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A role for Polycomb Repressive Complex 2 in the DNA damage response

by

Stuart Campbell

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ABSTRACT

Polycomb protein methyltransferase, Enhancer of Zeste 2 (Ezh2), is frequently overexpressed in human malignancy and is implicated in cancer cell proliferation and invasion. Recently, several other polycomb proteins have been implicated in modulating the response to DNA double strand breaks, and thus we hypothesize that Ezh2 is also involved in the DNA damage response pathway. We investigated the involvement of Ezh2 and its associated complex, polycomb repressive complex 2 (PRC2), in the DNA damage response pathway. We found that PRC2 proteins recruit to sites of DNA damage and knockdown of Ezh2 decreases double strand break repair efficiency and increases cellular sensitivity to ionizing radiation. Our data supports the hypothesis that PRC2 is involved in the DNA damage response and contributes to DNA double strand break signaling and repair.

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LIST OF ABBREVIATIONS

Abbreviation	Full name
^{o}C	degree Celsius
γ-H2AX	phosphorylated histone 2A variant X
μg	microgram
μL	microliter
μm	micrometer
%	percent
53BP1	protein 53-binding protein 1
A	adenine
ADP	adenosine diphosphate
APLF	aprataxin and PNKP like factor
AdOx	adenosine dialdehyde
APTX	aprataxin
ATM	Ataxia telangiectasia mutated
ATR	ATM and Rad3 related
ATR-IP	ATR interacting protein
Bmi-1	B lymphoma Mo-MLV insertion region 1
Brca (1, 2)	breast cancer related protein (1, 2)
Brct	Brca1 C-terminus
BrdU	bromodeoxyuridine
C	cytosine
c-myc	c- avian myelocytomatosis viral oncogene
Cbx	chromobox
CFGE	constant field gel electrophoresis
ChIP	chromatin immunoprecipitation
Chk (1, 2)	checkpoint kinase (1, 2)
CO_2	carbon dioxide
CSC	cancer stem cell
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
DNA-PK	DNA dependent protein kinase
DNA-PKcs	DNA dependent protein kinase catalytic subunit
DSB	double strand break
Dsp1	dorsal switch protein 1
DZNep	3-Deazaneplanocin A
EDTA	Ethylenediaminetetraacetic acid
Eed	embryonic ectoderm development
EMT	epithelial - mesenchymal transition
Esc	extra sex combs
E(z)	enhancer of zeste
Ezh (1, 2)	enhancer of zeste (1, 2)
FAR	fraction of activity released

FBS	fetal bovine serum
FRAP	fluorescence recovery after photobleaching
G	guanine
GATA4	GATA binding protein 4
GFP	green fluorescent protein
Gy	gray
h	hour
H (1, 2A, 2B, 3, 4)	histone (1, 2A, 2B, 3, 4)
H1K26	histone 1 lysine 26
H2AK119	histone 2A lysine 119
H2AK119ub	histone 2A lysine 119 ubiquitylation
H2AX	histone 2A variant X
H3K4	histone 3 lysine 4
H3K4me3	histone 3 lysine 4 trimethylation
H3K9	histone 3 lysine 9
H3K9me3	histone 3 lysine 9 trimethylation
H3K27	histone 3 lysine 27
H3K27me3	histone 3 lysine 27 trimethylation
H4K20	histone 4 lysine 20
HR	homologous recombination repair
HSC	hematopoietic stem cell
IgG	immunoglobulin G
IPS	induced pluripotent stem cell
IR	ionizing radiation
IRIF	ionizing radiation induced foci
J	joule
Klf4	Krueppel-like factor 4
KO	knockout
LMIR	laser micro-irradiation
Mcph1	microcephalin 1
Mdc1	mediator of DNA damage checkpoint 1
min	minute
mL	milliliter
mm	millimeter
MMSET	multiple myeloma SET domain
MNNG	Methylnitronitrosoguanidine
Mre-11	meiotic recombination 11
MRN	Mre-11 Rad50 NBS1
mW	milliwatt
N.A.	numerical aperature
Nbs1	Nijmegen breakage syndrome 1
ncRNA	non-coding ribonucleic acid
NHEJ	non-homologous end joining
nm	nanometer
Oct4	octamer binding transcription factor 4
P53	protein 53

Pc	polycomb
PcG	polycomb group
Pcgf (1-6)	polycomb group RING finger (1-6)
Ph	polyhomeotic
pH	potential hydrogen
Phc (1-3)	polyhomeotic like (1-3)
Pho	Pleiohomeotic
PRC (1, 2)	polycomb repressive complex (1, 2)
PRE	polycomb responsive element
Psc	posterior sex combs
PAR	poly (ADP-ribose)
PARG	poly (ADP-ribose) glycohydrolase
PARP	poly (ADP-ribose) polymerase
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
PI3	Phosphoinositide 3
PNKP	polynucleotide kinase phosphatase
Polβ	polymerase beta
PTM	post translational modification
Rap80	receptor associated protein 80
RepA	repeat A
RING	really interesting new gene
RIPA	radioimmunoprecipitation buffer
RNA	ribonucleic acid
RNF168	ring finger protein 168
RNF8	ring finger protein 8
RPA	replication protein A
ROS	reactive oxygen species
Sce	sex combs extra
shRNA	short hairpin RNA
Smc1	structural maintenance of chromosomes 1
Sox2	SRY (sex determining region Y) box 2
SSB	single strand break
SSBR	single strand break repair
ssDNA	single stranded DNA
SDS	sodium dodecyl sulfate
Suz12	suppressor of zeste 12
T	thymine
Tdp1	Tyrosyl-DNA phosphodiesterase 1
Tip60	tat-interactive protein 60
TopBP1	topoisomerase (DNA) II binding protein 1
U2OS	U2 osteosarcoma
UV	ultraviolet
WT	wildtype
Wrn	Werner syndrome ATP – dependent helicase

Xist	X inactive specific transcript
Xlf	XRCC4 like factor
XRCC(1, 4)	X-ray repair complementing defective repair in Chinese hamster cells (1, 4)
YY1	ying yang 1

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Chapter I: *Introduction*

1.1 DNA and Chromatin

The blueprint for life is stored within the nucleus of every living eukaryotic cell in the form of deoxyribonucleic acid (DNA) (Avery *et al.*, 1944). DNA is a polymer made from repeating nucleotides. There are four different nucleobases in DNA, adenine (A), guanine (G), cytosine (C) and thymine (T), and the order in which these four nucleobases appear in the DNA is known as the genetic sequence (Watson and Crick, 1953; Crick, 1968). The structure of these deoxyribonucleotides promotes complementary binding, whereby, A binds to T and G binds to C through hydrogen bonds (Watson and Crick, 1953). A sugar-phosphate backbone houses the bases, making a single-strand of DNA. Double-stranded DNA consists of two complimentary DNA strands bound anti-parallel to each other, forming the iconic DNA double helix. A single change to one DNA base-pair can result in a mutation and can be detrimental to the cell, which is why maintaining genomic integrity and protecting the genetic code is essential for both single-cell and multi-cell organisms. DNA is a vulnerable molecule and is constantly bombarded with both exogenous and endogenous agents that induce damage, necessitating the need for ways of protecting and repairing DNA. These mechanisms will be discussed later in the DNA damage and repair section.

Within the 3 billion base pairs of human DNA, there are approximately 25,000 – 30,000 genes that must be accessible for transcription; however, there are two meters of DNA being packaged into each human cell nucleus whose diameter averages between 5 - 10 μM (Pennisi, 2003). The cell must therefore

have a mechanism to organize and compact the length of the DNA. This organization and compaction is achieved by wrapping DNA around proteins called histones, forming a structure known as chromatin (Kornberg and Lorch, 1999). Chromatin enables DNA to be condensed in a reversible fashion so that DNA accessibility can be modulated depending on cell cycle, transcriptional status, or general DNA maintenance, such as repairing DNA damage. There are three broad types of chromatin: euchromatin, facultative heterochromatin, and constitutive heterochromatin. Euchromatin refers to the least condensed chromatin and is full of gene-rich regions of DNA that are actively transcribed. Constitutive heterochromatin refers to highly condensed regions of the genome that are gene-poor and have very low levels of transcription. Facultative heterochromatin refers to DNA sequences that can vary in condensation state and transcriptional status depending on cell type and differentiation status. DNA in heterochromatin or euchromatin is organized and condensed by the same histones; however, many additional proteins are instrumental in determining chromatin structure by either modifying or associating with histones.

1.1.1 Histones

147 base pairs of DNA wrap around a histone octamer composed of four core histones, histones 2A, 2B, 3 and 4, to form the first level of chromatin compaction, the nucleosome (Kornberg and Lorch, 1999). The nucleosomes can then be further compacted by the linker histone, H1, which binds the DNA at the entry and exit of the nucleosome to bring nucleosomes closer together (reviewed in (Raghuram *et al.*, 2009)). The structure of chromatin compaction during

interphase has been a source of speculation and has been debated through models, but it is still not clear what DNA-protein structures exist and the extent of compaction. However, it is quite evident during mitosis that chromatin can be extensively compacted. Compaction of chromatin beyond nucleosomes is achieved through histone post-translational modifications (PTM) and non-histone protein interactions with histones. Histones are subjected to many different types of PTMs including phosphorylation, acetylation, ubiquitylation, methylation, and SUMOylation (Figure 1).

As mentioned previously, chromatin can take on various levels of compaction, such as decondensed euchromatin, or condensed heterochromatin, and histone PTM's help define and regulate which regions of the chromatin are euchromatic or heterochromatic. Characteristic modifications found in euchromatin are histone acetylation and trimethylation of lysine 4 on histone 3 (reviewed in (Kouzarides, 2007)). Characteristic modifications of constitutive heterochromatin include an absence of histone acetylation, trimethylation of lysine 9 on histone 3, and trimethylation of lysine 20 on histone 4 (reviewed in (Kouzarides, 2007)). Facultative heterochromatin can contain all of these modifications, but is also very commonly marked with trimethylation of lysine 27 on histone 3 (reviewed in (Kouzarides, 2007)).

PTM of histones is important for not only chromatin compaction, but also for transcription, signaling and protein interactions (Kornberg and Lorch, 1999). Lysine 4 trimethylation on histone 3 is associated with active transcription and lysine 27 trimethylation on histone 3 is associated with transcriptional

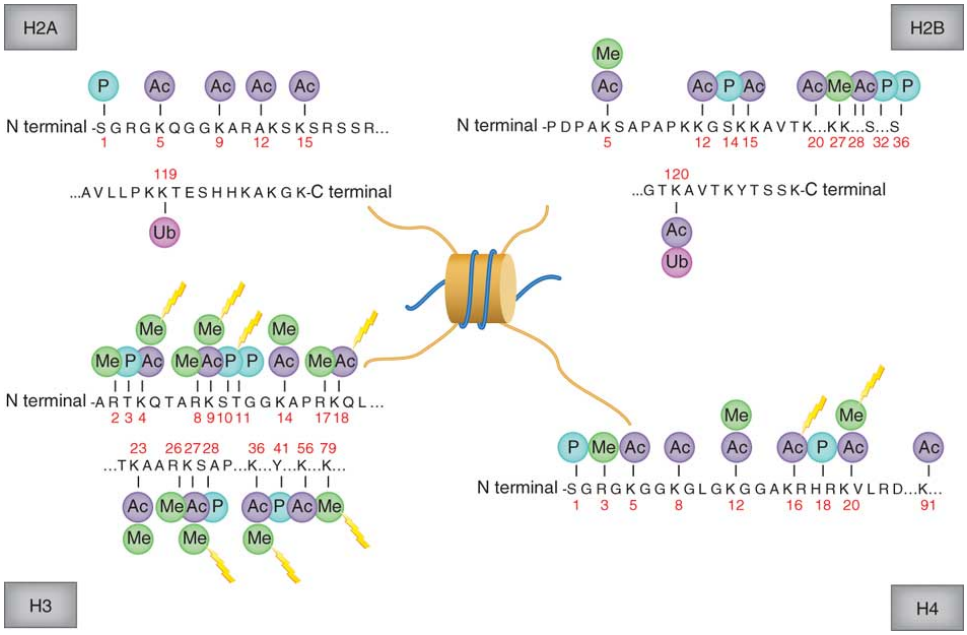

Figure 1. **Histones can undergo diverse post-translational modifications.** The combined affect of these modifications can alter chromatin structure and protein interaction changing chromatin condensation and transcriptional activity. Ac, acetylation; Me, methylation; P, phosphorylation; Ub, ubiquitination. Adapted by permission from Macmillan Publishers Ltd: Nature Medicine. Rodriguez- Paredes *et al.* copyright 2011.

repression (Cao *et al.*, 2002; Santos-Rosa *et al.*, 2002). The balance of these two marks throughout development is essential to regulate differentiation and expression of specific gene loci (Ringrose and Paro, 2004). Phosphorylation and ubiquitylation of histone variant H2AX is essential in the double-strand break repair pathway both for signaling the presence of DNA damage and retention of double-strand break repair proteins at the site of damage (reviewed in (van Attikum and Gasser, 2009)). The role of histone PTMs in DNA repair will be discussed further later in this chapter. Another extremely important role for histone PTMs is epigenetic regulation of gene expression. Epigenetics is the heritable changes in gene expression not associated with changes to the DNA sequences. Epigenetic gene regulation is an essential mechanism in cell differentiation, enabling organisms to express different genes in different tissues creating multiple cell types. This thesis will focus on one group of epigenetic gene regulators, Polycomb proteins.

1.2 Polycomb proteins

Polycomb group proteins (PcG) are epigenetic gene regulators originally discovered in *Drosophila* as key regulators of the *Hox* gene loci, whereby, mutations or knockout of the PcG proteins caused inappropriate expression of *Hox* genes in body segments, disrupting proper segmental development (Ringrose and Paro, 2004). PcG function is conserved in vertebrates, as PcG exert control of *Hox* expression and PcG mutants show skeletal defects indicative of improper development (Akasaka *et al.*, 1996; Core *et al.*, 1997). Biochemically, PcG proteins exist in two major multi-protein complexes, Polycomb Repressive

Complex 1 and Polycomb Repressive Complex 2 (PRC1 and PRC2 respectively). PRCs are responsible for the epigenetic regulation of many genes and function by modulating chromatin structure through PTMs of histones.

1.2.1 Polycomb repressive complex 1

PRC1 consists of the core proteins in humans (*Drosophila):* Pcgf1-6 (*Psc*), Cbx2,4,6,7,8 (*Pc)*, Phc1-3 (*Ph)*, and Ring1A/1B (*Sce/Ring*) (Shao *et al.*, 1999). In humans, there are several paralogs that exist for each PRC1 protein, making it much more diverse and complex than in *Drosophila*; however, the function is evolutionarily conserved. PRC1 consists of three RING-finger domain containing proteins, Pcgf, Ring1A, and Ring1B. The RING-finger domain is characteristic of E3-ubiquitin ligases and although all three of these proteins have been shown to have some E3-ubiquitin ligase activity *in vitro*, almost all PRC1 E3-ubiquitin ligase activity *in vivo* is attributed to Ring1B (Wang *et al.*, 2004; Buchwald *et al.*, 2006). Pcgf association with Ring1B greatly enhances the E3-ligase activity (Buchwald *et al.*, 2006). The main substrate for PRC1 ubiquitylation is lysine 119 on H2A, which confers the gene silencing ability of PRC1 (Wang *et al.*, 2004). PRC1 gene targeting is not yet fully understood, however, Cbx4 does contain a chromodomain that recognizes methylated lysine 27 and 9 on Histone H3, possibly directing PRC1 to regions of chromatin that are methylated on these residues (Paro and Hogness, 1991; Bernstein *et al.*, 2006). Cbx4 also contains E3-SUMO ligase activity, and has been found to sumoylate several targets, including Pcgf4 (Bmi-1) (Kagey *et al.*, 2003; Ismail *et al.*, 2012).

1.2.2 Polycomb repressive complex 2

PRC2 is the simpler of the two complexes, both because it contains fewer proteins and because there are fewer known functional homologs in humans. The core components of the PRC2 complex in humans (*Drosophila)* are Ezh2 (*E(z))*, Suz12 (*Su(z)12)* and Eed (*Esc)*. The enzymatic activity in PRC2 comes from the SET domain in Ezh2. The SET domain, named after three proteins it was originally found in, Su(var)3-9, Enhancer of Zeste, and Trithorax, is characteristic of lysine methyltransferases (reviewed in (Qian and Zhou, 2006)). In mammals, there exists a paralog of Ezh2, Ezh1, which can also function as a methyltransferase in the PRC2 complex. Ezh2's methyltransferase activity requires Suz12 and Eed *in vivo*, and trimethylates mainly lysine 27 on Histone H3 (H3K27me3) (Cao and Zhang, 2004; Pasini *et al.*, 2004). Although other methylation substrates of PRC2 have been identified, for example, lysine 26 on Histone H1 and transcription factor GATA4 (Kuzmichev *et al.*, 2004; He *et al.*, 2012), the major defined role for PRC2 is H3K27me3. There are several other proteins that can associate with PRC2, including histone deacetylases (HDACs) and DNA methyltransferases, both of which can contribute to the function and targeting of PRC2 (Vire *et al.*, 2006).

1.2.3 Polycomb-mediated gene repression

PcG-mediated gene repression is still poorly understood, although it is presumed that change in chromatin structure via histone PTMs facilitates PcG gene repression. One model of PcG-mediated gene repression first involves PRC2 marking chromatin with H3K27me3, which is then recognized by the chromodomain in PRC1, followed by ubiquitylation of H2AK119 (Cao *et al.*,

2002). These two PTM's cause the marked chromatin to be transcriptionally repressed (Figure 2).

One example where this is evident is in the inactive X-chromosome. The inactive X-chromosome is covered in both PcG mediated methylation and ubiquitylation, as well as bound by the PRC's (Plath *et al.*, 2003; de Napoles *et al.*, 2004). There are, however, genes that are marked and repressed by only H3K27me3 or H2AK119ub, indicating PRC1 and PRC2 can function independently in repressing gene targets (Ku *et al.*, 2008; Tavares *et al.*, 2012). PRC1 binding to chromatin can limit the accessibility of chromatin to chromatin remodelers (Shao *et al.*, 1999), thereby, inhibiting transcription. H3K27me3 can be bound by both PRC2 and PRC1 limiting the accessibility of transcription factors to promoters. PRC2 can also form a complex with histone deacetylases (HDAC), replacing the transcriptionally active, chromatin decondensing acetylation mark with the repressive methylation mark (van der Vlag and Otte, 1999). PRC2 can also assist in the recruitment of DNA methyltransferases (DNMT), another mechanism of repressing gene transcription (Vire *et al.*, 2006). Lastly, PRC2 has also been shown to have H1K26 methylation activity, which has the potential to increase chromatin compaction and facilitate the formation of heterochromatin (Kuzmichev *et al.*, 2004). These are all mechanisms that PcG proteins can employ to mediate transcriptional repression; however, it is still unclear how the cell mediates a gene-specific repression mechanism.

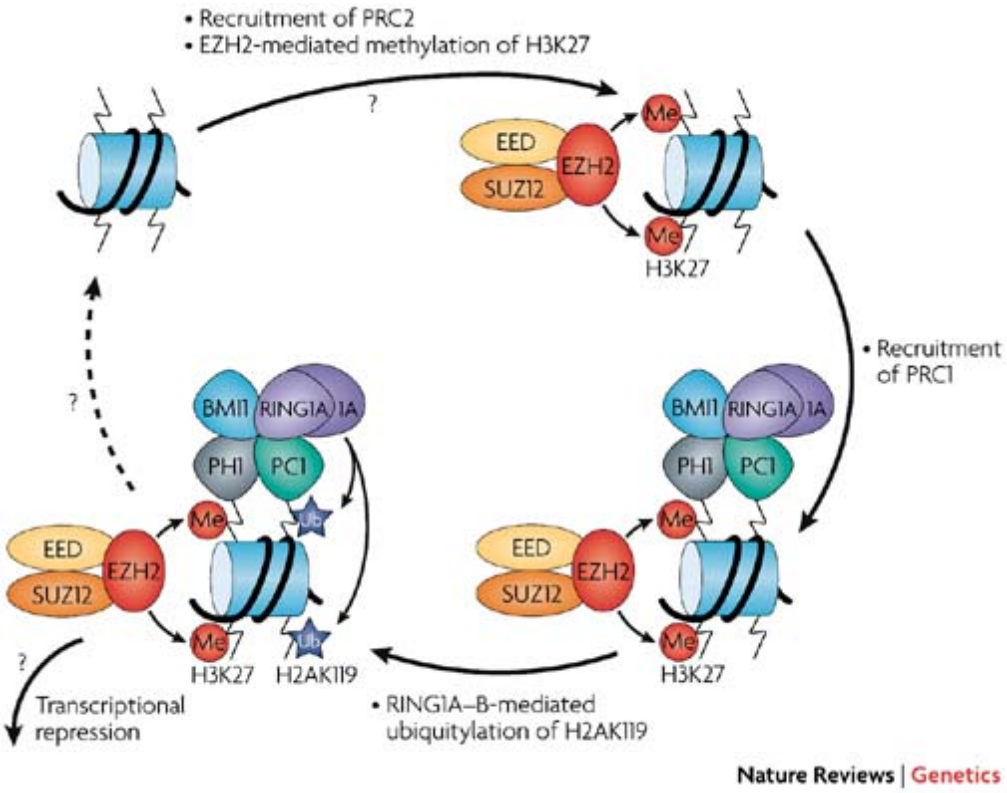

Figure 2. **Polycomb mediated gene repression.** The methyltransferase, Ezh2, is recruited as a part of the PRC2 complex to a targeted region of chromatin. Upon targeting, Ezh2 trimethylates histone 3 on lysine 27. The trimethylation mark is recognized by the PRC1 complex, which proceeds to ubiquitylate histone 2A on lysine 119. The association of both PRC2 and PRC1 and their respective post-translational modifications induces gene repression. Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Genetics. Spivakov and Fisher. Copyright 2007

1.2.4 Polycomb targeting

Polycomb proteins regulate the transcription of thousands of genes, and the gene targets change depending on cell type and degree of differentiation (reviewed in (Prezioso and Orlando, 2011)). The complexity and specificity of PcG gene repression is certainly reflected in the mechanism behind PcG targeting, and although there are still large gaps in our understanding of these mechanisms, we do have some insight into how PcG proteins target several genes. In *Drosophila*, specific DNA sequences termed Polycomb Response Elements (PRE) have been defined. PcG protein localization to these PRE's is dependent on many different sequence-specific DNA binding proteins including Zeste, GAGA, Pipsqueak, Grainyhead, Dsp1, as well as two that are officially classified as PcG proteins, Pho and Pho-like (Muller and Kassis, 2006; Ringrose and Paro, 2007). The hunt for PRE's in humans has been relatively unsuccessful, although recently a group discovered a vertebrate PRE that can recruit and stably bind PcG proteins regulating the gene expression of the mouse *MafB/Kreisler* gene (Sing *et al.*, 2009). This provides evidence that PREs are present in vertebrates and are responsible for at least some of the PcG gene targeting.

There are many proteins that associate with PcG proteins in humans that can influence both PRC enzymatic activity and target specificity. The Jumonji family protein Jarid2 associates with PRC2 via direct binding to Suz12 and promotes recruitment of PRC2 to specific gene targets (Peng *et al.*, 2009; Pasini *et al.*, 2010). Interestingly, although Jarid2 promotes recruitment of PRC2, associating with the complex reduces the methyltransferase activity of Ezh2,

possibly acting as a fine-tuner of gene repression (Peng *et al.*, 2009). In vertebrates, there is a functional ortholog of the PcG protein Pho, YY1, which is a transcription factor that can also associate with PRC2 (Satijn *et al.*, 2001; Caretti *et al.*, 2004). YY1 has DNA binding motifs that can direct PRC2 to specific gene targets (Caretti *et al.*, 2004). Assembling different complexes containing various PcG proteins or PcG homologs can also regulate PcG targeting (Ho and Crabtree, 2008). Changing the composition of the complex has been found to alter enzymatic activity and change substrate specificity (Kuzmichev *et al.*, 2005), but there is still very little known as to what determines the composition of the complex and how this influences PcG function.

Another mechanism of PcG gene targeting in humans that has been established is through non-coding RNAs (ncRNA). One ncRNA involved in PcG gene silencing is transcribed from the *HOXC* locus, known as HOTAIR (Rinn *et al.*, 2007). HOTAIR was shown to be essential for recruiting PcG proteins to the HOXD locus, providing the first evidence for PcG proteins targeted in *trans* by ncRNA (Rinn *et al.*, 2007). Chromosome X-inactivation is another example of an ncRNA providing a binding interface and targeting mechanism for PcG proteins. Xist is an ncRNA that coats the inactive X-chromosome, and within Xist exists a shorter ncRNA, RepA, that binds and localizes PRC2 to the inactive X-chromosome (Zhao *et al.*, 2008). RepA is necessary for the initial silencing of the inactive X-chromosome by targeting PRC2 methylation of H3K27 (Zhao *et al.*, 2008). The role of ncRNA in gene regulation is still a very new concept, and there

may be many more of these relationships with PcG proteins discovered in the future.

1.2.5 Polycomb and Stem cells

Polycomb proteins are extremely important during development and are essential in the maintenance of stem cells. PcG importance during development is evident in knockout mouse models, whereby, knockout of any PRC2 component or the catalytic component of PRC1, Ring1b, is early embryonic lethal (Faust *et al.*, 1998; O'Carroll *et al.*, 2001; Voncken *et al.*, 2003; Pasini *et al.*, 2004). These early mice embryos fail to differentiate properly while other PcG mutants show skeletal defects, consistent with PcG function in *Drosophila* regulating essential differentiation genes, such as *Hox* gene expression.

Many studies have focused on mapping global PcG target loci to determine the genes that are regulated by PcG proteins. These studies came to the same conclusion: PcG proteins repress many of the developmental genes that, if expressed, promote differentiation (Bracken *et al.*, 2006). It was found that many of these differentiation genes are bivalently marked with the repressive H3K27me3 and the activating H3K4me3 mark, poising these genes for quick transcription when necessary (Bernstein *et al.*, 2006). When PcG proteins are knocked out, these genes are then inappropriately transcribed resulting in improper differentiation (Chou *et al.*, 2011). Eed-null embryonic stem cells and Suz12-null embryonic stem cells can be cultured *in vitro*, indicating PRC2 activity may not be necessary to maintain cells in a pluripotent state (Boyer *et al.*, 2006; Pasini *et al.*, 2007; Chamberlain *et al.*, 2008); however, these cells do have

a higher level of spontaneous differentiation and are unable to properly differentiate (Boyer *et al.*, 2006; Pasini *et al.*, 2007). These embryonic stem cells are able to maintain pluripotency because they express the proper stem cell transcription factors. However, they also express elevated levels of differentiation genes due to abolished PcG-mediated gene repression, which prevents proper differentiation. Supporting this is the localization of PRCs with repressed genes occupied by Oct4, Sox2 and Nanog, three transcription factors essential for maintaining pluripotent stem cells (Avilion *et al.*, 2003; Boyer *et al.*, 2005; Lee *et al.*, 2006; Squazzo *et al.*, 2006). Oct4, Sox2 and Nanog could be involved in the recruitment of PRC to genes that need to be repressed, as knockout of Oct4 prevents PRC localization at these target genes (Squazzo *et al.*, 2006).

Adult stem cells have been found to exist in almost all tissues now, and PcG proteins play a key role in adult stem cell maintenance. Bmi-1 knockout mice are not embryonic lethal, however, these mice have severe phenotypes including ataxia and hematopoietic defects (van der Lugt *et al.*, 1994; Park *et al.*, 2003). Bmi-1 is essential for maintaining the hematopoietic stem cell (HSC) population, as knockout of Bmi-1 renders the HSCs unable to undergo self-renewal cell division (Park *et al.*, 2003). This is not unique to HSCs either, as knockout of Bmi-1 also causes depletion of neural stem cells (Molofsky *et al.*, 2003). Similarly to Bmi-1, Ezh2 is also a key contributor to maintaining stem cell populations, as conditional ablation of Ezh2 causes depletion of muscle stem cells (Juan *et al.*, 2011). PcG role in maintaining stem cell populations is not only evident in knockout models, but the reverse holds true for overexpression.

Conditional knock-in experiments that cause overexpression of Ezh2 in mouse hematopoietic cells increased the number and proliferation of the HSCs (Herrera-Merchan *et al.*, 2012). The role and importance of PcG proteins in stem cell self-renewal and proliferation will become more evident and discussed further in the PcG contribution to cancer section.

1.3 DNA Damage

The genetic code, stored via DNA, contains all the necessary information for cellular survival and function, and its integrity is essential to prevent mutations and disease. Cells have evolved many complex DNA repair pathways to protect and restore the genetic code since DNA is quite susceptible to damage from both endogenous agents, including errors in DNA replication, replication fork collapse, and reactive oxygen species, and exogenous agents, including ionizing radiation, topoisomerase poisons, and DNA cross-linking chemicals (Friedberg, 1995; De Bont and van Larebeke, 2004). There are several different DNA repair pathways, including direct reversal, base-excision repair, nucleotide excision repair, single-strand break repair (SSBR) and double-strand break repair (DSB), each pathway being very important to repair different types of DNA lesions, but none more important than DSB repair as DBS's are the most toxic form of DNA damage (Hakem, 2008) (Figure 3).

This thesis will focus on DSB repair, as it is the most toxic form of DNA damage and several cancer therapies induce DSBs as a treatment

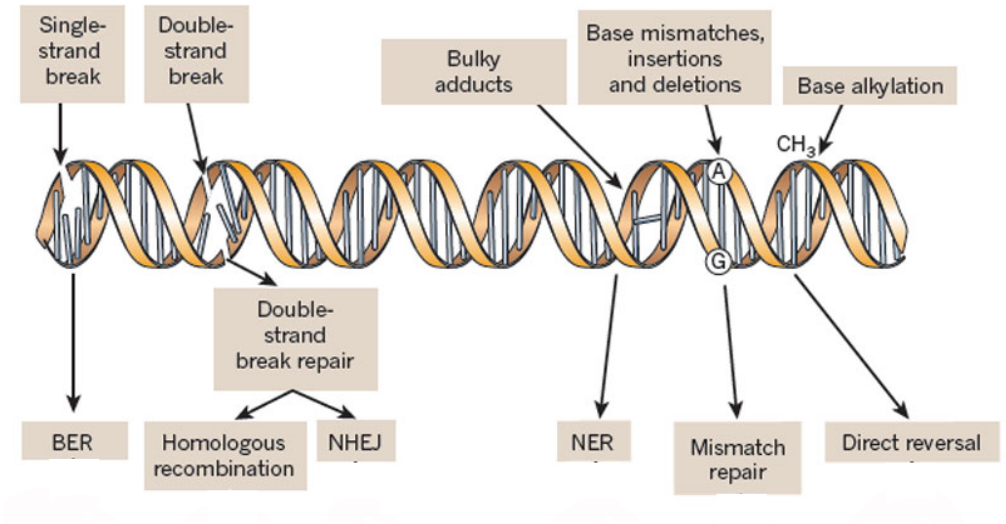

Figure 3. **DNA repair pathways are numerous and diverse.** DNA is continually exposed to many different types of DNA lesions. The cell has evolved repair pathways to contend with each type of lesion to ensure the integrity of the DNA. Adapted by permission from Macmillan Publishers Ltd: Nature. Lord and Ashworth, Copyright 2012.

modality (Helleday *et al.*, 2007; Chikamori *et al.*, 2010). SSBR will also be discussed because any SSB left unrepaired can become a DSB during replication. Each cell will encounter, on average, ten DSBs under normal circumstances each day, and a single DSB left unrepaired can lead to cell cycle arrest, apoptosis or worse, mutagenic translocations or deletions (Alison, 2005). Due to the toxicity of DSBs, cells have evolved multiple DSB repair pathways to ensure proper repair. There are two major DSB repair pathways, non-homologous end joining (NHEJ) and homologous recombination (HR) repair.

1.3.1 Single-strand break repair

Single-strand break repair is an extremely efficient process, and repairs SSBs with nearly perfect accuracy (Caldecott, 2008). This is important because each cell can encounter tens of thousands SSBs per cell per day via reactive oxygen species (ROS), intracellular metabolites, and spontaneous DNA decay (Caldecott, 2008). Failure to repair SSBs can lead to collapsed replication forks and the formation of more deleterious DNA damage, DSBs (Saleh-Gohari *et al.*, 2005). SSBR can be broken down into four main steps: damage recognition, DNA end-processing, DNA gap filling, and DNA ligation. The recognition and signaling of a DNA SSB is attributed to the poly(ADP)-ribose polymerase (PARP) family of enzymes (Satoh and Lindahl, 1992). PARP1 is considered the most active of the PARP enzymes and it strongly binds DNA SSBs, where upon binding DNA stimulates PARP1 to modify both itself and various other substrates with branches of poly(ADP)-ribose (PAR) (Satoh and Lindahl, 1992). Noteworthy, PARP1 has also been documented to bind strongly to DNA DSBs and contribute

to DSB recognition (Hochegger *et al.*, 2006; Wang *et al.*, 2006; Jorgensen *et al.*, 2009). The building of these PAR chains mediates recruitment of additional SSB repair machinery and promotes chromatin decondensation allowing for DNA repair machinery to access the DNA damage (Poirier *et al.*, 1982; El-Khamisy *et al.*, 2003; Ahel *et al.*, 2009). PAR moieties have a very short lifespan at SSBs, as they are quickly degraded by poly(ADP)-ribose glycohydrolase (PARG) to facilitate DNA repair (Lindahl *et al.*, 1995). The next step in SSB repair is DNA end-processing, which consists of a host of different enzymes depending on the type of damage, but an essential protein in this step is XRCC1. XRCC1 interacts with several other proteins, including PNKP, APTX, POLβ and DNA ligase III to ensure that DNA ends are properly processed so that POLβ can fill the gap and DNA ligase III can seal the gap, completing SSB repair (Caldecott *et al.*, 1996; Vidal *et al.*, 2001; Whitehouse *et al.*, 2001). Depending on the damage, other enzymes may be involved. For example, aborted topoisomerase I activity is processed by tyrosyl-DNA phosphodiesterase 1 (TDP1) (Yang *et al.*, 1996). Most SSBs are repaired within minutes of occurring; however, defects in SSB repair result in neurological diseases, genomic instability and an increased risk of cancer (Caldecott, 2008).

1.3.2 Non-Homologous End Joining

Non-homologous end joining is considered the major DSB repair pathway in higher eukaryotes and is functional throughout all stages of the cell cycle (Branzei and Foiani, 2008). NHEJ is also called the “error prone” DSB repair pathway because it does not restore the damaged DNA back to its original state; it

simply cleans up the damaged ends and ligates the DNA. Unfortunately, this can cause nucleotides to be lost at the DSB lesion and can cause oncogenic translocations. In both NHEJ and HR, the first step is recognition of the DSB. In NHEJ, the DSB is first recognized by the Ku70/Ku80 heterodimer, which binds the free DSB ends and tethers them together (Walker *et al.*, 2001). The PI-3 kinase-related kinase, DNA-PKcs, then binds the Ku70/Ku80 heterodimer and the free DNA ends, further tethering the free DNA strands together and protecting the DNA ends from nucleases (Yoo and Dynan, 1999; DeFazio *et al.*, 2002). Association of DNA-PKcs with the Ku complex and DNA begins a phosphorylation cascade, beginning with DNA-PK trans autophosphorylation (Meek *et al.*, 2007). The exact events occurring after this are not completely known, but it is known that phosphorylated DNA-PKcs can now dissociate from the break site and other NHEJ repair proteins including XRCC4, Artemis, XLF, and DNA ligase IV can process and repair the DSB (Mahaney *et al.*, 2009). Other DNA processing enzymes, such as PNKP, WRN and APLF may also be involved depending on the nature of the DSB and processing needs of the DNA ends for ligation. A cartoon of NHEJ is shown in figure 4.

1.3.3 Homologous recombination repair

Homologous recombination repair is also known as “error-free” repair because it uses the sister chromatid to replicate the damaged chromosome thereby keeping the genetic code intact. Because this pathway uses the sister chromatid, it

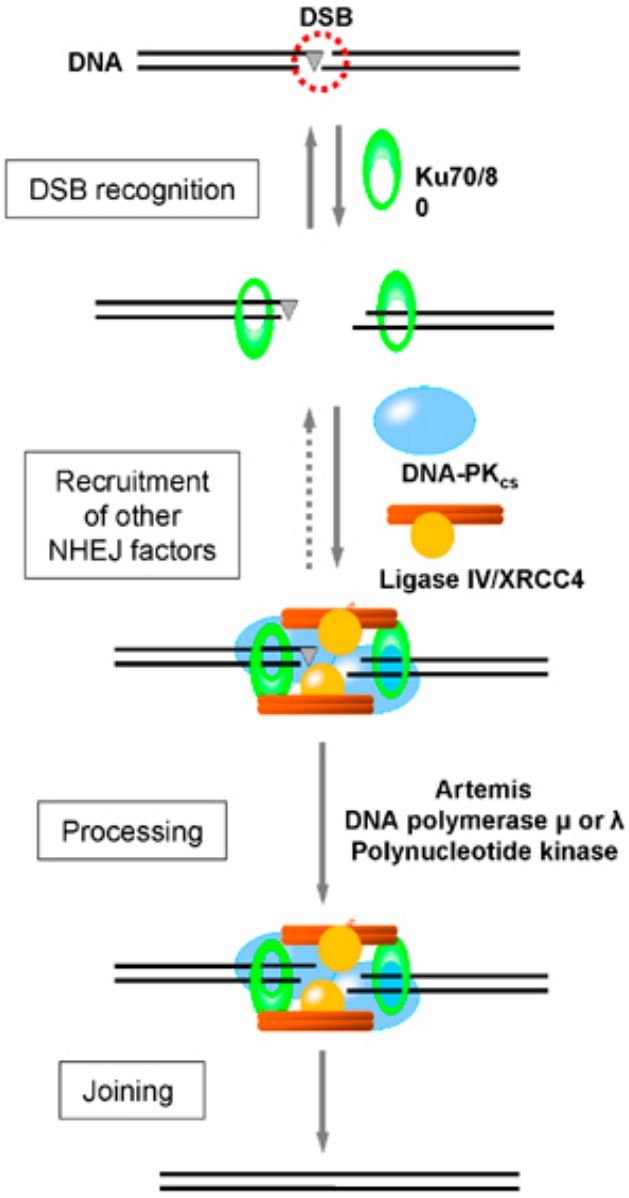

Figure 4. **A representative model of the non-homologous end joining repair pathway.** The DNA double-strand break is first recognized and bound by the Ku70/80 heterodimer. The DNA-PKcs kinase then binds the Ku heterdimer and DNA ends, followed by recruitment and binding of other DNA repair factors. The DNA ends are then processed to enable ligation, and the DNA ends are rejoined. Adapted by permission from Macmillan Publishers Ltd: Oncogene. Gent and Burg, Copyright 2007.

is restricted to late S-phase and G2 phase of the cell cycle (Mao *et al.*, 2008). Similarly to NHEJ, the first step of HR is recognition of the DSB, which is achieved mainly by the MRN complex (Mre-11, Rad50, NBS-1) with new evidence showing that PARP1 may also contribute (Haince *et al.*, 2008; Yuan and Chen, 2010). Upon recognition of the DSB, the MRN complex, along with other recruited nucleases and helicases digest back one strand of the DNA to create longer single-strand DNA overhangs, which are protected by RPA (Mimitou and Symington, 2008). The remaining steps of HR are very complicated and use numerous proteins, so this will be a much-simplified description. BRCA1 and BRCA2 proteins are mediators recruited to the DSB site and help RAD51 paralogs form filaments around the single-strand DNA to assist in sister chromatid invasion and the formation of a Holliday junction (Yang *et al.*, 2005; Holthausen *et al.*, 2010). DNA polymerase will then fill in the DNA gaps and DNA ligase can seal the gap and finish the repair process. A cartoon of HR repair is shown in figure 5.

1.3.4 Cellular response to DNA double-strand breaks

When the cell sustains damage in the form of a DSB, there are several possible outcomes including DNA repair, cell cycle arrest, and/or apoptosis (reviewed in (Shiloh, 2003)). The cell must first detect the DSB and start a signaling cascade to recruit DNA repair proteins. Meanwhile, the cell cycle has to be arrested to prevent DNA replication or mitosis while DSBs are present. Lastly, the DNA damage has to be assessed, and if the cell is unable to repair the damage,

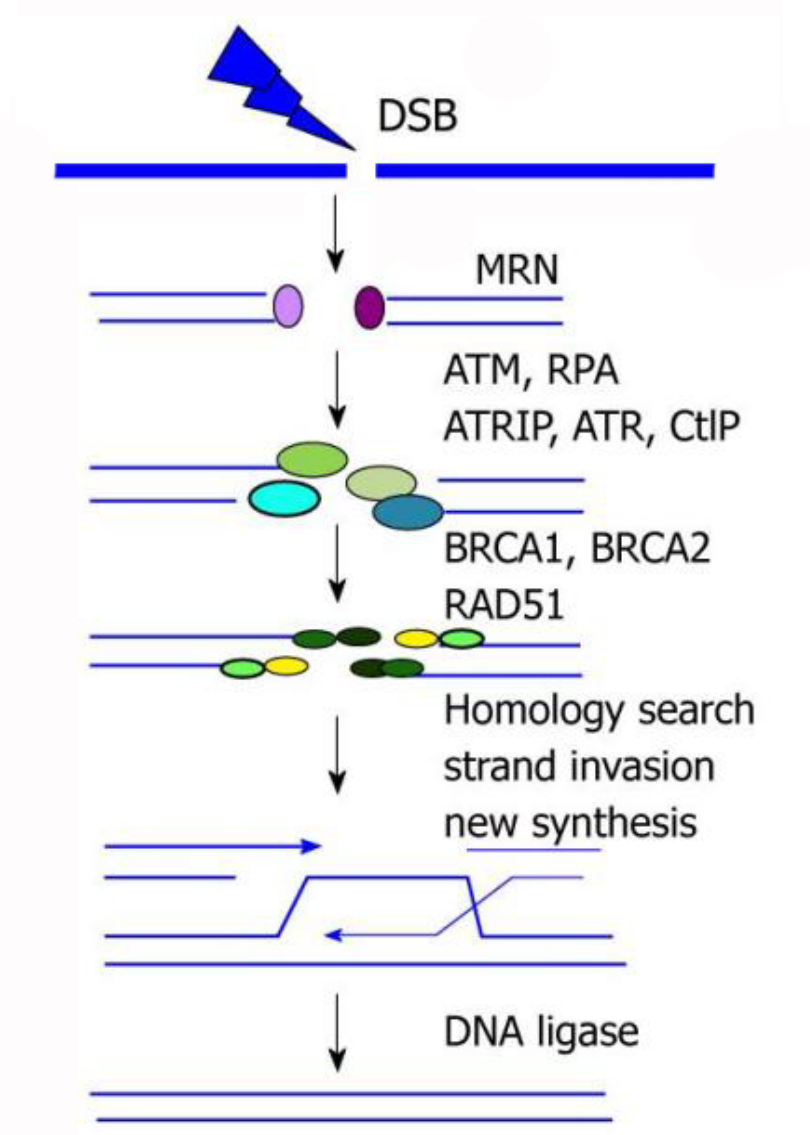

Figure 5. **A representative model of the homologous recombination repair pathway.** The DNA double-strand break is first recognized by the MRN complex. The ATM kinase is recruited and begins a phosphorylation cascade which recruits more double-strand break repair proteins. The Rad51 filament initiates homology search and strand invasion which is followed by DNA synthesis and ligation completing the repair process. Adapted from Peng and Lin. 2011.

apoptotic pathways will be activated. Mutations, translocations and improper cell division can occur if there is a failure to detect and/or signal the presence of a DSB within the cell.

There are three related signaling kinases that are essential for the DSB response: ATM (ataxia telangiectasia mutated), ATR (ATM- and Rad3 related) and DNA-PK (DNA-dependent protein kinase). ATR does not respond directly to DSBs, but does respond to agents that induce large segments of single-stranded DNA, such as interruptions to DNA replication causing stalled replication forks (Zou and Elledge, 2003). DNA-PK is essential for NHEJ and V(D)J recombination, but its role in DSB signaling is still controversial (reviewed in (Mahaney *et al.*, 2009)). ATM, however, is extremely important in DSB signaling, and as little as 0.5 Gy can activate almost every ATM molecule in the cell (Bakkenist and Kastan, 2003).

ATM is activated within minutes of inducing DNA damage by interacting with the MRN complex at the DSB lesion (Uziel *et al.*, 2003); however, there is also evidence that ATM can sense changes in chromatin structure and become activated (Bakkenist and Kastan, 2003). Upon inducing DNA damage, ATM phosphorylates itself in trans at serine 1981 (Bakkenist and Kastan, 2003). ATM then proceeds to phosphorylate over 30 downstream substrates, including histone 2A variant X (H2AX), NBS1, BRCA1, p53, SMC1, Chk1 and Chk2 (Shiloh, 2006). The main role of ATM phosphorylation is to amplify the DSB signal both at the site of DNA damage, where it helps recruit DSB repair proteins and inhibit transcription, and throughout the cell, where it acts to establish cell cycle arrest

and/or initiate apoptosis if necessary. ATM mutations cause several phenotypes including neurological defects, radiation sensitivity, and increased rates of developing cancer, all of which are phenotypes associated with decreased DSB signaling and repair (Bundey, 1994).

ATR activation occurs at stalled replication forks when large stretches of single-stranded DNA (ssDNA) are bound by RPA. ATR interacting protein (ATR-IP) and ATR bind to RPA at the ssDNA, followed by ATR activation by TopBP1 (Zou and Elledge, 2003; Kumagai *et al.*, 2006). ATR can also be phosphorylated by ATM, and thus can contribute to the DSB repair signaling cascade as well (Jazayeri *et al.*, 2006). ATM and ATR share many downstream targets, therefore it is difficult to determine the phosphorylation events that are independent of ATM; however, one important downstream target that is phosphorylated mainly by ATR is Chk1 (Liu *et al.*, 2000). ATR phosphorylation of Chk1 causes cell cycle arrest at the G1-S and G2-M phases of the cell cycle (Liu *et al.*, 2000).

1.3.5 Histone post-translational modifications at DSB sites

Posttranslational modifications are essential for cellular signaling, protein function, and protein interactions, all of which are necessary during DSB repair. The importance of ATM in the DSB repair pathway has already been highlighted, but one of its downstream targets, H2AX is also important during the DSB repair process. ATM phosphorylates H2AX at serine 139 in response to DNA damage, and this serves as a recognition site for downstream mediators MDC1 and MCPH1 (Burma *et al.*, 2001; Stucki *et al.*, 2005; Wood *et al.*, 2007).

Phosphorylated H2AX (γ-H2AX) is used as a marker for DSBs because it is highly enriched at DSBs, and forms distinct cellular foci that co-localize with other known DSB repair proteins. The phosphorylation of H2AX at sites of DNA damage increases the cellular response to a DSB, and retains DSB repair proteins at the site of DNA damage to ensure repair. H2AX is also ubiquitylated in response to DNA damage by PRC1, RNF8 and RNF168 (Mailand *et al.*, 2007; Doil *et al.*, 2009; Ismail *et al.*, 2010). DNA damage-induced ubiquitylation is not restricted to H2AX, as H2A is also ubiquitylated at sites of DNA damage (Mailand *et al.*, 2007; Doil *et al.*, 2009; Ginjala *et al.*, 2011). Much like phosphorylation of H2AX, H2A/H2AX ubiquitylation helps amplify DSB signaling and helps retain DSB repair proteins at the DNA damage site. H2A/H2AX ubiquitylation has also been shown to contribute to DSB induced transcriptional silencing (Shanbhag *et al.*, 2010). Histone methylation and histone acetylation also contribute to the signaling and repair of DSBs. Tip60 is an acetyltransferase that has been shown to be important in ATM activation at sites of DNA damage and necessary for efficient DSB repair (Sun *et al.*, 2005). It was shown that histone 3 lysine 9 (H3K9) trimethylation is recognized by the chromodomain on Tip60 and is essential for the activation of Tip60 at sites of DNA damage (Sun *et al.*, 2009). Global depletion of H3K9me3 prevented Tip60 and ATM activation causing defects in DSB repair and signaling (Sun *et al.*, 2009). It has also been shown that the Tudor domain of 53BP1 can recognize histone 4 lysine 20 (H4K20) dimethylation to facilitate 53BP1 recruitment and retention at sites of DNA damage (Sanders *et al.*, 2004). The histone

methyltransferase MMSET was shown to recruit to sites of DSBs and methylate H4K20 promoting the retention of 53BP1 at sites of DSBs (Pei *et al.*, 2011). Histone post-translational modifications are an important aspect of DSB recognition and signaling and are essential for the efficient, accurate repair of DSBs.

1.3.6 Polycomb proteins and DNA DSB repair

Polycomb proteins have a well-established role in gene regulation, development, and stem cell maintenance; however, recent evidence implicates polycomb proteins in the DSB repair pathway. PRC1 is responsible for the majority of H2A and H2AX monoubiquitylation in response to DNA damage, and knockout of PRC1 ubiquitin ligase activity causes decreased DSB repair capacity and increased sensitivity to ionizing radiation (Ismail *et al.*, 2010; Ginjala *et al.*, 2011). Another possible role for PRC1 at sites of DSBs is transcriptional silencing. Histone ubiquitylation was shown to be important for ATM mediated transcriptional silencing at sites of DSBs, and it is possible that PRC1 contributes by ubiquitylating H2A at sites of DNA damage (Shanbhag *et al.*, 2010). Recent reports place PRC1 signaling at DSBs upstream of ATM, highlighting the importance of PRC1 in DSB repair (Pan *et al.*, 2011).

PRC2 has also been implicated in DSB repair, however, there are conflicting reports and much less mechanistic insight into PRC2's role in the DSB repair pathway. One report shows that overexpression of Ezh2 causes a decrease in expression of Rad51 paralogs, thereby disabling HR and inhibiting DSB repair (Zeidler *et al.*, 2005). Another report states that Ezh2 is essential in cancer cells

for activating Chk1 in response to DNA damage, and knockout of Ezh2 promotes DNA damage-induced apoptosis (Wu *et al.*, 2011). As well, PRC2 members have been shown to recruit to sites of DNA damage and trimethylate H3K27 (O'Hagan *et al.*, 2008; Chou *et al.*, 2010). This thesis aims to clarify and define a role for PRC2 in the DSB repair pathway.

1.3.7 Techniques used to study DNA damage

There are several different methods commonly used to investigate the mechanisms of DNA repair and the DNA damage response pathway. As with most methods, there are both strengths and weaknesses to each technique, and thus, multiple methods are commonly used to determine how a protein is involved in the DNA damage response and repair pathway.

1.3.7.1 Ionizing Radiation Induced Foci

Microscopy is a great technique to directly visualize proteins within the cell to determine protein localization and response to various cellular stresses. Microscopy can be used in live cells or fixed cells, and various methods can be used to look at a protein of interest, whether it is immunostaining or tagging an endogenous protein with a fluorescent marker. A common characteristic of DNA damage response proteins is the accumulation at DSBs, which, when viewed using fluorescent microscopy, appear as large foci (Figure 6). These foci are named ionizing radiation induced foci (IRIF) because of their appearance after inducing DSBs by treating the cells with ionizing radiation. The exact organization of proteins within IRIF has yet to be resolved, but it is thought that IRIF are an accumulation of DSB repair proteins at a DSB. It is also important to

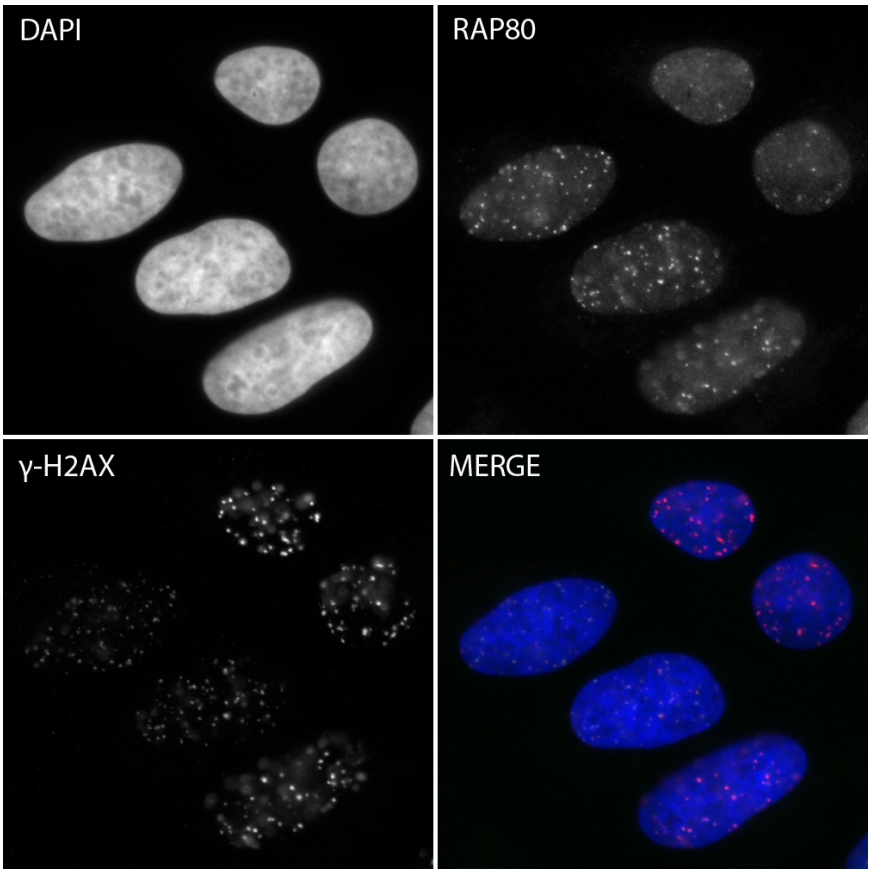

Figure 6. **Accumulation of double-strand break proteins at ionizing radiation induced foci.** U2OS cells were treated with 2 Gy and left to recover for 30 min. Cells were then fixed in 4% paraformaldehyde and stained using indirect-immunoflourescence with antibodies against γ-H2AX and Rap-80. The γ-H2AX modification and Rap-80 DSB repair protein co-localize at specific foci, termed ionizing radiation induced foci.

note that although many DSB repair proteins do accumulate at IRIF, not all DSB repair proteins form IRIF. Proteins involved in the NHEJ pathway do not form IRIF, including the Ku protein complex, which has been shown to be an essential DSB repair protein. Therefore, if a protein does accumulate at IRIF, it is likely involved in DSB repair; however, if a protein does not accumulate at IRIF, this does not exclude the protein as a possible DSB repair protein. Nonetheless, many studies have used the appearance of IRIF to determine the order and dependency of recruitment to DNA damage. In cells that lack H2AX, and are therefore unable to phosphorylate H2AX, MDC1 fails to recruit to IRIF (Stucki *et al.*, 2005). MDC1 contains a BRCT domain, which binds and recognizes the phosphate PTM added to H2AX (Stucki *et al.*, 2005). γ-H2AX IRIF are also used to determine the resolution of DSBs (Lobrich *et al.*, 2010). γ-H2AX foci assays look at the resolution of γ-H2AX foci over time and correlate this to the efficiency of DSB repair. It is still important to note that IRIF are not fully understood. The persistence of a γ-H2AX focus is largely assumed to indicate the persistence of a DSB, but this has not been proven.

1.3.7.2 Laser micro-irradiation

Laser micro-irradiation (LMIR) is a technique that allows the investigator to induce localized DNA damage within a select cell. The principle behind LMIR involves pre-incubating the cells with a DNA intercalator, followed by excitation of the DNA intercalator with a specific wavelength from a laser. The excitation of the intercalator by the laser causes the release of free radicals, which induce localized DNA damage. The investigator can then use microscopy techniques to

analyze the recruitment of proteins to the DNA damage. In our experiments, we use Hoechst dye as a DNA intercalator and a 750 nm laser to excite the Hoechst dye. Various excitation wavelengths from 355 nm to 405 nm have been used in the literature (Examples: 355 nm (Chou *et al.*, 2010), 375 nm (Ismail *et al.*, 2010), 405 nm (Hong *et al.*, 2008)). Bromodeoxyuridine (BrdU) is also commonly used instead of a DNA intercalator (Hong *et al.*, 2008; Chou *et al.*, 2010). The advantages of LMIR are the ability to create localized DNA damage and follow the recruitment of DNA damage response proteins in real-time. From this, recruitment kinetics can be obtained, and similar to IRIF, recruitment order and dependency pathways can be determined. Furthermore, NHEJ proteins, such as Ku70/80 (Koike and Koike, 2008), recruit to LMIR-induced DNA damage, and so many more DNA damage response proteins can be investigated using LMIR. Disadvantages to using LMIR include primarily that LMIR is an experimental form of DNA damage, and may not be clinically relevant. As well, LMIR induces a considerable amount of DNA damage in a very localized region, which again may cause a cellular response that is not clinically relevant. Lastly, LMIR induces multiple forms of DNA damage, including base damage, UV damage, SSBs, and DSBs, and each laser system creates these types of damage in a different ratio (Kong *et al.*, 2009). It can therefore be difficult to determine what type of DNA damage a protein is responding to and it can be difficult to compare results from one laser system to another. LMIR is, however, widely used in the DNA damage field, and can be a great system to determine if proteins are recruited to sites of DNA damage.

1.3.7.3 Gel electrophoresis

There are several different applications of gel electrophoresis for assessing DNA damage, all utilizing the same principle that when DNA is subjected to an electric field, the negatively charged DNA will migrate toward the positive end of the electric field. One application is constant-field gel electrophoresis (CFGE), whereby, DNA is subjected to a constant electric field and run through an agarose gel matrix (Wlodek *et al.*, 1991). The agarose gel matrix provides resistance to DNA mobility, so that DNA will be separated based on size; the shorter the DNA fragment, the faster it will migrate through the agarose. A variation to CFGE, is pulsed-field gel electrophoresis (PFGE), which uses a different instrument. The PFGE instrument constantly changes the direction of the electric field, and is better at resolving large DNA fragment sizes (Wlodek *et al.*, 1991). Both PFGE and CFGE can be used to determine the relative amounts of DSBs or SSBs. Using a neutral running pH will maintain the integrity of the DNA double-helix, and the DNA will remain double stranded. A more alkaline pH will cause the DNA strands to dissociate and become single stranded (reviewed in (Rojas *et al.*, 1999)). Neutral conditions will therefore only measure DSBs and alkaline conditions will measure both SSBs and DSBs. Gel electrophoresis can be performed on a single cell level, called the comet assay (reviewed in (McKelvey-Martin *et al.*, 1993)), or a cell population level, called the modified FAR assay (Ismail *et al.*, 2005). Gel electrophoresis can be used to assess the kinetics and efficiency of DNA repair by harvesting cells at different time points and under different conditions.

1.4 Cancer

Cancer is the leading cause of premature death in Canada, killing over 75,000 Canadians in 2011 (Canadian Cancer Statistics, 2011). Although there has been progress in cancer awareness and diagnosis, there is still a great need for improvements in cancer treatment, as 175,000 new cancer cases will present each year in Canada (Canadian Cancer Statistics, 2011). Treatment modalities mostly include surgery, radiation therapy and chemotherapy, which can also be used cooperatively to try and eliminate cancer cells. Most cancer therapies currently used in hospitals were developed in the mid-twentieth century and non-specifically target dividing cells. Almost all of these cancer therapies have significant and serious side effects, such as fatigue, digestive complications and immune suppression, which are driving a demand for new, less toxic therapies.

1.4.1 Chemotherapy

Chemotherapy is the administration of chemicals systemically to try to target mechanisms necessary for cancer cell proliferation and survival. There are many different classes of chemotherapies including alkylating agents, antimetabolites, topoisomerase inhibitors, mitotic inhibitors, and immunotherapeutics. Most chemotherapeutics used do not specifically target cancer cells, but instead target normal cellular functions that are more active in replicating cells, therefore targeting the rapid cell division commonly occurring in cancer. For example, taxol is a chemotherapy commonly used to treat solid tumors that inhibits microtubule function, thereby preventing proper chromosome alignment and segregation during mitosis (Horwitz, 1994). Unfortunately, taxol also targets the microtubules in normal cells, killing any rapidly dividing cell

population in the human body, including the hematopoietic system. Other examples include topoisomerase inhibitors and alkylating agents, which induce DNA damage in both normal cells and cancer cells. Topoisomerases release supercoiling strain in the DNA by inducing either a SSB or a DSB in the DNA and unwinding the DNA at the break site. When topoisomerases are functioning properly, the DNA is re-ligated and DNA damage does not occur. Topoisomerase poisons disable the re-ligation process or the release of topoisomerase causing SSBs, DSBs and/or bulky blocking lesions that impair DNA replication and transcription (reviewed in (Chikamori *et al.*, 2010)). Alkylating agents function differently than topoisomerase poisons but both chemotherapeutic groups have the same effect, damaging the DNA. Alkylating agents add methyl groups to DNA inhibiting transcription and DNA replication, as well as stimulating the DNA damage response. Unfortunately, much like the microtubule inhibitors, both alkylating agents and topoisomerase poisons also damage the DNA of normal cells, causing cell death and potential mutations in any normal cell population that is rapidly dividing. Again, this causes side effects such as harming the hematopoietic and digestive systems. The search for new and improved chemotherapies continues because of the toxic side effects involved with current treatments and also because many cancers tend to become resistant to these treatment modalities.

1.4.2 Radiation Therapy

In 1895, the discovery of X-rays by Rontgen fueled a new treatment modality for cancer(Rontgen, 1896). Scientists discovered that high-energy light

waves could reduce the size of tumor masses. Today, high-energy radiation is still used to treat patients, however, both technology and understanding of the principles behind radiation treatment have greatly increased the efficacy. Radiation therapy works by targeting high-energy radiation waves into the tumor mass, where the energy is deposited. The energy can be deposited directly into the DNA inducing DNA damage, or it can ionize water creating oxygen free radicals that can also compromise the structural integrity of the DNA, inducing base damage, SSBs and DSBs (Nunez *et al.*, 1996). Much like in chemotherapy, the damage to the DNA is a main contributor to cancer cell killing. The caveats to radiation therapy are dose administration and targeting. The high-energy radiation can also be absorbed by normal cells, causing DNA damage and possibly mutations in the normal tissue, and at too high a dose, cell death can occur. Current treatment modalities use "dose fractionation", which refers to giving small increments of radiation many times over that over time accumulates to a large targeted dose of radiation to the tumor. Dose fractionation gives the surrounding normal tissue time to repair while the tumor receives a much larger dose over time (Haffty, 2009). The second caveat to radiation therapy is tumor targeting. Delivering radiation to a tumor requires the physician to know where the tumor is located. Unlike chemotherapy, which is delivered systemically and travels everywhere in the body, radiation therapy is directly targeted to one location, and it is therefore useless against unknown metastasis and secondary tumors.

1.4.3 New target: Cancer Stem Cells

A revolutionary finding in the cancer field occurred when a group was studying acute myeloid leukemia, and found that the leukemia initiating cells shared cell surface markers of normal hematopoietic stem cells (Bonnet and Dick, 1997). It was concluded that normal stem cells were driving the cancer, and the cancer clones were organized into a hierarchy and capable of both self-renewal and differentiation (Bonnet and Dick, 1997). The hypothesis of cancer stem cells was further established when scientists separated a breast tumor into two populations based on cell surface markers, CD44+/- and CD24+/-, and injected these cells back into mice to test tumor forming potential. Surprisingly, they found that only the CD44+ / CD24-/(low) cells had tumor forming potential in mice, and that the resulting tumors from these cells contained mostly CD44-/CD24+ cells (Al-Hajj *et al.*, 2003). This showed that only a small subset of cancer cells are in fact capable of forming a tumor, and that these cells are able to re-establish the heterogeneity of the tumor. This gave rise to the first cancer stem cell model in solid tumors (Al-Hajj *et al.*, 2003), and the hypothesis that if cancer stem cells could be eradicated, the bulk of the tumor posed little to no threat. Although this last statement no longer holds true, the existence and importance of cancer stem cells is very apparent.

Cancer stem cells (CSC) have been found in breast, prostate, brain, colon, ovarian, and hematopoietic cancers (Bonnet and Dick, 1997; Al-Hajj *et al.*, 2003; Singh *et al.*, 2004; Patrawala *et al.*, 2006; Ricci-Vitiani *et al.*, 2007). The discovery of the induced pluripotent stem cell (IPS) greatly changed the understanding of differentiation and the CSC model. A group of scientists found

that through the expression of four factors: Oct3/4, Sox2, c-Myc, and Klf4, fully differentiated mouse fibroblasts could be reprogrammed into embryonic stem cells (Takahashi and Yamanaka, 2006). Differentiation was previously thought to be unidirectional, but this provided evidence that de-differentiation was very possible. Epithelial to mesenchymal transition (EMT) is another mechanism that allows cellular transformation from a more differentiated cell (an epithelial cell) to a less differentiated cell (a mesenchymal cell) (Mani *et al.*, 2008). EMT is a normal process that is necessary during wound healing and development, however, it was shown that through the process of EMT, normal breast epithelial cells could obtain stem-cell properties (Mani *et al.*, 2008). This newly acquired information had profound influence on the CSC theory and evolution of tumors. Any fully differentiated tumor could go through the process of de-differentiation and produce cancer cells that are more stem cell-like. This also means that any given cancer cell in a tumor has the potential to become a CSC, and thus not only must the CSCs be targeted by oncotherapy, the entire cancer population must be targeted.

1.4.4 Polycomb proteins and Cancer

Bmi-1 was the first PcG protein to be called a proto-oncogene and was discovered by its cooperation with E mu-myc in lymphomagenesis (van Lohuizen *et al.*, 1991). Overexpression of PcG proteins has now been observed in many different types of cancer, including breast, prostate, colon, brain, hematopoietic and others (Haupt *et al.*, 1993; Kleer *et al.*, 2003; Tateishi *et al.*, 2006; Crea *et al.*, 2010; Karanikolas *et al.*, 2010). The overexpression of PcG proteins in cancer is

also correlated with an aggressive, metastatic cancer and a poor prognosis (reviewed in (Crea *et al.*, 2011)) (Table 1). As discussed earlier, PcG proteins are essential for maintaining stem cells by repressing differentiation genes, and the function of PcG proteins may play the same role in cancer. Overexpression of PcG proteins in cancer leads to a less-differentiated phenotype, consistent with more aggressive cancers (Chang and Hung, 2012). PcG proteins represent a novel target for chemotherapies because it is expected that knocking out PcG function can drive the differentiation of cancers, thereby reducing aggressiveness and increasing the cancers susceptibility to current treatment modalities (reviewed in (Xiao, 2011) and (Cao *et al.*, 2011)) DZNep, a drug that targets PRC2 and causes PRC2 degradation, has been shown to be effective for reducing proliferation, migration, and tumorigenicity of several cancer cell types (Crea *et al.*, 2011; Chiba *et al.*, 2012; Kemp *et al.*, 2012). PRC2 inhibition appears to be a promising target for reducing the aggressiveness of many cancers.

1.4.5 Polycomb proteins, Cancer, and DNA damage

The relationship between PcG proteins, cancer, and DNA damage may provide another mechanism for trying to kill cancer cells. As mentioned previously, many current cancer therapies work by inducing DNA damage, namely DSBs via topoisomerase poisons, alkylating agents, and radiation therapy.

Protein overexpressed	Type of Cancer	Reference
Ezh2	B-cell non-Hodgkin lymphoma Bladder Breast Colon Hodgkin lymphoma Liver Lung Mantle cell lymphoma Melanoma Pancreas Prostate	(van Kemenade *et al.*, 2001) (Arisan *et al.*, 2005; Raman *et al.*, 2005; Weikert *et al.*, 2005) (Kleer *et al.*, 2003; Raaphorst *et al.*, 2003; Bachmann *et al.*, 2006; Collett *et al.*, 2006) (Mimori *et al.*, 2005) (Raaphorst *et al.*, 2000) (Sudo *et al.*, 2005) (Takawa *et al.*, 2011) (Visser *et al.*, 2001) (Bachmann *et al.*, 2006) (Ougolkov *et al.*, 2008) (Varambally *et al.*, 2002; Bachmann *et al.*, 2006)
Bmi-1	B-cell non-Hodgkin lymphoma Leukaemia Mantle cell lymphoma Medulloblastoma Neuroblastoma Non-small cell lung cancer	(van Kemenade *et al.*, 2001) (Sawa *et al.*, 2005) (Bea *et al.*, 2001) (Leung *et al.*, 2004) (Nowak *et al.*, 2006) (Vonlanthen *et al.*, 2001)

Table 1. **Polycomb proteins are overexpressed in many different types of cancer.** Polycomb proteins, Ezh2 and Bmi-1, and the types of cancer each protein has seen to be overexpressed in.

Targeting PcG proteins to treat cancer is currently being explored to try to force differentiation; however, targeting PcG proteins may also have another important therapeutic potential. PRC1 has already been show to have a very important role in DSB repair, and knockout of PRC1 causes sensitivity to ionizing radiation (Ismail *et al.*, 2010). Targeting PRC1 as a potential cancer therapy would, therefore, not only help drive differentiation in cancers, but also sensitize cancers to radiation and other DNA damaging therapies. It has also been shown that knocking down PRC2 components can sensitize cells to ionizing radiation and cisplatin, thereby furthering the therapeutic potential of targeting PRC2 in cancer (Chou *et al.*, 2010; Hu *et al.*, 2010).

One of the key factors contributing to cancer formation is genomic instability (reviewed in (Negrini *et al.*, 2010)). Most cancers have defects in DNA repair pathways that contribute to genomic stability and some of these defects can be exploited to try to kill the cancer cells. One example is BRCA-deficient cancers that lack the essential HR repair proteins BRCA1 and BRCA2. Some females are born heterozygous for BRCA function and are susceptible to developing breast cancer, because a single mutation in the functional BRCA gene can then result in the inability to perform HR, resulting in genomic instability (Snouwaert *et al.*, 1999; Moynahan *et al.*, 2001). In this instance, the same genetic mutation that causes genomic instability and cancer, can be exploited to kill the very cancer cells it helped create. PARP inhibitors can be used to generate SSBs by interfering with base excision repair (Strom *et al.*, 2011), and these SSBs accumulate through the cell cycle. As the cell tries to replicate its DNA, these

SSBs are converted into DSBs through replication fork collapse. Normal cells that have functional BRCA are able to repair these DSBs using HR, however, the cancer cells that are BRCA-deficient are unable to repair these DSBs and die (Bryant *et al.*, 2005; Farmer *et al.*, 2005). Individually, BRCA deficiency and PARP inhibition are not lethal, however, the two deficiencies combined are lethal. This is termed synthetic lethality. PARP inhibitors are currently undergoing clinical trials for BRCA-deficient breast cancer patients and are being investigated for therapeutic potential against other cancers (Rios and Puhalla, 2011). Other synthetic lethal relationships are being investigated, and there are potentially many more due to the inherent DNA repair defects present in most cancers.

1.5 Hypothesis

We hypothesize that PRC2 is recruited to sites of DNA damage and contributes to the signaling and/or repair of DSBs. We propose that impairing the function of PRC2 by inhibitors or by knocking down PRC2 protein levels will reduce the ability of cells to repair DSBs and cause sensitivity to ionizing radiation.

Chapter II: *Materials and Methods*

2.1 Cell culture and Transfections

U2OS (human osteosarcoma), H2AX WT (mouse fibroblast), and H2AX KO (mouse fibroblast) cells (a gift from Dr. Andre Nussenszeig) were cultured in DMEM supplemented with 10% FBS at 37°C and 5% CO_2. U2OS cells with the stably integrated p3216PECMS2β insert (263 cells) (gift from Dr. Susan Janicki) were cultured in High Glucose DMEM with 10% FBS and 100μg/mL hygromycin B. EBS, YZ5 (A-T patient cells transformed with SV-40, whereby EBS cells are ATM deficient.) and MO59J, MO59K (human glioma from a single patient, MO59J cells are DNA-PK deficient) were cultured in 50% DMEM/50% F12 supplemented with 10% FBS. All plasmid transfections were carried out with Effectene using the Qiagen protocol.

2.2 Laser micro-irradiation

Cells were plated on 35-mm glass bottom culture dishes (MatTek Corporation) 24 h before the experiment. Cells were treated with 1 μg/ml Hoechst 33258 for 15 min and then placed on a heated stage (37°C) of a laser-scanning confocal microscope (LSM510 NLO; Carl Zeiss, Inc.). DNA damage was generated along a 0.2–1 μm-wide region across the nucleus by excitation of the Hoechst 33258 dye using a 20 mW near infrared 750-nm titanium-sapphire laser line. The laser output was set to 15% and we applied 10 iterations to generate localized DNA damage using a Plan-Neofluar 40X/1.3 N.A. oil immersion objective. GFP fluorescence imaging was recorded after excitation with a 488-nm

argon laser using a 515–540 nm band-pass filter. Mean and standard error for a minimum of ten cells were plotted.

2.3 Fluorescence recovery after photobleaching (FRAP)

Cells were cultured on 35-mm glass bottom culture dishes (MatTek Corporation) 24 h before the experiment. Cells were placed on a heated stage (37°C) of a laser-scanning confocal microscope (LSM510; Carl Zeiss, Inc.). GFP fluorescence was bleached along a 1 μm-wide region across the nucleus using a 488-nm argon laser set to 100% output. GFP fluorescence imaging was recorded after excitation with a 488-nm argon laser using a 515–540 nm band-pass filter. Mean and standard error for a minimum of fifteen cells were plotted.

2.4 Immunofluorescence Microscopy

Immunofluorescence was performed as previously described (Ismail *et al*., 2010). Cells were fixed with 4.0% paraformaldehyde (PFA) in PBS, pH 7.5, for 5 min at room temperature. Cells were then permeabilized with PBS containing 0.5% Triton X-100 for 5 min, and then washed with PBS three times. Following permeabilization, cells were incubated with primary antibody for 30 min at room temperature. Cells were then rinsed with PBS containing 0.1% Triton X-100 and then washed three times with PBS. Cells were subsequently incubated with a secondary antibody for 30 min at room temperature. Next, cells were rinsed with PBS containing 0.1% Triton X-100 and washed three times with PBS. Finally, cells were mounted onto slides with a 90% glycerol/PBS-based medium containing 0.5 μg/mL DAPI and 1.0 mg/mL p-Phenylenediamine. Cells were observed using an Axiovert 200M microscope (Carl Zeiss, Inc.).

2.5 Chromatin Immunoprecipitation

263 cells were transfected 24 h prior to harvest with either mcherry-LacI-Fok-1 or catalytically dead mcherry-LacI-Fok-D480A. Cells were then fixed in suspension at 22^{o}C with 1% PFA for 10 min and then the PFA was quenched with 0.125 M glycine for 5 min. Cells were then centrifuged at 1400x *g* and the nuclei were released in Nuclei Isolation Buffer (250 mM sucrose, 150 mM NaCl, 20 mM Tris pH 8.0, 1.5 mM $MgCl_2$, 0.2 mM $CaCl_2$, 0.1% IGEPAL CA-630). Nuclei were centrifuged at 3200x *g* and resuspended in a modified RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% IGEPAL CA-630). Micrococcal nuclease was added for 10 min at room temp to digest the DNA (2000 gel units in modified RIPA plus 5 mM $CaCl_2$). The digested chromatin was then incubated with Dynabeads preincubated with the appropriate antibody according to the Invitrogen protocol. After elution, PFA crosslinks were reversed for 6 h at 65°C and proteinase K (3 mg/mL) was added. Quantitative PCR was performed using six previously described primer pairs (Shanbhag et al. 2010) and SYBR-green master mix. Calculations based on Ct values were done as stated by Invitrogen methods.

2.6 Constant field gel electrophoresis (CFGEP) Assay

The amount of DSBs remaining after exposure to IR was measured using a modified constant field gel electrophoresis (CFGE) protocol previously described (Ismail et al., 2005, Ismail et al., 2010). In brief, cells were exposed to 40 Gy IR and harvested immediately to measure initial DNA damage levels and after 5 h at 37°C to measure DNA damage remaining after repair. Cells were harvested using trypsin, centrifuged and washed with 1X PBS. 150,000 cells were mixed with low

melting point agarose (1.25% type VII in PBS with 5mM EDTA) and transferred to a plug mold (Bio-Rad cat# 170-3713). The cells were then lysed in the plug mold at 4°C for 24 h in lysis buffer (25mM EDTA pH 8.5, 0.5% SDS, with 3 mg/mL proteinase K added just before lysis). The cells were resolved using agarose gel electrophoresis (0.7%) in TAE (0.04mM Tris acetate, and 1mM EDTA, pH 8) at 4°C for 18 h at 1V/cm. Laser scanning equipment (Typhoon 9200 Variable Mode Imager; ImageQuant 5.2 software; GE Healthcare) was used to quantify the relative amounts of DNA that migrated into the gel and to calculate the amount of DSBs.

2.7 Colony Formation Assay

Cellular sensitivity to IR was determined using a previously described protocol (Ismail *et al*., 2010). In brief, U2OS cells were transfected with either control shRNA or Ezh2-shRNA 24 h prior to the experiment. Cells were plated at low density and exposed to different doses of IR (2, 4, or 6 Gy). Cells were then left to grow at 37°C for approximately 10 days (or until colonies of 40-50 cells were present). The cells were then fixed and stained with crystal violet. Colonies were counted to determine cellular viability. Error bars are from duplicate samples.

2.8 Flow cytometry cell cycle analysis

$1X10^6$ U2OS cells were seeded onto a 10 cm dish 24 h before the experiment and flow cytometry was performed as previously described (McManus and Hendzel, 2005). Cells were either untreated or drug treated 1 h before being exposed to ionizing radiation. Cells were left to recover for 1h 30 min after ionizing radiation before being harvested using trypsin. Cells were then washed three times in PBS,

and then centrifuged at 1500 x *g*. Cells were then resuspended in 100 μL PBS and fixed with 70% ice-cold ethanol. Fixed cells were maintained at 4°C for up to 1 week. Cells were immunofluorescently labeled with anti-Histone H3 phosphoserine 10 (1:10000) for 30 min, washed three times in PBS, and incubated with goat anti-rabbit Alexa Fluor 488 (1:200) for 30 min. Cells were stained for DNA content with 40 μg/mL (final concentration) propidium iodide (PI; Sigma-Aldrich, St. Louis, MO) containing 100 μg/mL RNase A (final concentration) (Sigma-Aldrich) for 30 min at 37°C. Cells were resuspended in 500 μl of PBS, and signal intensities were examined using a FACSort (Becton Dickson, Franklin Lakes, N.J.) and compared with controls (unstained, PI only, primary with secondary, and rabbit IgG1 isotype control; 1:200, Sigma-Aldrich). Graphs were exported as TIFF images and assembled in Photoshop.

2.9 Chromatin Fractionation

U2OS cells were seeded in a 10 cm dish 24 h prior to the experiment. Cells were treated or not with 5 μM AG14361 for 1 h and then exposed to 6 Gy or not and left to recover for 30 min. Cells were first harvested with trypsin and washed with PBS. Cells were then resuspended in Buffer A (10 mM Hepes pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.34 mM sucrose, 10% glycerol, and protease and phosphatase inhibitors). Then triton X100 was added to 0.1% and the cells were incubated on ice for 5 min. The cells were centrifuged for 4 min at 1,300 x *g*. The supernatent was collected and labeled cytoplasm. The pellet was washed once in Buffer A, and centrifuged again as earlier stated. The pellet was then resuspended in Buffer B (3 mM EDTA, 0.2 mM EGTA, and protease and phosphatase

inhibitors). We centrifuged again for 4 min at 1,700 x *g*, collected the supernatent and labeled it nuclear soluble. The pellet was washed in Buffer B and centrifuged again. The final pellet was resuspended in SDS running buffer and labeled chromatin bound. Each cellular fraction was then run on a SDS polyacrilamide gel, transferred to nitrocellulose paper, and probed by western blot for protein.

Chapter III: *Results*

3.1 PRC2 proteins recruit to laser microirradiation-induced DNA damage tracks.

We have previously shown that PRC1 recruits to DSBs and participates in DNA repair (Ismail *et al.*, 2010). Although PRC1 recruitment was not inhibited by Ezh2 knockdown, we wished to determine whether or not PRC2 also participates in the DNA damage response. We used laser micro-irradiation to introduce damage and monitored the recruitment of GFP-tagged PRC2 components to DNA damage sites in real-time. As mentioned previously, this approach is commonly employed to study the recruitment of DSB repair proteins (Kim *et al.*, 2007; Hong *et al.*, 2008; Chou *et al.*, 2010; Ismail *et al.*, 2010). We used Hoechst 33258 excited with two-photon excitation at 750 nm to introduce DNA damage. We transfected U2OS cells with GFP-tagged Ezh2, and then used laser micro-irradiation to determine whether or not GFP-Ezh2 recruits to DNA damage. We found that GFP-Ezh2 recruits to laser-induced DNA damage within two seconds and continues to recruit until a maximum fluorescence is reached at around one minute (Figure 7).

PRC2 also consists of two other essential subunits, EED and SUZ12. Therefore, we added a GFP tag to each of these proteins and proceeded with laser micro-irradiation experiments. Both GFP-EED and GFP-SUZ12 also recruit to laser-induced DNA damage (Figure 7). The recruitment kinetics of GFP-Ezh2 and GFP-Suz12 are almost identical, consistent with the proteins recruiting as a

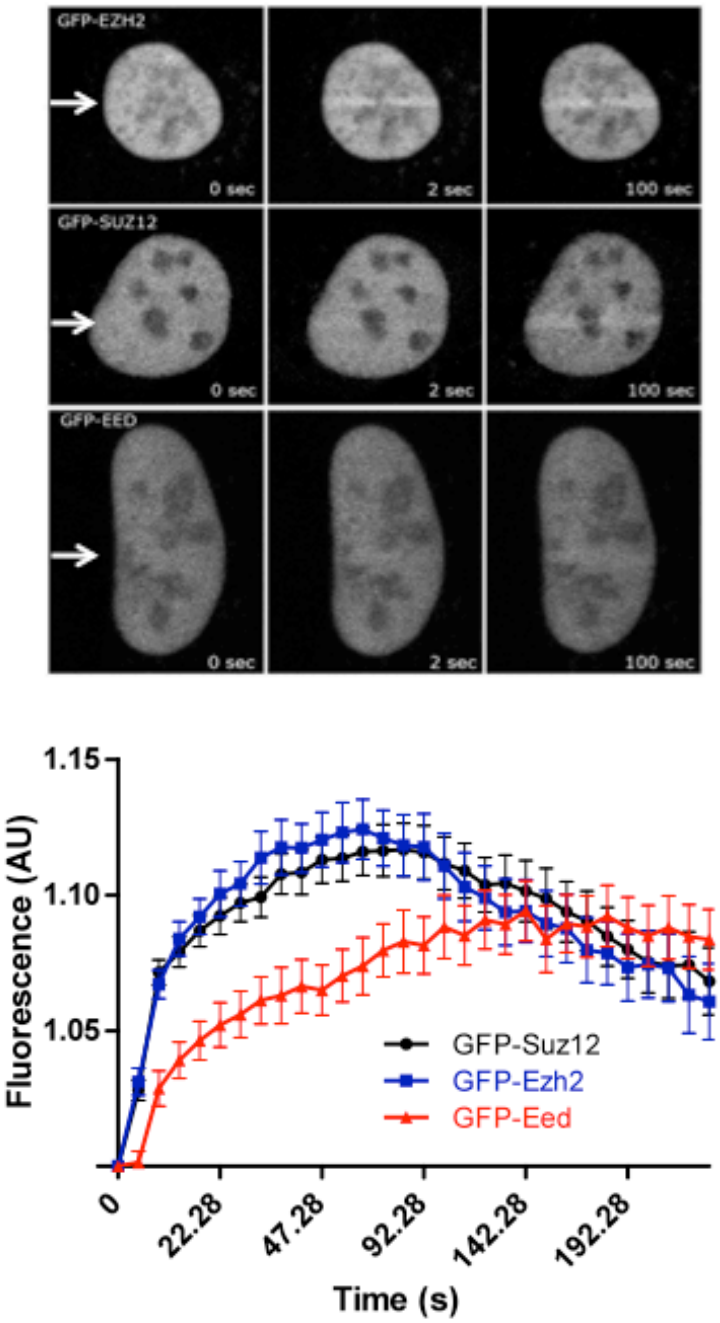

Figure 7. **Ezh2, Suz12 and Eed recruit to laser-induced DNA damage.** U2OS cells expressing GFP-Ezh2, GFP-Suz12 or GFP-Eed were laser micro-irradiated and monitored using time-lapse microscopy. Representative images at 0, 2 and 100 s are shown after laser micro-irradiation. Accumulation of GFP-Ezh2, GFP-Suz12 and GFP-Eed on the laser damage tracks over time were quantified and plotted (N=20). Increased fluorescence on the damage tracks is plotted over time.

complex. Interestingly, GFP-Eed has slightly delayed recruitment kinetics, which could be attributed to variations in the PRC2 complex, or protein interaction effects caused by the GFP tag. Nonetheless, the recruitment kinetics of the PRC2 proteins closely resembles the recruitment kinetics of other well-established DSB repair proteins, such as NBS1, MRE11, and RNF8 supporting the hypothesis that PRC2 acts early in the DNA damage response (Ismail *et al.*, 2010).

3.2 PRC2 does not recruit to UV induced DNA damage or single-strand breaks.

Laser micro-irradiation can induce several types of DNA damage; therefore, we tested the potential of the other major types of damage, UV and single-strand breaks, to recruit PRC2. We placed a 10 μm polycarbonate membrane over cells and exposed the cells to UV (1 J/s) for 2 minutes. Cells were allowed to recover for 20 minutes and then fixed and stained for Ezh2. We were unable to detect any Ezh2 enrichment at sites of UV damage (Figure 8a). We also performed fluorescence recovery after photobleaching (FRAP) to determine if treating cells with UV or an alkylating agent, MNNG, altered the mobility of Ezh2, reflecting its retention at sites of DNA damage. We found that neither UV (60 J) nor MNNG (0.1 mM) affected the kinetics of Ezh2 (Figure 8b). We were therefore able to conclude that Ezh2 is unlikely to be recruited directly by single-strand breaks or UV damage.

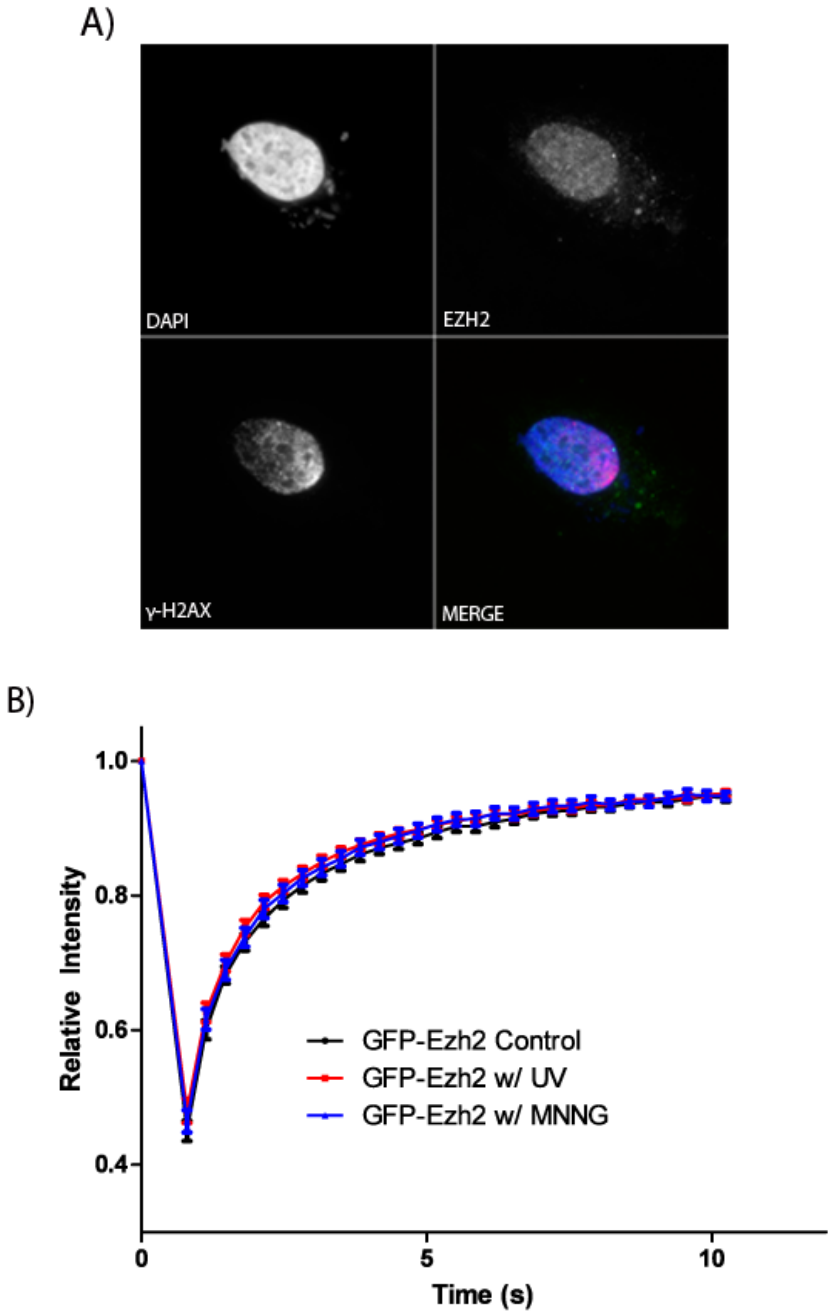

Figure 8. **Ezh2 does not recruit to UV damage or respond to single strand breaks.** A) U2OS cells were grown on a coverslip for 24 h prior to the experiment. The coverslip was then placed below a polycarbonate membrane and exposed to UV radiation (120 J/m^2). Cells were then left to recover for 20 min and then fixed with 4% PFA. Cells were immuno-stained with Ezh2 and γ-H2AX antibodies. B) U2OS cells were transfected with GFP-Ezh2 24 hrs before the experiment. Cells were either treated with UV (120 J/m^2), MNNG (100 μM) or left untreated and fluorescent recovery after photobleaching was then performed to determine the kinetics of GFP-Ezh2. Relative intensity of the bleached region was plotted over time.

3.3 Ezh2 does not significantly enrich at sites of ionizing radiation induced foci (IRIF)

A characteristic shared by many DSB repair proteins is the accumulation at IRIF after exposure to ionizing radiation. We wanted to determine if PRC2 proteins accumulate at ionizing radiation-induced foci so we treated U2OS cells with 2 Gy and examined Ezh2 accumulation at IRIF using indirect-immunofluorescence (Figure 9). We were unable to detect any enrichment of Ezh2 at IRIF marked with γ-H2AX. We were also unable to detect enrichment of Suz12 or Eed at IRIF (Data not shown). We wanted to determine if the Ezh2 antibody was recognizing Ezh2 at DNA damage sites, and so we stained endogenous Ezh2 on the laser micro-irradiation tracks. The Ezh2 antibody was able to detect endogenous Ezh2 on the DNA damage tracks, proving its functionality in this experiment (Figure 10). The absence of PRC2 IRIF does not exclude PRC2 as a DSB response protein, as there are several proteins essential to DSB repair that do not accumulate at IRIF (e.g., Ku70/80 (Kong *et al.*, 2009)).

3.4 Ezh2 recruits to Fok-1 endonuclease-induced DSBs.

Laser micro-irradiation induces a multitude of DNA lesions at the laser tracks, including DNA single-strand breaks, UV damage, and base damage. Given that we cannot detect Ezh2 enrichment in IRIF, we wanted to determine whether or not Ezh2 recruits to DNA DSBs using a more sensitive chromatin immunoprecipitation technique. We employed a previously established system for

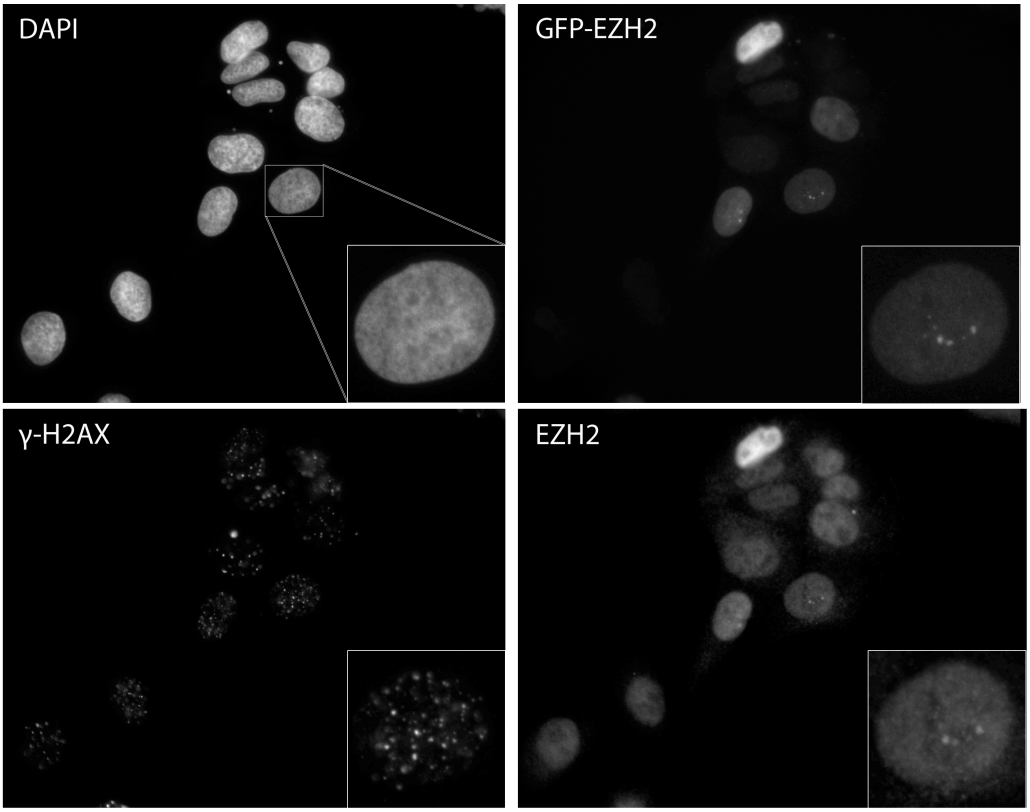

Figure 9. **Ezh2 does not accumulate at IRIF.** U2OS cells expressing GFP-Ezh2 were exposed to 2 Gy and left to recovery for 30min. Cells were then fixed in 4% paraformaldehyde and co-immunostained with γ-H2AX and Ezh2 antibodies.

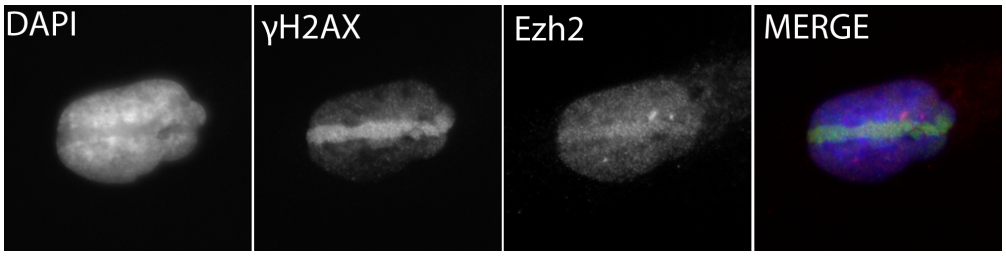

Figure 10. **Endogenous Ezh2 localizes to laser-induced DNA damage.** U2OS cells were laser micro-irradiated and then fixed immediately after DNA damage with 4% paraformaldehyde. Ezh2 and γ-H2AX are stained using indirect-immunofluorescence.

targeted DSBs (Shanbhag *et al.*, 2010), which targets the Fok-1 endonuclease to a specific integrated reporter site in the genome. Fok-1 generates a DSB at this site, enabling proteins to be mapped in the proximity of the DSB using ChIP. We transfected the transformed U2OS cells with either a functional Fok-1 construct or a mutated non-functional Fok-1(D450A) construct and performed ChIP to determine the relative increase of Ezh2. We saw increased levels of Ezh2 only when we transfected with functional Fok-1 endonuclease, confirming that Ezh2 is recruited and localized to DSBs (Figure 11). We were able to confirm the functionality of the ChIP assay and the presence of DSBs by demonstrating that this correlated with a significant enrichment in γ-H2AX. These results complement the laser micro-irradiation data and demonstrate that DSBs are amongst the types of damage that recruit Ezh2.

3.5 shRNA-mediated knockdown of Ezh2 decreases DNA double-strand break repair.

After confirming that Ezh2 recruits to DSBs, we wanted to determine whether or not the recruitment of Ezh2 to sites of DSBs had an effect on the ability of cells to repair the damage. We used U2OS cells transfected with shRNA directed against Ezh2 to determine the effect of Ezh2 knockdown on DSB repair efficiency. We first tested the effectiveness of the shRNA construct on knocking down Ezh2 protein levels. We saw that cells transfected with the shRNA targeting Ezh2 had significantly lower Ezh2 protein levels (Figure 12). Using the neutral constant field gel electrophoresis (CFGEP) assay, we quantified the proportion of DSBs that remained following gamma irradiation treatment at varying times after

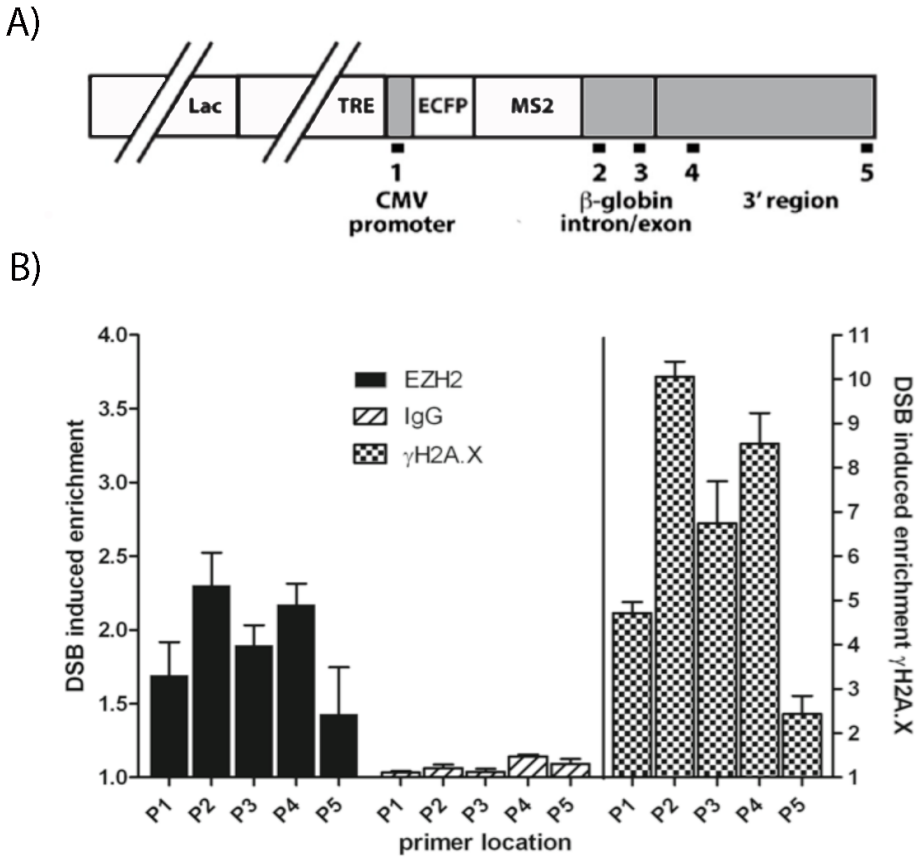

Figure 11. **Ezh2 accumulates at Fok-1 induced DSBs. A) Schematic of reporter locus with primer locations adapted from Shanbhag et al. 2010. B)** U2OS cells stably expressing the reporter locus were transfected with wild-type Fok-1 endonuclease. Cells were then fixed with 1% paraformaldehyde 18 h after transfection and harvested for chromatin immunoprecipitation (Ch-IP). Ch-IP was performed with Ezh2, γ-H2AX, and IgG antibodies. Quantitative PCR using five representative primers was done and the Fok-1 DSB induced enrichment for each primer and antibody is plotted.

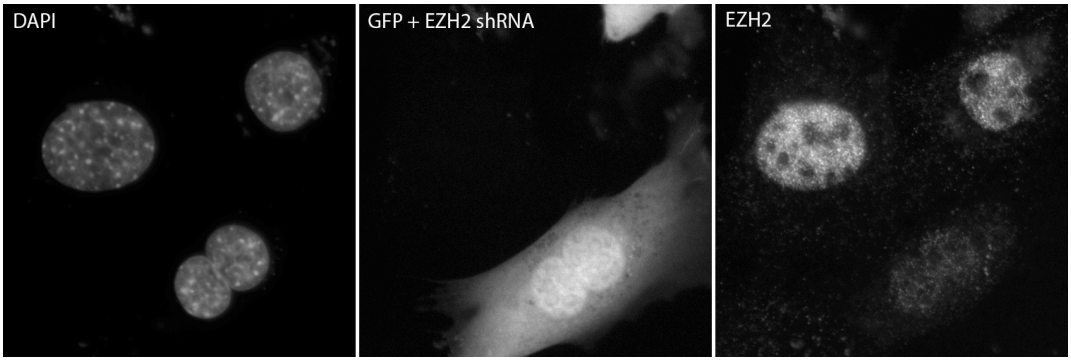

Figure 12. **Ezh2 shRNA reduces the endogenous Ezh2 protein levels.** 10T1/2 cells were transfected with shRNA targeting Ezh2 containing a GFP reporter. Cells were fixed and Ezh2 was stained using indirect immunofluorescence.

irradiation (Figure 13). After treating U2OS cells with 40 Gy, they were immediately harvested or left for 5 h to allow DSB repair. Compared to control U2OS cells where only 5% of the DSBs were not repaired after 5 h, more than 45% of the DSBs were not repaired in the Ezh2 knockdown cells. To confirm this result, we reconstituted the Ezh2 knockdown cells with GFP-Ezh2 that is resistant to the shRNA to determine if this could re-establish the DNA DSB repair capacity back to control levels. We observed that GFP-Ezh2 was able to restore DSB repair capacity back to control levels in the Ezh2-knockdown cells. This indicates that PRC2 is necessary for efficient repair of the DNA damage.

3.6 Ezh2 knockdown decreases cellular survival after exposure to ionizing radiation.

After determining that PRC2 is recruited to DSB and is necessary for efficient DSB repair, we wanted to determine whether or not PRC2 is necessary for survival in response to ionizing radiation. A previous study demonstrated a 20 to 40 percent decrease in IR-resistance upon knockdown of PRC2 subunits (Chou *et al.*, 2010). We used a colony formation cellular survival assay to determine whether Ezh2 plays an essential role in the cellular response to ionizing radiation induced damage. Using the colony formation assay and sequentially increasing doses of radiation from 0 Gy to 6 Gy, we determined the proportion of cells that survived each dose and continued to proliferate into a viable colony (Figure 14). Using U2OS transfected with shRNA directed against Ezh2, we determined that knockdown of Ezh2 decreased cellular viability by 50% after a 4 Gy dose compared to control cells. Reconstituting Ezh2 knockdown cells with GFP-Ezh2

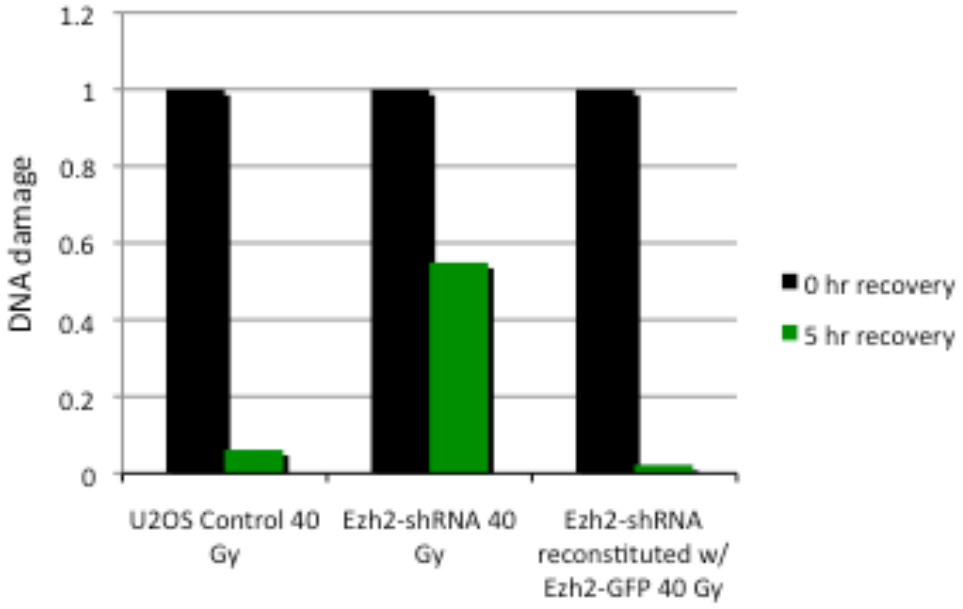

Figure 13. **Ezh2 knockdown decreases DSB repair efficiency.** U2OS cells, U2OS cells stably expressing Ezh2-shRNA, and U2OS cells stably expressing Ezh2-shRNA reconstituted with GFP-Ezh2 were exposed to 40Gy ionizing radiation. Cells were harvested immediately to determine total damage and after 5hrs to determine the DNA damage remaining. Total DNA damage immediately after IR is referred to as 100% DNA damage.

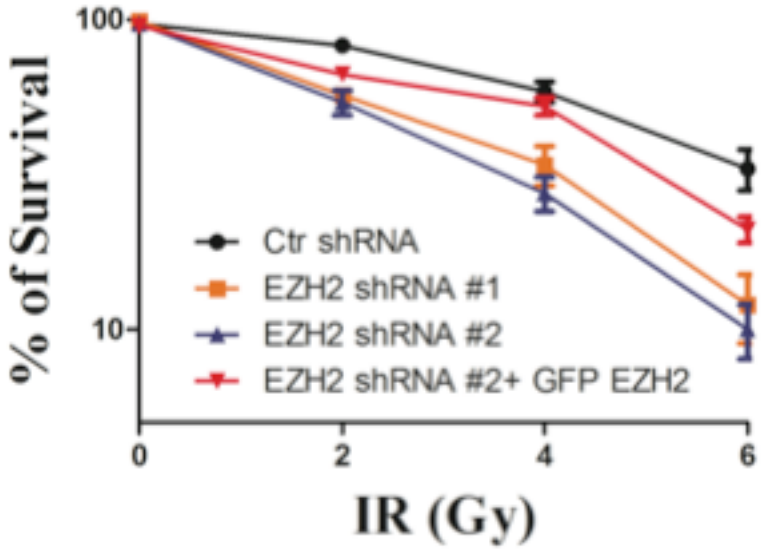

Figure 14. **Ezh2 knockdown increases cellular sensitivity to IR.** U2OS cells transfected with control shRNA, two different Ezh2-shRNAs, or Ezh2-shRNA reconstituted with GFP-Ezh2 were plated at low density. Cells were then exposed to 0, 2, 4, or 6 Gy and left to recover for two weeks. The cells were then fixed and stained and colonies were counted. Each colony represents a surviving cell, and the percent survival is plotted for each dose of IR.

restored cellular viability back to control levels. This result supports the constant field gel electrophoresis assay results and suggests that Ezh2 is required for the efficient repair of DNA double-strand breaks and that Ezh2 participates in radiation resistance.

3.7 Determination of the requirement for the recruitment of PRC2 to sites of laser-induced DNA damage

We next wanted to investigate the mechanism of PRC2 recruitment to sites of DNA damage. ATM and DNA-PKcs are two structurally related PI-3 kinases that coordinate and mediate the cellular response to DSBs. To determine if ATM and/or DNA-PKcs are required for the recruitment of Ezh2, we microirradiated cells that lack each kinase and examined the recruitment of Ezh2 in these cells using the GFP tagged Ezh2 construct. We found that neither of these kinases is responsible for the initial recruitment or retention of PRC2 at laser-induced DNA damage (Figure 15). The histone 2A variant, H2AX is a central platform for the accumulation of several DNA repair proteins at the sites of DNA damage. We therefore wanted to investigate the requirement for H2AX in the recruitment of PRC2 to laser-induced DNA damage. We found Ezh2 efficiently recruited in both H2AX WT and H2AX KO MEFs suggesting H2AX is dispensable for the recruitment of PRC2 to the sites of DNA damage (Figure 15). Similar results have been reported by ourselves and others for the recruitment of PRC1 to sites of laser micro-irradiation (Chou *et al.*, 2010; Ismail *et al.*, 2010). In both cases, the recruitment of PRC1 proteins to laser micro-irradiation-induced DNA damage was found to be PARP dependent (Chou *et al.*, 2010; Ismail *et al.*, 2012). To

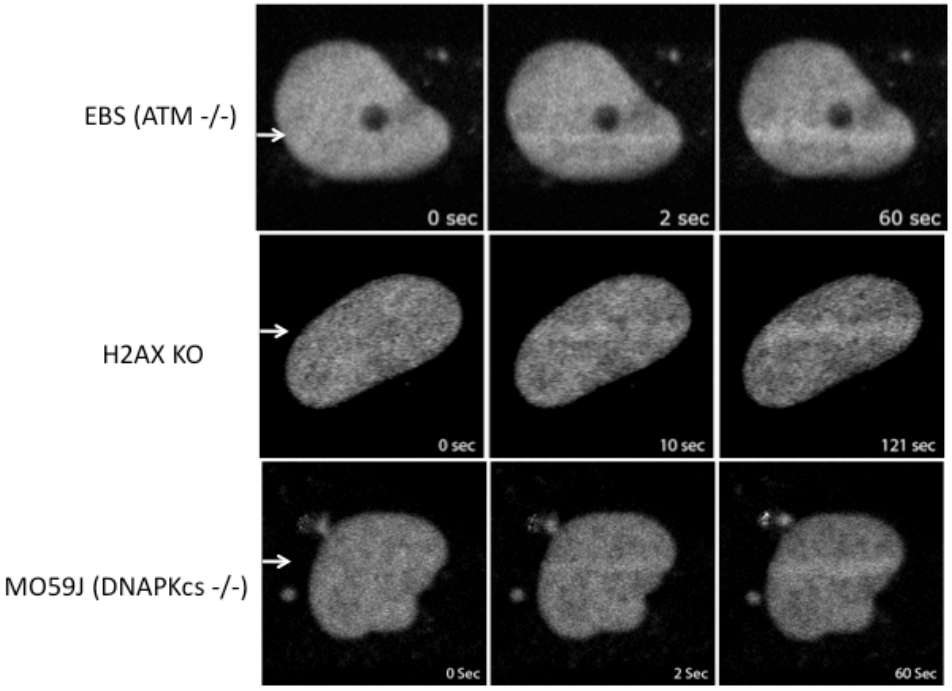

Figure 15. **Ezh2 recruitment is not dependent on H2AX, ATM or DNA PK.** GFP-Ezh2 was transfected in H2AX KO, EBS, or MO59J cells 24 h before the experiment. Cells were then laser micro-irradiated and imaged over time. Representative images at three different time points are shown.

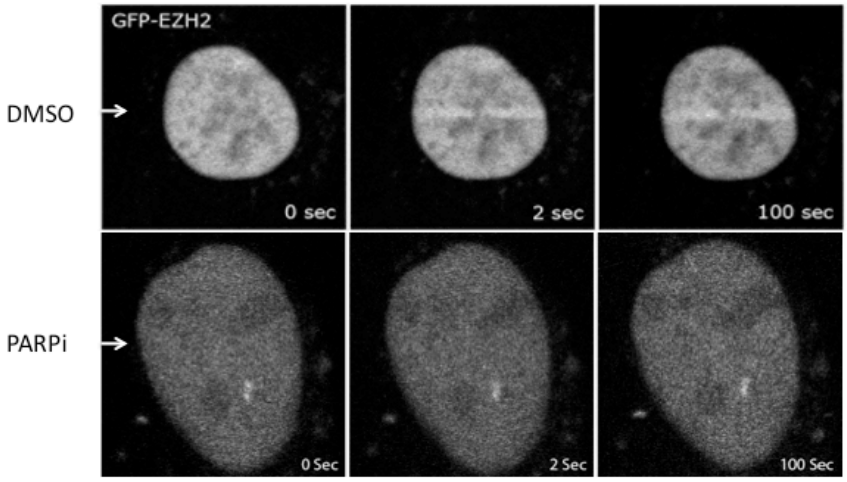

Figure 16. **Ezh2 recruitment to laser induced DNA damage is PARP dependent.** U2OS cells expressing GFP-Ezh2 were treated with PARP inhibitor AG14361 (5 uM) or DMSO (control) for 1 h prior to damage induction. Representative images at 0, 2 and 60 s are shown after laser micro-irradiation.

test the potential role of PARP in recruitment, we transfected U2OS cells with GFP-Ezh2 and treated the cells with 5 μM PARP1/2 inhibitor AG14361 for one hour prior to laser-microirradation. Our lab had previously established that 5 μM AG14361 completely abrogated PARP1/2 mediated poly (ADP-ribose) (PAR) formation in response to DNA damage. GFP-Ezh2 recruitment was completely abrogated in the presence of the PARP inhibitor (Figure 16). We obtained identical results with GFP-Suz12 and GFP-Eed further establishing the PARP dependency of PRC2 recruitment (Figure 17). Taken together these data indicate that PRC2 recruitment to laser-induced DNA damage is dependent on PARP1/2 activity.

3.8 H3K27me3 does not increase at laser micro-irradiation tracks.

Post-translational modifications are essential in the signaling cascade and repair of DSBs. It has been reported that methylation of H4K20 and H3K36 are important in the DSB repair pathway (Fnu *et al.*, 2011; Pei *et al.*, 2011). We next determined if the main substrate for PRC2 methylation, H3K27, is also methylated upon DNA damage. Using U2OS cells, we laser micro-irradiated and fixed cells immediately, 5 minutes, 30 minutes and 1 hour after damage and then stained the cells using a specific antibody to H3K27me3. Using indirect immunofluorescence, we were unable to detect an increase of H3K27me3 at the laser damage sites (Figure 18a). Similar results were obtained when cells were exposed to radiation (2 Gy) and examined for H3K27me3 ionizing radiation induced foci (IRIF) (Figure 18b). These results were further confirmed by

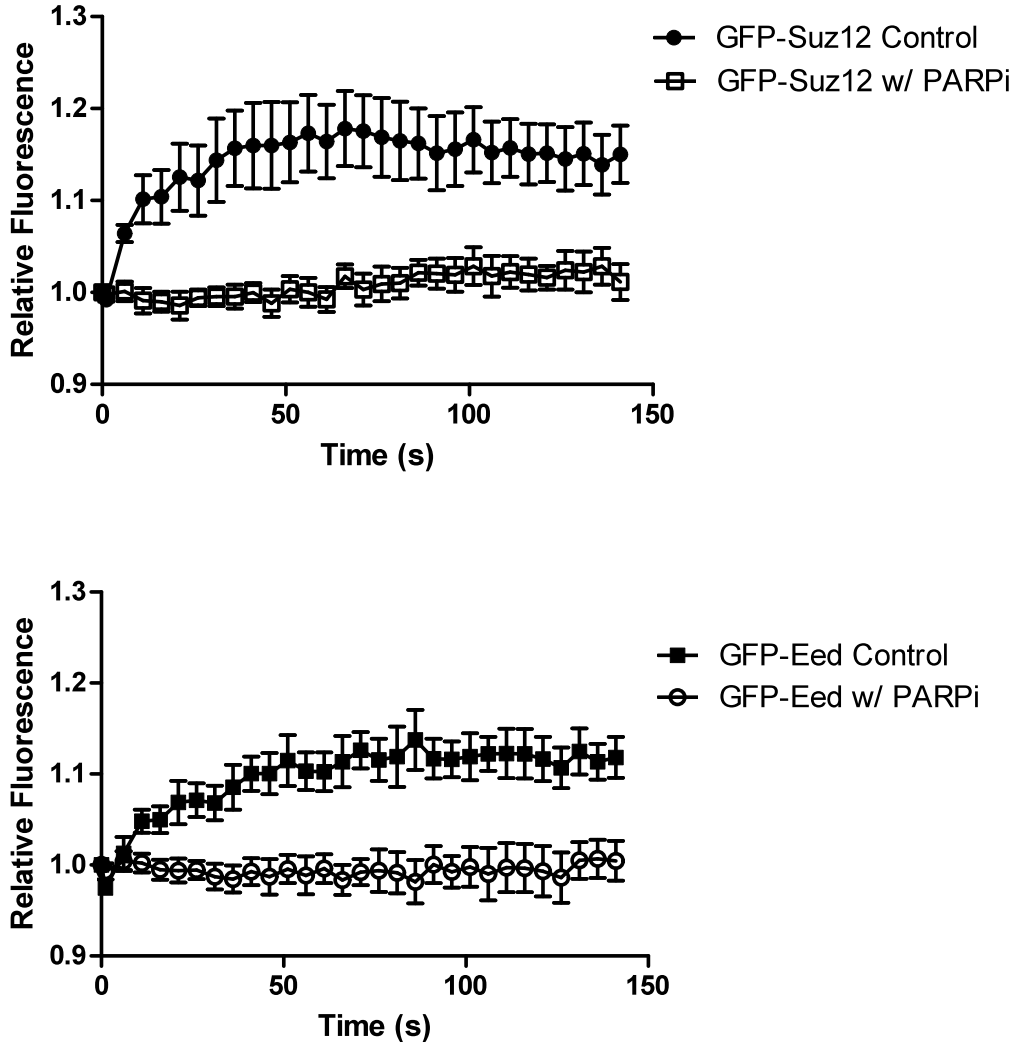

Figure 17. **GFP-Suz12 and GFP-Eed recruitment to laser induced DNA damage is PARP dependent.** U2OS cells were transfected with either GFP-Suz12 or GFP-Eed constructs 24 h prior to the experiment. Cells were either untreated or treated with 5 μM PARPi (AG14361) 1 h prior to laser- microirradiation. Error bars are plotted for a minimum of 10 cells.

immunoblot using H3K27me3 antibody. Immunoblotting of extracts prepared from cells exposed (6 Gy) or not exposed to radiation showed that H3K27me3 levels did not significantly increase after DNA damage (Figure 18c). Several separate commercial antibodies were additionally tested and all were found not to stain laser micro-irradiation sites.

3.9 DNA damage induced G2/M checkpoint does not require active methylation

We wanted to determine if Ezh2 recruitment and methyltransferase activity at sites of DNA damage is involved in activating the G2/M checkpoint. We used flow cytometry to examine the proportion of cells in various stages of the cell cycle. After treating cells with ionizing radiation, cells will stall in G2 until the DNA damage is fixed to prevent mitotic catastrophe. If the G2/M checkpoint is compromised, cells will fail to accumulate in G2 after receiving a dose of ionizing radiation. To determine if active methylation following ionizing radiation is important for the G2/M checkpoint, we treated cells with a general methylation inhibitor, 250 μM of adenosine dialdehyde (AdOx) (Ramakrishnan and Borchardt, 1987), one hour prior to treating cells with 8 Gy. As a control, we used an ATM inhibitor, 10 μM of Ku0055933, which is necessary for activating the G2/M checkpoint, or no inhibitor. We then analyzed the proportion of cells in each stage of the cell cycle to determine if the G2/M checkpoint was functional. We found that upon treatment with AdOx, the number of cells in mitosis greatly decreased compared to cells treated with ATM inhibitor (Figure 19). This indicates that in the presence of AdOx, the G2/M checkpoint was still active and

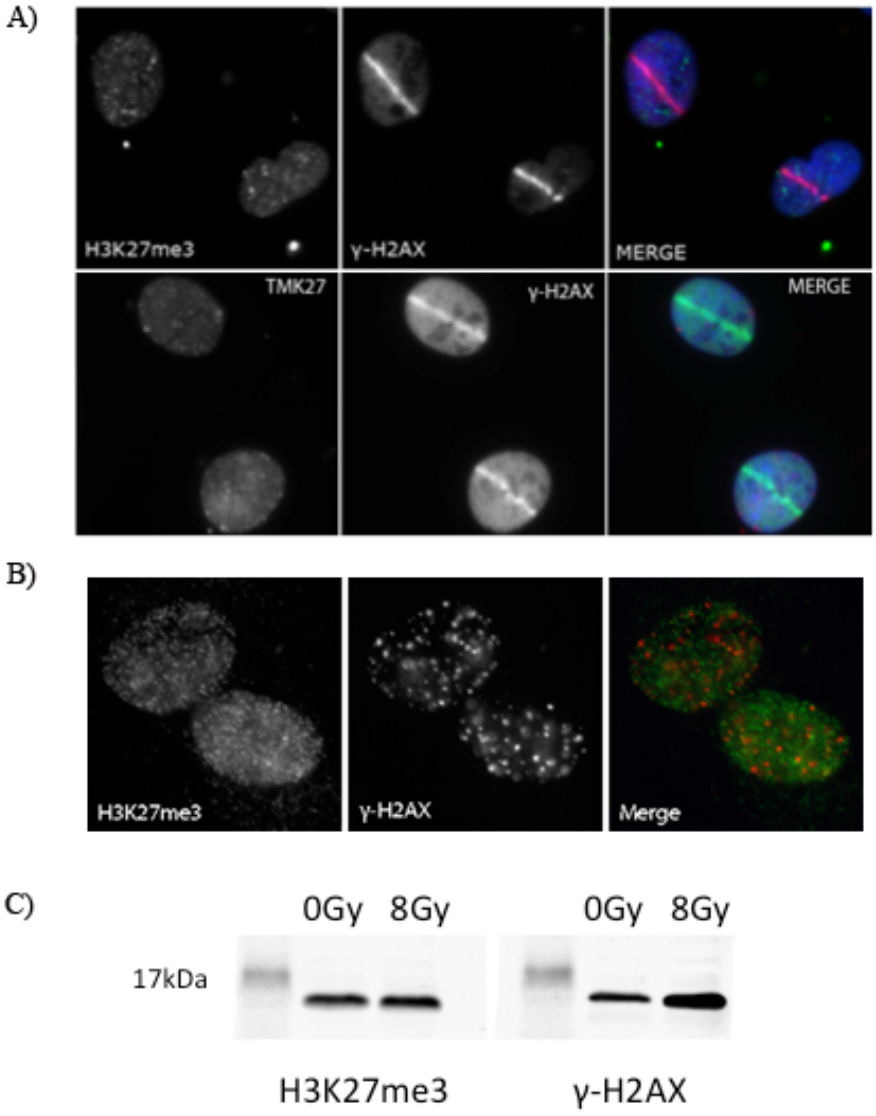

Figure 18. **H3K27 is not methylated upon laser induced DNA damage.** A) U2OS cells were laser micro-irradiated and then fixed with 4% paraformaldehyde 5 min (top) or 60 min (bottom) after laser induced DNA damage. Cells were then co-immunostained with H3K27me3 and γ-H2AX antibodies. B) U2OS cells were exposed to 2 Gy and then left to recover for 30 min. Cells were then fixed with 4% PFA and co-immunostained with H3K27me3 and γ-H2AX antibodies. C) U2OS cells were either not treated or treated with 8 Gy and left to recover for 30 min before harvesting. Nuclei were extracted, sonicated, and run on an 18% polyacrilamide gel. The western blots were stained with H3K27me3 and γ-H2AX to show the induction upon ionizing radiation.

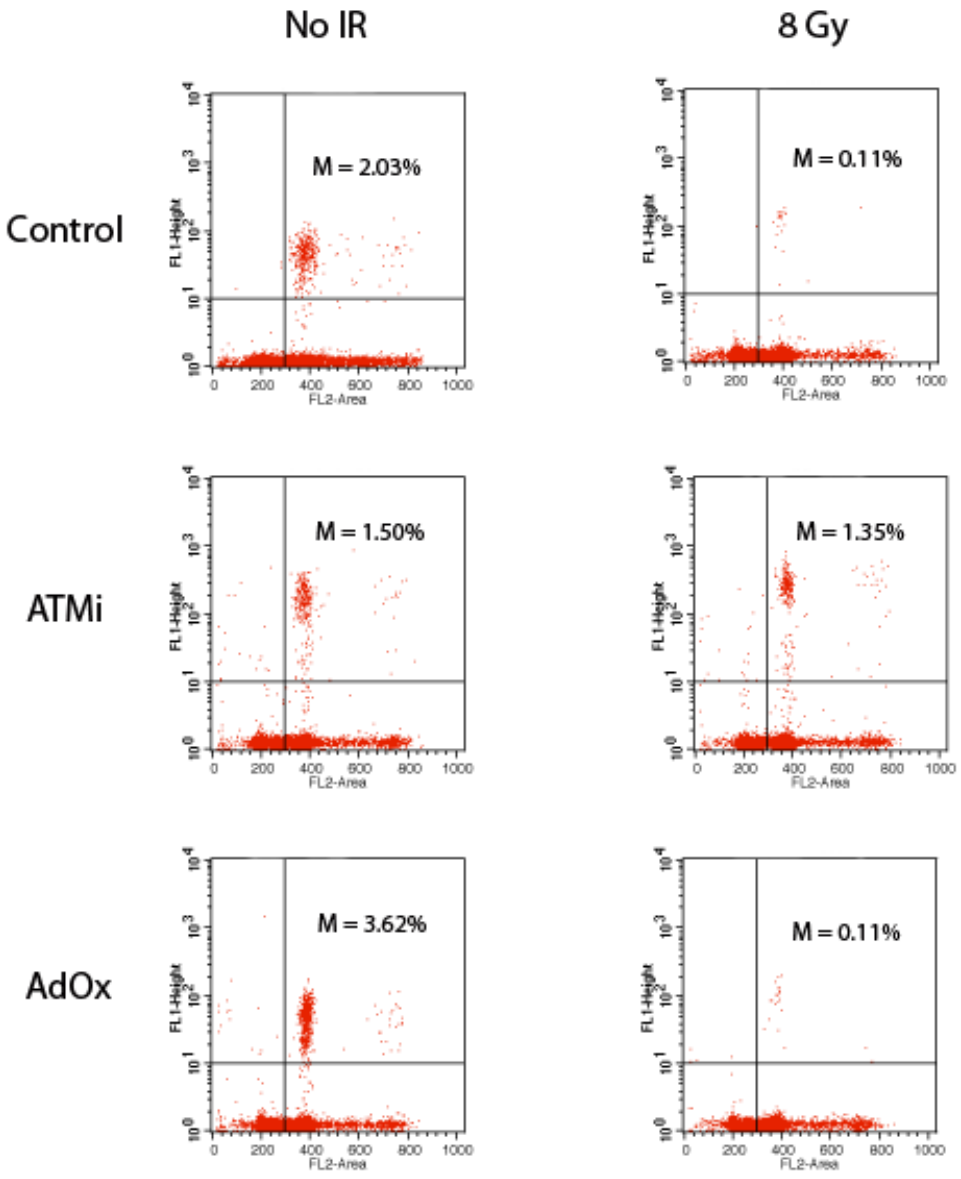

Figure 19. **Ionizing radiation induced methylation is not necessary to activate the G2/M checkpoint.** U2OS cells were either not treated or treated with 10 μM ATM inhibitor (Ku0055933) or 250 μM AdOx for one hour prior to being exposed to ionizing radiation. Cells were then exposed to 6 Gy and left to recover for 90 min. Cells were then analyzed using flow cytometry to determine the percentage of cells in mitosis. Mitotic populations are represented in the upper-right quadrant of each graph and the percentage of mitotic cells (M) is also shown.

that active methylation after ionizing radiation is not necessary for the G2/M checkpoint. This indirectly shows that Ezh2 methyltransferase activity is unlikely to contribute to the G2/M checkpoint in response to DNA damage.

Supplemental Data

3.10 Ezh2 kinetics do not change on the laser micro-irradiation tracks

In order for PRC2 to recruit to DNA damage, there must be an increase in binding partners or binding stability promoting the localization of the complex. In order to investigate the binding and stability of PRC2 at sites of DNA damage, we performed FRAP after laser micro-irradiation. FRAP after laser micro-irradiation allows us to evaluate and compare the kinetics of PRC2 at sites of DNA damage versus the general nucleus. To do this, we first laser micro-irradiated U2OS cells expressing GFP-Ezh2, and then rotated the cell 90^{o} and selected two regions for FRAP; one region on the DNA damage track and one in the general nucleus away from the DNA damage. We then compared the kinetics of GFP-Ezh2 in the two regions (Figure 20). We saw that there was no significant difference between GFP-Ezh2 on the laser tracks and in the general nucleus, indicating the binding stability and turnover is similar both at sites of DNA damage and in the general nucleus.

3.11 Ionizing radiation does not affect Ezh2 chromatin localization.

Many DNA damage response proteins become chromatin bound after DNA damage induction, and so we wanted to see if Ezh2 also increased chromatin affinity after DNA damage induction. We treated or did not treat cells

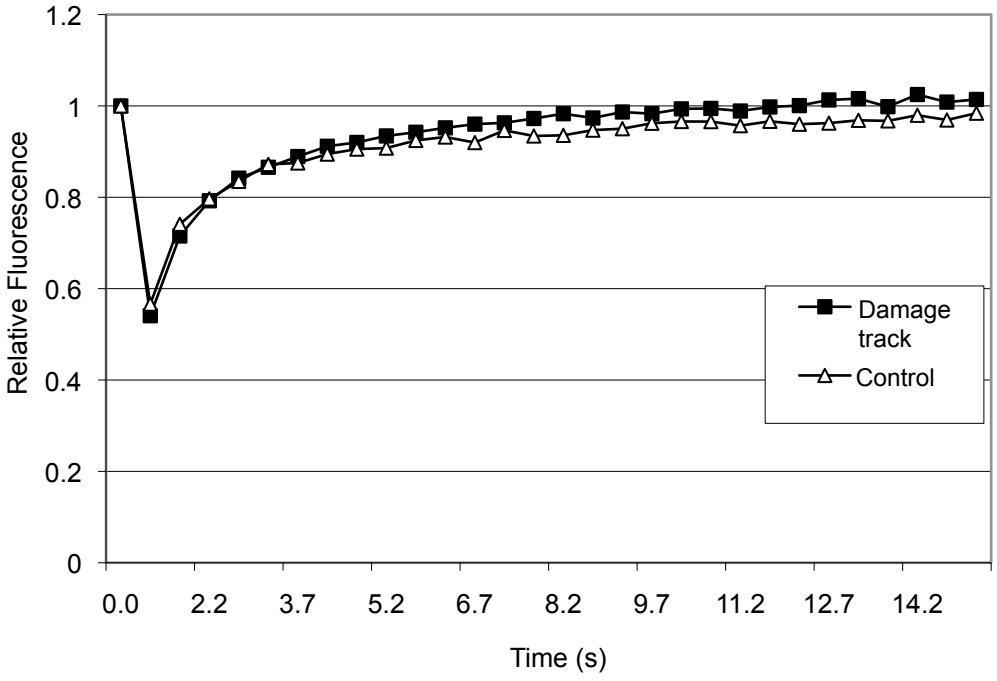

Figure 20. **GFP-Ezh2 kinetics is not significantly different at sites of DNA damage compared to the general nucleus.** U2OS cells expressing GFP-Ezh2 were laser-microirradiated and then immediately rotated 90^{O} and two regions were bleached; one region on the laser-microirradiation track and one region in the general nucleus. FRAP was performed on the two bleached regions and relative fluorescence of the regions in plotted versus time. A minimum of 15 cells is plotted.

with 5 μM PARP inhibitor (AG14361) for 1 hour and then either exposed cells or did not expose cells to 6 Gy and let the cells recover for 30 min. We then harvested the cells performed chromatin fractionation to separate the cytoplasm, soluble nuclear, and chromatin bound fractions. We then ran each fraction on an SDS polyacrylamide gel (split 15% / 8%) and performed an immunoblot to stain for Ezh2, H3K27, and γ-H2AX. We saw that under all conditions, Ezh2 is chromatin-associated; however, upon treating with ionizing radiation with or without PARP inhibitor, the fraction of Ezh2 bound to chromatin did not change (Figure 21). The γ-H2AX staining is a control to show the irradiated cell fractions, and the H3K27 is a loading control to show each lane has equal protein loads. This data further supports the FRAP data, showing the Ezh2 does not change kinetics or chromatin affinity after inducing DNA damage.

Additional Experiments

3.12 PRT4165 alters the chromatin association and mobility of PRC2

Recently, an inhibitor to PRC1, named PRT4165, has been characterized and has been shown to be effective for reducing PRC1 mediated ubiquitylation both *in vitro* and *in vivo* (Alchanati *et al.*, 2009). The mechanism of inhibition is still unknown; however, we wanted to determine if PRC1 ubiquitylation was involved in PRC2 recruitment to DNA damage. Interestingly, after adding 60μM PRT4165 for 1 hour, we noticed a drastic change in GFP-Ezh2 mobility within the nucleus. Using FRAP, we characterized the mobility of GFP-Ezh2 within the nucleus and found that, after treatment with 60 μM PRT4165 for 1 hour, GFP-Ezh2 recovers much slower than the control (Figure 22). This implies that upon

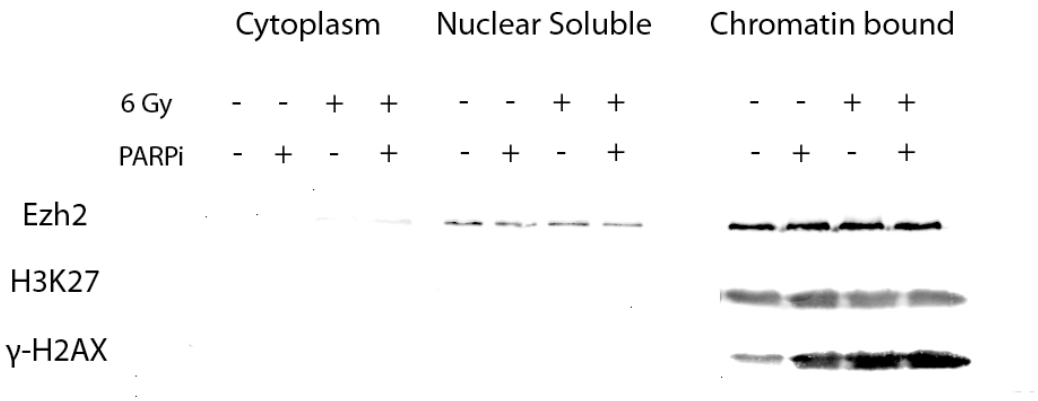

Figure 21. **Ezh2 does not change chromatin localization upon treatment with ionizing radiation.** U2OS cells were either treated or not treated with 5 μM PARP inhibitor (AG14361) for 1 h prior to irradiation. Cells were then exposed or not exposed to 6 Gy and left to recover for 30 min. Cells were then harvested with trypsin and fractionated into the cytoplasm, nuclear soluble, or chromatin bound fractions using the cellular fractionation protocol. Extracts were run on a split 15%/8% polyacrilamide gel. Western blots were performed using Ezh2, H3K27 and γ-H2AX primary antibodies.

treating with PRT4165, Ezh2 stability with binding partners is greatly increased. We wanted to determine if a general ubiquitylation inhibitor would also have the same effect on Ezh2 mobility, so we used another indirect, global ubiquitin ligase inhibitor, MG132, however, there was almost no effect on GFP-Ezh2 mobility (Figure 22). This indicates the effect on Ezh2 binding is somehow specific to PRT4165 mechanism of inhibiting the PRC1 complex.

3.14 PARP inhibition changes Rap80 recruitment kinetics to laser IR induced DNA damage

Rap80 is an ubiquitin binding protein that has been shown to be important for targeting BRCA1 to sites of DNA damage (Sobhian *et al.*, 2007). We used laser micro-irradiation and looked at the recruitment kinetics of GFP-Rap80 and saw that GFP-Rap80 recruited very similarly to other early DSB repair proteins. We were investigating the role of PARP in recruiting DSB repair proteins to laser-induced DNA damage and so we treated GFP-Rap80 expressing U2OS cells with PARP inhibitor for 1 hour. We saw that in the presence of the PARP inhibitor, recruitment of GFP-Rap80 to the laser micro-irradiation tracks was delayed compared to the control, however, the total recruitment levels were significantly higher in the presence of the PARP inhibitor (Figure 23). We also saw that the laser micro-irradiation track did not decondense in the presence of the PARP inhibitor, as seen by the width of damage track measured over time. It is evident that although PARP is not necessary for the direct recruitment of Rap80 to the laser induced DNA damage, PARP activity contributes to the kinetics and

dynamics of Rap80 at the laser micro-irradiation tracks, most likely through chromatin decondensation.

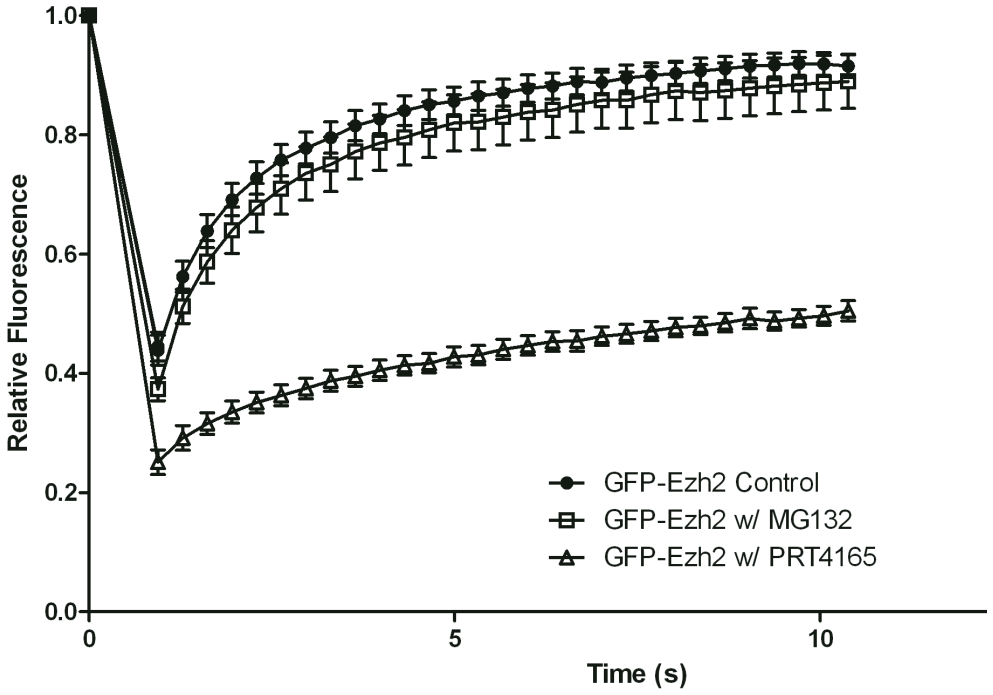

Figure 22. **PRT4165 increases the stability of Ezh2 binding in the nucleus.** U2OS cells expressing GFP-Ezh2 were either treated with 60 μM PRT4165, 5 μM MG132 or nothing for 1 h prior to FRAP. FRAP was performed with the drug still present. Relative fluorescence is plotted versus time for a minimum of 15 cells.

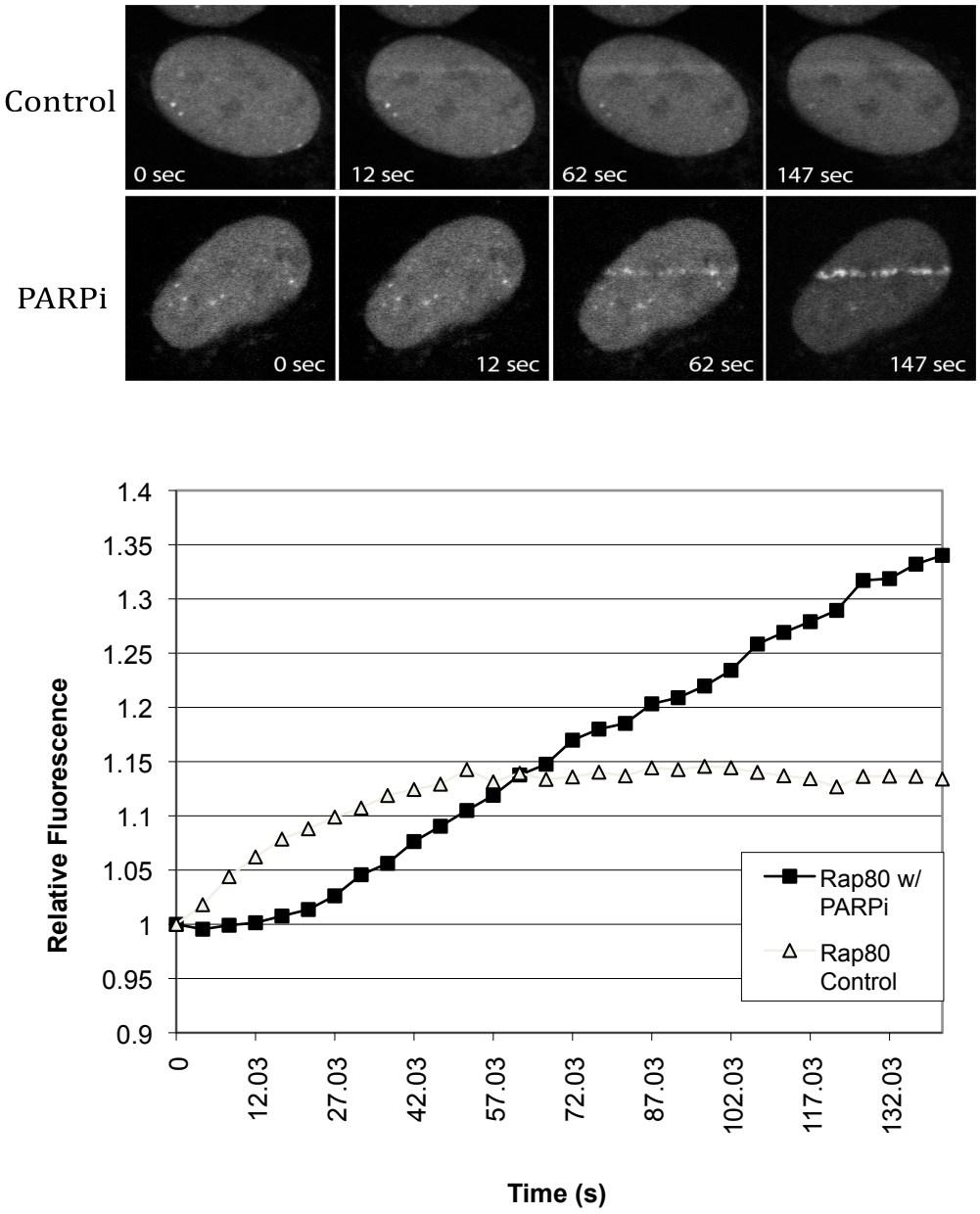

Figure 23. **PARP activity is not necessary for the recruitment of Rap80 to laser-induced DNA damage.** U2OS cells expressing GFP-Rap80 were either treated or not with 5 μM PARP inhibitor (AG14361) and laser micro-irradiated. The GFP-Rap80 recruitment to the laser micro-irradiation tracks is represented as relative fluorescence at the tracks over time. Representative images at select time points are shown.

Chapter IV: *Discussion, Future directions, and Significance*

Polycomb group proteins have recently been shown to play a role in the DNA damage response in addition to the previously established roles in development and stem-cell maintenance. Members of the PRC1 complex, Bmi-1, Ring1b and Cbx4 all recruit to sites of DSBs and are responsible for post-translational modifications that promote DNA damage signaling and DSB repair (Ismail *et al.*, 2010; Ginjala *et al.*, 2011; Ismail *et al.*, 2012). During gene repression, PRC2 is thought to promote the recruitment of PRC1. A previous study had reported that Ezh2 recruitment to sites of laser micro-irradiation increased lysine 27 methylation at the DNA damage sites and it was proposed that PRC2-mediated methylation was responsible for the recruitment of PRC1 to sites of DNA damage. However, we have previously shown that PRC1 recruitment is PARP-dependent but does not require PRC2 activity to recruit to DSBs. Therefore we further investigated the role of PRC2 in DSB repair. GFP-tagged Ezh2, Suz12 and Eed all recruit to the laser-induced DNA damage tracks with similar kinetics to other early DNA damage response proteins. In addition, we observed that Ezh2 recruits to Fok-1 endonuclease-induced DSBs. Furthermore, knockdown of the enzymatic component of PRC2, Ezh2, impaired the ability of cells to repair DSBs and increased their cellular sensitivity to ionizing radiation. These results are consistent with PRC2 functioning in the DNA damage response pathway. Surprisingly, and in contrast to recent publications (O'Hagan *et al.*, 2008; Chou *et al.*, 2010), we observed no increase in H3K27me3 at sites of DNA damage. While this contradicts previous reports, it is consistent with our finding

that PRC1 does not require PRC2 activity to recruit to sites of DNA damage (Ismail *et al.*, 2010).

Using laser microirradiation to induce DNA damage causes multiple types of DNA lesions, including base damage, UV damage, single-strand breaks as well as DSBs. To better define whether or not DSB damage recruits Ezh2, we utilized a construct that generates only DSBs via an endonuclease. We found that PRC2 recruits specifically to these endonuclease induced DSBs. We were also able to show that Ezh2 does not recruit to UV damage. As well, neither UV damage nor the alkylating agent MNNG changed the recovery kinetics of Ezh2 after FRAP. This indicates that Ezh2 does not respond to the UV damage response or SSBs caused during base excision repair. We also found that DSB repair efficiency is decreased when Ezh2 is knocked down in U2OS cells, which is consistent with the knockdown of PRC1 members. These results support the current working hypothesis that PRC2 is involved in the DNA damage response, and further establishes PRC2's role as an early, essential DSB repair complex.

Histone post-translational modifications are essential to the DNA damage response. They are responsible for the recruitment and retention of DSB proteins, as well as, propagation of the DSB signaling cascade. Histone methylation has been shown to be necessary for the recruitment and retention of 53BP1. We propose that the histone methyltransferase complex, PRC2, is also essential to the DSB repair pathway. It is still unclear what role PRC2 plays in facilitating the repair of DSBs. It will be important to determine the role of PRC2 mediated

methylation and the substrates that are targeted, as well as, the DSB repair proteins that may require PRC2 methylation within the repair pathway.

In response to DSBs, ATM mediates transcriptional silencing near the break site partly through the induction of histone ubiquitylation (Shanbhag *et al.*, 2010). These post-translational modifications observed at DSBs are consistent with a role for polycomb proteins in DSB induced gene repression. It may be that one role of PRC2 at DSB sites is to contribute to DSB-induced gene silencing through the methylation of H3K27. However, in the case of laser micro-irradiation, we failed to observe any increase in H3 lysine 27 methylation at the DNA damage site. Given that we employed the same antibody as previous publications (Chou *et al.*, 2010), we cannot explain this difference. However, it is consistent with our previous finding that Ezh2 is not required for the recruitment of PRC1 to DSBs (Ismail *et al.*, 2010).

In addition to PRC2 acting directly at sites of DNA damage, PRC2 has been implicated in several aspects of the DNA damage response. Overexpression of Ezh2 has been seen to confer resistance to cisplatin in ovarian cancer cells (Hu *et al.*, 2010); moreover, overexpression of Ezh2 downregulates RAD51 expression in breast epithelial cells, thereby impairing DNA repair (Zeidler *et al.*, 2005). Ezh2 has also been implicated in regulating cancer cell apoptosis in response to genotoxic stresses by regulating CHK1 activation (Wu *et al.*, 2011). The use of PRC2 inhibitors has been suggested for adjuvant chemotherapy (Xiao, 2011), but the mechanistic role PRC2 plays in DNA damage signaling and repair must be elucidated before PRC2 inhibitors can be used effectively. Our data, in

combination with the previous studies (Hong *et al.*, 2008; O'Hagan *et al.*, 2008; Chou *et al.*, 2010; Wu *et al.*, 2011), suggests a role for PRC2 in DSB repair and supports the hypothesis that PRC2 inhibitors could be used to sensitize undifferentiated cancers overexpressing PRC2 proteins to DNA damaging agents. The failure to detect PRC2 at sites of IR-induced DSBs while being able to detect it by chromatin IP at a defined DSB may mean that PRC2 is only required in low stoichiometric amounts, similar to what has been observed for Ku70 and Ku80, which are essential in DSB repair, recruit to sites of laser micro-irradiation, but cannot be detected in IR-induced foci (Koike and Koike, 2008).

Future Directions

It is important to determine the mechanistic role of PRC2 in the DNA damage response. It is evident that PRC2 recruits to DNA damage and is involved in the repair and signaling of DNA damage, but it is still unclear as to how PRC2 mediates the efficient repair and signaling of DNA damage. Determining the target substrates for PRC2 at sites of DNA damage will help define PRC2's role in the DNA damage response. It is also important to determine the downstream proteins regulated by PRC2 methylation. It has already been shown that Chk1 activation in cancer cells requires Ezh2 (Wu *et al.*, 2011), and it is important to determine what other signaling and repair pathways require PRC2 function. In addition to directly responding to DNA damage signals, PRC2 may also modulate the DNA damage response by changing gene expression. It has been shown that increasing expression of Ezh2 can downregulate the expression of Rad51 paralogs (Zeidler *et al.*, 2005). It is important to determine the changes in gene expression

mediated by altering PRC2 expression, as this may lead to changes in radiosensitivity.

In determining the mechanistic role for PRC2 in the DNA damage response, more synthetic lethality relationships may be discovered between PRC2 proteins and other DNA damage response proteins. There has already been a synthetic lethality relationship described between Ezh2 and BRCA proteins in breast cancer (Puppe *et al.*, 2009). Many cancers have defects in DNA repair pathways that contribute to genomic instability, and it is possible that these defects can be exploited through synthetic lethal relationships to selectively kill cancer cells.

Knocking down Ezh2 has been proven effective in reducing proliferation and aggressiveness in many types of cancer, and thus Ezh2 is a very attractive chemotherapy target. The role for PRC2 in the DNA damage response adds an additional therapeutic benefit to targeting PRC2 by sensitizing these cancers to both radiation and DNA damage inducing chemotherapeutics. It is important to investigate the benefits of combining PRC2 inhibitors with other types of cancer therapy. Furthermore, there are no known direct inhibitors of either Ezh2 or the PRC2 complex. DZNep is a general methylation inhibitor that can induce PRC2 degradation in cells. The mechanism of DZNep-induced PRC2 degradation still remains unknown. It is important to continue to search for a more specific PRC2 inhibitor to reduce off target effects. As well, the development of a specific inhibitor of PRC2 will help distinguish the direct PRC2 function in DNA damage versus the indirect gene regulatory functions of PRC2.

Significance

The purpose of this study was to elucidate and establish a role for PRC2 in the DNA damage response and repair pathway. It has been suggested that most cancers have a repair defect in at least one DNA repair pathway and that this contributes to genomic instability (reviewed in (Lord and Ashworth, 2012)). The defective DNA repair pathways present in cancer can be exploited through therapeutic synthetic lethality strategies, as seen in the sensitivity of BRCA-deficient breast cancers to PARP inhibitors. In order to fully utilize synthetic lethality relationships in cancer, it is important that we understand DNA repair pathways in their entirety. We have shown here that PRC2 is an important component in DSB repair and may provide an attractive target for future chemotherapeutics.

PRC2 is an attractive target for several reasons. Firstly, it has been shown that overexpression of PRC2 in cancer correlates with a poor prognosis and an aggressive cancer phenotype. It has been reported that knocking down Ezh2 can decrease the proliferative capacity and aggressiveness of several tumors. It has also been reported that overexpression of Ezh2 in cancers correlates with a less-differentiated tumor phenotype, which has been shown to be more resistant to both radiation and chemotherapy.

Our data supports the further investigation of PRC2 inhibitors as a potential adjuvant chemotherapy. In addition to decreasing the proliferative capacity of tumors and promoting differentiation in tumors with PRC2 inhibitors, we suggest that PRC2 inhibitors may also sensitize cancers to radiation and

chemotherapy. We also suggest that PRC2 inhibitors may have synthetic lethal relationships with various tumors defective in SSBR or DSBR pathways.

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