The emerging role of Polycomb repressors in the response to DNA damage

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Summary

Polycomb group (PcG) genes encode chromatin modifiers that are involved in the maintenance of cell identity and in proliferation, processes that are often deregulated in cancer. Interestingly, besides a role in epigenetic gene silencing, recent studies have begun to uncover a function for PcG proteins in the cellular response to DNA damage. In particular, PcG proteins have been shown to accumulate at sites of DNA double-strand breaks (DSBs). Several signaling pathways contribute to the recruitment of PcG proteins to DSBs, where they catalyze the ubiquitylation of histone H2A. The relevance of these findings is supported by the fact that loss of PcG genes decreases the efficiency of cells to repair DSBs and renders them sensitive to ionizing radiation. The recruitment of PcG proteins to DNA breaks suggests that they have a function in coordinating gene silencing and DNA repair at the chromatin flanking DNA lesions. In this Commentary, we discuss the current knowledge of the mechanisms that allow PcG proteins to exert their positive functions in genome maintenance.

Key words: Polycomb, DNA damage response, epigenetic gene silencing, histone ubiquitylation, DNA double-strand breaks (DSBs)

Introduction

The DNA of eukaryotes is associated with proteins in a highly organized and compacted structure known as chromatin. The chromatin environment has substantial impact on DNA-based processes, such as DNA replication, transcription and repair, as it intrinsically limits the degree of access to DNA. Chromatin-remodelling enzymes and post-translational modifications of the most abundant chromatin proteins, the histones, cooperate to overcome the chromatin barrier (Bell et al., 2011; Suganuma and Workman, 2011).

Even in normal homeostasis, DNA is constantly damaged. If unrepaired, DNA lesions have the potential to cause mutations that, eventually, may lead to cancer (Hoeijmakers, 2009). A plethora of molecular events that are triggered by DNA damage, collectively known as the DNA damage response (DDR), ensures that genome stability is maintained (Ciccia and Elledge, 2010; Lukas et al., 2011; Polo and Jackson, 2011), and is crucial to prevent tumorigenesis. Indeed, dysfunction of the DDR is a hallmark of human cancer (Negrini et al., 2010). The DDR involves detection of the DNA lesion, transduction of the damage signal and the establishment of conditions that promote DNA repair. These conditions include, but are not restricted to, cell cycle arrest and activation of the DNA repair process (Ciccia and Elledge, 2010; Lukas et al., 2011; Polo and Jackson, 2011). Chromatin serves diverse roles within the DDR. It acts as a signaling platform and provides the environment in which repair is coordinated with other DNA-based processes, such as ongoing transcription (Lukas et al., 2011). Chromatin rearrangements and histone modifications, including their phosphorylation, methylation and ubiquitylation, are necessary for accurate DNA damage signaling and repair (Al-Hakim et al., 2010; Lukas et al., 2011; Polo and Jackson, 2011).

In this Commentary, we focus on key chromatin modifiers, the Polycomb group (PcG) of transcriptional repressors. PcG proteins control various biological processes, including the maintenance of cellular identity and proliferation (Pietersen and van Lohuizen, 2008; Sauvageau and Sauvageau, 2010; Sparmann and van Lohuizen, 2006; Surface et al., 2010). Deregulation of PcG genes is frequently associated with aberrant maintenance of stem cell fate and with cancer (Bracken and Helin, 2009). There is rapidly growing evidence that implicates PcG proteins as novel regulators of the DDR in mammalian cells. After introducing the mechanisms of DDR and PcG silencing, we discuss the described functions of PcG proteins in DDR in greater detail. As most is known about the role of the Polycomb complex protein BMI1, we focus on its contribution in the ubiquitylation pathway at DNA double-strand breaks (DSBs). Finally, we discuss the hypothesis that PcG proteins have a role in the interplay between gene transcription and DNA repair.

The DNA damage response

DNA integrity is threatened by a number of possible DNA lesions (Hoeijmakers, 2009). The DDR responds to specific DNA lesions with the activation of distinct signal transducers that, subsequently, regulate all aspects of DDR. For instance, single-stranded DNA (ssDNA), which frequently occurs upon stalling of the replication fork, activates the ataxia telangiectasia mutated- and Rad3-related (ATR) kinase (Nam and Cortez, 2011), whereas ssDNA breaks activate poly[ADP-ribose] polymerase 1 (PARP1) (Ciccia and Elledge, 2010; Luo and Kraus, 2012; Polo and Jackson, 2011) and DNA DSBs activate the ataxia telangectasia mutated (ATM) kinase (Derheimer and Kastan, 2010). As the recent studies of the role of PcG proteins in DDR focus mainly on their function at DSBs, we will summarize here the cellular response to DSBs. A detailed description of DSB-induced signaling is provided in the legend to Fig. 1.

Active ATM at DSBs promotes checkpoint activation and apoptotic responses, for example through activation of p53 and

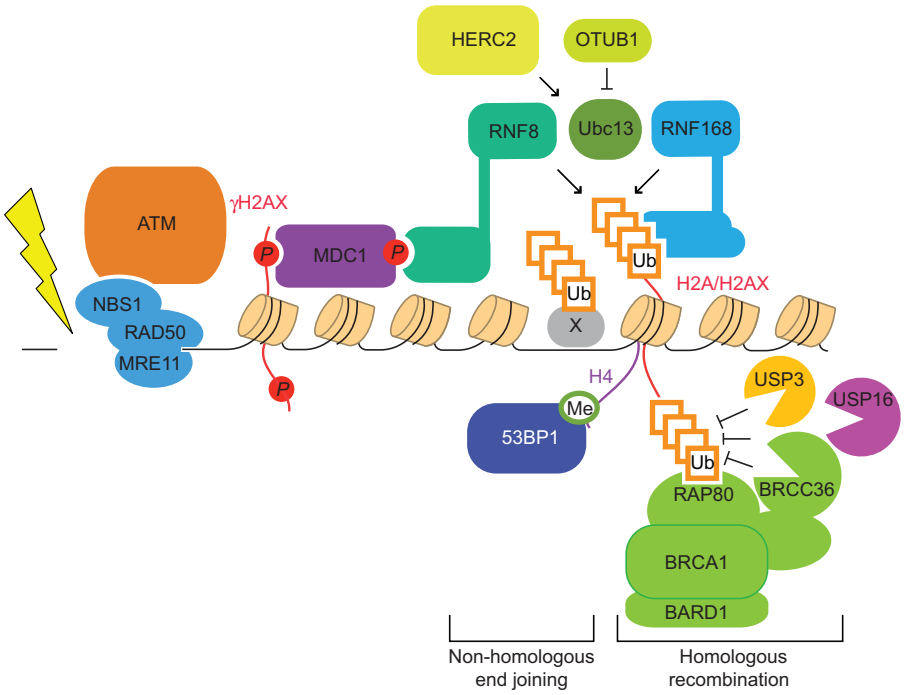

Fig. 1. Ubiquitin pathway at sites of DNA double-strand breaks. A multifaceted DDR is elicited by DNA double strand breaks. Local DDR integrates, among others, phosphorylation- and ubiquitylation-based signaling, and is initiated upon sensing of a DSB by the MRN complex – a complex of MRE11, NBS1 and RAD50 proteins (Williams et al., 2007b). NBS1 recruits ATM kinase (Williams et al., 2007b), which phosphorylates – among many other substrates – histone H2AX, yielding γH2AX (Burma et al., 2001). γH2AX is then bound by MDC1 (Stucki et al., 2005), which is also phosphorylated by ATM and serves as a scaffold for the recruitment of the ubiquitin E3 ligase RNF8 (Huen et al., 2007; Kolas et al., 2007; Mailand et al., 2007; Wang and Elledge, 2007). RNF8 is required for the recruitment of a second ubiquitin E3 ligase RNF168 (Doil et al., 2009; Stewart et al., 2009). The concerted action of RNF8 and RNF168, together with the E2 ubiquitin-conjugating enzyme UBC13 leads to the ubiquitylation of histones H2A and H2AX, and most probably also of other substrates at the site of damage (Doil et al., 2009; Pinato et al., 2009; Stewart et al., 2009; Zhao et al., 2007). UBC13 is inhibited in a non-catalytic manner by the deubiquitylating enzyme OTUB1 (Juang et al., 2012; Nakada et al., 2010; Wiener et al., 2012). The pathway is also regulated by the E3 ligase HERC2, which promotes the interaction between RNF8 and UBC13 (Bekker-Jensen et al., 2010). It has been shown that the main linkage of ubiquitin at sites of damage is through its lysine residue K63 (Al-Hakim et al., 2010; Doil et al., 2009; Lukas et al., 2011; Stewart et al., 2009; Tang and Greenberg, 2010). An increasing number of DDR factors have been reported to depend on the ubiquitin pathway at DSBs for their recruitment, including BRCA1 and 53BP1 (Al-Hakim et al., 2010; Lukas et al., 2011; Tang and Greenberg, 2010). A BRCA1-containing protein complex (shown in green) is recruited to DSBs through direct binding of K63-linked ubiquitylated histones by its RAP80 subunit (Kim et al., 2007; Wang and Elledge, 2007; Wang et al., 2007; Wu et al., 2009). BRCA1 has E3 ligase activity and functions as a heterodimer together with BARD1 (Hiom, 2010). 53BP1 has not been reported to have an ubiquitin-binding domain; instead, it binds H4K20Me3 at the site of damage (Botuyan et al., 2006; Pei et al., 2011). BRCA1 promotes homologous recombination-mediated DSB repair (Moynahan et al., 1999). 53BP1 promotes repair by non-homologous end joining (Bouwman et al., 2010; Bunting et al., 2010; Cao et al., 2009; Difilippantonio et al., 2008; Dimitrova et al., 2008). Three deubiquitylating enzymes have been reported thus far to negatively regulate the pathway, presumably by deubiquitylation of H2A and H2AX, USP3 and USP16, and BRCC36 (Nicassio et al., 2007; Shanbhag et al., 2010; Shao et al., 2009).

checkpoint kinase 2 (CHK2) (Derheimer and Kastan, 2010). In addition, ATM phosphorylates the histone H2A variant H2AX (Burma et al., 2001) at the site of damage. Phosphorylated H2AX (γH2AX) promotes the consecutive recruitment of the mediator of DNA damage checkpoint response protein 1 (MDC1) (Stucki et al., 2005), the ubiquitin E3 ligase ring finger protein 8 (RNF8) (Huen et al., 2007; Kolas et al., 2007; Mailand et al., 2007; Wang and Elledge, 2007) and, yet another E3 ligase, RNF168 (Doil et al., 2009; Stewart et al., 2009). The concerted action of RNF8 and RNF168 with the ubiquitin-conjugating E2 enzyme UBC13 leads to the ubiquitylation of histones H2A and H2AX, and probably also of other substrates at the chromatin surrounding the lesion (Doil et al., 2009; Pinato et al., 2009; Stewart et al., 2009; Zhao et al., 2007). The main linkage of ubiquitin chains at sites of DSBs is through its lysine residue 63 (K63) (Al-Hakim et al., 2010; Doil et al., 2009; Lukas et al., 2011; Stewart et al., 2009; Tang and Greenberg, 2010). RNF8 and RNF168 are the main E3 ligases at sites of DSBs, and their activity is required for the stable accumulation of, among others, breast cancer 1 early onset (BRCA1) and tumour protein p53 binding protein 1 (53BP1) (Al-Hakim et al., 2010; Lukas et al., 2011; Tang and Greenberg, 2010).

Importantly, the interplay between BRCA1 and 53BP1 determines the choice of DSB repair pathway employed by the cell (Bouwman et al., 2010; Bunting et al., 2010; Cao et al., 2009). BRCA1 promotes homologous recombination, which is considered error-free owing to the use of the intact sister chromatid as a template (Moynahan et al., 1999; Wyman and Kanaar, 2006). Conversely, 53BP1 favours non-homologous end joining (NHEJ) by several mechanisms (Bouwman et al., 2010; Bunting et al., 2010; Cao et al., 2009; Difilippantonio et al., 2008; Dimitrova et al., 2008). NHEJ repairs DSBs by rejoining the ends of a broken DNA molecule. Enzymatic resection of the broken ends may cause loss of genetic information, making this pathway error-prone (Ciccia and Elledge, 2010).

A growing number of factors that modulate the RNF8–RNF168–ubiquitin pathway are emerging. Modification by ubiquitin is reversible and deubiquitylating enzymes (DUBs) have been implicated in the negative regulation of H2A ubiquitylation at DSBs, such as the ubiquitin-specific proteases (USPs) USP3 and USP16, and the BRCA1–BRCA2-containing complex subunit 36 (BRCC36) (Nicassio et al., 2007; Shanbhag et al., 2010; Shao et al., 2009). Finally, the small ubiquitin-related modifier SUMO also accumulates at DNA damage sites and has been reported to positively regulate ubiquitin signaling by RNF8, RNF168 and BRCA1 (Galanty et al., 2009; Morris et al., 2009). For this review, we chose to discuss only the main players in the RNF8–RNF168–ubiquitin pathway; readers are referred to Fig. 1 for more details and to recent reviews for a more comprehensive overview of the ubiquitin and SUMO pathways in DDR (Al-Hakim et al., 2010; Luijsterburg and van Attikum, 2012; Lukas et al., 2011; Tang and Greenberg, 2010; Ulrich, 2012) or detailed information regarding ubiquitylation and sumoylation processes (Clague et al., 2012; Kerscher et al., 2006; Pickart and Eddins, 2004).

PcG proteins

PcG genes were initially discovered as genes that are involved in the regulation of morphogenesis in *Drosophila melanogaster*. PcG proteins are chromatin-associated transcriptional repressors that regulate bodyplan patterning by targeting homeotic (Hox) genes (Ringrose and Paro, 2004). PcG genes are conserved in sequence and function among vertebrates, as for instance analysis of mice with mutations in some PcG genes, such as *Bmi1* (van der Lugt et al., 1994), *Mel18* (Akasaka et al., 1996), and *Cbx2* (Core et al., 1997), has revealed skeletal abnormalities along the anterior-posterior axis.

Genome-wide mapping of PcG target sites in *Drosophila* and mammalian cells has greatly expanded our knowledge of PcG target genes. Besides the Hox genes, a large number of PcG targets have been identified that encode transcription factors and proteins with key roles in main developmental pathways, such as Wnt, Hedgehog and Notch pathways (Boyer et al., 2006). The deregulation of repression by PcG proteins also impacts on the development of various cancers. In fact, PcG genes and, most notably, BMI1 and enhancer of zeste homologue 2 (EZH2), are frequently overexpressed in human tumours (Bracken and Helin, 2009). An important PcG target is the CDKN2A locus, which encodes the tumour suppressor proteins p16 and p19 (Jacobs et al., 1999). These proteins block cell cycle progression and promote senescence by inhibiting the retinoblastoma-associated protein (Rb) and p53 pathways, respectively (Lowe and Sherr, 2003). Partly because of inappropriate repression of this locus, PcG proteins remove barriers in the process of oncogenic transformation (Bracken and Helin, 2009). Moreover, given that PcG proteins promote stem cell maintenance through repression of lineage-specific genes (Sauvageau and Sauvageau, 2010; Surface et al., 2010), it has been proposed that the observed deregulation of PcG genes in cancer causes the establishment of a more primitive differentiation state that is characteristic of many tumours and generally correlates with their clinical aggressiveness (Bracken and Helin, 2009). Readers interested in the details of the biological functions of PcG proteins are referred to some recent reviews (Bracken and Helin, 2009; Margueron and Reinberg, 2011; Sauvageau and Sauvageau, 2010; Simon and Kingston, 2009; Sparmann and van Lohuizen, 2006; Surface et al., 2010).

Polycomb-repressive complexes

PcG proteins are found to associate into two main Polycomb-repressive complexes (PRCs): PRC1 and PRC2. A detailed description of the components of PRC1 and PRC2 – which is required for a full understanding of this Commentary – is provided in Fig. 2. PRC2 specifically trimethylates histone H3 at lysine residue 27 (K27), resulting in H3K27Me3 (Cao et al., 2002; Czermin et al., 2002; Kuzmichev et al., 2002; Müller et al., 2002). PRC1 catalyzes histone H2A monoubiquitylation on lysine residue 119 (K119), which is the second PcG signature chromatin mark (Cao et al., 2005; Wang et al., 2004). Loss of the PRC1 components really interesting new gene 1A and 1B (RING1A and RING1B, respectively) results in a drastic reduction in basal levels of monoubiquitylated H2A (de Napoles et al., 2004; Wang et al., 2004), thereby demonstrating the exclusive requirement for these E3 ligases in catalyzing this particular modification. Besides ubiquitin E3 ligase activity, SUMO E3 ligase activity has been demonstrated for one of the PRC1 components, CBX4 (Kagey et al., 2003). Finally, in addition to its catalytic activity, PRC1 also possesses the ability to compact chromatin through binding of nucleosomes (Francis et al., 2004).

Mechanisms of PcG repression

PRC1 is considered to be responsible for gene silencing, which – at least in part – is exerted by controlling the RNA polymerase II (Pol II)-mediated elongation phase of transcription (Stock et al., 2007). In particular, chromatin immunoprecipitation (ChIP) experiments have shown that, although Pol II can still be detected at PRC1 target genes, it appears to be kept in a stalled configuration (Stock et al., 2007). Deletion of the RING1A and RING1B mouse homologues in mouse embryonic stem cells can release stalled Pol II and is accompanied by the derepression of PcG target genes (Stock et al., 2007). Two main mechanisms by which PcG complexes accomplish Pol II stalling have been described: chromatin compaction (Eskeland et al., 2010; Francis et al., 2004) and regulation of histone H2A monoubiquitylation (Gutiérrez et al., 2012; Scheuermann et al., 2010; Stock et al., 2007). However, the relative contribution of these mechanisms to PcG-mediated gene silencing remains to be investigated (Simon and Kingston, 2009).

PcG proteins in the DNA damage response

PcG genes directly and indirectly regulate various aspects of the DDR. Bmi1 is required for maintenance of the redox balance. *Bmi1* deficiency in mice leads to increased levels of reactive oxygen species, which results in DNA damage and DDR activation (Liu et al., 2009). With regard to the role of PRC2, cell-based assays support an involvement of EZH2 in the regulation of the G1/S and G2/M checkpoints upon treatment with clastogens (Wu et al., 2011b). In addition, in breast cancer cells, EZH2 might affect DSB repair indirectly through repression of the homologous recombination enzyme Rad51 (Chang et al., 2011) and of its paralogues (Zeidler et al., 2005), as well as through regulation of BRCA1 (Gonzalez et al., 2011; Gonzalez et al., 2009).

Recent work has begun to elucidate the involvement of specific PcG proteins in the local DDR at DSBs. A summary of PRC1 and PRC2 components that have been found to localize to these DNA damage sites is presented in Table 1.

The role of PRC1 and PRC2 at DSBs

Ubiquitylated H2A fulfils a dual function; it is a key epigenetic mark for transcriptionally silent chromatin (Cao et al., 2005; Wang

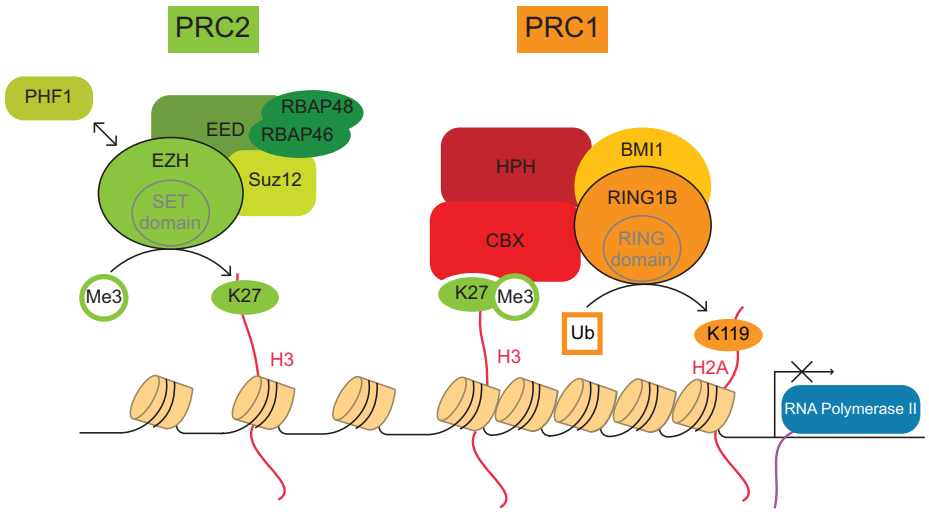

Fig. 2. Polycomb-repressive complexes (PRCs) and their enzymatic activities. The PcG transcriptional repressors are organized in two main PRCs, PRC1 and PRC2. PRC2 has four subunits: the Su(var)3-9, enhancer-of-zeste, trithorax domain (SET) domain protein EZH 1 or EZH2, the zinc finger protein SUZ12, the WD-repeat protein EED and the histone-binding proteins RBAP46 and RBAP48 (Cao et al., 2002; Czermin et al., 2002; Kuzmichev et al., 2002; Müller et al., 2002). EZH1 and its more intensively studied homologue EZH2 are responsible for the main enzymatic activity of PRC2, which is to trimethylate histone H3 at lysine 27, yielding H3K27Me3 (Cao et al., 2002; Czermin et al., 2002; Kuzmichev et al., 2002; Müller et al., 2002). In addition, a number of substoichiometric complex components of PRC2 have been described, such as, for example, PHF1 (Hong et al., 2008). PRC1 consists of the enzymatic subunit RING1A or RING1B, a RING-domain cofactor of the PCGF family such as BMI1 (also known as PCGF2) or MEL18 (also known as PCGF4), one of the human polyhomeotic homologues (HPH1, HPH2 or HPH3) and one of the chromobox proteins (CBX2, CBX4, CBX6, CBX7 or CBX8) (Simon and Kingston, 2009). RING1B and BMI1 form a heterodimer through the association of their RING domains (Buchwald et al., 2006; Li et al., 2006). RING-domain proteins are E3 ubiquitin ligases that catalyze the final step in ubiquitin conjugation (Pickart and Eddins, 2004). Indeed, PRC1 has E3 ligase activity and uses either RING1A or RING1B as its catalytic subunit and one of the PCGF proteins as an E3 ligase co-factor (Buchwald et al., 2006; Elderkin et al., 2007; Li et al., 2006). A hierarchical model that involves PRC2 recruitment followed by that of PRC1 has been proposed based on the fact that the CBX subunit of PRC1 binds to the H3K27Me3 histone mark (Fischle et al., 2003; Min et al., 2003). However, H3K27Me3 may not be sufficient for PRC1 recruitment, suggesting that additional mechanisms contribute to PcG protein recruitment (Gao et al., 2012; Margueron and Reinberg, 2011; Pasini et al., 2007; Schoeftner et al., 2006; Simon and Kingston, 2009; Tavares et al., 2012; Trojer et al., 2011; Yu et al., 2012). The repressive function of PRC1 is thought to, at least in part, depend on its ability to monoubiquitylate histone H2A at lysine 119 (K119) (Gutiérrez et al., 2012; Stock et al., 2007). In addition, PRC1 has chromatin-compaction activity (Francis et al., 2004).

et al., 2004), and also acts as a mark of DNA damage (Bergink et al., 2006; Doil et al., 2009; Huen et al., 2007; Ikura et al., 2007; Mailand et al., 2007; Marteijn et al., 2009; Nicassio et al., 2007; Stewart et al., 2009; Zhao et al., 2007). This raises the possibility that the PRC1 E3 ligase is also involved in DDR. Several laboratories confirmed this hypothesis by showing that both BMI1 and RING1B efficiently accumulate at ionizing radiation (IR)- and laser-induced DSBs (Chagraoui et al., 2011; Chou et al., 2010; Facchino et al., 2010; Gieni et al., 2011; Ginjala et al., 2011; Ismail et al., 2010; Nacerddine et al., 2012; Pan et al., 2011; Wu et al., 2011a) (Box 1 and Fig. 3). The kinetics of BMI1 and RING1B recruitment is similar to that of the early DDR factors, such as RNF8, meiotic recombination 11 (MRE11) and Nijmegen breakage syndrome 1 (NBS1) (Ismail et al., 2010), and their presence at DSBs is sustained for several hours after damage (Chou et al., 2010; Ginjala et al., 2011; Ismail et al., 2010). In contrast to BMI1, its homologue MEL18 is only transiently recruited to DSBs, suggesting that recruitment and maintenance of different PRC1 complexes occurs with distinct kinetics (Chou et al., 2010; Ginjala et al., 2011). The other PRC1 subunits, including human polyhomeotic homologue 1 and 2 (HPH1 and HPH2, respectively), as well as different chromodomain-containing proteins, such as CBX2, CBX4, CBX6, CBX7 and CBX8, are also found to localize to laser-induced DSBs (Chou et al., 2010; Ginjala et al., 2011; Ismail et al., 2012). Given the variety in the composition of PRC1 complexes (Gao et al., 2012; Gearhart et al., 2006; Ogawa et al., 2002; Sanchez et al., 2007; Tavares et al., 2012; Trojer et al., 2011; Vandamme et al., 2011; Yu et al., 2012), there is a large number of possible complexes that can be assembled at DSBs, which raises the question whether they perform distinct functions. Interestingly, depending on the experimental system used, in some cases the recruitment of BMI1 and RING1B was found to be independent from each other (Ginjala et al., 2011; Ismail et al., 2010), which suggests that in the absence of RING1B BMI1 can be recruited by RING1A and that in the absence of BMI1 other PCGF factors can recruit RING1B (Chou et al., 2010).

Besides PRC1, the recruitment of PRC2 core components, the PRC2-associated factor PHD finger protein 1 (PHF1), and the H3K27 methyl mark have also been detected at sites of DNA damage (Chou et al., 2010; Hong et al., 2008; O'Hagan et al., 2008; Seiler et al., 2011). This suggests that the entire PRC2 complex is present at DSBs.

The role of PRC1 in histone ubiquitylation and signaling at DSBs

As discussed above, the PRC1 E3 ligase is recruited to DSBs, which are main sites of ubiquitylation. These findings prompt the question whether PRC1 contributes to ubiquitylation at DSBs. Determining the contribution of PRC1 to overall ubiquitylation by immunofluorescence with an antibody that recognizes ubiquitin conjugates (FK2) has proven controversial (Ginjala

Table 1. Mammalian Polycomb group proteins that localize to DNA damage sites

	Protein	Protein motifs	Biochemical activity	Method of DNA damage induction	Function in DDR	References
PRC2	EZH1	SET domain	Histone methyltransferase	Laser micro-irradiation (GFP-tagged protein)	Prevent sensitivity to DSB-inducing agents	(Chou et al., 2010)
	EZH2	SET domain	Histone methyltransferase	UV; Laser micro-irradiation; endonuclease-induced DSBs (ChIP); H_2O_2 induced foci	Prevent sensitivity to DSB-inducing agents	(Chou et al., 2010; O'Hagan et al., 2008; O'Hagan et al., 2011)
	SUZ12	Zinc finger	Stimulates histone methyltransferase activity	UV	Prevent sensitivity to DSB-inducing agents	(Chou et al., 2010)
	PHF1 (interactor)	Two PHD fingers and a Tudor domain	Stimulates histone methyltransferase activity	Laser micro-irradiation (GFP-tagged protein)	Prevent sensitivity to DSB-inducing agents	(Hong et al., 2008)
PRC1	BMI1/PCGF4	RING domain	E3 ubiquitin ligase	Laser micro-irradiation; hydroxyurea-, camptothecin- and IR-induced foci; aphidicolin-induced fragile sites (ChIP); ZNF-induced DSBs (ChIP)	H2A/H2AX ubiquitylation; Prevent genomic instability; Prevent sensitivity to DSB-inducing agents	(Chagraoui et al., 2011; Chou et al., 2010; Ginjala et al., 2011; Ismail et al., 2010; Nacerddine et al., 2012; Pan et al., 2011)
	MEL18/PCGF2	RING domain	E3 ubiquitin ligase	Laser micro-irradiation	n.d.	(Chou et al., 2010; Ginjala et al., 2011)
	RING1A/RNF1	RING domain	E3 ubiquitin ligase	Laser micro-irradiation (GFP-tagged protein)	n.d.	(Chou et al., 2010)
	RING1B/RNF2	RING domain	E3 ubiquitin ligase	Laser micro-irradiation; IR-induced foci	H2A/H2AX ubiquitylation; Prevent sensitivity to DSB-inducing agents	(Chou et al., 2010; Ginjala et al., 2011; Ismail et al., 2010; Wu et al., 2011a)
	CBX2	Chromodomain	Methyl-lysine binding	Laser micro-irradiation; ZNF-induced DSBs (ChIP)	n.d.	(Ginjala et al., 2011)
	CBX4	Chromodomain	Methyl-lysine binding; SUMO E3 ligase	Laser micro-irradiation; IR-induced foci	SUMOylation of BMI1; Prevents sensitivity to DSB-inducing agents	(Chou et al., 2010; Ismail et al., 2012)
	CBX6, 7, 8	Chromodomain	Methyl-lysine binding	Laser micro-irradiation (GFP-tagged protein)	n.d.	(Chou et al., 2010)
	HPH1, 2	SAM and Zinc finger domain		Laser micro-irradiation (GFP-tagged protein)	n.d.	(Chou et al., 2010)

BMI1, B lymphoma Mo-MLV insertion region 1 homolog; CBX, chromobox; ChIP, chromatin immunoprecipitation; DSB, DNA double strand break; GFP, green fluorescent protein; EZH, enhancer of zeste homologue; HPH, human polyhomeotic homologue; PCGF, Polycomb group RING finger protein; PHD finger, plant homeodomain finger; PHF1, PHD finger protein 1; PRC; Polycomb repressive complex; RING, really interesting new gene; RNF, RING Finger protein; SAM, sterile alpha motif; SET domain, Su(var)3-9, Enhancer-of-zeste, Trithorax domain; SUMO, small ubiquitin-related modifier; Suz12, Suppressor of Zeste homologue 12; ZNF, zinc finger protein. n.d., not determined.

et al., 2011; Ismail et al., 2010), most probably because of the abundance of ubiquitin conjugates at DSBs and the limitations of this approach in measuring quantitative differences. The role of RING1B and BMI1 in DSB-induced histone ubiquitylation has been addressed specifically, both biochemically and by using immunofluorescence. Immunoblot analysis of H2A and H2AX suggests that loss of RING1B or BMI1, or mutation of the canonical target lysine of H2A or H2AX interfere with both basal and IR-induced monoubiquitylation (Ginjala et al., 2011; Ikura et al., 2007; Pan et al., 2011; Wu et al., 2011a). In addition, ubiquitylation of H2A – as detected using the antibody E6C5 – at laser-induced damage is found to depend on BMI1 (Ginjala et al., 2011). Although the specificity of this antibody is not undisputed, collectively these findings support a role for BMI1 in H2A monoubiquitylation at the chromatin that surrounds the DNA lesion (Fig. 3).

DSB-induced ubiquitin signaling entails a complex network of ubiquitin substrates, which are mostly regulated by RNF8–RNF168 (Al-Hakim et al., 2010; Lukas et al., 2011; Tang and Greenberg, 2010). The reported requirement of BMI1 for the monoubiquitylation of H2A and H2AX (Ginjala et al., 2011; Pan et al., 2011) and that of RNF8–RNF168 for the generation of K63-linked ubiquitin chains (Doil et al., 2009; Pinato et al., 2009; Stewart et al., 2009) suggest that these ubiquitin signals have distinct functions at DSBs and it will be of great interest to characterize these functions. Other important unanswered questions include: which lysine(s) on H2A or H2AX are modified by RNF8–RNF168 and do they overlap with the PRC1 target lysine? Do PRC1 and RNF8–RNF168 modify histone H2A molecules in the same nucleosome? Can they act on the same ubiquitin chains, and are there other ubiquitylation substrates that are targeted by these E3 ligases?

Another crucial question is whether PRC-complex-mediated histone modifications coordinate DDR signaling. As mentioned above, 53BP1 and also UIMC1 (also known as RAP80) are important DDR mediators, whose recruitment is dependent on the

Box 1. Methods to induce localized DNA lesions

Several methods can be used to induce localized DNA lesions in the nucleus of mammaliancells (Nagy and Soutoglou, 2009; Polo and Jackson, 2011). Laser lines are used to locally irradiate specific regions in the cell nucleus, thereby generating focal DNA damage. PcG protein recruitment to DNA lesions has been studied by using micro-irradiation with an UV-A laser (337 nm) on cells that were sensitized with halogenated nucleotide analogues such as BrdU or IdU (referred to as the UV-A laser scissor approach) (Chou et al., 2010; Ginjala et al., 2011; Nacerddine et al., 2012), and with a near-infrared laser line (750 nm) on cells sensitized with the DNA-intercalating dye Hoechst 33258 (Ismail et al., 2010; Ismail et al., 2012). A limitation of these laser-based methods is that, besides DSBs, they also give rise to a wide spectrum of other DNA lesions, including cyclobutane pyrimidine dimers, 6,4 pyrimidine-pyrimidones and ssDNA breaks (Bekker-Jensen et al., 2006; Dinant et al., 2007; Kong et al., 2009). Furthermore, structural changes in the DNA that are caused by intercalation of Hoechst dye may lead to non-physiological DDR (Dinant et al., 2007; Kong et al., 2009; Williams et al., 2007a). UV-A treatment, if applied at high power, can also elicit aberrant cellular responses by inducing protein damage or protein–protein and protein–DNA crosslinks. Thus, the type and relative contribution of DNA lesions need to be considered when comparing different studies, as they may affect the dynamics of DDR factor recruitment.

Nuclease-based systems are used to induce DSBs at defined genomic loci and are based on the use of endonucleases that recognize and cut highly specific DNA sequences. An inducible DSB-inducing system that uses endonuclease I-SceI (Rouet et al., 1994), as well as a zinc-finger nuclease (ZFN) strategy (Miller et al., 2007), has been employed to investigate the recruitment of PcG factors to DSBs (Ginjala et al., 2011; O'Hagan et al., 2008).

RNF8–RNF168–ubiquitin pathway (Al-Hakim et al., 2010; Lukas et al., 2011; Tang and Greenberg, 2010). Analysis of *Bmi1*-knockout cells shows that RAP80, as well as 53BP1, efficiently accumulate at DNA damage sites that were generated by using the UV-laser scissor approach (Box 1), suggesting that BMI1-mediated ubiquitylation of H2A does not affect the canonical ubiquitin pathway but, instead, impacts on a parallel pathway that involves the recruitment of yet unidentified factors (Ginjala et al., 2011). However, Ismail et al. reached the opposite conclusion; using a different method to induce DSBs (the near-infrared laser-based approach; see Box 1) and assessing recruitment at earlier time points, they found that *Bmi1* loss affects accumulation of 53BP1, RAP80 and BRCA1 at DNA damage sites (Ismail et al., 2010). Detailed time course experiments following induction of specified DNA damage are needed to clarify the exact contribution of BMI1 to DSB signaling.

Requirements of PRC1 and PRC2 for survival following clastogen treatment and for DSB repair

Work from several laboratories established that PcG proteins have functional relevance in DDR and in genome maintenance. Indeed, loss of components of PRC1 (Chagraoui et al., 2011; Ginjala et al., 2011; Ismail et al., 2010; Pan et al., 2011) and PRC2 (Chou et al., 2010; Hong et al., 2008) leads to increased sensitivity to IR and other clastogens (Table 1). Several authors examined a role for PcG proteins in DSB repair. In particular, experiments in which a GFP-based reporter assay was used to detect homologous recombination efficiency, support a contributing role of BMI1 in the homologous recombination repair pathway (Chagraoui et al., 2011; Ginjala et al., 2011; Nacerddine et al., 2012) (Fig. 3). Regardless of the exact underlying mechanisms, this provides an at least partial explanation for the requirement of BMI1 for cell survival upon treatment with DSB inducing agents (Chagraoui et al., 2011; Ginjala et al., 2011; Ismail et al., 2010; Pan et al., 2011). However, it should be noted that the effects on both homologous recombination and cell survival are relatively mild, which suggests that the PcG factors are not core repair factors, but rather have a modulatory role in the establishment of conditions

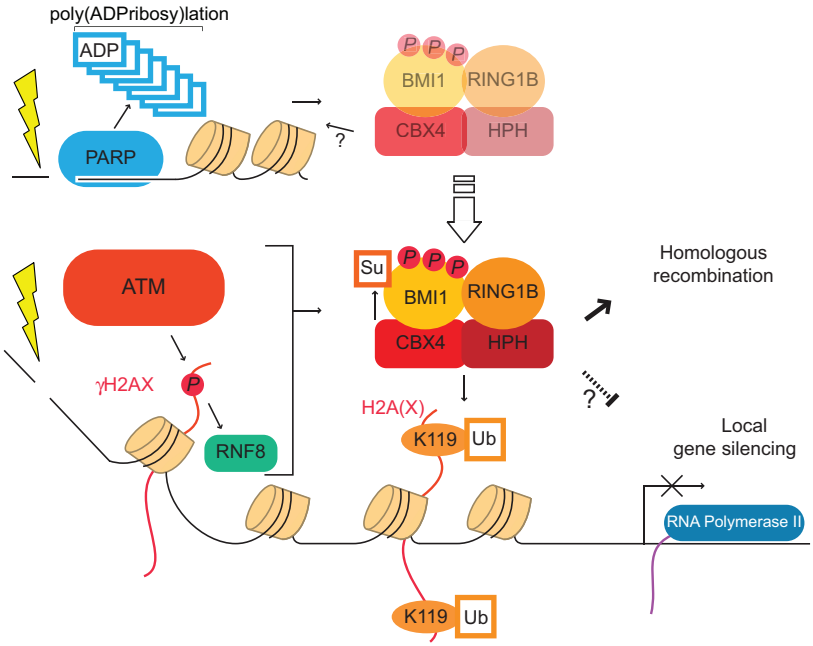

Fig. 3. Regulation of PRC1 recruitment and activity at DSBs. ATM kinase activity and H2AX are required for BMI1 recruitment to and its maintenance at DSBs, possibly through RNF8. Independently from ATM, poly(ADP ribosyl)ation by PARP enzymes supports the recruitment of the PRC1 subunits MEL18 and CBX4 to DSBs. The other components of PRC1 have also been detected at sites of DSBs, suggesting that the entire complex is recruited. The precise molecular mechanisms by which the ATM–H2AX–RNF8 pathway and PARP promote PRC1 recruitment are not fully understood. In particular, it is unknown whether components of PRC1 can bind directly to phosphorylated or ubiquitylated substrates. However, it has been suggested that CBX4 directly binds to poly(ADP-ribose), which would provide a mechanism by which PARP enzymes promote PRC1 recruitment. Recruitment of BMI1 to DSBs has been suggested to depend on SUMOylation (Su) by CBX4. Once recruited, BMI1 is involved in H2A and H2AX monoubiquitylation at DSBs. The ubiquitylation activity of BMI1 at DSBs is stimulated by phosphorylation, and one function of BMI1 at DSBs is to promote homologous recombination. Moreover, since H2A ubiquitylation has been implicated in transcriptional silencing of genes that are in direct proximity to the break, we speculate that PRC1 is also involved in this process (as shown by the question mark).

that favour repair. The potential involvement of PcG proteins in NHEJ awaits further investigation.

Regulation of PRC1 recruitment to sites of DSBs

The studies presented above support a broad involvement of PcG proteins in DDR but, nevertheless, raise several questions with regard to the underlying molecular mechanisms. For example, it remains to be determined which signaling events are required to induce the accumulation of PcG complexes at DSBs. With regard to PRC1, it has been shown that BMI1 recruitment at laser-induced DNA damage sites is dependent on ATM- and ATR-mediated signaling (Fig. 3) (Ginjala et al., 2011; Ismail et al., 2010). In response to ATM activation, a very early recruitment of BMI1 but not its persistence could be detected in mouse *H2ax*-knockout cells (Ginjala et al., 2011; Ismail et al., 2010). This suggests a biphasic mode of BMI1 accumulation, with an initial phase that is independent of H2AX, and a second phase that depends on ATM- or ATR-mediated phosphorylation and, possibly, ubiquitylation by RNF8 (Ginjala et al., 2011; Ismail et al., 2010) (Fig. 3). The DDR regulatory factors BRCA1 and 53BP1, which act further downstream in the ATM-RNF8–RNF168 pathway, were shown to be dispensable for BMI1 recruitment (Ginjala et al., 2011; Ismail et al., 2010).

In search for additional factors that influence the recruitment of PcG proteins to DNA damage sites, PARP-dependent signaling has also been investigated (Chou et al., 2010; Ginjala et al., 2011; Ismail et al., 2012). PARP inhibition or loss was reported to affect the recruitment of CBX4 (Ismail et al., 2012) and of the BMI1 homologue MEL18, but not of BMI1 itself (Chou et al., 2010; Ginjala et al., 2011) (Fig. 3). Taken together, the data discussed above suggest that multiple signaling pathways contribute to the recruitment of PcG proteins to DNA lesions (Fig. 3). In this context, it will be important to further elucidate the functional crosstalk between PcG proteins and the RNF8–RNF168–ubiquitin pathway, as well as the role of PARP-mediated signaling.

To dissect the molecular mechanism in detail it will be necessary to identify the molecular interactions that allow the recruitment of PcG complexes to damaged DNA, as PcG proteins do not have obvious binding domains for phosphorylated or ubiquitylated proteins. The observation that the H3K27Me3 mark has been detected at DSBs (O'Hagan et al., 2008) suggests that PRC2, to some extent, promotes PRC1 binding at these sites. However, against this hypothesis, recent experiments suggest that PRC1 recruitment to DSBs is not affected upon depletion of EZH2 or embryonic ectoderm development (EED) (Ismail et al., 2010), which are required for H3K27 trimethylation (Cao et al., 2002; Czermin et al., 2002; Kuzmichev et al., 2002; Müller et al., 2002). In addition, methyl-lysine binding by CBX4 appeared to be dispensable for recruitment to sites of microirradiation (Ismail et al., 2012).

Finally, recent data indicate that the function of BMI1 in DDR is also regulated by post-translational modification (Fig. 3). For example, modification of BMI1 with SUMO by CBX4 has been suggested to favor its recruitment to DSBs (Ismail et al., 2012). In addition, phosphorylation of BMI1 stimulates the ubiquitylation activity of PRC1 at DSBs (Nacerddine et al., 2012).

Taken together, is it clear that PcG proteins are required for cell survival upon the induction of DSBs and are physically recruited to DSBs, where BMI1 promotes histone H2A monoubiquitylation and efficient DDR. There are at least two ways by which PcG complexes might promote DSB repair. First, PcG proteins might contribute to DNA damage site recognition and, as we have discussed above, help to recruit DDR factors to the chromatin surrounding the break. Second, given that the PRC1-induced ubiquitylation of H2A acts as a repressive mark, an attractive means by which PcG proteins might facilitate DSB repair is by simply repressing transcription at the break, thereby coordinating the repair and transcriptional machineries (Chagraoui et al., 2011; Lukas et al., 2011). In the next section, we will discuss this possibility in detail.

Regulation of transcription in response to DNA double-strand breaks – a role for PcG proteins?

Several recent lines of evidence suggest that DDR-associated mechanisms exist to coordinate DSB repair and transcription within the chromatin environment (Lukas et al., 2011; Shanbhag and Greenberg, 2010). One such mechanism is local DSB-induced silencing. The existence of this phenomenon was demonstrated using stably integrated constructs that allow the induction of a defined DNA break in the direct vicinity of a transcriptional reporter gene (Cuozzo et al., 2007; O'Hagan et al., 2008; Shanbhag et al., 2010). The physiological relevance of these findings has recently been supported by ChIP-based studies that mapped the distribution of both γH2AX and Pol II in parallel with transcription occurring around endonuclease-induced breaks (Iacovoni et al., 2010; Pankotai et al., 2012). Importantly, the occurrence of a single DSB in the body of genes transcribed by Pol II results in inhibition of transcription (Iacovoni et al., 2010; Pankotai et al., 2012). The interplay between DDR and transcription could have more than one purpose. First, it might prevent the transcription of broken genes that would otherwise lead to the accumulation of truncated mRNAs. Second, it might facilitate DSB repair in the context of transcriptionally active chromatin, for example, by preventing collision between the repair and transcriptional machineries.

As PcG proteins are known transcriptional repressors and are recruited to DSBs, they are the ideal candidates to regulate transcription at these sites. A silencing role at DSBs has first been suggested for EZH2, and EZH2 and its chromatin mark H3K27Me3 have been detected by ChIP at DSBs (O'Hagan et al., 2008). DSB induction also results in DNA methylation and long-term silencing (Cuozzo et al., 2007; O'Hagan et al., 2008). These findings suggest that EZH2 contribute to DSB-induced epigenetic gene silencing (O'Hagan et al., 2008). The role of PRC1 in silencing at DSBs has not been extensively addressed yet (Chagraoui et al., 2011). However, a recent study suggests that H2A ubiquitylation has an important role in transcriptional silencing of genes in the vicinity of DSB sites (Shanbhag et al., 2010). There, the authors were able to visualize the inhibition of transcription in cis when a DSB was induced near a reporter gene (Shanbhag et al., 2010); they also showed that silencing is dependent on ATM-mediated activation of the RNF8–RNF168–ubiquitin pathway. Importantly, several lines of evidence support a functional involvement of monoubiquitylated H2A (Shanbhag et al., 2010). An open question is whether RNF8 and RNF168 are directly responsible for DSB-induced silencing, or whether another E3 ligase is involved. As mentioned above, although the RNF8 or RNF168 E3 ligases can clearly ubiquitylate histone H2A, their dominant modification is that of K63-linked ubiquitin

chains (Doil et al., 2009; Huen et al., 2007; Kolas et al., 2007; Mailand et al., 2007; Pinato et al., 2009; Stewart et al., 2009; Wang and Elledge, 2007). By contrast, BMI1 and RING1B catalyze the monoubiquitylation of H2A (Buchwald et al., 2006; Cao et al., 2005; Li et al., 2006; Wang et al., 2004). Thus, it will be of great interest to determine whether the described function of BMI1 in transcriptional repression, indeed, extends to sites of DSBs (Fig. 3). In that scenario, the silencing function of RNF8–RNF168 might be either indirect by stimulating BMI1 recruitment and, thus, H2A monoubiquitylation, or RNF8–RNF168 could act downstream of ATM in a distinct pathway that is parallel to that of BMI1. The most direct way to study this is by introducing the reporter systems mentioned above (Cuozzo et al., 2007; Shanbhag et al., 2010) into cells that are deficient of a particular PcG gene. Finally, as the above described efforts have only addressed short-term silencing, an interesting question is whether the silencing imposed by the ubiquitin pathway is epigenetically heritable – as it has been suggested for PRC2 (O'Hagan et al., 2008).

Concluding remarks

Taken together, the data from several laboratories have established PcG proteins as important new factors in the cellular response to DNA damage. However, as we have discussed here, there are still many unresolved issues with regard to the mechanistic aspects of PcG function, such as their recruitment to sites of DNA damage, the regulation of their enzymatic activities, their role in mediating DDR signaling and repair, and their potential role in transcriptional silencing at DNA breaks. An additional, yet unexplored question concerns the dynamics of PcG-mediated histone marks during and following repair. With regard to PcG-induced H3K27Me3 and ubiquitylation of H2A, it will be of interest to examine the roles of enzymes that remove these marks, such as H2A DUBs (Komander et al., 2009; Vissers et al., 2008) and H3K27 demethylases (Hubner and Spector, 2010). In particular, the H2A ubiquitylation signal at DSBs appears to be diverse and includes at least K63-linked chains as well as monoubiquitylation (Al-Hakim et al., 2010; Lukas et al., 2011; Tang and Greenberg, 2010). The dynamics of ubiquitylated H2A at DSBs might thus involve distinct DUBs. A number of DUBs have been implicated in DDR, of which USP3 (Nicassio et al., 2007) and USP16 (Shanbhag et al., 2010) can target histone H2A, and BRCC36 can target K63-ubiquitin chains (Shao et al., 2009). In the context of PcG proteins, the H2A DUB BRCA1-associated protein-1 (BAP1) stands out, as it has been implicated in both PcG repression and DDR (Jensen et al., 1998; Scheuermann et al., 2010; Ventii et al., 2008).

The deregulation of transcriptional programs and the loss of genome stability are key steps in the transformation of normal cells to cancer cells. PcG genes are involved in transcriptional repression and it is generally accepted that PcG-mediated repression of tumour suppressor genes is causally linked to tumorigenesis (Bracken and Helin, 2009). The recent finding that, besides their epigenetic function, PcG proteins are needed to maintain the genomic stability, provides encouragement for the putative development of small-molecule inhibitors of PcG activity. Perhaps the most effective use of such inhibitors would be in combination with other agents, such as standard chemotherapeutics and radiotherapy. The elucidation of PcG-mediated DDR thus has the potential to inspire new and more effective strategies to treat cancer.

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