Regulation of Polycomb Group proteins : the complex, the kinase and the interactor

Citation for published version (APA):

Niessen, H. E. C. (2009). *Regulation of Polycomb Group proteins : the complex, the kinase and the interactor*. [Doctoral Thesis, Maastricht University]. Maastricht University.
<https://doi.org/10.26481/dis.20091113hn>

Document status and date:

Published: 01/01/2009

DOI:

[10.26481/dis.20091113hn](https://doi.org/10.26481/dis.20091113hn)

Document Version:

Publisher's PDF, also known as Version of record

Please check the document version of this publication:

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Regulation of Polycomb Group proteins

**The complex, the kinase and the
interactor**

Printed by GVO printers & designers B.V. | Ponsen & Looijen
ISBN 978-90-6464-370-5
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Regulation of Polycomb Group proteins

The complex, the kinase and the interactor

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Universiteit Maastricht,
op gezag van de Rector Magnificus,
Prof. mr. G.P.M.F. Mols
volgens het besluit van het College van Decanen,
in het openbaar te verdedigen
op vrijdag 13 november 2009 om 12.00 uur

door

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Geboren te Roermond op 10 juni 1980

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This research was supported by NWO.

Printing of this thesis was financially supported by:

Greiner bio-one

GC biotech

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Chapter 1

General Introduction

Introduction

The human genome stores the information that a cell needs to produce the molecules it requires. Even though the DNA sequence is the same in all somatic cells of a human body, cells have a specific phenotype, depending on the developmental stage and tissue type in which they reside. The celltype-specific functions are determined by the set of genes that are transcribed and translated within the cell. Gene transcription in itself is not only determined by the availability and activity of transcriptional machinery, but also by the accessibility of the DNA and genes. Therefore, packing the genome into chromatin, making DNA accessible or inaccessible, is an important contributor to determining cell fate and function. The building blocks of chromatin are nucleosomes, which consist of DNA and histones¹. Each nucleosome contains two copies of each of the core histones (H2A, H2B, H3 and H4), together comprising an octamer, around which the DNA is wrapped (Fig. 1.1). The N-terminal histone tails protrude from the nucleosome². Condensation of nucleosomes into open or closed chromatin structures or states ultimately determines whether genes are accessible or not.

Figure 1.1. Organization of chromatin

DNA is stored in the nucleus as chromatin. The building unit of chromatin is the nucleosome, which consists of eight histones (two each of H2A, H2B, H3 and H4) with 147 base pairs of DNA wrapped around it. The histone tails protrude from the nucleosome and are available for post-translational modification.

Epigenetics

The study of heritable gene expression changes, and hence heritable phenotypes, that occur without changing the underlying DNA nucleotide sequence is referred to as epigenetics³. Modifications that (stably) alter the chromatin structure, and thereby gene expression are important epigenetic

regulators of development and disease. Epigenetic regulation occurs at several macromolecular levels: at the RNA, DNA and protein level. They all have in common that they ultimately affect gene expression. Non-coding RNA molecules play important roles in epigenetic regulatory mechanisms, of which the best known example is the coating of the inactive X chromosome by a large non-coding RNA: Xist⁴. There is increasing evidence that also small non-coding RNAs play an important role in epigenetic regulation of gene expression⁵. Perhaps the best known mechanism of epigenetic silencing is DNA methylation. It is catalyzed by DNA methyltransferases (DNMTs), occurs on cytosine bases in the context of CpG dinucleotides and is associated with repressed chromatin states⁶. At the protein level, nucleosomes are targets and coordinators of epigenetic regulation. Histones are mostly modified on their N-terminal tails, which are subject to *i.a.* acetylation, methylation, phosphorylation and ubiquitylation^{7, 8}. Another level of complexity is added since methylation can occur as mono-, di-, or trimethylation on lysines, and as mono- or di- (symmetric or asymmetric) methylation on argenines⁸. For some modifications the relation to transcription is understood. Histone acetylation mostly correlates with transcriptional activation. Histone methylation, however, correlates with both activation and repression, depending on the target residue. The multiple combinations of these histone modifications form a gene expression status index, which is also referred to as the histone code⁹. The 'writers' of this code are enzymes that catalyze the post-translational modifications of histones, such as histone acetyl transferases (HATs), histone methyl transferases (HMTs), kinases and ubiquitin ligases. The 'erasers' are enzymes that remove these modifications, such as histone deacetylases (HDACs), histone demethylases, phosphatases and de-ubiquitinating enzymes (DUBs). The 'readers' are proteins that through specific domains (*e.g.* PHD-, chromo- and bromo- domain) interact with specific histone modifications⁸. Many chromatin-associated complexes, being transcriptionally activating or repressive, either harbor or are associated with such catalytic activities.

Polycomb Group proteins

One chromatin-associated group of proteins are the Polycomb Group (PcG) proteins. They maintain transcriptional silencing via epigenetic mechanisms. PcG proteins fulfill a crucial role in antero-posterior patterning during development by repressing their classical targets, the HOX genes¹⁰. The mechanisms that contribute to PcG-mediated silencing are unfolding (Fig. 1.2). PcG proteins function in two complexes, Polycomb Repressive Complex 1 and 2 (PRC1, PRC2). The PRC2 complex contains EED, SUZ12 and EZH2, an HMT that installs trimethylation on lysine 27 of histone 3 (H3K27me3)¹¹⁻¹⁴. Members of PRC2 also

associate with DNMTs, HDACs, kinases and DUBs¹⁵⁻¹⁸. PRC1 is made up of several proteins, including BMI1, RNF2, PHC and CBX proteins^{19, 20}. Some CBX proteins specifically bind to the H3K27me3 mark, thereby recruiting the PRC1 complex^{21, 22}. RNF2 is a E3 ubiquitin ligase involved in the ubiquitylation of H2A on lysine 119²³⁻²⁵. Furthermore PRC1 also associates with another HMT²⁶. These catalytic activities, in combination with extensive interaction among PcG members and other chromatin factors, generate a repressive higher-order chromatin structure inaccessible to transcriptional activators.

Figure 1.2. Schematic drawing of Polycomb-mediated silencing.

Aim of the thesis

Although progress has been made in understanding the mechanisms that contribute to PcG-mediated repression, many aspects of Polycomb Group biology in mammals are still elusive: What processes control PcG complex formation, how is complex composition determined, what factors determine chromatin interaction at a specific site, and how is target gene specificity determined? And importantly: how is Polycomb Group function regulated?

We know that PcG proteins are post-translationally modified. At the beginning of this project it was known that at least some PcG proteins are phosphorylated and that this phosphorylation correlates with chromatin dissociation in a cell cycle-dependent fashion²⁷. Phosphorylation of PcG proteins might alter protein-protein interactions, subcellular localization, enzymatic activity and overall function. The working hypothesis of the research described in this thesis is that phosphorylation of PcG proteins leads to PcG dissociation from chromatin, giving the cell an opportunity to change its transcriptional status.

The research described in this thesis aimed at the identification of

- 1) kinases that are responsible for PcG phosphorylation;
- 2) PcG amino acid residues that are phosphorylated;
- 3) potential changes of the PcG complex composition upon phosphorylation and
- 4) the biological relevance of these phosphorylation events.

A detailed review of PcG complexes function and how PcG proteins contribute to tumorigenesis is presented in Chapter 2. The current knowledge of PcG post-translational modifications and PcG enzymatic activities are summarized in Chapter 3. The identification of a PcG kinase is presented in Chapter 4. The biological relevance of this kinase in terms of cellular function and PcG involvement is revealed in Chapter 5. The identification of PcG phosphorylation sites is described in Chapter 6, in which the complex composition of PRC1 was studied during phosphorylated conditions. The results obtained in these studies and their implications for PcG function are discussed in Chapter 7.

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Chapter 2

Polycomb Group proteins in tumorigenesis

Based on: Niessen H, Voncken JW. In: Tollefsbol T, ed. *Cancer Epigenetics*. Boca Raton: CRC Press; 2008:281-302

Short general introduction

The molecular mechanisms underlying cancer development have since long been a subject of study. Traditionally, cancer is thought of as a multi-step disease, largely based on accumulation of genetic abnormalities, such as amplifications, mutations and deletions, leading to gain-of-function of oncogenes, or loss-of-function of tumor suppressor genes¹. In recent years it has become evident that not only changes at the genomic level but also at the epigenomic level contribute to cancer development. It is now clear that abnormal epigenetic regulation may act as an alternative mechanism for, *e.g.*, loss of tumor suppressor function in cancer cells. One of the best-known examples is the inactivation of tumor suppressor genes, such as p16^{INK4A} and BRCA1, by DNA methylation². In this way, DNA-methylation-dependent gene silencing contributes to biallelic loss of tumor suppressor function, as proposed in the “two-hit” model, by Knudson³. Processes or proteins that orchestrate epigenetic regulation may affect expression or regulation of tumor suppressor or oncogenes, and in doing so contribute to tumorigenesis. In this chapter, we specifically focus on a group of epigenetic regulators, the Polycomb group proteins and their role in normal development and in tumorigenesis.

PcG introduction

Epigenetic regulation of gene expression in essence centers on processes that modify chromatin structure (Box 2.1). Epigenetic chromatin remodeling may take place at any higher-order chromatin structure level (*i.e.*, ranging from posttranslational changes on nucleosomes to chromatin compaction levels beyond the 30 nm solenoid fiber). As all DNA-templated processes require modification of chromatin structure in order to allow access to DNA, the epigenetic mechanisms that regulate DNA access and gene expression are pivotal to maintenance of cell identity throughout differentiation and hence normal development. PcG proteins play a key role in the maintenance of gene silencing patterns, whereas their biological counterparts, the trithorax Group (trxG) proteins, generally are or associate with transcription activators⁴. PcG proteins were first identified as repressors of Hox genes in *Drosophila melanogaster*. The descriptive name Polycomb derives from a class of homeotic phenotypes in the fruit fly, which have the common property of determining the number or positioning of a structure called the sex comb, of which male flies normally have only one on each front leg. The phenotypic characteristics of fly extremities (*e.g.*, antennae, legs, wings, halteres) and their positioning (*i.e.*, anteroposterior (A-P) axial patterning) are controlled by the homeotic complex of genes (HOM-C)^{5, 6}. An oversimplified delineation of HOM-C dependent A-P patterning argues that segment/extremity identity is determined by the

combination of HOM-C genes active in any given A-P position. The fly HOM-C genes are structurally conserved throughout evolution. In addition, the organization of HOM-C is conserved from primitive chordates to humans, as is the co-linear regulation of HOM-C gene expression⁵. PcG proteins were identified as a HOM-C repressive activity almost 30 years ago⁷. Whereas initiation of silencing or activation is not dependent on PcG proteins, *Drosophila* PcG mutants typically mis-express specific HOM-C genes at later stages; this, combined with unaltered expression initiation of HOM-C genes in PcG-mutants led to the proposition that maintenance of HOM-C expression boundaries (*i.e.*, silencing) was affected in these mutants. Also PcG and trxG proteins appeared conserved throughout evolution and even their antagonistic properties^{8, 9}. Hence, it would seem likely that their regulatory function and mode of action on the mammalian Hox gene clusters (counterparts of the fly HOM-C) would also be conserved. Indeed Hox and PcG genes are involved in A-P axial patterning in vertebrates, exemplified by typical skeletal abnormalities in PcG mutant mice, which are accompanied by defective maintenance of Hox-gene expression boundaries^{10, 11}. Also it is now clear that besides Hox genes, PcG proteins repress many other genes, among which genes that are directly relevant for cell proliferation and survival, and thus oncogenesis. Relevantly, the first mammalian PcG gene, BMI1, was identified as an oncogene in a murine model for oncogene cooperativity in leukemia^{12, 13}. Besides transcriptional regulation, PcG proteins are implicated in diverse biological processes, such as X inactivation, genomic imprinting, cell cycle regulation, and stem cell maintenance¹⁴⁻¹⁸. Some of these properties, when deregulated, are likely to contribute to tumorigenesis. Our understanding of the molecular details of PcG repression is still incomplete, however, in recent years our insight in the silencing mechanisms that PcG proteins impinge on has increased significantly.

PcG biology

Mechanisms in PcG silencing

We now know that PcG proteins function in large multi-protein complexes. PcG biochemistry appears highly conserved among fruit flies and higher eukaryotes, like mice and men. The two most studied Polycomb repressive complexes (PRCs) are PRC1 and PRC2^{19, 20}. Reconstitution studies using recombinant proteins identified the core complex of *Drosophila* PRC1, which consists of Polycomb (Pc), Polyhomeotic (Ph), Posterior sex combs (Psc), and dRing/Sex combs extra (Sce)²¹⁻²³. As was the case with evolutionary duplication of HOM-C between *Drosophila* and human (the human genome harbors four Hox gene clusters), mostly single fly PcG genes were also duplicated. Mammalian PRC1 genes comprise multiple orthologues of the *Drosophila* core components: HPC2 and 3,

Box 2.1. Epigenetic mechanisms in gene expression

DNA, the macromolecule in which our genetic make-up is hardwired, is tightly packed inside the eukaryotic nucleus. This packing fits 2 m of DNA into a nucleus of approximately 2 mm in diameter. The macromolecular DNA/protein structure in which DNA is packed is called chromatin. The consequence of this packing is that DNA (genes) is not naked and therefore not freely accessible. For transcription factors and DNA-dependent RNA polymerases to access DNA, for example to activate gene expression, chromatin structure has to be remodeled. Thus, chromatin structure regulation is essential for many, if not all, important biological processes, which are DNA templated (*e.g.*, DNA replication, transcription, recombination, repair, chromosome segregation). The direct consequence of chromatin packing is that expression of genes and genetic traits is not solely dependent on the genetic blueprint, as it is stored in the base pair sequence in the DNA. Complex eukaryotic cells have a second, epigenetic, layer of control on top of the genetic blue print; access to DNA (*i.e.*, expression of genetic traits) is controlled by chromatin structure modification: whether a gene is used or not is what matters. Epigenetic regulation explains cellular diversity in our body (despite genetically identical cells). Epigenetics is also what makes genetically identical monozygotic twins different in terms of susceptibility to disease (*i.e.*, neurological, cancer, metabolic disorders). Evidence is accumulating that epigenetic information is also heritable, and that epigenetic inheritance may even contribute to heritable, multifactorial disease. Epigenetic regulation of gene expression occurs at least at three macromolecular levels: (1) at the DNA level—covalent modification of DNA by coupling methyl-groups to specific nucleotides in DNA (2) at the RNA level—noncoding RNAs may either target genes or mRNAs, and directly regulate their expression, and (3) at the protein level, for instance by covalent modification of the N-terminal tails of histone proteins. DNA is wrapped almost twice around a nucleosome, an octamer of four different histone proteins. The protruding N-terminal histone tails are accessible to many regulatory proteins. Depending on the modification (*i.e.*, methyl, acetyl, phosphoryl, ubiquityl groups), novel protein binding sites are generated and protein complexes are recruited that either open-up chromatin or compact its structure. Combined, these histone-tail modifications function as an epigenetic register. The epigenetic register is “written” by histone-modifying enzymes. Many proteins have been identified which can “read” the register and thereby become recruited to chromatin to write additional modifications or change its configuration. Many histone-modifying complexes combine writing and reading properties. Recent studies suggest that at least some of these histone-tail-marks recruit nucleosome-remodeling machineries. For a gene to become transcriptionally active, RNA-polymerase has to gain access to the naked DNA. For this to occur, nucleosomes are quite literally moved aside, by ATP-dependent nucleosome remodeling machineries, to bear the DNA elements required for binding, and transcription initiation. Gene silencing reverses most of these processes.

HPH1, 2, and 3, BMI1, and RING1A and RING1B/RNF2, respectively (Box 2.2)²⁴. Biochemical purification of *Drosophila* PRC2 members identified Enhancer of zeste (E(z)), Extra sex combs (ESC), and Suppressor of zeste-12 (Su(z)12) as the main core complex members²⁵. The mammalian orthologues of PRC2 are EZH2, EED, and SUZ12. As PcG proteins are equipped with multiple protein-interaction domains, and many if not all mammalian homologues are known to homo- or heterodimerize, many different protein–protein binding modalities are expected to contribute to complex composition and diversity. Several observations suggest that also spatio-temporal expression patterns of PcG proteins may not

only determine PcG complex composition but also allow composition to change throughout development [20]. Studies of early fly development suggest that the PRC2 complex initiates PcG-mediated repression. The most compelling evidence for this is provided by temporally restricted sequential expression patterns of the fly PRC2 and PRC1 complexes, respectively²⁶⁻²⁸. One study revealed a transient physical interaction between PRC2 and PRC1 complexes, which was interpreted as perhaps being required to transfer PcG-mediated transcriptional silencing from initiation to maintenance state²⁹. Mammalian PcG complex collaboration may not be entirely similar, yet a sequential collaborative action was suggested by recent biochemical studies. EZH2 contains histone methyl transferase (HMT) activity within its SET domain, which mediates methylation of other proteins. *Drosophila* E(z) and its mammalian orthologue EZH2 specifically trimethylate histone H3 on lysine K27 (H3K27me3) and, to a lesser extent, lysine 9 (H3K9me3)^{25, 30-32}. EZH2 thus writes the epigenetic register (Box 2.1). Other PRC2 proteins read epigenetic markings on other histones. As WD40 domains have been implicated in binding specific histone methyl-lysine marks in other proteins³³⁻³⁵, EED may likewise read specific methylated histone marks via its WD40 repeat and contribute to sequential recruitment of other histone-modifying enzymes and, as such, provide additional regulation³³. The H3K27me3 mark is a PcG-associated silencing mark, as it recruits the PRC1 maintenance complex via binding of the chromodomain of the *Drosophila* Pc protein^{36, 37}. Crystallography studies have provided compelling evidence for a differential fit of the H3K9me3 and H3K27me3 in the chromodomains of HP1 and Pc, respectively^{36, 37}. This represents a functional biochemical bridging mechanism between PRC2 and PRC1 complexes. Biochemical interactions and repressive mechanisms in mammalian systems are assumed to function similarly to some extent (based in part on experimental data, in part on inference), in that the SET domain HMTases Ezh1/2 write an epigenetic H3K27me3 mark, to which mammalian Pc orthologs HPc2/3 and possibly other CBXs are recruited^{19, 38}. Recent studies suggest, however, that the H3K27me3 mark by itself may not be sufficient for HPC2 recruitment, as RNF2 depletion also disrupts HPC2 chromatin binding, whereas the H3K27me3 motif remains³⁹. HPC2 itself is a SUMO-E3 ligase, which sumoylates transcription factors and, as such, may provide additional regulation^{40, 41}. RING finger domain proteins within PRC1 may all be E3 ubiquitin ligases, yet so far only RNF2 was shown to be responsible for histone H2A monoubiquitylation of K119 (H2AK119ub1)^{39, 42, 43}. Although its exact enzymatic substrate is currently not known, BMI1-association somehow stimulates the E3 ligase activity of RNF2 toward H2A^{44, 45}. In addition, recent studies of X-inactivation revealed that RNF2 is recruited to and marks nucleosomes on the inactivated X-chromosome in female cells, independent of prior PRC2 activity⁴⁶. Clearly, the above studies have only just begun to unravel

the complexity and multiple layers of regulation of PcG repressive activity. Finally, PRC complexes also associate with other chromatin-modifying enzymes (Box 2.2). The EED protein interacts with histone deacetylases (HDACs) and deubiquitinases (DUBs), and HPC2 brings HMT SUV39H1 activity to local chromatin, which may help silencing marks spread-out over regulatory and into coding sequences^{33, 47, 48}. EZH2 recruits DNA methyltransferases (DNMTs) to specific target genes, suggesting intersection of epigenetic mechanisms at the chromatin level, which may ultimately serve to stabilize repression^{49, 50}. Two PRC2-like complexes were identified recently, termed PRC3 and PRC4, which incorporate different EED isoforms (RNA splice variants)^{51, 52}. These isoforms were suggested to differentially target HMT activity toward histone H3K27me or histone H1K26me⁵¹, although other studies found no support for such concept⁵³. PcG expression may also influence complex composition through mechanisms other than spatio-temporal presence of complex members: PRC4 is formed when EZH2 is overexpressed in cultured cells and it comprises the NAD⁺-dependent histone deacetylase SirT1 and EED-isoform 2. PRC4 appears preferentially expressed in cancer and undifferentiated embryonic stem (ES) cells⁵².

Recently, a third PRC complex, Pleiohomeotic (Pho) repressive complex (PhoRC) has been identified in *Drosophila*⁵⁴. It contains Pho and the SFMBT protein (Scm-related gene containing four malignant brain tumor domains). Pho is one of the two PcG proteins, together with pleiohomeotic-like (PhoI) that has sequence-specific DNA-binding properties⁵⁵⁻⁵⁷. The MBT repeats of SFMBT bind mono- and dimethylated H3-K9 and H4-K20⁵⁴. So far, the only mammalian orthologue with apparent direct DNA-binding activity is YY1. YY1 interacts with both PRC2 and PRC1 complexes^{58, 59}. Whether this dual interaction acts in integrating or bridging silencing by PRC2 and PRC1 is still speculative.

PcG-mediated higher-order chromatin structure remodeling

The PRC1 complex inhibits chromatin remodeling and transcription *in vitro*^{21, 23, 24, 60}. In addition, a reconstituted PRC1 core complex induced *in vitro* compaction of nucleosomal arrays⁶¹. Although EEDs' ability to target PRC2-HMT activity to linker histone H1 may point to a possible involvement in higher-order remodeling, there is to date no *in vivo* data that suggests PcG binding leads to transcriptional silencing by higher-order chromatin compaction. In *D. melanogaster* elements have been identified in regulatory regions of PcG target genes (*i.a.* HOX genes) that are necessary for PcG-mediated silencing, termed Polycomb response elements or PREs. PREs are cis-acting elements typically several hundred base pairs in size. Although there is apparent variation between PREs in sequence, size, or distance from possible PcG-target genes, they share

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the functional characteristic that they confer PcG-responsiveness on transgenes⁶². Multiple studies have concentrated on the role of PREs in PcG-mediated silencing. Strangely, PREs appear to be nearly void of nucleosomes and hence of PcG-mediated trimethyl marks^{62, 63}. Recent insights may, however, have uncovered a mechanistic role for PREs in PcG-dependent silencing in the fly: the PhoRC complex is recruited to PREs, via DNA consensus recognition; Pho biochemically interacts with PRC2 and PRC1⁶⁴. The PRC2 complex mediates trimethylation of H3K27 and H3K9 in upstream regulatory regions, the promoter region and in the coding region of target genes. This in turn would recruit PRC1 complexes via chromodomain binding; via recruitment of yet additional HMTase activity, PcG-dependent silencing would spread over larger chromatin areas⁶³⁻⁶⁶. The Pho-PRC2 interaction at PREs may provide PREs with the capacity to function as cis- or trans-acting repressive nucleation sites for adjacent or remote

Box 2.2. Polycomb Group genes and functions

PcG complex	<i>Drosophila</i>	Human	Protein domains	Biochemical activity
PRC2	E(z)	EZH1 EZH2	SET	Histone methyl transferase for H3K27
	Esc	EED	WD40	Cofactor for E(z); methyl-lysine binding motif?
	Su(z)12	SUZ12	Zinc-finger	
PRC1	Pc	CBX2/HPC1 CBX4/HPC2 CBX6 CBX7 CBX8/HPC3	Chromodomain	HPc2: binding to H3K27me3, RNA binding, Sumo-E3 ligase
	Ph	HPH1/EDR1/ PHC1 HPH2/EDR2/ PHC2 HPH3/EDR3/ PHC3	SAM	Currently unknown
	Sce/dRing	RING1 /RNF1/RING1A RNF2 /RING1B/RING2	RING-finger	E3 Ubiquitin ligase for H2AK119
	Psc	BMI1 /PCGF4 /RNF51 PCGF2 /RNF110/MEL18	RING-finger	Cofactor for RING proteins
	PhoRC	YY1	Zinc-finger	Sequence specific DNA binding

Due to the large number of PcG orthologues, this table is limited to core complex members. Gene names according to HUGO gene nomenclature are shown in bold. BMI, B lymphoma mo-MLV insertion region; CBX, chromobox homologue; EDR, early development regulator; EED, embryonic ectoderm development; ESC, extra sex combs; E(z), enhancer of zeste; HPC, homolog of polycomb; HPH, homolog of polyhomeotic; Pc, polycomb; PCGF, polycomb group ring finger; Ph, polyhomeotic; PHC, polyhomeotic-like; Pho, pleiohomeotic; Psc, posterior sex combs; RING, really interesting new gene; RNF, ring finger protein; SAM, self association motif; Sce, sex combs extra; Su(z), suppressor of zeste; YY, yin-yang transcription factor.

Box 2.2. Continued

promoters. This mode of action would show resemblance to Sir protein-mediated telomere-proximity silencing in yeast^{63, 67, 68}. Paradoxically, many active promoters in *Drosophila* appear “preoccupied” with Pc, as if on standby, waiting on other local epigenetic events that signal (progressive) silencing; indeed many *Drosophila* PRE and promoters simultaneously bind PcG and trxG proteins, in support of this “bivalent standby” notion^{66, 69}. Indeed the presence of the trxG protein Ash1 within the promoter and coding regions of a transcriptionally active Ubx locus (one of *Drosophila*’s HOM-C clusters) correlates with loss of H3K27, H3K9, and H4K20 methylation marks and suggest a model in which recruitment of trxG proteins prevents silencing⁶⁶. To date, no mammalian PREs have been identified. However, recent studies have revealed highly conserved noncoding elements (HCNE) in higher eukaryotes, which display specific PcG protein association and may orchestrate PcG-dependence in regulation of developmental programs⁷⁰. The epigenetic indexing on and around these HCNEs is peculiar in that it contains both activating and inactivating histone modifications (e.g., H3K4me3/H3K27me3), and hence represent “bivalently” marked nuclear elements (see below). These findings are

supported by reports that H3K27me3-marking does not necessarily correlate with transcriptional silencing but rather precedes silencing^{71, 72}. A somewhat antiquated model suggested PcG-mediated silencing involved sterical blockage of DNA-access for transcription factors; recent insights, however, have superseded this view and suggest a role in interference with events that either control pre-initiation complex formation and transcription initiation, despite transcription machinery recruitment and elongation processes^{62, 66, 73}. Recent technological advances have enabled us to directly examine PcG and trxG occupation around and within genes and interrogate epigenetic marking and promoter occupation, PcG complex composition and other relevant aspects of chromatin mediated silencing. Although PcG-mediated transcriptional silencing already appears a much more complex process than anticipated, ongoing studies will help refine positioning of PcG function in the proper cellular context.

PcG in stem cells and differentiation

Stem cells have two key properties: the ability to self-renew and the ability to differentiate along any given differentiation program. Through asymmetric division, a dividing stem cell will give rise to one new stem cell and one cell that will differentiate into a mature cell of a certain lineage. Two main categories of mammalian stem cells are ES cells and adult stem cells. ES cells are pluripotent, which refers to their capacity to differentiate into any cell type of the adult organism. Adult stem cells, such as hematopoietic stem cells, in contrast, are restricted in their differentiation range. PcG proteins are involved in the maintenance of embryonic and adult stem cells^{18, 74}. In adult stem cells PcG proteins regulate self-renewal of hematopoietic and neuronal stem cells. BMI1, RAE28/MPH1, and EZH2 all have a role in murine hematopoietic stem cell (HSC) self-renewal. HSCs have the capacity to regenerate fully functional bone marrow, as illustrated by serial transplantation experiments with limited numbers of cells in mice. Both BMI1 and RAE28/MPH1 deficient HSCs are defective in their proliferative and self-renewal capacity, whereas EZH2 overexpression in HSCs prevents hematopoietic stem cell exhaustion⁷⁵⁻⁷⁹. BMI1 is also required for the self-renewal of stem cells in the peripheral and central nervous systems⁸⁰. The dependence of self-renewal of these stem cells on BMI1 is believed to contribute to the murine BMI1^{-/-} phenotype; besides skeletal transformations, mice show a gradual depletion of progenitor and mature cells in haematopoietic and neural lineages⁸¹. The observation that EZH2 deficient ES cells cannot be established *in vitro*, indicates involvement of PcG in ES cell biology⁸². Functional PcG gene ablation studies in mice show that EZH2, EED, SUZ12, and RNF2 are essential for early embryonic development⁸²⁻⁸⁵. Even though both EED^{-/-} and SUZ12^{-/-} ES cells can be established in tissue culture, they display aberrant activation of differentiation specific genes^{86, 87}. For a long time we have known

that PcG proteins repress Hox clusters. Due to their involvement in diverse biological processes, it was expected that PcG proteins bind to and repress many other genes in addition to HOX genes. A number of studies have recently identified genome-wide binding patterns for PcG proteins in human, mouse, and *Drosophila* cells^{70, 87-92}. Indeed, in all organisms PcG proteins target a large number of loci. Most PcG target genes, as studied in human embryonic fibroblasts and both human and mouse ES cells, constitute developmental regulators⁸⁷⁻⁸⁹. Among the PcG target genes transcription factors and components of signaling pathways are highly represented. Transcription factor PcG target genes include homeodomain genes of the *Dlx*, *Irx*, *Lhx*, and *Pax* gene families, and *FOX*, *SOX*, and *TBX* gene families, which all control differentiation. Signaling pathways, identified as PcG-regulated, include Wnt, Hedgehog, Notch, TGF- β , FGF, and BMP signaling. Differentiation activates some of these signaling pathways, which corresponds to decreased H3K27me3 and PcG binding at the PcG target genes that regulate the specific developmental process^{70, 87-89, 93}. Recent studies have focused on identifying molecular signatures for pluripotency⁹⁴. In ES cells many promoters of developmental regulators are occupied by the transcription factors OCT4, SOX2, and NANOG, all key factors in maintaining ES cell pluripotency⁹⁵. Interestingly, almost all repressed developmental regulators that are occupied by OCT4, SOX2, and NANOG are co-occupied by PcG proteins⁸⁹. Many nontranscribed genes in ES cells have a bivalent chromatin state, harboring both H3K4me3 (catalyzed by trxG proteins) and H3K27me3 epigenetic marks (a PRC2-installed mark), normally associated with gene transcription and gene repression, respectively. Many of these bivalently marked regulatory regions occur in CpG-rich promoters, which in turn are often found in genes expressed during embryogenesis and in “housekeeping” genes^{70, 71, 93}. Housekeeping genes are, however, mostly marked with H3K4me3 only (*i.e.*, void of PRC-association) and their transcriptional status is mostly “on.” Bivalent marking is thought to keep developmental control genes (*e.g.*, key developmental factors, morphogens, surface molecules, microRNAs) transcriptionally silent in ES cells, while preserving their potential to become activated upon lineage commitment and during differentiation^{70, 71, 93}. So PcG proteins occupy a set of developmental genes in ES cells that need to be repressed to maintain pluripotency and that are poised for activation during ES cell differentiation. A model of PcG protein functioning during differentiation, which is gaining acceptance and support by empirical observation, is that in undifferentiated cells PcG proteins activate and maintain gene repression by instating and binding to H3K27me3 marks. Upon lineage commitment or during differentiation, PcG proteins are displaced from critical developmental control genes by as yet unidentified mechanisms. These genes will now become actively transcribed; corresponding regulatory regions

will lose their bivalent marking; lineage “nonrelevant” developmental genes will lose their activating marks (*i.e.*, H3K4me3) and will become truly PcG-repressed targets; a third class of high CpG-content promoters loses both me3 marks; these will eventually be shut-down transcriptionally. Despite this emerging modus operandi many questions remain. Several studies have reported that transcriptional activity is lost upon loss of Polycomb function or that transcription occurs, despite PRC-occupance, suggesting involvement of other spatio-temporal factors or aspects of chromatin status^{71, 96-98}. In addition, some PcG proteins may harbor as of yet unknown biochemical activity which may not be directly linked to epigenetic indexing of genes; in breast cancer cells, Ezh2 appears to transactivate gene expression in estrogen and Wnt signaling pathways in a SET domain independent manner⁹⁹. Combined, these findings clearly stress that PcG-mediated repression is controlled by mechanisms currently far from completely understood. Delineation of chromatin contexts, identification of interaction partners, and associated enzymatic activity in a cell context-dependent manner will undoubtedly help further our understanding of the molecular nature of PcG-mediated processes.

PcG regulation

Signaling molecules in cancer pathways often comprise both oncogenic as well as tumor suppressive activity. Knowing the biochemical relationships between signaling proteins and their regulation is crucial for our understanding of how mutations in these gene products contribute to cancer and how oncogenesis signaling may be averted. Regulation of PcG function is currently poorly understood. Several levels of regulation may be anticipated: transcriptional, posttranscriptional, translational and posttranslational. Very little is known about transcriptional regulation of PcG genes themselves. A number of reports address possible pathways and transcription factors that control expression of specific PcG genes^{98, 100-102}. Some of these proteins are known to be involved in cancer as well and may therefore be involved in exposing tumorigenic properties of PcG proteins. Activated JNK signaling in *Drosophila* during trans-determination of imaginal discs (larval structures that form extremities) leads to downregulation of PcG genes¹⁰³. Studies in the flies and mammalian systems also indicate that expression of PcG genes is dependent on PcG proteins^{88, 104-106}. Changes in cellular expression levels may drive differential PcG complex composition, consequently overexpression of particular PcG proteins in cancer may contribute to tumorigenesis. At the posttranscriptional level, *i.e.*, mRNA processing, stability or translation, virtually nothing is known about PcG mRNA regulation. Interestingly though, genetic ablation of Sf3b1, a factor involved in mRNA splicing, evokes a Polycomb phenotype in mice, suggesting a connection with either translation of PcG proteins or their targets⁹. Also TrxG members

have been associated with RNA processing: the association of the SWI/SNF-protein Brm with snRNAs and spliceosome factors and the observation that loss of Brm function affects mRNA elongation and inclusion of variant exons, suggests a direct role in RNA-splicing and possibly points toward a more fundamental role of PcG and trxG proteins in chromatin structure dependent processes¹⁰⁷. Regulation of PcG function at the posttranslational level is slowly emerging. As discussed in the preceding paragraphs, PcG complex composition varies between cell types and over time (hematopoiesis). A little over a decade ago, large, functionally distinct complexes, like PcG or trxG complexes, which shared conserved interaction modules and other domains, were tentatively viewed as large bulky multiprotein masses that modified chromatin structure at least in part by binding and physically interfering with accessibility. As we begin to uncover and understand more about the properties of individual PcG proteins, it is now clear that many PcG proteins have specific catalytic or binding activities, relevant to epigenetic regulation. Studies on their posttranslational regulation are slowly emerging, but our knowledge on PcG-regulation is of yet far from complete. Several PcG proteins are regulated by posttranslational modification. Self-ubiquitylation of RNF2 is required to monoubiquitylate H2A at lysine 119, which in turn is required for PRC1 repression^{39, 108}. RNF2 and BMI1 are both ubiquitylated by an as of yet unknown E3 ligase; this results in their proteolytic breakdown¹⁰⁸. As RNF2 and BMI1 are often found overexpressed in cancers, abnormal ubiquitylation is likely to contribute to tumorigenic processes. EZH2 is phosphorylated by AKT, which controls survival signaling in cells¹⁰⁹. Although EZH2 overexpression marks malignant progression in some tumors, paradoxically, the tumorigenic action of EZH2 may not solely depend on its chromatin repressive properties, as phosphorylation by AKT inhibits its HMT-activity by impeding H3 binding¹⁰⁹. Two recent mass spectrometric studies identified kinase activity associated with PRC1 complex members CBX8 and RNF2^{110, 111}. In addition, a recent study identified the PRC1 complex as targets of canonical MAPK signaling pathways, and identified MAPKAPKs as interaction partners of the PcG complexes (¹¹², see Chapter 4 of this thesis). Relevantly, phosphorylation of PRC1 correlates with chromatin dissociation, and activation of repressed tumor suppressors¹¹². Although the above observations suggest a link between tumorigenic pathways and regulation of PcG function, clearly much work remains to fully understand the significance of these connections with cancer, and whether or not these regulatory processes provide entries for therapeutic applications.

Cross-talk between PcG and other epigenetic mechanisms

Several levels of epigenetic regulation exist. At its most fundamental level, epigenetic regulation of gene expression occurs at the level of DNA (*e.g.*, DNA methylation), RNA (*e.g.*, RNA interference) and at the protein level (*e.g.*, histone modifications; chromatin structure; Box 2.1). Recent epigenetic studies suggest cross-talk between these processes. PcG function most directly impinges on epigenetic regulation at the protein level. However, several reports indicate that epigenetic mechanisms involving DNA and RNA may intersect with PcG-mediated epigenetics. In *Arabidopsis* and *C. elegans* RNA interference is defective in a PcG mutant background¹¹³⁻¹¹⁵. In *Drosophila* PcG-chromatin association and silencing is dependent on components of the RNAi machinery¹¹⁶. Chromodomains, which are also used by PcG CBX proteins (Box 2.2), are RNA binding moieties; chromatin association of HP1, a structural relative (chromodomain protein which binds H3K9me3) of the PcG CBX proteins, is dependent on RNA components¹¹⁷⁻¹¹⁹. X-inactivation is dependent on coating of the inactive X chromosome with a noncoding RNA, Xist, which is at least in part responsible for recruitment of PRC1⁴⁶. At the DNA-methylation level, several observations suggest a direct link with PcG-mediated silencing: EZH2 binds directly to DNMTs (de novo DNA methyl transferases) and DNA methylation at EZH2 target genes is dependent on EZH2⁵⁰. Notably, PcG target genes are up to 12 times more likely to have cancer-specific promoter DNA hypermethylation than nontargets^{120, 121}. RNA, DNA, and protein-mediated epigenetic repressive mechanisms have all been implicated in cancer; therefore these biochemical connections are likely to become increasingly relevant for our understanding of tumorigenic processes and treatment options.

PcG in cancer

Cancer is also an epigenetic disease. It is well established that tumorigenesis is accompanied by global hypomethylation of DNA at CpG dinucleotide sequences, and local hypermethylation at CpG-islands. Loss or mutation of tumor suppressor genes is one of the hallmarks of cancer; tumor suppressors are also targets of aberrant DNA methylation in cancers^{1, 2, 122}. The first indication that PcG proteins are involved in tumorigenesis came from studies that searched for oncogenes that cooperated with c-Myc in lymphomagenesis. In this way the *BMI1* gene (B lymphoma Mo-MLV Insertion region 1) was identified as an oncogene^{12, 13}. Subsequently, the murine knock-out model revealed an inverse correlation between *BMI1* expression and *INK4A/ARF* expression¹²³. *INK4A/ARF* encodes two cell cycle inhibitory proteins, P16^{INK4A} and P14^{ARF} (p19^{Arf} in mice)¹²³, that regulate the PRb and TP53 pathways, respectively (Box 2.3)¹²⁴. P16^{INK4A} is a cyclin-dependent kinase inhibitor (CKI), which inhibits cyclinD-CDK4/6-mediated

phosphorylation of PRb, whereas P14^{ARF} inhibits interaction between MDM2 and TP53, thereby stabilizing TP53. BMI1's ability to downregulate *INK4A/ARF* expression has obvious etiological consequences for tumorigenesis. Indeed this property provided an explanation for its cooperative oncogenic action with c-Myc, as c-Myc-induced P14^{ARF}-dependent apoptosis was suppressed by BMI1, effectively favoring proliferation¹²⁵. Although it is believed that overexpression of BMI1 may be functionally equivalent to loss of PRb and TP53, and therefore reflects distinctive tumor etiology, recent reports on simultaneous overexpression of P16INK4A as well as BMI1 in some tumors clearly suggests other relevant players^{126, 127}. Recent studies revealed an antagonistic action of MEL18 and BMI1 on AKT/PKB-activation, a known tumor promoting signal transduction molecule, suggesting multiple genetic interactions of PRC function and the PI3KAKT/mTOR pathway^{109, 128}. The intuitive notion that there must be many more oncogenic PcG targets is further supported by the recent identification of several thousand Polycomb targets in human fibroblasts⁸⁸.

Box 2.3. Basic mechanisms in cancer

Cumulative scientific evidence over the last decades demonstrates that multiple steps are required for a normal cell, with a limited proliferative life span, to by-pass this limitation and eventually become a fully transformed cancerous cell. Proliferative longevity and controlled proliferation of human cells are kept in check by multiple so-called checkpoints. These checkpoints evaluate and integrate input from a myriad of pathways, which collectively provide crucial information on the cell and its relationship with its environment. This input includes replicative age of a cell, the presence of DNA damage, availability of nutrients, metabolic status, environmental cues important for cell cycle entry, arrest or exit, such as growth factor signaling-input, cell-cell, or cell-matrix contacts, etc. Checkpoints provide failsafe mechanisms to protect a cell from genome integrity breaches and unwarranted, potentially malignant proliferation. Inevitably, the outcome of checkpoint activation is cell cycle arrest/quiescence, senescence, or apoptosis. As a cancer becomes increasingly malignant, many of these checkpoints appear to have been compromised. Weinberg's Hallmarks of cancer describe a biologically defined set of common tumorigenic events which ultimately occur in most if not all cancers¹. Human cells need to lose at least three checkpoints to gain proliferative immortality and require a fourth oncogenic event (*e.g.*, activation of H-RAS) to become fully transformed. A common property of many cancer cells is enhanced telomerase (hTERT) activity, which protects telomere repeats at the ends of chromosomes from further "erosion." Telomere length effectively mirrors replicative age and telomere erosion eventually leads to replicative senescence¹²⁹. Senescence bypass is an important mechanism in the onset and development of cancer. Two tumor-suppressor pathways are pivotal in establishing arrest/quiescence, senescence, or apoptosis in response to many potentially dangerous situations (including non-telomeric signals) when left unchecked. These are the PRb and the TP53 pathways, which control cell cycle entry and progression. Loss or inactivation of PRb and TP53, or mutation of effector or effector molecules in the PRb and TP53 pathways are common to most cancers. These same antitumor pathways, which control senescence in normal cells, also limit the life span of stem cells. Increasing scientific evidence supports the presence of cancer stem cells (CSC) in many different tumors. These CSC are held responsible for tumor maintenance,

Box 2.3. Continued

growth, and ultimately malignant progression, and because of particular properties inherent to stem cells, are most often difficult to treat with current anticancer therapy. The prototype mammalian PcG-protein BMI1 impinges on several antitumor checkpoints described above, but likely does so in a cell-type dependent fashion (see main text).

Simplified representation of check-points in cell cycle regulation and cancer; possible role for Polycomb Group protein Bmi1 in these checkpoints

BMI1 is not the only PcG protein that has been linked to tumorigenesis. Since EZH2 was recognized as a marker for the progression of prostate cancer, it has been linked to a variety of other cancers, such as breast cancer and several lymphomas¹³⁰⁻¹³⁵ (see Table 2.1). Although BMI1 and EZH2 have received most attention, a general notion of the tumorigenic potential of other PcG proteins is slowly emerging. The use of tissue microarrays to study PcG expression in normal and tumor tissues will greatly enhance our understanding of PcG in cancer¹³⁶. This particular study analyzed PcG-expression immuno-histologically in different normal and tumor tissues; several tumors show very distinctive and sometimes unique PcG expression profiles, pointing to specific etiology. To fully understand the role of PcG in tumorigenesis, it will be crucial to chart all PcG proteins implicated in tumor development, and thoroughly examine their mode of action, and their relative contribution to different types of cancer in years to come. Not only do PcG proteins appear overexpressed in various cancer types

Table 2.1 Deregulated PcG Expression in Human Cancers

PcG complex	PcG Protein	Cancer type	References
PRC2	EZH2	B-cell non-Hodgkin lymphoma	137
		Bladder	138-140
		Breast	130-133
		Colon	141
		Endometrium	130
		Hodgkin lymphoma	134
		Liver	142
		Mantle cell lymphoma	135
		Melanoma	130
		Prostate	130, 143-146
		Stomach	147, 148
	SUZ12	Breast	149
		Colon	149
		Liver	149
PRC1	CBX7	Follicular lymphoma	150
	RING1	Prostate	146
	RNF2	Colon	136
		Diffuse large B cell lymphoma	136
		Hodgkin lymphoma	136
		Stomach	136
	BMI1	B-cell non-Hodgkin lymphoma	137
		Breast	151
		Colon	152
		Diffuse large B cell lymphoma	136, 153
		Hodgkin lymphoma	134, 136, 154
		Leukaemia	155
		Mantle cell lymphoma	136, 156
		Medulloblastoma	102
		Neuroblastoma	157
		Non-small cell lung cancer	158
		Oral squamous cell carcinoma	159
		Parathyroid adenoma	136
		Pituitary adenoma	136
		Prostate	144, 146
	PHC1	Acute lymphoblastic leukaemia	160

but also expression is often correlated with specific aspects of tumor biology: a more progressive disease state, metastasis, and ultimately poor prognosis. In prostate cancer, EZH2 staining intensity increased from benign, prostatic atrophy, prostatic intraepithelial neoplasia, clinically localized prostate cancer to

metastatic prostate cancer¹⁴³. In this study, EZH2 expression was a better predictor of clinical outcome than surgical margin status, maximum tumor dimension, Gleason score, and preoperative PSA¹⁴³. Indeed, malignant tumor progression could be mimicked *in vitro* by overexpression of EZH2. Thus EZH2 protein expression in prostate specimens may be a valuable prognostic marker. EZH2 overexpression is associated with a poor prognosis in prostate, breast, gastric, and colon cancers as is BMI1 overexpression in different leukaemia's^{132, 143, 147, 153, 161}. Moreover, EZH2 was even upregulated in preneoplastic lesions in breast tissue¹⁶². In this respect EZH2 may help identify patients at risk for developing breast cancer¹⁶³. Recently, microarray-based gene expression profiling was used to identify a death-from-cancer signature, which is partly based on BMI1- regulated genes and consists of 11 genes¹⁶⁴. A stem cell-like expression profile of this signature in primary tumors was a powerful predictor of a short interval to disease recurrence, distant metastasis, and death after therapy in 11 different cancer types¹⁶⁴. Interestingly, EZH2 also embodies a direct bridge between epigenetic mechanisms at the protein level (histone-tail modifications) and the DNA level; recent reports provided experimental evidence for a biochemical and functional link between PcG-mediated silencing and DNA methylation at tumor suppressor genes, persuasively arguing that the PRC2 complex may contribute to carcinogenesis through disabling check-points by silencing tumor suppressor genes^{50, 165}. Although sparse information is available on overexpression of YY1 in human cancers, its positive association with DNA-repair and inverse correlation with cellular TP53 homeostasis suggests a potential oncogenic role for this PcG and trxG associated factor^{166, 167}. These observations together imply that screening for altered PcG expression in certain cancer types may in the near future enable physicians to more accurately predict prognosis and personalize patient treatment strategies.

PcG and senescence

Cellular senescence, an irreversible proliferative arrest, can be induced *in vitro* by diverse stresses, such as telomere shortening, DNA damage, and strong mitogenic signaling by activated oncogenes^{168, 169}. As cellular senescence limits the proliferative capacity of cells, bypass of senescence is an important step in tumorigenesis (Box 2.3). Senescence was recently shown to occur *in vivo* in premalignant or benign conditions¹⁷⁰. Although diverse stimuli induce senescence, its regulation converges on the TP53 and PRb pathways. Recent studies advanced our knowledge of how PcG proteins regulate expression of the *INK4A/ARF* locus and senescence. Until recently, mechanistic insights in repression of the *INK4A/ARF* locus by PcG proteins as BMI1 and CBX7 was inferred from inverse correlation between PcG and *INK4A/ARF* expression^{123, 171}. However, chromatin immunoprecipitation (ChIP) experiments identified both

PRC2 members, SUZ12 and EZH2, and PRC1 members, BMI1, CBX4, CBX7, CBX8, and RNF2 as direct interactors of the *INK4A/ARF* locus^{110, 172, 173}. In senescing fibroblasts EZH2 expression was downregulated, which coincided with decreased H3K27me3 levels, BMI1 displacement and activation of P16^{INK4A} transcription¹⁷². Thus, PcG binding to *INK4A/ARF* regulates senescence. Overexpression of the PcG proteins BMI1, CBX7, and CBX8 can overcome senescence^{110, 123, 171}. CBX7 can promote proliferation in the absence of BMI1, whereas the function of CBX8 is dependent on BMI1, indicating that CBX7 might be part of PRC1 complexes that do not include BMI1^{110, 171}. BMI1 is also implicated in a different mechanism of bypassing senescence. In human mammary epithelial cells (HMECs) overexpression of BMI1 led to activation of human telomerase reverse transcriptase (hTERT) transcription and induction of telomerase activity¹⁷⁴. For now this has only been detected in HMECs, and does not appear to contribute to immortalization in human fibroblasts or in normal human oral keratinocytes^{175, 176}, and even so, telomere shortening does occur in BMI1-immortalized HMEC¹⁷⁴. Clearly, PcG proteins regulate senescence via *INK4A/ARF*-dependent and independent mechanisms and they do so in a cell type-dependent fashion, presumably reflecting cell type-specific molecular networks and underlying epigenetic profiles and chromatin status. As PcG proteins are upregulated in many different cancers, it is tempting to speculate that PcG-mediated senescence bypass represents an early event in tumorigenesis.

PcG and cancer stem cells

Stem cells have also been detected in cancers, where they can give rise to more cancer stem cells (CSCs) and also nontumorigenic cancer cells¹⁷⁷. This explains at least in part the heterogeneity often observed in tumors. The identification of cancer stem cells has direct implications for cancer treatment. As CSCs are emerging as the main driving tumorigenic entities, to treat cancer effectively, preferably CSCs should be targeted. Hence, it is crucial to understand the processes that define stem cellness, and the pathways or proteins that are deregulated in CSCs. PcG proteins are also implicated in the self-renewal of CSCs. In a recent study, BMI1^{-/-} bone marrow cells were transduced to develop acute myeloid leukemia (AML). Importantly, primary recipients developed AML, whereas BMI1^{-/-} AML, in contrast to wild-type derived AML, failed to repopulate secondary recipients⁷⁷. This establishes BMI1 as an important regulator of the proliferative activity of not only normal but also leukemic HSCs. As overexpression of BMI1 is often seen in leukemias, a comprehensive understanding of the regulation of stem cell biology by PcG proteins is a very important aspect of hematologic oncology.

Perspectives

Recent research position PcG proteins as master regulators of development. Their suppressive action is necessary for proper embryonic development, cell proliferation, and stem cell biology. The initial view that PcG proteins act as stable repressors throughout cell division and differentiation has been adapted to a more dynamic regulation of PcG binding and repression. As with all proteins that regulate normal proliferation, deregulated PcG protein expression levels have been detected in cancer. Putative mechanisms of how PcG proteins contribute to tumorigenesis are bypass of senescence and maintenance of CSCs. Many questions remain. How do PcG proteins exactly function in tumorigenesis? How are PcG binding patterns changed upon differentiation? Which enzymatic activities are associated with PcG complexes and which unknown enzymatic activities do they harbor themselves? How do posttranslational modifications alter PcG function? Answers to these questions will determine whether PcG proteins can become suitable targets for therapeutic intervention. In this respect it is promising that *e.g.*, loss of BMI1 seems to promote cancer specific cell death¹⁷⁸. For now, the use of PcG expression as a biomarker for tumor progression and patient prognosis is a far more attainable concept. First these concepts have to be validated and detection methods require standardization before it can be translated to clinical application. Considering the speed at which the PcG research field is developing, it can be anticipated that many novel aspects of PcG function and regulation will be elucidated in the next few years.

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**Talking to chromatin:
post-translational modulation of
Polycomb Group function**

Based on: Niessen HE, Demmers JA, Voncken JW.
Epigenetics Chromatin. 2009;2(1):10.

Abstract

Polycomb Group proteins are important epigenetic regulators of gene expression. Epigenetic control by Polycomb Group proteins involves intrinsic as well as associated enzymatic activities. Polycomb target genes change with cellular context, among which lineage commitment and differentiation status, revealing dynamic regulation of Polycomb function. It is currently unclear how this dynamic modulation is controlled and how signaling affects Polycomb-mediated epigenetic processes at the molecular level. Experimental evidence on regulation of Polycomb function by post-translational mechanisms is steadily emerging: Polycomb Group proteins are targeted for ubiquitylation, sumoylation and phosphorylation. In addition, specific Polycomb Group proteins modify other (chromatin-) associated proteins via similar post-translational modifications. Such modifications affect protein function by affecting protein stability, protein-protein interactions and enzymatic activities. We here review current insights in covalent modification of Polycomb Group proteins in the context of protein function and present a tentative view of integrated signaling to chromatin in the context of phosphorylation. Clearly, available literature reveals the tip of the iceberg, and exact molecular mechanisms in, and biological relevance of post-translational regulation of Polycomb function await further elucidation. Our understanding of causes and consequences of post-translational modification of Polycomb proteins will gain significantly from *in vivo* validation experiments. Impaired Polycomb function has important repercussions for stem cell function, development and disease. Ultimately, increased understanding of signaling to chromatin and mechanisms involved in epigenetic remodeling will contribute to development of therapeutic intervention in cell fate decisions in development and disease.

Introduction

Polycomb Group (PcG) proteins preserve transcriptionally silenced states through epigenetic marking of target genes in higher eukaryotes. Currently at least two biochemically and functionally distinct Polycomb Repressive Complexes (PRC) are recognized, PRC2 and PRC1, which contribute to establishment and maintenance of gene repression profiles (Fig. 3.1A)¹⁻³. As such PcG function equips the cell with a transcriptional memory throughout development and differentiation. It is becoming increasingly clear that PcG-chromatin association is subject to dynamic regulation; PcG complex composition and chromatin association change throughout eukaryotic development^{4, 5}. In a constantly changing environment (for example during differentiation) cells respond to a plethora of extracellular and intrinsic cues. Mechanistic insight in epigenetic regulation in response to signaling is essential to understand how cell fate, function and physiology are controlled. It is, however, still largely unknown how cells “talk” to chromatin to facilitate appropriate cellular responses. Physiological adaptation of cells is initially mediated by mostly transient and reversible covalent post-translational modifications (PTMs) at specific amino acid residues including ubiquitylation, sumoylation, and phosphorylation (see Box 3.1). Through altered protein-protein interaction, subcellular localization, enzyme activity and protein stability, PTMs ultimately also affect gene expression. We review current established PTMs on PcG proteins and the effect they have on interaction and enzymatic activity in the paragraphs below. In addition, we extracted PcG specific PTM from published analyses and used these to predict upstream kinase pathways signaling to Polycomb. The final section presents a tentative integrated view on signaling to chromatin in the context of PcG PTM.

Polycomb-mediated Ubiquitylation

Ubiquitylation plays a central role in PRC-mediated silencing. Histone 2A (H2A) is one of the most abundant ubiquitylated nuclear proteins, and H2AK119ub1 is required for PcG-mediated gene repression⁶. Published data shows that the PRC1 protein RNF2 (RING1B) ubiquitylates H2A, as loss of RNF2 dramatically decreases global H2Aub levels and derepresses PcG-controlled genes. The RING1 (RING1A) RING domain substitute for that of RNF2 *in vitro*⁷. Consistently, H2AK119ub1 is maintained in RING1 or RNF2 single null cells, but not in double knockout cells, supporting functionally redundant roles for these proteins in certain biological contexts⁸. RNF2-mediated H2A ubiquitylation is important in X chromosome inactivation^{8, 9}. The exact role of RNF2 and H2AK119Ub1 in X-inactivation is currently under debate, however, as recent studies suggest that H2AK119ub1 may not be sufficient for X-inactivation and conversely, Xist-

Box 3.1. Most common PTM in Polycomb biology

Ubiquitylation.

Besides in protein degradation ubiquitylation fulfills many non-proteolytic roles, such as in regulating DNA repair, transcription and signal transduction⁷⁴. Ubiquitin (Ub) is a small ubiquitous 76 amino acid polypeptide which is covalently bound as a monomer (monoubiquitin) to proteins or as a Ub-polymer (polyubiquitin). In polyubiquitin, chains are linked by conjugation to one of seven lysines (Lys, K) of a pre-existing ubiquityl-moiety, *e.g.* Lys48 or Lys63. Polyubiquitylation via Lys48 linkage is mostly implicated in 26S proteasome-mediated protein degradation. Covalent linkage of ubiquitin to a substrate requires sequential action of activating (E1), conjugating (E2) and ligating (E3) enzymes⁷⁵: E1 activates ubiquitin in an ATP-dependent manner, which is then conjugated to the E2, that, assisted by an E3 ligase, transfers ubiquitin to a lysine residue onto the substrate protein⁷⁴. The diversity and combination of these enzymes ultimately determines substrate specificity and biological responses. Two classes of E3 ligases are recognized: the HECT domain E3s and the RING-domain E3s; RING-type E3 ligases are thought to function as bridging factors between the E2 and substrate⁷⁵. Ub-protein substrates are de-ubiquitylated by DUBs (deubiquitylases). The large number of different E2 and E3 enzymes and DUBs identified to date and their involvement in a great variety of processes underscores the complexity of regulation by ubiquitylation⁷⁵.

Sumoylation.

Small ubiquitin-related modifier (SUMO) proteins are approximately 10 kDa in size⁷⁶. Like ubiquitylation, sumoylation involves three distinct enzymatic activities: an E1 activating enzyme (AOS1/UBA2 heterodimer) an E2 conjugating enzyme (UBC9) and an E3 ligase. Currently three classes of SUMO E3 ligases are distinguished: SP-RING motif E3 ligases, such as PIAS (protein inhibitor of activated STAT), a second class containing RanBP2, and a third class with no apparent homology to either other class comprising CBX4 (HPC2). In contrast to ubiquitylation, sumoylation takes place at SUMO-acceptor sites: ΨKXE (Ψ and X represent hydrophobic and any amino acids).

Phosphorylation.

Phosphorylation is probably the most widespread and best studied PTM in cellular signaling. Protein kinases catalyze the transfer of γ-phosphate from adenosine triphosphate (ATP) to a substrate protein, thereby generating ADP⁷⁷. Phosphorylation is reversible: phosphatases remove the attached phosphate moiety. An estimated ~ 30% of all proteins are phosphorylated on at least one residue. Phosphate is most commonly linked to Ser (S), Thr (T) or Tyr (Y)), but in addition occurs on His (H), Lys (K), Arg (R), Asp/Glu (D/E), and Cys (C)⁶⁶.

initiated silencing occurs in the absence of RNF2 and H2AK119ub1^{10, 11}. Also histone variant H2A.Z may be a target for RNF2-mediated ubiquitylation, as knockdown of RNF2 reduces H2Aub and H2A.Zub levels *in vitro*¹².

Whereas in *Drosophila melanogaster* multiple E3-ligase complexes contribute to H2A ubiquitylation, the PRC1 protein RNF2 is currently considered the major E3 ligase for H2AK119 (Fig. 3.1B)^{13, 14}. Although within the PcG core complex, containing RING1, RNF2, BMI1 and PHC2, most *in vitro* H2A-E3 ligase activity is attributed to RNF2⁸, other PcG RING-type E3 ligases control H2A-directed

Figure 3.1. Examples of post-translational modulation of Polycomb Group and associated proteins.

(A) Simplified model of PcG-mediated repression. The histone methyltransferase EZH2 trimethylates histone H3 at lysine 27, this mark is recognized by CBX proteins via the chromodomain. RNF2/RING1 homologs are E3 ubiquitin ligases for H2A; RYBP binds H2AK119Ub1. Combined these activities induce/maintain transcriptional repression. Gray boxes depict PRC-associated, epigenetically relevant enzymatic activities. (B) RNF2 E3-ligase activity is significantly enhanced in the presence of BMI1, or of phosphorylated PCGF2. (C) DNA damage-induced phosphorylation of HIPK2 leads to phosphorylation of CBX4. Phosphorylation of CBX4 at T495 in turn enhances the HIPK2 sumoylation. (D) AKT-induced phosphorylation of EZH2 on S21 impairs its binding to histone H3, thereby inhibiting H3K27 trimethylation. ph: phosphorylation, su: sumoylation; ub: ubiquitylation, me: methylation.

ubiquitylation and affect *HOX* gene silencing: BMI1 (PCGF4) homologues PCGF2 (MEL18) and PCGF1 (NSPc1) enhance H2A ubiquitin E3 ligase activity when complexed to RNF2 (Fig. 3.1B)¹⁵⁻¹⁸. In addition, PcG ubiquitin E3 ligase activity is enhanced within the molecular context of an intact PRC1 complex: fully reconstituted complexes containing RNF2, RING1, BMI1 and CBX8 show highest activity compared to RNF2 alone or subcomplete PRC1 complexes¹⁵. Crystal structure analyses of interacting RING domains of mammalian BMI1 and RNF2 reveals extensive contacts between the RNF2 and BMI1 RING domains: the N-terminal 'arm' of RNF2 embraces the BMI1 RING domain^{7, 19}. RING-protein partnerships occur frequently in cell biology²⁰; RING domain proteins function as adapters, bringing together E2 conjugases and their substrates (see Box 3.1)²¹. Based on structural analogy to a BRCA1-BARD1 complex, it was suggested that

RNF2 contains an E2 binding site, whereas BMI1 is involved in substrate binding¹⁹. E2 conjugase UbcH5 subtypes a, b, c and UbcH6 promote H2A ubiquitylation, although these Ubc's do not bind the RING RNF2/BMI1 complex⁷. Which E2 conjugases contribute to H2A ubiquitylation *in vivo* is currently not known. A summary of PcG-related PTMs and their functional relevance is provided in Table 3.1.

Ubiquitylation of PcG proteins

The above relatively simple picture is complicated by additional levels of PTM. Both RNF2 and BMI1 are ubiquitin-proteins and differential auto-ubiquitylation of RNF2 is required for H2AK119ub1^{7, 22}. Poly-ubiquitylation of RNF2 requires Lys6, Lys27 and Lys48 linkage on the same ubiquitin molecule and RNF2 auto-ubiquitylation is promoted by Ubc5 *in vitro*²². Some of these K-residues are involved in epigenetic silencing, as RNF2s' ability to promote H2AK119ub1 relates to the availability of UbK6 and UbK27, not UbK48²². Although inhibition of Ub-dependent degradation with proteasome inhibitor increases RNF2 levels, auto-ubiquitylation-mutant RNF2(I53S) proteins are still efficiently degraded, suggesting the involvement of other E3 ligase and/or Ub-site(s). BMI1 inhibits ubiquitylation of RNF2 and coexpression of RNF2 and BMI1 blocks its degradation in a RING domain dependent manner²². Unlike RNF2, BMI1 lacks auto-ubiquitylation activity. However, like RNF2, it is stabilized by proteasome inhibition. As for RNF2, the identity of the ubiquitin E3 ligase responsible for proteasomal degradation of BMI1 is not known. Whereas RNF2K112ub appears dispensable for H2A E3-ligase activity⁷, mutant RNF2 with an intact RING domain, however missing most ubiquitylation sites (K92-198R) still binds BMI1, but lacks E3 ligase activity²². Thus, seemingly at odds with each other: although RNF2 autoubiquitylation is required for H2A ubiquitylation, it is inhibited by BMI1, yet overall, BMI1 promotes RNF2 H2A-E3 ligase activity and blocks its proteolytic degradation²². A possible explanation for this discrepancy may involve number, length and linkage-type of ubiquitin chains, aside from molecular context.

A candidate E3 ubiquitin ligase for BMI1 is the CULLIN3/SPOP complex: *in vitro* and *in vivo* analyses confirmed that CULLIN3 and SPOP are required for BMI1-ubiquitylation in cells. As RNAi-mediated knockdown of CULLIN3 or SPOP does not affect BMI1 protein levels, CULLIN3/SPOP mediated ubiquitylation of BMI1 most likely has no bearing on protein stability²³. Interestingly, a human BMI1 polymorphism resulting in a C18Y substitution increases ubiquitylation and proteasomal degradation²⁴. Whether or not this sorts any effect on human health is currently not clear.

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Table 3.1. PTMs in PcG biology

PcG protein	PTM	Interacting protein	Biological effect
RNF2 (and RING1)	Ub	H2A (K119), H2A.Z	Transcriptional repression, X-inactivation
+ BMI1			Histone H2A ubiquitylation
+ phosphorylated MEL18			
+ PCGF1			
RNF2	Ub (mono, poly)	-	Protein stability,
BMI1	Ub	-	Protein stability
		CULLIN/SPOP3	X-inactivation
RYBP	Ub	-	-/Histone H2A binding
PRC1	Ub	Geminin	Protein stability
CBX4	Sumo	CtBP	
		Dnmt3a	Abolishes repression
		HIPK2	DNA damage response
		SIP1	Relieves E-cadherin repression
		CBS	Inhibition CBS activity
PCGF2	Prevention of Sumo	HSF2	Decreased interaction during mitoses
		RanGAP1	Increased interaction during mitosis
CBX4	Sumo	-	-
CtBP1	Sumo	-	-
SUZ12	Sumo	PAISX β	-
EZH2	Sumo	-	-
BMI1	Ph		Chromatin dissociation
CBX2	Ph	-	Nuclear localization
PCGF2/MEL18	Ph	PKC	Blocks dimerization
Esc (<i>Drosophila</i>)	Ph		Homodimerization, complex formation/stabilization
EED	Ph		Homodimerization
EZH2	Ph	-	Histone H3 binding
EZH1	Ph	ZAP70	Interaction
RNF2	Proteolytic cleavage	Caspases 3 and 9	Apoptosis
Ph (<i>Drosophila</i>) – PHC3	O-GlcNAc	Sxc/Ogt	Repression - unknown
RNF2	Ac	-	unknown

Listed are published biochemical connections between PcG and associated proteins; see main text for details on functional relationships.

The PcG protein RYBP (RING1 and YY1 binding protein) is mono-ubiquitylated by RING proteins, and binds H2AUb1, among other proteins, *in vitro*, through a zinc finger Ubiquitin binding domain (UBD) of the NZF type (Npl14 zinc finger).

Although a UBD-NZF mutant still interacts with RING1 and RNF2, it prevents formation of Polycomb-bodies in osteosarcoma cells, suggesting a dominant negative role in PcG recruitment²⁵. Thus, RYBP may play a role in engagement of specific transcription factors, and hence direction of PcG complexes to specific target genes^{17, 26}.

Combined, the above data shows that PRC proteins are engaged in numerous ubiquitin-dependent regulatory mechanisms, and that ubiquitylation is important for PcG-mediated silencing at multiple levels.

Polycomb-mediated Sumoylation and Polycomb Sumoylation

Although mechanistically not completely understood, one of the potential functional outcomes of PcG protein sumoylation is induction of transcriptional repression²⁷. In support of a biologically relevant role in the context of transcriptional repression, sumoylation appears conserved throughout evolution. A genome-wide RNA interference screen in *Drosophila* cells identified proteins that, when absent, relieve sumo-dependent inactivation of the transcription factor Sp3²⁸. Among interactors identified were the PcG protein Sfmbl, the zinc-finger protein MEP-1 and dMi-2, an ATP dependent chromatin remodeler which shows genetic interaction with PcG²⁹; all three proteins bind Sp3-SUMO *in vitro* and all are recruited to promoters in a Sp3-sumoylation dependent manner²⁸. Also in *C. elegans* a link was established between sumoylation and PcG proteins: the PcG-like protein SOP-2 interacts with UBC9 via its conserved SAM (sterile alpha motif/self association motif)³⁰. SOP-2 sumoylated is required for *in vivo* localization to nuclear bodies and repression of *Hox* genes³⁰.

The identification of CBX4 as a SUMO E3 ligase forged the first link between PcG function and SUMO. CtBP (carboxyl-terminus binding protein, an interaction partner of RING1 and other PcG proteins³¹) are sumoylated^{32, 33}. CBX4 interacts with E2 Ubc9 and CtBP and sequesters both proteins to PcG bodies. Multiple biological CBX4 targets have been identified which begin to link PcG-mediated sumoylation to relevant biological processes^{32, 34-38}. Among these targets is the *de novo* DNA methyltransferase (Dnmt3a). Despite its highly conserved nature among the PcG proteins CBX2, CBX6, CBX7 and CBX8, only the C-terminal COOH box of CBX4 interacts with Dnmt3a in a yeast two-hybrid setting. Dnmt3a is poly-sumoylated; sumoylation terminates the interaction of Dnmt3a with HDAC1/2 and completely abolishes its repressive ability *in vitro*, suggesting a role for PTMs in dynamic epigenetic regulation of gene expression^{35, 36}. DNA damage-induced homeodomain interacting protein kinase 2 (HIPK2) phosphorylates and activates CBX4 E3 sumo activity, whereas CBX4-mediated sumoylation of HIPK2 in turn enhances its ability to repress genes in response to

Table 3.2. Site-specific Polycomb sumoylation and ubiquitylation sites

Complex	Name	Alternative symbol	Accession Number	Total nr AA	Modification Site	Conservation in Mouse	Ref
PRC2	SUZ12		Q15022	739	K72 SUMO	K74	42
					K73 SUMO	K75	42
					K75 SUMO	K77	42
PRC1	CBX4	HPC2	O00257	558	K492 SUMO	K490	38, 79, 80
	Rnf2	Ring1b	Q9CQJ4	336	K112 Ub	K112*	7
PhoRC	YY1		P25490	414	K288 SUMO	K288	81

The table lists PTM confirmed by mass spectrometric and/or mutational analysis. AA: Amino Acid; SUMO: Sumoylation site; Ub: Ubiquitylation site.

DNA damage (Fig. 3.1C)³⁸. This auto-regulatory feedback loop is likely relevant in the context of cellular DNA damage responses. Sumoylation of SIP1 (SMAD interacting protein 1) interferes with CtBP interaction and relieves repression of E-cadherin, an important regulator of EMT (epithelial mesenchymal transition) during development and tumorigenesis³⁷. Furthermore CBX4 targets cystathionine beta-synthase (CBS), an enzyme involved in homocysteine to cysteine-conversion³⁴. Sumoylation of CBS inhibited its enzymatic activity³⁴. As homocysteine to cysteine conversion is an important step in the synthesis of S-Adenosylmethionine (SAM), a major methyl-donor reagent for essential methylation reactions³⁹, this may have obvious implications for local and global epigenetic regulation.

In the context of PRC1 function, the PcG protein PCGF2, appears to oppose sumoylation. PCGF2 binds, both *in vitro* and *in vivo*, heat shock factor 2 (HSF2), which is predominantly sumoylated during mitosis⁴⁰. PCGF2 dissociates from HSF2 during mitosis and, conversely, recombinant PCGF2 inhibits *in vitro* sumoylation of HSF2. Thus PCGF2 may act as an anti-SUMO regulator⁴⁰. PCGF2 also inhibits sumoylation of RanGAP1, which is independent of its RING domain⁴¹. Intriguingly, in contrast to HSF2, the interaction between PCGF2 and RanGAP1 increases during mitosis, suggesting a sumo-dependent switch of interaction partners of PCGF2⁴¹.

PRC2 complex function is associated with sumoylation as well: SUZ12 and EZH2 (Fig. 3.1A) are both sumoylated; the E3 ligase for SUZ12 appears to be PAISX β , not CBX4. The exact biological role of SUZ12 sumoylation is not clear, as wild type or a non-sumoylatable SUZ12 3KR mutant both show similar H3K27me3 in a SUZ12^{-/-} background, and 3KR co-localization with EZH2 and EED is not affected⁴². An overview of experimentally confirmed site-specific sumoylation and ubiquitylation sites on PcG proteins is presented in Table 3.2.

In summary, sumoylation, like ubiquitylation emerges as a PTM relevant for regulation of gene expression. In contrast to ubiquitylation, PcG-mediated

sumoylation has not directly been linked to histone modifications yet. In stead, currently available data suggest a relevant role for sumoylation in dynamic interaction with non-PcG proteins in the context of cell physiology.

Phosphorylation of PcG proteins

Although thus far no kinases have been identified as PcG proteins, phosphorylation is a common PTM on PcG proteins. Recent observations begin to show that signaling, which triggers downstream phosphorylation events, affects subcellular localization, protein-interactions within complexes, enzymatic activity and chromatin-association of several PcG proteins and other factors. The first report of PcG protein phosphorylation suggested a meaningful regulatory role for PcG phosphorylation, as phosphorylated BMI1 dissociates from chromatin at late S-phase, when chromatin is assembled “*de novo*”⁴³. Also MBLR (MEL18 and BMI1-Like RING finger protein; PCGF6) is predominantly phosphorylated in G2/M⁴⁴. *In vitro* kinase assays suggested PCGF6 as a substrate for CDK7⁴⁴. Interestingly, also Trithorax orthologs are phosphorylated in a cell cycle dependent manner⁴⁵, suggesting phosphorylation as a common mechanism to temporarily relocate chromatin associated proteins. Phosphorylation may affect individual PcG proteins in other biologically relevant ways. CBX2/M33 phosphorylation affects nuclear localization: high-mobility (unmodified) CBX2 resides in the cytoplasm in mouse livers, whereas low-mobility isoforms localize to the nucleus⁴⁶. Dimerization of PCGF2, is blocked in the presence of protein kinase C (PKC)⁴⁷. Also NSPc1/ PCGF1 is functionally targeted by phosphorylation: a synthetic phospho-mutant GAL4-DBD-NSPc1 no longer transcriptionally represses a GAL4-LUC-reporter⁴⁸.

Phosphorylated *Drosophila* PRC2 protein *Extra sex combs* (Esc) is preferentially found in a 600 kDa complex with PcG protein *enhancer of zeste* (E(z)) and Esc phosphorylation is required for formation and stability of a larger Esc/E(z) complex containing PCL and RPD3^{49, 50}. Both Esc and EED (its mammalian orthologue) are phosphorylated in a domain responsible for Esc/EED homodimerization⁵⁰. CK1 and CK2 may be the responsible kinases, as they phosphorylate Esc and EED *in vitro* and promote Esc/EED homodimerization. In addition, phosphorylation appears to regulate EZH2, a PRC2 histone methyl transferase (HMT) which trimethylates Lysine 27 on histone 3 (H3K27me3)⁵¹: AKT (PKB)-induced EZH2 Ser21ph suppresses its methyltransferase activity by impeding EZH2 binding to histone H3 (Fig. 3.1D)⁵². In addition, phosphorylation affects self-recruitment of EZH2 to its own mark, which may have bearing on maintenance of repression throughout cell divisions^{53, 54}. Also the PcG EZH2 homologue EZH1 is phosphorylated: the tyrosine kinase p56^{lck} is required for the phospho-dependent association between EZH1 and zeta associated protein-70

(ZAP-70) in activated T cells⁵⁵; whether phospho-residues within EZH1 and ZAP-70 are directly involved in binding awaits experimental validation.

Figure 3.2. Classification of S/T phosphorylation sites into general kinase recognition sequence categories.

At present, not all consensus phosphorylation motifs are known for all kinases. A number of general phospho-site classes have been annotated based on amino acid sequences surrounding S/T phosphorylation sites: proline-directed, acidic, basic and otherwise⁷⁸. (A-C) Sequence logos for PcG phosphorylation motifs of Proline-directed (A; n=42) acidic (B; n=36) and basic (C; n=15) categories where the phosphorylated residue (S/T) is centered were generated with Weblogo (<http://weblogo.berkeley.edu>). Only serine and threonine phosphorylation sites were taken into account when a full 15-mer sequence was available. To avoid sequence bias only non-overlapping human and mouse PcG phosphorylation sites were used. Centered 15-mer sequences were assigned to a motif class sequentially by following a binary decision tree as follows: P at +1 (Pro-directed), 5 or more E/D at +1 to +6 (acidic), R/K at -3 (basic), D/E at +1/+2 or +3 (acidic), 2 or more R/K at -6 to -1 (basic), otherwise. (A) Although phospho-site numbers are low and thus no solid deductions are warranted, it is apparent for the proline-directed class (classified by a P in the +1 position), that prolines are not limited to +1, but are abundant in various – and + positions. (B) In the acidic motif, characterized by the presence of the amino acids glutamic acid (E) and aspartic acid (D) in the + residues, E/D's are present in the – positions. (C) In basic motifs, Arginine (R) is predominantly found in -3 positions, in addition to -5.

Classification of PcG-phospho-sites

Most current published information on PcG phosphorylation neither unequivocally identifies responsible kinases nor targeted amino acid residues, and the majority of existing *in vitro* data will have to be confirmed and

functionally tested *in vivo*. Over recent years several large-scale phospho-proteomic analyses were published. We have extracted PcG-related data from these and our own studies; this reveals numerous PcG phosphorylation sites: to date 118 human (Table 3.3) and 31 mouse (data not shown) PcG phosphorylation sites have been published; so far 14 of these phosphorylation events are shared by both species (Table 3.4). Amino acids surrounding phosphorylation sites direct kinase-binding and hence define kinase-substrate specificity. Classification of currently known PcG phosphorylation sites into predicted phospho-site categories shows that most sites group in the proline-directed or acidic class (Table 3.5; Fig. 3.2A-C). Kinases that recognize/bind the proline-directed residues belong to the MAPkinase family; acidic motifs are phosphorylated by casein kinase II (CK2); kinases targeting basic motifs belong to the AKT/PKB and PKA types⁵⁶. Consistent with our analysis, published PCGF2/6 (CK1/2) and EZH2 phospho-sites (AKT/PKB) classify in the acidic and basic target group, respectively.

Although it is not known at present whether PcG phosphorylation directly affects chromatin association, their correlation (*i.e.* signaling induced phosphorylation and chromatin dissociation) suggests cells use phosphorylation cascades to relay environment cues to chromatin, where reprogramming of gene activity includes epigenetic remodeling events⁵⁷. Consistent with a functional link between MAPK signaling and PcG function, we recently showed that PRC1/chromatin association is disrupted downstream of MAPK activation⁵⁷. In summary, it is clear that phosphorylation is a relevant part of PcG biology. Further characterization and functional validation of these phosphorylation sites will be pivotal for our understanding of the cellular conditions under which PcG phosphorylation take place, the regulatory pathways involved and how cells use these pathways to modulate chromatin structure to allow biologically appropriate responses.

Other PTMs in PcG biology

GlcNAcylation

Recently, the *Drosophila* PcG gene *super sex combs* (*sxc*) was reported to encode the enzyme O-linked N-acetylglucosamine (O-GlcNAc) transferase (Ogt)⁵⁸. PRC1 protein Polyhomeotic (Ph), is GlcNAcylated by *sxc*/Ogt *in vivo*. PRC1-mediated repression is dependent on functional *sxc*/Ogt, possibly via GlcNAcylation of Ph⁵⁸. Interestingly, the process appears conserved across species, as the mammalian orthologue PHC3 is O-GlcNAcylated as well⁵⁹. Analogous to PcG, GlcNAcylation of the histone methyltransferase MLL5, a trithorax-like protein, generally assumed to counteract PcG function, regulates

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Table 3.3. Human Polycomb phosphorylation sites

Complex	Name	Alternative symbol	Accession Number	Total nr AA	Phospho-site	Conservation in Mouse	Ref
PRC2	EZH1		Q92800	747	-	-	-
							52
					S21	S21	82
					T339	T339	83
					S362	S362	83
					S363	S363	82, 83
					S366	S366	82, 83
					T367	T367	84
	EZH2		Q15910	746	S380	S380	82, 83, 85-90
					T487	T487*	82, 83, 85-90
							82, 83, 85-90
					S25	S25	82, 83, 85-90
					S34	S34	82, 83, 85-90
					S43	S43	82, 83, 85-90
					S46	S46	82, 83, 85-90
					T50	T50	82, 83, 85-90
	EED		O75530	441	T55	T55	82, 83, 85-90
					T57	T57	82, 83, 85-90
							82, 83, 85-90
					S37	S37	82, 83, 85-90
					T131	T133	82, 83, 85-90
					S139	S141	82, 83, 85-90
					S382	S384	82, 83, 85-90
					T418	NC	82, 83, 85-90
	SUZ12		Q15022	739	S541	S543	82, 83, 85-90
					S546	S548*	82, 83, 85-90
					S583	S585*	82, 83, 85-90
							82, 83, 85-90
							82, 83, 85-90
							82, 83, 85-90
							82, 83, 85-90
							82, 83, 85-90
PRC1	CBX2	HPC1	Q14781	532	-	-	-
							93
					Y205	Y207	82, 83, 85-90
					S347	S350	82, 83, 85-90
					T349	T352	82, 83, 85-90
					S413	S427	82, 83, 85-90
					T415	T412	82, 83, 85-90
					S430	S427	82, 83, 85-90
	CBX4	HPC2	O00257	558	T435	T432	82, 83, 85-90
					T495	NC	82, 83, 85-90
							82, 83, 85-90
					S107	S107	82, 83, 85-90
					S301	S302	82, 83, 85-90
					S303	S304	82, 83, 85-90
							82, 83, 85-90
							82, 83, 85-90
	CBX6		O95503	412			82, 83, 85-90
							82, 83, 85-90
	CBX7		O95931	251			82, 83, 85-90
							82, 83, 85-90
	CBX8	HPC3	Q9HC52	389	S110	S110	82, 83, 85-90
					S130	NC	82, 83, 85-90

Talking to chromatin: post-translational modulation of PcG function

Complex	Name	Alternative symbol	Accession Number	Total nr AA	Phospho-site	Conservation in Mouse	Ref
					T234	T207	⁸² ^
					S256	S229*	^{82, 83}
					S265	S238*	^{82, 83, 88} , #
					S311	S284*	#
					S332	S305	^{82, 87}
					S352	S325	⁸²
					S354	S327	⁸² ^
	PHC1	HPH1/EDR1	P78364	1004	Y32	Y32	⁹³
					S195	S195	#
					S645	S653	#
					S653	S661	⁹⁴
					S657	S665	⁹⁴
					S664	S672	⁹⁴ , #
					S669	S677	⁹⁴ , #
					S786	S794	#
					S895	S903	#
					T918	T926	⁸³
					T922	T930	⁸³ , #
	PHC2	HPH2	Q8IXK0	858	S591	S583	⁸²
					T619	T611	⁸²
					S621	S613	⁸³
					S701	S693	⁸⁴
					S704	S696	⁸⁴
					S733	S725	⁹⁵ , #
					S745	S737	⁸⁵
	PHC3	HPH3/EDR3	Q8NDX5	983	S229	S227	⁹⁵
					S263	S261	^{85, 90}
					S315	S312	⁸² , #
					T609	T607*	^{82, 83, 85, 87, 89, 90} , #
					T614	T612*	⁸² ^ ⁸⁷
					S616	S614*	^{82, 83, 85, 87, 89, 90} , #
					S724	S722	⁸² ^
					S761	S759	⁸²
					S762	S760	⁸²
					S862	S860	#
	RING1	RING1A/RNF1	Q06587	406	S38	S38	^{82, 87} ⁸³ , #
					S187	S187	^{82, 83}
					S188	S188	^{82, 83}
					S190	S190	^{82, 83}
					S229	S229	#

Chapter 3

Complex	Name	Alternative symbol	Accession Number	Total nr AA	Phospho-site	Conservation in Mouse	Ref
	RNF2	RING1B	Q99496	336	S41	S41	63
					S168	S168	90
	BMI1	PCGF4	P35226	326	S251	S249*	90
					S253	S251*	90
					S255	S253*	90
	PCGF2	MEL18	P35227	344	Y197	Y197	93
					Y205	Y205	93
					Y206	Y206	93
					T344	gap	82
	PhoRC	YY1	P25490	414	S118	S120*	89
					S184	S184	84
					S247	S247*	82, 83, 85, 88, 89
					Y251	Y251	82
					T348	T348	82, 96
					T378	T378	82, 83
Other	PCGF1	NSPc1	Q9BSM1	259	-	-	-
	PCGF6	MBLR	Q9BYE7	350	S30 [§]	S34	44
	SCMH1		Q96GD3	660	S649	S653	90, 94
	SCML1		Q9UN30	208	S15	N.A.	82 ^
					S17	N.A.	82
					S117	N.A.	88
	SCML2		Q9UQR0	700	S255	N.A.	82 ^
					S256	N.A.	82
					S258	N.A.	82
					S267	N.A.	82
					S299	N.A.	82
					S300	N.A.	82
					T305	N.A.	82
					S495	N.A.	82 ^
					S499	N.A.	82, 83, 85, 86
					T503	N.A.	82
					S511	N.A.	82, 83, 85, 86
					S570	N.A.	95
					S576	N.A.	82
					T581	N.A.	82 ^
					S583	N.A.	82
					S590	N.A.	82, 83
					S594	N.A.	82, 83
					S606	N.A.	82
	RYBP		Q8N488	228	S127	S127	82

Talking to chromatin: post-translational modulation of PcG function

Complex	Name	Alternative symbol	Accession Number	Total nr AA	Phospho-site	Conservation in Mouse	Ref
					S130	S130	⁸²
					S203	S203	⁸² ^
					T215	T215	⁸² ^

Asterix (*) indicates sites that were detected as phosphorylation sites in mouse cells as well; AA: Amino Acid; NC: non-conserved; N.A.: Not available; # marks unpublished results (HN,JD,JWV); \$: Site was referred to as S32 in original article; ^: These putative phosphorylation sites do not reach a sufficiently high probability based on prediction algorithms used.

methylation of H3K4⁶⁰. These findings begin to show that GlcNAcylation of chromatin factors, such as PcG proteins, is relevant for epigenetic regulation and provide important leads for future study.

Proteolytic cleavage

Other than ubiquitin-26S proteasome dependent protein degradation, as holds for PcG proteins RNF2 and BMI1²², proteolytic cleavage may also occur via activated caspases⁶¹. Caspase substrates include *i.a.* structural proteins, such as actin, vimentin and nuclear lamins and proteins involved in transcription and translation, such as NFkB and translation initiation factors⁶¹. Recently RNF2 was identified as a direct substrate of caspases-3 and 9 in apoptotic cells⁶². The exact function of RNF2 cleavage during apoptosis remains illusive, but it is possibly a prerequisite for nuclear condensation and DNA fragmentation to occur. Whether PcG protein cleavage is relevant outside the context of apoptosis is currently unclear.

Acetylation

Currently acetylation of only one PcG protein has been reported: RNF2 is acetylated at S2, which is accompanied by a N-terminal methionine excision⁶³. The significance of this modification in relation to PcG function is not known.

Table 3.4. Summary of PcG phosphorylation sites

residue	Human	Mouse	Human + Mouse non-overlapping	Human + Mouse overlapping
Serine	87 (74%)	26 (83%)	101 (75%)	12 (86%)
Threonine	25 (20%)	5 (16%)	28 (21%)	2 (14%)
Tyrosine	6 (6%)	-	6 (4%)	-
Total	118 (100%)	31 (100%)	135 (100%)	14 (100%)

Classification of PcG phospho-sites based on common phospho-amino acids.

Signaling to Chromatin: Post-translational Cross-talk to Polycomb

It is evident that PcG proteins are subject to numerous different PTM (*i.a.* ubiquitylation, sumoylation, phosphorylation and GlcNAcylation) and, besides, harbor intrinsic enzymatic modifying activities themselves (HMT, ubiquitin and SUMO E3 ligase, O-GlcNAc transferase). How these PTM relate to each other is currently far from clear, however, several studies begin to unveil an intricate interplay between different PTMs, their effect at the molecular level and their biological relevance (Fig. 3.1). Complex formation between PCGFs and RNF2 regulates H2A ubiquitin E3 ligase activity¹⁶. PCGF2 phosphorylation is required for substrate recognition in a relevant chromatin context, as dephosphorylated RNF2/PCGF2 complexes no longer efficiently ubiquitylate nucleosomes¹⁶. Similar regulation likely holds true for its close relatives BMI1 and PCGF1. Phosphorylated EZH2 has reduced methyltransferase activity toward histone H3, as a result of reduced substrate binding⁵². Phosphorylation and sumoylation mediated auto-regulatory feedback between CBX4 and HIPK2 has obvious relevance for DNA damage responses³⁸. Recently the PRC1 complex was identified as the E3 ubiquitin ligase for Geminin, an inhibitor of replication licensing factor Cdt1. PHC1 (Rae28) deficiency in mice impairs ubiquitin/S26-proteasome mediated degradation of Geminin and has direct consequences for cell cycle progression in the haematopoietic lineage⁶⁴.

In keeping with differential chromatin association of Ph as compared to other PRC1 proteins, GlcNAcylated Ph does not co-purify with other PRC1 components in larval extracts, whereas it does in embryonic nuclear extracts⁵⁸. Phosphorylation and GlcNAcylation mostly affect the same amino acids (S/T), hence an intricate interplay between these modifications may be predicted, either via competitive occupancy at the same site or alternative occupancy at adjacent sites⁶⁵.

Although clearly numerous PTM affect PcG biology, molecular mechanisms in signaling to chromatin, down-stream modulation of epigenetic marking and the establishment and transfer of heritable epigenetic states remain largely elusive. Specific consensus/motif based kinase-substrate interactions most likely define and direct signaling-induced remodeling and/or other epigenetically relevant events at the chromatin level. In contrast, context-dependent variation in sumoylation sites is less well-defined and the role of select consensus motifs in ubiquitylation is largely unknown. Although speculative, this may suggest that once signaling is triggered, a hierarchical sequence of PTMs is initiated, the target-specificity (*i.e.* networks, pathways, complexes) of which is defined by phosphorylation events, whereas down-stream effects (*i.a.* altered protein interactions, activity or stability) are 'merely' consequential, and controlled by numerous other PTM. Indeed ubiquitylation, sumoylation, GlyNAcylation and

phosphorylation are probably functionally linked in PcG biology, as important cross talk between these PTMs exists in many ways in other systems⁶⁶. In this context important open issues are for instance how exactly PcG PTMs functionally relate to each other, *i.e.* whether PTM act separately, processively or combinatorially. Signaling-induced PTMs are generally reversible, proteolytic cleavage excluded, hence many chromatin-associated, epigenetic regulators are presumably rapidly converted to their initial post-translational status upon signal recession. However, at the receiving end, stable, heritable covalent histone modifications appear somehow exempt from such regulation. Notions like these should provide important basis for future hypothesis-driven research.

Table 3.5. Classification of PcG S/T phosphorylation sites

class	Human + Mouse non-overlapping
Pro-directed	42 (37%)
Acidic	36 (32%)
Basic	15 (13%)
Other	20 (18%)
Total	113 (100%)

Classification of PcG phosphorylation sites in relation to amino acid context. Only putative Ser and Thr phosphorylation sites were taken into account when a full 15-mer sequence was available and a sufficiently high phosphorylation probability was predicted

Conclusions

It is evident that chromatin-associated protein complexes, like PcG proteins, are targets for cell signaling. These signaling events lead to PTMs that may affect chromatin binding, complex composition and catalytic activity. We and others found that multiple kinases target PcG proteins. In addition, PcG proteins are subject to ubiquitylation, sumoylation and additional PTM. Studies recapitulated in this Chapter have only just begun to unravel the complexity and multiple layers of regulation of PcG function. PcG-mediated transcriptional silencing already appears a much more complex process than the antiquated view that PRCs physically obstruct transcription factor binding to DNA, and ongoing studies refine positioning of PcG-function in the proper cellular context. Current conservative estimates predict the existence of anywhere between 60-100 or more mammalian PcG(-related) proteins, each likely with multiple phosphorylation and/or ubiquitylation, sumoylation and potentially numerous other PTM sites, including acetylation and methylation^{67, 68}. In stark contrast with this: at the moment only two PcG phospho-specific antisera exist^{44, 52}. Clearly there is a need for additional experimental tools and approaches. Ultimately PTMs are aimed at concerted regulation of a number of chromatin-based processes in which PcG proteins play a role, including dynamic

transcriptional regulation, long term silencing, DNA replication and DNA damage responses, to ensure proper regulation of cell fate and survival. Increased insight into mechanisms employed by cells to target chromatin and chromatin-associated factors, among which PcG, and their physiological consequences at the chromatin level will be important for further development and application of epigenetic strategies in for instance regenerative medicine. As many of the processes targeting and involving PcG function are etiologically linked to disease (*e.g.* overexpression in cancer, bypass of replicative senescence⁶⁹⁻⁷²), a better fundamental understanding of gene-environment interactions at the molecular level will eventually contribute to the development of therapeutic and preventive strategies relevant for western type diseases, including obesity, diabetes and cancer.

Acknowledgements

Collection of PcG annotations was based on www.uniprot.org and www.phosphosite.org. Cross-species sequence conservation was verified by aligning human and murine homologous sequences in Geneious software (Biomatters, Auckland New Zealand), using default settings. PTM-related protein nomenclature was based on published consensus⁷³. The authors thank M. Vidal (Madrid, Spain) and R.Shiri-Sverdlov for critically reading the manuscript.

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**Kinase 3pK phosphorylates and
regulates chromatin association of
the Polycomb Group protein Bmi1**

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Abstract

Polycomb group (PcG) proteins form chromatin-associated, transcriptionally repressive complexes, which are critically involved in the control of cell proliferation and differentiation. Although the mechanisms involved in PcG-mediated repression are beginning to unravel, little is known about the regulation of PcG function. We showed previously that PcG complexes are phosphorylated *in vivo*, which regulates their association with chromatin. The nature of the responsible PcG kinases remained unknown. Here we present the novel finding that the PcG protein Bmi1 is phosphorylated by 3pK (MAPKAP kinase 3), a convergence point downstream of activated ERK and p38 signaling pathways and implicated in differentiation and developmental processes. We identified 3pK as an interaction partner of PcG proteins, *in vitro* and *in vivo*, by yeast two-hybrid interaction and co-immunoprecipitation, respectively. Activation or overexpression of 3pK resulted in phosphorylation of Bmi1 and other PcG members and their dissociation from chromatin. Phosphorylation and subsequent chromatin dissociation of PcG complexes were expected to result in de-repression of targets. One such reported Bmi1 target is the *Cdkn2a/INK4A* locus. Cells overexpressing 3pK showed PcG complex/chromatin dissociation and concomitant de-repression of p14^{ARF}, which was encoded by the *Cdkn2a/INK4A* locus. Thus, 3pK is a candidate regulator of phosphorylation-dependent PcG/chromatin interaction. We speculate that phosphorylation may not only affect chromatin association but, in addition, the function of individual complex members. Our findings linked for the first time MAPK signaling pathways to the Polycomb transcriptional memory system. This suggests a novel mechanism by which a silenced gene status can be modulated and implicates PcG-mediated repression as a dynamically controlled process.

Introduction

Members of the mitogen-activated protein kinase (MAPK) family (ERK, JNK/SAPK, and p38 kinases) are implicated in many biological processes, such as adaptation to environmental changes, differentiation, immune activation, inflammatory responses, cell cycle modulation, transformation, and apoptosis¹. These phosphorylation cascades may function as switching mechanisms that modulate gene activity². 3pK, also known as MAPK-activated protein kinase 3 (MAPKAPK3), belongs to a growing family of kinases that are activated by one or more members of the MAPK family³. To better understand processes regulated by 3pK, we aimed to identify interactors and potential novel substrates of 3pK. Here, we report the finding of a group of novel interaction partners of 3pK, the Polycomb group (PcG) proteins, including the mammalian PcG proteins HPH2 and Bmi1^{4,5}. PcG protein complexes maintain a transcriptionally repressed gene status in dividing and differentiating cells throughout developing eukaryotes⁶⁻⁸. The mechanisms that contribute to PcG-mediated silencing are beginning to unravel. PcG proteins form large multimeric protein complexes that associate with chromatin (reviewed in Refs.^{9,10}). The current view is that PcG-mediated silencing in mammals involves generation and recognition of histone modifications; a subset of PcG complexes, the EED/EZH complex (hPRC2 complex), associates with histone deacetylases, implicating histone tail deacetylation in PcG-mediated repression¹¹. Within this complex, EZH2 was identified as a histone methyltransferase with preference for histone H3 lysine 27¹². A biochemically distinct Polycomb-repressive complex (hPRC1 in human, PRC1 in *Drosophila*^{13,14}) recognizes and binds the epigenetic trimethyl(3m)lysine 27 mark produced by the (h)PRC2 complex; recognition and maintenance of repressed chromatin may involve additional histone modifications, such as histone ubiquitination or methylation^{12,15-18}. The current view is that PcG-mediated repressional maintenance involves a combination of (associated) catalytic activities responsible for posttranslational modifications on histone tails and extensive interactions among PcG members and other chromatin (-bound) factors, which generate a repressive higher order chromatin structure inaccessible to transcriptional activators and chromatin remodeling factors^{8,19-21}.

Little is known about how chromatin association and, potentially, function of PcG complexes is controlled. In the present study, we identified Polycomb group (PcG) proteins as *in vivo* interaction partners of the MAPKAP kinase 3pK. We have provided evidence that MAPK signal transduction cascades target PcG protein complex/chromatin interaction through phosphorylation. 3pK acts as a Bmi1 kinase *in vitro* and *in vivo*. Of relevance, 3pK overexpression causes PcG/protein dissociation from chromatin and de-repression of the *Cdkn2a*/

INK4A locus. Our data support a model in which Polycomb-mediated repression is modulated by stress- and mitogen-activated protein kinase cascades. These findings point to a molecular mechanism by which a transcriptionally silenced gene status can be reprogrammed and implicates PcG-mediated repression as a dynamically controlled process. These observations are expected to have important implications in understanding epigenetic mechanisms in development and disease.

Experimental procedures

Plasmid construction

Eukaryotic expression vectors for untagged and tagged wild type or mutant 3pK have been described elsewhere^{3, 22}. The nucleus-retained 3pK mutant 3pkΔC was created by deleting the C-terminal (aa 308–383) regulatory region from Gal4DB-3pK. pGAD10-HPH2 (C-298 aa) and pGAD10-HPH2 (432 aa) were described elsewhere⁴. HDII/SPM/SAM domain mutations within the C-198 HPH2 construct were generated by PCR-directed deletion of the 3'-most sequences or by site-directed mutagenesis (QuikChange; Stratagene Europe, Amsterdam, The Netherlands) within the fifth C-terminal-most α -helix. MT2SM-HA-Bmi1 (mouse) was kindly provided by Dr. M. van Lohuizen⁵. Glutathione S-transferase (GST)-tagged 3pK^{3, 22} was recloned into pBABE- or LZRS-based retroviral expression vectors^{23, 24}. The murine ecotropic receptor was recloned from an original construct by Dr. J. M. Cunningham (Harvard Medical School, Boston, MA) and shared by Dr. A. Lund (Netherlands Cancer Institute, Amsterdam, The Netherlands). RNA interfering 3pK sequences 5'-CGGCAAAGTGTCTGGAGTGC-3', 5'-GCAGCTGATCCGCTCCTG-3', and 5'-GGAGGAGATGACCAGTGCC-3' (Fig. 4.5C, respectively, *lanes 1–3*) were cloned into stable hsRNA vectors described elsewhere²⁵.

Yeast two-hybrid system

A kinase-inactive 3pK mutant (K73M) was used as bait in two-hybrid screens against a human heart MATCHMAKER cDNA library cloned into the pGAD10 vector (Clontech). Yeast strain CG-1945 (Clontech) was manipulated according to the manufacturer's instructions. Positive clones were monitored by growth on SD/-Trp/-Leu/-His plates and activity of the *lacZ* reporter gene in filter assays. In direct two-hybrid assays, reselected SD/-Trp/-Leu/-His filters were incubated for 12–24 h at 30 °C before the β -Gal assay was performed. The liquid culture β -Gal assay with *o*-nitrophenyl- β -D-galactopyranoside (Sigma) as substrate was performed according to the manufacturer's instructions using the yeast strain Y190.

Cell culture and cell cycle synchronization

Indicated cell lines were cultured at 37 °C, 5% CO₂, 100% humidity in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS), antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin), 200 mM L-glutamine and 4.5 g/liter L-glucose. The TIG3-Bmi1.2PY has been described elsewhere²⁶. Human U2OS osteosarcoma cells and TIG3 primary human fibroblasts expressing the murine ecotropic receptor were provided by Dr. D. Shvarts (Utrecht Medical Center, Utrecht, The Netherlands) and Dr. D. Peeper (Netherlands Cancer Institute, Amsterdam, The Netherlands), respectively. Titers of LZRS-IRES-GFP (Bmi1, 3pK, or control) viral supernatants were sufficiently high to achieve near 100% infection; infection with pBABE-GST (3pK or control) virus was followed by selection with puromycin. G0/G1 arrest of cells was established by contact inhibition and/or a 48-h serum starvation period (in Dulbecco's modified Eagle's medium supplemented with 0.1% FCS). M phase enrichment was achieved by adding colcemid (0.01 µg/ml) to S phase-synchronized cells, acquired by a double thymidine block (2 x 2 mM; 15–15 h) essentially as published previously²⁷. M phase enrichment was accomplished by mitotic shake-off.

Immunofluorescence

Immunofluorescence on methanol-fixed U2OS cells was carried out as described previously²⁶. For immunocytochemical detection of cell cycle inhibitors, cells were infected on glass slides (Superfrost; Menzel GmbH & Co. KG); fixation was accomplished by a 3–5-min incubation in 100% methanol at -20 °C, or a 20-min 3.7% formaldehyde fixation at ambient temperature, followed by a 7-min 100% acetone treatment at -20 °C, and subsequent washing and staining were carried out 3–5 days following infection. Polyclonal rabbit antiserum against recombinant 3pK and GST antisera were obtained from rabbits immunized with purified GST. The anti-Bmi1 mAb F6 and anti-MPh 1 (HPH1) pAb 70092 were kindly provided by Dr. M. van Lohuizen. Other antisera used were anti-2Py mAb (MMS-115R; Babco, Richmond, CA); anti-Bmi1 mAb (6C9; Bmi1O), anti-HPc2 mAb (M9), anti-Ring1A mAb (M3) kindly provided by Dr. A. Otte²⁸; anti-Rnf2 (Ring1B) mAb²⁹; anti-dinG (Ring1B) pAb (Dr. M. Dyer, University of Leicester, Leicester, UK); anti-HPH1 mAb (Dr. H. Koseki, RIKEN Yokohama Institute, Yokohama, Japan), anti-Gsk3β pAb (catalog number 9332; Cell Signaling), anti-AKT pAb (catalog number 9272; Cell Signaling), anti-p14^{ARF}/p16β pAb (Ab-1; LabVision Corp., Fremont, CA), anti-pSer10 pAb (Upstate Biotechnology, Lake Placid, NY), and anti-pSer28 pAb (rat) kindly provided by Dr. M. Inagaki³⁰. Monoclonal primary antibodies were detected with goat-anti-mouse Texas Red polyclonal primary antisera with goat-anti-rabbit fluorescein isothiocyanate.

Transfections and 3pK activation

HEK293 and U2OS cells were transfected at 10–30% confluency. Transfections were done by standard calcium phosphate co-precipitation. Production of infectious viral particles was carried out as described previously³¹. Cells were starved in Dulbecco's modified Eagle's medium with 0.1% (U2OS) or 0.3% (HEK293) FCS for 48 h. For activation of 3pK, the cells were stimulated with 0.2–0.5 mM sodium-*meta*-arsenite (Sigma) or with 5 or 10% FCS with or without 100 ng/ml tetradecanoyl phorbol acetate (TPA) for 1 h before harvesting, unless indicated otherwise. Preincubation with kinase inhibitor UO126³² or SB202190³³ was 20–30 min before activation of the cells.

Immunoprecipitation and differential extraction

Cell lysates were prepared as described previously^{3, 5}. For immunoprecipitation, cells were extracted with ELB buffer^{5, 26}; immune complexes were precipitated with protein A- or G-agarose. Differential extraction was carried out as described previously³⁴. Briefly, the cells were washed twice in phosphate-buffered saline and carefully suspended in 200 µl of phosphate-buffered saline supplemented with 1 mM NaVO₄ and 50 mM NaF and extracted in IP0.1 buffer (60 mM KCl, 15 mM NaCl, 5 mM MgCl₂, 0.1 mM EGTA, 15 mM Tris-HCl, pH 7.4, 300 mM sucrose, 1 mM NaVO₄ and 50 mM NaF) supplemented with 0.3% non-ionic detergent Igepal (Nonidet P-40). The cells were incubated for 3 min at ambient temperature, gently pelleted, and the supernatant was collected (soluble fraction). The pellet was carefully washed once in nuclear buffer and extracted in IP0.1 buffer (20 mM HEPES, pH 7.6, 10% glycerol, 25 mM MgCl₂, 0.1 mM EDTA, 0.2% Nonidet P-40, 0.1 M Potassium acetate, 1 mM NaVO₄, 50 mM NaF; chromatin-bound fraction) as described previously³⁴. Protein extracts were analyzed by standard Western blot technology.

Immune complex kinase assay

GST-3pK and HA-Bmi1 immune complexes were immunoprecipitated separately and combined and washed twice in high salt radioimmune precipitation assay buffer (500 mM NaCl) and kinase buffer (10 mM MgCl₂, 25 mM β-glycerophosphate, 25 mM HEPES, pH 7.5, 5 mM benzamidine, 0.5 mM dithiothreitol, 1 mM NaVO₄³). In control experiments, GST-3pK-containing immune complexes were incubated with Hsp27 as a substrate. The kinase assays were performed at 30 °C for 30–120 min in kinase buffer supplemented with 3–5 µCi [γ-³²P]ATP and 0.02–0.1 mM cold ATP. *In vitro* kinase assays with recombinant protein utilized 1 µg of kinase (3pK-EE) and 5 µg of substrate (His- or GST-tagged Bmi1).

3pK phosphorylates and regulates chromatin association of Bmi1

Figure 4.1. 3pK and PcG proteins associate in yeast and in mammalian cells.

(A) schematic depiction of the HPH2 clones interacting with 3pK (hit-frequency, *left*; arbitrary staining intensity, *right*). Homology domains (*HD-I* and *HDII* boxes) and the zinc finger (*Zn*) and interaction domains required for homo- and heterotypic interactions (---) are shown. Arrows demarcate the coding sequences. Direct two-hybrid binding assay between 3pK(K73M) and the various GAL4-AD-HPH2 fusion constructs are identified in the figure. The relative binding strength (*3pK-binding*), as determined by liquid culture β -gal assay, is depicted ($\pm$). Empty control vectors were negative in all experiments. Mutational analysis of the HDII/SPM/SAM domain in the C-198 aa construct shows that the α 5 motif is crucial for HPH2/3pK interaction. Quantitation is as follows: +++, 30 min (C-73 aa); +, 60–150 min (C-198 aa); $\pm$, 150–360 min (C-198 aa, L417A). (B) HA-tagged full-length Bmi1 co-immunoprecipitates with GST-3pK (*lane 1*) and *vice versa* (*lane 3*) from HEK293 (human embryonic kidney) cell extracts; no interaction is observed in control samples (*lanes 2 and 4*). (C) endogenous PcG complexes from U2OS (osteosarcoma) cell extracts co-precipitate GST-3pK (*left panel*); Bmi1 is co-precipitated with 3pK (*right panel*); overexpression of GST-3pK (GST-3pK-IRES-GFP retroviral vector) is indicated (+). (D) 3pK associates with PcG proteins in HeLa (cervical carcinoma) cells overexpressing Bmi1-PY; Bmi1 precipitates with its binding partners hRnf2 and HPH1. (E) co-immunoprecipitation of endogenous proteins, only, from HeLa cell extracts. Antisera used for immunoprecipitation (IP) are indicated. *ns*, normal serum. Two unrelated kinases, AKT and GSK3 β , were used in control immunoprecipitations. Immunoprecipitation was confirmed by reduced detection in the depleted extract. Antisera used in Western detection are as indicated (arrows): anti-GST (for GST-3pK) and anti-Bmi1 (F6; for either endogenous or overexpressed Bmi1).

Results

3pK binds the C terminus of HPH2 in vitro

In two independent yeast two-hybrid screens using an inactive 3pK (K73M) variant as bait, 20 overlapping clones containing cDNA of the human Polyhomeotic protein (HPH2) were isolated (Fig. 4.1A). These clones represented 73, 145, 198, or 209 C-terminal amino acid fragments of HPH2 (Fig. 4.1A). In a direct yeast two-hybrid test, 3pK also interacted with a larger HPH2 C-terminal fragment of 298 aa and the putative full-length HPH2 (432 aa) (Fig. 4.1, A and B). The C-terminal 3pK interaction domain of HPH2 (Fig. 4.1A) overlaps with the α -helical HDII/ SPM/SAM domain involved in hetero- and homotypic interactions between several Polycomb proteins^{4, 5, 35, 36}. Mutational analysis of the C-198 fragment was used to further investigate the role of the HDII domain in the HPH2/3pK interaction. The HDII domain contains at least five distinct α -helical (α 1– α 5) structures³⁷. C-terminal deletions removing these α -helices prevent HPH2/3pK interaction (Fig. 4.1A, *lower inset*). Removal of the C-terminal-most helix (α 5) is sufficient to completely abolish interaction. The requirement of an intact α 5 was confirmed by the introduction of selected point mutations in α 5 (Fig. 4.1A, *asterisks on lower panel*), which are predicted to be present on the surface of the protein and therefore may be required for the interaction with 3pK. Removing the basic side chain through exchange of K422A prevented interaction between HPH2 C-198 and 3pK completely, whereas exchange of L417A led to a reduction of protein association. 3pK (K73M) displays high affinity for the shorter HPH2 fragments, in particular for the C-terminal 73 fragment (Fig. 4.1A). This may relate to binding-site masking in full-length HPH2. Interestingly, mutation of known phosphorylation sites on 3pK (*e.g.* T313E, T313A, T201E/T313E) does not alter binding affinity as compared with the wild type kinase (not shown), suggesting that HPH2 binding is independent of Thr201/Thr313 phosphorylation. Thus, the HDII/ SPM/SAM domain plays an important role in HPH2/3pK interaction.

3pK complexes with PcG proteins in vivo

Interaction between 3pK and the individual HPH2 fragments in mammalian cells was confirmed by co-immunoprecipitation experiments (data not shown) with results consistent with the quantitative yeast two-hybrid data (Fig. 4.1A). The finding that 3pK is a binding partner of HPH2 suggests that the kinase may be part of a PcG complex and may also be associated with other PcG proteins. Biochemical analysis indeed confirms that HA-tagged Bmi1, a direct binding partner of HPH1/2^{4, 5}, is specifically co-immunoprecipitated with GST-3pK and vice versa (Fig. 4.1B). *In vivo*, GST-3pK co-precipitates with endogenous PcG

proteins, such as (h)Rnf2 (Fig. 4.1C), which is part of the hPRC1 complex^{38, 39}. Exposure of cells to a 3pK-activating agent, the stress-inducer arsenite³, does not alter the interaction quantitatively in these cells (not shown), again suggesting that phosphorylation of 3pK does not affect PcG association. This is in line with the comparable interaction of 3pK phosphorylation- site mutants with HPH2 described above. In a reciprocal experiment, Bmi1 was immunoprecipitated with an anti-3pK antiserum in both U2OS cell extracts (Fig. 4.1C) and HeLa cell extracts with high Bmi1 levels (Fig. 4.1D). Finally, we showed that endogenous proteins co-precipitate (Fig. 4.1E); this interaction was specific, because two unrelated kinases, AKT and Gsk3 β , did not co-precipitate hPRC1 core members (Fig. 4.1E). These biochemical data demonstrated association of 3pK with PcG proteins *in vivo*, without the need for overexpressed binding partners.

Mitogen- and stress-induced phosphorylation of Bmi1 in vitro and in vivo

3pK is a kinase activated by both stress stimuli and mitogenic signals. Activation by mitogens, such as serum and TPA, occurs almost exclusively via the ERK pathway, whereas stress stimulation with arsenite recruits the MAPKAPK to the p38 MAPK cascade³. Thus, we next examined whether PcG proteins may represent downstream targets of these MAP kinase cascades by applying the respective stimuli. We focused on Bmi1, because chromatin association of Bmi1 correlates with its phosphorylation status²⁶. ERK activation, via mitogenic stimulation of serum-starved cells, leads to a rapid and readily detectable phosphorylation of Bmi1 *in vivo*; activation of p38, via arsenite, induces a much stronger hyperphosphorylation of Bmi1 as compared with mitogenic stimulation (Fig. 4.2A). Importantly, serum starvation prior to activation in these experiments precludes cell cycle-dependent phosphorylation²⁶ from interfering with these assays. Phosphatase treatment of PcG proteins extracted from arsenite-exposed cells establishes phosphorylation as the main posttranslational modification on PcG proteins in response to p38 activation (Fig. 4.2B). We then studied the effect of protein phosphorylation on PcG core complex/protein interaction. As indicated in Fig. 4.2C, interaction of known PcG core complex partners, such as HPH1 and HPc2¹⁴, is at least in part preserved, as they still co-immunoprecipitate with Bmi1. Stress (Fig. 4.2D) or mitogenic (data not shown) stimulation result in Bmi1 phosphorylation in HeLa and 293T cells and, importantly, also in primary human TIG3 fibroblasts, showing that the observation is not cell-type restricted or linked to a specific cellular phenotype. Ground-state phosphorylation comparison in the different cell types also reveals that Bmi1 is already phosphorylated in some serum-starved established cell lines (Fig. 4.2, A and D). This would be consistent with a constitutive activation of MAP kinase signaling pathways in some cancer cell lines. Arsenite-induced

Figure 4.2. Mitogen-activated and stress-induced phosphorylation cascades target Bmi1.

(A) signaling through ERK1/2 or p38/SAPK, via serum/TPA or arsenite stimulation respectively, results in Bmi1 phosphorylation *in vivo*. U2OS cells were serum-starved prior to activation. (B) arsenite-induced phosphorylation of Bmi1 and HPH1 in U2OS cells. Immunoprecipitated protein complexes were subjected to phosphatase treatment and detected by Western blot. (C) PcG/protein interactions in response to p38 activation in U2OS cells. Co-precipitation of nonphosphorylated or phosphorylated core complex members from arsenite-stimulated cells shows the PcG core complexes remain largely intact. (D) phosphorylation of Bmi1 following arsenite exposure in 293T, HeLa, and TIG3 (human primary fibroblasts) cells; the latter two cell lines overexpress Bmi1. Western detection was done with anti-Bmi1 (F6). (E) the p38 inhibitor SB202190 partially blocks Bmi1 phosphorylation in U2OS cells. Stress kinase cascade was activated by 0.2 mM sodium arsenite. (F) the ERK inhibitor UO126 blocks MAPK-mediated Bmi1, HPc2, and HPH1 phosphorylation. U2OS cells (E and F) were serum-starved (0.1% FCS) for 2 days prior to stimulation. The MAPK cascade was activated as indicated under "Experimental Procedures." *indicate phosphorylated proteins.

Bmi1 phosphorylation is reduced in the presence of the p38 inhibitor SB202190 (Fig. 4.2E), providing additional evidence for specific catalytic targeting of Bmi1 by the stress-activated phosphorylation cascade, which acts through p38/ SAPK. Arsenite also activates the JNK pathway upstream of 3pK⁴⁰, which may explain the incomplete reduction of Bmi1 phosphorylation upon treatment with the p38 inhibitor. The mitogen-activated protein kinase/extracellular signal-regulated kinase kinase inhibitor UO126 reduces mitogen-induced phosphorylation of Bmi1 (Fig. 4.2F) and of HPH1 and HPc2, two known PcG core complex factors. Finally, the degree of Bmi1 phosphorylation downstream of mitogen- or stress-

activated kinase signaling (Fig. 4.2, A, E, and F), correlates well with the reported difference in 3pK activation by these respective stimuli⁴⁰.

Figure 4.3. Phosphorylation-dependent PcG/chromatin interaction.

(A) Bmi1 visibly dissociates from chromatin upon mitogenic or stress signaling. U2OS cells were serum-starved for 48 h prior to serum/TPA (15 min) or arsenite (45 min) treatment. PcG/chromatin release is partially inhibited by the p38 inhibitor SB202190 (*far right*; compare with Fig. 4.2E). (B) antibodies against a number of different PcG proteins show PcG/chromatin association under serum-starved conditions and PcG/chromatin dissociation following exposure to arsenite. Cells were fixed and permeabilized in 100% methanol, which allows for the detection of chromatin-bound PcG proteins²⁶. (C) differential extraction (see “Experimental Procedures”) of soluble fractions shows extensive chromatin dissociation of Bmi1 upon arsenite stimulation (*ars*), whereas HPH1 release is substantially less. Synchronized G1 and M phase control cell extracts reveal the predicted chromatin association/dissociation, respectively²⁶; loading was corrected for cell numbers. See page 198 for colour print.

Signaling-induced phosphorylation abolishes Polycomb/chromatin association

Next, the effect of MAPK signaling on PcG proteins/chromatin association was examined by immunocytochemistry. Serum-starved/refed cells (ERK1/2-mediated signaling) show a clearly visible decrease of chromatin-associated nuclear Bmi1 domains (Fig. 4.3A)^{26, 41}. Likewise, arsenite exposure (p38-mediated signaling) relieves Bmi1/chromatin association by a process that is partially inhibitable by blocking p38 activity (Fig. 4.3A, *right panel*). In addition to Bmi1, a number of other PcG proteins are released from chromatin. These

include HPc2, hRing1, and hRnf2 (Fig. 4.3B). Diminished fluorescence can be explained in a number of ways. Proteolytic breakdown was excluded by comparing straight lysates in the presence or absence of proteasome inhibitors; detection was unaltered, independent of physiological condition or inhibition of proteolysis (data not shown). Epitope masking by phosphorylation is rather unlikely, because a number of PcG proteins were detected by multiple independent antisera (data not shown). Differential extraction of soluble and chromatin-bound PcG proteins confirms the above immunocytochemical findings. As in M phase control extracts, which mainly contain soluble phosphorylated Bmi1²⁶, upon p38 activation (arsenite), a substantial amount of phosphorylated Bmi1 is retrieved in the soluble fraction (Fig. 4.3C). Interestingly, HPH1 appears to undergo a nuclear redistribution upon exposure of cells to arsenite; consistently, less HPH1 is released from chromatin as compared with other PcG core proteins (Figs. 3B, 4A, and 5E). Also, less phosphorylated HPH1 is extractable as soluble protein from arsenite-stimulated cells, as compared with M phase cells (Fig. 4.3C). Despite this HPH1 “chromatin retention,” a fraction of HPH1 interacts with Bmi1, even when phosphorylated (Fig. 4.2C). Multiple distinct interaction partners for HPH1 may indeed exist in various nuclear (sub)compartments; *Drosophila* polyhomeotic was found in multiple protein interactions, some of which were free from posterior sex combs and polycomb⁴².

p38/SAPK activation may affect chromatin remodeling more directly through phosphorylation of histones⁴³. In analogy to the reported interference of p(phospho)Ser10 with heterochromatin protein (HP1)/3m(trimethyl)Lys9 interaction⁴⁴, it is possible that simultaneous histone H3 phosphorylation and PcG/chromatin association are mutually exclusive. We therefore studied the dynamics of PcG/chromatin dissociation *versus* histone H3 Ser28/10 phosphorylation, as trimethylation at the adjacent lysine 27 residue is required for Polycomb complex/ histone H3 interaction^{12, 15, 16}. Serum-starved U2OS cells showed very low histone H3 phospho-Ser (pSer28; Fig. 4.4A, and pSer10, not shown) levels (Fig. 4.4A and not shown); arsenite exposure led to the accumulation of both pSer10 and pSer28 (Fig. 4.4). There was a good correlation between histone H3 Ser28 phosphorylation and loss of PcG/chromatin association (Fig. 4.4A). Global histone H3 Ser28 phosphorylation varied significantly between cells, whereas individual PcG proteins dissociated or relocated uniformly (Fig. 4.4A). This suggests PcG/chromatin dissociation may occur relatively fast in response to direct phosphorylation by 3pK (or other arsenite-activated kinases) or in response to local histone H3 Ser28 phosphorylation. Kinase assays on N-terminal peptides, however, suggest that H3 and H4 are not direct 3pK phosphorylation targets *in vitro* (HN, VD and JWV, unpublished data). In contrast, pSer10 and PcG/chromatin association are not

mutually exclusive (Fig. 4.4B), consistent with the observation that histone H3 pSer10 phosphorylation is thought to interfere with recognition and binding of the 3mLys9 silencing mark by HP1 chromodomain proteins but not by HPc chromodomain proteins⁴⁴.

Figure 4.4. Post-translational histone H3-tail modifications during stress signaling. (A) histone H3 Ser28 phosphorylation correlates with loss of PcG/chromatin association in U2OS cells. Arsenite-exposed cells show loss of Ring1B binding and the characteristic relocation of HPH1 (see also Fig. 4.3B). Phosphorylation of Histone H3 Ser28 is not detectable in serum-starved cells (*control*), whereas it clearly is upon arsenite exposure. (B) histone H3 Ser10 phosphorylation in cells does not correlate with PcG/chromatin dissociation in arsenite-exposed cells. Shown is an example of cells in which histone H3 Ser10 phosphorylation in response to arsenite, as well as the typical punctate nuclear PcG staining, is clearly visible. Histone H3 Ser10 phosphorylation in control cells (serum starved) is non-detectable (not shown). *Arrows* indicate cells positive for both pSer10 and chromatin-bound PcG complexes; cells were exposed to arsenite on glass slides and fixed in formaldehyde/acetone. DAPI, 4',6-diamidino- 2-phenylindole. See page 198 for colour.

Identification of 3pK as a candidate Bmi1 kinase

We studied 3pK as a candidate Bmi1 kinase by *in vitro* kinase assays and by stimulation of cells with altered 3pK expression levels. Using recombinant proteins, we showed that Bmi1 is a direct phosphorylation target for 3pK *in vitro* (Fig. 4.5A). For additional *in vitro* kinase assays, active 3pK was obtained by mitogen or arsenite stimulation of cells³. Immunoprecipitated Bmi1 is strongly phosphorylated in the presence of the active kinase (Fig. 4.5B, *lower panel*),

similar to levels obtained with the control substrate Hsp27 (Fig. 4.5B, *lower panel*). Preliminary data obtained with PcG complexes purified by immunoprecipitation suggest that other PcG complex members (HPH1, HPc2) are also directly phosphorylated *in vitro* by 3pK (BN and SL, unpublished data). To provide additional evidence for phosphorylation of Bmi1 by 3pK *in vivo*, genetically matched cells with increased or diminished 3pK expression were used in the phosphorylation assays. The expression of a nucleus-retained active 3pK mutant results in more intensive Bmi1 phosphorylation after serum stimulation compared with wild type cells (Fig. 4.5C). Serum stimulation reveals subtle but clear differences in phosphorylation between control and 3pK-overexpressing cells (Fig. 4.5D); Bmi1 is phosphorylated more rapidly and more intensely in 3pK-overexpressing cells. We were able to relate these findings to specific biological effects of 3pK overexpression (HN and JWV, manuscript in preparation). Conversely, in cells in which 3pK is knocked down by RNA interference²⁵ using two independent RNA interfering sequences (Fig. 4.5E, *lanes 1 and 3*), decreased phosphorylation of Bmi1 correlates nicely with decreased 3pK levels. These findings connect 3pK expression levels and Bmi1 phosphorylation *in vivo*. Cells knocked down for 3pK expression retain Bmi1 and HPH1/chromatin association upon arsenite stimulation (Fig. 4.5F), whereas cells overexpressing 3pK show a more rapid Bmi1 release (data not shown), again directly implicating 3pK in this process. Combined, these findings strongly suggest that PcG proteins are phosphorylated downstream of activated MAPK (ERK/p38) pathways and identify 3pK as a Bmi1 kinase.

Overexpression of 3pK reduces PcG complex/chromatin association

To qualify as a PcG complex-associated Bmi-1 kinase, 3pK would be expected to associate with chromatin. Indeed, differential nuclear extraction of cells overexpressing human 3pK constructs shows, in line with the immunoprecipitation data presented above, that a fraction of the wild type 3pK protein is associated with chromatin (Fig. 4.6A). Neither constitutively active nor kinase-inactive mutants of 3pK associate with chromatin to the extent wild type 3pK does (Fig. 4.6A), in concordance with observations that such mutants are mainly found in the cytoplasm⁴⁵⁻⁴⁸. Combined with the immunoprecipitation data, this demonstrates that at least a fraction of cellular MAPKAP kinase 3 is associated with PcG complexes at the chromatin level.

Because repressive PcG complexes are intimately associated with chromatin and MAPK-induced PcG phosphorylation results in chromatin dissociation, it would follow that 3pK activation or overexpression causes PcG/chromatin dissociation, which would then result in de-repression of PcG target genes. To begin to understand the biological relevance of PcG complex phosphorylation by 3pK, we studied the cellular function of 3pK. 3pK overexpression induces a profound

3pK phosphorylates and regulates chromatin association of Bmi1

Figure 4.5. Identification of 3pK as a candidate Bmi1 kinase.

(A) 3pK directly phosphorylates Bmi1. Recombinant GST-3pK (T201E/T313E) and recombinant His-tagged Bmi1 protein were used in *in vitro* kinase assays. (B) *in vitro* kinase assays show that immunoprecipitated Bmi1 is phosphorylated by 3pK. Active GST-3pK was obtained from HEK293 cells (arsenite- or serum/TPA-stimulated for 60 min). HA-Bmi1 substrate and kinase were immunoprecipitated separately and then combined in kinase assays. Hsp27, a known 3pK substrate, was used in control reactions (*lower panel*). (C) increased *in vivo* phosphorylation of Bmi1 by a nucleus-retained (NR) 3pK mutant, which lacks the C-terminal (aa 308–383) regulatory region. Lane c, serum-starved cells; control cells or cell expressing NR-3pK were serum-stimulated (10% FCS), following which Bmi1 was immunoprecipitated from cell extracts. P32 incorporation was determined by autoradiography. (D) exposure to serum (5%) reveals differences in the kinetics of Bmi1 phosphorylation between control and 3pK overexpressing U2OS cells; time (min) between start of stimulation and harvest is indicated. (E) reduced Bmi1 phosphorylation in response to hsRNA-mediated 3pK reduction. Various stably expressed hsRNAs (*lanes 1–3*; see “Experimental Procedures”) reduce 3pK protein levels (*upper panel*); diminished Bmi1 phosphorylation upon mitogenic stimulation correlates well with lowered 3pK expression levels (*lower panel*). Lane 0 is an empty vector control. U2OS cells were serum-starved prior to activation (*lower panel, far left lane c*). (F) reduced chromatin dissociation of PcG proteins Bmi1 and HPH1 upon arsenite stimulation of cells correlates with 3pK expression in U2OS cells. Cells were treated as described in the legend to Fig. 4.3A. RNA interfering sequence used: 1 (see Fig. 4.5C and “Experimental Procedures”). See page 195 for colour print.

effect on cell proliferation; cell cycling slows down significantly or arrests, depending on the cell type studied.⁴ We therefore asked whether overexpression of 3pK induces de-repression of the *Cdkn2a/INK4A* locus, a well established Bmi-1 target^{49, 50} that is transcriptionally silent in U2OS cells⁵¹. 3pK-

overexpressing cells showed reduced (or loss of) nuclear PcG staining (Fig. 4.6C, *middle panel*). In agreement with this, in U2OS cells overexpressing 3pK, less Bmi1 is present in the chromatin-bound fraction as compared with control cells (Fig. 4.6B), which supports the idea that 3pK overexpression results in chromatin release of Bmi1. Cells that have lost PcG/chromatin association show re-expression of p14^{ARF}, one of the gene products of the *Cdkn2a/INK4A* locus (Fig. 4.6C). The above findings are consistent with the notion that PcG repression needs to be lost to de-repress a target gene.

Discussion

We here identify 3pK as a component of PcG complexes. Initially identified in a yeast two-hybrid screen as a binding partner of HPH2, 3pK was subsequently shown to associate with chromatin and PcG complexes by biochemical analysis. The region within HPH2 responsible for 3pK binding is the HDII/SPM/SAM domain, which also mediates homo- and heterotypic interactions with other PcG proteins, such as SCM and Bmi1^{4, 5, 35, 36}. We established an intact $\alpha 5$ motif as a crucial structure for HPH2/3pK interaction within the HDII domain. MAPKAP kinase 2, a close homolog of 3pK, was recently found to bind HPH2 in a yeast two-hybrid screen⁵². The interaction region was mapped to the C-terminally located HDII domain as well. In addition, full-length MPh1 (mouse Polyhomeotic) interacts with 3pK in direct interaction assays.³ This combined information supports the notion that the HDII/ SPM/SAM domain plays an important role in HPH/3pK interaction and suggests that complex composition may vary with cell type or physiology, or alternatively, that there may be ternary complex formation between HPH1/2, Bmi1, and 3pK or other binding partners *in vivo*.

3pK phosphorylates Bmi1 and possibly other PcG proteins *in vitro*, and 3pK overexpression or knockdown in genetically matched cell lines results in enhanced and reduced Bmi1 phosphorylation *in vivo*, respectively. In addition, we showed that the typical punctate nuclear PcG staining pattern is lost upon ERK or p38 activation. This implicates a connection between MAP kinase signaling and chromatin structure remodeling through modulation of PcG complex function. A simple mechanistic model proposes that PcG-associated 3pK is an integral regulatory element within PcG complexes; 3pK phosphorylation and activation by upstream nuclear shuttle kinases (ERK, p38 and possibly JNK) results in phosphorylation and subsequent chromatin dissociation of PcG complexes from known chromosomal binding sites²⁶. Although chromatin dissociation is expected to result in reactivation of repressed genes, it is currently unknown what the molecular effect of protein phosphorylation is on PcG members. Phosphorylation is thought to affect

Figure 4.6. Overexpression of 3pK releases chromatin-associated Bmi1 and de-represses *Cdkn2a/INK4A*.

(A) GST-tagged wild type 3pK is chromatin-associated. Cells expressing wild type GST-3pK or mutant GST-3pK (K73M, TT201/313EE, respectively. *KM* and *EE* in figure)³ were differentially extracted. Shown are the chromatin-bound protein fractions. In concordance with published data⁴⁸, only wild type 3pK resides in the nucleus. (B) 3pK overexpression reduces chromatin association of Bmi1 (*right panel*). Shown is the chromatin-bound fraction of differential nuclear extracts. Cells were kept under high selection pressure (puromycin) for two passages. (C) reduced PcG/chromatin association (*middle panel*) and increased p14^{ARF} expression (*right panel*) in 3pK-overexpressing U2OS cells. U2OS cells were infected on glass slides with GST-3pK-IRES-GFP retrovirus; infection was confirmed by fluorescence. Under these conditions, 16–20% ($n > 150$) of the cells have lost the typical nuclear PcG domains (*arrowhead*). The majority of these cells is positive for p14^{ARF} expression. Control cells, infected with empty vector, showed neither PcG dissociation nor p14^{ARF} expression ($n > 250$). (D) connection between cell signaling and epigenetic regulation of gene expression via chromatin remodeling factors. MAPK phosphorylation cascades are known to target transcription factors (*TF*, *right*) and chromatin (nucleosomes, *left*) directly. We propose that activated MAPK cascades (schematically depicted by *circles on arrow*) also target chromatin (*beads*) through modulation of chromatin remodeling complexes (*gray structures*), such as PcG complexes. Activation of 3pK leads to phosphorylation of PcG complexes; subsequent chromatin dissociation results in de-repression of silenced loci. See page 199 for colour print.

subcellular localization or homodimerization of some PcG proteins^{53, 54}. We find some relocation of PcG proteins to the cytoplasmic fraction (JWV, unpublished data). However, the fate of dissociated PcG complexes, as well as that of the activated MAPKAP kinase, is unclear, as studies with proteasome inhibitors do not show marked proteolytic breakdown. Given our observation that at least

part of the mammalian PcG protein core complex¹⁴ remains intact upon phosphorylation, it is unlikely that Bmi1 phosphorylation directly causes chromatin dissociation. A more likely scenario is that PcG/chromatin binding is mediated by critical amino acid residues on a (limited number of) PcG complex member(s) in a phosphorylation-dependent manner. Potentially interesting candidates are the mammalian orthologs for Pc (HPc1/2/3), which may directly bind histone H3 3 m (trimethyl) Lys27-silencing imprints through its chromodomain. It is possible that, besides chromatin association, protein/protein interaction and/or (associated) catalytic activity of individual PcG proteins is altered upon phosphorylation. In this light, the redistribution of HPH1 is interesting. Although the exact implications of this HPH1-redistribution are currently not clear, it may well serve to stall DNA replication and or gene expression upon activation of the stress kinase pathway through its interaction with DNA replication inhibitors^{55, 56}. Likewise, recently identified enzymatic activities of PcG proteins (*i.e.* histone methyltransferase activity of EZH2)¹², small ubiquitin- like modifier E3 ligase activity of HPc orthologs⁵⁷, and ubiquitin E3 ligase activity for the Ring finger proteins (such as Ring1B⁵⁸ and possibly Bmi1) are likely to be affected by phosphorylation. Clearly, identification of relevant cellular substrates of these enzymes and mutation analysis of relevant phosphorylation sites are essential to increasing our understanding of the exact role of phosphorylation in PcG-mediated silencing.

ERK and/or p38 and JNK MAP kinases and downstream MAPK effector kinases (one of which is 3pK) are implicated in a variety of fundamental cellular processes, including proliferation, differentiation, and cell cycle regulation¹. The involvement of PcG and trxG proteins in development and cancer is well documented (reviewed in Ref.⁷). Similar to 3pK, we have also observed *in vitro* HPH2 binding (as previously reported⁵²), Bmi1 phosphorylation by MAPKAP kinase 2, and reduced chromatin dissociation upon knockdown of MAPKAP kinase 2 protein levels (BN, SL, URR and JWV, unpublished data). These observations suggest that additional kinases may target PcG proteins downstream of active MAPK cascades. Interestingly, other kinases within the MAPKAP kinase family act on histones⁵⁹, further supporting the idea that such kinases, besides directly activating transcription factors, also target chromatin components and chromatin remodeling factors (Fig. 4.6D). Phosphorylation of residues (*e.g.* histone H3 pSer10 or pSer28) adjacent to silencing marks (*e.g.* histone H3 3 m Lys9 or 3 m Lys27) could act as a (re-)activating event⁴⁴ and serve to release repressive (HP1 or PcG, respectively) effector complexes and effectively de-repress target genes. In accordance with this “binary switch” model⁴⁴, our data suggest that PcG/chromatin dissociation is a relatively early event and may be directly connected to local phosphorylation of histone H3 Ser28; *i.e.* histone H3 Ser28 phosphorylation at target loci is sufficient to release

PcG complexes from chromatin. The co-existence of pSer10 and Polycomb complexes in arsenite-stimulated cells, however, seems to rule out a major role for histone H3 pSer10 in PcG/chromatin dissociation, in line with a specific role for H3 3mLys9 in HP1-mediated heterochromatin formation⁶⁰. However, to formally exclude the possibility that these respective phosphorylation events are independent, detailed examination of the histone H3 Ser28 status at target genes in relation to PcG/chromatin association is required.

Bmi1 and its direct binding partners, Rnf2, MPh1 and M33, through transcriptional control of the *Cdkn2a/INK4A* locus, are involved in proliferative control during embryogenesis and postnatal life and in stem cell renewal in hematopoiesis and neurogenesis⁶¹⁻⁶⁵. We have studied the effect of 3pK on cell cycle progression and its genetic interaction with PcG proteins in detail.4 Relevantly, overexpression of 3pK de-represses the *Cdkn2a/INK4A* locus, further supporting the interaction of MAPK signaling and PcG-dependent chromatin remodeling. Both mitogenic as well as stress activation lead to PcG/chromatin dissociation. Clearly, different target genes are predicted to be (de)activated in response to these different physiological stimuli. Recent findings indicate that 3pK activation and translocation kinetics are dependent on the pathway through which it is activated^{3, 48}; this is expected to influence the type of substrates and genes targeted by activated kinases downstream of mitogens or cellular stressors.

In summary, we have described a novel link between signaling through MAP kinase cascades and an epigenetic transcription regulatory system. We propose this as a molecular mechanism by which cells modulate gene expression profiles to adequately respond to changes in their microenvironment; consequentially, PcG-mediated silencing may be subject to dynamic modulation. Chromatin association of both trxG and PcG protein complexes has been reported to depend on their phosphorylation status^{26, 34}. We propose that MAPK signaling impinges on gene expression through functional regulation of chromatin-modifying complexes via PcG and trxG kinases and/or phosphatases (Fig. 4.6D). Unraveling the nature of kinase/PcG and PcG/chromatin association and the exact biological significance of phosphorylation for PcG function presents an exciting and relevant challenge for future research. It will be of particular interest to examine whether and how this molecular connection relates to the proposed role of MAPK pathways as switching mechanisms that modulate gene activity². PcG, trxG proteins, and MAPK signaling pathways are connected to tumorigenesis; the hereindescribed findings will improve our understanding of how cells modulate gene expression at the epigenetic level in response to extracellular cues and have the potential to yield therapeutic strategies to fight cancer.

Acknowledgments

We are much indebted to R. Agami, T. Brummelkamp, M. Dyer, M. Inagaki, H. Koseki, A. Lund, A. Otte, D. Peeper, D. Shvarts, M. van Lohuizen, M. Vidal, and M. Ummelen for sharing reagents and M. van Lohuizen and B. Wouters for useful comments to the manuscript.

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MK3 controls proliferation by an epigenetic switch-mechanism involving Polycomb function

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Abstract

Polycomb (PcG)-chromatin association, although crucial for maintenance of gene repression profiles, is subject to dynamic regulation during development. Molecular insight in processes that control PcG-chromatin association is limited. We previously showed that activation of MAP/SAPK signaling triggers phosphorylation of PcG proteins through the kinase MK3. We report here that cells regulate proliferation and replicative life span through MK3. Importantly, MK3 alleviates PcG-chromatin binding at target loci without concomitant changes in H3K27 trimethylation. Our studies uncover a novel MK3-dependent epigenetic switching mechanism at PcG target loci in cell cycle control and support a role for MK3 in chromatin structure dynamics.

Introduction

During its lifetime a cell integrates multiple signals, both internally and from its environment to generate appropriate biological responses. These responses are often initiated through changes in gene expression that arise as a result of changes in the rates of synthesis, activity, stability and sub-cellular localization of RNAs and proteins. At the chromatin level, gene-environment interactions are, besides activation and/or recruitment of transcription factors, determined by epigenetic control over chromatin structure and nucleosome movement.

Polycomb Group (PcG) repressive complexes (PRC) act as part of a cellular transcriptional maintenance system; as such they play an important role in cell fate determination. Recent genome-wide ChIP studies revealed that PcG-chromatin association throughout development is subject to dynamic changes: lineage commitment of *e.g.* embryonal stem cells is accompanied by redistribution of PcG complexes across chromatin and establishment of novel lineage-relevant transcriptionally active domains^{1, 2}. The molecular mechanisms that underlie this dynamic relocation of repressive PcG complexes are currently unknown.

PcG proteins are functionally subdivided in two complexes. Polycomb repressive complex 2 (PRC2) contains SUZ12, EED and EZH2, the latter harbors histone lysine methyl transferase (HMT) activity toward histone 3 on lysine 27³. The PRC1 core consists of at least 4 PcG subclasses: BMI1/Mel18 (PCGF4/2), CBX2 (HPC1), CBX4 (HPC2), CBX6, CBX7 or CBX8, PHC1-3 (HPH1-3, EDR1-3) and RING1/RNF2 (Ring1A/1B)^{4, 5}. PRC1 and PRC2 function were shown to be linked in *Drosophila melanogaster*, in that the CBX homologue Pc binds to H3K27me3^{6, 7}. Recently we demonstrated that mitogen-activated protein kinase-activated protein kinase 3 (MAPKAPK3; also known as MK3 or 3pK) phosphorylates PcG proteins⁸. Mitogenic signaling through GFRs (growth factor receptors) activates p21RAS. This subsequently triggers various downstream signaling cascades, among which MAPK/MAPKAPK pathways⁹, leading to changes in gene expression. Signaling to MK3 through SAPKs (JNK, p38) or MAPK (ERK)¹⁰ triggers distinct cellular responses (*i.a.* cell cycle arrest and proliferation). In cancer, oncogenic p21RASV12-signaling ultimately induces senescence as part of a fail-safe mechanism to oncogenic transformation¹¹. Both MAPK and SAPK signaling have been implicated in replicative life span¹². Given its crucial positioning at convergent mitogenic and stress signaling, it is conceivable that MK3 fine tunes signaling in the context of cell cycle regulation.

Insight in signaling pathways that cells use to affect chromatin responses is limited. As PcG-phosphorylation correlates with dissociation from chromatin^{8, 13}, it is tempting to speculate on a function for MK3 in regulating PcG complex-mediated transcriptional repression. Here we hypothesized that MK3 activity

Figure 5.1. MK3 induces cell cycle arrest and a senescence-like state. (A,C) Overexpression of MK3 reduces cell proliferation of primary human TIG3 fibroblasts (A) or osteosarcoma U2-OS cells (C) expressing murine Bmi1 (open triangles) or MK3 (open circles) or an empty vector control (filled circles). (B, D) TIG3 (B) or U2-OS (D) cells overexpressing MK3 (MK3) display a replicative block and large flat cell phenotype. MK3 expressing large flat cells are positive for senescence-associated β -Galactosidase (SA- β Gal; B: panel bottom right). Arrows (D) mark the outline a large flat cell. (E) The effect of MK3-OE is dependent on the intensity of mitogenic stimulation: decreased serum concentrations reduce proliferation in MK3 overexpressing U2-OS cells (squares: 10% FCS; circles: 5% FCS; triangles: 2% FCS; filled symbols: con; open symbols: MK3). Indicated are percentages growth reduction relative to control cells. (F) MK3.i-res.GFP-OE in U2-OS cells induces senescence. Arrows: large flat cells positive for GFP (left panel) and SA- β Gal (right panel). See page 199.

regulates PRC1-chromatin association, which results in derepression of PcG target genes. We show that overexpression of MK3 expression suppresses cell proliferation and induces a senescence-like state similar to loss of Polycomb function. We observe that MK3 binds to known PcG target gene promoters in human fibroblasts, and that activation of MK3 induces dissociation of the PRC1 protein complex, without the need for concomitant changes in local H3K27 methylation. Our data suggest a novel rapid epigenetic molecular switching-mechanism mediated by MK3 phosphorylation of PcG proteins leading to a dynamic regulation of relevant target genes in response to activated signaling cascades.

Results & Discussion

MK3 overexpression reduces proliferation and induces a senescence-like arrest

Overexpression of MK3 (MK3-OE) in primary human TIG3 fibroblasts significantly reduces proliferative capacity (Fig. 5.1A, 5.S1A,B). MK3-OE in TIG3 cells induces a large flat cell morphology that was accompanied by expression of the senescence-associated marker SA- β Gal (SA- β -Galactosidase; Fig. 5.1B). Although MAPKs and SAPKs have been linked to regulation of hTERT-function^{14, 15}, the observation that MK3-induces a similar morphology in hTERT-immortalized human fibroblasts cells (Fig. 5.S1C), suggests that MK3 acts either downstream or independent of hTERT. The human cancer cell lines U2-OS (Fig. 5.1C) and HeLa (data not shown) also show reduced proliferation following MK3-OE. Remarkably, also the U2-OS and HeLa cell lines show an enhanced flat cell phenotype (Fig. 5.1D, 5.S1D) upon MK3-OE and concomitant SA- β Gal activity (Fig. 5.1F). Little to no apoptosis or cell death was observed in response to MK3-OE (Fig. 5.S1B). The MK3-OE phenotype resembles that of loss of PcG function: loss of PRC1 proteins such as Bmi1 or PHC2 induces a flat cell phenotype in U2-OS cells (Fig. 5.S1E,F) and has been associated with premature senescence in mouse fibroblasts^{16, 17}. Our observations suggest a potential role for MK3 in regulation of replicative life span.

Modulation of MK3 proteins levels is expected to result in a proliferative-signaling imbalance. Acute loss (stable short hairpin (sh)RNA-assisted knock-down) of MK3 function (MK3-KD) in primary human fibroblasts consistently results in a cell cycle arrest (Fig. 5.S2A,B,C). In addition to an expected mitogen concentration-dependent reduction in proliferation rate (Fig. 5.1E), serum reduction enhanced the large flat cell phenotype in both primary human fibroblasts as well as in U2-OS cells overexpressing MK3 (Fig. 5.S2D,E). Hence, mitogenic signaling appeared to balance-out a senescence program set-off by MK3-OE.

Figure 5.2. Expression analysis of cell cycle regulators in MK3-expressing senescent cells. (A) Upregulation of the check point regulator proteins P53 and p21^{CIP1/WAF1} in response to MK3-OE in primary fibroblasts. (B, D) Co-expression of P53 (B), p21^{CIP1/WAF1} and p14^{ARF} (D) in MK3 positive senescent TIG3 cells. (C) TIG3 fibroblasts deficient for P53 (shP53) are insensitive to the 3pK-induced cell cycle arrest (open squares: MK3-OE + shP53; closed squares (empty vector control + shP53)). Compare proliferation profiles to those in Fig. 5.1a. (E) Co-expression of p21^{CIP1/WAF1} and p14^{ARF} and P53 and P14^{ARF} in MK3 positive senescent (arrow) U2-OS cells. See page 200 for colour.

MK3 induced senescence is mediated by p53 and the INK4A locus

We studied the role of MK3 in RASV12-oncogene induced senescence (OIS), a known senescence program. Whereas either RASV12-OE or shMK3 induce replicative arrest in primary fibroblasts (Fig. 5.S3A), MK3-KD transiently prevents

RASV12-induced proliferative arrest in a dosage-dependent manner, indicating that MK3 relays oncogenic signaling by RASV12. RASV12-induced senescence involves p53 signaling¹¹. P53 expression is upregulated in MK3-OE cells (Fig. 5.2A,B, 5.S3B) and loss of P53 bypasses MK3-OE dependent proliferative arrest (Fig. 5.2C). P53 and p21^{CIP1/WAF1} are both upregulated in MK3-OE cells (Fig. 5.2A,D) in accordance with a P53/p21^{CIP1/WAF1} dependency of early senescence¹⁸. Cells expressing nuclear P53 or p21^{CIP1/WAF1} are also positive for p14^{ARF} (Fig. 5.2D,E), which is, like p16^{INK4A}, associated with replication check-points in human cells¹⁹. Although p14^{ARF} may stabilize P53²⁰, we can not formally rule out possible independent roles for P53 and p14^{ARF} and p21^{CIP1/WAF1} in the MK3-OE induced replicative arrest^{21, 22}. Senescent fibroblasts show clear nuclear p16^{INK4A} accumulation under these experimental conditions (Fig. 5.S3C); this was confirmed by immuno-blotting (Fig. 5.4C). The upregulated expression of p16^{INK4A} upon prolonged MK3 overexpression is in good agreement with a crucial role for p16^{INK4A} in establishing irreversible senescence²³⁻²⁵.

We previously reported that MK3-OE reduces chromatin association of PRC1-complex members Bmi1, CBX4 and RNF2 and induced re-expression of p14^{ARF}, one of the gene products encoded by the CDKN2A/INK4A locus⁸ and a confirmed PcG-target²⁶. We used chromatin immunoprecipitation (ChIP) to prove a direct association of MK3 to CDKN2A/INK4A in primary human TIG3 fibroblasts. H3K27me3 and CBX8 enrichment at the p16^{INK4A} promoter and at the p16-exon1 were confirmed: MK3 enrichment is observed at the CDKN2A/INK4A locus and increases when MK3 is overexpressed (Fig. 5.4A).

Genetic interaction between MK3 and Polycomb function in senescence

The PRC2 protein EZH2, generates the repression-associated H3K27me3 mark at target genes³. EZH2 levels are reduced in MK3-OE primary TIG3 cells (Fig. 5.4C, 5.S5A), in line with a previously reported senescence-associated decline in EZH2 levels²⁶. In accordance with this, MK3-OE reproducibly decreases global H3K27me3 in HeLa cells (Fig. 5.3A). Bmi1-OE counters the global loss of H3K27me3 (Fig. 5.3B). We further explored a possible genetic interaction between PRC1 and MK3 in the context of cell cycle regulation. Co-expression of Bmi1 and MK3 in primary human fibroblasts cells restores proliferation rate (data not shown) and bypassed senescence-associated morphological changes induced by MK3-OE alone (Fig. 5.3C). In line with the above data simultaneous Bmi1-KD and MK3-OE in U2-OS cells causes a strong additive effect on induction of flat cell morphology (Fig. 5.S3D). Relevantly, serially transduced Bmi1+MK3 fibroblast cultures auto-select for increased Bmi1 levels over time (Fig. 5.3D), demonstrating that cells utilize Bmi1 to overcome the adverse effects of MK3-OE on cell proliferation. Our findings support a strong genetic and biochemical interaction between MK3 and PRC1-function, with clear biological relevance to

Figure 5.3. Genetic interaction between MK3 and Bmi1. (A) MK3-overexpressing HeLa cells show reduced global H3K27me3. Quantification of H3K27me3 was done by densitometry and normalized against β-actin levels. (B) Overexpression of Bmi1 in MK3 transduced HeLa cells corrects H3K27me3 levels. (C) Reversal of MK3-induced proliferative and morphological alterations by overexpression of Bmi1 in primary human fibroblasts. Cells were sequentially transduced with either Bmi1.i^{res}.GFP (Bmi1) or control (con) virus, and MK3 (MK3) or control virus (con) at 48 hour intervals. (D) Serially transduced cultures (see C) were grown for 25 days and analyzed for MK3 and Bmi1 expression. See page 201 for colour print.

proliferative control. MK3 activates cell cycle checkpoints involving known tumorsuppressor pathways and counteracts the oncogenic effects of Bmi1. This is consistent with MK3 loss-of-function in several malignant cancers and hence a potential tumorsuppressive role²⁷. However, our findings are also consistent with a pro-oncogenic role for MK3: MK3-OE induces similar cellular responses as RASV12, RASV12-induced OIS is dependent on MK3. A recent study on NMYC

suggested that the outcome of oncogenic signaling imbalance, *i.e.* proliferation (oncogenic) or apoptosis (anti-oncogenic), is dependent on critical threshold NMYC expression levels and, equally relevant, on the cells' intrinsic (*i.a.* genetic) state and its microenvironment²⁸. In line with this notion, we show that changes in the mitogenic environment of MK3-OE cells significantly affects MK3 induced senescence. MK3 mutations were recently suggested to be potentially oncogenic, in contrast to MK2 mutations²⁹. Although the exact role of these mutations in tumorigenesis awaits further analysis, our data support differential cellular functions for MK3 and MK2. This is corroborated by our finding that, in contrast to MK2³⁰, MK3 is not required for UV induced arrest (Fig. 5.S4A,B,C). Combined with the recent finding that MK5 (PRAK), although required for RAS-OIS, by itself is not capable of inducing senescence³¹, these data further support the idea that MAPKAP kinases although related, may have acquired divergent cellular context dependent functions.

MK3-PRC1: an epigenomic dimmer switch

We next studied regulatory protein occupation at known PcG-target loci using ChIP. Overexpression of MK3 only minimally affects H3K27me3 levels at the *p16* promoter, whereas H3K27me3 is slightly reduced at *p16* exon 1 (Fig. 5.4A). Relevantly, the MK3-OE TIG3 cells used in this experiment express more *p16*^{INK4} and display the distinctive senescence phenotype (Fig. 5.4C, 5.S5A,B). Non-PcG-targets (*i.a.* *p15*exon1, *CCNA2*) show little to none CBX8 occupation (Fig. 5.4A, 5.S5D), in line with earlier reports^{26, 32}. Remarkably, although CBX8 and PHC1 are both part of the PRC1 complex, they show distinct chromatin association in response to MK3-OE: whereas local CBX8-chromatin occupation is substantially reduced, (*e.g.* *ATF3*, *p16* exon1), PHC1 binding appears mostly increased (Fig. 5.4A, 5.S5D), indicating PHC1 occupation occurs without the need for simultaneous CBX occupation, and suggesting differential regulation between members of the same complex. This is consistent with our previous report that PHC behaves differently in response to MK3 activation compared to PRC1 proteins like BMI1, CBX4 and RNF2⁸. In agreement with this, PHC-association to the PRC1 core complex was reported to be comparatively weak³³ and purified PHC1 was shown to reside in multiple complexes, some of which are non-PRC complexes³⁴. Hence, nuclear PHC1 may fulfill multiple functions. Interestingly, MK3 and PHC1 show a very similar enrichment profile (Fig. 5.4A, 5.S5D), also at non-PcG targets (*e.g.* *p15* and *CCNA2*). This is in good agreement with our previously reported direct interaction between MK3 and PHC proteins⁸. To obtain evidence for direct phosphorylation of PRC1 proteins by MK3, we performed an *in vitro* kinase assay using a PRC1 peptide spot array, covering potential phosphorylation sites in Bmi1, Cbx4, Phc2, Rnf2 and histone H3.3, that adhere to the common MK phosphorylation motif RxxS (Fig. 5.S6A). We

Figure 5.4. MK3-overexpression or activation regulates PRC1 chromatin association and gene activity. (A) ChIP analysis at known PcG target loci: CDKN2A/INK4A and ATF3 locus in MK3-OE (MK3) and control (con) TIG3 cells. p15 exon1 acts as a negative control. Enrichments are presented as percentages of total input. (B) ChIP analysis in TIG3 cells, after 48hrs of serum starvation with 0.1%FCS followed by either no or 45 min. stimulation with 15% serum and 100ng/ml TPA. (C) Western analysis of cell lysates prepared from TIG3 cells expressing MK3 or control cells. P14^{ARF} was not detectable in either control or MK3 OE TIG3 cells. (D) real time quantitative ATF3 mRNA analysis upon 45 minutes and 90 minutes serum stimulation.

previously established that MK3 activation leads to PRC1 phosphorylation⁸. We now confirm that PRC1 peptides, in this specific assay are, targets for MK3 phosphorylation (Fig. 5.S6B). A much stronger MK3-induced phosphorylation is detected on 3 Phc2 peptides and 1 Cbx4 peptide. Peptides in which the R in the RxxS-motif is substituted by an A or synthetically pre-phosphorylated are not phosphorylated in the kinase assay (not shown), confirming the specificity of the assays and indicating that indeed the Serine residues within the RxxS MK3-motif are phosphorylated. Neither Rnf2 nor H3.3 peptides or full length H3.3 were phosphorylated by MK3 *in vitro*. These data show that MK3 targets multiple PRC1 proteins for phosphorylation. It is therefore tempting to speculate that MK3-OE affects PRC1-chromatin occupancy through phosphorylation and disruption of complexes.

As decreased EZH2 protein levels in MK3-OE TIG3 (Fig. 5.4C) may contribute to loss of global H3K27me3 and indirectly cause CBX8 displacement, we also studied PRC1-chromatin occupation in the context of a relevant physiological mitogenic stimulus. Bmi1 and CBX8 protein levels do not change under these conditions, and Bmi1-phosphorylation was confirmed upon mitogenic stimulation (Fig. 5.S5C). MK3-chromatin occupation is overall reduced in response to mitogenic stimulation (Fig. 5.4B, 5.S5E). Importantly, a relatively short mitogenic stimulus (45 minutes) does not change H3K27me3 levels at the *CDKN2A/INK4A* locus, but is sufficient to reduce CBX8 binding at all Polycomb targets (Fig. 5.4B). Changes in PHC1 occupation do not match that of CBX8: PHC1 increases at a number of loci (*p16* promoter, *p14^{ARF}* exon1) (Fig. 5.4B, S5E). Conversely, chromatin at confirmed PRC1-targets (*ATF3*, distal promoters *HOXA* cluster;³²) clearly reveals CBX8 displacement in response to stimulation, despite unchanged H3K27me3 levels (Fig. 5.4B, S5E). When combined with gene expression measurements, ChIP analysis revealed a clear correlation between loss of both CBX8 and PHC1 and activation of gene expression at the *ATF3* locus (Fig. 5.4D). In contrast, loss of CBX8 only (PHC1 increased or unchanged) did not correlate with gene expression at other loci (Fig. 5.S5F). Hence, this data suggests that initial loss of PRC1-chromatin association but not removal of H3K27me3, correlates with gene expression activation and that all PRC1 core complex members may need to dissociate at crucial regulatory regions to allow for active transcription. We propose that signaling through MK3-PRC1 changes the local epigenomic state through progressive loss of PRC1 members from a repressed/poised state^{1, 2, 32}, to a 'de-repressed' and eventually fully transcriptionally active state (Fig. 5.5). Intergenic similarities in dynamic PcG/chromatin changes as well as intragenic variation in response to MK3-OE and serum stimulation, may point to global chromatin structure regulation in addition to complex local regulation, respectively. Genome-wide analysis of

Figure 5.5. Hypothetical model for MK3/PcG interaction at target genes. (A) Bmi1, Ring, CBX and PHC proteins together form the PRC1 core complex, CBX proteins bind H3K27me3 marks at loci poised for transcriptional activation via the chromodomain. MK3 interacts with the PRC1 complex via PHC⁸. (B) Upon activation (e.g. by MAPK or SAPK activation) the PRC1 complex dissociates from chromatin. H3Ser28 phosphorylation is a very early event which correlates with PRC1-chromatin dissociation⁸. Part of the core-complex remains intact upon chromatin dissociation; in contrast, PHC partially dissociates from PRC1 but remains largely chromatin-associated. PHC1 may serve as an interface (e.g. epigenomic marking) for core-complex (re)binding or play an additional role. (C) Additional loss of PHC1 and acquisition of active epigenomic marks may be required for local gene expression. PRC1 protein phosphorylation (p) is expected to affect protein-protein interaction and/or enzymatic activity of members within the complex.

MK3 occupation and chromatin-protein redistribution analyses are currently performed to explore these issues.

In summary, our findings suggest that signaling-imbalance is at the basis of cellular responses to MK3 and that an activated stress-program is responsible for the MK3-induced senescence-like replicative arrest. The data presented here

suggests that signaling upstream of MK3 disrupts PRC1-complexes, and consequently alleviates PRC1-repression which results in transcription of relevant target genes.

The observation that MK3 reactivates 'dormant' senescence checkpoints in cancer cells with compromised pRB and P53 function (*i.e.* *CDKN2A/INK4A* locus methylation in U2-OS cells *cq.* constitutive HPV-E6/E7 mediated inactivation of pRb and P53 in HeLa cells^{35, 36}) is quite remarkable and warrants further investigation into MK3-dependent epigenomic regulation of gene transcription in cancer.

We show here that MK3 activation releases PRC1-components from chromatin without changing the repressive PcG-associated histone H3K27me3 mark. Consistent with this, H3K4me3 does not change either (data not shown). MK3-PRC1 may act as a molecular switch to allow for rapid dynamic regulation of gene activity. It is conceivable that disruption and/or dissociation of the PRC1 complex is dependent on post-translational modification, such as phosphorylation. In support of this notion, we obtained *in vitro* evidence that MK3 directly phosphorylates a number of PcG proteins. Global MK3-chromatin co-association analysis and PcG phospho-site mutation will help clarify the underlying molecular effects of MK3-to-Polycomb signaling.

Materials and methods

Tissue culture and expression systems

Indicated cell lines were cultured at 37 °C, 5% CO₂, 100% humidity in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS). Tissue culture plastics were from Greiner Bio-One. Serum starvation was carried out at 0.05% FCS for cancer cell lines and at 0.1-0.5% for human fibroblasts. Retroviral expression vectors were used throughout these studies to maximize percentages of expressing cells and to minimize integration effects. Retroviral vectors (pBABE-PURO, pBMN-LZRS.ires.GFP, pBMN-LZRS.ires.NEO) expressing murine Bmi1 and human MK3 have been described elsewhere⁸. Criteria used for shRNA sequence design and the retroviral expression system were as described before³⁷. Targeting sequences are listed in Supplementary Table 5.1. Transduced cells were selected for 1 week on 4-16 µg/ml puromycin and then used in experiments. Growth curves were performed as described before¹¹

Immuno-histochemistry and cell staining

Cell were seeded at 5-10% confluence on glass slides, infected at low MOI ($\pm$ 1:1) and subjected to a short, intense selection (*i.e.* 8 (Tig3) -16 µg (U2-OS) puromycin/ml) when applicable. Cells were fixed for 10 minutes at ambient

temperature in 2% formaldehyde/PBS solution followed by permeabilization in 0,2% triton-X/PBS or directly fixed and permeabilized for 20 minutes in 100% methanol at -20°C. Antibody dilution was done in blocking buffer (5% normal goat serum, 5% fetal bovine serum, 0.02% triton X in PBS). Polyclonal (Pab) rabbit antiserum glutathione S-transferase (GST) and monoclonal antiserum (Mab) against MK3 were kindly provided by S. Ludwig (Münster, Germany). anti-2Py-tag Mab (MMS-115R; Babco, Richmond, California); anti-p14^{ARF}/p16^β Pab (Ab-1; LabVision corp., Fremont, California), Anti p16INKA mab (E6H4, MTM CINTec laboratories, Heidelberg, Germany), anti p21 Pab (C-19; Santa Cruz biotechnology), anti P53 Mab (DO-7; M7001; DAKO, Denmark). Primary Mabs were detected with goat-anti-mouse TexasRed, primary Pabs with goat-anti-rabbit FITC. SA-β-gal assays were performed as previously described³⁸.

ChIP assays

ChIPs were performed and analyzed essentially as described³⁹. The antibodies used were rabbit anti-H3K27me3 (07-449; Upstate Biotechnology), anti-HA (sc-805; Santa Cruz Biotechnology), an equal mix of anti-CBX8 "LAST" and "GALD"³², anti-MK3²⁷, or anti-PHC1⁴⁰. The immunoprecipitated DNA was quantified by real-time qPCR. The sequences of the primers used are listed in Supplementary Table 5.1.

Acknowledgments

We are greatly indebted to a many colleagues for research materials and support of the study (please refer to *Methods* section). We thank Frédérique Peronnet, Ronit Sverdlov and Anders Lund for critically reading the manuscript and members of the MAASTRO and molecular genetics department for helpful discussions. These studies received financial support from the European Molecular Biology Organization (Germany) 186.00-06; the Dutch Science Organization (ZonMW-NWO): Research Support grant 908-02-040 to JWV and VIDI grant 016.046.362; the National Rheuma Foundation (Reumafonds) grant LLP14 to JWV and a Netherlands Genome Initiative (NGI) fellowship 050-72-422 to HN.

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Supplemental Materials and Methods

Cell lines

Human U2-OS osteosarcoma cells were kindly provided by D. Shvarts (Utrecht Medical Center, Utrecht, The Netherlands); BJ-hTERT and primary human TIG3 fibroblasts expressing the murine ecotropic receptor were a courtesy of D. Peeper (Netherlands Cancer Institute, Amsterdam, The Netherlands). Expression vectors encoding the murine ecotropic receptor were kindly provided by R. Bernards, (Amsterdam, The Netherlands),

ChIP assays

MK3 overexpression: TIG3 cells were transduced with pBABE-puro GST or GST-3pK and initially selected with 4µg/ml puromycin. Cells were propagated without selection and finally plated with 16µg/ml puromycin before fixation.

3pK activation: TIG3 cells were serum-starved for 48 hours in medium containing 0.1% FCS and subsequently stimulated or not for 45 min. with 15% FCS and 100 ng/ml tetradecanoyl phorbol acetate (TPA) before fixation. TIG3 cells were fixed in 1% formaldehyde. Cross-linking was allowed to proceed for 10 min at room temperature and stopped by addition of glycine at a final concentration of 0.125 M, followed by an additional incubation for 5 min. Fixed cells were washed twice with PBS and harvested in SDS Buffer (50 mM Tris at pH 8.1, 0.5% SDS, 100 mM NaCl, 5 mM EDTA), supplemented with protease inhibitors (Aprotinin, Antipain and Leupeptin all at 5µg/mL and 1mM PMSF). Cells were pelleted by centrifugation, and suspended in IP Buffer (100 mM Tris at pH 8.6, 100 mM NaCl, 0.3% SDS, 1.7% Triton X-100, and 5 mM EDTA), containing protease inhibitors. Cells were disrupted by sonication, yielding genomic DNA fragments with a bulk size of 200-500 bp. For each immunoprecipitation, 1 mL of lysate was precleared by addition of 35 µL of blocked protein A beads (50% slurry protein A-Sepharose, Amersham; 0.5 mg/mL fatty acid-free BSA, Sigma; and 0.2 mg/mL herring sperm DNA in TE), followed by clarification by centrifugation. 10µl aliquots of precleared suspension were reserved as input DNA and kept at 4°C. Samples were immunoprecipitated overnight at 4°C with antibodies for either HA as a negative control (sc-805; Santa Cruz), H3K27me3 (07-449; Upstate), an equal mix of anti-CBX8 "LAST" and "GALD"³², anti-MK3²⁷, or anti-PHC1⁴⁰. Immune complexes were recovered by adding 40 µL of blocked protein A beads and incubated for 4 h at 4°C. Beads were washed three times in 1 mL of Mixed Micelle Buffer (20 mM Tris at pH 8.1, 150 mM NaCl, 5 mM EDTA, 5% w/v sucrose, 1% Triton X-100, and 0.2% SDS), twice in 1mL of Buffer 500 (50 mM HEPES at pH 7.5, 0.1% w/v deoxycholic acid, 1% Triton X-100, and 1 mM EDTA), twice in 1mL of LiCl Detergent Wash Buffer (10 mM Tris at pH 8.0, 0.5% deoxycholic acid, 0.5% NP-

40, 250 mM LiCl, and 1 mM EDTA), and once in 1mL of TE. Immuno-complexes were eluted from beads in 250µL elution buffer (1% SDS; and 0.1M NaHCO₃) for 2 h at 65°C with continuous shaking at 1000 rpm, and after centrifugation supernatants were collected. 250µL elution buffer was added to input DNA samples and these were processed in parallel with eluted samples. Crosslinks were reversed overnight at 65°C followed by a 2h digestion with RNase A at 37°C and 2h proteinase K (0.2µg/µL) at 55°C. DNA fragments were recovered using QIAquick PCR purification columns, according to manufacturers' instructions. Samples were eluted in 75 µL EB buffer and then further 1/5 diluted in TE buffer. The immunoprecipitated DNA was quantified by real-time qPCR. The sequences of the primers used are listed in Supplementary Table 5.1. Each experiment was performed three times. Results of one representative experiment are shown.

Western analysis

Cells were lysed in RIPA buffer containing 5 mM Benzamidine, 5 µg/ml Antipain, 5 µg/ml Leupeptin, 5 µg/ml Aprotinin, 1 mM Sodium Vanadate, 10 mM Sodium Fluoride, 10 mM Pyrophosphate, 10 mM β-glycerophosphate, 0.5 mM DTT and 1mM PMSF. After centrifugation for 10 min at 13.2 krpm, protein concentration was determined using a BCA protein assay kit (Pierce). Equal amounts of protein were boiled in Laemmli buffer and loaded on 9-15% polyacrylamide gels. Proteins were transferred to polyvinylidene fluoride (PVDF) membranes. After blocking with 5% non-fat dry milk in PBS containing 0.1% Tween-20, membranes were incubated o/n at 4°C with the following antibodies: anti β-actin Mab (C4, 691001, MP Biomedicals), anti EZH2 Mab (BD43; courtesy D. Pasin; Copenhagen, Denmark), anti p16 Mab (DCS50), anti histone H3 Mab (Ab24824 Abcam), and anti P53 Mab, anti p21 Pab, anti CBX8 Pab, anti Bmi1 Mab (F6; courtesy M. van Lohuizen; Amsterdam, The Netherlands), anti MK3 Mab, anti MK3 Pab, anti H3K27me3 Pab and anti-2Py-tag Mab as described in the main text. After extensive washing, membranes were incubated with corresponding horseradish peroxidase conjugated secondary antibodies for 1 h at room temperature. Signals were detected using enhanced chemoluminescence.

Quantification of mRNA levels by qPCR

Total RNA was isolated using Tri-Reagent (Sigma). Quantity and quality of the RNA were checked on the nanodrop (Witec AG, Germany) according to the manufacturer's instructions. RNA (500ng) was converted into cDNA using the iScript™ cDNA synthesis kit (Bio-Rad) according to the manufacturer's protocol. MyIQ analysis was performed on 10 ng of cDNA using the qPCR iQ™ Custom SYBR® Green Supermix (Bio-Rad) and 300nM primer. Analysis was performed on an iCycler® thermal cycler (Bio-Rad). Cyclophilin A was used as a control gene

for normalization. The sequences of the primers used are listed in Supplementary Table 5.1.

BrdUrd pulse labeling & DNA profiling

Tig3 cells were pulse labelled with 10 μ M BrdUrd for 45 min. Cells were fixed in cold methanol overnight at -20°C immediately after labeling, DNA was denatured by incubation with 0.4 mg/ml pepsin in 0.1 N HCl for 30 min followed by a 30 min incubation with 2N HCl at 37°C, pH was readjusted to 7-8 by addition of 0.1M sodium borate buffer pH 8.5, subsequently cells were incubated with 5 μ l anti-BrdUrd IIB5 for 1-1.5 h at RT. Following washing, cells were incubated with 5 μ l FITC-conjugated anti-mouse Ig over night at 4°C, washed and resuspended in PBS containing 100 μ g/ml RNase and 20 μ g/ml PI for 2-3 hours at RT in dark environment, immediately prior to FACS analysis. Analysis was performed using a Becton Dickinson FACS machine with CellQuest software.

After UV irradiation (20 J/m²) U2OS cells were fixed in 70% ethanol overnight at -20°C, simultaneously permeabilized with PBS containing 0.2% Triton X-100 for 20 min at 4°C and blocked with 2% FBS in PBS, incubated with anti-phosphohistone H3 for 60 min at RT. Following washing, cells were incubated with FITC-conjugated goat anti-rabbit antibody (diluted 1:500) for 30 min at RT, washed, and resuspended in PBS containing 100 μ g/ml RNase and 20 μ g/ml PI for 2-3 hours at RT in dark environment, immediately prior to FACS analysis. Analysis was performed using a Becton Dickinson FACS machine with CellQuest software.

In vitro kinase assay on peptide arrays

Custom arrays were produced by spotting peptides in triplicate on etched glass surface (PhosphoSiteDetector; Jerini peptide technology, Germany). Spotted 13-mer peptides corresponded to 27 Bmi1, 27 Cbx4, 27 Phc2, 10 Rnf2 and 7 Histone H3.3 peptides. Positive control peptides of HSP27 and CREB were included. *In vitro* kinase assay was performed with constitutively active recombinant MK3-EE. The kinase was pre-incubated for 10 minutes at 30°C in kinase buffer (10mM MgCl₂, 25mM β -glycerophosphate, 25mM HEPES pH 7.5, 5mM benzamidine, 0.5mM DTT and 1mM sodiumvanadate) supplemented with 2 μ M ATP. Subsequently 45 μ Ci radioactively labeled gamma-ATP³² (Amersham 250 μ Ci/25 μ l; 3000Ci/mmol) was added; the reaction mix was wedged between one peptide array and one glass slide with the peptide spot surface facing each other and incubated for 2 hours at 30°C. Slides were washed 5 x 4 min with 0.1M phosphoric acid and 5 x 4 min with MQ. Washing solutions were filtered through 0.4 μ m particle filters before use. The slide was rinsed in methanol and air dried. The slide was incubated overnight in a PhosphorImager cassette and images were acquired with a PhosphorImager.

Supplemental Figures

Figure 5.S1. MK3 offsets cell proliferation in different cell types. Representative DNA profiling (A) and quantitative analysis (B) shows G1 accumulation and concomitantly reduced S-phase entry MK3 overexpressing low passage TIG3 cells. The effect of MK3-OE on primary cells positively correlated with MK3 expression levels and passage number (not shown). (C) MK3 overexpression induces a senescence-like phenotype in TIG3-hTERT (C) and hTERT-BJ fibroblasts (not shown). (D) Proliferative differences and morphological changes in MK3-overexpressing human cervical carcinoma HeLa cells. (E,F) Induction of a senescent state in TIG3 (E) and U2-OS (F) cells via stable shRNA-mediated knock down of Bmi1 (upper panels) and HPC2 (middle panels). Large flat cells are positive for SA- β GAL (not shown).

Figure 5.S2. MK3 expression level affects proliferation responses. (A) shRNA mediated knockdown of MK3 (shMK3) induces a replicative arrest in TIG3 cells. (B,C) Corresponding representative DNA profile (B) and quantitative analysis (C) of shMK3 TIG3 cultures versus control. shMK3.1 and 3.3 represent two independent MK3 target sequences. (D) Low serum concentrations substantially increased the number of flat cells in low PDL (30-35) TIG3 MK3-OE cultures (D) and U2-OS cultures (E); (con = empty vector control cells). See page 201 for colour print.

Figure 5.S3. Role of MK3 in senescence pathways. (A) shMK3 transiently reduced RASV12 provoked proliferative arrest. TIG3 cells were simultaneously transduced with a fixed MOI of RASV12-virus and increasing MOIs of shMK3 virus (gradient left to right: RASV12:MK3-KD 1:1, 1:3, 1:10). Population doubling was determined 4 days and 8 days post-infection (pi). The early and late effects of RASV12 oncogene induced senescence were reflected in cell death and strong vacuolization, respectively; both were less pronounced/absent in shMK3 cultures (not shown). Eventually RASV12 cells in culture senesced. (B) Expression of P53 in MK3 positive senescent (arrow) U2-OS cells. (C) Co-expression of p16^{INK4A} and p14^{ARF} in primary human TIG3 fibroblasts overexpressing MK3. Successful transduction was confirmed by GFP-fluorescence prior to methanol fixation; all large flat cells were GFP-positive; empty vector control TIG3 fibroblasts did not show the senescent phenotype. (D) Simultaneous knockdown of Bmi1 (shBmi1) and overexpression of MK3 (MK3) increases senescent phenotype in U2-OS cells. See page 201 for colour print.

Figure 5.S4. MK3 is not required for G2/M arrest following UV-induced DNA damage. (A) Western blot analysis of cell lysates prepared from U2OS cells transduced with shMK3 or control shGFP. (B,C) shGFP (B) or shMK3 (C) expressing U2-OS cells were exposed to 20 J/m² UV (+UV) or sham treated (-UV) and then placed in 50 ng/ml nocodazole-containing media for an additional 16hr. Cells were analyzed by flow cytometry for DNA content by PI staining and for H3S10p staining as a marker for mitotic entry.

Figure 5.S5. MK3 overexpression or activation differentially affects PRC1 chromatin occupation and mRNA expression of target genes. (A) Top: qRT-rtPCR analysis of EZH2 and INK4A/ARF mRNA levels in MK3-OE and control TIG3 cells; bottom: schematic overview of human INK4A/ARF locus and primers used in this study. Due to overlap in mRNA sequences of P14^{ARF} and p16^{INK4A} detection of only p16^{INK4A} mRNA expression was not possible and was therefore calculated. (B) Microscopic morphology of cell cultures corresponding to experiments in A and C. (C) Starvation-serum restart induces Bmi1 phosphorylation (p/ppBmi1); Western blot analysis of TIG3 cells expressing tagged Bmi1-2PY and CBX8 after 48hrs of serum starvation with 0.1% FCS followed by stimulation with 15% FCS and 100 ng/ml TPA for various amounts of time. Endogenous PHC1 was not detected.

Figure 5.55. Continued

(D,E) MK3-OE or activation regulates PRC1 chromatin association. (D) ChIP analysis in MK3-OE TIG3 cells of PcG target and non-target loci (as in Fig. 5.4A): CCNA2 and p14exon1 and a region 15 kilobases downstream of ARF (ds ARF) are not occupied by PRC1 complexes in TIG3 fibroblasts; HOXA10 and HOXA11 are PcG targets, as defined by H3K27me3 and CBX8 binding profiles. (E) ChIP analysis of PcG target and non-target loci in TIG3 cells after 48hrs of serum starvation with 0.1% FCS followed by either no or 45 min. stimulation with 15% serum and 100ng/ml TPA (as in Fig. 5.4B). (F) real time quantitative mRNA measurements of (non) PcG-target genes upon 45 minutes and 90 minutes serum/TPA stimulation.

Figure 5.S6. MK3 phosphorylates PRC1 proteins *in vitro*. (A) layout of peptide array (left) and phosphorimager result (right). (B) Intensity analysis of phosphorylated peptides (top) and peptide sequence of corresponding peptides, centered around the RxxS motif (bottom)

Supplemental Table 5.1. Primers used for ChIP and mRNA expression analysis

qPCR Primers for ChIP analysis		sequence
p14ARF exon1	Forward	GTGGGTCCCAGTCTGCAGTTA
	Reverse	CCTTTGGCACCAGAGGTGAG
15kb downstream of ARFpromoter	Forward	GCACTTGCCCTTCCAGGTATA
	Reverse	TGATAGTTCAAGGCCCTATGCC
p16INK4A promoter	Forward	ACCCCGATTCAATTTGGCAG
	Reverse	AAAAAGAAATCCGCCCCCG
p16INK4A exon1	Forward	AGAGGGTCTGCAGCGG
	Reverse	TCGAAGCGCTACCTGATTCC
CCNA2	Forward	TGACGTCATTCAAGGCGACAG
	Reverse	GCTCAGTTTCCTTGGTTTACCC
p15 exon1	Forward	GGAACCTAGATCGCCGATGTAG
	Reverse	TGTTTTACGCGTGAATGCAC
ATF3	Forward	TGTTTTTCTTTTGC GTTTGGC
	Reverse	TCGTGGCAACCAAATCTAAACAG
HOXA10	Forward	CCCGAGCTGATGAGCGAGTC
	Reverse	GCCAAATTATCCACAACAATGTC
HOXA11	Forward	AATCTATCCCCATCCTTAGCAGG
	Reverse	TTGTCAATTTCAACATCGGGTC
qPCR Primers for mRNA expression analysis		sequence
EZH2	Forward	GGGACAGTAAAAATGTGTCCTGC
	Reverse	TGCCAGCAATAGATGCTTTTTG
p14ARF	Forward	CCCTCGTGCTGCTGATGCTACTG
	Reverse	CCCATCATCATGACCTGGTCTT
p14ARF+p16INK4A	Forward	GAAGGTCCCTCAGACATCCCC
	Reverse	CCCTGTAGGACCTTCGGTGAC
Cyclophilin A	Forward	TTCCTGCTTTCACAGAATTATTCC
	Reverse	GCCACCAAGTGCCATTATGG
ATF3	Forward	TCACTGTCAGCGACAGACCC
	Reverse	CTACCTCGGCTTTTGTGATGG
HOXA10	Forward	GCCCTTCCGAGAGCAGCAAAG
	Reverse	AGGTGGACGCTGCGGCTAATCTCTA
HOXA11	Forward	TGCCAAGTTGTA CTACTACGTC
	Reverse	GTTGGAGGAGTAGGAGTATGTCA
CCNA2	Forward	CGCTCCAAGAGGACCAGGA
	Reverse	CCCTCCTGCAGATATCCCG
shRNA Target sequences		sequence
3pK-1		CGGCAAAGTGCTGGAGTGC
3pK-3		GGAGGAGATGACCAAGTGCC
BMI1		GAATGGTCCACTTCCATTG
PHC2		GTTCAAGCGTCCAAGCGC
p53 ³⁷		GACTCCAGTGGTAATCTAC

**Cell stress-induced association of
Polycomb Repressive Complex 1
with the chromatin factor KAP1**

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Abstract

Polycomb Repressive Complex (PRC) proteins constitute a functional transcriptional memory during growth and development. We previously reported a correlation between phosphorylation of the PRC1 protein Bmi1 and its chromatin dissociation. Little is known about the molecular consequences of PRC-phosphorylation. Here we show that the cell stressors arsenite (As) and selenite (Se) induce phosphorylation of and association between Bmi1 and KAP1, a nuclear corepressor. Signaling pathways involved in As and Se-induced phosphorylation of Bmi1 and KAP1 include p38, ATM/ATR, PKC, JNK and GSK3 β pathways. As- or Se-induced Bmi1-KAP1 interaction requires kinase signaling, however, once established, it is no longer dependent on phosphorylation. The establishment of Bmi1-KAP1 association is enhanced by inhibition of PKC and JNK signaling and reduced by inhibition of GSK3 β signaling under cell stress; however, inhibition of p38 and ATM/ATR signaling reduces binding only in response to Se, not to As. This is the first report of a stress-specific interaction of Polycomb Repressive Complex 1 with the thus far unrelated chromatin-associated protein KAP1. Possible functional implications of the Bmi1-KAP1 interaction are discussed in the context of our findings.

Introduction

PcG proteins are part of an epigenetic memory system that contributes to maintenance of gene expression profiles in differentiation, development and stem cell self renewal¹⁻³. PcG proteins act in two functionally distinct multi-protein complexes: Polycomb repressive complex 1 and 2 (PRC1; PRC2). The PRC2 complex, comprising EED, SUZ12 and EZH2, catalyzes trimethylation of histone 3 on lysine 27 (H3K27me3)^{4,5}. PRC1 consists of BMI1, CBX2, CBX4, CBX6, CBX7, CBX8, PHC1-3, RING1 and RNF2⁶. RNF2 and RING1 ubiquitinate H2A, which is enhanced by Bmi1⁷⁻¹⁰. In transcriptional regulation, PRC1 and PRC2 may, in part, act in succession in that CBX-chromo domain proteins are recruited to H3K27me3 marks. However, PRC1 can act independently of PRC2 as well¹¹. Transcriptional repression by PcG proteins is currently viewed as a dynamic process. PRC complex composition and repressive activity are regulated throughout development and likely respond to specific stimuli. Cellular differentiation is accompanied by a redistribution of PcG binding patterns². How this redistribution occurs is currently unknown. Several PcG proteins are regulated by post-translational modifications (PTMs). For instance, phosphorylation of EZH2 by AKT suppresses its methyltransferase activity by hindering binding to histone 3¹². We recently identified the PRC1 complex as a target of canonical MAPK signaling pathways, and identified MAPKAPK3 (3pK, MK3) as an interaction partner of the PRC1 complex¹³. MK3 is activated downstream of mitogenic and stress response signaling¹⁴. Relevantly, phosphorylation of PRC1 correlates with chromatin dissociation, and activation of PcG-repressed tumor suppressors (Chapter 5 of this thesis).

To gain insight into regulation of Polycomb function, we set out to determine how PTMs regulate complex composition. To identify changes in PRC1 complex composition upon phosphorylation, immunoprecipitation and mass spectrometric analysis were combined. We report here an association between PRC1 and the transcriptional corepressor Krüppel-associated box (KRAB)-associated protein1 KAP1 (TRIM28, TIF1 β , KRIP-1), a chromatin factor known to be involved in chromatin structure remodeling in transcriptional repression and DNA damage responses.

We show here that KAP1 associates with the PRC1 complex in the context of cellular stress. The induced interaction is stress stimulus-specific, and requires several signaling pathways. We show that both KAP1 and Bmi1 are phosphorylated in response to several stress stimuli. In addition, once established maintenance of PRC1-KAP1 interaction is not dependent on phosphorylation. This is the first report that links these thus far distinct chromatin associated proteins.

Results

Arsenite induces interaction of PRC1 and KAP1

To induce PTMs on PcG proteins, cells were exposed to arsenite (As), a stimulus that induces phosphorylation of BMI1 and other PcG proteins¹³. To identify phosphorylation events on PcG proteins, immunoprecipitations (IPs) of ectopically expressed Polyomavirus epitope-tagged Bmi1 (Bmi1-Py) in U2-OS cells were performed, and subjected to mass spectrometry. In keeping with published data most of the known mammalian PRC1 complex members, among which PHC, RING and CBX proteins^{6, 15, 16}, are readily identifiable (Table 6.S1). We identified 9 previously described and 11 novel PcG phosphorylation sites on CBX8, PHC1, PHC2, PHC3, RING1 and Bmi1 (Table 6.S2). A number of additional phosphorylated peptides were detected, for which the exact phosphorylation sites could not be determined. As the used detection method is non-quantitative, we cannot formally exclude any relative changes in phosphorylation at these residues. In addition, many PRC1 members are relatively arginine (R) and lysine (K) rich around potential phosphorylation sites which interferes with detection of tryptic peptides. This data confirms that PRC1 members are phospho-proteins, and that a number of PRC proteins are poly-phosphorylated.

Although detection of the PRC1 complex remained largely unchanged upon As treatment, KAP1 was consistently detected as a novel associated factor in stimulated extracts (Table 6.S1). The As-induced PRC1-KAP1 association was confirmed by IP and western blotting (Fig. 6.1A). Cells were serum-starved to prevent interference by cell cycle-dependent phosphorylation of PcG proteins¹⁷. Upon As treatment higher molecular weight forms of both Bmi1 and KAP1 appear, consistent with reduced electrophoretic migration as a result of phosphorylation (Fig. 6.1A). The novel interaction was validated by reciprocal IP against KAP1, which co-precipitates overexpressed Bmi1-Py (Fig. 6.1B). As-induced phosphorylation as well as association of both Bmi1 and KAP1 increases over time and is dependent on the intensity of the stress stimulus. The association of Bmi1 with CBX4, however, is largely unaffected (Fig. 6.1C), which is in good agreement with the mass-spectrometric findings and our previous report¹³. In keeping with our earlier findings that phosphorylated BMI1 dissociates from chromatin, immuno-fluorescence (IF) analysis of nuclear KAP1 and BMI1 in U2-OS cells reveals that KAP1 and BMI1 both dissociate from chromatin in response to As: cells in which the BMI1 containing PcG bodies disappear are the same cells that show a decreased nuclear staining of KAP1 (Fig. 6.1D). This suggests that mainly dissociated BMI1 engages KAP1. We observe co-localization of endogenous PRC1 complexes with KAP1 in U2-OS cultures upon shorter As exposure (Fig. 6.1E). Co-localization of PRC1 and KAP1

Cell stress-induced association of PRC1 with KAP1

Figure 6.1. Bmi1-Py and KAP1 phosphorylation and association upon Arsenite exposure. (A) Immunoprecipitation (IP) western analysis of U2-OS cells expressing Bmi1 with Py-tag (Bmi1-Py) or not (control). Cells were serum-starved and subjected to Arsenite (As) stimulation as indicated. IPs were performed using an antibody against the Py epitope. Antibodies used for the immunoblotting are indicated by (IB). Longer exposure of film to the same western blot is indicated by t+. (B) Western analysis of IPs performed with an anti-KAP1 antibody. KAP1 was not fully immuno-depleted due to inferior IP-quality of the KAP1 immuno-serum (data not shown). (C) anti-Py IP western analysis of serum-starved U2-OS Bmi1-Py cells treated with indicated As concentrations for various amounts of time. (D-E) Immunofluorescence analysis of serum starved U2-OS cells treated with As for 90 min (D) or 45 min (E). See page 202 for colour print.

occurs at the borders of nucleoli (Fig. 6.S1), perhaps suggesting a role in functional partitioning of the genome. At longer As exposures and higher concentrations, protein stability of Bmi1 and CBX4 appear compromised, we therefore used 75-90 min 0.2mM As exposure for most co-interaction studies. Collectively our data suggest that PRC1 and KAP1 associate upon As treatment and that this occurs when both Bmi1 and KAP1 are phosphorylated.

To further validate the PRC1-KAP1 interaction, we performed immunoprecipitations i) with endogenous proteins ii) in cell types other than U2-OS and iii) with other types of expression vectors. Although we were unable to detect association between endogenous proteins, most likely due to limiting availability of (free) binding partner and or the transient nature of the interaction (data not shown), the Bmi1-KAP1 association was reproduced in cervical carcinoma cells (HeLa) and primary human fibroblasts (Tig3) in support of a valid PRC1-KAP1 association (Fig. 6.S2A-C). Furthermore, the use of differently tagged PRC1-protein expression vectors confirmed the PRC1-KAP1 interaction and excluded potential interference by protein tags (Fig. 6.S3A-B). From these data we conclude that KAP1 is a valid interaction partner of PRC1 under certain stress-induced conditions.

Dependence of arsenite-induced PRC1-KAP1 interaction on phosphorylation

To gain further insight into the requirement of phosphorylation for PRC1-KAP1 interaction, co-IPs were subjected to phosphatase-treatment and vigorously washed. Migration of As-induced slower migrating forms of Bmi1, KAP1, CBX4 and PHC1 was largely restored by dephosphorylation (Fig. 6.2A), confirming phosphorylation as the main PTM under these conditions. Surprisingly, removal of phosphorylation does not affect interaction between Bmi1 and KAP1 or other PRC1 members (Fig. 6.2A). This suggests that the interaction between PRC1 and KAP1, once established, is not phosphorylation dependent.

To identify the kinase pathways involved in establishing the PRC1-KAP1 interaction, several kinase inhibitors were used in combination with stressors. We previously showed that the As-induced phosphorylation of Bmi1 is p38-dependent¹³. KAP1 is known to be phosphorylated as part of a DNA damage response in an ATM-dependent manner^{18, 19}. Therefore kinase inhibitors of p38 (SB202190) and ATM/ATR (caffeine) were used. As-induced phosphorylation of Bmi1 and KAP1 is dependent on both p38 and ATM/ATR signaling (Fig. 6.2B); a combined pretreatment with both inhibitors further reduces the migration of Bmi1 and KAP1 (Fig. 6.2B), indicating that these pathways function independently in Bmi1 and KAP1 phosphorylation. Use of inhibitors only marginally affects the Bmi1-KAP1 association (Fig. 6.2B). It is therefore unlikely that the interaction is dependent on p38 and ATM/ATR signaling. Employment of additional kinase inhibitors (NaSalicylate and Wortmannin) demonstrated

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treatment with Staurosporin and SP600125 enhance the association between Bmi1 and KAP1, whereas LiCl treatment has the opposite effect (Fig. 6.2C). Thus progressive phosphorylation of Bmi1 and KAP1 downstream of PKC and JNK signaling negatively interferes with their association, whereas GSK3 β signaling increases association, supporting a role of one or more PKC/JNK phospho-sites in regulating the Bmi1-KAP1 interaction. In remarkable contrast, interaction between PRC1 core members Bmi1 and CBX4 is not affected. To further address the role of Bmi1 phosphorylation in the interaction, Bmi1 mutants were implemented, in which 7 putative RXXS/T consensus phosphorylation sites were mutated to obtain less-phosphorylatable Bmi1 (Bmi7A) or a phospho-mimetic form of Bmi1 (Bmi7E). RXXS/T are potential consensus sites for a number of kinases, among which MAPKAPKs and PKCs. As Bmi7E does not bind to KAP1 under basal conditions, and Bmi7A still interacts with KAP1 upon As exposure (Fig. 6.S4A), it is unlikely that phosphorylation at these sites are instrumental in initiating the association. There may, however, be a role for these sites in combination with other PTMs in affecting Bmi1 function or interactions. Consistent with this, we find that the 7E mutant exhibits spontaneous additional changes, which apparently result in protein instability, as the 7E mutant is stabilized by proteasome inhibitors. In keeping with a role for RXXS/T phosphorylation in protein turn over, a 7A mutant does not display this instability (Fig. 6.S4B). Our findings demonstrate that As-induced activated p38, ATM/ATR, PKC, JNK and GSK3 β signaling cascades target Bmi1 and KAP1. Bmi1-KAP1 association is differentially affected by signaling through these pathways: GSK3 β activity positively affects the association, whereas p38 and ATM/ATR have no effect, and PKC and JNK inhibit interaction. Collectively this data points to a specific involvement of signaling cascades and PTM-events in PRC1 protein biochemistry.

Stimulus-specific phosphorylation of BMI1 and KAP1

To chart physiological conditions that induce PRC1 and KAP1 phosphorylation, U2-OS cells were subjected to several stressors, including arsenite (As), selenite (Se), ionizing radiation (IR) or ultra violet (UV) treatment. As, Se and UV exposure phosphorylate BMI1, whereas IR does not (Fig. 6.3A-D). For Se and IR treatment, KAP1 phosphorylation was undetectable at the level of the total protein (Fig. 6.3B,C), whereas As exposure readily induces KAP1 phosphorylation (Fig. 6.3A). Upon UV exposure KAP1 is initially rapidly phosphorylated, after which it gradually declines over time (Fig. 6.3D). However, by using a phospho-specific antibody that recognizes KAP1 phosphorylation at S824, Se, IR and UV, but not As, are shown to specifically induce KAP1 phosphorylation at Ser824 (Fig. 6.3A-D), in line with ATM-dependent KAP1-phosphorylation in response to double strand DNA damage¹⁸⁻²⁰. KAP1 phosphorylation at S473, which correlates

with KAP1-HP1 β binding²¹, is potently induced by UV, and to a lesser extent by As, Se and IR (Fig. 6.S5 A-D). Thus, our data demonstrate that phosphorylation of BMI1 and KAP1 is stimulus-specific and confirm that both proteins are phosphorylated at multiple residues.

Figure 6.3. Stimulation specific phosphorylation of BMI1 and KAP1. (A-B) Western analysis of serum-starved U2-OS cells treated with As (A) and Se (B) for indicated amounts of time. (C-D) Western analysis of confluent U2-OS cells treated with IR (C) and UV (D) where indicated minutes refer to the time-period between the stimulus and generation of protein extracts. Actin serves as loading control.

Selenite induces interaction of PRC1 and KAP1

We next investigated Bmi1-KAP1 association in response to various stressors. As KAP1 is phosphorylated upon DNA damage, we initially focused on DNA damage inducing treatments. Surprisingly, next to As, only Se appears a potent inducer of this interaction (Fig. 6.4A). IR, camptothecin and UV treatment did not elicit an interaction under the conditions tested (Fig. 6.S6A-C). Even though KAP1 is phosphorylated on S824 upon Se exposure, this modification is not readily detected in the IP (Fig. 6.4A), suggesting that this PTM is not required for the PRC1-KAP1 association and that pS824-KAP1 localizes to a different subcellular compartment (see below).

Figure 6.4. More potent association of Bmi1-Py and KAP1 after Selenite stimulation compared with Arsenite stimulation.

(A) IP western analysis of U2-OS cells expressing Bmi1 with Py-tag (Bmi1-Py) or empty vector (EV). Cells were serum-starved and subjected to Selenium (Se) stimulation as indicated. IPs were performed using an antibody against the Py epitope. Longer exposure of film to the same western blot is indicated by t+. (B) anti-Py IP western analysis of serum-starved U2-OS Bmi1-Py cells treated with indicated Se concentrations for various amounts of time. (C) anti-Py IP western analysis of serum-starved U2-OS Bmi1-Py cells treated with As, Se or a combination of As and varying concentrations of Se as indicated.

The Se-induced association between Bmi1 and KAP1 is time-dependent and concentration-dependent (Fig. 6.4B). In comparison to As, Se evokes a more intense association between Bmi1 and KAP1 (Fig. 6.4B,C). As and Se have been reported to exert antagonistic effects on cellular signaling²². Remarkably, simultaneous exposure of As and Se reduces the association between Bmi1 and

KAP1 as compared to Se only (Fig. 6.4C). Furthermore, KAP1 phosphorylation is consistently less profound with Se compared to As (Fig. 6.4B,3, S2), suggesting that As inhibits or overrules signaling by Se directly or indirectly.

Dependence of selenite-induced PRC1-KAP1 interaction on phosphorylation

Analogous to As, when IP's were phosphatase-treated the interaction between Bmi1 and KAP1 was not lost (Fig. 6.5A). Again, although a very weak band is seen for pS284-KAP1, it was by no means enriched in the co-IP experiment (Fig. 6.5A), further substantiating that it is not pS824 KAP1 that associates with PRC1. Pretreatment of cells with kinase inhibitors revealed a different pattern for Se than for As treatment. Whereas p38 and ATM/ATR inhibition did not affect As-induced association (Fig. 6.2B), application of these kinase inhibitors prior to Se exposure completely prevented Bmi1-KAP1 association (Fig. 6.5B). Inhibition of PKC and JNK signaling reduces the Se-induced phosphorylation of KAP1, yet increases the association of KAP1 with Bmi1 (Fig. 6.5C). In addition, inhibition of GSK3 β signaling decreased binding (Fig. 6.5C). Therefore the Se-induced interaction between Bmi1 and KAP1 is negatively affected by phosphorylation downstream of PKC and JNK signaling and positively affected by p38, ATM/ATR and GSK3 β signaling.

Immuno-fluorescence analysis of KAP1 and BMI1 binding in U2-OS cells reveals that although KAP1 and BMI1 both dissociate from chromatin in response to As or Se (Fig. 6.1D; Fig. 6.S7), pS824-KAP1 is chromatin associated (Fig. 6.S7). Consistent with a separate task for pS824, IF on Se stimulated cells shows that PcG dissociation and pS824 inversely correlate: approximately half of nuclei positive for PcG bodies are pS824 negative or weak, whereas approximately half of KAP1-positive nuclei lack PcG bodies (Fig. 6.S8). In support of functionally separate KAP1 pools, phosphorylated S824 KAP1 was reported to associate with chromatin¹⁹.

Se-induced S824-KAP1 phosphorylation and H2AX phosphorylation (γ H2AX), both known ATM-catalyzed events in response to DNA damage, are blocked by caffeine (Fig. 6.S9A,B). As predicted, p38 inhibition partially blocks PRC1 dissociation (Fig. 6.S7), but has no discernable effect on KAP1-S824 or H2AX phosphorylation. Interestingly, while As alone does not induce DNA damage under these conditions, simultaneous exposure of cells to As and Se blocks phosphorylation as efficiently as caffeine does, suggesting As counteracts Se at least in part through a mechanism involving ATM inhibition (Fig. 6.S9B). Conversely, KAP-1 dissociation appears not depend on either p38 or ATM activation alone; combined As and Se treatment, however, blocks KAP1 dissociation (Fig. 6.S9).

These immuno-histological data are therefore complementary to the interaction data and, combined, demonstrate that multiple kinase pathways differentially

Figure 6.5. Kinase pathways involved in Selenite-induced PRC1-KAP1 interaction. (A) IP western analysis of U2-OS cells expressing Bmi1 with Py-tag. IPs were performed using an antibody against the Py epitope. Cells were serum-starved and subjected to Se stimulation as indicated. IPs were either subjected to phosphatase or mock treatment. (B) IP western analysis of serum-starved Bmi1-Py overexpressing U2-OS cells pretreated with a p38 inhibitor (SB202190) and/or an ATM/ATR inhibitor (caffeine) before Se application. Longer exposure of film to the same western blot is indicated by t+. (C) IP western analysis of serum-starved Bmi1-Py overexpressing U2-OS cells pretreated with a PKC inhibitor (staurosporin), a JNK inhibitor (SP600125) or a GSK3β inhibitor (LiCl) or their corresponding controls before Se exposure.

affect PRC1 and KAP1. Even though both As and Se exposure induce phosphorylation and binding of Bmi1 and KAP1, the dependence of these two cellular stressors on signaling cascades is clearly different. Only Se signaling results in pS824-KAP1. Furthermore, the interaction between Bmi1 and KAP1 is

dependent on p38 and ATM/ATR signaling only when induced by Se, and support the idea that these combined phosphorylation events are involved in chromatin dissociation of both interaction partners, rather than establishing the interaction.

Biological relevance of PRC1-KAP1 interaction.

To begin to understand the biological relevance of the PRC1-KAP1 interaction, we initiated a number of studies. As KAP1 acts as co-repressor, and through its interaction with HP1 proteins is part of the heterochromatic compartment in the nucleus, we investigated its potential role in PcG mediated repression. Mitogen-stimulated primary human TIG3 fibroblasts initiate a rapid increase in ATF3 mRNA (Fig. 6.6A); ATF3 was shown to be a genuine PRC-target in TIG3 cells²; we have recently obtained experimental evidence for PRC1-chromatin dissociation at this locus in response to mitogenic signaling (Chapter 5 of this thesis). Consistent with its transcriptionally repressive properties, loss of KAP1 leads to an exaggerated transcriptional response (Fig. 6.6B). In good agreement with our finding that several kinase pathways converge on Bmi1 and KAP1 interaction, pretreatment of cells with p38 and/or ATM inhibitors increases basal ATF3 expression, whereas PKC inhibition reduces basal ATF3 levels (Fig. 6.6A). It is tempting to interpret these findings in the context of reduced and increased availability of PRC for local chromatin binding, respectively. Remarkably, staurosporin abolishes mitogen-induced ATF3 expression completely, whereas caffeine does not and p38 or MEK inhibition slightly, but substantially reduce ATF3 transcription. Without further insight into the contribution and interaction of individual phosphorylation events, it is impossible to pinpoint an exact mechanism. Combined these findings however indicate progressive and sequential phosphorylation events in PRC1/KAP1 regulation.

Discussion

In this study we demonstrate that multiple PRC1 proteins (Bmi1, CBX4 and PHC1) are phosphorylated upon induction of cell stress via As or Se stimulation. In addition we demonstrate for the first time that PRC1 complexes associate with KAP1 (alternative names TRIM28, TIF1 β , KRIP-1) upon induction of cell stress. PRC1 proteins are phosphorylated by various kinase pathways, including: p38, ATM/ATR, PKC, JNK and GSK3 β signaling. In addition, some PRC1 proteins, like Bmi1 and RNF2, appear phosphorylated under basal conditions, when cells are serum-starved, and arrested in G1 (Fig. 6.2A,5A). In total 20 PcG phosphorylation sites were identified by mass spectrometry, three of which were on CBX8, twelve on PHC proteins, two on RING1 and three on Bmi1.

Figure 6.6. Mitogenic signaling-induced transcription of PcG target gene ATF3 is dependent on PKC and enhanced by KAP1 knockdown.
(A) Real time quantitative mRNA measurements of PcG target gene ATF3. Serum-starved TIG3 cells were incubated with indicated inhibitors prior to 45 minutes serum/TPA stimulation.
(B) Real time quantitative mRNA measurements of PcG target gene ATF3 expression in serum-starved TIG3 cells containing pRETROSUPER-based knockdown constructs of GFP (con) and KAP1. Cells were treated with serum/TPA stimulation as indicated (left). Western analysis confirming KAP1 knockdown (right).

We found that some, but not all DNA damage-inducing stimuli lead to Bmi1 phosphorylation. Previously phosphorylation of PHC2 on S733 and PHC3 on S227 was shown to result from DNA damage²³. In addition, it was reported that DNA damage induction by adriamycin treatment induced CBX4 phosphorylation, which was dependent on HIPK2 kinase activity²⁴. This suggests that the

phosphorylation of PcG proteins is a common feature of DNA damage responses and suggests a role for PcG proteins in damage responses.

The association between Bmi1 and KAP1 appears to be direct, as high stringency washing (1.5 M NaCl) upon IP does not release the interaction. Phosphatase treatment of protein complexes once the interaction is established, does not affect Bmi1-KAP1 binding. Combined, our findings specify a role for phosphorylation events in initiation of PRC1-KAP1 association, whereas maintenance is no longer dependent on phosphorylation. In keeping with our earlier reports, this data supports functional compartmentalization of PRC1 proteins through phosphorylation-induced novel associations. The finding that PRC1 core complex members are quantitatively retrieved in IPs, suggests that either PRC1-core complex interactions are not compromised by KAP1 binding and/or Bmi1 is part of more than one complex. In some mass spectrometric analyses two other tripartite motif-containing proteins were detected, TRIM21/RO52 and TRIM25/Efp, suggesting the PRC1-association with this protein family is more widespread. The tripartite motif consists of a RING domain, one or two B-box motifs and a coiled-coil region, also known as the RBCC domain²⁵. KAP1 carries C-terminal a PHD and a BROMO domain; both are also found in other TIF1 proteins (transcription intermediary factor). PHD and BROMO domains are often found in chromatin associated proteins and some have histone binding properties²⁶. However, the PHD finger and bromodomain of KAP1 do not display methyllysine and acetyllysine binding properties, respectively²⁷. Since the more C-terminally located domains in the three TRIM proteins are not similar, we speculate that the interaction between PRC1 and KAP1 is established via the RBCC domain of KAP1. An interaction between a PcG protein and a TRIM protein has been described before. RET finger protein (RFP; TRIM27) interacts via its coiled coil region with Enhancer of Polycomb (EPC)²⁸. Efforts to identify the KAP1 region that is responsible for the PRC1-KAP1 interaction are underway. Initial attempts using C-terminally FLAG-tagged KAP1 deletion constructs failed due to inability of these constructs to localize to the nucleus and did not co-precipitate with Bmi1 (data not shown).

KAP1 binds to certain KRAB-containing zinc finger proteins²⁹⁻³¹. It regulates transcriptional repression by interacting with several proteins and complexes including HP1 proteins³²⁻³⁴, a N-CoR-1 complex, containing the nuclear receptor corepressor (N-CoR), HDAC3 and several members of the SWI/SNF protein family³⁵, a NuRD complex containing a form of Mi-2 α , HDAC1 and RbAp48³⁶, and SETDB1, a H3K9 methyltransferase³⁷.

Sumoylation of KAP1 is essential for its transcriptional repressive properties³⁸⁻⁴⁰, and is necessary for HDAC, SETDB1 and CHD3/Mi2 recruitment^{39, 40}. DNA damage induces phosphorylation of KAP1 on S824, which is necessary for DSB-induced chromatin relaxation^{18, 19}. Furthermore ATM-induced KAP1 dissociation

is specifically required for damage repair in heterochromatin²⁰. Phosphorylation of KAP1 on S824 by ATM upon DOX treatment correlates with reduced sumoylation and an increased expression of several KAP1-repressed genes such as p21 and Gadd45⁴¹. Whether S824 phosphorylation interferes with sumoylation is unclear as of yet. S473 phosphorylation, however, was shown to regulate KAP1 binding with HP1²¹.

In an attempt to determine the biological relevance of KAP1-PRC1 interaction we looked at common targets of PRC1 and KAP1. Although our loss of KAP1 experiments indicate a potential role in PRC1 mediated repression, a comparative analysis of published ChIP-on-chip analysis of KAP1 and PRC1 binding in TIG3 cells^{1-3, 42} revealed overlap between only few PRC1 and KAP1 sites (not shown). Of the top 2000 KAP1 targets approximately 11% were also bound by H3K27me3⁴². Our comparison did not identify ATF3 as a common PRC1/KAP1 target. This strongly suggests that the effect of KAP1 on PRC1 targets maybe indirect, via more global chromatin responses. Chromatin immunoprecipitation analysis will have to point out whether the dynamics of PRC1-chromatin dissociation is altered in the absence of KAP1. Clearly, whether and how KAP1-PRC1 interaction impinges on PcG-mediated repression should be the focus of future investigations.

Prolonged exposure to several stress stimuli seems to promote Bmi1 degradation. Previously it has been established that Rnf2 and Bmi1 are ubiquitinated and targeted for degradation by an as of yet unknown E3 ubiquitin ligase⁴³. Interestingly, some TRIM proteins are E3 ubiquitin ligases. TRIM25/Efp targets 14-3-3 σ for proteasomal degradation. The TRIM25 RING domain interacts with the E2 Ubch8, whereas the B-boxes and coiled-coil domain mediate substrate interaction⁴⁴. Interestingly, the KAP1 PHD domain is a E3 sumo ligase for its adjacent bromodomain⁴⁰. Based on this it is intriguing to speculate that KAP1 will function as an ubiquitin E3 ligase for Bmi1; this and the specific role of phosphorylation in protein stability warrant further research.

In conclusion, we have detected a novel interaction between PRC1 and KAP1, both involved in gene repression via epigenetic mechanisms. We here identify multiple phosphorylation events on PRC1 proteins, which illustrates the complexity of PcG regulation. Combined these findings indicate progressive and potentially sequentially hierarchical phosphorylation events in PRC1/KAP1 regulation. Further characterization of the circumstances under which novel associations occur, what the exact molecular nature of the interaction is, what phospho-residues are involved and what their biological significance is, is clearly needed. This will provide us with new insights in how regulation of PcG phosphorylation and chromatin dissociation contributes to processes such as development, differentiation and possibly tumorigenesis.

Materials and methods

Tissue culture and expression systems

Human osteosarcoma cells (U2-OS), human cervix carcinoma cells (HeLa) and human primary fibroblasts (TIG3) were cultured at 37°C, 5% CO₂, 100% humidity in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS), antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin), 200 mM L-glutamine and 4.5 g/liter L-glucose. Serum starvation was carried out at 0.1% FCS for 48 hrs. Retroviral expression vectors were used throughout these studies to maximize percentages of expressing cells and to minimize integration effects. Expression constructs encoding murine Bmi1-2Py, MPH2-flag-TAP and Bmi1-TAP were constructed in pBABE-PURO, pBMN-LZRS.ires.GFP or pBMN-LZRS.ires.NEO). The pRETRO-SUPER shRNA system used for KAP-1 (or GFP control) knockdown was obtained from A. Goodarzi²⁰, with permission of Ziv et al.¹⁹. Phosphomutants of Bmi1 were made with the Quikchange XL site-directed mutagenesis kit according to manufacturers protocol (Stratagene). Primers are listed in Table 6.S3.

Cell treatment

Sodium (meta)arsenite (S7400, Sigma), sodium selenite (S5261, Sigma) and 15% FCS and 100 ng/ml tetradecanoyl phorbol acetate (TPA) were used to stimulate cells. Pretreatment with kinase inhibitors prior to stimulation was performed as follows: 30 min 20µM SB202190 (Calbiochem), 60 min 5mM caffeine (C8960, Sigma), 30 min 1µM Staurosporin (S4400, Sigma), 60 min 25µM SP600125 (Biomol), 30 min 20mM LiCl (L9650, Sigma) or 30 min 10µM U0126. 20µM Proteasome inhibitor (539160, Calbiochem) or solvent (EtOH) was added 135 min prior to collection of cellular extracts.

Cellular protein extracts

Cells were lysed in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS, 50 mM Tris (pH 8), 5 mM EDTA) supplemented with 5 mM Benzamidine, 5 µg/ml Antipain, 5 µg/ml Leupeptin, 5 µg/ml Aprotinin, 1 mM Sodium Vanadate, 10 mM Sodium Fluoride, 10 mM Pyrophosphate, 10 mM β-glycerophosphate, 0.5 mM DTT and 1mM PMSF. 10mM n-ethylmaleimide (NEM, E38760, Sigma Aldrich) or solvent (EtOH) was added to the cell lysis buffer. Extracts were sonicated on ice and centrifuged for 10min at 4°C at 13.2 Krpm.

Immunoprecipitations

Cells were lysed in ELB buffer (250mM NaCl, 0.1% Igepal (NP40), 5mM EDTA, 50mM Hepes pH 7.0) supplemented with 5 mM Benzamidine, 5 µg/ml Antipain, 5 µg/ml Leupeptin, 5 µg/ml Aprotinin, 1 mM Sodium Vanadate, 10 mM Sodium

Fluoride, 10 mM Pyrophosphate, 10 mM β -glycerophosphate, 0.5 mM DTT and 1mM PMSF. Extracts were sonicated on ice and centrifuged for 10min at 4°C at 13.2 Krpm. 1/10th of the supernatant was taken as input. Appropriate amount of antibody was added and incubated minimally 1hr at 4°C on a spinning wheel. Antibodies used are anti-2Py-tag Mab (MMS-115R; Babco), α -KAP1 Pab (A300-275A, Bethyl laboratories), α -FLAG M2 (F3165, Sigma), α -Bmi1 (F6) Mab (kind gift of M. van Lohuizen), α -Bmi1 Mab (AF27)⁴⁵, or no antibody for TAP constructs. Appropriately washed beads were added (ProtA, ProtG or IgG beads) and incubated for minimally 4 hrs at 4°C on a spinning wheel. The mixture was centrifuged 2min, 4°C, 3000 rpm and supernatant was collected as depleted fraction. Beads were washed 4x in ELB buffer with supplements and stored in -80°C until analysis. For phosphatase treatment, IPs were washed an additional 2x in phosphatase buffer, phosphatase was added and IPs were incubated for 30min at 37°C. Afterwards beads were washed 4x in ELB buffer with supplements and stored in -80°C until analysis. IPs for mass spectrometry were performed differently. After taking input samples, cell lysates were precleared for 30 min at 4°C on a spinning wheel with an equal mix of ProtA and ProtG sepharose and subsequently incubated with α -Py resin (affinity matrix AFC-115P, Covance) for minimally 4 hrs at 4°C on a spinning wheel. The mixture was centrifuged 2min, 4°C, 3000 rpm and supernatant was collected as depleted fraction. Beads were washed 4x in ELB buffer with supplements and subsequently 4x washed in modified ELB buffer with supplements, containing 1.5M NaCl, and stored in -80°C until analysis.

Proteolytic digestion and mass spectrometric analysis

Beads were washed three times with a 50 mM Tris buffer (pH 8.0). Trypsin was added to the beads suspension in a 1:20 ratio to the substrate and the suspension was then incubated o/n at 37°C while shaking. Beads were spun down and the supernatant was used for mass spectrometry analysis. Phosphorylated peptides were selectively enriched for in an offline chromatographic manner using a titanium dioxide (TiO₂, Titansphere) packed fused silica capillary that is used as a trap and which acts as an first-dimension separation step in a two-dimensional chromatography system⁴⁶. Phosphorylated peptides were separated from non-phosphorylated peptides by trapping them under acidic conditions on the TiO₂ column and ultimately desorbed under alkaline conditions, dried and dissolved in 0.1 M formic acid. Subsequently, nanoflow LC-MS/MS was performed on an 1100 series capillary LC system (Agilent Technologies) coupled to an LTQ-Orbitrap mass spectrometer (Thermo) operating in positive mode and equipped with a nanospray source. Peptide mixtures were trapped on a ReproSil C18 reversed phase column (Dr Maisch GmbH; column dimensions 1.5 cm × 100 μ m, packed in-house) at a flow rate of

8 µl/min. Peptide separation was performed on ReproSil C18 reversed phase column (Dr Maisch GmbH; column dimensions 15 cm × 50 µm, packed in-house) using a linear gradient from 0 to 80% B (A = 0.1 M formic acid; B = 80% (v/v) acetonitrile, 0.1 M formic acid) in 70 min and at a constant flow rate of 200 nl/min using a splitter. The column eluent was directly sprayed into the ESI source of the mass spectrometer. Mass spectra were acquired in continuum mode; fragmentation of the peptides was performed in data-dependent mode. Peak lists were automatically created from raw data files using the Mascot Distiller software (version 2.1; MatrixScience). The Mascot search algorithm (version 2.2, MatrixScience) was used for searching against the NCBI nr database (release 20070617; taxonomy: *Mus musculus* and *Homo sapiens*). The peptide tolerance was set to 10 ppm and the fragment ion tolerance to 0.8 Da. A maximum number of 2 missed cleavages by trypsin were allowed and carbamidomethylated cysteine and oxidized methionine were set as fixed and variable modifications, respectively. The Mascot score cut-off value for a positive protein hit was set to 60. Individual peptide MS/MS spectra with Mowse scores below 40 were checked manually and either interpreted as valid identifications or discarded.

Western blot analysis

Immunoblotting was performed with the following antibodies: anti β-actin Mab (C4, 691001, MP Biomedicals), anti-2Py-tag Mab (MMS-115R; Babco); α-KAP1 Pab (A300-275A, Bethyl laboratories), α-p-KAP1S824 pAb (A300-767A, Bethyl laboratories), α-Bmi1 (F6); α-CBX4 (HPC2 M9, A. Otte), α-PHC1 Pab (α-HPH1, 70092, M. van Lohuizen) α-PHC2 Mab (α-HPH2, H. Koseki, RIKEN Yokohama Institute, Yokohama, Japan) α-Histone H3 (ab 1791-100, Abcam) and α-RNF2 Pab (anti-dinG (Ring1B) pAb (Dr. M. Dyer, University of Leicester, Leicester, UK).

Immunofluorescence and cell staining.

Cells were grown on 6W culture plates and either fixed for 10 minutes at ambient temperature in 1-2% formaldehyde/PBS solution followed by permeabilization in 0,2% triton-X/PBS or directly fixed and permeabilized for 20 minutes in 100% methanol at -20°C. Antibody dilution was done in blocking buffer (5% normal goat serum, 5% fetal bovine serum, 0.02% tritonX in PBS). Antibodies used were: α-gamma H2AX Mab JBW301, Upstate), α-Bmi1 (F6) Mab; α-KAP1 Pab (A300-275A, Bethyl laboratories), α-p-KAP1S824 pAb (A300-767A, Bethyl laboratories). Primary Mabs were detected with goat-anti-mouse TexasRed, primary Pabs with goat-anti-rabbit FITC.

Quantification of mRNA levels by qPCR

Total RNA was isolated using Tri-Reagent (Sigma). Quantity and quality of the RNA were checked on the nanodrop (Witec AG, Germany) according to the manufacturer's instructions. RNA (500ng) was converted into cDNA using the iScript™ cDNA synthesis kit (Bio-Rad) according to the manufacturer's protocol. MyIQ analysis was performed on 10 ng of cDNA using the qPCR iQ™ Custom SYBR® Green Supermix (Bio-Rad) and 300nM primer. Analysis was performed on an iCycler® thermal cycler (Bio-Rad). Cyclophilin A was used as a control gene for normalization. Primers sequences are: ATF3: Forward TCACTGTCAGCGACAGACCC; Reverse CTACCTCGGCTTTTGTGATGG; Cyclophilin A: Forward TTCCTGCTTTCACAGAATTATTCC; Reverse GCCACCAGTGCCATTATGG.

Acknowledgments

We thank members of the MAASTRO and molecular genetics department for helpful discussions. We thank C. Ramaekers, J. Luiken, R. Langen and N. Kubben for sharing materials. The authors received financial support from the European Molecular Biology Organization (Germany) 186.00-06; the Dutch Science Organization (ZonMW-NWO); Research Support grant 908-02-040 to JWV and VIDI grant 016.046.362.

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Supplemental figures

Figure 6.S1. PHC1 and KAP1 concentrate around nucleolar structures. Immuno-fluorescence detects KAP1 and PHC1 compared to phase-contrast in serum-starved U2-OS cells treated with As for 45 min. Single colour photographs correspond to photographs in Fig. 6.1E. Photographs on the left show fluorescent images merged with phase-contrast images. See page 202 for colour print.

Figure 6.S2. Bmi1-Py-KAP1 association is not cell-type specific.

(A) IP western analysis of serum-starved HeLa cells expressing Bmi1 with Py-tag (Bmi1-Py) or empty vector (control). Cells were exposed to two As concentrations for various amounts of time as indicated. IPs were performed using an antibody against the Py epitope. Longer exposure of film to the same western blot is indicated by t+. (B-C) Anti-Py IP western analysis of serum-starved TIG3 cells transduced with either LZRS (B) or pBMN (C) based vectors; empty vector (EV), overexpression of Bmi1-Py or Bmi1-Py 7E. Cells were treated with As and Se as indicated.

Figure 6.S3. PRC1-KAP1 association is not dependent on Bmi1 or Py-tag.

(A) IP western analysis of serum-starved U2-OS cells expressing MPH2 with a FLAG-TAP-tag exposed to As as indicated. IPs were performed using an antibody against the Flag epitope. Longer exposure of film to the same western blot is indicated by t+. (B) IP western analysis of serum-starved U2-OS cells expressing Bmi-TAP exposed to As as indicated. IPs were performed using IgG beads. The TAP-tag contains ProtA; therefore IP can be performed with IgG beads, and detection can be performed by using only secondary antibody (R α MPO).

B

Figure 6.S4. KAP1 interaction and stability of phosphomutant forms of Bmi1.

(A) IP western analysis of U2-OS cells transduced with LZRS empty vector (EV) or vector containing Bmi1 with Py-tag (Bmi1-Py) in an unmutated, wildtype (WT) form, or with 7 possible phospho-acceptor sites mutated to either Alanine (7A) or glutamic acid (7E). Cells were serum-starved and subjected to Arsenic (As) stimulation as indicated. IPs were performed using an antibody against the Py epitope. Longer exposure of film to the same western blot is indicated by t+. (B) Western analysis of serum-starved U2-OS cells containing pBMN Bmi1-Py WT, 7A or 7E, treated with or without proteasome inhibitors. NEM was added to the lysisbuffer to prevent de-sumoylation or de-ubiquitylation.

Figure 6.S5. Stimulation specific phosphorylation of KAP1 on S473.

(A-B) Western analysis of serumstarved U2-OS (A) or U2-OS Bmi1-Py (B) cells pretreated with a p38 inhibitor (SB202190) and/or an ATM/ATR inhibitor (caffeine) before As (A) or Se (B) application. (C-D) Western analysis of confluent U2-OS cells treated with IR (C) and UV (D) where indicated minutes refer to the time-period between the stimulus and generation of protein extracts. Equal protein loading was validated by immunoblotting against actin (data not shown).

Figure 6.S6. Double-strand DNA damage does not induce Bmi1-KAP1 association. (A-B) IP western analysis of U2-OS cells expressing Bmi1 with Py-tag (Bmi1-Py) or empty vector (EV). Confluent contact-inhibited cells were subjected to IR (A) or camptothecin (B) as indicated. IPs were performed using an antibody against the Py epitope. (C) Anti-Py IP western analysis of confluent U2-OS Bmi1-Py cells or HeLa Bmi1-Py cells treated with UV and harvested after various amounts of time.

Figure 6.S7. Localization of BMI1 and KAP1 in stressed cells. Immunofluorescence detecting BMI1 and pS824-KAP1 in serum-starved U2-OS cells incubated with indicated stimuli and inhibitors. See page 203 for colour print.

Figure 6.S8. BMI1 and pS824-KAP1 do not colocalize
(A) Scoring of PRC1 dotted staining pattern and pS824-KAP1 staining intensity in serum-starved U2-OS cells exposed as indicated to Se. A small number of small cells lacking PcG foci were probably in the early G1 phase of the cell-cycle (denoted as eG1). (B) Examples of scored fields. Immunofluorescence detection of Bmi1 and pS824-KAP1. See page 204 for colour print.

Figure 6.S9. Localization of DNA damage and KAP1 in stressed cells. Immunofluorescence detecting (A) γ H2A.X and KAP1 or (B) γ H2A.X and pS824-KAP1 in serum-starved U2-OS cells incubated with indicated stimuli and inhibitors. See page 205 for colour print.

Figure 6.S9. Continued
See page 206 for colour print.

Table 6.S1. Mass spectrometric analysis of Bmi1-Py interacting proteins

Treatment	Score	Protein	Da	cover (%)	uniq pept.	total pept.
- As	547	Bmi1	37254	22.2	6	9
	509	PHC1	115996	9.9	6	6
	429	RNF2	38088	24.1	6	9
	399	RING1	42973	19.2	4	6
	368	RYBP	25065	22.8	3	3
	297	PHC2	44862	11.9	4	5
	286	CBX2	56388	11.1	4	4
	147	CBX8	43483	10.3	3	3
	121	PHC3	106910	2.5	2	2
	429	KAP1	90247	12.3	6	6
	394	RNF2	38088	19.9	5	6
+ As	384	PHC1	115996	8.6	5	5
	272	CBX2	56388	11.1	4	4
	237	Bmi1	37254	10.2	3	3
	237	PHC2	44862	15.1	4	4
	201	RYBP	25065	18.9	2	3
	193	CBX8	39893	14.1	4	4
	154	RING1	42973	7.6	2	4

Cell stress-induced association of PRC1 with KAP1

Table 6.S2. Identified PcG phosphorylation sites

Organism	Protein	Accession number	Total nr AA	Phospho-site	Described
Human	CBX8	Q9HC52	389 AA	S110	47
				S265	47
				S311	-
Human	PHC1	P78364	1004 AA	S195	-
				S645	-
				S664	48
				S669	48
				S786	-
				S895	-
				T922	-
Human	PHC2	Q8IXK0	858 AA	S733	23
Human	PHC3	Q8NDX5	983 AA	S315	47
				T609	47, 49-52
				S616	47, 49-52
				S862	-
Human	RING1	Q06587	406 AA	S38	47, 50
				S229	-
Mouse	Bmi1	P25916	324 AA	S110	-
				S251	-
				S314	-

Table 6.S3. Primer sequences for site-directed mutagenesis of Bmi1-Py

Mutation	Forward	Reverse
T5A	GCATCGAACAGCCAGAATCAAGATC	GATCTTGATTCTGGCTGTTTCGATGC
T5E	GCATCGAACAGAGAGAATCAAGATC	GATCTTGATTCTCTCTGTTTCGATGC
T126A	GGAGAAGAGGATTATAGCTGATGATGAG	CTCATCATCAGCTATAATCCTCTTCTCC
T126E	GGAGAAGAGGATTATAGAGGATGATGAG	CTCATCATCCTCTATAATCCTCTTCTCC
T228A	GTTCGCCAGCTTGCAAAAGAATG	CATTCTTTTGCAAGCTGGCCGAAC
T228E	GTTCGCCAGAGTGCAAAAGAATG	CATTCTTTGCACTTGCCCGAAC
SS313314AA	GACCTCGAAAAGCAGCACTAAATGGGTCATC	GATGACCCATTTAGTGCTGCTTTTCGAGGTC
SS313314EE	GACCTCGAAAAGAAGAACTAAATGGGTCATC	GATGACCCATTTAGTTCTTTTCGAGGTC
SS318319AA	ACTAAATGGGGCAGCAGCACTTCATCTGGTGG	CCACCAGATGAAGTTGCTGCTGCCCCATTAGT
SS318319EE	ACTAAATGGGGAAGAAGCACTTCATCTGGTGG	CCACCAGATGAAGTTGCTTCTTCCCCATTAGT

General Discussion

PcG proteins play a key role in repressing genes involved in development, differentiation and stem cell self renewal²⁸⁻³⁰. Little is known about the mechanism by which PcG/chromatin-binding is regulated. Based on earlier findings that PcG-phosphorylation correlates with chromatin-dissociation from late-S-phase throughout M-phase²⁷, we hypothesized that phosphorylation of PcG proteins plays an important role in PcG dissociation from chromatin. We speculated that PcG-dissociation is required for cells to change their transcriptome and hence, cell fate. In this thesis various techniques have been implemented to determine phosphorylation sites on PcG proteins, the kinases responsible for PRC1 phosphorylation, potential changes of PRC1 complex composition upon phosphorylation and the biological relevance of PcG phosphorylation.

Phosphorylation sites of PRC1 proteins

Several approaches were taken to identify PRC1 protein phosphorylation. Experiments relying on a candidate approach, in which 7 potential phosphorylation sites of Bmi1 were mutated to alanine (A) or glutamic acid (E) residues, revealed that Bmi1 could still be phosphorylated, indicating that additional phosphorylation sites exist at other Bmi1 residues (Chapter 6). Another strategy we used was an *in vitro* kinase (IVK) assay with kinase MK3 on PRC1 peptides (Chapter 5). Several phosphorylation sites were detected on Phc2, Cbx4 and Bmi1 peptides. By combining immunoprecipitation (IP) experiments with mass spectrometry (MS) we have identified in total 20 phosphorylation sites on CBX8, PHC1, PHC2, PHC3, RING1 and Bmi1 proteins (Chapter 6). Of those 20, 9 had been previously described and 11 are newly identified. Until now in total 118 human and 31 murine PcG phosphorylation sites have been detected, including ours (Chapter 3). This indicates that PcG proteins are heavily phosphorylated. Since the majority of PcG phosphorylation sites has been identified in large-scale phospho-proteome analyses, not specifically aimed at PcG proteins, the biological relevance of those phospho-sites has thus far not been addressed. Interestingly, we have detected Bmi1-S314 phosphorylation independently by IVK and MS. Furthermore, the putative CBX4-S350 phospho-site, as detected by our IVK, is conserved on its human homologue CBX4 and a target of phosphorylation³¹ (S347; chapter 3). Thus, our findings confirm and extend the relevance of published data.

Kinases involved in PcG phosphorylation

Using a yeast-two hybrid assay we identified MK3 (MAPKAPK3, 3pK) as a direct interactor of PHC2/HPH2 (Chapter 4). IPs confirmed MK3 as a genuine interactor of the PRC1 complex (Chapter 4). Recombinant MK3 phosphorylates PcG

proteins *in vitro* (Chapter 5) and mass spectrometric analysis confirmed at least one of these phosphorylation sites *in vivo* (Chapter 6). Independently we established that MK3 is present at PcG target genes using Chromatin immunoprecipitation experiments (ChIP) and that part of the PRC1 complex dissociates from chromatin upon MAPK-signaling (Chapter 5). MK3 is not the only MAPKAPK that associates with PRC1 proteins. MAPKAPK2 (MK2) interacts with HPH2 in a yeast two-hybrid system³². Recently MK2 was also shown to maintain self-renewal capacity of hematopoietic stem cells, which was dependent on the binding of MK2 to PHC2/HPH2³³. Since MK2 and MK3 share most of their phosphorylation targets^{34,35}, it is likely that MK2 is also a PRC1 kinase.

By using specific inhibitors we have established that P38, ATM/ATR, PKC, JNK, and GSK3 β signaling pathways all contribute to Bmi1 phosphorylation under certain conditions. PKC has already been described as a PcG related kinase^{36,37} (Chapter 3). Whether these kinases directly phosphorylate PRC1 proteins is currently not known. It does however substantiate the notion that multiple kinases are involved in PcG protein phosphorylation and that possibly sequential (*i.e.* progressive and interdependent) phosphorylation events takes place.

PcG complex composition changes upon phosphorylation

Phosphorylation of PcG proteins also appears to affect PRC1 complex composition and specific protein-protein interactions. Several observations underscore this hypothesis. First of all immuno-fluorescence studies showed different staining patterns for PHC1 compared to other PRC1 proteins upon arsenite treatment³⁸ (Chapter 4). PcG-chromatin association, normally visible in G1-phase cells, is released upon arsenite treatment. In contrast to CBX4, RING1 and RNF2, whose chromatin association appears to be lost, PHC proteins appear to redistribute in the nucleus³⁸ (Chapter 4). Although the involved post-translational modifications (PTMs) are currently not fully mapped, the relocation is a phosphorylation-induced event. Secondly, PHC1/2 proteins are not stably associated to the PRC1 core complex as other PcG members under conditions of increased phosphorylation, whereas for instance levels of co-precipitated CBX4 are unchanged. Instead PHC1/2 proteins appear to show increased heterotypical interaction (Chapter 6). Thirdly, PHC and CBX show distinct chromatin enrichment/occupation profiles in response to physiological stimuli in ChIP studies (Chapter 5). Whereas CBX8 is mostly found on genes where also H3K27me3 is present, PHC1 enrichment profiles closely resemble those for the PHC1 interacting kinase MK3 (Chapter 5). Taken together, our own and other published observations clearly support differential regulation within the PRC1 complex upon activation of signaling cascades, implicitly suggesting

differential roles for and/or regulation of PHC proteins and other members of the PRC1 complex in response to cellular signaling.

Importantly, we also identified a new interaction partner of the PRC1 complex, KAP1. The KAP1/PRC1 interaction is dependent on phosphorylation events, in our experimental systems, induced by the cell stressors arsenite and selenite (Chapter 6). Both stressors show an increased association of Bmi1-KAP1 by inhibition of PKC and JNK signaling and a reduced association by inhibition of GSK3 β signaling. However, inhibition of p38 and ATM/ATR signaling reduces binding only in response to Se, not to As. Our findings clearly demonstrate that multiple kinase pathways target PRC complexes and substantiate our previous findings. The biological relevance of the KAP1-PRC1 interaction is as of yet elusive. As both KAP1 and PcG proteins play a role in transcriptional repression, the interaction could have an effect on repression of certain genes. Indeed KAP1 knockdown enhances mitogen-induced expression of a PcG target gene, ATF3, suggestive of some form of co-repression. Surprisingly, ATF3 was not identified as a KAP1 target in promoter tiling array experiments³⁹ (Chapter 6). This could relate to limited analysis in the reported study (many regulatory elements can be found in intragenic regions and at locations more remote from the transcription start site). It is possible, however, that KAP1 fulfills a function in chromatin modulation at a level which is currently not understood. Clearly several functions converge in KAP1: it is a reported co-repressor, and has a distinct function in damaged heterochromatic DNA repair⁴⁰. Likewise PRCs unite transcriptional regulation with DNA replication and perhaps also DNA damage responses^{41, 42}. Furthermore, KAP1 could be involved in determining PRC1 protein stability: KAP1 is a sumo E3 ligase⁴³, and has a RING finger, a domain which is often involved in ubiquitylation⁴⁴. Our preliminary data indicate that Se and As induce proteolytic breakdown of some PRC1 proteins. Also, a phospho-mimic form of Bmi1 appears instable; this is enhanced by Se exposure (Chapter 6 and *unpublished observations*). A possible link between KAP1 and destabilization of Bmi1 is therefore likely, although at this moment speculative.

Control of chromatin association of PcG proteins

The correlation between PcG phosphorylation and chromatin dissociation is currently widely accepted, both in a cell-cycle dependent and cell-cycle independent context^{27, 38, 45}. The exact molecular mechanism underlying the effect of phosphorylation on PcG/chromatin binding is currently not known. However, several hypothetical mechanisms can be formulated by analogy to another histone binding protein family, HP1 (heterochromatin protein 1). Three HP1 isoforms exist (HP1 α , β and γ , respectively also referred to as CBX5, CBX1 and CBX3), which associate with constitutive heterochromatin and regions of

facultative heterochromatin⁴⁶. HP1 and PcG CBX proteins share a chromodomain, which interacts with specific histone 3 trimethyl-marks. HP1 proteins specifically interact with H3K9me3^{47, 48}, whereas the PcG CBX homologues display differential binding to both H3K9me3 and H3K27me3 *in vitro*⁴⁹. Both chromodomain protein classes are released from chromatin during mitosis. HP1 dissociates from chromatin in M-phase, despite unchanged H3K9me3 levels. Phosphorylation on H3S10 adjacent to the K9me3 mark strongly reduces HP1 binding *in vitro* and releases HP1 from chromatin *in vivo*. The kinase responsible for the transient M-phase related phosphorylation is Aurora kinase B⁵⁰. As release of HP1 occurs without concomitant loss of H3K9 trimethylation, rather than de-methylation of the binding mark, phosphorylation was suggested to act as part of a “binary switch” mechanism of the “methyl/phos”-type: phosphorylation of a site adjacent to a methyl mark leads to induced loss of binding of a factor or complex, normally engaged by the methyl mark^{51, 52}. It has been suggested that this switching mechanism is not limited to the K9/S10 region of H3, but may be more widespread: both K9 and K27 in histone 3 reside in very similar amino acid contexts (ARKS); hence this switching mechanism may be involved in controlling CBX binding to H3 as well (Fig. 7.1). Indeed, H3S28 is also phosphorylated in M-phase^{53, 54}. In addition we have reported a close correlation between PRC1 dissociation and H3S28ph, whereas this was not seen in relation to H3S10ph (Chapter 4)³⁸. The dynamic control of protein binding by “methyl/phos” switching also provides a tentative explanation for how HP1 and CBX binding can be correctly re-established at the end of mitosis. H3-(ARK)S-phosphorylation also occurs downstream of signaling, in a cell-cycle independent fashion. Physiological stimuli studied include arsenite, ultraviolet B, TPA and anisomycin. Arsenite induced H3S10ph is dependent on Akt1, extracellular signal-regulated kinase 2 (ERK2) and ribosomal S6 protein kinase 2 (RSK2), but not on mitogen and stress-activated protein kinase 1 (MSK1)⁵⁵. Exposure of cells to ultraviolet B (UVB) induces H3S10ph and H3S28ph, albeit with different kinetics. UVB induced phosphorylation of H3S10 is dependent on ERK and p38 MAP kinases⁵⁶; H3S28 is targeted by ERK, p38, JNK1-MAPK signaling and by MSK1^{57, 58}. Mitogenic signaling by TPA and stress signaling by anisomycin, via the ERK and p38 pathways respectively, lead to the phosphorylation of H3S10 and H3S28 by MSK2 and, to a lesser extent, MSK1⁵⁹. Hence, the larger family of MSK, ERK and RSK kinases appear to integrate regulation of gene expression at several functional levels by targeting transcription factors, chromatin binding complexes and nucleosomal components. Strikingly, the subnuclear distribution of H3S10ph is different from H3S28ph, they form distinct nuclear foci^{60, 61}. Even though MSK1 can phosphorylate H3S10 and S28 within the same peptide *in vitro*, this apparently does not happen *in vivo*⁶¹. The spatial distribution of these H3 PTMs is likely

controlled by kinase recruitment (by *e.g.* HP1 or PcG complexes), substrate availability, and local regulation of kinase and phosphatase activity.

A combinatorial effect of PTMs on histone and non-histone proteins can be predicted. DNA damage induces HP1- β T51 phosphorylation by casein kinase 2 (CK2), which mobilizes HP1- β , and facilitates H2AX phosphorylation⁶². The dissociation mechanism involves disruption of hydrogen bonds required for HP1- β chromodomain folding around H3K9me3. Sequence alignment of HP1 and PcG CBX proteins reveals that T51 is conserved in human and mouse CBX1-8 proteins and in HP1 and Pc of the fruitfly (in human and murine CBX2 this amino acid is a serine). Although CBX protein phosphorylation at these conserved residues has not been reported yet, it is possible that, by analogy to HP1, these residues function in release of methyl-binding interfaces (Fig. 7.1). Interestingly, two recent studies identified subunits of CK2 as interactors of RNF2 and CBX8^{63, 64}. It is currently unclear whether and how CK2 plays a role in complex dissociation. Likewise, determination of which (combinations of) phospho-sites within and outside chromo-boxes and other domains control PcG protein-protein and PRC-chromatin interactions will be subject of future research.

Another level of regulation of PcG CBX chromatin binding occurs via the establishment and erasure of histone methylation. EZH2 of the PRC2 complex achieves H3K27 tri-methylation, to which PRC1 can bind. For a long time demethylating enzymes for this and every other histone methylation mark were unknown. Recently numerous histone demethylases have been identified, that display specificity for the position (*e.g.* H3K4 or H3K9) and the number of methyl groups (mono- di- or tri-methylation)⁶⁵. UTX and JMJD3 have been identified as H3K27me2/3 demethylases⁶⁶⁻⁷⁰. Recruitment of UTX to promoters of the anterior genes of the HOXA and HOXB clusters during retinoic acid-induced differentiation of NT2/D1 cells leads to the loss of H3K27me3, SUZ12 and EZH2 from these promoters^{66, 71}. This indicates that a histone demethylase can regulate the chromatin association of PcG proteins. Finally, published data also suggest that the H3K27trimethyl mark *per se* is not sufficient for chromobox recruitment *in situ*, as in *Drosophila* cells loss of dRing and hence H2AK119Ub, also abolished Pc binding⁷². The PRC1 protein RNF2, in collaboration with Bmi1, acts as a ubiquitin E3-ligase for H2AK119⁷²⁻⁷⁵; the H2AK119ub1 mark is closely associated with polycomb repressive action⁷²⁻⁷⁵.

Ultimately the binding of PRC1 to chromatin is determined by the interplay of multiple factors, including histone methylation, histone phosphorylation, PcG CBX protein phosphorylation and possibly other PRC1 phosphorylation. This in turn is controlled by signal transduction cascades influencing the expression, localization and activity of HMTs, HDMs, kinases and phosphatases. Future research will identify which enzymes are involved and which genes are then

differentially regulated, depending on the biological context of the stimulus (*e.g.* stress or mitosis).

Figure 7.1. Proposed mechanisms for dissociation of PRC1 from chromatin.

The chromodomain of CBX interacts with H3K27me3. Loss of H3K27me3 or phosphorylation of H3S28 and/or conserved residue(s) within the chromodomain of CBX may contribute to chromatin dissociation.

Future research directions

Although we have identified phosphorylation sites on PcG proteins, the limitations of the used techniques are such that we are unable to exactly determine which phosphorylation event was induced by what stimulus or kinase. The development of antibodies, recognizing specific PcG phospho-sites would be of great help to clarify this. The proposed approach, however, will not be trivial, as there are more than 100 PcG-phosphorylation sites to start off with. By implementing quantitative methods, like stable isotope labeling by amino acids in cell culture (SILAC), a more detailed overview could be extracted of the PcG phospho-proteome⁷⁶. MS, in combination with the use of specific kinase inhibitors could in principle help narrow down and identify the number

of putatively involved kinases. If then these kinases and phosphorylation sites were further analyzed by the *in vitro* kinase strategy on peptide arrays, the PcG kinome-phosphoproteome could be further unraveled. Biochemical analysis, including ChIP assays in combination with transcriptional regulation of PcG gene targets and *in vitro* ubiquitylation assays will be pivotal in mapping the biological consequences of PRC-phosphorylation for transcriptional repression. Finally, functional assays involving (combinations of) phospho-mutant PRC proteins will ultimately help establish exact molecular mechanisms. To dissect these mechanisms it is important to map common targets of kinases and PcG proteins in a genome-wide approach, for instance by ChIP-on-chip or ChIP-seq analysis. Common targets would represent likely candidate genes for regulation by PcG phosphorylation by specific upstream kinase signaling pathways.

Many questions regarding the PRC1-KAP1 interaction still remain. It is not clear yet which region within KAP1 is responsible for the interaction and Bmi1 is not necessarily the PRC1 protein KAP1 interacts with. Immunoprecipitations performed with deletion mutants of KAP1 could solve this issue. The question whether KAP1 functions as a sumo or ubiquitin E3 ligase for PRC1 proteins is still unresolved, but should be addressed in future experiments.

Conclusions

The research in this thesis has started to unravel the dynamics of PcG phosphorylation. Combined, these recent observations begin to show that signaling, which triggers downstream phosphorylation events, affects subcellular localization, protein-interactions within complexes, enzymatic activity and chromatin-association of several PcG proteins. Further characterization and validation of these phosphorylation sites will increase our understanding of the cellular conditions under which PcG phosphorylation take place, which kinases and phosphatases are involved and how cells use these pathways to modulate chromatin structure to allow biologically appropriate responses.

PcG proteins are important for regulating embryonic development, cell proliferation and stem cell biology and PcG proteins are deregulated in many cancers (Chapter 2). Therefore regulation of PcG function by post-translational modification is likely to have an important impact on these processes. MK2 was recently reported to control self-renewal of hematopoietic stem cells; this is dependent on the interaction of MK2 and PRC1³³. This finding supports the notion that the interaction of a kinase with PcG proteins is a key determinant of stem cell biology. Whether MK3 is involved in stem cell self renewal needs to be addressed in future studies. Furthermore MK3 is involved in cancer development. The MK3 locus is often deleted in cancer and MK3 mutations

have been reported to contribute to oncogenesis^{77, 78}. Although this would appear to qualify MK3 as a gene with potential tumor suppressive capacity, and we show that indeed MK3 has a negative effect on cell proliferation and proliferative life span, our studies suggest these adverse effects on cell proliferation may occur via processes akin to oncogenic signaling (Chapter 5). Recently, potential driver mutations in MK3 were identified, consistent with an oncogenic gain-of-function⁷⁷. However the potential role in tumorigenesis of these mutations awaits functional validation. Interestingly, MK3 activation activates dormant check-points in cancer cells (*i.a.* cervical carcinoma, osteosarcoma). This suggests MK3 and efferent/afferent signaling modules could be targeted for therapeutic purposes. Loss of PRC1 members often correlates with reduced proliferative life span. Conversely, overexpression of PRC1 members is tumorigenic. We have shown that phosphorylation of PRC1 complexes and subsequent loss of chromatin-association, may be functionally equivalent to PRC1 loss-of-function. Arsenic and selenite both reduce PcG association via increased phosphorylation (Chapters 4 and 6). Arsenic has been applied as chemotherapy in diverse cancers⁷⁹⁻⁸¹. We speculate that the anti-oncogenic properties of arsenite and possibly related non-metallic bivalent ions may in part mechanistically involve Polycomb Group proteins. This novel idea may open further avenues for development of anticancer therapies.

It is expected that regulation of PcG function by phosphorylation, which has been studied in this thesis on a cellular level, contributes to proper development on an organismal level. The relevance of post-translational modifications of PcG proteins and the effect of signaling to PcG proteins are relevant topics in the field of gene-environment interactions that in addition to normal embryogenesis will contribute to our understanding of cancer and other Western-type diseases.

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Summary

The specific function of a cell is mostly determined by the genes and proteins it expresses. The information required to make a protein is stored in DNA and is called a gene. Through transcription and translation of genes proteins are produced. As cell types with different functions express different sets of proteins, it is important to regulate which genes are transcribed, and equally important, which genes are not. The latter is in part controlled by Polycomb Group (PcG) proteins, which are an important part of the transcriptional memory system of a cell: they mark certain genes with repressive signals during developmental and differentiation processes. The repressive marking includes post-translational modifications (PTMs) on histone proteins, such as trimethylation of Lysine 27 on histone 3 (H3K27me3) and ubiquitylation of Lysine 119 on histone 2A (H2AK119ub1). Two main Polycomb Repressive Complexes are recognized, namely PRC1 and PRC2. The histone methyltransferase activity of PRC2 member EZH2 is responsible for the H3K27me3 mark, to which CBX proteins of the PRC1 complex can bind. Other PRC1 proteins, such as RNF2 ubiquitylate H2A. Together this, in general, maintains transcriptional repression. The basic biological concepts that are used in PcG biology, such as histones, chromatin and epigenetics, are described in Chapter 1.

Although the mechanisms that contribute to PcG-mediated silencing, as described above, are relatively well-known, there are still many aspects of PcG biology that are not understood. One important question formed the basis of the research described in this thesis: how is Polycomb Group function regulated? Answering this question is important, as PcG proteins control several biological processes. PcG proteins are essential for embryonic development and stem cell renewal. Among the genes that are targeted by PcG proteins are transcription factors, components of signalling pathways and tumor suppressor genes. Since PcG proteins control epigenetic silencing of many genes involved in cell fate decisions, it is not surprising that deregulated expression of PcG proteins is frequently found in cancer. The role for PcG proteins in the development of cancer is discussed in Chapter 2. Putative mechanisms of how PcG proteins contribute to tumorigenesis are bypass of senescence and maintenance of cancer stem cells.

PcG proteins are not only involved in post-translational modification of chromatin-associated proteins, they are targeted for PTMs, such as ubiquitylation, sumoylation and phosphorylation themselves (Chapter 3). These modifications affect protein function by affecting protein stability, protein-protein interactions and enzymatic activities. The exact molecular mechanisms in, and biological relevance of the majority of PTMs on Polycomb function are

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not known. The research described in this thesis aimed to identify PcG phosphorylation sites, involved kinases and the relevance of PcG phosphorylation in terms of complex composition, chromatin association and gene repression.

At the beginning of this project it was known that at least some PcG proteins are phosphorylated and that this phosphorylation correlates with chromatin dissociation in a cell cycle-dependent fashion. The identification of MAPKAPK3 (MK3) as an interaction partner of the PRC1 complex member PHC2 provided an interesting lead to further elucidate the functional relevance of PcG phosphorylation (Chapter 4). Stimulation of the ERK and p38 MAPK cascades, of which MK3 is a downstream target, leads to the phosphorylation of the PRC1 members PHC1 and BMI1. *In vitro* data establish that MK3 can directly phosphorylate BMI1. The induced phosphorylation of most PRC1 proteins correlates with both dissociation from chromatin and appearance of H3S28 phosphorylation. PHC1 staining, however, is not lost upon phosphorylation but is redistributed within the nucleus. This indicates a differential response of PRC1 complex members to phosphorylation. Manipulation of MK3 levels in cells confirms that Bmi1 phosphorylation is, at least in part, dependent on MK3. Furthermore the overexpression of MK3 results in expression of p14^{ARF}, a cell-cycle inhibitor, indicating derepression of the PcG target gene CDKN2A. These results are in line with the hypothesis that activation of MAPK signalling cascades lead to phosphorylation and chromatin dissociation of PcG proteins, thereby relieving transcriptional repression and allowing for gene activation.

This hypothesis was further tested in Chapter 5 in which MK3 overexpression was studied in the context of cell cycle regulation. Overexpression of MK3 reduces cell proliferation and induces a senescence-like state. This is accompanied by upregulation of several cell cycle regulatory proteins, such as p16, p14^{ARF}, p21 and p53. Knockdown of relevant PcG proteins also induces senescent-like states, indicating that MK3 overexpression and PcG knockdown have the same functional implication. Concomitant overexpression of BMI1 with MK3 reverses the MK3-induced cell-morphological changes. Combined with the observation that BMI1 and MK3 overexpressing cells select for high BMI1 expression this indicates a genetic interaction between BMI1 and MK3. Chromatin Immunoprecipitations (ChIP) further demonstrated that MK3 binds to the promoters of several PcG target genes, including the *CDKN2A* locus. Activation of MK3 is sufficient for a partial release of PRC1 proteins CBX8 and PHC1 from certain genes and transcriptional activation of these genes. Surprisingly this occurs despite an unchanged H3K27me3 mark. The MK3-PRC1

interaction may act as a molecular switch, allowing the cell to regulate PcG-mediated gene expression and rapidly respond to changes in its environment. Chromatin association of PHC1 in ChIP does not mirror CBX8 enrichment, but closely resembles the MK3 occupation dynamics. This observation supported the atypical behaviour of PHC1 compared to other PRC1 proteins. To study direct phosphorylation of PRC1 proteins by MK3 *in vitro* phosphorylation studies were conducted on spotted PRC1 peptides. The strongest *in vitro* MK3 phosphorylation occurs on PHC2 peptides. The signaling-induced differences in PHC chromatin binding compared to other PRC1 proteins might be explained by (1) the direct binding of MK3 to PHC proteins and (2) the direct phosphorylation of PHC proteins by MK3.

To gain insight into regulation of Polycomb function PRC1 complex composition was studied in the context of stress-signaling (Chapter 6). Next to the identification of several PcG phosphorylation sites, a new PRC1 interacting protein, KAP1, was identified, which only binds to PRC1 under certain stress conditions. Although phosphorylation occurs on both Bmi1 and KAP1 when this interaction occurs, the phosphorylation itself is not necessary for the established interaction. Signaling pathways involved in arsenite (As) and selenite (Se) -induced phosphorylation of BMI1 and KAP1 include p38, ATM/ATR, PKC, JNK and GSK3 β pathways. The establishment of Bmi1-KAP1 association is enhanced by inhibition of PKC and JNK signalling, yet is reduced by inhibition of GSK3 β signaling under cell stress; however, inhibition of p38 and ATM/ATR signaling reduces binding only in response to Se, not to As. PcG mediated transcriptional repression is potentially regulated by this interaction. Mitogenic signalling leads to a higher mRNA expression of PcG target gene ATF3, which is potentiated by concomitant knockdown of KAP1. This might, however, occur via more global chromatin responses. The biological relevance of this interaction is therefore currently not clear.

The significance of the results and their interrelationship are discussed in Chapter 7. The research described in this thesis begins to show how activation of signaling cascades leads to phosphorylation of PcG proteins and affects their subcellular localization, protein interactions within the PRC1 complex and their binding to certain target genes, whose expression is thus regulated. Future studies on the regulation of PcG proteins by phosphorylation and other post-translational modifications will determine which impact it has on both physiological processes, such as embryonic development and stem cell renewal, and pathological processes like cancer.

Samenvatting

De specifieke functie van een cel wordt grotendeels bepaald door expressie van genen en eiwitten. De informatie die nodig is om een eiwit te maken, ligt opgeslagen in het DNA en wordt een gen genoemd: de DNA code van genen wordt overgeschreven tot een RNA code (transcriptie) en vertaald in een eiwit code (translatie). Verschillen in celtype worden bepaald door welke eiwitten tot expressie worden gebracht en welke niet. Dit laatste wordt gedeeltelijk bepaald door Polycomb Groep (PcG) eiwitten; PcG eiwitten vormen een belangrijk transcriptioneel geheugensysteem: ze markeren bepaalde genen met epigenetisch repressieve signalen tijdens ontwikkeling en differentiatieprocessen. Dergelijke repressieve markering bestaat deels uit post-translationele modificaties (PTMs) op histon eiwitten, waaronder trimethylering van Lysine 27 op histon 3 (H3K27me3) en ubiquitylering van Lysine 119 op histon 2A (H2AK119ub1). Er bestaan twee belangrijke Polycomb Repressieve Complexen: PRC1 en PRC2. De histon methyltransferase activiteit van het PRC2-eiwit EZH2 is verantwoordelijk voor de H3K27me3 markering, waaraan CBX eiwitten van het PRC1 complex kunnen binden. Andere PRC1 eiwitten, waaronder RNF2, ubiquityleren H2A. In het algemeen leidt dit tot behoud van transcriptionele repressie. De algemene biologische concepten die gebruikt worden in de PcG biologie, zoals histonen, chromatine en epigenetica, worden uitgelegd in hoofdstuk 1.

Ofschoon een aantal mechanismen, dat betrokken is bij PcG-afhankelijke repressie, zoals hierboven beschreven, ontrafeld is, blijven er nog steeds veel aspecten van PcG biologie onbegrepen. Ondermeer de vraag hoe PcG-functie gereguleerd wordt, stond aan de basis van het onderzoek beschreven in dit proefschrift. Het beantwoorden van deze vraag is relevant, omdat PcG eiwitten belangrijke fundamentele biologische processen reguleren. PcG eiwitten zijn essentieel voor embryonale ontwikkeling en stamcel-vernieuwing. Onder de genen die gereguleerd worden door PcG eiwitten zijn transcriptiefactoren, onderdelen van signaaltransductie cascades en tumor suppressor genen. Aangezien veel van dergelijke genen betrokken zijn bij het differentiatie en celdeling, is het niet verrassend dat ontregeling van PcG-eiwitten vaak voorkomt in kanker. De rol van PcG eiwitten bij het ontstaan van kanker wordt belicht in hoofdstuk 2. Mechanismen die bijdragen aan kankerontwikkeling zijn het voorkomen van een verouderingsrespons (senescentie) en de instandhouding van kanker stamcellen.

PcG eiwitten zijn niet alleen betrokken bij het aanbrengen van post-translationele modificaties op chromatine-geassocieerde eiwitten, maar worden zelf ook post-translationeel gemodificeerd, ondermeer door ubiquitylering, sumoylering en fosforylering (hoofdstuk 3). Deze modificaties beïnvloeden de

functie van eiwitten door aan te grijpen op eiwitstabiliteit, eiwit-eiwit interacties en enzymatische activiteit. De exacte gevolgen van PTMs op PcG functie op moleculair nivo en hun biologische relevantie zijn niet bekend. Het onderzoek beschreven in dit proefschrift had o.m. tot doel PcG specifieke gefosforyleerde aminozuren en betrokken kinases te identificeren en de gevolgen van PcG fosforylering in termen van complex compositie, chromatine associatie en gen repressie te bepalen.

Bij aanvang van dit project was het bekend dat op zijn minst enkele PcG eiwitten gefosforyleerd worden en dat deze fosforylatie op een cel-cyclus-afhankelijke manier correleerde met een PcG-dissociatie van chromatine. De identificatie van MAPKAPK3 (MK3) als interactie partner van PHC2, dat deel uitmaakt van PRC1, zorgde voor een aanknopingspunt om de functionele relevantie van PcG fosforylatie te bestuderen (hoofdstuk 4). Stimulatie van ERK en p38 MAPK cascades, van welke MK3 een target is, leidt tot de fosforylatie van PRC1 leden PHC1 en BMI1. *In vitro* data bevestigen dat MK3 direct BMI1 kan fosforyleren. De geïnduceerde fosforylatie van de meeste PRC1 eiwitten correleert met zowel de dissociatie van chromatine als de verschijning van H3S28 fosforylatie. Chromatine associatie van PHC1 gaat daarentegen niet verloren na fosforylering, maar vertoont een redistributie in de nucleus. Deze observatie correleert met een differentiële respons van PRC1 eiwitten na fosforylatie. Manipulatie van MK3 eiwit niveaus in cellen bevestigt dat BMI1 fosforylatie, op zijn minst gedeeltelijk, afhankelijk is van MK3. Verder resulteert de overexpressie van MK3 in de expressie van p14^{ARF}, een celcyclus remmer, wat een derepressie van het PcG target gen CDKN2A aangeeft. Deze resultaten zijn in overeenstemming met de hypothese dat activering van MAPK-signalering leidt tot fosforylatie en chromatine dissociatie van PcG eiwitten, waardoor transcriptionele repressie wordt opgeheven en de mogelijkheid tot genactivatie ontstaat.

Deze hypothese werd verder getoetst in hoofdstuk 5 waarin MK3 overexpressie bestudeerd werd in de context van celcyclus regulatie. Overexpressie van MK3 vermindert celdeling en induceert een op celveroudering lijkende status: cellen worden groot en plat en delen niet meer. Deze verandering gaat gepaard met activering van verschillende celdelings remmers, waaronder p16, p14^{ARF}, p21 en p53. Experimenteel geïnduceerd verlies van relevante PcG eiwitten resulteert eveneens in een verouderingsrespons. Dit geeft aan dat MK3 overexpressie en PcG verlies vergelijkbare functionele gevolgen hebben. Gelijktijdige overexpressie van BMI1 en MK3 draait de MK3-geïnduceerde celmorfologische veranderingen terug. Samen met de waarneming dat BMI1 en MK3 overexpresserende cellen selecteren voor een hoge BMI1 expressie, duidt dit op

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een genetische interactie tussen BMI1 en MK3. Chromatine Immunoprecipitaties (ChIP) demonstreerden dat MK3 bindt aan promotoren van PcG doelwitgenen, waaronder de CDKN2A locus. Activering van MK3 is voldoende voor een gedeeltelijk verlies van chromatine-associatie van de PRC1 eiwitten CBX8 en PHC1 op bepaalde genen; dit gaat gepaard met een verhoogde transcriptie van deze genen. Opmerkelijk is dat dit gebeurt met behoud van de epigenetisch repressieve H3K27me3 markering. Onze bevindingen suggereren dat MK3-PRC1 als een moleculair schakelmechanisme werken, waardoor een cel PcG-gemedieerde gen repressie snel kan aanpassen en daarmee kan reageren op veranderingen uit de cellulaire omgeving. Chromatine-associatie veranderingen van PHC1, bepaald met behulp van ChIP, volgen niet de CBX8-associatie profielen, maar lijken eerder op die van MK3. Deze waarneming ondersteunt het eerder gevonden atypische gedrag van PHC1 in vergelijking tot andere PRC1 eiwitten. Om fosforylering van PRC1 eiwitten door MK3 te bestuderen hebben we *in vitro* fosforylerings studies gedaan op geïmmobiliseerde PRC1 peptiden. De duidelijkste *in vitro* MK3 fosforylering blijkt plaats te vinden op PHC peptiden. De signalerings-geïnduceerde verschillen in PHC-chromatine binding in vergelijking tot andere PRC1 eiwitten worden mogelijk verklaard door (1) de directe binding van MK3 aan PHC eiwitten en (2) de directe fosforylering van PHC eiwitten door MK3.

Om inzicht te krijgen in de regulering van Polycomb functie werd de complex samenstelling van PRC1 bestudeerd in de context van stress-signalering (hoofdstuk 6). Naast identificatie van verschillende PcG fosforyl-aminozuren ontdekten we een nieuw PRC1-bindend eiwit, KAP1, dat alleen aan PRC1 bindt onder stress condities. Ofschoon fosforylering plaatsvindt op zowel Bmi1 als KAP1 wanneer deze interactie plaatsvindt, blijkt fosforylering niet noodzakelijk om de interactie in stand te houden. Signaleringscascades die betrokken zijn bij Arseen (As) en Seleen (Se)-geïnduceerde fosforylering van BMI1 en KAP1 zijn onder andere P38, ATM/ATR, PKC, JNK en GSK3 β cascades. De totstandkoming van BMI1-KAP1 binding onder cel-stress condities, wordt gestimuleerd door remming van PKC en JNK signalering, en verhinderd door remming van GSK3 β . Echter, blokkering van p38 en ATM/ATR signalering vermindert de binding alleen als gevolg van Se, en niet As. Polycomb gemedieerde transcriptionele repressie wordt mogelijk beïnvloed door deze interactie. Mitogene signalering leidt tot hogere mRNA expressie van PcG target gen ATF3, die bovendien versterkt wordt door verlies van KAP1. Ofschoon de biologische relevantie van deze interactie nog niet duidelijk is, is het mogelijk dat hierbij een meer globale chromatine structuurverandering betrokken is.

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De betekenis van de resultaten en hun onderlinge verband worden bediscussieerd in hoofdstuk 7. Het onderzoek beschreven in dit proefschrift laat zien hoe activering van signaaltransductie cascades leidt tot fosforylering van PcG eiwitten en daardoor hun subcellulaire locatie, eiwit interacties binnen het PRC1 complex en binding aan bepaalde target genen beïnvloed wordt, waardoor de expressie van deze genen wordt gereguleerd. Verder onderzoek naar de regulering van PcG eiwitten door fosforylering en andere post-translationele modificaties zijn nodig om de invloed van dergelijke veranderingen op zowel normale fysiologische processen, waaronder embryonale ontwikkeling en stamcel vernieuwing, als pathologische processen zoals kanker, te bepalen.

Dankwoord

Dankwoord

Na de afgelopen jaren hard gewerkt te hebben om mijn promotieonderzoek af te ronden, ben ik toegekomen aan het schrijven van dit dankwoord. Aan iedereen die van plan is om alleen dit gedeelte te lezen wil ik toch even zeggen dat de rest ook best de moeite waard is. Er zijn veel mensen die mij de afgelopen jaren geholpen hebben, en die ik wil bedanken voor hun steun (en ook ik hoop dat ik niemand vergeet).

Ten eerste mijn co-promotor en promotores.

Beste Willem, jouw enthousiasme voor de wetenschap is de grote drijfveer achter het Polycomb Groep-groepje. Tijdens werkbijeenkomsten werden alle foto's, grafieken, staaf-diagrammen (waar zijn de blips?) en vooral blotjes (nee, ik heb niets omgewisseld) tot in detail besproken, en leverden een stortvloed aan nieuwe ideeën op. Je hoge standaard heeft, buiten wat frustraties hier en daar, het beste uit me naar boven gehaald. Bedankt voor alles wat ik van je heb geleerd.

Beste Joep en Marten, bedankt voor de begeleiding, vooral tijdens de laatste fase.

Vivian, ik weet niet wat er van mijn promotie terecht was gekomen zonder jou. Je hebt niet alleen bergen werk verzet, maar je stond ook altijd klaar met een goed advies of luisterend oor als het wetenschappelijk gezien even tegenzat. Jouw efficiëntie en nauwkeurigheid op het lab zijn ongeëvenaard, en altijd een voorbeeld voor mij geweest. Buiten dat vond ik je altijd een gezellige collega. Ik ben blij dat ik jou straks als paranimf achter me heb staan. Ik vraag me trouwens af hoe lang het nog duurt, voordat we onafhankelijk van elkaar exact dezelfde schoenen kopen.

De rest van het VWO groepje (Claudia, Céline, Frank, Peggy, Nard, Iris, Elena, Raymond en Juliette) wil ik bedanken voor de goede sfeer op het lab (en dat jullie me nooit verboden hebben om met de radio mee te zingen). Claudia, jij hebt zonder morren altijd de meest vreselijke klusjes uitgevoerd (cellen tellen, mutanten maken, virussen maken etc). De uren in de celkweek vlogen met jou erbij om! Céline, I enjoyed having you as a colleague. I admire your ability to stay strong, even though your research-life was tough on you. Frank (Frenkieeee!), onze eeuwige optimist (wat goed van pas komt in de wetenschap en als Ajax-fan). Met jou als labbuurman wist ik dat ik mijn spullen nooit meer dan een halve labtafel verderop moest zoeken. Ondanks dat het je niet gelukt is om mijn muzieksmaak te beïnvloeden, kon ik je muzikale heropvoedingspogingen wel waarderen. Succes met de laatste loodjes! Peggy, ook met jou heb ik altijd een gezellige tijd gehad. Met jou erbij valt er nooit een pijnlijke stilte. Ik ben blij dat je verder gaat met waar ik gebleven was. Met jouw perfectionisme moet het wel goed komen. Mocht je nog wat adviezen kunnen gebruiken van je

Dankwoord

AIO-mamma, dan hoor ik het wel! Nard, je behoort misschien niet tot het core complex, maar je bent zeker een geassocieerde factor. Ik vind het leuk om op de hoogte te blijven van je avonturen in Washington. Succes met afronden.

Verder kwamen er elk jaar een heleboel studenten voorbij die ons groepje hebben versterkt. Zonder volledig te zijn (onmogelijke taak) wil ik Caroline, Chantal, Guus, Joyce, Marjolein, Martin, Martijn, Merel, Petra H., Rene, Sjoerd, Yvette en in het bijzonder Petra C. en Stefanie bedanken voor de gezelligheid en (in meer of mindere mate) voor de hulp bij het totstandkomen van dit proefschrift.

De soms lange dagen op het lab vlogen voorbij dankzij de gemoedelijke sfeer op het lab en tijdens de koffiepauzes. Aangezien ik best wel lang bij moleculaire genetica heb gewerkt, heb ik veel collega's zien komen en gaan. Groot was mijn verbazing toen bleek dat mijn voormalige stageplaats verhuisde naar moleculaire genetica, en we ineens collega's werden. Iedereen bedankt! In willekeurige volgorde: Veerle B., Veerle C., Wilma, Will (ho, ho), Daniëlle C., Ellen L. (denk eraan, alles komt goed), Ellen D., Guillaume (ik kon het niet meer verplaatsen), Marjon (jouw onbezonnen uitspraken zijn vaak hilarisch), Jan, Pieter, Patrick (altijd netjes; ondanks mijn 116^e vraag), Daphna, Daniëlle vd V., Nicole, Petra, Joost, Chantal, Robert, Inge, Arjen, Laura, Sander, Le (how could every song somehow be related to Burt Bacharach?), Ronit, Monique (wie is hier blauw?), Wino (wat kan cellen kweken toch frustrerend zijn!), Menno, Sofia (Hey Sofiaaaa!), Kristiaan, Mathijs (wanneer zorgen we nu eens voor die integratie?), Stijn, Jana, Olivier, Debby, Roy, Susan, Remco, Maurice en Mohammed.

Dan zijn er nog de mensen buiten de afdeling die hun steentje hebben bijgedragen.

Dear Adrian, thanks for showing me how to perform a decent ChIP experiment. It was a crucial part of my PhD studies. Even though it was a short stay, I learned a lot from the meaningful discussions.

Ik wil iedereen van de Maastricht besprekingen bedanken voor hun bijdrage aan mijn "oncologische-ontwikkeling". Door deze samenwerking heb ik geleerd verder te kijken dan mijn Polycomb-neus lang is. Dus bedankt Brad, Marianne, Jan, Roland, Ludwig, Michael, Maud, Younan, Asferd, Sherry, Barry, Kim, Kim, Natasja, Chantal (voor je DNA schade expertise), Kasper (dat er ge-ChIP'ed moest worden), Twan (dat jij daar ook nog wat aan had), Ramon en Jos.

Natuurlijk mag ik mijn nieuwe collega's niet vergeten. Alle "Angels" (man/vrouw) bedankt voor jullie oprechte interesse en bemoedigende woorden bij het afronden van dit boekje. In het bijzonder Manon (bedankt dat ik bij je aan de slag mocht en voor het duwen richting de streep), Sarah (al aan het

Dankwoord

samenwerken voordat we collega's werden), Kim en Veerle (mijn huidige roomies; hoe goed kan het klikken tussen collega's?).

Natuurlijk is het belangrijk om naast het werk nog een sociaal leven te hebben. Saskia, of eigenlijk Sas. Onze vriendschap bestaat al zo lang, dat we het begin niet eens kunnen herinneren. Van peuterspeelzaal tot promoveren, samen stonden we sterk. De bezoeken over en weer zijn de laatste tijd wat sporadisch geworden, maar ik hoop dat nu weer wat meer op te kunnen pakken. Wat denk je, Utrecht, Maastricht of Roermond?

Susan, lieve Suus, van stagiaire naar collega's naar vriendinnen. Ik heb er bewondering voor hoe jij altijd het positieve in mensen kunt zien. Hopelijk kunnen we nog lang samen lunchen.

Mijn sportvriendinnen. Inge, wat een geduld had jij toen mijn conditie nog niet-bestaand was. Zonder jou als stok achter de deur had ik het hardlopen nooit volgehouden. Bedankt ook voor je feilloze tips qua muziek. Imke, met jou erbij is rennen nooit saai. Simultaan rennen en praten is voor jou een makkie. Ik hoop dat we snel weer kunnen beginnen (en anders slaan we dat rennen over en gaan we meteen door met eten en bijkletsen). Anouk, onze wekelijkse tennis of badminton avonden waren altijd erg gezellig (vooral het naborrelen). Met je nuchtere blik kun je de af en toe rare randvoorwaarden van het onderzoekswereldje in het juiste perspectief plaatsten. Nu kan ik je eindelijk ook eens uitnodigen voor een feestje!

Waar zou je zijn, zonder je familie? Lieve pap en mam (Jeanneke en ziene Jan), jullie liefde voor ons vind je terug in al jullie woorden en daden. Ik voel me gezegend met jullie als ouders. Bedankt voor alles!

Petra, lieve Peetje, van jongs af aan kunnen wij het altijd goed met elkaar vinden. Je bent een schat! Waar anderen zich niet konden voorstellen om ooit met hun broer/zus te moeten samenwerken, was dat voor ons totaal geen probleem. Bedankt voor al je kalmerende woorden wanneer de proefschriftstress weer eens in volle hevigheid toesloeg. Ik ben blij dat ik zo'n lieve zus, en paranimf, heb. Sander, tijdens mijn promotie ging jij van collega naar schoonbroer. Ik had me geen betere kunnen wensen. Wat wordt het volgende concert?

Al zijn ze tegenwoordig qua opzet veranderd, de zondagmiddagen staan in mijn geheugen gegrift. Opa en oma, Frank, Marion, Dennis, Frits, Margriet, Wouter, Marjolein en Ewout, bedankt voor al het heerlijke gebak en eten, het ongezoeten voetbalcommentaar, de ongeordende discussies, maar vooral voor de gezelligheid.

Hanneke

Curriculum Vitae

Hanneke Niessen werd geboren op 10 juni 1980 in Roermond. In 1992 begon zij aan de gymnasium opleiding van het Bisschoppelijk College Broekhin te Roermond. Net als haar zus ontwikkelde ze een gezonde belangstelling voor het vak biologie. Aangezien ze niet nog een opleiding wilde doorlopen waar de docenten haar betitelden als “het zusje van” besloot ze in 1998 Biomedische Gezondheidswetenschappen te studeren in Nijmegen. Later koos ze voor pathobiologie als hoofdvak en moleculaire levenswetenschappen als bijvak. Tijdens haar stages bij de vakgroepen Matrix Biochemie (Nijmegen Centre for Molecular Life Sciences, UMC St Radboud) en Fysiologie (Universiteit Maastricht) bleek haar voorkeur uit te gaan naar de moleculaire biologie. Deze interesse kwam goed van pas tijdens haar promotie-onderzoek op het redelijk ambitieuze project “the identification and characterization of Polycomb-associated kinase activity” dat ze eind 2002 onder supervisie van Dr. J.W. Voncken startte bij de vakgroep Moleculaire Genetica in Maastricht, niet geheel toevallig de afdeling waar haar zus al werkzaam was. Door de toekenning van een NGI en een short-term EMBO fellowship kon ze in 2006 naar het Biotech Research & Innovation Centre in Kopenhagen, waar zij de chromatine immunoprecipitatie techniek leerde van Dr. A. Bracken.

Sinds augustus 2008 is ze als postdoc werkzaam bij de vakgroep Pathologie, waar ze onder begeleiding van Dr. M. van Engeland onderzoek doet naar de relevantie van GATA4 en GATA5 promotermethylering in darmtumoren.

List of publications

Papers

Niessen HEC, Prickaerts P*, Salvaing J*, Spaapen F*, Dahlmans V, Geijselaers C, Bartels S, Takihara Y, Hansen K, Wouters B, Bracken A, Rapp U, Voncken JW. MK3 controls proliferation by an epigenetic switch-mechanism involving Polycomb function. *Submitted*

Rouschop KMA, van den Beucken T, Dubois L, **Niessen H**, Bussink J, Savelkoul K, Keulers T, Mujcic H, Landuyt W, Voncken JW, Lambin P, van der Kogel AJ, Koritzinsky M, Wouters BG. The unfolded protein response protects cells during hypoxia through regulation of the autophagy genes LC3b and ATG5. *Submitted*

Niessen HEC, Dahlmans V, Prickaerts P, Spaapen F, Geijselaers C, Lee S-C, Wouters BG, Demmers J and Voncken JW. Cell stress-induced association of Polycomb Repressive Complex 1 with the chromatin factor KAP1. *In preparation*

Spaapen F, van den Akker G, Caron M, Prickaerts P, **Niessen H**, Dahlmans V, Surtel D, Paulis Y, Schweizer F, Rofel C, Welting T, Eijssen L, Voncken JW. The immediate early gene EGR1 controls epigenetic reprogramming through Polycomb Group proteins during chondrogenesis. *In preparation*

Niessen HE, Demmers JA, Voncken JW. Talking to chromatin: post-translational modulation of polycomb group function. *Epigenetics Chromatin*. 2009;2(1):10.

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Derks S, Bosch LJ, **Niessen HE**, Moerkerk PT, van den Bosch SM, Carvalho B, Mongera S, Voncken JW, Meijer GA, de Bruine AP, Herman JG, van Engeland M. Promoter CpG island hypermethylation- and H3K9me3 and H3K27me3-mediated epigenetic silencing targets the deleted in colon cancer (DCC) gene in colorectal carcinogenesis without affecting neighboring genes on chromosomal region 18q21. *Carcinogenesis*. 2009;30(6):1041-1048.

List of publications

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Voncken JW*, **Niessen H***, Neufeld B*, Rennefahrt U, Dahlmans V, Kubben N, Holzer B, Ludwig S, Rapp UR. MAPKAP kinase 3pK phosphorylates and regulates chromatin association of the polycomb group protein Bmi1. *J Biol Chem*. 2005;280(7):5178-5187.

(*equal contribution)

Book contribution

Niessen H, Voncken JW. Polycomb Group Proteins in Tumorigenesis. In: Tollefsbol T, ed. *Cancer Epigenetics*. Boca Raton: CRC Press; 2008:281-302.

Abbreviations

Abbreviations

3pK	MAPK-activated protein kinase 3
A	Alanine
AA	Amino acid;
AML	Acute myeloid leukemia
A-P	Anteroposterior
As/Ars	Arsenite
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
ATR	Ataxia telangiectasia mutated- and Rad3-related
BMI1	B-cell-specific Moloney murine leukemia virus integration site 1
CBX	Chromobox homologue
ChIP	Chromatin Immunoprecipitation
CK2	Casein kinase 2
CKI	Cyclin-dependent kinase inhibitor
CSC	Cancer stem cells
CtBP	Carboxyl-terminus binding protein
DAPI	4',6-diamidino- 2-phenylindole
DNMTs	DNA methyltransferases
DUBs	De-ubiquitinating enzymes
E	Glutamic acid
EDR	Early development regulator
EED	Embryonic ectoderm development
<i>e.g.</i>	Exempli gratia
EMT	Epithelial mesenchymal transition
ERK	Extracellular signal-regulated kinase
ES	Embryonic stem
ESC	Extra sex combs
EV	Empty vector
E(z)	Enhancer of zeste
FCS	Fetal calf serum
GFP	Green fluorescent protein
GFRs	Growth factor receptors
GSK3 β	Glycogen synthase kinase-3 β
GST	Glutathione S-transferase
H2A	Histone 2A
H2AK119ub1	Histone H2A monoubiquitylation of K119
H2B	Histone 2B
H3	Histone 3
H4	Histone 4
H3K27me3	Trimethylation on lysine 27 of histone 3
HA	Hemagglutinin
HATs	Histone acetyl transferases
HCNE	Highly conserved noncoding elements
HDII/SPM/SAM	Homology domain II/sex combs on midleg, polyhomeotic, I(3)mbt/self-association motif
HDACs	Histone deacetylases
HIPK2	Homeodomain interacting protein kinase 2
HMECs	Human mammary epithelial cells
HMTs	Histone methyl transferases
HOX	homeobox

List of publications

HP1	Heterochromatin protein 1
HPC	Homolog of polycomb
HPH	Homolog of polyhomeotic
HSC	Hematopoietic stem cell
HSF	Heat shock factor
hTERT	Human telomerase reverse transcriptase
<i>i.a</i>	Inter alia
<i>i.e.</i>	Id est
IF	Immuno-fluorescence
IP	Immunoprecipitation
IR	ionizing radiation
IRES	Internal ribosomal entry site
IVK	<i>In vitro</i> kinase
JNK	c-Jun NH2-terminal kinase
K	Lysine
KAP1	KRAB-associated protein 1
KD	Knock down
KRAB	Krüppel-associated box
mAb	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
MAPKAP	Mitogen activated protein kinase activated protein
MBLR	MEL18 and BMI1-Like RING finger protein
MEL18	Melanoma nuclear protein 18
MK2	MAPKAP kinase 2
MK3	MAPKAP kinase 3
MS	Mass spectrometry
MSK1	Mitogen and stress-activated protein kinase 1
OE	Overexpression
O-GlcNAc	O-linked N-acetylglucosamine
Ogt	O-GlcNAc transferase
OIS	Oncogene induced senescence
pAb	Polyclonal antibody
Pc	Polycomb
PcG	Polycomb Group
PCGF	Polycomb group ring finger
Ph	Polyhomeotic
PHC	Polyhomeotic-like
Pho	Pleiohomeotic
PhoI	Pleiohomeotic-like
PhoRC	Pho repressive complex
PKC	Protein kinase C
PRC1	Polycomb Repressive Complex 1
PRC2	Polycomb Repressive Complex 2
Psc	Posterior sex combs
PTM	Post-translational modification
RING	Really interesting new gene
RNF	Ring finger protein
RSK2	Ribosomal S6 protein kinase 2
RYBP	RING1 and YY1 binding protein
S	Serine

Abbreviations

SA-βGal	Senescence-associated β-Galactosidase
SAM	Self association motif
SAPK	Stress-activated protein kinase
Sce	Sex combs extra
Se	Selenite
SILAC	Stable isotope labeling by amino acids in cell culture
SIP1	SMAD interacting protein
SUMO	Small ubiquitin-related modifier
Su(z)	Suppressor of zeste
Sxc	Super sex combs
T	Threonine
TPA	12-O-tetradecanoylphorbol-13-acetate
TRIM28	Tripartite motif-containing 28
TrxG	Trithorax Group
Ub	Ubiquitin
UBD	Ubiquitin binding domain
UVB	Ultraviolet B
Y	Tyrosine
YY	Yin–yang transcription factor
ZAP-70	Zeta associated protein-70

Colour figures

Figure 4.3. Phosphorylation-dependent PcG/chromatin interaction.

Figure 4.4. Post-translational histone H3-tail modifications during stress signaling.

Figure 4.5.F Identification of 3pK as a candidate Bmi1 kinase.

Figure 4.6.C Overexpression of 3pK releases chromatin-associated Bmi1 and derepresses *Cdkn2a-INK4A*.

Figure 5.1.B+F MK3 induces cell cycle arrest and a senescence-like state.

Figure 5.2. Expression analysis of cell cycle regulators in MK3-expressing senescent cells.

Figure 5.3.C Genetic interaction between MK3 and Bmi1.

Figure 5.S2.D MK3 expression level affects proliferation responses.

Figure 5.S3.B+C Role of MK3 in senescence pathways.

Figure 6.1.D+E Bmi1-Py and KAP1 phosphorylation and association upon Arsenite exposure.

Figure 6.S1. PHC1 and KAP1 concentrate around nucleolar structures.

Figure 6.S7. Localization of BMI1 and KAP1 in stressed cells.

A

B

Figure 6.S8. BMI1 and pS824-KAP1 do not colocalize

Figure 6.S9.A Localization of DNA damage and KAP1 in stressed cells.

Figure 6.S9.B Localization of DNA damage and KAP1 in stressed cells.

Seldom, very seldom, does complete truth belong to any human disclosure;
seldom can it happen that something is not a little disguised, or a little mistaken.

From Emma by Jane Austen