 103]. Specifically, this auto-phosphorylation event is known to be triggered by PLK4 to prevent centriole amplification [103]. A follow-up study by Holland *et al.* (2012) further revealed that this auto-regulation of PLK4 persists endogeneously to maintain appropriate levels of PLK4 and limit centrosome replication to once per cell cycle [104]. Loss of PLK4 kinase activity culminates in centriole amplification, insults to genomic integrity, and interestingly, a proliferation-arrest in a p53-dependent context [104].

Alternative modes of regulation also exist in cells to modulate PLK4 levels and activity. Classically, the activity and stability of protein kinases are governed *via* phosphorylation by upstream regulatory kinases in a temporally-dependent manner. Phospho-dependent stabilization of PLK4 by Tec tyrosine kinase has been reported to prompt an activating PLK4 auto-phosphorylation event [105]. More recently, as previously mentioned, stress-induced MTK1 SAPKKK is able to stimulate PLK4 activity. Transcriptional control of *PLK4* by p53-mediated histone deacetylation has also been identified as an additional mechanism to tightly regulate PLK4 levels and the numeral integrity of centrosomes [99].

**Figure 1.4. Polo-box domain-mediated functions and regulation of PLK4.** Polo-like kinase 4 has three structurally unique polo-box motifs. Collectively, polo-box 1 (PB1) and polo-box 2 (PB2) cassette are responsible for homo-dimerization of PLK4 molecules, interaction with Asterless and Cep152 for centriole duplication, and robust centrosome localization. PB1 and PB2 are important for *trans* auto-phosphorylation of PLK4 for subsequent ubiquitination and degradation by the SCF ubiquitin ligase. This occurs to limit centriole duplication to once per cell cycle. Polo-box (PB3) contributes to the localization of PLK4 to the centrosomes. *Figure adapted from Slevin et al., 2012.* 



Figure 1.4

### Epigenetic aberrancies in cancer

Classically, the term 'epigenetics' refers to heritable alterations to gene expressions that occur through non-genetic changes [106]. The field of epigenetics has received much attention over the past few decades with the knowledge that epigenetic aberrations, which often contribute to the initiation and progression of diseases, can ensue upon exposure to environmental factors. Interestingly, unlike genetic abnormalities, the epigenomic landscape which is regulated by DNA methylation and histone modifications can be reversed in a clinical setting to treat patients.

### DNA methylation and histone modifications

Methylation, catalyzed by DNA methyltransferases (DNMTs), takes place at cytosine residues found in CpG dinucleotides, where methyl groups are added on to the 5' carbon position of the cytosine ring [107]. Enriched sites with CpG nucleotides, known as CpG islands, are located at approximately 60% of gene promoters in the human genome [108]. Generally, accumulation of 5-methylcytosines at CpG promoter islands is associated with transcriptional repression and gene silencing [109]. Suppression of transcriptional activity is speculated to occur through three mechanisms. Firstly, the bulky methyl groups can hinder the binding of transcriptional factors to recognition sites in the promoter regions and directly inhibit transcriptional activity [110]. Secondly, the methyl groups are able to recruit and bind to methyl cytosine binding (MBD) proteins. These proteins have methyl-CpG binding domains along with a transcriptional repressor domain which can inhibit the assembly of essential components of transcription, including the initiation complex [111]. Lastly, histone deacetylase (HDAC) complexes,

which displace acetyl groups on histone tails and remodel the DNA structure into a condensed and inactive form, are also known to associate with methyl binding proteins (Fig. 1.5) [112, 113]. Therefore, methyl groups are able to prompt chromatin remodelling *via* covalent histone modifications to form a compact repressive chromatin structure that is inaccessible by transcription factors. In the absence of methyl groups, histone acetylases (HATs) can place acetyl groups and lower the affinity between histones and the DNA backbone, allowing the chromatin to be in an open, transcriptionally-active state.

### Aberrant DNA methylation contributes to cancer

Nearly all tumor types have significant hypermethylation of CpG islands at promoter regions of genes while CpG sites are generally unmethylated in normal tissues. Genome-wide methylation screening has identified important proteins involved in cell cycle regulation, DNA repair, apoptosis, cell adhesion, and hormonal homeostasis to be deregulated through epigenetic means in human cancers [114]. Classifying the methylation pattern, or the methylotype, for each tumor type and even, subtypes has been an invaluable diagnostic and prognostic tool for clinicians. Hypermethylation of *BRCA1* is a hallmark of ovarian and breast cancer [114]. More prominent is the methylationassociated inactivation of tumor suppressors p16INK4a and p15INK4b in lymphomas and leukemias, respectively [114-116]. Interestingly, silencing of p15INK4b is associated with high-risk cases of myelodysplastic syndromes (MDS) and MDS-derived leukemia and greater degree of disease progression while patients with hypomethylated p15INK4b have a promising clinical outcome [116].

**Figure 1.5.** Methylation-associated transcriptional inactivation of genes. DNA methylation of the CpG sites at the promoter region is closely associated with transcriptional silencing of the gene. Methyl groups attract methyl binding proteins (MBDs) which further recruit histone deacetylases (HDACs). HDACs facilitate transcriptional repression by remodelling the chromatin to a closed and condensed state, preventing access of DNA to transcription factors. In contrast, absence of methyl-cytosines allow for histone acetylases (HATs) to transform the chromatin to an open and transcriptionally active condition. *Figure adapted from Grønbaek et al.*, 2007 [131].





There is substantial crosstalk between epigenetics and genetics. Loss of genomewide methylation in DNA methyltransferase-null mice causes destabilization of the centromeric and pericentromeric regions, predisposing cells to loss of heterozygosity and genomic recombination [117, 118]. Similarly, genomic aberrations such as chromosome translocation have also been known to attract methylation [119]. Epigenetic and genetic defects synergistically act as causative agents of malignant transformation and augment tumor development [120]. In accordance with Knudson's multiple-hit hypothesis, accumulated mutations that confer growth and survival advantages are requisites for tumorigenesis. A number of studies have demonstrated that the initial step towards development of cancer occurs through the epigenetic lesions that will further propagate the collection of genome-wide mutations [121, 122]. Aside from focal epigenetic changes that alter the expression of oncogenes and tumor suppressors, there are two modes through which changes in DNA methylation can promote cancer: 1) point mutation of genes via spontaneous deamination of methylated cytosine sites and 2) chromosome instability as a result of genome-wide demethylation. Methyl-cytosine sites can serve as endogenous mutagens as they are susceptible to spontaneous deamination to thymine [123]. If the C-to-T transitions are not corrected appropriately by DNA repair machinery, the point mutation can alter the function of the gene and contribute to cancer [124, 125]. Intriguingly, Rideout and colleagues (1990) have shown that many of mutations occurring in p53 take place at CpG regions as a result of C-T transitions [125]. In contrast to their normal counterparts, analysis of a wide-range of neoplastic cells and tissues has also revealed global loss of methylation in the genomic DNA accompanied by hypermethylation of local regions [126-128]. Importantly, lower levels of methylcytosine content have been associated with chromosomal rearrangements, gene dosage imbalances, and loss of imprinting, all of which contribute to disease initiation and progression [127]. Given that a number of studies examining tumorigenic cell lines and patient specimens have demonstrated that aberrant DNA methylation is a product of deregulated DNA methyltransferase activity, it underscores that this epigenetic phenomena may be a key driver of tumor development [129, 130].

# The polo-like kinases are epigenetically-regulated

The canonical hypermethylation-mediated silencing of tumor suppressors has been evident in a number of cancers including lung, bladder, ovarian, breast, lymphoma, myelodysplasia, gastric, and colon (Reviewed in [131]). The polo-like kinases, known to be critical for cell cycle regulation and preservation of mitotic and genomic integrity, are genes that are susceptible to such epigenetic regulation. Fluctuations in the PLK protein levels as well as abnormal regulation of their functions cause mitotic infidelities that have been linked to oncogenesis and tumor development. Perturbations in PLK2 protein levels are associated with malignancies such as lymphomas, myelodysplastic syndromes and leukemias [69, 70, 132]. Syed et al. (2006) has reported that transcriptional silencing of *PLK2 via* methylation is a frequent event in Burkitt's lymphoma [69]. Interestingly, promoter methylation of *PLK2* has been correlated with resistance to chemotherapy with paclitaxel and carboplatin and greater susceptibility for relapse in patients with ovarian cancer [132]. Promoter-associated CpG islands of *PLK1-4* are epigenetically modified in human hepatocellular carcinoma. Pellegrino and others (2010) have shown that in human hepatocellular carcinoma, *PLK1* is hypomethylated and up-regulated, while *PLK2-4* have promoter methylation and are down-regulated [133]. In brain cancers such as

glioblastomas and astrocytomas, *PLK5* gene also undergoes extensive promoter methylation that abrogates gene expression [85].

Our lab has demonstrated that the *Plk4* gene promoter is also a recipient of epigenetic modifications with increasing age in *Plk4* mutant mice [134]. Down-regulated expression of Plk4 as a result of hypermethylation was associated with the development of hepatocellular carcinomas in these mice. Interestingly, livers isolated from young  $Plk^{+/-}$  mice display significantly higher levels of global methylation in comparison to wild-type mice; in contrast, aged *Plk4* heterozygous male mice exhibit lower levels of global methylation in comparison to its wild-type counterpart [134]. More recently, Ward and Hudson (2014) have shown that changes in epigenetic marks of the *PLKs* can be induced in conditions of oxidative stress including exposure to hypoxia and reactive oxygen species which are key elements of the tumor microenvironment [135]. These findings underscore that not only can epigenetically-induced changes of the *PLKs* contribute to the establishment and progression of cancer, but that the methylation marks and expression of the *PLKs* can also have clinical value.

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### Chapter 2

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

This chapter includes data generated by Alejandra Ward, Dr. Jordan Nantais, Dr. Anna Kozarova, and Sharon Yong.

#### **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, PLKs 1-4 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  $\alpha$ -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  $\chi$ -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 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<sup>-/-</sup>* 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<sup>+/-</sup> 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 deregulation of Plk4 levels has downstream impacts on normal epigenetic modifications in vitro and in vivo [23, 25]. Plk4<sup>+/-</sup> MEFs have higher levels of DNA methyltransferase 3A (DNMT3A), an enzyme responsible for de novo methylation, before and after oxidative stress [25]. Furthermore, Plk4<sup>+/-</sup> 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 down-regulated through DNA hypermethylation [23]. It is therefore of importance to identify PLK4 downstream targets 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 coimmunoprecipitation indeed showed Flag-PLK4 was interacting with PRMT5 under these conditions (Fig. 2.1a). 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. 2.1b). 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 polobox domain [27]. This tri-polo-box architecture facilitates PLK4 activities such as oligomerization, auto-phosphorylation 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. 2.1c). Based on our co-immunoprecipitations, all of the N-terminal mutant constructs interacted with PRMT5 (Fig. 2.1d) 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. 2.1e). Flag-YVH1, a phosphatase, was used as an additional control for non-specific binding (Fig. 2.1d). 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 nonhistone proteins at arginine residues, and as a type II arginine methyltransferase, it primarily catalyzes the symmetric transfer of methyl groups onto arginine [30, 31]. PRMT5 can mediate transcriptional repression at the histone level by methylation of H3R8 and H4R3, [32] which in turn, recruits the DNA methylating protein, 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].

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Figure 2.1. PRMT5 interacts with PLK4's N-terminal domain. (a) PRMT5 Coimmunoprecipitations 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) 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. (c) 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.  $\Delta PB$  encompasses the entire kinase domain without the polo box domain. 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. (d) To characterize the interaction between PRMT5 and PLK4, transiently transfected N-terminal mutants were pulled down with PRMT5 antibody. (e) A PRMT5 co-immunoprecipitation with lysates from transiently transfected C-terminal domain truncation mutants. (f) Endogenous PLK4 co-immunoprecipitation was performed with PRMT5 antibody with lysates from p53 null cells.





Additionally, PRMT5 has a role in the DNA 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. 2.1f), indicating that p53 was not required for the interaction.

# PRMT5 is a novel 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 targeted by both wild type and kinase active forms of PLK4, thus indicating that PRMT5 is a substrate of PLK4 (Fig. 2.2a). 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. 2.2a). As expected, auto-phosphorylation of both full length and kinase active forms of PLK4 were also observed (Fig. 2.2a) 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. 2.2b).

### 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 Tim barrel at the aminoterminal lobe of one monomer and the carboxy-terminus  $\beta$ -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 methyltransferase 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 aminoterminal of PRMT5 provides a platform for substrate interaction through MEP50, while the carboxy-terminal domain encompasses the salient catalytic core possessing the Sadenosylmethionine (AdoMet)-dependent arginine methyltransferase activity of PRMT5 [40]. Undoubtedly, the active site of PRMT5 is indispensable for its functions in several cellular processes, including the modulation of signaling pathways and gene expression.

**Figure 2.2. PRMT5 is a 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 [γ-<sup>32</sup>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.





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  $\Delta N$ -terminal (amino acids 1-291)-PRMT5, a deleted arginine methyltransferase domain  $\Delta$ MTD (amino acids 365-369)-PRMT5, which causes the protein to lose its enzymatic activity, and a  $\Delta C$ -terminal (amino acids 292-637)-PRMT5 (Fig. 2.2c). Surprisingly, results from the immunoprecipitations displayed PLK4 interaction with all three mutated constructs of PRMT5 (Fig. 2.2d). 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 Cterminus possesses four Ser and four Thr phosphorylatable sites. It is 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 axis 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 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 assay to determine whether PLK4 is detected in the immuno-complexes of proteins with symmetric arginine methylation. Our results showed PLK4 to be undetectable, suggesting that under these conditions PRMT5 does not confer any post-translational arginine methylation marks on PLK4 (data not shown). This suggests that the functional 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. 2.3a). 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 cytokinesis [29]. In wild type MEFs, PRMT5 was also detected in the nucleus and the cytoplasm, but PRMT5 also colocalized to the centrosomes in more than 85% of the cells examined (Fig. 2.3b, g). **Figure 2.3. Plk4 heterozygosity impacts Prmt5 localization.** Mouse fibroblasts were used to examine the endogenous localization of Prmt5. Hoechst 33342 was used to stain the nucleus, γ-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-f) *Plk4*<sup>+/-</sup> MEFs were transiently transfected with Flag empty vector, full length PLK4, and the KD PLK4 mutant. (g) 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.



In contrast in  $Plk4^{+/-}$  MEFs, we found that on average, that approx. 10% of the cells examined had PRMT5 localizing to the centrosome (Fig. 2.3c,g) with the majority of PRMT5 localized to the nucleus in  $Plk4^{+/-}$  MEFs (Fig. 2.3c,g). In order to further establish a role for Plk4 levels in PRMT5's localization patterns, we examined whether Plk4 over-expression on 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. 2.3e,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. 2.3f, g). The PRMT5 localization in  $Plk4^{+/-}$  MEFs transfected with Flag-vector alone remained primarily in the nucleus similar to untransfected cells (Fig. 2.3d, 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. 2.4a). This indicates that 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. 2.4a). PRMT5 protein levels were 50% lower in *Plk4* heterozygous MEFs than Plk4 wild type MEFs (Fig. 2.4b), 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<sup>+/-</sup> MEFs had almost no detectable arginine methylated proteins (Fig. 2.4c, d) [52]. PRMT5 catalyzes the formation of symmetric dimethyl arginine residues in proteins. Our findings indicate 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 playing a role in both the localization of PRMT5 and its levels.

 $Plk4^{+/-}$  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*<sup>+/-</sup> 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].

**Figure 2.4.** *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) 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. (c, d) 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 2.4

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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. 2.5a). 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. 2.5a), 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. 2.5b). The role of p53 during DNA damage is to either mediate DNA damage repair pathways, or, if the damage is too great, 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 (yH2AX), a marker of p53 activity, which generally increases in response to DNA damage [53, 54]. In  $Plk4^{+/+}$  MEFs, yH2AX levels peaked at 40mJ/UV dose, while in *Plk4*<sup>+/-</sup> MEFs, yH2AX was initially high and protein levels became depleted with increasing dosage of UV (Fig. 2.5a), suggesting that *Plk4*<sup>+/-</sup> 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<sup>+/+</sup> MEFs displayed an increase in the sub-G0 population compared to the untreated cells by 12%, suggestive of apoptosis (Fig. 2.5c), whereas, *Plk4*<sup>+/-</sup> 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. 2.5c).

Figure 2.5. Lowered levels of Prmt5 inhibit p53 activation during DNA damage 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  $\gamma$ -H2AX post DNA damage in *Plk4*<sup>+/+</sup> and *Plk4*<sup>+/-</sup> 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. Specifically, the distribution of cell populations in the Sub-G0 phase of the cell cycle is shown here. The data is representative of three independent experiments and error bars are the standard deviation of the mean. \*represents a p<0.001.









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<sup>2</sup> UV, Plk4<sup>+/-</sup> 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 could surmise that this tripartite grouping could be acting collaboratively to disseminate the appropriate response to DNA damage prior to the onset of mitosis (Fig. 2.6).

Here, we demonstrated that PLK4 is required for the normal functioning of PRMT5 through direct interaction, phosphorylation, and co-localization at the centrosomes as well as maintenance of normal PRMT5 levels. Interestingly, in the data obtained from human hematological malignancies study, PLK4 and PRMT5 followed a comparable pattern where downregulation of PLK4 *via* promoter methylation was also associated with lower levels of PRMT5 (Sivakumar *et al.*, Chapter 2 dissertation; Ward *et al.*, Chapter 5 dissertation). Moreover, it may be that the opposite scenario is also viable; 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.

Figure 2.6. Potential mode of action by which PLK4 haploinsufficiency 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 phosphorylate 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 2.6

Some of the processes that are 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, Hif1 $\alpha$ , and NF- $\kappa\beta$ . [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 investigations surrounding Plk4 deregulation in malignancy have 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 surrounding PRMT5 suggests that PRMT5 may be an ideal therapeutic target in cancers since its expression or localization is deregulated in many malignancies [33, 63-65]. And 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,  $7.5 \times 10^{-4}$ % v/v formaldehyde). Developer solution (28.3 mM sodium carbonate, 5.0x10<sup>-4</sup>% v/v formaldehyde, 2.6x10<sup>-2</sup> 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  $\mu$ L 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  $\mu$ L 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 ( $\approx 20\mu$ L). 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°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°C and 220rpm shaking for 45 minutes. The resulting digestion buffer and protein extractions were then concentrated by volume reduction to  $\approx$ 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 bioinformatics 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<sub>2</sub>. 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<sup>TM</sup> transfection kit. The transfections were carried out according to manufacturer's recommendations. In order to establish inducible cell lines, T-REx<sup>TM</sup> 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  $\mu$ g/mL blasticidin 24 hours post-transfection. Following this, inducible colonies were established by maintaining transfected cells in growth medium with 5  $\mu$ g/mL blasticidin and 500  $\mu$ g/mL Zeocin<sup>TM</sup>. Once inducible cell lines were established, Flag-PLK4 expression was induced using tetracycline at a final concentration of 1  $\mu$ g/mL.

To carry out the co-transfection of two plasmids, a total of 13  $\mu$ g 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-γ-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<sup>™</sup> 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  $\mu$ L 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 non-specific interactions during the co-immunoprecipitation any 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°C for 2-3 hours with gentle rocking. Protein from transfections was incubated with beads at 4°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<sup>TM</sup> XL-1000 UV Crosslinker (Spectronics Corporation) at specified doses ranging from 10-40 mJ/UV. 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  $\Delta$ Methyltransferase domain,  $\Delta$ C-terminal, and  $\Delta$ N-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 30°C 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 4°C. 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<sub>2</sub>, 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  $\mu$ g of anti-Flag antibody (Sigma) and 20  $\mu$ l of calibrated Protein G

Sepharose beads 4 Fast Flow (GE Healthcare Life Sciences) at 4°C overnight. Immunocomplexes 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<sub>2</sub>, 3 mM MnCl<sub>2</sub>, 50 mM NaF, 25 mM dithiothreitol, 125  $\mu$ M cold ATP and Roche protease inhibitor pellet). Bacterialpurified, thrombin cleaved GST-PRMT5 protein was incubated with purified immunocomplexes of Flag-PLK4 constructs. For each kinase reaction, 8  $\mu$ g of GST-PRMT5 and 10  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>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- $\gamma$ -tubulin antibodies (Sigma) staining was incubated for an hour at room temperature at a dilution of 1:100. Secondary antibodies Alexa Fluor<sup>®</sup> 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 Hoechst

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 analysis

Statistical analysis was conducted using Statsoft Statistica software Version 7. A Oneway 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).

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#### Chapter 3

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

This chapter was done in collaboration with Alejandra Ward, Dr. Sindu Kanjeekal, Dr. Caroline Hamm, and Brayden Labute

#### **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 has endeavoured to identify novel biomarkers and potential indicators of prognosis and response to therapies [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 neoplasms. 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 in gene expression at the DNA and histone level without modification to the primary sequence of a gene. This encompasses 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 3.1a,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. 3.1a,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. 3.1 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. 3.1 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. 3.1e). A similar trend was observed in MDS/Leukemia samples (Fig. 3.1e). Strikingly, in more than 90% of the cases, PLK4 promoter methylation status correlated to protein expression (Fig. 3.1d), suggesting that in blood

neoplasms aberrant promoter methylation of Plk4 is an important method of regulating 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 replication resulting in centrosome abnormalities, aneuploidy and thus contributing to genomic instability in dividing cells [25, 26].

Figure 3.1 Promoter methylation analyses 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 amplification for *PLK2* with either methylated or unmethylated 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, \* represents statistical significance p<0.05. Error bars represent the standard deviation from the raw values obtained from densitometry analysis.


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 Bcell 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 did observe 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. 3.1a,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* and *PLK4* promoter regions were hypermethylated (Fig. 3.2a). Upon subjecting the bone marrow biopsies for all three family members to MSP analysis, the same promoter methylation profile was revealed in all three individuals for *PLK4* with an increase in *PLK4* promoter methylation (Fig. 3.2b), the same methylation profile as the initial peripheral blood sample. However, *PLK1* promoter was hypomethylated in these samples unlike what was seen in the peripheral blood of the mother.

#### 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) over-expression 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. 3.3a, 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. 3.3b). We divided the polarized levels of JAK2 into elevated or downregulated 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. 3.3a,c). This is suggestive of a regulatory relationship between these two proteins. We 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. 3.3b).

**Figure 3.2** *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.



-135 bp -235 bp

N+

N-

Σ

PIk1-PIk4-

Controls

Mother

Peripheral blood

a.

-235 bp

Figure 3.2

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 (Sivakumar *et al.*, Chapter 2 dissertation; 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-eleventranslocation 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 downregulated 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. 3.4a,b). This was also evident in the familial cases of MDS (Fig. 3.4b). The lowest levels of PLK4 were associated with the most depleted levels of TET2 (Fig. 3.4a-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 be a recipient of these methylation errors in conjunction with increases in methylation at the genome level.

**Figure 3.3 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 samples for each of the proteins noted. In each case their level was normalized to the housekeeping protein, GAPDH. Error bars represent the standard deviation from raw densitometry values. (c) A comparative analysis of JAK2 expression 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.





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. 3.4c). 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 used to diagnose MDS and acute myelogenous leukemia (AML) [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. 3.4a,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. This suggests that PLK4 expression may have a role to play in the development and progression of hematological neoplasms. **Figure 3.4 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 3.4

a.

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 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 leukemia and lymphoma cell lines under standard cell culture conditions *via* methylation-specific PCR analysis (Fig. 3.5a) (Supplementary Table 3.1). Remarkably, examination of patientderived 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 leukemia and lymphoma cell lines displayed hypermethylation of the PLK2 promoter-associated CpG islands in accordance with the previous findings by Benetatos et al. (2011) (Fig 3.5a) [10]. However, uncharacteristic of most malignant cells, *PLK1* promoter methylation was detectable in these in vitro models of neoplasia (Fig. 3.5a). Moreover, 7/9 and 5/9 of neoplastic cell lines had 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. 3.5b). 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. 3.5c). 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. 3.6a, b). This antagonistic correlation between the notorious oncogene and tumor suppressor has been previously reported, as aforementioned [13].

Figure 3.5 Methylation profiles of leukemia- and lymphoma-derived patient cell lines at the *PLK* promoter regions pre- and post-hypoxia. (a) Leukemia 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 realtime PCR to examine HIF1 $\alpha$  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 3.6 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.



-62kDa

RPMI-8226 - + -95kDa

-75kDa

-68kDa -34kDa







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, is 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, prompts 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 3.2). 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 can mediate aberrant epigenetic modifications of the *PLKs* as we have shown and contribute to oncogenic transformation and disease progression.

# Effect of epigenome-targeting drugs on the methylotype and expression of the Polo-like kinases

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<sup>INK4B</sup>, p73, and E-cadherin in myeloid leukemia cells [44]. 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 and leukemic patients. A deoxycytosine 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 genomewide 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 domain protein 2dependent 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. 3.1a-c). To highlight the heterogeneity of blood-derived neoplasms, drug treatment data from two leukemia and two lymphoma cell lines have been shown here.

Post-drug administration, there was a remarkable sensitivity of the polo-like kinases to these drugs largely at the 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. 3.7a). 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 (Sivakumar *et al.*, Chapter 2 dissertation; 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.

Figure 3.7 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 3.7

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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. 3.7b). 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. 3.8a). 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. 3.8a). PLK1 is down-regulated when PLK4 levels are the elevated and vice versa.

**Figure 3.8 Impact of DNA-modifying drugs on PLK protein levels in lymphoma cells.** (a, b) *PLK4* responded to the long-term five-day treatment with epigenomemodifying 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.





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 (Fig. 3.8b). 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 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. 3.8a). 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, depending 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 3.1). 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 3.1). 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 to 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 enhance the expression of the oncogenic PLK1. Understanding how epigenetic therapy can influences the underlying epigenetic lesions of the *PLKs* in leukemia and lymphomadiseased patients may be a valuable predictor of prognosis in a clinical setting. Moreover, it seems that the hypoxic physiological conditions associated with cigarette consumption may potentially impact how well a patient responds to epigenetic therapy.

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<sup>INK4B</sup> and p16<sup>INK4A</sup>, 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, especially that of PLK4 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<sup>™</sup> 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].

#### <u>Cell culture</u>

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<sub>2</sub> (normoxia) or at 5% O/5% CO<sub>2</sub> (hypoxia).

### Hypomethylation and histone-modifying treatments

To perform treatments with epigenome-targeting agents, cells were treated with either 0.5  $\mu$ M 5-aza-2'deoxycytidine (Decitabine -DAC; Sigma), 1 mM valproic acid (VPA; Sigma), or 0.5  $\mu$ M 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. Colorimetric analysis was carried out on a Perkin Elmer Wallac Victor<sup>3</sup> 1420 Multilabel Counter.

## 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 3.1. 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 druginduced 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.













10.0µM DAC 5.0µM DAC

Day 0 Day 1 Day 2 Day 3 Day 4 Day 5

2.5µM DAC

40 20 0

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**Supplementary Table 3.1. 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.

3.1
Table
Supplementary

a.

	Cell line Description	Disease type	Age/Gender	Characteristics
	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
/ SCIW	Meg-01	Chronic myelogenous leukemia	55 year old male	Karyotype: hyperdiploid; the Philadelphia chromosome is present Antigen expression: CD41 +; CD61 +; CDw14 +
Leukemia	K-562	Chronic myelogenous leukemia	53 year old female	Karyotype: triploid; occurrence of spontaneous dicentrics; X chromoso is disomic Tumorigenic: Yes; tumors developed in nude mice at 100% frequency w inoculation Antigen expression: CD7
	Reh	Acute lymphocyticleukemia (non-T; non-B)	None provided	Antigen expression: CD3 A (17%) B (17%) C (20%), CD4 (15%), CD10 (55%
	Ramos	Burkitt's lymphoma	3 year old male	Antigen expression: CD23+ Receptor expression: interleukin 4 (IL-4); Iow 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); Iow 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
Lymphoma	ST-486	Burkitt's lymphoma	Female	Derived from parent line Ramos; Antigen expression: CD23+ Receptor expression: interleukin-4 (IL-4); Iow 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 +

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**Supplementary Table 3.2 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* 

**Supplementary Table 3.2** 

а.

Population information	n=51	%
Average age	64.75	
Number of patients with a smoking history	24	47.0
Patients with low O <sub>2</sub> Saturation	6	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  $\mu$ l 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 4

# Polo-like kinase 4 phosphorylates the p53- and BRCA1-induced stress response protein, GADD45a and also interacts with the apoptotic regulator, Nucleophosmin/B23

This chapter includes data generated by Bing Wu in her Master's thesis.

# **Introduction**

Regulators of cell cycle, DNA damage repair, and apoptosis collectively help to maintain controlled growth, proliferation, mitotic fidelity, and genomic stability. Perturbations to the function of these putative elements disrupts cell cycle checkpoints, counteracts the trigger of DNA repair, cell cycle arrest, and apoptotic circuits, and ultimately, promotes the genesis of cancer (Reviewed in [1, 2]). The polo-like kinase (plk) family are the epitome of molecular regulators involved in these cardinal cellular processes. Functioning as serine/threenine kinases, the Plks have been remarkably safeguarded during the course of biological evolution as noted by their omnipresence in the simplest of organisms like yeast to highly evolved eukaryotes [1]. The plks are responsible for a myriad of cellular processes including mitotic entry, spindle formation, chromosome segregation, cytokinesis, centrosome dynamics, and DNA repair to warrant harmonious cellular division (Reviewed in [3]). While yeast possess only one Plk gene, complex eukaryotes, such as mammals, bear five PLKs which encompass PLK1, PLK2, PLK3, PLK4, and the recently discovered, PLK5 [4]. It is speculated that with increasing sophistication and intricacy of organisms, additional PLKs evolved over time to provide stringent cell cycle controls during ontogenetic processes, differentiation, and exposure to cellular or environmental stressors (Reviewed in [1]).

The polo-related kinases orchestrate a broad range of biological functions through a signature kinase domain located at the N-terminus and two conserved polo-box motifs at their C-terminus which mediate substrate interaction and subcellular localization [5, 6]. Although PLK4 possesses the basic canonical architecture of the polo kinases, this protein kinase has a weak sequence homology in comparison to the others. In addition to possessing several PEST sequences that regulate its stability, this kinase has three polo box motifs, rendering it the most divergent member of the Plk family [7, 8]. Subjugated by complex temporal and spatial signaling determinants in a context-dependent manner, the PLKs have refined, unique functions and substrate specificities [5].

Polo-like kinase 4 protein levels and catalytic activity is well-understood to be essential for cell viability, centrosome integrity, and more recently, the DNA damage response [9-13] [Bonni, 2007 Master's thesis] [Ward *et al.*, 2014 Chapter 4 dissertation]. Disconcerted Plk4 expression as a result of epigenetic modifications *via* methylation, loss of heterozygosity, or haploinsufficiency in *Plk4+/-* mice have previously been reported to predispose cells to carcinogenesis [14-16]. The incidence of dysregulated centrosome replication, formation of supernumerary centrosomes, abnormal spindle formation, chromosome missegregation, and tetraploidy due to aberrant PLK4 protein levels increases susceptibility to spontaneous lung, liver, and colorectal cancers [14-17]. While PLK1-3 are known to have unbridled interactions with a large array of substrates, the interacting partners and substrate repertoire of PLK4 remains obscure despite its discernible functions in chief molecular pathways.

As yet, PLK4 is known to interact with and phosphorylate proteins involved in cell cycle progression, namely Cdc25C, and in the DNA damage signaling pathway as

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evident by the phosphorylation of p53 and Chk2 [13, 16, 18, 19]. PLK4 binds and phosphorylates Ect2 to regulate cytokinesis and chromosome stability; it also activates GCP6 and Cep152 to permit centrosome biogenesis [20, 21]. Intriguingly, PLK4-dependent phosphorylation of the transcription factor, Hand1, has also been identified as an important event to trigger cell-fate determination during early development [22]. In view of the fact that PLK4 is a centrosome-associated protein, it is astonishing that it has few characterized centriolar substrates.

present study, provide evidence that GADD45a In the we and Nucleophosmin/B23 (NPM), both of which are known to localize to the centrosomes, are novel interacting partners of PLK4. GADD45a is a stress-induced protein regulated by BRCA1 and p53 which is closely intertwined with the DNA damage signaling network, epigenetic directives, and centrosome regulation [23, 24]. On the other hand, NPM is a nucleolar protein that acts as a nuclear import chaperone to various proteins, including GADD45a, and is known to be a key regulator of p53 during the early phases of DNA damage [25, 26]. PLK4 phosphorylates GADD45a, likely at the centrosomes, while the interaction between Nucleophosmin/B23 needs to be further characterized. Our findings implicate PLK4 in an elaborate role in a signaling network that may tether its functions in centrosome dynamics and the intrinsic cellular response to stress cues.

# **Results and Discussion**

GADD45a is one of the three members in the family of growth arrest and DNA damage-inducible proteins whose expression is known to be regulated by both genotoxic and non-genotoxic stress including UV radiation, methyl methanesulfonate, and ionizing radiation [27]. Mediated by p53 and BRCA-1 signaling pathways, this stress response

protein, has a number of important cellular functions in the DNA damage response, growth suppression, and the execution of cell cycle checkpoints [28]. There have also been controversial reports of a role for GADD45a in active DNA demethylation *via* nucleotide excision repair to reactivate gene expression [29, 30]. Most interestingly and relevant to our investigation, GADD45a plays a role in centrosome duplication and the maintenance of genomic stability. In fact, the phenotypes seen in *Gadd45a-/-* mice are strikingly reminiscent of *Plk4+/-* mice, notoriously harbouring abnormal cytokinesis, an excessive number of centrosomes, multipolar spindles, multinucleation, mitotic infidelity, and aneuploidy, all of which ultimately feed cancer progression [9, 15, 31].

PLK4 physically associates with GADD45a, a novel binding partner, in a p53independent fashion

As GADD45a null mice closely resemble features of *Plk4* +/- mice, we sought to establish whether PLK4 and GADD45a had an affiliation. To ascertain whether PLK4 associates with GADD45a endogenously, we performed a co-immunoprecipitation (Co-IP) assay with a GADD45a antibody using protein extracts from HEK 293T cells. Immuno-precipitates of GADD4a bound PLK4 protein, as noted by a robust interaction (Fig. 4.1a). To rule out the possibility of nonspecific interactions, beads and IgG were included as negative controls; PLK4 protein was undetectable in these immune-complexes.

Figure 4.1. GADD45a interacts with PLK4 independently of p53. (a) To establish whether GADD45a interacts with PLK4 endogenously, whole cell lysates from human embryonic kidney cells (HEK) 293T cells were used for a co-immunoprecipitation assay with Protein G Sepharose beads and GADD45a antibody. Beads and mouse IgG, in the first and second lane respectively, were used as negative controls. The heavy chain of antibodies is detected at 55 kDa. (b) Co-immunoprecipitation (Co-IP) assays were repeated using the GADD45a antibody in 293T cells which possess normal levels of p53 and in Hep3B cells which are p53-deficient. Western blot analysis was used to confirm the p53 status in these cells. GAPDH was used as a loading control. (c) Wild-type *Plk4*<sup>+/+</sup> (WT) and heterozygous *Plk4*<sup>+/-</sup> (HET) mouse embryonic fibroblasts were used to examine Gadd45a protein levels *via* Western blot analysis. Protein levels were quantified using densitometry. \* represents a p < 0.01.



Figure 4.1

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While Gadd45a-/- mice have numerous shared abnormalities with the Plk4+/mice, it is also important to note that these effects are also hallmarks of p53-deficient mice. Indeed, loss of p53 in mice is also an impetus for cell cycle checkpoint perturbations, gene amplification, reduced apoptotic activation, diminished DNA repair, and genomic instability [32-34]. In context with these observations, it has been established that PLK4 phosphorylates the DNA damage response protein, p53 at a residue which generally corresponds with its activation [35]. Studies in our lab have shown reduced p53 activity in Plk4+/- mouse embryonic fibroblasts (MEFs) during UV damage [Bonni, 2007 Master's thesis; Ward et al., 2014 Chapter 4 dissertation]. PLK4 may also contribute to the stimulation of p53 by phosphorylating protein arginine methyltransferase 5 (PRMT5) which is an enzyme that methylates and activates p53 [36] [Ward *et al.*, 2014 Chapter 4 dissertation]. Taken together, there is evidence for PLK4 as a potential modulator of p53 activity and it may be possible that PLK4, p53, and GADD45a may function synergistically in a trichotomous network to maintain cellular homeostasis during stress. Naturally, we tested whether the ability of PLK4 and GADD45a to interact with one another in a p53-dependent context. Utilizing hepatocellular carcinoma-derived Hep3B cells which are p53-deficient, Co-IP assays were repeated with the GADD45a antibody. Interestingly, we discovered that the presence of p53 was not essential for the interaction between PLK4 and GADD45a under these normal conditions (Fig. 4.1b).

Decreased Gadd45a levels reflect reduced dosage of Plk4 in Plk+/- heterozygous MEFs

To characterize the relationship dynamic between GADD45a and PLK4, we analyzed Gadd45a levels in *Plk4*+/+ wild-type MEFs and *Plk4* +/- heterozygous MEFs *via* Western blot analysis. Decreased dosage of Plk4 in the heterozygous mice caused approximately a 60% reduction in the basal level of Gadd45a in comparison to the wild-type MEFs (Fig.4.1c). In accordance with our findings, Gramantieri *et al.* (2005) found GADD45a mRNA to be down-regulated in 20/26 patients with hepatocellular carcinoma (HCC) [37]. Interestingly, previous work in our lab underscored the higher risks of developing HCCs in the *Plk4* heterozygous mice in comparison to the wild-type counterpart [14]. Taken as a whole, these findings provide compelling evidence for an important interplay between PLK4 and GADD45a.

#### GADD45a is a novel substrate of PLK4

As Plk4 levels in mice had a prominent effect on Gadd45a protein levels, we sought to establish whether this stress-inducible protein could be a substrate of PLK4. Initially we performed Co-IP assays to determine whether GADD45a interacted with the Nterminal kinase module of PLK4. To do this, HEK 293T cells were transfected with a variety of Flag-PLK4 truncation mutants and lysates were co-immunoprecipitated with the GADD45a antibody (Fig. 4.2a). The interaction between PLK4 and GADD45a was detected in all segments of the N-terminal domain including Flag-PLK4-T170D kinase active (KA), Flag-PLK4-K41M kinase dead (KD), and Flag-PLK4-D154N alternate kinase dead (KD2). GADD45a also interacted with Flag-PLK4ΔPB (lacking polo-box 3 domain) and Flag-PLK4R1 (containing both polo-box 1 and 2 of the cryptic polo box) which are constituents of the C-terminus of PLK4 (Wu B, Master's thesis) (Fig. 4.2b). However, the conserved polo-box motif PB3, Flag-PLK4PB, did not interact with immuno-precipitated GADD45a, deeming this region of PLK4 irrelevant for interaction with GADD45a under these standard conditions (Wu B, Master's thesis).

Figure 4.2. GADD45a association with PLK4 domains. (a) Schematic diagram depicting the domains of full-length PLK4 as well as the derived truncation mutants. PLK4-T170D KA represents the kinase active form of PLK4 where replacement of the threonine 170 residue with aspartic acid results in an activating mutation in the T-loop of the kinase. With conversion of lysine 41 to methionine, PLK4 has a mutated ATP binding domain, rendering PLK4-K41M to be a kinase dead mutant. PLK4-D154N is also a kinase dead version of PLK4 with an asparagine in place of a lysine residue at amino acid position 154. PLK4 $\Delta$ PB encompasses all the domains of PLK4 except for polo-box domain 3 (PB3), which is a conserved motif amongst the Polo-like kinases. PLK4PB possesses only the PB3 domain of PLK4. Lastly, PLK4R1 is composed of the region previously known as the cryptic polo-box which is now known to contain two additional polo-box motifs, PB1 and PB2. (b) HEK 293T cells were transiently transfected with Flag-tagged wild-type PLK4 and the various PLK4 constructs. To confirm successful transfection, Western blot analysis was conducted with whole cell lysates. Co-IPs were performed using GADD45a antibody. The cells lane is a negative control and represents un-transfected cells whose lysates were incubated with beads and GADD45a antibody. Flag antibody was used to detect interaction of PLK4 mutants with GADD45a.





PLK4

Kinase Domain

e.

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Given that GADD45a interacts with the catalytic kinase domain of PLK4, we pursued *in vitro* kinase assays to ascertain whether GADD45a is a substrate of PLK4. In order to do this, HEK 293T cells were transiently transfected with Flag-conjugated empty vector, Flag-PLK4 (wild-type), Flag-PLK4 KA, and Flag-PLK4 KD. Whole cell lysates were immuno-precipitated with Flag antibody and incubated with purified GADD45a. Initially, we had employed histidine-tagged GADD45a to conduct the kinase assays through which we observed PLK4 phosphorylation of GADD45a (data not shown). However, reports demonstrated non-specific PLK4 phosphorylation of His-tag. Utilizing bacterially-expressed and purified GST-fused GADD45a, kinase assays were repeated with immuno-precipitated PLK4 protein in the presence of  $[\gamma^{-32}P]$ . We observed phosphorylation of GST-GADD45a in conjunction with auto-phosphorylation of PLK4, which served as a positive control for the kinase reactions. This confirmed GADD45a as a novel substrate of PLK4 (Fig. 4.3a). Kinase assays were also performed with purified GST protein to exclude the possibility of false-positives (Fig. 4.3b).

# Figure 4.3. PLK4 phosphorylates and localizes with GADD45a at the centrosomes. (a) To elucidate whether GADD45a could be substrate of PLK4, kinase assays were performed. 293T cells were transiently transfected with Flag, wild-type (PLK4), kinase active (KA), and kinase dead (KD) versions of PLK4. The kinase dead construct of PLK4 refers to the K41M mutant. Lysates were immunoprecipitated with Flag antibody. Bacterially expressed and purified GST-tagged GADD45a protein and the immune-complexes of PLK4 were incubated in the presence of [ $\gamma$ -<sup>32</sup>P]. Phosphorylated bands were visualized *via* autoradiography. Immunoblots show transfection of PLK4 constructs and loading of GST-GADD45a protein. (b) *In vitro* kinase assays were also repeated with GST protein alone as a negative control. Immunoblots confirm transfection of PLK4 and its mutants along with the loading of GST protein. (c) Wild-type *Plk4* MEFs were used to determine the subcellular localization of GADD45a.





# PLK4 and GADD45a co-localize at the centrosomes

PLK4 is predominantly concentrated at the centrosomes, although its translocation into the nucleus, midbody, and cleavage furrow has been observed under specific contexts [9, 20, 22, 38]. In contrast, while GADD45a is known to localize to the cytoplasm and centrosomes, GADD45a is predominantly found in the nucleus where it potentiates the DNA damage response signaling cascade [39]. Although further experiments need to be performed to truly understand the functional significance of the unison between GADD45a and PLK4, some aspect of their association can be dissected by identifying their subcellular co-localization. Immunofluorescent staining was performed with MEFs isolated from wild-type Plk4+/+ mice. Cells were stained with anti-y-tubulin, which specifically localizes to centrosomes, in addition to the anti-GADD45a antibody to detect endogenous GADD45a. Interestingly, we found that GADD45a is situated at the centrosomes, similar to PLK4 (Fig 4.3c). As previously mentioned, GADD45a has been implicated in the centrosome cycle where its expression and function seem to be cardinal to the maintenance of centrosome integrity and genomic fidelity.

Nucleophosmin/B23, a nuclear chaperone of GADD45a, interacts with Polo-like kinase 4

Nucleophosmin/B23 (NPM/NPM1), also called numatrin or NO38, is a ubiquitous nucleolar phosphoprotein from the NPM family of proteins which also include NPM2 and NPM3 [41]. Nucleophosmin has diverse functions including roles in ribosome biogenesis [42], cell proliferation [43], nucleic acid binding [44], ribonuclease activity, suppression of apoptosis via p53 [25, 45], centrosome duplication [46], and nucleocytoplasmic molecular trafficking [47]. Depletion of NPM cripples cell proliferation and exacerbates the induction of apoptosis, whereas, ectopic expression of B23 prompts cell cycle arrest and a senescence-like phenotype mediated by p53 in normal cells [Reviewed in [48]). However, in cells lacking functional p53, the overexpression of NPM results in a push through S-phase [25, 49, 50]. Given that NPM confers proliferation and survival advantages, both tumorigenic and rapidly dividing cells express high levels of this protein. This is exemplified in a subset of cancers including gastric, colon, ovarian, and prostate where NPM levels are strikingly elevated (Reviewed in [48]). Moreover, in blood neoplasms such as myelodysplastic syndromes/acute myeloid leukemia, promyelocytic leukemia, and anaplastic large cell lymphomas, the *NPM* gene often undergoes chromosomal translocation, mutation, and deletion [51]. Much effort is underway to validate NPM as a biomarker or a prognostic indicator for certain types of cancers. Nevertheless, NPM seems to harbour characteristics of both an oncogene, as witnessed by its implication malignancies, and a tumor suppressor, as noted by its focal roles in upholding centrosome integrity, genomic fidelity, and stress response.

NPM is governed *via* differential phosphorylation at distinct sites by a number of kinases including protein kinase C, mitotic protein kinase cdc2, and Cdk2/CycE

throughout the cell cycle to facilitate necessary biological outcomes [52, 53]. Interestingly, PLK1 and PLK2 have been identified to target NPM in the context of centrosome dynamics [46, 54]. The centrosome replication cycle is evoked in G1/S phase and completed within the S-phase synchronously with DNA replication [55]. NPM monitors centrosome duplication by binding to the centrosomes prior to duplication and dissociates from the structure upon phosphorylation by Cdk2/cyclinE at T199 to initiate new centriole growth [52, 56]. Interestingly, in addition to phosphorylation by Cdk2/cyclinE, a concomitant PLK2 phosphorylation of NPM on Ser 4 during S-phase is also required to trigger initiation of centrosome duplication [46]. Furthermore, mitotic-specific PLK1 phosphorylation of Ser 4 on NPM is indispensable specifically for mitotic fidelity, normal centrosome profile, and completion of cytokinesis [54]. Many substrates are common among the Polo-like kinases and are differentially regulated in a temporally-and spatially-dependent context by each kinase. Perhaps, NPM is also an interacting partner of PLK4?

To answer this question, we investigated the possibility of an interaction between endogeneous NPM and PLK4. Co-IP assays were performed with HEK 293T cells using the PLK4 antibody. In the pull down assay, NPM was detected in the immune-complexes of PLK4 whereas this interaction was not observed in the negative controls (Fig. 4.4a). As a way of further substantiating this interaction, HEK 293T cells were transfected with Flag empty vector and Flag-PLK4. Again, we observed interaction of NPM with Flagtagged PLK4, but not with Flag alone, beads, or co-immunoprecipitation with IgG (Fig. 4.4b). Indeed, nucleophosmin was found to be a novel interacting partner of Polo-like kinase 4.

Figure 4.4. Nucleophosmin (NPM), a known nuclear chaperone of GADD45a, also interacts with PLK4. (a) In order to ascertain whether PLK4 and nucleophosmin interacted with one another, protein extracts from HEK 293T cells were used in a Co-IP assay. Endogenous NPM was detectable in the immunoprecipitates of PLK4. Beads and mouse IgG were used as negative controls to ensure that the interaction is not nonspecific. Heavy chain and light chain of the antibody are visible at 55 kDa and 25 kDa, respectively. (b) NPM association with PLK4 was also confirmed with ectopic expression of PLK4 in cells. HEK 293T cells were overexpressed with empty Flag vector and Flag-PLK4. Lysates were co-immunoprecipitated using the Flag antibody. NPM specifically interacted with Flag-PLK4 and not with immune-complexes of Flag alone, mouse IgG, or beads. (c) As an initial step to understand the impact of Plk4 heterozygosity on NPM expression, we examined NPM levels in lysates from wild-type  $Plk4^{+/+}$  (WT) and heterozygous Plk4<sup>+/-</sup> (HET) MEFs. In addition, HET MEFs were also transfected with Flag, Flag-Plk4, and Flag-Plk4 K41M kinase dead mutant to determine the effect of ectopic expression of PLK4 and lack of kinase activity on NPM levels. (d) Densitometry analysis was performed to quantify changes in NPM levels. Error bars represent the standard deviation from three independent trials. \* represents a p < 0.001; \*\* represents a p < 0.0001.





Amongst the many proteins for which an NPM interaction is essential for nuclear import is the stress-inducible protein, GADD45a. As an important regulator of cell cycle checkpoints, GADD45a-induced G2/M arrest occurs specifically through nuclear interaction with Cdc2, abrogation of Cdc2/cyclinB1 interaction, and hampered Cdc2 kinase activity [26, 57-60]. Lacking a nuclear localization signal, nuclear import of GADD45a is accomplished solely through association with NPM [26]. Naturally, given the newly established relationship between PLK4 and GADD45a, it was of interest to determine whether NPM interacted with the PLK4-GADD45a protein complex. Endogenous Co-IP assays were performed with HEK 293T cell lysates from standard conditions using the GADD45a antibody. Curiously, while PLK4 interaction was evident, NPM was undetectable in the immuno-complexes of GADD45a (data not shown). Taking into consideration that DNA damage signals are essential to trigger GADD45a translocation to the nucleus and mediate G2/M cell cycle arrest, a potential multilateral interaction between GADD45a, nucleophosmin, and PLK4 may require DNA damage.

# Down-regulation of Plk4 is mirrored through decreased NPM expression

To determine the relevance of the physical association of PLK4 and NPM, we initially compared NPM expression in *Plk4*+/+ wild-type and *Plk*+/- heterozygous MEFs *via* Western blot analysis. *Plk4* heterozygosity resulted in approximately a 40% decrease in NPM expression in comparison to the wild-type counterpart (Fig. 4.4c, 4.4d). Next, we examined whether NPM expression can be rescued by transiently transfecting *Plk4*+/- MEFs with Flag-Plk4. We also ectopically introduced Flag-Plk4 kinase dead (KD) mutant to examine whether the catalytic activity of Plk4 was relevant to maintain NPM

protein levels. As expected, Plk4 overexpression caused significant two-fold increase in NPM protein levels (p<0.05). Interestingly, ectopic expression of a kinase-inactive form of Plk4 displayed an additional 1.5 fold increase in relation to MEFs transfected with wild-type Plk4 (p<0.05). Collectively, these results provide evidence for nucleophosmin as a novel interacting partner of PLK4. PLK4 levels are consistently correlated with NPM levels as changes in PLK4 expression are echoed through parallel changes in NPM levels. We are currently testing whether PLK4 is an upstream regulator of NPM.

The possibility that this correlation has physiological relevance is intriguing. For example, in a previous study by Macmillan and colleagues, neoplastic colorectal mucosa displayed elevated levels of PLK4 mRNA [17]. Similarly, NPM levels are also considerably amplified, both at the mRNA and protein level, in colorectal adenomas [61]. In addition, recent studies conducted within our lab alluded to a possible role for PLK4 in the development and progression of a subset of myelodysplastic syndromes (MDS), acute myeloid leukemia (AML) and lymphomas. In 82.0% and 80.5% of bone marrow samples from lymphoma and MDS/leukemia patients, respectively, displayed aberrant DNA modification of *PLK4 via* methylation and exhibited down-regulation of PLK4 levels [Ward *et al.*, Chapter 5 dissertation]. Indeed, methylation of the *PLK4* promoter region and decreased PLK4 protein levels was associated with proteins that have been correlated with poor prognosis in MDS, leukemia, and lymphoma patients. Similarly, Sportoletti et al. (2008) have implicated NPM haploinsufficiency as a driver of hematological malignancies, specifically MDS [62]. While the functional relevance of NPM/PLK4 interaction remains to be elucidated, dysregulation of either protein contributes to similar disorders. These studies, in conjunction with our findings, suggest that PLK4 and nucleophosmin are integrated together in an unknown molecular network.

#### **Conclusions**

A conglomeration of studies on centrosome structure and function suggests that this organelle may be a mecca for proteins from regulatory pathways to oversee cellular activities. This component of the microtubule organizing center appears to be a platform where multi-directional cues from acute/chronic stress and signal transduction pathways can be exchanged to facilitate cellular homeostasis (Reviewed in [55]). More than a hundred proteins are anchored at the centrosomes *via* organized multi-protein scaffolds and molecular complexes (Reviewed in [55]). Specifically, there seems to be a considerable amount of crosstalk between proteins associated with the centrosome machinery and that of DNA damage pathways [63, 64]. In the event of DNA damage, cells halt centrosome activities while DNA repair processes are triggered in order to prevent segregation of damaged chromosomes [65]. DNA-damage associated centrosome inactivation has been described in *Drosophila* whereby Chk2 migrates to the centrosomes to inhibit cell cycle progression and spindle pole assembly to ensure genomic fidelity [66].

Aside from GADD45a, nucleophosmin, and PLK4, much research provides rapport for the localization of p53 to the centrosomes [67]. Naturally, it is possible that there could be multifaceted interactions between these key cell cycle regulators, especially during stress. Perhaps, in a damage-control pathway, these regulatory proteins allow for the coupling of centrosome function and repair pathways to block the propagation of

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unrepaired, mutated DNA? Furthermore, given the intersecting pathways between NPM and GADD45a, it is likely that PLK4 mediates the regulation of these proteins in a series of cellular processes that may include centrosome amplification, DNA repair, cell cycle arrest, apoptosis, and even, cell survival [12, 26, 39, 45, 60, 68, 69] [Ward et al., 2014 Chapter 4 dissertation). PLK4 has been implicated in the etiology of HCC, colon cancers, and more recently, in hematological malignancies. It is intriguing that deregulation of GADD45a and nucleophosmin results in the development of the same tumor profiles. Characterizing the functional importance of PLK4 substrate interactions can elucidate the mechanism through which PLK4 establishes cellular homeostasis in the context of DNA damage and centrosome regulation. More importantly, given that PLK4 is deregulated during tumorigenesis through aberrant epigenetic modifications and loss of heterozygosity [14, 15] [Sivakumar et al., 2014 Chapter 3 dissertation; Ward et al., 2014 Chapter 5 dissertation], understanding its downstream targets can underscore the need for re-establishing appropriate PLK4 expression in cancer patients. In fact, this work may further substantiate the use of PLK4 as a biomarker in the clinical setting.

# **Materials and Methods**

#### <u>Cell culture and transfections</u>

Mouse embryonic fibroblasts (MEFs) were cultured in Dulbecco's Modified Eagle's Media (DMEM) supplemented with 20% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 0.5% gentamicin. MEFs used for experiments were between passages 2-5 unless otherwise stated. MEFs were transfected using the Qiagen Effectine<sup>TM</sup> transfection kit. The transfections were carried out as per the manufacturer's recommendations. Human embryonic kidney (HEK) 293T cells were grown in DMEM with 10% FBS. HEK 293T cells were transiently transfected with 1 mg/mL polyethylenimine (Sigma) and 10  $\mu$ g of purified plasmid DNA (Qiagen Maxiprep kit) for 18-24 h. Whole cell lysates were collected post-transfection as described below. All cells were maintained in standard conditions at 37°C with 5% CO<sub>2</sub>.

#### Western blot analysis

Protein levels were analyzed and quantified using Western blot analysis. Whole cell protein was extracted using a lysis buffer (50mM Tris-Cl pH 7.5, 150mM NaCl, 1% Triton-X, 0.1% SDS) supplemented with EDTA-free protease inhibitor cocktail (Roche). For Western blot analysis, a total of 40 µg of protein was loaded per sample. The following primary antibodies were used: anti-PLK4 and anti- GADD45a (Santa Cruz Biotechnology); anti-GAPDH (Cell Signalling); anti-Flag (Sigma-Aldrich); anti-NPM/B23 (Life Technologies). Secondary antibodies, anti-rabbit HRP (Cell Signalling) and anti-mouse HRP (Sigma), were also used during protein analysis. Protein bands were acquired visualized using an Alpha Innotech Multimage<sup>™</sup> Light Cabinet and densitometry analysis was carried out using ImageJ software Version 1.47.

### Co-Immunoprecipitations

Protein G-sepharose beads (GE Healthcare) were equilibrated and washed three times in 50 mM Tris-HCl pH 7.4, 300 mM NaCl, 0.1% triton X-100, 0.1% SDS, and protease inhibitor cocktail (Roche). Cell lysates were incubated with beads and 1  $\mu$ g of appropriate antibody at 4°C for 16 h. Protein complexes were washed seven times and subsequently, separated by SDS-PAGE.

# Plasmid clones and mutagenesis

To construct the GST-GADD45a plasmid, a Cre-loxP cloning reaction was performed using 250ng each of pDNR-Dual GADD45a donor vector (DNASU), and V621 pGEX-4T-LP acceptor vector (DNASU). In addition, the reaction was supplemented with 1 µl of Cre-loxP recombinase, and 1 µl of 10x Cre Recombinase Reaction Buffer in a total reaction volume of 10 µl. The reaction mixture was incubated at room temperature for 15 minutes, followed by an incubation step at 70°C for 5 minutes to heat-inactivate the Cre recombinase enzyme. Up to 5  $\mu$ l of the reaction mixture was used to transform heatshock competent Top10 F' cells and the resulting transformants were plated on agar plates containing 30 µg/mL Chloramphenicol and 7% sucrose to select and screen for successful recombinant colonies containing the GADD45a insert in the GST vector. Sitedirected mutagenesis of the GST-GADD45a plasmid was subsequently performed using the QuikChange multi-site directed mutagenesis kit (Stratagene) to add a stop codon upstream of the 6x HN tag present at the 3' end of the insert. Forward primer 5'-CAGTGATTAATCTCCCTGAACGGTAGTGACTAGTTCTAGACCATTCGTTTGGC GC-3' and reverse primer 5'-GCGCCAAACGAATGGTCTAGAACTAGTCACTACCG

# TTCAGGGAGATTAATCACTG-3' were used.

# Expression and purification of recombinant protein

pGEX-4T-LP vector containing GADD45a was expressed in BL21 DE3 *E.coli* (Stratagene) with the addition of 0.5 mM IPTG at 30°C for 4 h. Bacterial pellets were resuspended in a lysis 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). Protein lysates were subjected to sonication after 20 min of incubation on ice and centrifuged at 12,000 rpm for 30 min. The cleared lysate was incubated with glutathione *S*-transferase beads on a nutator at 4°C for 2 h. The sepharose 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). GST-fusion protein was released from the filtrate with the addition of elution buffer (50 mM Tris HCl pH 7.5, 100 mM NaCl, 10 mM glutathione). Following collection, the eluate was filtered and concentrated using Amicon Ultra centrifugal filters (Millipore). Empty GST vector was also expressed in *E.coli* and purified as per the protocol above.

# In vitro kinase assays

Cell extracts were collected and incubated with 1 µg of anti-Flag antibody (Sigma) and calibrated Protein G Sepharose beads (GE Healthcare Life Sciences) at 4°C overnight. Protein-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, twice with kinase buffer (60 mM Hepes pH 7.5, 3 mM MgCl<sub>2</sub>, 3 mM MnCl<sub>2</sub>, 50 mM NaF, 25 mM dithiothreitol, 125 µM cold ATP and Roche protease inhibitor
pellet). Purified GST- GADD45a protein was incubated with immunoprecipitated Flag-PLK4 constructs. For each kinase reaction, 8  $\mu$ g of GST- GADD45a and 10  $\mu$ Ci of ( $\gamma$ -<sup>32</sup>P) (Perkin Elmer) were supplemented to the kinase buffer. Reactions were incubated at 30°C for 30 min. Kinase reactions were stopped by addition of 6x SDS protein sample buffer and boiling. Protein samples were resolved by SDS-PAGE and subjected to autoradiography. Phosphorylated bands were visualized using Cyclone Plus Phosphor Imager (Perkin Elmer) and the Optiquant software Version 5.0.

#### Immunofluorescence microscopy

MEFs were cultured and grown on glass slides in 6-well plates to 80-90% confluency. Cells were fixed in 3.7% PFA and 0.1% triton X-100. The cells were then incubated with GADD45a antibody (Santa Cruz Biotechnology) at a 1:50 dilution for 16 hours at 4°C. Cells were also subjected to anti-γ-tubulin antibody (Sigma) staining at room temperature for 1 h at a dilution of 1:100. Secondary antibody staining with Alexa Fluor<sup>®</sup> 568 (Invitrogen) and FITC (Vector laboratories) was conducted at room temperature for 1 hour at a dilution of 1:500. Fluorescent staining of nuclei was also performed DAPI at a dilution of 1:10,000 by incubating slides at room temperature for 2 min. Images were resolved on a Zeiss Axioskop 2 mot plus using Northern Eclipse software.

#### <u>Statistical analysis</u>

Statsoft Statistica software Version 7 was used to conduct statistical analysis. P-values were determined using a One-way ANOVA test. \* represent a p<0.01. All results are the average of values obtained from three independent trials. Error bars are representative of the standard deviation (SD) of the mean value.

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#### Chapter 5

#### DISCUSSION

## PRMT5, an epigenetic modifier, is a substrate of PLK4

The protein arginine methyltransferase 5, PRMT5, catalyzes the symmetric arginine di-methylation on target proteins to regulate cellular functions ranging from transcription and ribosome biogenesis to signaling processes during development, processing differentiation, cell death, and tumor development [1, 2]. Interesting to our work, PRMT5 has also been characterized as a writer of repressive epigenetic marks that modulates gene expression. Arginine dimethylation of histone H4 arginine 3 (H4R3me2s) is a magnet for the binding of DNA methyltransferase 3A (DNMT3a) and histone deactetylases to methylate DNA and produce a transcriptionally incompetent chromatin structure, respectively, to silence gene expression [2, 3]. I have identified PRMT5 as a novel substrate of PLK4 (Sivakumar et al., 2014 Chapter 2 dissertation). In wild-type *Plk4* MEFs, Prmt5 localized to the centrosomes where PLK4 is known to be found. In contrast, heterozygous *Plk4* MEFs have suppressed PRMT5 expression and in addition, the reduced dosage of Plk4 aberrantly directs Prmt5 localization to the nucleus which has been previously associated with a global loss in protein arginine methylation [Ward et al. 2014 Chapter 4 dissertation]. Moreover, we have consistently observed perturbed p53 levels and activity in heterozygous MEFs in response to environmental stressors (Bonni, Master's thesis; Ward *et al.* 2014 Chapter 2 and 4 dissertations). More than likely, this is a biological outcome of reduced capacity of PRMT5 to append arginine methylation marks to activate p53 [4].

Previous work by us and others has shown *PLK4* to be epigenetically regulated in the context of cancer development and progression (Sivakumar *et al.*, Chapter 3 dissertation; Ward *et al.*, 2011; Ward *et al.*, Chapter 2, 3, and 5 dissertations). Here, we have demonstrated a potential for PLK4-mediated epigenetic control. Intriguingly, there is also evidence for a direct interaction between DNMT3A, a *de novo* methyltransferase enzyme, and PLK4 and like Prmt5, *Plk4+/-* MEFs display down-regulated levels of Dnmt3a (Ward and Cerghet, unpublished). We can speculate that deregulation of these key proteins as a result of reduced Plk4 levels can lead to inappropriate gene expression and activation, loss of genomic fidelity, and carcinogenesis.

#### PLK4 promoter methylation as a potential biomarker in blood neoplasms

Over the past few years, research conducted within our lab has identified the novel epigenetic regulation of *PLK4* via promoter-associated hypermethylation in hepatocellular carcinoma [5]. In the present study, we also demonstrate methylation-mediated down-regulation of *PLK4* in blood neoplasms. Analysis of approximately eighty-percent of bone marrow samples derived from lymphoid and myeloid malignancies revealed a methylated *PLK4* promoter in comparison to its normal counterpart (Ward *et al.* 2014 Chapter 5 dissertation). These methylation marks translated into suppressed PLK4 levels, collectively suggesting that this may be a common event or play an important role in the development and progression of blood neoplasms. In addition, we observed an interesting trend between *PLK1* and *PLK4* methylation and expression. While *PLK4* was methylated and down-regulated in neoplastic bone marrow samples, *PLK1* was hypomethylated and up-regulated. JAK2 and TET2 have recently

emerged as biomarkers for hematological malignancies. The JAK2-STAT pathway is often up-regulated in myeloid neoplasia, conferring cells with proliferative and growth advantages [6-9]. TET2 is a demethylating enzyme that is dysregulated in both lymphomas and leukemias and produces an aberrant methylome [9, 10]. Interestingly, in bone marrow aspirates, JAK2 expression coincided with PLK4 downregulation while depletion of TET2 correlated with reduced PLK4 levels. Considering the interesting relationship between JAK2 and PLK4, I performed co-immunoprecipitation assays and determined that there was a remarkable interaction between these two proteins in a leukemia cell line (Sivakumar, unpublished) (Appendix A Fig. A.1). While the functional relevance of this association is to be characterized, we can speculate that in a tumorigenic state JAK2 might target the stability and activity of PLK4.

While it is unknown whether aberrant TET2 and JAK2 regulation is a driving factor for tumourigenesis, they possess diagnostic and prognostic value in a clinical setting. Given that PLK4 levels in patient-derived bone marrow samples seem to be closely tethered to JAK2 and TET2 expression, there may be potential for PLK4 as a novel biomarker of hematological malignancies. This may have potential clinical value as in the case for *PLK2* which has recently been established as an epigenetic determinant of clinical response to chemotherapeutic drugs [11, 12]. Hypermethylation-related gene silencing of *PLK2* provides resistance to drugs such as paclitaxel and carboplatin to epithelial ovarian cancers. Perhaps, methylation of *PLK4* provides selective advantages for growth, survival, or chemo-sensitivity in cancer cells?

The deregulation of *PLKs* was further substantiated *in vitro* using patient-derived leukemia and lymphoma cell lines. As the initial development and progression of blood neoplasm originate in the bone marrow, we sought to determine the methylation state of the PLKs in 5% oxygen conditions which mimicked the bone marrow microenvironment. Initial analysis of the promoter region in these neoplastic cell lines under standard oxygen conditions revealed a characteristic pattern where the *PLKs* were largely identified to be methylated (Sivakumar et al. 2014 Chapter 3 dissertation). However, some cell lines displayed unmethylated promoter regions for *PLK3* and *PLK4*. Interestingly, promoter methylation was induced in these cell lines post-hypoxia, suggesting that environmental stressors including low oxygen tension can target the PLK promoters for epigenetic regulation. In accordance with these findings, Ward et al. (2014) has recently demonstrated that the *PLKs* are susceptible to epigenetic modifications with exposure to oxidative stressors including hypoxia and ROS [5]. Furthermore, health history data of our patients with diseased bone marrows revealed that approximately 50% of patients were chronic cigarette smokers. These patients have low oxygen saturation levels of <95%. Given that long-term smoking is associated with hematological malignancies, perhaps, the low oxygen tension and reactive oxygen species in these individuals further induces aberrant methylation and promotes disease initiation?

Epigenome-targeting chemotherapeutics that are commonly used in the clinic include Trichostatin A (TSA), Valproic Acid (VPA), and Decitabine (DAC). While TSA and VPA prompt the remodelling of the chromatin architecture into a transcriptionally competent state, DAC depletes DNA methyltransferases and inhibits the addition of methylation marks. We performed an *in vitro* drug study, under standard and bone-

marrow environment conditions, to elucidate whether these epigenetic drugs impact PLKs at the DNA or protein level. We found that at large there was a wide-breadth of responses in the expression of the PLKs between each cell line, drug, and oxygen condition, underscoring the heterogeneity of these blood disorders. Interestingly, we observed several expression patterns that were consistent with our *in vivo* study. Down-regulated TET2 levels correlated with reduced PLK4 levels, over-expression of JAK2 was associated with decreased PLK4, and lastly, high levels of PLK1 was linked to low levels of PLK4 (Sivakumar *et al.* 2014 Chapter 3 dissertation). Further experiments are necessary to provide insight into the dynamic relationship between JAK2, TET2 and PLK4.

#### The molecular regulation and functions of GADD45a and Nucleophosmin

In face of DNA damage, cell cycle processes such as centrosome duplication and DNA replication come to a pause in order to stimulate and complete DNA repair. Without proper coordination between centrosome and repair machinery during stress, genomic instability ensues and mutated DNA is propagated in the cell population. Polo-like kinase 4 has been extensively studied to understand its functions in centrosome homeostasis. Deregulation of PLK4 can occur through loss of heterozygosity, haploinsufficiency, and DNA methylation [5, 13, 14] [Ward *et al.* 2014, Chapter 4 and 5 dissertation). Aberrant levels of PLK4 have been a causative factor of centrosome abnormalities including supernumerary centrosomes, multipolar spindles, improper mitotic assembly, and chromosome missegregation, all of which culminate in loss of genomic integrity and lead to tumorigenesis [5, 15, 16].

In order to gain insight into the pleiotropic functions of PLK4, identification and characterization of its physiological substrates is essential. In this study, we have provided evidence for two novel interacting partners of PLK4, both of which are implicated in the DNA damage response and maintenance of centrosome stability. GADD45a is a substrate of PLK4 which was found to co-localize at the centrosomes. It remains to be determined whether reduced GADD45a levels translate into decreased GADD45a activity. GADD45a activates the MTK1/MEKK4 SAPKKK pathway under stress, which in turn actuates SAPK pathways via c-Jun N-terminal kinase, p38, and PLK4 [17]. p38 is specifically known to phosphorylate p53 at Ser15, Ser33, and Ser37 under UV damage to increase its stability and transcriptional activity [18]. Essentially, upon UV exposure, GADD45a is responsible for the stabilization of p53 [19]. Attenuation of p53 function, a phenotype prominent in *Plk4* heterozygous MEFs, during UV damage can be attributed to decreased GADD45a levels. However, decreased p53 activity may also be tethered to the lower levels of PRMT5 that is observed in *Plk4+/-*MEFs [13][Ward et al., 2014 Chapter 4 dissertation]. More notably, Ko et al. (2005) has shown Plk4 to directly target p53 at S392, a residue which has been associated with activation and of course, this may also contribute to the depressed p53 function in heterozygous MEFs.

Nucleophosmin was identified as a binding partner of PLK4 and our findings suggest that appropriate levels of PLK4 are essential to maintain NPM expression. In order to fully characterize the functional relevance of the PLK4-NPM association, it is necessary to perform *in vitro* kinase assays to establish whether NPM is a substrate of PLK4. Alternatively, NPM may interact with PLK4 as a scaffold or docking protein to

mediate substrate interaction or subcellular localization of PLK4. NPM has been implicated in the DNA damage response through several mechanisms. Stress-induced binding of GADD45a to NPM is essential for nuclear translocation of GADD45a and GADD45a-mediated G2/M cell cycle arrest [20]. Paradoxically, NPM antagonizes the tumor suppressor, p53 during the early response to genotoxic stress [21]. NPM inhibits UV-induced Ser 15 phosphorylation of p53 via physical association with p53 and reduces its transcriptional capacity by more than 70% to prevent premature activation of cell cycle arrest and apoptosis [21]. This, in turn, largely prevents transcriptional activation of p53-effectors like p21, Bax, and GADD45a (Reviewed in [22]). Considering that both PLK4 and NPM have regulatory functions in the p53 signaling network, it would be imperative to test whether formation of the NPM-PLK4 complex is dependent on p53 status in cells. If so, *in situ* immunofluorescence assays can be conducted to determine the subcellular region where these three proteins interact. Additionally, in the nucleus, NPM functions to antagonize apoptotic stimuli by binding to caspase-activated DNAse and preventing DNA fragmentation [23]. Given the dual roles of nucleophosmin under stress, upstream signaling by a stress sensor may dictate whether NPM will promote arrest, DNA fragmentation, and cell death or alternatively, cell survival.

Novel regulation of PLK4 by stress-activated protein kinase kinase kinases (SAPKKKs) of the MTK1/MEKK4 pathway has been shown to occur in response to stress stimulus. MTK1 phosphorylates and activates PLK4, prompting the downstream phosphorylation of AKT at its catalytic site T308 by PLK4 [24]. As AKT governs a wide range of pro-growth and pro-survival processes, this kinase is tightly regulated by differential phosphorylation [25]. Some of the mechanisms through which AKT confers

protection against growth suppression and apoptosis include phosphorylation of CREB to increase transcription of cell survival factors and interestingly, stabilization of NPM *via* SUMOylation to prevent caspase 3-dependent degradation of NPM, respectively [26, 27]. Strikingly, this regulation of NPM by AKT is solely dependent on T308-specific phosphorylation on AKT and not its inherent kinase activity. We can speculate that PLK4 directly binds to NPM to not only regulate NPM expression, but also stabilize NPM levels through AKT and ensure cell survival.

#### A testable model for the PLK4 signaling network

Here, we provide a model to speculate the functional consequences of PLK4 interaction with GADD45a and nucleophosmin along with its other substrates including p53 and PRMT5 (Fig. 5.1). An amalgamation of the overlapping pathways of NPM, GADD45a, p53, PRMT5 and PLK4 appear to underscore two distinct roles of PLK4 during DNA damage signal transduction.

In the early phase of DNA damage or other stress stimuli, GADD45a-mediated stimulation of MTK1 evokes PLK4 activity [24]. Despite the initial trickle of stress cues, I propose that PLK4 promotes cell survival through phosphorylation of AKT to initiate transcription of cell survival factors *via* CREB and protect NPM [24, 26, 27]. This allows for NPM to interact with p53 to suppress its transcriptional activity and also inhibit DNA fragmentation [21, 23]. Considering these findings, the initial biological response of PLK4 during damage may be important to protect cells from premature cell cycle arrest or apoptosis. In support of this theory, we have previously demonstrated that

overexpression of PLK4 in cells results in recovery from UV-induced G2/M arrest (Bonni, Master's thesis 2007).

Exposure to chronic stress stimuli, in contrast, can trigger a disparate response from PLK4. PLK4 may halt the dissemination of signals promoting cell cycle progression and cell survival once a definite threshold of stress has been reached. I propose that in the later phase of stress response, where unabated stress may have caused irreversible damage to cells, PLK4 resolves to target p53 and PRMT5. Concrete evidence supports that as p53 is stabilized *via* arginine methylation and phosphorylation by PRMT5 and MTK1-activated p38, respectively [18] [Ward et al. 2014 Chapter 4 dissertation]. Subsequently, transcriptional activation of p53-response genes, such as GADD45a and Bax, is augmented (Reviewed in [22]). Not only do I suggest that prolonged stress elicits p53-mediated cell cycle arrest and apoptosis specifically by a cooperative PLK4, but also that subsequently, to safeguard cells from its own hyperactivity, PLK4 shrewdly selfdestructs through auto-phosphorylation of a number of residues to allow itself to be recognized by the SCF-Slimb/E3 ubiquitin ligase complex for degradation [28]. Moreover, p53 recruits DNMT3a and HDACs to transcriptionally repress to inhibit centrosome hyper-amplification by PLK4, recall that [29]. The timing of these events needs to be well-delineated.

With regards to the newly characterized interacting partners, we can contemplate that GADD45a phosphorylation by PLK4 may occur to alter the stability of the protein or perhaps, modulate its apoptotic function during the late phase of stress response. We have already established that the PLK4/GADD45a complex is associated with the centrosomes. Interestingly, aside from implications with centrosome replication, it has

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been shown that GADD45a localizes to the centrosomes to trigger mitochondriamediated cell death. GADD45a allows for BIM, a BCL-2 family member, to dissociate from the microtubule organizing centers, re-localize to the mitochondria, and interact with BCL-2 [30]. As a pro-survival protein, BCL-2 prevents BAX, an apoptotic protein, from inducing cytochrome-c release-mediated cell death [31]. However, BIM/BCL-2 interaction releases BAX, eliciting cellular death [30]. Perhaps, PLK4 mediates this apoptotic function of GADD45a? Intriguingly, Ko *et al.* (Dissertation) has shown that haploinsufficiency of *Plk4* in mouse embryonic fibroblasts abrogates apoptosis during UV-mediated DNA damage, resulting in the propagation of cells with damaged DNA which can lead to tumorigenesis [32]. It still remains possible that PLK4 regulates GADD45a in the late response of stress via post-translational modification of NPM, permitting NPM-mediated GADD45a nuclear import to induce G2/M cell cycle arrest and DNA damage repair. The functional relevance of these interactions remains to be characterized. **Figure 5.1. A testable model for the signaling network of PLK4.** An illustration amalgamating the well-characterized interactions and functions of GADD45a and Nucleophosmin in the context of PLK4 and other PLK4 substrates. This model speculates the potential functional relevance of PLK4 interaction with NPM, GADD45a, and PRMT5 specifically in the milieu of DNA damage. An early stress response may trigger pro-survival pathways by PLK4 to prevent premature cell cycle arrest and apoptosis. Conversely, with chronic exposure to DNA damaging agents, damage-control pathways are initiated to preserve genomic integrity *via* inhibition of centrosome amplification, G2/M arrest to facilitate DNA repair, and cell death. Blue arrows indicate events that are likely to occur in immediately in the early stages of stress response to DNA damage. Red arrows represent molecular events that take place with longer exposure to stressors. While all solid lines are biological events that have been previously characterized and published, dotted red lines refer to the potential and speculated involvement of PLK4 in specific biological triggers.



Figure 5.1

#### **Conclusions**

Our current knowledge of molecular biology underscores the roles of genetic and epigenetic alterations in the pathogenesis of cancer. The need to identify diagnostic markers to determine onset of disease and prognostic markers to predict response to cancer therapy is intensifying. While certain drugs elicit astonishing therapeutic response in patients, the same agents seem to prompt progression and relapse of the disease, and also worsen the clinical outcome in other patients. In addition, inter- and intra-tumoral genetic and epigenetic heterogeneity further augments the complexity of cancer treatment. The field of epigenetics has been booming in the recent decades for there is great potential for epigenetic abnormalities in the DNA methylome or histone codes to be reversed using epigenetic agents. Moreover, understanding the deregulation of the epigenetic landscape in cancer, especially at specific loci, may be fundamental to characterize the parameters of molecular and clinical response. Our lab focuses on the function and regulation of the omnipresent Polo-like kinases (PLKs). A number of studies suggest that PLKs2-5 function as tumor suppressors while PLK1 has notorious roles as an oncogene. Abnormal regulation of the *PLKs* predisposes cells to inappropriate growth, proliferation, and aberrant responses to cellular insults, culminating in malignant transformation and tumor development (Reviewed in [33-35]).

*PLK1* and *PLK2* have been established as potential therapeutic targets and provide clinical value for a number of malignancies [11, 12, 36]. Very little focus has been placed on PLK4, a key member of the polo family, as a potent tumor suppressor. In fact, much research is underway to use inhibitors of PLK4 as part of an anti-cancer regimen [37]. In this present study, we have shown that methylation-associated down-

regulation of PLK4 may be a common event in blood disorders (Sivakumar *et al.*, 2014 Chapter 3 dissertation; Ward *et al.*, 2014 Chapter 5 dissertation). In addition, I have demonstrated that the *PLKs* are targets of clinically-used drugs that target the epigenetic machinery. Moreover, we have identified a novel role for PLK4 in the DNA damage signaling network. Appropriate levels of PLK4 seem to be necessary for PRMT5mediated p53 activation during UV damage (Ward *et al.*, Chapter 4, dissertation; Sivakumar *et al.*, 2014 Chapter 2 dissertation). I have also characterized another substrate of PLK4, GADD45a, which is implicated in epigenetic gene activation [38-40], centrosome regulation [41, 42], and DNA damage-control pathways [22]. Lastly, I have discovered two interacting partners of PLK4, JAK2 and Nucleophosmin, proteins which are often deregulated in hematological malignancies [6, 8, 9, 43] and have known functionalities in the regulation of DNA-damage response proteins [4, 44].

Much evidence exists to show that the centrosomes act as a liaison for communication between different regulatory networks. In fact, the centrosomal structure may serve as a pinnacle for molecular regulators of the centrosome and DNA damage pathway to establish a platform to communicate stress signals [45, 46]. Our findings implicate PLK4 in a novel signaling network and it can be speculated that PLK4 is a master regulator of stress response with a dichotomous response to physiological or environmental stress.

#### **Future Directions**

Here, I propose experiments that need to be conducted to further the findings of my master's research.

With regards to the PRMT5, we are currently in the process of performing mass spectrometry of phosphorylated PRMT5 to determine the precise site(s) of phosphorylation by PLK4. By determining the phosphorylation sites, we can use sitedirected mutagenesis of PRMT5 to create phosphomimetic mutants. We have already shown that ectopic expression of Plk4 in Plk+/- MEFs can rescue the subcellular localization of PRMT5 and p53 activity. By overexpressing these mutants in heterozygous MEFs and examining levels and localization of Prmt5, we may be able to further substantiate that PLK4-dependent phosphorylation is essential and indispensable for appropriate Prmt5 activity. It would also be logical to expose phosphomimetic mutant-transfected Plk4+/- MEFs to DNA damaging agents and determine whether the p53 response is re-activated. As aforementioned, DNMT3a interacts with PRMT5 to mediate gene silencing and PLK4 also interacts with both of these proteins. It would be of interest to determine whether these proteins together simultaneously and monitor their subcellular localization in normal and heterozygous *Plk4* MEFs. *In vitro* kinase assays are also necessary to establish whether DNMT3A is a substrate of PLK4.

MDS patients often receive decitabine as the mainstay treatment regimen if not as an additional component of their cancer therapy. If provided the opportunity, it would be of great value to assess changes in *PLK* methylation status post-decitabine treatment in bone marrow or peripheral blood samples. The *in vitro* drug treatment studies did not identify notable changes in the methylation status of *PLK* promoters. As MSP primers only amplify a subset of the CpG sites in the promoter region, it would be prudent to perform bisulfite sequencing with unbiased primers to assess the methylation state of each CpG site in the *PLK* promoter. It will be interesting to ascertain whether the deregulated expression of the PLKs during drug treatment confers selective advantages for survival and growth in these MDS and lymphoma cell lines. To do this, we can examine changes in the ability of a cell to grow into a colony *via* colony formation assays and perform quantitative cell proliferation assays with a fluorescent marker. As a way of characterizing the interaction between JAK2 and PLK4, it would be necessary to perform in vitro kinase assays with JAK2 and PLK4 as a substrate. There is potential for a bidirectional functional regulation with these proteins. Both normal and mutant *Plk4* MEFs need to be assessed for changes in JAK2 levels to elucidate whether changes in Plk4 dosage impacts JAK2. In conjunction with this, localization studies need to be performed in wild-type *Plk4+/+* and heterozygous *Plk4+/-* MEFs using immunofluorescence to identify the region of their interaction. Moreover, it would be prudent to determine whether the JAK2-PLK4 liaison is dependent on PRMT5. It will be interesting to see whether RNAi-dependent knockdown of PRMT5 interferes with their interaction. More simply, we can examine whether the JAK2-PLK4 immuno-complexes have detectable PRMT5.

Although it is evident that Gadd45a levels are depressed in *Plk4* heterozygous MEFs, it remains to be determined whether lower levels of Gadd45a translate into reduced activity of this stress-response during damage. It is necessary to examine the levels and activity of GADD45a targets, such as MTK1 and p38 in the context of *Plk4* heterozygosity. GADD45A has role in a G2 checkpoint and may have implications for G2 to M cell cycle progression (Reviewed in [41]). Similarly, PLK4 levels peak in G2 and M phases of cell cycle (Reviewed in [35]). Using flow cytometry and co-immunoprecipitation assays, arrests at each stage of cell cycle need to be performed to

examine the phase at which GADD45a interacts with PLK4. Given that the networks of GADD45a and NPM are intertwined, it is important to determine whether they interact concomitantly with PLK4 in a complex. While I have tested this under standard conditions and did not detect a three-way interaction between these proteins, it may be worthy to determine whether such a multi-protein complex forms in cells with exposure to DNA damaging agents.

While the functional consequence of the direct interaction between NPM and PLK4 is still elusive, it is well-established that a T308 phosphorylation is necessary for AKT regulation of NPM and that PLK4 activation of AKT occurs at the same residue. To ascertain this, we need to delineate the timing of these specific events and establish whether PLK4 is essential for AKT-mediated NPM functions. Utilizing Plk4 heterozygous MEFs exposed to DNA damage by etoposide or UV, we can examine the levels of AKT phosphorylation at T308, the downstream SUMOylation of NPM, and caspase activity using specific antibodies. In addition, using a BrdU terminal deoxynucleotidyl transferase-dUTP nick end labeling (TUNEL) assay and Annexin V staining, we can quantitatively assess the degree of DNA fragmentation and apoptosis, respectively, in heterozygous MEFs in comparison to the wild-type counterpart. Moreover, using MEFs that have been exposed to UV irradiation, coimmunoprecipitation assays can also be performed to determine whether PLK4 acts as an intermediary protein between NPM and p53 to repress p53 transcriptional activity. Elucidating the region of PLK4-NPM co-localization can also serve to dissect some aspects of the biological relevance of their interaction. NPM expression has been welldocumented in the centrosomes, nucleus, nucleolus, nucleoplasm, and cytoplasm

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(Reviewed in [47]). As aforementioned, PLK4 has a dynamic pattern of subcellular localization including occupancy at the centrosomes, midbody, cleavage furrow, and nucleus/nucleolus and in addition, interacts with many proteins, like MTK1/MEKK4 SAPKKK, in the cytoplasm [24, 28, 48, 49]. It is possible that PLK4 and NPM may interact with one another in one of their common subcellular compartments.

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## **APPENDICES**

## Appendix A

## MISCELLANEOUS DATA/RESULTS

Figure A.1. Impact of epigenome-targeting drugs on MEG-01 and RAMOS-2G6 cell lines, leukemia and lymphoma-derived cells, respectively. MEG-01 and RAMOS-2G6 cells were treated with drugs under standard and hypoxic conditions. Western blot analyses show the effect of chronic drug treatment on PLK protein levels, PRMT5, and p53. TET2 levels and DNMT3a levels were also assessed.





Figure A.2. JAK2, a biomarker of myeloid and lymphoid malignancies, interacts with PLK4. (a) Co-immunoprecipitation assays were performed with lysates from Jurkats, a T-cell leukemia cell line, with a JAK2 antibody. Immuno-blotting identified PLK4 as a novel binding partner of JAK2. (b) To further confirm that this interaction was reciprocal, Co-IPs were repeated using a PLK4 antibody. JAK2 was identified in the immuno-complexes of PLK4.





Figure A.2.

# Appendix B

Letters of consent from collaborators and co-authors

Date: Tuesday, April-22-14

I, Rosa Alejandra Ward, hereby give my consent to Gayathri Sivakumar to use data I generated in both "Chapter 2 PRMT5, A novel substrate of PLK4 is deregulated in *Plk4* heterozygous MEFs" or "Chapter 3- The deregulated methylation of the PLKs in hematological malignancies as a potential clinical biomarker" to be used in her dissertation. I am aware that she has given me credit for my contributions.

Specifically, I contributed the following data:

Chapter 2 Characterizing Prmt5 interaction with PLK4

Chapter 2 Localization studies

Chapter 2 p53 levels and activity in wild-type and heterozygous MEFS

Chapter 5 in vivo work with bone-marrow samples

Sincerely,

R. Alejandra Ward PhD Candidate, University of Windsor 971 Jefferson Blvd. Windsor, Ontario, N8S2P9 vallej1@uwindsor.ca I, <u>Brayden LaBute</u>, hereby give my consent to Gayathri Sivakumar to use data I generated in "Chapter 3- The deregulated methylation of the PLKs in hematological malignancies as a potential clinical biomarker" to be used in her dissertation. I am aware that she has given me credit for my contributions.

Specifically, I contributed the following data: Chapter 3, western blot analysis of p53 protein expression in lymphoma patients.

Sincerely,

Brayden LaBute M.Sc. Candidate University of Windsor labuteb@uwindsor.ca I, Sharon Yong, hereby give my consent to Gayathri Sivakumar to use data I generated in "Chapter 2 PRMT5, A novel substrate of PLK4 is deregulated in *Plk4* heterozygous MEFs" to be used in her dissertation. I am aware that she has given me credit for my contributions.

Specifically, I contributed the following data: Chapter 2 localization studies

Sincerely,

Sharon Yong Research Assistant University of Windsor Room 300, Biological Sciences yongs@uwindsor.ca I, Anna Kozarova, hereby give my consent to Gayathri Sivakumar to use data I generated in "Chapter 2 PRMT5, A novel substrate of PLK4 is deregulated in *Plk4* heterozygous MEFs" to be used in her dissertation. I am aware that she has given me credit for my contributions.

Specifically, I contributed the following data:

Chapter 2 mass spectrometry analysis

Sincerely,

Anna Kozarova, PhD Research Associate University of Windsor Windsor, Ontario, Canada kozarova@uwindsor.ca Date: April 24, 2014

I, Jordan Nantais, hereby give my consent to Gayathri Sivakumar to use data I generated in "Chapter 2 PRMT5, A novel substrate of PLK4 is deregulated in *Plk4* heterozygous MEFs" to be used in her dissertation. I am aware that she has given me credit for my contributions.

Specifically, I contributed the following data:

Chapter 2 Mass spectrometry analysis: Specifically, MALDI-TOF spectra were provided which identified PRMT5 as a possible interacting partner of PLK4

Sincerely,

Dr. Jordan Nantais PGY-2 General Surgery Dalhousie University <u>nantaisj@gmail.com</u>
## VITA AUCTORIS

NAME:	Gayathri Sivakumar
PLACE OF BIRTH:	Bangalore, Karnataka, India
YEAR OF BIRTH:	1990
EDUCATION:	Vincent Massey Secondary School, Windsor, ON, 2007
	University of Windsor, Bachelor of Science with Honours, 2011
	University of Windsor, Master of Science, 2014